The Divergent Inflammatory and Anti-inflammatory Effects of Adiponectin upon Kupffer Cells.

Dr Briohny Winnett Smith
BSc MBBS FRACP

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School of Medicine and Pharmacology.
Queen Elizabeth II Medical Centre
Nedlands, Western Australia.

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Abstract

**Background**

Adiponectin is a highly abundant adipokine with serum levels that are inversely correlated with adiposity; obese individuals are generally found to have low circulating adiponectin levels and lean individuals have high circulating adiponectin concentrations. Adiponectin possesses multiple anti-inflammatory, anti-atherogenic, anti-fibrotic and insulin sensitizing properties, and as such, low adiponectin levels have been associated with the metabolic syndrome and insulin-resistant states including non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). In animal models, adiponectin has been shown to protective against hepatic steatosis, steatohepatitis and fibrosis. In view of these findings, adiponectin may represent a therapeutic modality for the treatment of NAFLD and NASH in humans. Despite these known beneficial effects of adiponectin, recent reports suggest that adiponectin may be adversely associated with several immune mediated diseases including rheumatoid arthritis and renal disease. In addition there have been increasing findings of adiponectin promoting an inflammatory response in *in vitro* studies of macrophages and monocytes.

**Aims**

The aim of this thesis is to describe both the anti- and pro-inflammatory effects of adiponectin upon Kupffer Cells and to describe the mechanisms responsible for eliciting these possible divergent effects of adiponectin.

**Hypothesis**

In this thesis the hypothesis is that adiponectin will initially evoke the release of pro-inflammatory and pro-fibrotic cytokines in Rat Kupffer Cells. This initial pro-
inflammatory and pro-fibrotic cytokine release will be down regulated with ongoing adiponectin exposure and will be regulated by adiponectin-induced IL10 production.

Methods

Kupffer Cells were isolated from Sprague-Dawley rats using the two-stage pronase-collagenase digestion and centrifugal elutriation technique. Day 1 cultured Kupffer Cells were treated with adiponectin and/or LPS for 18 hours, and in some experiments were re-exposed to adiponectin and/or LPS for a further treatment period of up to 4 hours. To determine the mechanisms by which adiponectin acts on Kupffer Cells, cells were co-cultured with exogenous IL10 peptide, neutralizing IL10 antibody or MAPK pathway inhibitors. Cytokine changes were assessed with qPCR or culture media ELISA. Signalling pathways and receptors were analysed using Western blot.

Results

Initial adiponectin treatment induced increased TNFα, IL6 and IL10, and decreased TGFβ, CTGF and PDGF in Kupffer Cells. Adiponectin pre-treatment however abrogated both LPS and adiponectin-induced TNFα and IL6 but not IL10 on repeated exposure. Kupffer Cells exposed to both adiponectin and exogenous IL10 failed to elicit an inflammatory response. Conversely, IL10 neutralization was associated with restoration of the inflammatory response suggesting IL10 mediates the development of adiponectin tolerance in Kupffer Cells. Adiponectin failed to up regulate TNFα when Kupffer Cells were cultured with inhibitors to ERK/2, P38 or JNK, with adiponectin pre-exposure and exogenous IL10 both preventing phosphorylation of these MAPK pathways.
Conclusion

Adiponectin exerts an initial inflammatory response in rat Kupffer Cells followed by the development of tolerance to repeated adiponectin exposure with an associated anti-inflammatory response. This tolerance is mediated by IL10, which feeds back to inhibit adiponectin-induced ERK1/2, P38 and JNK and subsequent TNFα generation. Further understanding of this initial inflammatory response to adiponectin is required to facilitate the use of adiponectin as a therapeutic agent in NAFLD and NASH.
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Statement of Candidate Contribution

This thesis comprises of my original work that has been prepared for publication, some of which has been co-authored.

Contributions from others have been acknowledged in my thesis.
Publications Arising From This Thesis


Smith BW, Ramezani M, George J, Hebbard L. Initial adiponectin exposure induces TNFα and IL6 in Kupffer Cells prior to tolerance development via an IL10 dependent mechanism.

Prepared for publication


In progress.
Abstracts


Smith BW, Hebbard L, George J. Adiponectin Induces Pro-inflammatory Cytokines In Kupffer Cells and Tolerance to Itself via the Generation of IL-10. J Gastroen Hepatol. 2009 Oct; 24(Suppl. 2) A274

Smith BW, Brymora J, George J. Adiponectin induces IL10 and abrogates the LPS-induced release of pro-inflammatory cytokines by Kupffer Cells. J Gastroen Hepatol. 2008 Oct; 23(Suppl. 4) A274
Presentations

Smith BW, Hebbard L, George J. Adiponectin induces pro-inflammatory cytokines in Kupffer Cells via the activation of MAPK Pathways.


Smith BW, Hebbard L, George J. Adiponectin Induces Pro-inflammatory Cytokines In Kupffer Cells and Tolerance to Itself via the Generation of IL-10.


Smith BW, George J, Hebbard L. Adiponectin Induces Pro-inflammatory Cytokines In Kupffer Cells and Tolerance to Itself via the Generation of IL-10.


Smith BW, Brymora J, George J. Adiponectin induces IL10 and abrogates the LPS-induced release of pro-inflammatory cytokines by Kupffer Cells.

## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACC</td>
<td>Acetyl coenzyme-A carboxylase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate activated protein kinase</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>aP2</td>
<td>Adipocyte protein 2</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>APPL1</td>
<td>Adapter protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α- Smooth muscle actin</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>BME</td>
<td>Beta-mercaptoethanol</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCl₄</td>
<td>Carbon tetrachloride</td>
</tr>
<tr>
<td>ChREBP</td>
<td>Carbohydrate responsive element binding protein</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanacavlin A</td>
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<td>CPT1</td>
<td>Carnitine palmitoyltransferase-1</td>
</tr>
<tr>
<td>Cₜ</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>CXCL9</td>
<td>Chemokine (C-X-C motif) ligand 9</td>
</tr>
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<td>CXCL10</td>
<td>Chemokine (C-X-C motif) ligand 10</td>
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<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle media</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>Egr-1</td>
<td>Early growth response protein-1</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated kinase 1 and 2</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
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<td>FCS</td>
<td>Foetal calf serum</td>
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<td>FFA</td>
<td>Free fatty acid</td>
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<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
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<tr>
<td>GalN</td>
<td>D-Galactosanine</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GBSS</td>
<td>Geys balanced salt solution</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte antigen</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase-1</td>
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<td>HSC</td>
<td>Hepatic Stellate Cell</td>
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<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
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<tr>
<td>IFNβ</td>
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<td>IFNγ</td>
<td>Interferon-γ</td>
</tr>
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<td>IGF-1</td>
<td>insulin like growth factor</td>
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<tr>
<td>IL13</td>
<td>Interleukin 13</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IRAK-M</td>
<td>IL1 receptor associated kinase</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of κB JNK c-Jun NH(_2)-terminal kinase</td>
</tr>
<tr>
<td>KRH</td>
<td>Krebs, Ringer, Haselite</td>
</tr>
<tr>
<td>LMW</td>
<td>Low molecular weight</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
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<td>MIP-2</td>
<td>Macrophage inflammatory protein-2</td>
</tr>
<tr>
<td>MMP</td>
<td>Metalloproteinase matrix</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>MCD</td>
<td>Methionine choline deficient</td>
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<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>MAMPs</td>
<td>Microbe-associated molecular patterns</td>
</tr>
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<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa-light chain enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer Cell</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural Killer T-lymphocytes</td>
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<tr>
<td>P/S</td>
<td>Penicillin-Streptomycin</td>
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<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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</table>
PI3K Phosphoinositide 3-kinase
PI3K/AKT Phosphoinositide 3-kinase/protein kinase B
PPARα Peroxisome proliferator activated receptor α
PRR Pattern recognition receptor family
PVDF Polyvinylidene fluoride
qPCR Quantitative polymerase chain reaction
Rab5 RAS-associated protein 5
RIPA Radioimmunoprecipitation assay buffer
RNS Reactive nitrogen species
ROS Reactive oxygen species
RT-qPCR Real time quantitative polymerase chain reaction
SDS sodium dodecyl sulphate
SEC Sinusoidal endothelial cell
SNP Single nucleotide polymorphism
SOCS Suppression of cytokine signalling
SREBP Sterol regulatory element-binding protein
STAT3 Signal transducer and activator of transcription-3
TEMED Tetramethylethylenediamine
TGFβ Tumour growth factor β
Th-1 T-helper cell-1
Th-2 T-helper cell-2
TIMP Tissue inhibitors of metalloproteinase
TIR Toll/IL1 Receptor
TLR Toll-like receptor
TNBS 2,4,6-trinitrobenzene sulfonic acid
TNFα Tumour necrosis factor α
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>TRIF</td>
<td>TIR-domain containing adapter inducing IFNβ</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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Chapter 1 Background, Introduction and Literature Review.
1.1 Background to the Thesis. Non-Alcoholic Fatty Liver Disease: An Evolving Worldwide Epidemic.

Non-alcoholic fatty liver disease (NAFLD) is a common disease state in which lipid accumulates in the liver in the absence of excess alcohol consumption [1]. It represents a spectrum of liver disease ranging from simple steatosis through to non-alcoholic steatohepatitis (NASH), with or without fibrosis, cirrhosis and end stage liver failure. Patients with bland steatosis alone rarely develop progressive liver disease where as those with NASH are at risk of developing cirrhosis, liver failure and hepatocellular carcinoma (HCC) [2]. NAFLD represents the hepatic manifestation of the metabolic syndrome, being strongly associated with its components including obesity, hypertension, dyslipidaemia and insulin resistance states such as type II diabetes mellitus. The pathogenesis of NAFLD and NASH is poorly understood, and as the underlying basis for this thesis, will be discussed in detail in later sections. Further insight into the pathogenesis of NAFLD and NASH is required in order to develop rational and improved management strategies, which are currently sub-optimal.

1.1.1 Epidemiology

NAFLD occurs worldwide in both developing and developed countries and in all ethnicities. It is thought to be the most common liver disease in the world. Within developed countries such as the United States, Italy and Israel, population based studies estimate the prevalence of NAFLD as between 20-30% [3, 4]. In developing countries, the prevalence of NAFLD has been reported to be 13-17% in China [5, 6] and 9% in rural India [7]. NAFLD affects both adults and children. In the United States the childhood prevalence of NAFLD rises from 0.7% in 2-4 year old to 17% in teenagers [8].

In particular, the prevalence of NAFLD is increased by the presence of central obesity, impaired glucose tolerance and type 2 diabetes, dyslipidaemia and hypertension. An
Italian study found that the prevalence of NAFLD increased from 16.4% in healthy weight patients with a body mass index (BMI) <25 kg/m$^2$ to 75.8% in obese patients with a BMI >30 kg/m$^2$ [9]. NAFLD prevalence has been observed to increase with increasing levels of fasting blood glucose with a prevalence of 27% in those with normal fasting glucose (<6.1 mmol/l), 43% in those with impaired fasting glucose (110-126 mg/dl) and 62% in those with diabetes (>126 mg/dl) [10]. The prevalence of NASH is less well defined. In healthy adults who had a liver biopsy performed as a work up for living donor liver transplantation, NAFLD was found to be present in 15-52% of subjects with NASH in 3-15% of subjects [11, 12]. An autopsy study demonstrated that the prevalence of steatohepatitis increased from 2.7% amongst healthy weight patients to 18.5% in morbidly obese patients and 12.2% in those with diabetes [13]. In morbidly obese patients undergoing bariatric surgery, benign steatosis has been found in 47% of subjects, NASH in 42% and cirrhosis in 2.9-3.9% [14]. It is likely that the overall prevalence of NASH in Western Countries is between 5-12% [15].

1.1.2 Clinical Features

The vast majority of patients with NAFLD are asymptomatic, often coming to medical attention following incidental abnormalities being detected in serum transaminases or on abdominal imaging [16]. A small group of patients with NASH related cirrhosis present with signs and symptoms of hepatic decompensation and portal hypertension. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are typically mildly elevated and rarely greater than three times the upper level of normal [17, 18]. Abdominal ultrasound, computer tomography and magnetic resonance imaging are all useful imaging modalities to detect moderate to severe (>30%) hepatic steatosis [15]. Unfortunately, none of these imaging modalities are able to differentiate NAFLD from NASH or stage the level of hepatic fibrosis [19].
Liver histology is important for the diagnosis of NAFLD, differentiating between NAFLD and NASH and for grading fibrosis [20]. Hepatocellular triglyceride accumulation is universally present in cases of NAFLD. In cases of NASH, lobular inflammation consisting of mononuclear and polymorphonuclear cell infiltrate is present. NASH may also be associated with hepatocyte ballooning, necrosis and Mallory's hyaline [20]. Fibrosis generally develops in zone three of the hepatic lobule and tends to be laid down in the pericellular and perisinusoidal areas. As disease progresses fibrotic septae develop which go on to form nodules and eventually cirrhosis.

1.1.3 Natural History

NAFLD patients have an increased risk of death in comparison to the general public with one community based cohort of NAFLD subjects having a 34% increase in hazard ratio for overall mortality [21]. The prognosis of subjects with NAFLD is dependent upon the histological sub-type of disease. Subjects with fatty liver alone tend to have a relatively benign disease course and are subsequently at low risk of developing liver related morbidity or mortality [22, 23]. In contrast, NASH is frequently progressive and is known to lead to cirrhosis with complications of HCC, liver failure and liver related death or requirement for liver transplantation. In a Swedish cohort of 71 NASH patients, 10% developed end-stage liver disease over a mean time period of 13.7 years [24]. In addition to the increased liver related morbidity and mortality, subjects with NASH are at increased risk of cardiac morbidity and mortality [24, 25].

1.1.4 Management

The management of NAFLD involves both the treatment of the fatty liver itself, as well as all the associated metabolic risk factors. Unfortunately, studies investigating pharmacological interventions for NAFLD are generally small and poorly designed therefore ongoing well-designed studies are required before definitive recommendations can be made.
Weight loss and exercise are ideal treatments for overweight patients with NAFLD in that both hepatic triglyceride accumulation and metabolic risk factors are targeted. Weight loss though lifestyle modification and bariatric surgery has been shown to improves liver biochemistry and reduce hepatic steatosis [26-30], however no study of lifestyle modification has been able to demonstrate an improvement in liver fibrosis.

Improving hepatic insulin sensitivity through the use of metformin or the thiazolidinediones has demonstrated mixed results with metformin resulting in no consistent improvement in patients with NAFLD [31]. The thiazolidinediones were shown to improve steatosis and inflammation but not fibrosis [31, 32]. Unfortunately thiazolidinediones are poorly tolerated due to the side effects of fluid retention, weight gain and osteoporosis; and furthermore rosiglitazone has been associated with a high cardiovascular risk which limits its use [33]. Anti-oxidants have also demonstrated some promise; in a recent large randomised controlled trial pioglitazone or vitamin E were significantly better than placebo alone at improving hepatic steatosis, inflammation but not fibrosis in individuals with NASH [32]. Vitamin E has been associated with increased risk of haemorrhagic stroke and cardiovascular disease, which may limit its use [34].

1.1.5 Summary

The worldwide increase in obesity prevalence is associated with increased prevalence of all obesity related illnesses including NAFLD. NAFLD and NASH related complications, both hepatic and non-hepatic, present a significant global health problem. The pathogenesis of NAFLD, and in particular NASH is not completely understood and certainly treatments for NASH are sub-optimal. In the following sections I will be exploring the role of adiponectin in the pathogenesis of NAFLD and NASH, and particularly exploring the effect of adiponectin on the Kupffer Cell, an important hepatic cell mediator of NAFLD and NASH. This will contribute to both
furthering our current understanding of the pathogenesis of NAFLD and NASH, which in turn may contribute to designing treatments for this burgeoning health problem.
1.2 Liver Structure, Function and Response to Injury.

1.2.1 Normal Liver Structure and Function.

The liver is the largest internal organ of the human body and is located in the right hypochondrial region of the abdominal cavity directly below the diaphragm. Hepatocytes make up 80% of the total liver volume and perform the major metabolic functions of the liver. Non-parenchymal hepatic cells only occupy 6.5% of the liver volume however comprise 40% of the total hepatic cell number. Non-parenchymal hepatic cells are mostly responsible for regulating hepatocyte function via both cytokine and mitogen signalling.

The functional unit of the liver is the sinusoid; this is composed of an endothelium of fenestrated sinusoidal endothelial cells (SECs), which are separated from a plate of hepatic cells by the space of Disse. Multiple functional sinusoid units are arranged into a liver lobule as illustrated in Figure 1.1. Each lobule is supplied via the peripherally placed hepatic artery and hepatic portal vein. Blood flows through the sinusoids to a central vein thus ensuring all hepatocytes are in direct contact with the blood.

The liver is unique in that the majority of the blood supply is venous, derived from the nutrient and antigen rich portal vein. Nutrients that have been absorbed by the gastrointestinal tract flow directly to the liver where they are extracted and stored by hepatocytes. Hepatocytes also play an important role in multiple regulatory processes including carbohydrate, lipid and protein metabolism, vitamin and mineral storage and synthesis and removal of toxins. The liver is also responsible for the synthesis of multiple proteins, hormones and bile. Non-parenchymal hepatic cells including the Kupffer Cell, Hepatic Stellate Cells (HSC) and SEC as outlined below, also contribute to normal liver structure and function.
1.2.2 Non-Parenchymal Hepatic Cells

1.2.2.1 Hepatic Stellate Cell

The HSC, formally known as the fat-storing cell, Ito cell, lipocyte, perisinusoidal cell or vitamin A-rich cell, is the resident extra-sinusoidal cell within the space of Disse. A quiescent HSC within the healthy liver is laden with Vitamin A droplets. However following a hepatic insult, the HSC becomes activated in doing so, loses its Vitamin A droplets, expresses α Smooth Muscle actin (α-SMA) and synthesises multiple components of the extracellular matrix (ECM). In doing so, the HSC becomes the major fibrogenic cell type within the liver [35]. The role of the HSC in a healthy liver is unclear however the HSC has been demonstrated to activate natural killer (NK) cells by acting as an Antigen Presenting Cell (APC) [36].

HSCs become phenotypically active in response to multiple stimuli following hepatocyte injury and apoptosis, these include both hepatocyte-derived and immune cell, particularly Kupffer Cell-derived cytokines, mitogens and growth factors. Guo and Friedman [37] have described HSC activation as two phases; initiation which renders the HSC responsive to cytokines and perpetuation in which the HSC responds to cytokines by enhancing scar tissue formation. HSC perpetuation involves multiple responses to cytokines that occur in seven distinct though often simultaneous pathways. 1) Proliferation following exposure to platelet-derived growth factor (PDGF) and other mitogens. 2) Chemotaxis and migration in response to PDGF, tumour growth factor β1 (TGFβ1) and other cytokines. 3) Fibrogenesis driven by TGFβ1. 4) Release of pro-inflammatory, fibrogenic and mitogenic cytokines that stimulate the accumulation of inflammatory cells and ECM production. 5) Increased contractility via endothelin-1 (ET-1) which in turn constricts sinusoids and reduces blood flow. 6) Break down of the normal liver matrix that interrupts normal liver function. 7) Loss of vitamin A droplets;
Figure 1.1 Hepatic Lobule Structure.

Blood enters the lobules through the portal vein and hepatic artery branches then flows through the sinusoids to the central vein where blood exits the hepatic lobule. The hepatocytes remain in direct contact with blood; in doing so extracting nutrients, metabolising and excreting waste products and synthesising and releasing proteins.

Source: http://www.as.miami.edu/chemistry/2086/Chapter%2024-newPART2.htm
the function of which is unclear. Additionally it has been demonstrated that hyperinsulinaemia can directly activate HSCs [38].

1.2.2.2 Sinusoidal Endothelial Cell

SECs make up the wall of the hepatic sinusoid and as such permit diffusion of substances between the blood and hepatocyte via fenestrations, a type of transcellular pore, and carry out a filtration function. They also synthesise ECM components type IV collagen and laminin and a splice form of cellular fibronectin, all of which contribute to capillarization of the sinusoid [39]. ET-1, vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF), all of which contribute to HSC activation, are secreted by the SEC. Recent evidence also suggests that SECs have an important role in the innate immune system by acting as a non-professional APC, which in turn contributes to cytokine release and hepatic inflammatory response [40].

1.2.2.3 Kupffer Cell

Kupffer Cells are the resident liver macrophages that reside within the hepatic sinusoid. Kupffer Cells account for 80-90% of the total body fixed tissue macrophages and are responsible for the phagocytosis of insoluble wastes, thus forming an important part of the innate immune system [41]. As the liver receives 80% of its blood supply directly from the gut via the portal vein, the liver is continually exposed to high levels of bacterial products, environmental toxins and food antigens and therefore acts as a barrier between the gut and the rest of the body. Kupffer Cells reside within the portal sinusoid where they encounter blood-borne microbes and toxins, which they phagocytose and remove from circulation. Kupffer Cells are also responsible for maintaining liver homeostasis, tissue remodelling following liver injury and regulating metabolic functions [42]. As the major focus of this thesis, the important functions of the Kupffer Cell will be expanded in further sections.
1.2.3 Hepatic Fibrosis; Response and Repair Following Injury

Hepatic fibrosis is a wound healing response following either an acute or chronic liver insult [37]. Hepatic fibrosis can occur following multiple insults including alcoholic or non-alcoholic induced steatosis, chronic viral hepatitis, autoimmune and metabolic disorders. Hepatic fibrosis results when the rate of ECM deposition exceeds the rate of ECM degradation. Following acute injury, these changes are often transient and over time normal hepatic architecture is returned. Cirrhosis occurs in the setting of ongoing liver insult and represents end-stage hepatic fibrosis whereby the normal hepatic architecture is replaced with bands of fibrotic scar that encircle hepatocytes, thus giving rise to cirrhotic nodules.

As discussed above, the hepatic sinusoid is composed of an endothelium of fenestrated SECs, which are separated from a plate of hepatic cells by the space of Disse. A healthy liver contains low-density type IV collagen basement membrane within the space of Disse. As demonstrated in figure 1.2, in cirrhosis this is replaced by fibril containing collagens in particular collagen types I and III as well as a total increase in type IV collagen, laminin, proteoglycans, fibronectin and hyaluronic acid [37]. Further changes that develop as a result of ECM accumulation include decreased SEC fenestrae, the development of a continuous basal SEC barrier and the development of hepatic shunts between the portal vein and artery and hepatic vein [43-45]. These collective changes contribute to heightened hypoxia, which can further drive the fibrotic process and portal hypertension.

1.2.3.1 Fibrogenic Cytokines

As chronic inflammation is the usual prequel to hepatic fibrosis, it is expected that inflammatory cytokines are key modulators of the fibrotic process. Multiple inflammatory cell types secrete cytokines following cell injury including Kupffer Cells, hepatocytes, HSCs, NK cells, lymphocytes and dendritic cells. Key mediators of
Figure 1.2 The Liver Sinusoid in Health and Hepatic fibrosis.

A. Normal liver contains a SEC lined sinus which is separated from hepatocytes by the space of Disse. The normal space of Disse contains a collagen IV, low density basement membrane which enables normal metabolic exchange as well as quiescent Vitamin A rich HSCs.

B. In disease, activated HSC secrete excessive ECM, which results in the loss of the normal hepatic basement membrane, decreased fenestrations, decreased hepatic exchange, hypoxia and the development of portal hypertension.

Source: [46]
hepatic fibrosis include TGF-β1, PDGF and connective tissue growth factor (CTGF) are focussed on in this thesis and will be discussed in more detail. These mediators trigger a myriad of signal transduction pathways in both an autocrine and paracrine manner that contribute to HSC activation and the initiation and perpetuation of the fibrotic response as illustrated in figure 1.3.

1.2.3.1.1 TGF-β1

TGF-β1 is up regulated in the fibrotic livers of rats and is a potent trigger of HSC activation [47]. TGFβ1 blockage decreases hepatic fibrosis in animal models however this has not been translated to human studies [48]. More recently, TGFβ signalling has been found to be inhibited by BAMBI, a transmembrane receptor that lacks an intracellular tyrosine kinase domain and acts as a TGFβ pseudoreceptor [49]. It has been demonstrated that BAMBI is down regulated following lipopolysaccharide (LPS)-induced toll-like receptor (TLR)-4 signalling thus further sensitizing HSCs to further TGFβ1 [50]. In addition, adiponectin has been shown to increase BAMBI expression in primary human hepatocytes [51].

1.2.3.1.2 PDGF

PDGF is a dimeric protein composed of two polypeptide chains that combine to form PDGF-A and PDGF-B [52]. PDGF-B signals through the tyrosine kinase receptor PDGFRβ and is the most potent mitogen for HSCs [53]. All PDGF isoforms and their receptors have been demonstrated to be up-regulated in liver fibrosis and in fact, PDGF levels actually correlate with the fibrosis grade [54, 55]. Fibrosis is prevented when PDGF signalling is blocked by a dominant-negative soluble receptor in rodents [56].

1.2.3.1.4 CTGF

CTGF has been implicated in the pathogenesis of hepatic fibrosis in humans as a downstream mediator of the TGFβ pathway [57, 58]. Patients with NASH have been
Figure 1.3. Cellular Interactions in the Pathogenesis of Hepatic Fibrosis.

Hepatic fibrosis involves multiple interactions between hepatic and immunological cells, cytokines and cell-derived products in a paracrine and autocrine manner. Central to this process is inflammation with resultant ECM secretion from the HSC.

IGF-1, insulin like growth factor; MMPs, matrix metalloproteinases; RNS, reactive nitrogen species; ROS, reactive oxygen species; MCP-1, monocyte chemotactic protein-1; MIP-2, macrophage inflammatory protein-2; TIMPs, tissue inhibitors of metalloproteinases; Source: [37]
shown to have overexpression of CTGF in their liver biopsy samples and in further studies glucose and insulin have both been demonstrated to increase CTGF mRNA in cultured HSCs [59]. Thus CTGF is likely to play an important role in the pathogenesis of NASH related fibrosis.

1.2.3.2 Management of hepatic fibrosis

As fibrosis is a dynamic process of ECM deposition and resorption, all stages of fibrosis are potentially reversible. The ideal treatment of fibrosis is to eliminate the underlying disease or fibrotic trigger [60]. However as this is not always possible, a direct anti-fibrotic agent would be desirable. Several potential targets for anti-fibrotic therapy have been identified including inhibition of collagen synthesis, matrix deposition interruption, stimulation of matrix degradation, blocking HSC activation and induction of HSC death.

Several anti-fibrotic agents have been studied in animal models, however very few of these agents have been transitioned into human studies. Agents that have been examined in human studies include colchicine, angiotensin receptor blockers and pirfenidone. [60-69]. To date, these studies have been small and no conclusive data as to their effectiveness has been established. An ideal anti-fibrotic agent would have combined effects of being able to "turn off" the excess production of ECM as well as have remodelling qualities thus allowing the liver to reabsorb excess ECM and return to pre-insult architecture, ongoing research into so called "global" treatments including adiponectin and interleukin 10 (IL10), as included in this thesis, is ongoing.
1.3 Pathogenesis of Non-Alcoholic Fatty Liver Disease.

The classical model of NAFLD and NASH pathogenesis has been that of the "two hit" hypothesis with the first hit being the accumulation of hepatic lipid and the second hit being hepatocyte death and scar tissue accumulation [70]. Recent hypotheses reflect a paradigm shift with the "multi-parallel hits" hypothesis, in which NAFLD and NASH may reflect different disease entities, gaining favour [71]. Regardless of the exact underlying disease mechanism, hepatic lipid accumulation remains the cornerstone pathogenic event in NAFLD and NASH.

1.3.1 Obesity and Insulin Resistance Drives Hepatic Lipid Accumulation

Hepatic steatosis results from an imbalance in lipid flux resulting in increased lipid influx from adipose tissue via lipolysis, a high fat diet, increased hepatic de novo lipid synthesis and decreased hepatic lipid export by reduced very low density lipoprotein (VLDL) secretion [71]. An insulin resistant state, an almost universal finding in NAFLD and NASH, is a major driver of hepatic lipid accumulation [72, 73]. Lipogenic transcription factors including sterol regulatory element-binding protein (SREBP) 1c and carbohydrate responsive element binding protein (ChREBP) are up regulated in hyperinsulinaemic, hyperglycaemic states, thus promoting de novo hepatic lipogenesis [74]. Furthermore, visceral adipose lipolysis increases in insulin resistant states which results in a net influx of free fatty acids (FFAs) into the liver [75].

1.3.2 Oxidative Stress and Cell Injury.

Multiple connecting and diverging pathways contribute to the progression of NAFLD to NASH. In particular, an excess of hepatic FFAs increases mitochondrial and peroxisomal β-oxidation, which leads to increased reactive oxygen species (ROS) generation [76]. High concentrations of hepatic ROS directly increase lipid peroxidation and inflammation which leads to further organelle damage [77]. Furthermore excessive ROS oxidises mitochondrial DNA, proteins and lipids which
stimulates tumour necrosis factor α (TNFα) synthesis and release [78]. TNFα acts locally to activate Kupffer Cells, thus potentiating further ROS and adipokine release.

1.3.3 Visceral Adipose Tissue Contributes to Hepatic Necroinflammation and Fibrosis.

Visceral obesity is a key determinate which increases the likelihood of hepatic inflammation and fibrosis [79]. Rather than being a storage compartment for excess lipid alone, adipose tissue is a metabolically active organ with multiple endocrine and immune functions that exerts its effect on multiple distant organs including the liver. In obese individuals, adipose tissue becomes more vascular and infiltrated with macrophages. [80]. The adipose tissue, macrophages interaction (part of the innate immune system) contributes towards the low grade inflammatory state and insulin resistance which is frequently observed in patients with NASH [81]. This inflammatory state is characterised by elevated levels of circulatory inflammatory markers, cytokines and adipokines, and adipokine-secretion dysregulation [82]. The ensuing adipokines, including TNFα, interleukin-6 (IL6), adiponectin, leptin, and resistin, may act locally in an autocrine or paracrine manner or exert their effects on distant organs such as the liver and pancreas and the cardiovascular system.

1.3.4 Adipokines

Adipokines are important homeostatic proteins generated predominantly by adipose tissue, they include TNFα, IL-6, leptin and adiponectin. There are over 100 described adipokines; with adiponectin the major focus of this thesis to be described in detail in the following sections. In lean individuals, adipose tissue preferentially secretes anti-inflammatory cytokines such as adiponectin, IL10, interleukin-4 (IL4), interleukin-13 (IL13) and apelin, where as adipose from obese individuals secretes inflammatory adipokines including TNFα, IL6, leptin, visfatin, resistin, angiotensin II and plasminogen activator inhibitor 1 (PAI-1) [83]. All adipokines flow directly to the liver
via the portal vein and have all been implicated in the pathogenesis of NASH. In particular, TNFα, IL6, leptin and adiponectin have been demonstrated to play a major role in the pathogenesis of NAFLD and are briefly discussed below, a detailed description of the role of adiponectin and the closely intertwined interleukin, IL10 will be included later. As adipokines have multiple important metabolic and inflammatory functions, therapeutic manipulation of an individual’s adipokine profile is an exciting potential future therapeutic modality for NAFLD and NASH.

1.3.4.1 TNFα

TNFα is associated with multiple physiological and pathological conditions in every human organ. TNFα acts in either a direct or indirect manner to induce cytotoxicity, cell differentiation and growth; as well as possessing multiple immune-modulation and pro-inflammatory effects [84]. Elevated levels of TNFα have been observed in fulminant hepatic failure, viral hepatitis, alcoholic liver disease and biliary obstruction and have been strongly associated with the development of NASH [85]. TNFα is also secreted by adipocytes and is well recognised to be elevated in obesity and NAFLD [86]. TNFα is also secreted by macrophages including the Kupffer Cell in response to tissue injury [87] and induces IL6 and TGFβ1 synthesis and release in both a paracrine and autocrine manner, and is also implicated in hepatocyte apoptosis [84, 85, 88, 89].

In rodent models of carbon tetrachloride (CCL₄)-induced fibrosis, elevated levels of TNFα with associated activation of Kupffer Cells have been observed [90]. Further rodent models have found that interruption of TNFα signalling by a soluble TNFα receptor prevents CCL₄-induced fibrosis [91] and TNFα receptor knock out mice fail to develop CCL₄-induced fibrosis [92]. More recently, infliximab an anti-TNFα antibody has been shown to decrease CCL₄-induced necro-inflammation and fibrosis in a rodent model [93].
1.3.4.1 Lipopolysaccharide stimulates TNFα synthesis and release.

Intestinal bacterial overgrowth is associated with NAFLD and NASH [94], with increase prevalence of intestinal bacterial overgrowth present in those with NASH [95] and increased endotoxin levels found in those with NAFLD [96]. In addition, elevated levels of TNFα have been shown to be associated with increased fibrosis in NAFLD patients [97]. Together, these findings suggest a pathological role of LPS-induced TNFα in the development of NAFLD and NASH.

In healthy individuals minimal amounts of LPS enters the enteric circulation where it is rapidly removed by hepatic Kupffer Cells. However in individuals with NAFLD, Kupffer Cells have reduced capacity to remove LPS, which can therefore lead to increased serum LPS and perpetuate further liver damage [96, 98]. Large doses of LPS have been shown to cause liver injury with Kupffer Cells being required to cause liver damage [99-102]. Kupffer Cells are thought to be the major hepatic source of TNFα following LPS administration; when Kupffer Cells are inactivated, mice and rats are protected from liver injury [103].

LPS induces rapid Kupffer Cell activation, [104-106], following interaction with either the TLR-4, the main LPS ligand and a member of the pattern recognition receptors (PRR) family, or indirectly via complement activation [107]. TLR-4-mediated signals transduce through a either myeloid differentiation primary response gene 88 (MyD88) dependent or independent pathway, and in doing so, signal through nuclear factor kappa-light-chain enhancer of activated B cells (NFκB), mitogen-activated protein kinases (MAPK) and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) resulting in increased TNFα release, inflammation and insulin resistance [41, 108-111].

1.3.4.2 IL6

IL6 has a complex role in liver disease with both hepato-protective and hepatotoxic associations described [112]. IL6 is closely associated with insulin resistance. Obese
mice treated with IL6 antibodies demonstrated improved insulin resistance [113] and in obese patients undergoing bariatric surgery, a decrease in serum IL6 levels was found to be associated with improved insulin resistance [114]. IL6 knockout mice develop less diet-induced NASH than their wild-type counterparts [115]. Human subjects with NAFLD have been observed to have higher serum IL6 levels [109, 116, 117] and furthermore IL6 levels have been shown to correlate with severity of NASH [79].

1.3.4.3 Leptin

The adipokine leptin has wide-ranging effects and is involved in energy homeostasis, immune function and reproduction. Obese individuals exhibit leptin resistance and thus leptin levels increase with adiposity and have been implicated in the development of NAFLD and NASH [118]. Leptin is required for hepatic fibrogenesis in animal models [119] and leptin deficient ob/ob mice develop a phenotype of obesity, insulin resistance and fatty liver [120]. However, in human studies, the association of leptin with NAFLD and NASH independent of obesity and insulin resistance is less clear with no correlation observed between leptin levels and fibrosis stage in 88 adults with NAFLD [121].

1.3.5 Visceral Adipose Tissue and Insulin Resistance

In addition to the direct secretion of adipokines into the systemic circulation, excess visceral adipose tissue can trigger the further development of insulin resistance. Visceral adipose has been found to be more cellular than subcutaneous adipose and has increased insulin resistance and lipolysis rates thus leading to increased hepatic free fatty acids [122]. In the same manner as hepatic fat, FFA oxidation within visceral fat generates oxygen radicals with subsequent lipid peroxidation, cytokine induction and mitochondrial dysfunction [123]. In addition, specific adipose tissue inflammatory pathways, particularly the c-Jun NH2-terminal kinase (JNK)-1 pathway which is activated by FFAs, cytokines including TNFα, endoplasmic reticulum stress and a high fat diet play a major role in the pathogenesis of NAFLD and insulin resistance [82,
Similarly, obesity and high fat diets leading to hepatic steatosis and induction of NFκB, may initiate inflammatory cytokine cascades such as TNFα, IL6 and suppression of cytokine signalling (SOCS), which are insulin-antagonising molecules [109, 125, 126]. Therefore, the induction of NAFLD may potentially exacerbate insulin resistance, thereby creating a self-perpetuating cycle of insulin resistance leading to NASH. This ensuing hyperinsulinaemia and hyperglycaemia may act directly on HSCs to stimulate fibrosis [59, 127].

1.3.6 Summary

The pathogenesis of NAFLD and NASH involves a complex array of interactions between the liver, adipose tissue and the innate immune system. NAFLD and NASH is unique in that inflammatory events in the remotely placed adipose tissue results in liver damage. To enable this significant cross-talk between the liver, adipose tissue and cells of the innate immune system, a circulating inflammatory milieu of cytokines, adipokines and ROS develops. Understanding the interactions between cytokines and adipokines and parenchymal and non-parenchymal hepatic cells, adipocytes and monocytes and tissue macrophage will assist in better understanding the underlying causes of NAFLD/NASH and enable better therapy in the future. In view of this, this thesis will explore the interactions of the adipokine adiponectin upon the resident liver macrophage, the Kupffer Cell in detail.
1.4 The Kupffer Cell

As previously mentioned, Kupffer Cells are the resident liver macrophages that reside within the hepatic sinusoid. Kupffer Cells are capable of isolating and clearing both exogenous and host derived-endogenous material that is recognised as foreign and potentially harmful [128]. As described by Baffy; in response to toxins Kupffer Cells become activated, enabling them to carry out multiple processes including 1) release biological mediators such as cytokines, chemokines, reactive oxygen species and nitric oxide which enables interaction with other liver cells; 2) recruit non-resident cells including neutrophils, natural killer cells and blood-derived monocytes to the liver; 3) engulf, ingest and eliminate particles such a microbiota, apoptotic cells and cell debris; and 4) process and present antigens to T-cells [129].

Kupffer Cell activation results in a phenotypic shift in the Kupffer Cell. Kupffer Cells respond to encountered stimuli by undergoing polarization to either an M1 (classical) or M2 (alternative) activated phenotype [42]. The M1 phenotype is characterised by high expression of pro-inflammatory cytokines, reactive nitrogen and oxygen species, promotion of a T-helper cell-1 (Th-1) response and associated anti-microbial and anti-tumourcidal activity. The M2 Kupffer Cell phenotype has a tendency towards parasite control, high phagocytic activity, hepatic remodelling, tumour progression and immunoregulation [42]. Toll-like receptor ligands, interferon-γ (IFNγ), IL4 and IL13 polarize Kupffer Cells towards an M1 phenotype where as IL10, glucocorticoid hormones, apoptotic cells and immune complexes polarize Kupffer Cells towards an M2 phenotype [130].

Kupffer Cells generate various products including cytokines, prostanoids, nitric oxide and ROS that regulate not only the phenotype of the Kupffer Cell that produce them, but neighbouring cells including hepatocytes, HSC, SECs and other non-resident immune cells [131, 132]. This activation modulates both acute and chronic liver injury,
including hepatic fibrosis and cirrhosis. In support of this, soluble CD163, a marker of Kupffer Cell activation, was found to be three-fold higher in subjects with cirrhosis compared to normal controls [133].

1.4.1 Kupffer Cells form an Integral Component of the Innate Immune System.

The innate immune system is the first line of attack against infection and consists of lymphocytic cells, phagocytic cells, physical barriers and humoral factors. The portal blood flow from the intestines to the liver is rich in bacterial products, environmental toxins and food antigens, and as such, the liver acts as a barrier designed to protect the body from these potential harmful particles. In doing so, the liver is an important component of the innate immune system that is capable of protecting the liver and body from harm, whilst preventing a potentially damaging immune response.

The liver contains large number of innate immune cells including the phagocytic cells (Kupffer Cells and SECs) and lymphocytic cells (NK Cells and Natural Killer T-lymphocytes (NK T)). In addition the liver biosynthesises multiple innate proteins including acute-phase proteins, complement factors and secreted PRR [134]. All parenchymal and non-parenchymal hepatic cells contribute towards innate immunity.

Membrane bound PRRs including the TLRs are a family of proteins that recognise non-eukaryocyte pathogen-associated molecular patterns (PAMPs). TLRs recognise different PAMPs and in doing so, activates specific antimicrobial responses [135]. All liver cells express TLRs [136] which contribute to liver injury and repair. Although the role of hepatocyte TLRs in protecting against pathogens has not been fully elucidated [41], Kupffer Cell membrane-bound TLRs have been shown to play important immuno- and hepato- protective roles as well as contribute towards hepatic fibrosis.

1.4.2 Inflammatory Pathways and Innate Immunity

Kupffer Cells and Macrophages mount an inflammatory response following PRR activation. Despite variations in proximal signalling pathways, activation of both the
MAPK and NFκB pathway is the eventual outcome of this receptor binding [137]. Although fourteen MAPKs have been described in mammalian cells, extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 and JNK are particularly important in macrophages. Multiple PAMPs as well as TNFα and other inflammatory cytokines, osmotic stress, hormones and heat shock have the ability to trigger MAPK activation [138]. As a result of the MAPK pathway activation multiple pro-inflammatory cytokines including TNFα, IL6, Interleukin 1 (IL1) and interleukin 12 (IL12) are synthesised and released, and cell proliferation, differentiation and apoptosis may eventuate [139].

Each MAPK pathway appears to have specific cellular functions and physiological response. ERK1/2 regulates cytokine transcription and release in macrophages. In particular ERK1/2 has been associated with up regulation of TNFα, interleukin-1β (IL1β) and IL10 and down regulation of IL12, interferon-β (IFNβ) and inducible nitric oxide synthase (iNOS) [137]. P38 activity is associated with autoimmune and inflammatory illness including rheumatoid arthritis [140]. As such, P38 activation and perpetuation appears to be closely associated with TNFα and IL10 [137]. JNK1 and 2 have a role in the regulation of insulin function and the development of insulin resistance, with myeloid specific JNK knockout mice failing to develop insulin resistance in response to a high fat diet [141]. JNK has also been associated with the development of the M1 inflammatory phenotype in macrophages [142].

NFκB is the major pathway responsible TNFα generation and plays a major role in inflammation and oncogenesis. A full discussion of the NFκB pathway is beyond the realms of this review, however in brief, NFκB is activated following phosphorylation and degradation of inhibitor of κB (IκB), NFκB subsequently translocates to the cell nucleus where it binds to DNA response elements and promotes cytokine mRNA
transcription [143]. Multiple activators of NFκB have been identified including ROS, TNFα, LPS and ionising radiation [144-146]

1.4.3 Kupffer Cells Promote Immune Tolerance

The liver is a unique organ in that it is immune-privileged; it is able to induce peripheral immune tolerance in preference to induction of immunity [147, 148]. Liver tolerance contributes towards the survival of human leukocyte antigen (HLA) mismatched allogeneic liver transplants in the absence of adequate immune suppression [149], however may also contribute to chronic hepatic intracellular infections including Hepatitis B virus, Hepatitis C virus and Plasmodium infections [148].

The mechanism by which hepatic tolerance develops is not well understood however evidence for multiple cell types contributing towards tolerance is accumulating. In particular, the major hepatic lymphocyte population has a different make-up to systemic lymphocyte populations. In the liver CD8\(^+\) T-cells, NK cells and NKT cells are the predominant lymphocytes, and with their propensity to secrete IL10 may contribute towards hepatic tolerance [150]. Further studies of antigen presenting dendritic cells suggests that co-culture of human monocytes with rat hepatic SECs promotes a T-helper cell-2 (Th-2) response again, likely to be driven by IL10 [151]. Additionally, hepatic SECs act as semi-professional antigen presenting cells which are able to activate T-cells in response to either an orally or parentally administered antigen and elicit a tolerant CD8\(^+\) T-cell response [152].

The role of Kupffer Cells in hepatic tolerance has been demonstrated by their reaction to LPS. Isolated Kupffer Cells have been demonstrated to synthesise IL10 in response to LPS, [153] thus supporting a model of tolerance to orally ingested antigens. In further studies small doses of LPS have been shown to activate Kupffer Cells without any associated liver injury, suggesting that either the levels or duration of cytokine release may be below the required threshold for liver injury [154]. Animal studies have
highlighted the important role that Kupffer Cells play in the induction of tolerance to allografts [155, 156] and suggest that Kupffer Cells fail to elicit T-cell activation and in fact inhibit T-cell responses induced by other APCs [157].

1.4.4 Kupffer Cells and NAFLD

Kupffer Cell activation is a vital role in the pathogenesis of NASH with emerging animal and human studies expanding knowledge into this area. A high fat diet has been shown to be associated with hepatic steatosis and inflammatory activation of Kupffer Cell in mice [158, 159]. In a mouse model of methionine/choline deficient (MCD) diet induced NAFLD; Kupffer Cell depletion prevented NASH [160]. In a similar mouse study of high fat diet-induced hepatic steatosis, insulin resistance was associated with Kupffer Cell activation, moreover, when Kupffer Cells were depleted; an improvement in hepatic insulin resistance was demonstrated [161]. Further studies have demonstrated that the TNFα signalling pathway NFκB within hepatic myeloid cells contributes to hepatic insulin resistance in animal models of fatty liver disease [162]. TNFα signalling in rodent models of fatty liver disease has also been demonstrated to cause Kupffer Cell dysfunction thereby increasing hepatocyte sensitivity to LPS resulting in hepatic inflammation and fibrosis [163, 164].

Kupffer Cell polarisation into either an M1 or M2 phenotype has now been shown to be an important determinant of the development of liver injury in both alcohol-associated and non-associated fatty liver disease [165]. M1 macrophages have been demonstrated to be important in the pathogenesis of multiple chronic inflammatory diseases including atherosclerosis [166] and insulin resistance [167], both of which are both closely associated with NAFLD. In morbidly obese patients undergoing bariatric surgery, those with less hepatic steatosis had an increased expression of the M2 markers CD206 and CD163 as compared to those with higher levels of steatosis [168]. The authors then went on to demonstrated that mice fed a high fat diet, developed hepatocyte steatosis
and apoptosis with associated increased M1 polarisation; treatment with resveratrol improved steatosis and switched the Kupffer Cell polarisation towards M2 [168].

Further *in vitro* studies of these isolated M2 Kupffer Cell found that they were capable of inducing M1 Kupffer Cell apoptosis IL10-induced arginase activation [168]. Increased CD68-positive Kupffer Cells correlates with more severe NAFLD in human liver biopsy samples [169] with a perivenular distribution of large ED-2 Kupffer Cells noted in NASH as compared to diffuse distribution in NAFLD [170]. It has been proposed that impaired phagocytic mechanism in Kupffer Cells results in decreased clearance of LPS and other hepatic toxins, which may accelerate liver injury [129]. Supporting this hypothesis, super-paramagnetic iron oxide magnetic resonance imaging studies have demonstrated impaired Kupffer Cell phagocytosis in NAFLD patients [171].

Alcohol also induces fatty liver disease in humans with many pro-inflammatory cytokines produced by Kupffer Cells being responsible for the onset of alcoholic liver disease [172, 173]. Elevated levels of TNFα, IL1β and IL6 have been observed in human patients and animal models of alcoholic liver injury [174, 175]. Depletion of Kupffer Cells prevents alcohol induced liver injury and suppresses alcohol-induced microvesicular and macrovesicular steatosis in an animal model [176, 177]. The plasma levels of IL10 are decreased by alcohol and IL10 knockout mice have more susceptibility to alcohol induced liver injury [178].
1.5 Adiponectin

Adiponectin, also known as gelatin-binding protein-28 (GBP28), AdipoQ, adipocyte complement-related protein (ARCP30) or apM1, is present in human plasma at levels of 1.9-17 µg/ml, one of the most abundant plasma hormones in humans [179]. Despite original reports that adiponectin is a protein exclusively secreted from adipose tissue [180], adiponectin has also been found to be synthesised and secreted by mononucleocytes and placental tissue. Adiponectin has multiple anti-diabetic, anti-atherosclerotic and anti-inflammatory functions and as such plays a major role in the pathogenesis of the metabolic syndrome and hepatic insulin sensitivity. Despite adiponectin being secreted by adipose tissue, adipose is inversely related to the degree of obesity or overweight in humans and animals [179] and is decreased in individuals with insulin resistance [181] and type 2 Diabetes mellitus. [182], cardiovascular disease [182, 183], hypertension [184] and metabolic syndrome [185]. Weight loss has been demonstrated to lead to both a significant increase in plasma adiponectin levels with an associated increase in insulin sensitivity [186].

1.5.1 Adiponectin Structure

Adiponectin structure is highly conserved amongst mammalian species. Human adiponectin is a 244 amino acid peptide that is made up of an N-terminal variable region, a collagenous domain that shares substantial homology with complement factor sub unit c1q and a C-terminal globular domain as illustrated in figure 1.4. The collagenous domain is made up of 22 G-X-Y or G-X-X collagen repeats, which are involved in forming a collagen triple helix. The globular domain three-dimensional structure shows marked similarity to TNFα despite the two proteins sequences being unrelated [187].
Human adiponectin consists of an N-terminal domain, collagenous domain and a C-terminal globular domain. A cysteine residue is found within the variable N-terminal domain; this forms a disulphide bond allowing adiponectin multimer formation. The collagenous domain contains conserved proline, hydroxylated proline and hydroxylated and/or glycosylated lysine residues which have a role in adiponectin multimerisation and function. The globular domain shares homology with C1q.

Source [188]
As shown in figure 1.5, adiponectin circulates as multimers formed by interactions between the collagenous domains [189]. Initially adiponectin oligomerize to form a trimer or low molecular weight (LMW) adiponectin. These trimers are capable of combining to form hexamers (6 adiponectin monomers) and high molecular weight (HMW) adiponectin that may consist of 12, 18 or 24 individual adiponectin molecules.

Adiponectin isomers have variable biological activity; with HMW adiponectin been shown to have enhanced metabolic effects. Recent studies have demonstrated that insulin sensitivity and the metabolic syndrome correlates more closely to HMW adiponectin than total adiponectin alone [190, 191]. This is despite conflicting reports however, of HMW adiponectin activating NFκB with resultant upregulated IL6 production [192]. Trimeric adiponectin is thought to mediate inflammatory response by signalling through adenosine monophosphate activated protein kinase (AMPK) with resultant increased IL10 and decreased IL6 generation [193, 194]. Adiponectin gene mutations that result in impaired adiponectin multimer formation are associated with in increased risk of type 2 diabetes [195].

In addition to LMW and HMW forms of adiponectin, adiponectin may also exist as a putative proteolytic cleavage fragment consisting of the globular c-terminal [196]. The globular fragment is generated by proteolytic cleavage by leukocyte elastase, which is secreted from activated monocytes or neutrophils [197]. The globular fragment circulates at low concentrations and plays an important role in in energy homeostasis by increasing muscle tissue FFA oxidation [196].
Figure 1.5. Adiponectin Multimer Formation

Separation of adiponectin multimers into LMW and HMW fractions by centrifugation on a 5-20% sucrose density gradient. The basic circulating structure of adiponectin is a trimer. Trimers can oligomerise with other adiponectin trimers via an N-terminal cysteine residue to form hexamers and HMW adiponectin. Fractions 2 to 7 are LMW adiponectin where as HMW adiponectin are within fractions 8-11.

Source [188]
1.5.2 Adiponectin Receptors and Signaling

Adiponectin signals through 3 surface receptors; AdipoR1 and AdipoR2 [198] and T-cadherin [199]. AdipoR1 is abundantly expressed on skeletal muscle where as AdipoR2 is predominantly found in the liver. AdipoR1 has a high affinity for the globular fraction of adiponectin and low affinity for standard full-length adiponectin, where as AdipoR2 has an intermediate affinity for both forms of adiponectin [198]. An adapter protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif (APPL1) acts as a downstream target for AdipoR1 and AdipoR2 in mammalian cells [200]. T-cadherin is present on endothelial cells of large blood vessels and myofibroblasts; it is weakly expressed on SECs but not expressed by hepatocytes [201]. T-cadherin mRNA is absent on RAW264.7 cells [192] and is absent on vascular monocytes, macrophages and foam cells [202]. T-cadherin only binds HMW adiponectin [199]. Currently the functional role of the T-cadherin is poorly understood, though recently a single nucleotide polymorphism rs12051272 in the CDH13 gene encoding T-cadherin has been shown to affect adiponectin plasma levels [203].

Recent insights into post-adiponectin receptor signalling mechanisms have contributed towards increased knowledge of the multiple homeostatic mechanisms that adiponectin regulate as shown in figure 1.6. APPL1 is stimulated by adiponectin-adiponectin receptor binding, and has been shown to mediate the downstream effects of adiponectin with AAPL1 over expression and inhibition resulting in enhanced and down regulated effects of adiponectin respectively. AAPL1 has an additional role in facilitating the interaction between adiponectin and insulin signalling [200].

The insulin sensitising effects of adiponectin in liver, skeletal muscle and adipose tissue come about through activation of AMPK and peroxisome proliferator activated receptor α (PPARα) [197, 204, 205]. Activation of AMPK by adiponectin leads to phosphorylation and inhibition of acetyl coenzyme-A carboxylase (ACC) in cultured
Adiponectin binds to its receptors AdipoR1, AdipoR2 and T-cadherin resulting in post-receptor cell signalling. T-cadherin downstream signalling is poorly understood. AdipoR1 and 2 interact with AAPL1 which binds to the N-terminal of the receptors. Major downstream targets include the AMPK, Glut 4 and PPARα pathways; all of which mediate important metabolic and homeostatic pathways. p38MAPK (p38 mitogen activated protein kinase), Rab5 (RAS-associated protein 5), aP2 (adipocyte protein 2), LPL (lipoprotein lipase).

Source [206]
hepatocytes [204]. Globular adiponectin has been demonstrated to activate APMK in skeletal muscle, again resulting in ACC inhibition [205]. ACC inhibition stimulates fatty acid oxidation by suppressing acetyl-CoA to malonyl-CoA conversion [207]. Evidence of enhanced PPARα signalling was demonstrated when C2C12 myocytes were cultured with adiponectin, this resulted in both increased PPARα ligand activity and fatty acid oxidation [208]. When obese mice are supplemented with adiponectin, they have increased hepatic expression of PPARα and associated enhanced fatty acid combustion, energy consumption and decreased hepatic and skeletal muscle triglyceride [209].

1.5.3 Adiponectin and Human Disease

1.5.3.1 Type 2 Diabetes Mellitus

Adiponectin is intimately associated with insulin resistance and as such, adiponectin levels are closely related to type 2 diabetes mellitus. In a longitudinal study of Pima Indians, those with high plasma adiponectin had a lower risk of developing type 2 diabetes when compared to those individuals with low adiponectin levels [210]. This negative correlation between adiponectin concentration and development of type 2 diabetes has been confirmed in multiple study populations including Indian Asians, Europeans and Koreans [211-213]. Although low adiponectin levels are also associated with obesity [179], itself a risk factor for diabetes, the inverse relationship between adiponectin and diabetes appears to be somewhat independent of obesity.

1.5.3.2 Cardiovascular Disease

Cardiovascular disease and in particular coronary artery disease, once again highly prevalent in obese individuals, is characterised by low adiponectin levels [214]. Hypoadiponectinaemia has also been shown to be a predictor of coronary artery disease in men [183].
1.5.3.3 Cancer

Adiponectin levels are inversely related to the rates of several different cancers including breast, endometrial, prostate, gastric, liver, pancreatic, haematological and colon cancers [215]. Male patients with colorectal cancer have low serum concentrations of adiponectin [216]; furthermore low adiponectin is associated with larger and higher numbers of colonic adenomas [217].

1.5.3.4 Adiponectin and Non-NAFLD Liver Disease

Adiponectin as previously mentioned plays a major role in the pathogenesis of NAFLD and NASH. Knowledge surrounding the role of adiponectin in non-NAFLD liver disease and liver cancers is now increasing. This is particularly evident in the area of alcoholic liver disease, which shares in part, similar pathological changes, and in chronic viral hepatitis C for which eradication treatment success has been closely linked to patient body mass index.

In chronic hepatitis C genotype 4 infection, low adiponectin levels are associated with steatosis and high serum adiponectin levels with fibrosis [218]. Further studies found that Chinese patients with chronic hepatitis C had increased insulin resistance and decreased adiponectin when compared to a control population [219] and serum adiponectin levels in a group of patients from Egypt are inversely correlated with steatosis grade, histological activity score and fibrosis grade [220].

In patients with alcohol-related hepatic steatosis, hypoadiponectinaemia was a risk factor for increased hepatic dysfunction assessed by ultrasound and biochemistry [221]. Alcohol induced liver disease is also associated with altered levels of adiponectin and impaired adiponectin receptors which is likely to contribute to ongoing steatosis and fibrosis [222].

The increasing incidence of HCC in the United States is being attributed to the parallel increasing incidence of obesity and NAFLD [223]. In the United States, metabolic
syndrome was also found to be a significant risk factor for the development of primary liver cancer with an odds ratio=2.13; 95% confidence interval=1.96-2.31, P<0.0001) [224]. In vitro studies have demonstrated that adiponectin exerts anti-malignancy effects on the HCC cell lines HepG2 and Huh7 via inhibiting leptin signalling [225].

1.5.4 Adiponectin induces IL10

The full anti-inflammatory and anti-fibrotic effects of adiponectin are not completely understood; however increasing data supports the theory that multiple effects of adiponectin are mediated by increased expression of IL10. IL10 has multiple anti-inflammatory effects and as such is an important immunomodulatory cytokine. Adiponectin pre-treatment reduced Concanavalin A (ConA)-induced hepatic injury in mice and was associated with increased hepatic IL10 production; this protective effect was lost when IL10 was neutralised [226]. In cell culture work, adiponectin has been demonstrated to stimulate IL10 synthesis and release in porcine macrophages, isolated rat Kupffer Cells, human monocytes, macrophages and dendritic cells and RAW 264.7 macrophages [227-232].

IL10 is a potent inhibitor of antigen presentation and production of pro-inflammatory cytokines by macrophages. Macrophages have been described to respond to toxic insults by initially synthesising and secreting inflammatory cytokines, then subsequently synthesising and secreting IL10 which contributes to macrophage tolerance [230, 233]. IL10 itself suppresses macrophage function and therefore inhibits the ability of macrophages to secrete cytokines; thus it may have a self-regulatory effect [234, 235].

IL10 is produced by several cell types including Th2 cells, type I regulatory T cells, macrophages and mast cells. In macrophages and dendritic cells, IL10 expression occurs following PRR triggering by pathogen-derived products, with TLR-2 being particularly important for IL10 generation [236]. TLR-signalling activates MyD88 and
Toll/IL1 receptor (TIR)-domain containing adapter protein inducing IFNβ (TRIF), which leads to IL10 expression [237]. MAPK and NFκB activation occurs following TLR signalling through MyD88 [135] with ERK and P38 activity particularly important to IL10 generation [238, 239]. Importantly, all major inflammatory cytokines including IL1, IL6, IL12 and TNFα are inhibited by IL10 [240]. The major anti-inflammatory effects of IL10 are likely to be mediated by the induction of heme oxygenase-1 (HO-1) via p38 phosphorylation [241]. HO-1 is a catalyst in the biosynthesis of heme which amongst other things, results in the production of biliverdin, an inhibitor of pro-inflammatory cytokines [242].

Several mechanisms by which IL10 exerts immunomodulatory and anti-inflammatory effects have been identified. IL10 decreases the stability of granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and TNFα mRNA [243]. Kishore and colleagues explored this concept further, finding that the 3’ untranslated region of genes which contain a cluster of AU rich elements, may render genes sensitive to IL10 degradation [244]. IL10 has also been found to inhibit TNFα and IL6 in Trypanasoma cruzi infected cardiomyocytes by upregulating SOCS-3, which prevented NFκB nuclear translocation and ERK1/2 phosphorylation [245]. IL10 also inhibits antigen presentation thereby limiting ongoing inflammatory cytokine generation [246]. IL10 has multiple modulatory effects on T-cells. IL10 induces T-cell suppression by inhibiting the CD28 co-stimulatory signal thus contributing towards an anergic T-cell response [247]. IL10 acts on macrophages and dendritic cells to inhibit Th-1 responses as well as Th-2 and allergic responses [248, 249].

Evidence for the protective role of IL10 in the pathogenesis of liver injury include; IL10 knockout mice develop hepatic polymorphonucleocyte infiltration, microabscesses and higher serum TNFα levels in response to ethanol and LPS challenge [178]. In CCl₄
induced fibrosis, rats that were supplemented with IL10 had reduced expression of TGFβ and PDGF [250]. IL10 gene therapy via a human IL10 plasmid reversed thioacetamide-induced liver fibrosis in mice with associated decreased liver expression of TGFβ, TNFα, collagen α1, intracellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and TIMP-1 and 2 [251].

1.5.5 Inflammatory Effects of Adiponectin

Despite overwhelming evidence that supports the insulin sensitising, anti-atherogenic and anti-inflammatory effects of adiponectin, there are increasing reports of adiponectin being associated with or even contributing towards inflammatory and/or immune dysfunctional disease states [252]. In particular, elevated circulating adiponectin levels have been reported in patients with systemic lupus erythematosus, rheumatoid arthritis, active autoimmune idiopathic recurrent pericarditis, type 1 diabetes mellitus, chronic heart failure, end stage renal failure, liver cirrhosis and chronic obstructive pulmonary disease [253-260].

*In vitro* cell culture work has demonstrated an inflammatory response to adiponectin in THP-1 cells, RAW 264.7 macrophages and human macrophages [230, 261, 262]. This inflammatory response contradicts other findings and is expanded on later in this chapter. Variable effects of adiponectin upon adiponectin knockout mice have been observed which may be a reflection of inter-strain variability [252]. Factors that may contribute towards these contradictory inflammatory findings include adiponectin source ie *E. coli* derived versus from a mammalian cell line, potential Adiponectin-LPS binding, variable adiponectin types ie HMV versus LMW versus globular adiponectin and the potential for adiponectin tolerance to develop particularly in macrophages [263]. Further study into the potential inflammatory effects of adiponectin is required to facilitate a possible transition of adiponectin to a therapeutic modality.
1.6 Adiponectin and the Pathogenesis of NAFLD

Serum levels of adiponectin are inversely proportional to obesity and insulin resistance, both major contributors to NAFLD [206]. In particular, adiponectin levels have been demonstrated to be lower in both adults and children with NAFLD than healthy controls [264-271]. Together, these observations have led to extensive investigation of the role of adiponectin on the pathogenesis of NAFLD. Adiponectin has been shown to act in both a direct and indirect manner upon the liver in the pathogenesis of NAFLD. Adiponectin-activated AMPK inhibits biosynthetic pathways including fatty acid, glucose and sterol synthesis and promotes catabolic pathways such as fatty acid oxidation; all of which contribute to the prevention of NAFLD [272]. In addition, the downstream mediator of adiponectin, PPARα, has an important role in modulating hepatic triglyceride accumulation by regulating fatty acid oxidation and adipose derived inflammatory cytokine production, thus preventing NAFLD [74, 273]. Furthermore, adiponectin has been shown to be negatively associated with the pro-inflammatory and NASH-associated cytokines IL6 and TNFα, but positively associated with the anti-inflammatory cytokine IL10 [274]. As will be discussed in further detail below, adiponectin also exerts insulin-sensitizing, anti-inflammatory and anti-fibrotic effects directly upon hepatocytes, Kupffer Cells, HSCs and SECs.

1.6.1 Animal Studies of Adiponectin and NAFLD

Animal models have been used extensively to further understand the role of adiponectin in NAFLD and NASH with multiple studies demonstrating the hepato-protective activities of adiponectin. In a hallmark study of the in vivo effects of adiponectin, Xu and colleagues found that exogenously administered recombinant adiponectin abrogated hepatomegaly, steatosis and ALT abnormalities, decreased circulating FFAs and decreased hepatic TNFα synthesis in the obese ob/ob mouse [275]. The authors attributed these effects to increased carnitine palmitoyltransferase-1 (CPT1) activity,
enhanced hepatic fatty acid oxidation, a decrease in ACC and fatty acid synthetase and suppression of hepatic TNFα generation. A further study of hepatic steatosis demonstrated that adiponectin suppressed SREBP1c in the db/db mouse via AdipoR1 and the AMPK signalling pathway, thus regulating hepatic fatty acid synthesis [276]. Adiponectin knockout mice developed a more severe liver injury following D-Galactosamine (GalN)/LPS exposure than wild-type mice with associated elevated plasma levels of TNFα, decreased IL10 and IFNγ [277]. A further study by Masaki and colleagues demonstrated that KK-Ay obese mice pre-exposed to adiponectin prior to GalN/LPS – induced liver injury, had a reduction in mortality, serum ALT and AST, hepatocyte apoptosis and necrosis, focal and massive hepatic necrosis and serum TNFα [278]. Kamada and colleagues demonstrated the efficacy of adiponectin in preventing hepatic fibrosis with adiponectin knockout mice developing extensive hepatic fibrosis following CCl₄ administration, with mice injected with an adiponectin-producing adenovirus having attenuated hepatic fibrosis [279]. Neumeier and colleagues investigated changes in serum adiponectin and adiponectin receptors in rodent models of fatty liver disease and NASH cirrhosis. They found low circulating adiponectin levels in rats fed a high fat diet, and increased adiponectin in mice that had undergone bile duct ligation [280]. The mechanisms underlying the observed increase in adiponectin levels in the bile-duct ligation model, whereby mice develop extensive fibrosis, is likely to be similar to those observed in human cirrhotic subjects who also have high circulating adiponectin levels. In the fatty liver rat model AdipoR2 mRNA expression was increased when compared to the control and in the cirrhosis, bile-duct ligation mouse model, both AdipoR1 and R2 expression was reduced in comparison to the control animal [280]. In a MCD-induced fibrosis mouse model, inhibition of AdipoR2 increased all histological changes of NASH, whereas over expression of AdipoR2 decreased all NASH changes [281].
AdipoR1 or AdipoR2 in the liver by an adenovirus was demonstrated to reverse steatosis induced insulin resistance in diabetic mice [282].

1.6.2 Human Studies of Adiponectin and NAFLD

Adiponectin levels have been demonstrated to be lower in both adults and children with NAFLD than healthy controls [264-271]. These initial studies were flawed in that healthy controls were not always well matched to BMI. More recently adiponectin levels have been demonstrated to be inversely associated with hepatic steatosis independently of insulin sensitivity, abdominal obesity and ethnicity [283]. Differences in adiponectin levels in individuals with NAFLD compared to those with NASH have been described. Bugianesi and colleagues were able to correlate adiponectin levels with hepatic fat content but not with necroinflammation or fibrosis [264]. Other groups have since described a correlation between adiponectin levels and NAFLD or NASH. Adiponectin was inversely associated with steatosis in 19 subjects when matched with healthy BMI controls, but not obese controls, however the same study showed adiponectin to be inversely associated with NASH compared to simple steatosis and obese and non-obese controls [284]. In individuals undergoing bariatric surgery, those with NASH had lower serum adiponectin compared to those with bland steatosis [285]. Low adiponectin levels in NASH compared to NAFLD were described in 108 patients, with adiponectin correlating with the degree of steatosis and necroinflammation but not fibrosis [286]. In 30 morbidly obese patients, hepatic expression of adiponectin but not adiponectin receptors was reduced in those with NASH compared to those with NAFLD alone [287]. Further studies have found serum levels of adiponectin to be lower in individuals with early-stage, or non-cirrhotic NASH compared to those with simple steatosis [285, 288]. A meta-analysis of 27 studies encompassing 2243 subjects by Polyzos et al found that controls had higher adiponectin levels compared to those with NAFLD [CI=3.0 (1.57-4.43)] and NASH [CI=4.75 (3.78-
NASH subjects had lower adiponectin levels than NAFLD patients [CI=1.81 (1.09-2.53)] [289]. The above observations have led several authors to hypothesise that low adiponectin may be an important mediator in the transition from NAFLD to NASH. In patients with NASH-related cirrhosis however, elevated levels of adiponectin have been observed, with this elevation in adiponectin possibly contributing to fat loss that is observed in NASH cirrhosis [260, 280].

Adiponectin gene polymorphisms have also been detected and attributed to human disease states. The adiponectin single nucleotide polymorphisms (SNPs) at +45 of exon 2 and +276 of intron 2 were sequenced in NAFLD patients and controls [290]. The investigators found the +276 G/G phenotype to be significantly higher in females with NAFLD and the +45 G/G phenotype to be significantly higher in those with severe fibrosis with the +45 G/G phenotype to be an independent risk factor for advanced hepatic fibrosis following multivariate analysis.

The role of adiponectin receptors expression in NAFLD pathogenesis is unclear with variable study findings reported. Similar AdipoR1 expression was reported between controls and NAFLD subjects [291], NAFLD and NASH subjects [287] and controls, NAFLD and NASH subjects [292]. In only one study by Nannipieri and colleagues was AdipoR1 expression found to be increased in those with NASH compared to those with NAFLD or control subjects [293]. Reduced hepatic expression of AdipoR2 has been observed in patients with NAFLD compared to controls with no difference observed in hepatic AdipoR2 expression between NAFLD and NASH patients [291] and a further study found significantly decreased levels of AdipoR2 mRNA in individuals with NASH compared to those with simple steatosis [271]. AdipoR2 expression was similar in NAFLD and NASH patients [287] and controls, NAFLD and NASH subjects [292]. Higher AdipoR2 expression in NASH compared to NAFLD or controls was found in a single study [293].
1.6.3 The Effect of Adiponectin on Monocytes and Macrophages

Kupffer cells as the resident liver macrophages, are derived from the mononuclear and monocyte cell line and as such possess similar cell receptors and signalling mechanisms. Macrophage and monocyte cell lines, and peripherally-derived mononucleocytes provide researchers with an invaluable tool to further understand adiponectin biology. This allows further insight into possible effects of adiponectin on Kupffer Cells in both an *in vitro* and *in vivo* setting.

Blood derived mononuclear cells and their tissue counterparts the macrophages, form an important cellular component of the innate immune system. As previously discussed, peripheral blood mononucleocytes and macrophages play an important role in the pathogenesis in the metabolic syndrome, particularly the adipocyte-macrophage interaction within adipose tissue and the monocyte/foam cell-vascular endothelial cell interaction within atheromatous plaques. These cells express both AdipoR1 and AdipoR2 receptors and appear to be exquisitely responsive to the effects of adiponectin. Adiponectin appears to modulate the early macrophage responses to noxious stimuli, thereby acting to inhibit both the growth of myelomonocytic cells and decreasing the ability of mature macrophages activate and develop an inflammatory phenotype. [227, 294]. As such, significant research into the effect of adiponectin on both tissue-derived monocytes and macrophages, and cell lines has contributed towards understanding the pathogenesis of the metabolic syndrome. The effect of adiponectin on Kupffer Cells, the resident liver macrophage, and the hepatic manifestation of the metabolic syndrome NAFLD and NASH, will be discussed below.

1.6.3.1 Adiponectin decreases LPS-induced Pro-inflammatory Cytokine Release in Macrophages.

Several groups have investigated the effect of adiponectin on monocyte and macrophage derived inflammatory cytokines. Elevated serum LPS in NAFLD is
important in the pathogenesis of NAFLD and NASH by stimulating TNFα via TLR4 signalling [295]. Several investigators have used LPS in cell culture work as both a “model” of NAFLD and as a mechanism to stimulate pro-inflammatory cytokine synthesis and release. Overnight culture in adiponectin has been demonstrated to decrease LPS-induced TNFα synthesis and release in cultured human macrophages, RAW 264.7 macrophages and human leukocytes [227, 229, 230, 294, 296-298]. Yokaota and colleagues demonstrated that cultured human macrophages treated with adiponectin for 24 hours prior to exposure to LPS had decreased TNFα mRNA and peptide synthesis but unchanged IL1β and IL6 synthesis, when compared to macrophages that were not pre-treated with adiponectin [294]. In porcine, blood-derived macrophages, adiponectin was again found to suppress LPS-induced TNFα and IL6 synthesis in an ERK1/2 and p38 dependent manner, and by preventing NFκB nuclear translocation [227]. RAW 264.7 cells that were transfected with a TNFα-promoter luciferase reporter were found to have reduced LPS-induced TNFα luciferase activity following overnight culture in globular adiponectin by decreasing early growth response protein-1 (Egr-1), NFκB and activator protein-1 (AP-1) binding and decreasing ERK1/2 and p38 phosphorylation, preventing IkB loss and reducing TNFα mRNA stabilisation [296]. Similarly, pre-treatment of RAW 264.7 macrophages with globular adiponectin reduced LPS-induced p38, c-JNK, ERK1/2 and IkB phosphorylation [297]. A study of primary human macrophages suggested that globular adiponectin inhibited LPS-induced pro-inflammatory cytokine synthesis by inducing the inactive form of the IL1 receptor associated kinase (IRAK-M) thereby activating the mitogen activated protein 3k8 gene, Tpl2/ERK, and PI3K/AKT signalling pathways [298].
1.6.3.2 Adiponectin induces IL10 synthesis in Macrophages.

Both LPS and adiponectin were shown to increase IL10 expression in porcine macrophages, and interestingly, macrophages exposed to LPS and adiponectin together in culture had an even greater IL10 expression [227]. In Kupffer Cells, maximal IL10 mRNA synthesis occurred 2 hours following LPS stimulation, this was inhibited by actinomycin D suggesting de novo RNA synthesis, however further IL10 synthesis was blocked by exogenous IL10, and neutralizing endogenous IL10 with a monoclonal antibody caused an increased IL10 response to LPS (Knolle, Uhrig et al. 1998). IL10 also suppresses TNFa synthesis and production in macrophages, this is overcome with the addition of IL10 antibodies [228].

Adiponectin induces the anti-inflammatory mediators IL10 and interleukin 1 receptor agonist (IL1RA) in human monocytes, dendritic cells and macrophages in a PI3k dependent manner [229]. The same study also found that LPS potentiated IL10 production in monocytes but not in macrophages or dendritic cells [229]. RAW 264.7 macrophages cultured with either full length or globular adiponectin initially had an increase in TNFα synthesis, prior to the generation of IL10 [230]. When cells were transfected with siRNA against the TNFα receptor, IL10 production was decreased confirming the investigators hypothesis that TNFα was required for increased IL10 expression that subsequently led to LPS tolerance [230]. Further studies found that the initial TNFα expression occurred via ERK1/2 and early growth response protein-1 (Egr-1) pathways [230]. The same research group also demonstrated that globular adiponectin increased IL10 mRNA in both primary rat Kupffer Cells and RAW 264.7 macrophages by phosphorylating a cyclic AMP response element binding protein which was downstream of protein kinase A and Erk1/2 [231]. In addition, adiponectin-induced IL10 mediates down regulation of LPS-induced inflammatory cytokines in rat Kupffer Cells and RAW264.7 macrophages [230, 232]. A somewhat contradictory
study by Folco and colleagues however, found that adiponectin-induced inhibition of LPS, TNFα or signal transducer and activator of transcription-3 (STAT3) inflammatory pathways was not altered when macrophages were cultured with IL10 neutralizing antibodies or transfected with IL10 siRNA [299].

1.6.3.3 Adiponectin inhibits macrophage-foam cell transformation.
Lipid-rich macrophages, foam cells are a major cellular component of atherosclerotic plaques. Modulating foam cell formation by inhibiting lipid accumulation within macrophages is a potential management strategy to prevent coronary arterial disease, cerebrovascular accidents and peripheral vascular disease. Cultured human macrophages that were exposed to adiponectin had reduced intracellular cholesterol content and down regulated macrophage scavenger receptor when compared to untreated macrophages [300]. A further study demonstrated that adiponectin reduced Acyl-coenzyme A: cholesterol acyltransferase-1 activity and cholesterol ester accumulation in cultured human macrophages [301]. Transgenic mice over-expressing AdipoR1 had decreased lipid accumulation and reduced macrophage foam cell formation within atherosclerotic lesions when compared to wild-type (WT) mice [302].

1.6.3.4 Adiponectin inhibits Macrophage phagocytic activity.
Adiponectin decreased the phagocytic activity of mature human derived monocytes; when the complement receptor C1qRp was blocked, phagocytic inhibition by adiponectin was lost suggesting that adiponectin acts on this receptor [294].

1.6.3.5 Adiponectin induces macrophage phenotypic transformation.
Adiponectin was found to promote the activation of human monocytes to the alternative M2 anti-inflammatory macrophage with adiponectin exposed human monocytes differentiating into macrophages that expressed M2 cell markers including the mannose receptor and CC chemokine-1 [303]. However human macrophages exposed to 24 hours of adiponectin were found to express 28 of 46 M1 markers and only 3 of 42 M2
markers as analysed by microarray [262]. In a mouse in vivo study, adiponectin knock-out mice were found to have increased Kupffer Cell infiltrate with associated decreased M2 cytokine markers after 2 weeks of an MCD diet, when compared to WT mice [304]

1.6.3.6 Pro-inflammatory effects of Adiponectin upon Macrophages.

Despite the well-described anti-inflammatory effects of adiponectin upon macrophages, several research groups have described an inflammatory response to adiponectin.

Initial treatment of the macrophage cell line THP-1 cells with globular adiponectin resulted in TNFα and IL6 secretion [261]. However the authors found that the THP-1 cells developed tolerance to globular adiponectin; when cells were treated repeatedly with globular adiponectin they had no further increase in TNFα or IL6 secretion. Globular adiponectin pre-treated cells subsequently exposed to LPS or polyI:C had a decreased production of IL6 and TNFα and when cells were treated with a low level of globular adiponectin then re-exposed to a high level of globular adiponectin, an increased production of IL6 and TNFα was observed. TLR-3 was down regulated by globular adiponectin, however no change was seen in TLR-4, therefore the authors concluded that the mechanism by which tolerance to LPS occurs is by adiponectin preventing the translocation of NFκB.

In the paper by Park and colleagues, previously described above, Raw 264.7 macrophages that were incubated overnight in adiponectin-containing media had a 4-fold increase in TNFα mRNA when compared to basal cells [230]. The authors found TNFα promoter-driven luciferase activity increased within 4 hours of exposure to adiponectin and continued to peak over 24 hours. When the TNFα promoter was truncated by deleting two κB sites and the Egr-1 site, luciferase activity was reduced initially, but not after 24 hours, thereby demonstrating the important role of NFκB and Egr-1 in the initial adiponectin-induced TNFα expression. After 24 hours, increased
activator protein (AP)-1 DNA binding was observed, thus AP-1 activity is likely to be more important in late adiponectin-induced TNFα expression.

Human macrophages were found to have maximal expression of TNFα and IL6 at following 3-6 hours exposure to adiponectin with AdipoR1 and/or AdipoR2 knockdown with siRNA abrogating this effect [262].

1.6.4 The Effects of Adiponectin upon Kupffer Cells.

Few studies have investigated the effect of adiponectin on primary Kupffer Cells. The Kupffer Cell is a specialised macrophage and as such, may behave differently to the monocyte and macrophage models that have been more extensively studied. Many Kupffer Cell studies use alcoholic liver disease as a model for inducing Kupffer cell dysfunction. Alcoholic liver disease shares several pathogenic similarities to NAFLD, but are unlikely to be completely representative of a NAFLD model. Several studies however do provide some insight into the anti-inflammatory effects of adiponectin upon the Kupffer Cell.

Isolated rat Kupffer Cells that were pre-exposed to adiponectin-containing culture media prior to LPS stimulation had reduced TNFα mRNA and increased IL10 expression than those Kupffer Cells that were not pre-exposed to adiponectin [277]. In a similar study which examined a rodent model of alcohol induced liver injury, chronic ethanol feeding increased the sensitivity of Kupffer Cells to LPS resulting in increased TNFα synthesis; this increase was abrogated by the pre-treatment of Kupffer Cells with globular adiponectin [305]. A similar effect was found in chow-fed rats, however to a lesser degree of change. To understand the mechanism underlying this TNFα suppression, signalling pathways were examined, with adiponectin alone being found to have no effect on IκB signalling, adiponectin however suppressed LPS-stimulated p38 phosphorylation in Kupffer Cells from ethanol-fed but not chow-fed rats, LPS-
stimulated ERK1/2 phosphorylation in both ethanol and pair-fed rat Kupffer Cells and LPS induced ROS generation in both ethanol and pair-fed rat Kupffer Cells.

To investigate the link between IL10 and TNFα, this same research group demonstrated that adiponectin failed to suppress LPS induced TNFα synthesis in Kupffer Cells when IL10 or its downstream mediator HO-1 expression was knocked down via siRNA or the HO-1 inhibitor zinc protoporphyrin [232]. The same authors then investigated the effects of adiponectin on TLR-4 signalling pathways in Kupffer Cells; finding that both adiponectin and HO-1 inhibited TLR-4-dependent cytokines and the TLR-4 signalling mediator MyD88 in both a dependent and independent manner [306].
1.7 Summary

Rates of NAFLD and NASH with the complications of cirrhosis, liver failure and liver cancer are rapidly increasing worldwide. The pathogenesis of NAFLD and the progression to NASH has not been fully elucidated, however adipocyte-macrophage-hepatocyte interactions with associated cytokine and adipokine signalling are likely to be major contributors. Gaining further insight into the pathogenesis of NAFLD and NASH is vital to not only understand this common condition better, but to assist with developing effective, safe and tolerable treatments.

Adiponectin has multiple metabolic, homeostatic, anti-inflammatory and anti-fibrotic effects including insulin sensitising properties, the ability to polarise inflammatory cells towards a non-inflammatory phenotype and anti-inflammatory effects due to TNFα and IL6 inhibition and IL10 induction. The Kupffer cell is an important cellular mediator of NAFLD, NASH and liver fibrosis, and modulation of its pro-inflammatory and pro-fibrotic effects by adiponectin may prevent NAFLD/NASH progression. However increasing reports of inflammatory effects of adiponectin upon macrophages may limit the therapeutic usefulness of adiponectin; therefore further insights into the pro-inflammatory effects of adiponectin are required.

Adiponectin has been shown in some studies to reduce Kupffer Cell derived inflammatory mediators, however these studies are limited in number and are generally in the setting of alcoholic liver disease. In macrophage *in vitro* studies, conflicting inflammatory and anti-inflammatory effects of adiponectin have been observed. In this thesis I will be further exploring the effect of adiponectin on the Kupffer Cell, with particular emphasis on the cytokines that have been demonstrated to have an important role in potentiating steatohepatitis and fibrosis including TNFα, IL6, IL10, TGFβ, CTGF and PDGF. This will further contribute to our understanding of the effects of adiponectin in the pathogenesis of NAFLD, which in the future, may contribute towards
the use of adiponectin or its downstream targets to both prevent and treat NAFLD related liver fibrosis.
1.8 Philosophy, Hypotheses and Aims of The Thesis

1.8.1 Philosophy of Study

This thesis consists of three studies, which together test the major hypothesis of this thesis; adiponectin may induce divergent anti-inflammatory/fibrotic or pro-inflammatory/fibrotic effects on the Kupffer Cell depending upon previous exposure. The Kupffer Cell is a central mediator of hepatic inflammation and fibrosis due to its ability to respond to blood-derived compounds and mount a locally acting response. Adiponectin has a negative association with the metabolic syndrome and NAFLD along with multiple anti-inflammatory properties and has been shown to act directly on both macrophages and Kupffer Cells to dampen LPS-induced inflammatory response. Evidence however does suggest that the initial responses of macrophages to adiponectin, is in fact an inflammatory response, and this inflammatory response persists until tolerance to adiponectin develops, and in doing so an anti-inflammatory response ensues. There is no current effective treatment for NAFLD or NASH and adiponectin offers promise as a future biological treatment. Understanding the effects and mechanisms of action of adiponectin on the Kupffer Cell is important, particularly as an initial inflammatory response could be deleterious in many individuals.

1.8.2 Study 1: The Modulatory Effects of Adiponectin upon LPS-induced cytokine changes in Kupffer Cells.

1) Hypotheses

1. Acute adiponectin exposure induces an initial rapid cytokine release from Kupffer Cells comprising of both anti-inflammatory and pro-inflammatory cytokines.

2. Acute adiponectin exposure potentiates the synthesis and release of LPS-induced pro-inflammatory and anti-inflammatory cytokines in the Kupffer Cell.

3. Adiponectin primed (pre-treated) Kupffer Cells will have a down-regulation in LPS induced pro-inflammatory cytokine release.
2) Aims

1. To assess the effect that acute adiponectin in the presence or absence of LPS, has on primary Rat Kupffer Cell synthesis and release of the major mediators of hepatic necro-inflammation and fibrosis, TNFα, IL6, TGFβ, CTGF and PDGF and anti-inflammatory cytokine IL10.

To achieve this aim, isolated rat Kupffer cells will be exposed to adiponectin and/or LPS and/or vector for 18 hours. Cytokine changes will be measured by quantitative polymerase chain reaction (qPCR) and ELISA.

2. To assess the effect of adiponectin pre-treatment on subsequent LPS-induced cytokine synthesis and release in primary Rat Kupffer Cells cytokine synthesis and release.

Primary Rat Kupffer Cells will be primed with adiponectin or cultured in vector alone for 18 hours, prior to exposure to LPS +/- adiponectin for 4 hours. Cytokine changes will be analysed by qPCR and ELISA.

**1.8.3 Study 2: Kupffer Cell Tolerance to Adiponectin occurs in an IL10-Dependent Manner.**

1) Hypothesis

1. Adiponectin primed (pre-treated) Kupffer Cells will have a down-regulation in adiponectin induced pro-inflammatory cytokine release.

2. Kupffer Cells develop tolerance to adiponectin by the induction of IL10, which abrogates ongoing inflammatory cytokine synthesis.

2) Aims

1. To assess the effect that adiponectin pre-treatment has on subsequent adiponectin-induced pro-inflammatory cytokines TNFα and IL6.

To achieve this aim, primary Rat Kupffer Cells will be primed with adiponectin or cultured in media alone for 18 hours. All media will be replaced with fresh
adiponectin-containing media and cells will be cultured for a further 4 hours. Cytokine changes will be assessed by qPCR and ELISA.

2. To assess whether IL10 mediates the disproportionate expression of TNFα and IL6 from adiponectin primed and non-primed Kupffer Cells.

To achieve this aim, primary Rat Kupffer Cells will be primed in adiponectin or cultured in media alone for 18 hours. Cells will then be re-exposed to fresh media containing adiponectin and IL10 antibody or recombinant IL10. Changes in TNFα and IL6 will be measured with qPCR and ELISA.

1.8.4 Study 3: Mechanisms of Adiponectin-Induced Inflammatory Response in Rat Kupffer Cells.

1) Hypothesis

1. Adiponectin initially induces TNFα in Kupffer Cells via activation of the NFκB, ERK1/2, P38 and JNK pathways.

2. Adiponectin pre-exposure abrogates, adiponectin-induced ERK1/2, P38 and JNK phosphorylation in an IL10-dependent manner.

2) Aims

1. To determine whether adiponectin-induced TNFα in Kupffer Cells occurs via activation of the ERK1/2, P38 and JNK pathways.

To achieve these aims, non-primed Kupffer Cells will be exposed to adiponectin and/or the ERK1/2 inhibitor PD98059 and/or the P38 inhibitor SB203580 and/or the JNK inhibitor for 4 hours. TNFα mRNA will be assessed using qPCR.

2. To determine whether adiponectin-primed or non-primed Kupffer Cells elicit different patterns of ERK1/2, P38 or JNK phosphorylation when acutely treated with adiponectin and whether IL10 facilitates this different pattern.

To achieve this aim, primary Rat Kupffer Cells will be primed in adiponectin-containing media or non-primed in media alone for 18 hours. A time course experiment
will then be carried out whereby Kupffer Cells will be exposed to adiponectin +/- soluble IL10 antibody +/- IL10 peptide for various time periods from 0 to 60 minutes. At each time period, total and phosphorylated forms of ERK1/2, P38 and JNK will be determined by Western Blot.
Chapter 2 Materials and Methods
2.1 Materials and Reagents

Recombinant murine Adiponectin and Adiponectin antibodies (Catalogue number RD281023100) were from Biovendor (Hong Kong). Full-length adiponectin (Catalogue number BW001-02) was produced in the HEK293 human embryonic kidney cell line and globular adiponectin (Catalogue number BW001-0) was produced in E.coli. IL-10 recombinant protein (Catalogue number 522-RL) and Rat IL-10 antibody (Catalogue number AF519) were from R&D (Australia). Antibodies to AMPKα (Catalogue number 2793), Phospho-AMPKα (Catalogue number 2531), p38 (Catalogue number 9228), phospho-p38 (Catalogue number 4511), ERK1/2 (Catalogue number 9107), phospho-ERK1/2 (Catalogue number 4370), SAPK/JNK (Catalogue number 9258) and phospho-SAPK/JNK (Catalogue number 4668) were from Cell Signalling (Australia). AdipoR1 (Catalogue number ADIPOR11-A) and AdipoR2 (Catalogue number ADIPOR22-A) antibodies were purchased from Alphadiagnostic International (San Antonio, USA). T-cadherin (Catalogue number sc-7940), IκB (Catalogue number sc-4327) and Phospho-IκB (Catalogue Number 4094) antibodies were purchased from Santa Cruz Biotechnology (Dallas, USA). JNK-inhibitor was from Calbiochem. PD98059 and SB203580 were from Promega (WI, USA). Geys Balanced Salt Solution (GBSS), Histodenz, 6-mercaptopurine, bovine serum albumin (BSA), latex beads (0.81 µM diameter), Radioimmunoprecipitation assay (RIPA) buffer, Protease Inhibitor Cocktail 1 and Phosphatase Inhibitor Cocktail 2 were from Sigma (Australia). Pronase E, collagenase B, DNAse I were from Roche (Australia). Penicillin-streptomycin (P/S), and foetal calf serum (FCS) were supplied from Gibco BRL (Mulgrave, Australia). Molecular reagents were primarily purchased from Invitrogen (Mulgrave, Australia). These included, Superscript III First Strand synthesis system kits, random hexamers, deoxynucleotides and DTT. RNeasy kit for column extraction of RNA was from Qiagen (Venlo, Netherlands). Enzyme-linked immunosorbent assay (ELISA) kits were
purchased from R&D Systems (MN, USA). Molecular weight standards were from Fermentas Life Science (Scorsbey, Australia). Oligo primers were supplied by GeneWorks (Hindmarsh, Australia). Heparin was obtained from David Bull Laboratories (Melbourne, Australia). Anaesthetic agents, ketamine and xylazine, were purchased from Ilium (Smithfield, Australia). ECL Western Blotting Detection kits were from Amersham, GE Healthcare (Pittsburgh, USA). Restore Western Blot Stripping Buffer was from Thermoscientific (Scorsbey, Australia). Dulbecco’s Modified Eagle Media (DMEM) was from GIBCO BRL (Grand Island, NY). All other chemicals and all solvents were obtained as analytical grade reagents from Ajaz chemicals (Sydney, Australia).
2.2 Liver Perfusion and Cell Isolation Solutions

Ca\(^{2+}\) and Mg\(^{2+}\) free Geys Balanced Salt Solution (GBSS) was prepared with the addition of 7.0 g NaCl, 370 mg KCl, 150 mg NaH\(_2\)PO\(_4\).2H\(_2\)O, 227 mg NaHCO\(_3\), 30 mg KH\(_2\)PO\(_4\), 210 mg MgCl\(_2\).6H\(_2\)O and 1.0 g glucose and made up to 1 litre with double distilled water, the pH was adjusted to 7.4 with 0.1 M NaOH. The solution was filter sterilised through a 0.22 µm filter (EMD Millipore Corporation, Billerica, MA).

Pronase E perfusion solution was prepared at the concentration of 3.0 mg Pronase E per ml GBSS and filter sterilised through a 0.22 µm filter (EMD Millipore Corporation, Billerica, MA).

Collagenase B perfusion solution was prepared at the concentration of 0.3 mg collagenase B per ml of GBSS and filter sterilised through a 0.45 µm filter (EMD Millipore Corporation, Billerica, MA).

NaCl-free GBSS was prepared with the addition of 370 mg KCl, 70 mg MgSO\(_4\).7H\(_2\)O, 150 mg NaH\(_2\)PO\(_4\).2H\(_2\)O, 220 mg CaCl\(_2\).2H\(_2\)O, 227 mg NaHCO\(_3\), 30 mg KH\(_2\)PO\(_4\), 210 mg MgCl\(_2\).6H\(_2\)O and 1.0 g glucose and made up to 1 litre with double distilled water; the pH was adjusted to 7.4 with 0.1 M NaOH. The solution was filter sterilised through a 0.22 µm filter (EMD Millipore Corporation, Billerica, MA).

Krebs, Ringer, Heselte (KRH) Elutriation Buffer was prepared by HEPES 5.96 g, NaCL 7.2 g, KCl 370 mg, KH\(_2\)PO\(_4\) 140 mg, EDTA 190 mg and D-glucose 450 mg and made up to 1 litre in double distilled water and adjusted to pH 7.3 with 10 M NaOH then filter sterilised (0.22 µM filter, EMD Millipore Corporation, Billerica, MA).
2.3 Animals

2.3.1 Animal care and ethics

All animal studies were approved by the Western Sydney Area Health Service Animal Ethics Committee. All animals were housed in the Westmead Hospital Animal House under conditions of a constant ambient temperature of 22 degrees Celsius and stable humidity with a 12-hour light/dark cycle (light on 0600-1800).

Male Sprague-Dawley weighing 250-400 g were obtained from the Australian Resource Centre (ARC, Canning Vale, Western Australia). Rats were fed a commercial rat pellet diet (Allied Foods, Sydney Australia) and were allowed food and water *ad libitum*. Animals were housed in plastic cages (550 x 370 x 170 mm) with open wire lids and wood shavings for bedding (Boral, Australia). Rats were housed at no more than 3 animals per cage. Rats were sacrificed by exsanguination whilst under anaesthesia for liver perfusion.

2.3.2 Anaesthesia

Animals were not fasted prior to anaesthesia and surgery. Anaesthesia was induced by placing the rat in a bell jar containing diethyl ether, then maintained by intramuscular injection of 100 mg/kg body weight ketamine and 1 mg/kg body weight xylazine into the lateral aspect of the upper hind leg.
2.4 Primary culture of rat Kupffer cells

2.4.1 General surgical technique

The anaesthetised rat was secured in the supine position. The abdominal surface was sterilised with 70% ethanol and the abdominal cavity was opened by performing a laparotomy via a midline vertical incision. Using aseptic technique, the small and large bowel were retracted away from the liver. The liver was identified and the left lobe was gently retracted and lifted superiorly and packed posteriorly with sterile gauze to expose the portal vein. 1000 IU of heparin was injected into the inferior vena cava with a 25G needle; the portal vein was then cannulated with either a 16 or 18-gauge cannula that was fastened in situ using a silk tie. The liver was perfused in situ via the portal vein. The inferior vena cava was severed once perfusion had begun to facilitate outflow for the perfusion solutions.

2.4.2 Perfusion of rat liver

Kupffer cells were isolated by the two-stage collagenase and pronase digestion followed by centrifugal elutriation as described by Knook [307] and Zahlten [308]. The liver was perfused in situ via a cannula inserted into the portal vein as described above using a non-recirculating peristaltic pump (Gilson, Middleton, WI). The perfusate was maintained at 37°C by placing all perfusion solutions in a 37°C water bath. Initial perfusion was 150 ml of Ca$^{2+}$ and Mg$^{2+}$ free GBSS at 20 ml/min. This was followed by perfusion of 150 ml Pronase E solution then 150 ml of Collagenase B solution at 20 ml/min. The liver was prevented from drying out by regular application of warmed sterile sodium chloride solution to the external surface of the liver. Following perfusion of all solutions, the liver was excise from adjacent membranes and diaphragm and placed in a sterile container.
2.4.3 Isolation of Kupffer Cells from rat liver

All procedures were carried out in a sterile laminar flow hood. The liver was minced with scissors until homogenous. The liver was then incubated with 50 ml of GBSS containing Pronase E (0.2%) and DNAse I (0.002%). The cells were continuously agitated for 30 minutes at 37°C. The resultant cell suspension was passed through a 100um nylon mesh filter to remove cellular aggregations and connective tissue debris. The filtered cell suspension was centrifuged at 80 g for 2 minutes to sediment all hepatocytes. The cell pellet was discarded and the remaining supernatant was made up to 50 ml with GBSS containing 0.002% DNAse and centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended and washed in 40 mL of GBBS containing DNAse I (0.002%) twice with centrifugation at 2000 rpm for 5 minutes between washes to sediment non-parenchymal hepatic cells. The final cell pellet was re-suspended in 10 ml of GBSS containing DNAse I (0.002%). This non-parenchymal hepatic cell suspension was then combined with 6 ml of NaCl-free GBSS containing 30% Histodenz resulting in a 11.25% gradient solution. Two, 3-layer gradients were constructed in 15 ml Falcon Tubes. As illustrated in figure 2.1A, these consisted of a 2.5 ml base of 20% Histodenz in NaCl-free GBSS, followed by a middle layer 8 ml of non-hepatic parenchymal cell suspension and a top layer of 1.5 ml GBSS containing 0.002% DNAse. These gradients were then centrifuged for 20 min at 1400 g at slowest acceleration and deceleration to prevent mixing of layers. After the spin, the Kupffer Cells settled in a layer between the 20% and 11.25% gradients as shown in figure 2.1B. This layer was collected and washed in GBSS containing 0.002% DNAse and centrifuged at 2000rpm for 5 min to pellet the cells. The cells were then resuspended in 10 ml DMEM media containing 20% FCS for elutriation. This cell suspension contains in addition to Kupffer cells, hepatic SECs and varying contaminating cells predominantly red blood cells, and therefore requires further
purification by means of centrifugal elutriation. HSCs settle in a layer between the 11.25% gradient and GBSS.

2.4.4 Centrifugal elutriation

Counter-current centrifugal elutriation is a process that allows a heterogeneous cell suspension to be separated into individual cell populations. Centrifugal elutriation consists of two separate processes to separate cells; the process of centrifugation which involves the process of sedimentation under centrifugal force, and elutriation which is a process of separation by washing. The separation occurs in a funnel-shaped elutriation chamber. A cell suspension is pumped at a pre-set flow rate into the rotor to the elutriation chamber whilst the rotor is spinning within the centrifuge. The centrifugal force and the fluid velocity act upon each individual cell within the elutriation chamber. These act in opposition with the centrifugal force pushing the cell away from the centre of rotation and the fluid velocity pushing the cell towards the centre of rotation. Cells eventually settle in a position within the chamber where the effects of the two opposing forces become balanced. This causes large cells to settle close to the chamber inlet where the centrifugal force and velocity are high and small cells to settle in the wider section of the elutriation chamber close to the outlet. As shown in figure 2.2, by balancing centrifugal force against the opposing flow rate, cell populations of any uniform size can selectively removed from the cell suspension; initially small cells followed by increasingly larger cells. As the cells are washed out they can be collected in fractions.

Kupffer cells were isolated using a J-6M/E centrifuge fitted with a JE 6.0 elutriation rotor as shown in Figure 2.3. Prior to use the chamber was sterilised with 250 ml of 70% ethanol followed by 250 ml sterile phosphate-buffered saline (PBS). The elutriation buffer was a KRH buffer that was supplemented with fresh DNAse I (0.002%) and FCS (10 ml/l) prior to use. FCS was used to minimise cell injury and
Figure 2.1. Histodenz and Cell Suspension Gradients Prior to Centrifugation (A) and Following Centrifugation (B).

A. A 30% histodenz gradient is overlayed by a 11.25% histodenz and cellular suspension. GBSS is carefully layered above the cellular layer. The gradient is centrifuged at 1400 G for 20 minutes.

B. Following centrifugation Hepatic Stellate Cells settle in a layer between the GBSS and 11.25% histodenz and a combination of Kupffer Cells and Sinusoidal Epithelial Cells settle between the 30% and 11.25% histodenz layers.
Figure 2.2. Diagrammatic representation of the elutriation process.

Cells are distributed within the chamber according to their size with small cells being flushed out of the chamber at lower pump speeds and larger cells requiring higher pump speeds to be flushed from the chamber.

Source: Beckman, Ca, USA.
DNAse I prevents cell clumping that impairs the separation purity and yield. The initial pump rate at which the cell suspension was loaded was 10 ml/min. Once the cell suspension settled in the elutriation chamber, the pump speed was progressively increased to flush out red blood cells (18 ml/min) and hepatic SECs (21-25 ml/min). Kupffer cells were collected at a flow rate of 45-48 ml in a 180 ml aliquot which consisted of three Falcon Tubes.
Figure 2.3. The JE-6B Elutriator System.

Essential elements of the system are shown. The elutriation system is primed with buffer then the cell suspension is introduced into the elutriation chamber at 10 ml/min via a closed circuit injection into a 3-way tap. The cells can be seen to settle in the elutriation chamber by direct vision through the viewing port. Once all cells have settled in the elutriator chamber, the pump speed is increased to flush out red blood cells and sinusoidal endothelial cells. Kupffer Cells are flushed out and collected in a flask at a flow rate between 45 ml-48 ml/minute.

Source: Beckman, California, USA.
2.5 Cell Culture Techniques

2.5.1 Culture of Kupffer Cells

Kupffer Cells were pelleted from the elutriate by centrifuging at 2000 RPM for 5 minutes. Pellets from each Falcon Tube were combined and resuspended in DMEM containing 10% FCS and P/S 50 U/ml. Cell number was determined using a Neubauer hemocytometer and light microscopy. The cell suspension was diluted to a final concentration of 1.5 x 10^6 cells/ml culture media. Kupffer Cells were cultured onto either a plastic 6 well plate at a density of 3 x 10^6 cells per well or a plastic 12 well plate at a density of 1.5 x 10^6 cells per well. Plates were incubated at 37 degrees in 5% CO2 overnight, the media was then aspirated to remove non-adherent cells and fresh media was added prior to treating cells.

2.5.2 Determination of cell purity

As Kupffer cells are capable of phagocytosing 0.81 µm diameter latex beads, a latex bead phagocytosis assay can easily identify Kupffer Cells. Following culture with latex beads, Kupffer Cells containing latex beads within their cytoplasm are easily identified with light microscopy [309]. The other hepatic cell that is capable of phagocytosis, the sinusoidal endothelial cell, only phagocytoses particles up to 0.33 µm [310].

Kupffer Cells were isolated and cultured using the method described above. Following overnight incubation, media was aspirated from the wells and replaced with fresh DMEM containing 10% FCS and P/S and a 1:10 dilution of 1 µM sterile 0.81 µm latex bead particles. The cells were incubated at 37 degrees for 1 hour then cells were washed three times with sterile PBS to remove excess beads. Cells were examined under light microscopy as shown in figure 3.4 with purity described as number of cells containing particles divided by total number of cells per high power field. Repeated examination of cultured Kupffer Cells had a greater than 95% purity.
2.5.3 Trypan blue exclusion

Viable cells with an intact functional cell membrane are capable of excluding particular dyes including trypan blue. When cells become non-viable and lose their intact cell membrane, dye is able to permeate into the cell and develop a blue colour. Thus viable cells remain clear under light microscopy whereas non-viable cells are blue in colour.

100μl of 0.4% Trypan blue was added to 1 ml of Kupffer Cell suspension. After 5 minutes, the cell suspension was loaded onto a Neubauer hemacytometer and cells were counted under light microscopy. Dividing the number of blue cells by the total cell number per grid view assessed the percentage of non-viable cells. Viability was consistently between 90% and 95%.
Figure 2.4. Kupffer Cells Containing Phagocytosed Latex Beads.

Isolated Kupffer Cells were incubated at 37 degrees for 1 hour in DMEM containing 1:10 dilution of 0.81 µm latex bead particles. The cells were washed and examined under a light microscope. Cell purity was determined by counting the number of cells containing latex particles divided by the total number of cells and then multiplied by 100.
2.6 Molecular Methods

2.6.1 Extraction of RNA from Kupffer Cells.

Total RNA was extracted from Kupffer Cells using the Qiagen RNeasy kit according to manufacturer's instructions. In brief, all media was aspirated from the wells and Kupffer Cells were washed twice with PBS with the final wash being completely aspirated from the well. 350ul of RLT lysis buffer (provided) was added to one well of cultured Kupffer Cells, the wells were thoroughly scraped to lyse all cells. The cell lysate was transferred to an eppendorf tube and combined with 350ul of 70% ethanol.

This solution was applied to a RNeasy spin column with a collection tube fitted below, and centrifuged for 15 seconds at 10 000 RPM. The flow through was discarded. The column was then washed with 700ul of RW1 buffer (provided) and centrifuged at 10 000 RPM for 15 seconds. The spin column was then washed twice with 500ul of RPE buffer (provided) and centrifuged for 15 seconds at 10,000 RPM. Prior to elutriation of RNA, the column was spun at 10 000 RPM for 2 minutes to prevent carryover of buffer. The RNA was elutriated from the spin column by adding 30ul of RNase free water (provided) and centrifuging the column, with a fresh eppendorf tube, at 10,000 RPM for 1 minute.

2.6.2 Determination of RNA concentration

RNA concentration and quality was determined using the nanodrop ND-1000 spectrophotometer (ThermoScientific, Wilmington, DE). A blank was then performed with 1 µl of RNA-free water. Each RNA sample was analysed by pipetting 1 µl of sample onto the pedestal then lowering the lever arm. Between each analysis, the pedestal was wiped clean with a lint-free wipe.

The provided software calculated an RNA concentration for each sample and each samples absorbance at 230 nm, 260 nm and 280 nm wavelengths. A 260/280 ratio of
greater than 2.0 signifies RNA purity and a 260/230 ratio of between 1.8 and 2.2 signifies low level of guanidine salt contamination.

2.6.3 cDNA synthesis

cDNA was reverse transcribed from mRNA using an Invitrogen Superscript First Strand Synthesis system according to the manufacturer's instructions. RNA was diluted to a final concentration of 500 ng mRNA in 11 ml of water. 11 µl of mRNA, 1 µl of dNTPs (as provided) and 1 µl of oligo-dT primer (as provided) were combined well, incubated at 65°C for 5 minutes and then left on ice for 1 minute. A reaction mixture containing 4 µl of first strand buffer, 1 µl of 0.1 M DTT, 1 µl of RNase inhibitor and 1 µl of SuperScript III Reverse Transcriptase was prepared and mixed with the mRNA/oligo-dT mixture prepared above. This was incubated at 25°C for 5 minutes and then 50°C for 60 mins; incubating at 70°C for 15 minutes inactivated the reaction. The resultant cDNA was made up to 50 µl with diethylpyrocarbonate (DEPC) - treated water and stored at 4 degrees C.

2.6.4 Real time quantitative Polymerase Chain Reaction

Real time (RT)-qPCR was performed using a Stratagene Mx3000 QPCR system with Platinum® SYBR Green qPCR Master Mix (Invitrogen). SYBR Green fluoresces when bound to double-stranded DNA, and allows the accumulation of a PCR product to be monitored over time. The Platinum® SYBR Green qPCR SuperMix-UDG contains Platinum® Taq DNA Polymerase and SYBR® Green I dye.

The diluted cDNA from was used as the DNA template, while housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeper gene to normalise the RT qPCR results. Internal standards were used for all experiments; a pooled cDNA sample was made by adding 5 µl of each cDNA sample to be analysed, this was serially diluted 1:5 with DEPC water construct a total of 7 standards that were used to construct a standard curve during qPCR analysis. Each
reaction well contained a 20 µl reaction made up of 10 µl of Platinum® SYBR Green Master Mix, 0.4 µl of a 10 µM forward primer and 0.4 µl of a 10 µM reverse primer, 4.2 µl of water and 5 µl of cDNA. The forward and reverse primers sequences for all genes are listed in Table 2.1. The primer pairs all crossed introns. Each gene analysis experiment contained a no-treatment control and a standard curve. Each value was performed in duplicate.

The cycling conditions used were 50°C for 2 minutes then 95°C for 2 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 45 seconds. Melt curve dissociation occurred at the completion of the run to confirm that specific products were generated and that fluorescence was not a result of primer-dimer formation.

Results were analysed using the Stratagene software program. A baseline was set using the linear view and then the log scale was used to automatically detect the cycle threshold (Ct) line. A numerical Ct value for each sample was calculated with the stratagene software. The Ct values for all genes were normalised with the average value of the duplicated reading of the GAPDH housekeeping gene. The fold change of each gene from baseline was then calculated using ΔΔCt data analysis method.
<table>
<thead>
<tr>
<th>GENE</th>
<th>Forward (F) or Reverse (R)</th>
<th>Primer sequences</th>
</tr>
</thead>
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<td>GAPDH</td>
<td>F</td>
<td>5'-TGG GAA GCT GGT CAT CAA C-3'</td>
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<tr>
<td></td>
<td>R</td>
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2.7 Western Blot

2.7.1 Protein Extraction for Western Blot
Cultured Kupffer Cells were washed twice with ice cold PBS following which, all remaining PBS was aspirated from the wells. Cells were lysed by the addition of 200µl of cold RIPA buffer containing 1:100 protease inhibitors and 1:100 phosphatase inhibitors and thorough scraping of the wells with a plastic scraper to loosen adherent cells. The cell lysate was transferred to an eppendorf tube and kept on ice prior to protein quantification.

2.7.2 Protein Quantification
Proteins were quantified using the Bio-Rad DC Protein Assay as per the manufacturers instructions. A series of 5 Bovine Serum Albumin (BSA) standards ranging from 0.1 mg/ml to 1.5 mg/ml were diluted to enable a standard curve. 5 µl of either a standard or protein lysate was pipetted into a Microtiter plate. 25 µl of the prepared Reagent A (supplied), copper tartrate solution and 1:50 Reagent S (supplied) was added to each well and the plate was gently agitated. After 15 minutes the absorbances were read at 750 nm.
A standard curve constructed using the standards and concentration of protein samples was subsequently determined.

2.7.3 Polyacrylamide Gels for Western Blot
A 7.5% or polyacrylamide separation gel was made up with 20 ml of 30% acrylamide, 48.8 ml of distilled water, 10 ml of 3 M Tris (pH8.8), 850 µl of 10% sodium dodecyl sulphate (SDS), 400 µl of 10% ammonium persulphate (APS) and 35 µl of tetramethylethlenediamine (TEMED). To make a 10% gel, 26.6 ml of acrylamide and 42.2 ml of distilled water were used. The solution was poured between 2 glass sheets held together in a gel cassette leaving a 3cm space at the top for the sample well comb and stacking gel. The gel was left to set at room temperature. Once the separation gel
was set, a stacking gel was made using 2.6 ml of 30% acrylamide, 14.7 ml of distilled water, 2.5 ml of 1 M Tris (pH 6.8), 200 µl of 10% SDS, 100 µl of APS and 20 µl of TEMED. The stacking gel was poured on top of the separation gel and a comb was inserted.

### 2.7.4 Western Blot Buffers

Loading buffer was made by adding 188 µM Tris (pH 6.8), 30% glycerol, 3% SDS and 5% bromophenol blue in distilled water.

Running Buffer was made by adding 30 g of Tris, 44 g of glycine, 10 g of SDS and making up to 1 litre with distilled water.

Transfer Buffer was made by adding 3 g of Tris, 14.4 g of glycine, 200 ml of methanol and making up to 1 litre with distilled water.

TBST was made with 60.6 g of TRIS and 87.2 g of NaCl made up to 1 litre with distilled water and supplementing with 0.1% TWEEN.

### 2.7.5 Preparation and Loading of Proteins

20 µg protein was loaded into each well of a large gel and 10 µg of protein was loaded into each well of a mini gel. 3x loading buffer was prepared by adding 75 µl of beta-mercaptoethanol (BME) to 550 µl of 3x buffer. Samples were diluted 2 parts sample to 1 part 3x loading buffer and denatured at 37°C for 5 minutes prior to being loaded into a well. 20 µl of Page Ruler plus pre-stained protein ladder were loaded into one well of each gel.

### 2.7.6 Protein Separation by Electrophoresis

The loaded gel cassette was inserted into the electrophoresis tank and the tank was filled with running buffer. The gel was ran at 145V for approximately 30 minutes or until the samples were through the stacking gel and then at 170V for approximately 2 hours or until the samples had reached to bottom of the gel. Once all proteins were separated, the cassette was disassembled and the gel was carefully removed for protein transfer.
2.7.7 Protein Transfer

Polyvinylidene fluoride (PVDF) membranes were activated by soaking in ethanol for 2 minutes. The gel was placed over a membrane and air bubbles were removed by gentle rolling. The gel and membrane were sandwiched between transfer buffer-soaked filter paper and a sponge, air-bubbles were removed and placed in a transfer cassette. A transfer was performed overnight at 50V in a 4 degree Celsius cool room. Membranes were checked for full transfer of the marker ladder.

2.7.8 Antibody Conjugation

Membranes were washed 3 times with TBST prior to blocking as in table 2.2 below for 1 hour at room temperature with gentle agitation. Primary antibody conjugation was performed overnight at 4 degrees Celsius with gentle agitation. Membranes were then washed with TBST 3 times to remove any non-adherent antibody then exposed to the secondary antibody for 1 hour with gentle agitation at room temperature then washed 3 times with TBST.

2.7.9 Detection and Quantification of Protein Bands

Amersham reagent was applied to membranes for 2-5 minutes. Protein bands were detected with by exposing the membrane to photographic film that was developed in the dark room. Band quantification was determined using the Kodak M1 quantification.
2.8 ELISA

2.8.1 Collection of Media

Media was aspirated from Kupffer Cells and transferred to Eppendorf tubes. To pellet any contaminating particular matter, tubes were centrifuged at 1500 RPM for 10 minutes at 4 degrees Celsius. Supernatant was collected and stored in a fresh eppendorf tube at -80 degrees Celsius.

2.8.2 ELISA Technique

Commercially available ELISA kits for IL10, TNFα and IL6 were purchased from R&D Systems (MN, USA). The manufactures instructions were followed. Culture media used for TNFα detection was diluted 1:3 with the supplied Calibrator Diluent RD5-17 (supplied). IL10 and IL6 media was not diluted. For each gene tested, the prepared standard was serially diluted 1:2 six times to construct standards. 50 µl of assay diluent was added to each well of the ELISA plate followed by 50 µl of each standard, sample and negative control in each well. Samples were performed in duplicate. The plate was gently tapped to ensure mixing, then covered and left to incubate for 2 hours at room temperature. Each well was aspirated and washed five times, then 100 µl of conjugate was added to each well for a further 2 hours incubation time. The wells were aspirated and washed five more times then 100 µl of substrate solution was added away from light. The plate was left for 30 more minutes at room temperature then 100 µl of stop solution was added. The plate was read at 450 nm and corrected at 540 nm using the microplate reader.
2.9 Statistical Methods

All experiments were repeated 3 to 6 times. Statistical analysis was performed using ANOVA for comparison of more than 3 treatment groups or the Student’s t-test to compare isolated data between treatment and control groups only (Prism 6 for Macintosh, version 6.0e, Graphpad Software Inc, La Jolla, Ca, USA). The probability level of \( p \leq 0.05 \) was set for statistical significance. Results are expressed as mean values relative to the control non-primed group with the standard error of the mean.
Chapter 3 The Modulatory Effects of Adiponectin upon LPS-induced cytokine changes in Kupffer Cells.
3.1 Introduction

NAFLD is the most common liver disease in developed nations with a prevalence estimated to be between 20-30% [3, 4]. Whilst the majority of individuals with NAFLD have benign steatosis and an associated low rate of liver-related morbidity and mortality, a small sub-set of individuals develop NASH with an associated high rate of liver-related morbidity and mortality. Multiple pathological processes are likely to contribute towards the development of NASH including LPS and modulation of its effect by adiponectin.

Endotoxaemia is a common finding in NAFLD, with NASH patients have higher circulating LPS levels than those with bland hepatic steatosis or no hepatic steatosis [94, 96]. Serum adiponectin levels are inversely associated with NAFLD [283] with several studies describing lower circulating adiponectin levels in those with NASH when compared to those with benign steatosis alone [284, 285]. In contrast to LPS, adiponectin is negatively associated with the pro-inflammatory cytokines IL6 and TNFα and positively associated with the anti-inflammatory cytokine IL10 [274].

Previous studies investigating the effects of adiponectin on LPS-induced pro-inflammatory cytokine synthesis and release in macrophages and Kupffer Cells are limited to models in which cells were exposed to adiponectin prior to LPS exposure. In these cell culture models, adiponectin pre-exposure was demonstrated to decrease LPS-induced TNFα in cultured human macrophages, RAW 264.7 macrophages, human leukocytes and rat Kupffer Cells [227, 229, 230, 277, 294, 296-298]. The direct “additive” effects of adiponectin and LPS upon macrophages or Kupffer Cells have not been well described with only a single study describing an increase in the anti-inflammatory cytokines IL10 and IL1RA in primary human macrophages treated with adiponectin and LPS, compared to those treated with either adiponectin or LPS alone [229].
The aims of the studies presented in this chapter are therefore to describe the direct and additive effects of LPS and adiponectin on Rat Kupffer Cell cytokine synthesis and the effect of adiponectin pre-treatment upon LPS-induced cytokines in Rat Kupffer Cells. Cytokines measured are those that have been demonstrated to have important pro-inflammatory, pro-fibrotic or anti-inflammatory roles in NASH; these include TNFα, IL6, TGFβ, CTGF, PDGF and IL10.
3.2 Methods

3.2.1 Isolation and Culture of Primary Rat Kupffer Cells.
Primary Kupffer Cells were isolated from male Sprague-Dawley Rats using the previously described two-stage collagenase and pronase digestion followed by centrifugal elutriation.

3.2.2 Cell Culture Technique
Kupffer Cells were plated into plastic 6 well cell culture plates at a density of $3 \times 10^6$ cells per well with 2 ml of DMEM media containing 10% FCS and P/S per well. Kupffer Cells from more than one rat were not combined. Cells were incubated overnight at 37 degrees with 5% CO$_2$.

In the first experimental protocol, Day 1 Kupffer Cells were treated with vehicle PBS (control group), adiponectin (variable concentrations) and/or LPS (50 ng/ml) and/or polymixen (33 µg/ml). After 18h of incubation, Kupffer Cell media was aspirated and stored for ELISA analysis, and cells were lysed for mRNA extraction.

In the second experimental protocol (Figure 3.1), Day 1 Kupffer Cells were treated with vehicle (control group) or adiponectin (1 µg/ml). After 18h of incubation, Kupffer Cell media was aspirated and replaced with 1 ml of fresh media containing LPS (50 ng/ml) or LPS and Adiponectin (1 µg/ml). Kupffer Cells were incubated for a further 4 hours. Media was then aspirated and stored for ELISA and cells were lysed for mRNA extraction.
Figure 3.1 Experimental Protocol: Adiponectin Primed or Non-Primed Kupffer Cells.

Kupffer Cells were primed in 1 µg/ml adiponectin containing media or remained non-primed in culture media alone. After 18 hours, Kupffer Cells were washed and fresh media containing 50 ng/ml LPS +/- 1 µg/ml adiponectin was applied for 4 hours.

KC: Kupffer Cells
3.2.3 Molecular Techniques

mRNA was extracted from Kupffer Cells using the Quigen RNeasy kit according to the manufacturers instructions. RNA yield and purity was determined using the Thermo Scientific Nanodrop. 200 ng of RNA was reverse transcribed to cDNA using the Invitrogen Superscript First Strand Synthesis System using the manufacturers instructions.

3.2.4 Quantitative Real Time Polymerase Chain Reaction

Standards were diluted 1 in 50, 1 in 10 (IL10) or 1 in 2 (IL6) for analysis. The Stratagene Mx3000 QPCR System with Platinum SYBR Green qPCR Master Mix (Invitrogen) was used for all samples. GAPDH was the housekeeper gene and primer sequences were as described in table 2.1. All samples were performed in duplicate. Results were analysed using the Stratagene software program.

3.2.5 ELISA

TNFα, IL6 and IL10 were measured in cell culture media using Rat ELISA Quantikine® Kits (R&D Systems, Minneapolis, MN, USA) according to manufacturers instructions.

3.2.6 Statistical Analysis

All experiments were repeated 3 to 6 times. Statistical analysis was performed using ANOVA to compare data between different treatment groups (Prism 6 for Macintosh, version 6.0e, Graphpad Software Inc, La Jolla, Ca, USA). The probability level of p≤0.05 was set for statistical significance. Results are expressed as mean values relative to the control group with the standard error of the mean.
3.3 Results

3.3.1 Adiponectin Dose

As IL10 is consistently up regulated in adiponectin-stimulated macrophages and Kupffer Cells, it was used as a marker of response in this dose finding experiment [229-231]. IL10 mRNA was measured following Kupffer Cell stimulation with variable adiponectin doses or forms (globular or full-length) to obtain the ideal adiponectin form and dose to use in this thesis.

IL10 expression was significantly increased in Kupffer Cells that were treated with full-length adiponectin or globular adiponectin at doses of 1 µg/ml, 2.5 µg/ml or 5 µg/ml (Figure 3.2). Compared to control Kupffer Cells, globular adiponectin stimulation resulted in a 23.15 fold-increase in IL10 mRNA at a media concentration of 1 µg/ml (p<0.005), 28.66 fold-increase at 2.5 µg/ml (p<0.05) and 31.1-fold increase when used at 5 µg/ml (p<0.05). Full-length adiponectin stimulation resulted in a 23.13 fold-increase in IL10 mRNA when used at media concentrations of 1 µg/ml (p<0.05), 23.65 fold-increase at a concentration of 2.5 µg/ml (p<0.005) and 30.22 fold increase at 5 µg/ml (p<0.05) compared to controls.

As there was no significant difference in IL10 expression between either full-length or globular adiponectin or between adiponectin doses; the lowest effective dose 1 µg/ml of full-length adiponectin was used for the remainder of the experiments.
Figure 3.2. Kupffer Cell IL10 mRNA expression is up-regulated by variable doses of globular or full-length adiponectin

Cultured Rat Kupffer Cells were stimulated with variable concentrations of either globular or full-length adiponectin for 18 hours. IL10 mRNA levels were determined by qPCR using GAPDH as the housekeeper gene. Results are expressed as fold-change from the non-stimulated control group.

n=4
3.3.2 The anti-inflammatory cytokine IL10 is induced by adiponectin and LPS in Rat Kupffer Cells.

In primary Rat Kupffer Cells that were exposed to adiponectin or LPS for 18 hours, IL10 mRNA and peptide were significantly increased in Kupffer Cells compared to non-exposed control Kupffer Cells (Figure 3.3). IL10 mRNA expression was up regulated 19.48 fold (p<0.05) in adiponectin-treated Kupffer Cells and 20.12 fold (p<0.05) in LPS treated cells when compared to control Kupffer Cells. When Kupffer Cells were treated with both adiponectin and LPS, a non-statistically significant 36.48 fold increase in IL10 mRNA was observed compared to the control group. There was no statistically significant difference in IL10 mRNA expression between groups.

Both adiponectin and LPS treatment resulted in a statistically significant increase in Kupffer Cell-derived IL10 peptide. Media concentration of IL10 peptide in control Kupffer Cells was 133.1 pg/ml; 412.7 pg/ml in adiponectin treated Kupffer Cells (p<0.05 compared to control); 335.9 pg/ml in LPS treated Kupffer Cells (p<0.05 compared to control) and 317.4 pg/ml in both adiponectin and LPS treated Kupffer Cells. There was no statistically significant difference in IL10 peptide between treated Kupffer Cell groups.

3.3.3 The Pro-inflammatory Cytokines TNFα and IL6 are induced by Adiponectin and LPS in Rat Kupffer Cells.

In Kupffer Cells that were exposed to adiponectin and/or LPS for 18 hours an increase in TNFα and mRNA and peptide was observed (Figure 3.4). TNFα mRNA expression was up regulated 3.29-fold (p<0.005) in adiponectin-treated Kupffer Cells and 2.38-fold (p<0.05) in LPS treated cells compared to non-exposed control Kupffer Cells. Kupffer Cells treated with both adiponectin and LPS together had a non-statistically significant 1.34 fold increase in TNFα mRNA expression. There was no statistically significant
Figure 3.3 Kupffer Cell IL10 increases following exposure to adiponectin and/or LPS.

Cultured Rat Kupffer Cells were cultured for 18h in adiponectin (ADN) 1 µg/ml and/or LPS 50 ng/ml. A: IL10 mRNA expression was determined by qPCR, with normalization to GAPDH housekeeper gene. Results are expressed as fold-change from the non-stimulated control group. B: IL10 concentration was determined in Kupffer Cell media by ELISA.
difference in TNFα mRNA expression between treatment groups. The mean TNFα concentration in the media of non-treated Kupffer Cells was 65.86 pg/ml; 3168 pg/ml in adiponectin treated Kupffer Cells (p<0.005 compared to control group); 3290 pg/ml in LPS treated Kupffer Cells (p<0.0005 compared to control group) and 2966 pg/ml in both adiponectin and LPS stimulated Kupffer Cells (p<0.005 compared to control group). Again, there was no statistically significant difference in TNFα peptide between treated Kupffer Cell groups.

IL6 mRNA and peptide was increased in Kupffer Cells that were exposed to 18h of adiponectin and/or LPS (Figure 3.5). IL6 mRNA expression was up regulated 4.12 fold (p<0.05) in adiponectin-treated Kupffer Cells and 5.49 fold (p<0.05) in LPS treated cells compared to control Kupffer Cells. Kupffer Cells treated with both adiponectin and LPS together had a non-statistically significant 3.19 fold increase in IL6 mRNA expression, compared to the control group. There was no significant difference in IL6 mRNA expression between treatment groups. After 18h, the mean IL6 concentration in the media of non-treated Kupffer Cells was 116.6 pg/ml; 3614 pg/ml in adiponectin treated Kupffer Cells (p<0.005 compared to control); 4010 pg/ml in LPS treated Kupffer Cells (p<0.0005 compared to control) and 3983 pg/ml in combined adiponectin and LPS stimulated Kupffer Cells (p<0.0005). There was no statistically significant difference in IL6 peptide between treated Kupffer Cell groups.
Figure 3.4 Kupffer Cell TNFα is up-regulated following exposure to Adiponectin and/or LPS.

Cultured Rat Kupffer Cells were exposed to 18h adiponectin (ADN) 1 µg/ml and/or LPS 50 ng/ml. TNFα mRNA expression was determined by qPCR using GAPDH as the housekeeper gene. Results are expressed as fold-change from the non-stimulated control group (A). Kupffer Cell media TNFα concentration was determined through ELISA (B).
Cultured Rat Kupffer Cells were exposed to 18h adiponectin (ADN) 1 µg/ml and/or LPS 50 ng/ml. A: IL6 mRNA expression was determined by qPCR using GAPDH as the housekeeper gene. Results are expressed as fold-change from the non-stimulated control group. B: Kupffer Cell media IL6 concentration was determined through ELISA.

Figure 3.5 Kupffer Cell IL6 is up-regulated following exposure to Adiponectin and/or LPS.
3.3.4 The Pro-Fibrotic Cytokines TGFβ, CTGF and PDGF are suppressed by

Adiponectin and LPS in Rat Kupffer Cells.

When Kupffer Cells were exposed to 18h of either adiponectin and/or LPS; TGFβ, PDGF and CTGF mRNA synthesis was reduced (Figure 3.6).

Adiponectin-treated Kupffer Cells had a 1.49-fold relative reduction in TGFβ mRNA, LPS-treated Kupffer Cells had a significant 2.32-fold (p<0.05) relative reduction in TGFβ mRNA and combined adiponectin and LPS-treated Kupffer Cells had a 1.92-fold (p<0.05) relative reduction in TGFβ mRNA when compared to non-treated control Kupffer Cells.

Compared to the control group, adiponectin-exposed Kupffer Cells had a significant 5.36-fold (p<0.0005) relative reduction in PDGF mRNA, LPS-treated Kupffer Cells had a significant 5.77-fold (p<0.0005) relative reduction in PDGF mRNA and adiponectin and combined adiponectin and LPS-treated Kupffer Cells had a significant 6.52-fold (p<0.0005) relative reduction in PDGF mRNA when compared to non-treated control Kupffer Cells.

Adiponectin-treated Kupffer Cells had a significant 4.54-fold (p<0.005) relative reduction in CTGF mRNA when compared to control Kupffer Cells, LPS-treated Kupffer Cells had a significant 5.77-fold (p<0.0005) relative reduction in CTGF mRNA compared to control Kupffer Cells and combined adiponectin and LPS-treated Kupffer Cells had a significant 3.41-fold (p<0.005) relative reduction in CTGF mRNA when compared to non-treated control Kupffer Cells.
Figure 3.6 Kupffer Cell TGFβ, PDGF and CTGF mRNA is down-regulated following exposure to Adiponectin and/or LPS.

Rat Kupffer Cells were cultured in adiponectin (ADN) 1 µg/ml and/or LPS 50 ng/ml for 18h. TGFβ (A), PDGF (B) and CTGF (C) mRNA expression was determined by qPCR using GAPDH as the housekeeper gene. Results are expressed as fold-change from the non-stimulated control group.
3.3.5 Adiponectin is not contaminated with LPS.

Polymyxin B is an antibiotic with bactericidal properties against gram-negative bacteria and has been used in research settings to ensure LPS-clearance of reagents. To ensure that LPS did not contaminate the adiponectin used in these experiments, TNFα mRNA was determined following Kupffer Cell treatment with adiponectin and polymyxin B. Cultured primary rat Kupffer Cells were treated for 18 hours with PBS vehicle, adiponectin 1 µg/ml or adiponectin 1 µg/ml and polymyxin B 33 µg/ml. Kupffer Cells treated with adiponectin had a significant 3.93-fold (p<0.05) increase in TNFα mRNA compared to controls (Figure 3.7). Kupffer Cells treated with adiponectin and polymyxin B had a significant 2.73-fold (p<0.05) increase in TNFα mRNA compared to controls. There was no significant difference in TNFα mRNA between the two adiponectin-treated groups of Kupffer Cells thereby excluding LPS-contamination.

3.3.6 Adiponectin pre-treatment has no effect on LPS-induced IL10 by Kupffer Cells.

As Kupffer Cells are typically tolerant, the effects of 18h adiponectin pre-exposure prior to subsequent LPS exposure were examined in a two-stage protocol. In Kupffer Cells that were not pre-exposed to adiponectin (non-primed Kupffer Cells), 4 hours of LPS exposure resulted in a statistically significant 206.2-fold (p<0.05) increase in IL10 mRNA compared to non-exposed control Kupffer Cells (Figure 3.8). This was similar to the finding of a 244.1-fold (p<0.05) increase in IL10 mRNA in adiponectin pre-exposed (primed Kupffer cells) prior to LPS treatment. Keeping Kupffer Cells in adiponectin-containing media during LPS treatment did not alter IL10 mRNA synthesis, with a 241-fold (p<0.005) increase observed in IL10 mRNA when primed Kupffer Cells were treated with subsequent adiponectin and LPS. There was no difference detected between any of the treatment groups.
Figure 3.7 Adiponectin-induced TNFα mRNA is not down regulated by polymyxin B in Kupffer Cells.

Primary Rat Kupffer Cells were cultured for 18h in adiponectin (ADN) 1 µg/ml and polymyxin B 33.3 µg/ml. TNFα mRNA expression was determined by qPCR and normalized to GAPDH. Results are expressed as fold-change from the non-stimulated control group.
In assessing IL10 peptide; control Kupffer Cells had an IL10 media concentration of 47.02 pg/ml. Compared to control Kupffer Cells, non-primed Kupffer Cells had a non-significant increase in IL10 peptide to 353.3 pg/ml following 4h of LPS exposure, adiponectin primed Kupffer Cells had a statistically significant increase in IL10 media concentration to 219.9 pg/ml (p<0.05) and adiponectin primed Kupffer Cells exposed to 4h of LPS and adiponectin together had an IL10 media concentration of 300.3 pg/ml. There was no statistically significant difference between any of the treatment groups.

3.3.7 Adiponectin pre-exposure decreases LPS-induced pro-inflammatory cytokines TNFα and IL6 in Kupffer Cells.

Adiponectin pre-exposure decreased Kupffer Cell TNFα and IL6 in response to LPS exposure (Figure 3.9).

Non-adiponectin primed Kupffer Cells had a significant 506.4-fold (p<0.05) increase in TNFα mRNA following 4h of LPS compared to controls. This was significantly reduced to 10.06-fold (p<0.05) when Kupffer Cells were primed with adiponectin for 18 hours prior to LPS exposure. Exposing primed Kupffer Cells to 4h of combined LPS and adiponectin did not alter TNFα mRNA at 7.12-fold from baseline, compared to 4h of LPS alone.

Non-primed and non-LPS exposed, control Kupffer Cells had a TNFα media concentration of 71.54 pg/ml. When non-primed Kupffer Cells were exposed to LPS, the resultant media concentration of TNFα was 2991 pg/ml; this decreased significantly to 1247 pg/ml (p<0.05) in adiponectin primed Kupffer Cells.
Figure 3.8 LPS Induces IL10 in Both Adiponectin Primed and Non-Primed Kupffer Cells.

Cultured Rat Kupffer Cells were primed for 18h in adiponectin (ADN) 1 µg/ml or remained non-primed (cultured in media alone) prior to treatment with LPS 50 ng/ml +/- adiponectin 1 µg/ml. A: IL10 mRNA expression was determined by qPCR and normalized to GAPDH. Results are expressed as fold-change from the non-stimulated control group. B: IL10 peptide concentration in Kupffer Cell media was determined by ELISA.
Non-adiponectin primed Kupffer Cells had a significant 16.71-fold (p<0.05) increase in IL6 mRNA following 4h of LPS exposure when compared to control, non-stimulated Kupffer Cells. This was significantly reduced to 3.21-fold (p<0.05) when Kupffer Cells were primed with adiponectin prior to LPS exposure. Again, exposing primed Kupffer Cells to 4h of combined LPS and adiponectin did not alter IL6 mRNA compared to LPS alone.

Control Kupffer Cells had an IL6 peptide concentration of 44.53 pg/ml. Non-primed Kupffer Cells exposed to LPS had statistically significant increase in IL6 peptide of 2991 pg/ml (p<0.05) compared to control cells. When compared to non-primed Kupffer Cells, adiponectin-primed Kupffer Cells had a significant decrease in TNFα concentration to 565.2 pg/ml (p<0.05) following LPS treatment.

3.3.8 Adiponectin pre-treatment further reduces the pro-fibrotic cytokines TGFβ, CTGF and PDGF in Kupffer Cells.

Adiponectin primed Kupffer Cells have suppressed expression of the pro-fibrotic cytokines TGFβ, CTGF and PDGF (Figure 3.10). Non-primed Kupffer Cells had a significant 2.03 fold (p<0.05) increase in TGFβ mRNA following LPS exposure compared to control cells. However, when adiponectin-primed Kupffer Cells were exposed to LPS, TGFβ mRNA decreased significantly to 0.52 fold (p<0.005) of baseline or 0.57 fold (p<0.005) when exposed to LPS in the ongoing presence of adiponectin.

Non-adiponectin primed Kupffer Cells had a non-significant 1.76 fold increase in CTGF mRNA following LPS treatment compared to control Kupffer Cells. When Kupffer Cells were primed with adiponectin prior to LPS exposure, CTGF mRNA decreased significantly to 0.14 fold (p<0.05) of baseline or 0.19 fold (p<0.05) when exposed to LPS in the ongoing presence of adiponectin.
Figure 3.9 LPS-Induced TNFα and IL6 is down-regulated in Adiponectin Primed Kupffer Cells.

Rat Kupffer Cells were primed for 18h in adiponectin (ADN) 1 µg/ml or cultured in media alone prior to treatment with LPS 50 ng/ml +/- adiponectin 1 µg/ml. A: TNFα mRNA expression was determined by qPCR. B: TNFα concentration in media was determined by ELISA. C: IL6 mRNA expression by qPCR. D: IL6 media concentration was by ELISA. qPCR normalized to GAPDH and results are expressed as fold-change from the non-stimulated control group.
Non-adiponectin primed Kupffer Cells had a 1.87 fold increase in PDGF mRNA following exposure to 4 hours of LPS compared to control Kupffer Cells. This upregulation was abrogated when Kupffer Cells were primed with adiponectin prior to LPS exposure and PDGF mRNA decreased to 0.07 fold (p<0.05) of baseline or 0.06 fold (p<0.05) when exposed to LPS in the ongoing presence of adiponectin.
Figure 3.10 LPS-Induced TGFβ, PDGF and CTGF are down-regulated in Adiponectin Primed Kupffer Cells.

Cultured Rat Kupffer Cells were primed for 18h in adiponectin 1 µg/ml or cultured in media alone then exposed to LPS 50 ng/ml +/- adiponectin 1 µg/ml. TGFβ (A), CTGF (B) and PDGF (C) mRNA expression was determined by qPCR and normalized to GAPDH. Results are expressed as fold-change from the non-stimulated control group.
3.4 Conclusion

The pathogenesis of NAFLD and NASH is multi-factorial, which under the classic “two hit” hypothesis consists of initial lipid accumulation within the hepatocytes followed by an inflammatory and fibrotic response with associated hepatocyte necrosis and development of hepatic fibrosis [70]. Multiple “liver hits” including insulin resistance, altered levels of adipokines, small bowel bacteria and anti-oxidant deficiency have been suggested as contributing towards NAFLD and NASH. In particular, recent studies suggest that high serum levels of adiponectin [179] and low serum levels of LPS [311] are associated with low incidence of NAFLD and NASH. In an effort to elucidate the potential anti-inflammatory and anti-fibrotic effects of adiponectin upon Kupffer Cells, cultured rat Kupffer Cells were stimulated with LPS and either pre or co-currently treated with adiponectin and subsequent cytokine changes were measured.

The studies included in this chapter demonstrate that adiponectin has varied effects on Kupffer Cells; in particular both pro- and anti-inflammatory effects; and anti-fibrotic effects were observed. These findings are consistent with conflicting reports of adiponectin effects upon macrophages in the literature. In this study 18 hours of adiponectin treatment was found to increase Kupffer Cell IL10, TNFα and IL6 synthesis and release, and decrease TGFβ, CTGF and PDGF synthesis. In addition, 18 hours of LPS treatment was found to up regulate IL10, TNFα and IL6, and down regulate TGFβ, PDGF and CTGF. Kupffer Cells that were cultured in both adiponectin and LPS together did not alter any measured cytokine synthesis and release when compared to adiponectin or LPS stimulated Kupffer Cells alone. The anti-inflammatory effects of adiponectin were observed when Kupffer Cells were exposed to adiponectin and LPS in two sequential treatments. 4 hours of LPS treatment resulted in increased synthesis and release of IL10, TNFα, IL6 and TGFβ. All LPS-induced cytokines including both the pro-inflammatory cytokines TNFα and IL6, and pro-fibrotic cytokines TGFβ, CTGF
and PDGF were down regulated when Kupffer Cells were primed with adiponectin for
18 hours prior to LPS treatment. As adiponectin is known to bind LPS; LPS
contamination as a possible cause of these abhorrent inflammatory effects was excluded
by co-culturing Kupffer Cells in adiponectin and the antibiotic polymyxin B.
The results presented in this thesis are novel in that an inflammatory response to
adiponectin has not previously been described in Kupffer Cells. These results are
consistent however with the described inflammatory response to adiponectin in
monocytes and macrophages. Globular adiponectin has been shown to induce TNFα
and IL6 secretion in the THP-1 differentiated macrophage cell line, isolated human
peripheral monocytes and mouse thioglycollate-induced peritoneal macrophages [261].
In the RAW 264.7 macrophages cell line, globular adiponectin induced TNFα mRNA
synthesis and peptide secretion with TNFα mRNA synthesis peaking following 2 hours
of treatment and peptide peaking after 8 hours of treatment [230]. In differentiated
THP-1 cells and primary human monocytes, both high molecular weight and low
molecular weight adiponectin resulted in increased LPS-induced IL6 release [194].
TNFα and IL6 were both up regulated in human macrophages cultured for 24 hours
with full length adiponectin with the authors describing a predominantly M1 response
[262]. In addition, NF-κB pathway activation has been demonstrated following full
length and globular adiponectin exposure in U937 monocyte cell line [312] and full
length but not globular adiponectin in the monocyte C2C12 cell line [313].
In contrast to the above inflammatory effects of adiponectin upon Kupffer Cells,
adiponectin was also found to up regulate the immunomodulatory and anti-
inflammatory cytokine IL10 in Kupffer Cells. Adiponectin induced up regulation of
IL10 has been previously described with results in this thesis consistent with, and
reinforcing current knowledge. In previously described work, both globular and full-
length adiponectin have been shown to induce IL10 synthesis in Kupffer Cells with a
dose-dependent response observed with globular adiponectin [232]. In this thesis a dose dependent change in IL10 synthesis was not observed however this is likely to be due to the stepped adiponectin doses from 1 to 5 µg/ml used in this study compared to the multiple 10-fold dose changes used in Mandal’s study ranging from 0.01 to 1 µg/ml of globular adiponectin.

In previous studies of investigating the effects of adiponectin upon monocytes and macrophages, findings were similar to those observed in Kupffer Cells. Both LPS and adiponectin were shown to induce IL10 in porcine macrophages, with LPS and adiponectin together potentiating IL10 production [227]. Similarly in this thesis, a trend towards increased IL10 synthesis and release was observed when Kupffer Cells were treated with both adiponectin and LPS together. In differentiated THP-1 cells, low molecular weight but not high molecular weight adiponectin resulted in increased LPS-induced IL10 secretion [194]. However in contrast to the two previously mentioned studies where adiponectin induced IL10 in THP-1 cells, Wolf and colleagues found that adiponectin induced IL10 in human monocytes, macrophages and dendritic cells but not THP-1 cells, and potentiated LPS-induced IL10 release in monocytes, macrophages and dendritic cells [229]. Further studies have shown that globular adiponectin induces IL10 synthesis by RAW 264.7 macrophages, with prior TNFα secretion and ERK1/2 phosphorylation and subsequent cyclic-AMP response element binding protein activation being required to enable subsequent IL10 synthesis [230, 231]. The studies described in this thesis also demonstrated that both globular and full-length adiponectin induced IL10 synthesis and release in Kupffer Cells. This is consistent with Kupffer Cells possessing leukocyte esterase that enables globular adiponectin to be cleaved from full-length adiponectin [197]. Variability in findings between studies are likely to be due to minor phenotypic differences in monocytes and macrophages and their activation state, as well as in the source of adiponectin used by different research group. The
adiponectin used in this thesis is full length mouse adiponectin derived from human embryonic kidney cell line with 100% homology to the amino acid sequence of mouse serum adiponectin (Biovendor, Hong Kong), in contrast globular adiponectin expressed by E. coli lacks post-translation modification which allows high molecular weight multimer formation [314].

The conflicting findings described above of adiponectin inducing both a pro- (TNFα, IL6) and anti-inflammatory (IL0) response in Kupffer Cells is likely to be a typical feature of cellular components of the innate immune system. Immune cells have been demonstrated to adapt to their environment following exposure to prior “danger signals” [315] thereby mounting an inefficient or “tolerising” response to subsequent insults. This so-called memory effect appears to be characteristic for macrophages and their tissue counterparts [263] with Kupffer Cells being shown to synthesise IL10 in response to LPS and subsequently develop tolerance to further LPS [235]. Consistent with the hypothesis of innate immune cells developing memory, the subsequent studies in this chapter demonstrate that adiponectin pre-exposure prevented subsequent LPS-induced TNFα, IL6, TGFβ, CTGF and PDGF in Kupffer Cells.

Consistent with the findings of this thesis, in rat Kupffer Cells, pre-treatment with globular adiponectin prevented subsequent LPS induced TNFα [232, 305, 306]. This research group went on to demonstrate that adiponectin pre-treatment suppressed LPS-induced IκB degradation, ERK1/2 phosphorylation and early growth response-1 binding activity [305] and the effects of adiponectin are dependent upon an IL10 and HO-1 pathway with adiponectin signalling through AdipoR1 [232, 306]. In addition, adiponectin was shown to suppress LPS-induced TLR4 mRNA and surface expression [306]. There are no published reports of adiponectin pre-treatment down regulating LPS-induced IL6, TGFβ, CTGF or PDGF.
The novel observation in this thesis, that adiponectin prevents LPS-induced IL6 in Kupffer Cells is consistent with described effects of adiponectin upon LPS-activated monocytes and macrophages. Studies of the effect of adiponectin upon LPS-induced inflammatory changes in human monocytes have shown that adiponectin down regulates LPS-induced TNFα, chemokine (C-X-C motif) ligand 10 (CXCL10) and chemokine (C-X-C motif) ligand 9 (CXCL9) [316], TNFα, IL6 and inhibits IκB, JNK and P38 phosphorylation [299] and TNFα but not IL6 [294]. In LPS activated porcine macrophages, adiponectin pre-exposure abrogated TNFα and IL6 with IL6 suppression occurring via ERK1/2 inhibition and TNFα inhibition occurring via inhibition of NFκB [227]. In cell line models, low molecular weight but not high molecular weight adiponectin down regulated LPS-induced IL6 in THP-1 cells [194], globular adiponectin down regulated LPS-induced TNFα and IL6 in THP-1 differentiated macrophages [261], globular adiponectin down regulated LPS-induced TNFα in RAW 264.7 macrophages [230, 296] and adiponectin pre-treatment reduced LPS-activated NFκB activity in U937 monocytes [312].

The studies included in this thesis demonstrate that adiponectin decreases both basal and LPS-induced TGFβ, CTGF and PDGF mRNA synthesis by Kupffer Cells. This finding greatly expands current knowledge into the anti-fibrotic effects of adiponectin as no previous studies have described the anti-fibrotic effects of adiponectin upon Kupffer Cells. In an in vivo study, Kamada and colleagues found that adiponectin knockout mice developed extensive fibrosis in response to CCl₄ that was attenuated when mice were injected with an adiponectin-producing adenovirus [279]. Whilst direct anti-fibrotic effects of adiponectin upon the hepatic stellate cell have been described [317], this is the first report of adiponectin directly modulating Kupffer cell pro-fibrotic cytokines. This has important implications in hepatic fibrosis as these pro-fibrotic factors act in paracrine manner on hepatic stellate cells.
In summary, the observations described in this chapter highlight the inflammatory, anti-inflammatory and anti-fibrotic effects of adiponectin upon the Kupffer Cell. Clinically, adiponectin has an association with a predominantly anti-inflammatory state with NAFLD, type 2 diabetes and cardiovascular and elevated levels of C-reactive protein being associated with low serum levels of adiponectin [318]. There are several reports though of an association between elevated adiponectin levels and inflammatory disease including rheumatoid arthritis, chronic obstructive pulmonary disease and end stage renal disease, which adds, further confusion to this contentions issue [252, 319]. As suggested by the findings in this chapter that adiponectin pre-exposure sensitizes Kupffer Cells to subsequent LPS exposure, others have hypothesized that adiponectin may induce an initial inflammatory response that is closely followed by desensitization to subsequent stimuli [263]. To understand these contradictory effects of adiponectin, subsequent chapters of this thesis will explore further the effects of adiponectin tolerance to itself and potential mechanisms by which Kupffer Cells “switch” from inflammatory to anti-inflammatory upon adiponectin exposure.
Chapter 4 Kupffer Cell Tolerance to Adiponectin occurs in an IL10-Dependent Manner.
4.1 Introduction

Adiponectin has been demonstrated to have beneficial effects on metabolic diseases due to its insulin sensitizing and anti-inflammatory effects. In particular low adiponectin levels are associated with human obesity [179], type 2 diabetes mellitus [213], NAFLD [283] and NASH [284, 285]. Despite these well documented insulin-sensitizing and anti-inflammatory effects of adiponectin, there is increasing reports of inflammatory and fibrotic disease states being associated with elevated adiponectin levels; examples include rheumatoid arthritis, chronic obstructive pulmonary disease, end-stage renal disease and liver cirrhosis [252, 319, 320].

At a cellular level, there are reports of adiponectin promoting both an inflammatory and anti-inflammatory response. In the previous chapter, I demonstrated that 18 hours of adiponectin exposure induced a significant increase in TNFα and IL6 in isolated rat Kupffer Cells. This finding is consistent with other studies, which have demonstrated this initial increase in TNFα and IL6 in THP-1 and RAW 264.7 macrophage cell lines [261, 296]. Conversely, I also described in the previous chapter anti-inflammatory effects of adiponectin where by adiponectin pre-exposed Kupffer Cells abrogated further TNFα, IL6, TGFβ1, CTGF and PDGF release in response to LPS. Again, these finding are in keeping with published reports of adiponectin down regulating TNFα synthesis in cultured human macrophages and leukocytes and RAW 264.7 macrophages [227, 229, 230, 294, 296-298].

Recent commentary on the conflicting effects of adiponectin have focused on the theory that adiponectin may stimulate an initial inflammatory response in cells followed by desensitization of the cell to ongoing stimuli [263]. Kupffer Cells like other tissue macrophages are characteristically tolerant cells [153]. Consistent with this theory, tolerance to adiponectin with failure to continue to secrete TNFα and IL6 following
repeated adiponectin exposure has been demonstrated in THP-1 macrophages, though the underlying mechanism for this tolerance has not been fully elucidated [261]. In macrophages, IL10 is important in mediating the “switch” from a cell that produces inflammatory cytokines in response to toxic stimuli, to a cell that is tolerant to ongoing toxic insult and ceases to continue producing inflammatory cytokines [233-235]. As described in the previous chapter, IL10 is produced by Kupffer Cells in response to adiponectin and therefore has the potential to contribute towards adiponectin tolerance in Kupffer Cells by creating an auto-feedback loop.

Alterations in cell surface receptors represent another potential mechanism for cellular tolerance. Adiponectin signals through 3 different receptors, AdipoR1, AdipoR2 and T-cadherin and although alterations in adiponectin receptors has been described in various disease states, alteration in Kupffer Cell surface receptors has not been previously described.

In this chapter the development of adiponectin tolerance in primary Kupffer Cells will be studied, with focus on the inflammatory cytokines TNFα and IL6. To understand the mechanism by which tolerance to adiponectin develops in Kupffer Cells, changes in adiponectin receptor synthesis, alterations in adiponectin form and the role of IL10 in the development of tolerance will be explored.
4.2 Methods

4.2.1 Isolation and Culture of Primary Rat Kupffer Cells.

Primary Kupffer Cells were isolated from male Sprague-Dawley Rats using the previously described two-stage collagenase and pronase digestion followed by centrifugal elutriation.

4.2.2 Cell Culture Technique

Kupffer Cells were plated into plastic 6 or 12 well cell culture plates at a density of 3 x 10^6 cells per well (6 well plate) or 2 x 10^6 cells per plate (12 well plate) with 2 ml of DMEM media containing 10% FCS and P/S per well. Kupffer Cells from different rats were not combined and were plated to enable one experiment consisting of multiple paired treatments. Cells were incubated overnight at 37 degrees with 5% CO₂.

In the experimental protocol (Figure 4.1), Day 1 Kupffer Cells were treated with vehicle (control group) or adiponectin (1 µg/ml). After 18h of incubation, Kupffer Cell media was aspirated; cells were washed with PBS and 1 ml of fresh media containing Adiponectin (1 µg/ml) +/- recombinant IL10 peptide or IL10 antibody was added. Kupffer Cells were incubated for a further 4 hours. Media was then aspirated and stored at -80°C for analysis by ELISA, cells were washed twice prior to lysis for mRNA or protein extraction.

4.2.3 Molecular Techniques

mRNA was extracted from Kupffer Cells using the Quiagen RNeasy kit according to the manufacturers instructions. RNA yield and purity was determined using the Thermo Scientific Nanodrop. 200 ng of RNA was reverse transcribed to cDNA using the Invitrogen Superscript First Strand Synthesis System using the manufacturers instructions.
Figure 4.1 Experimental Protocol: Adiponectin Primed or Non-Primed Kupffer Cells.

Kupffer Cells (KC) were primed in adiponectin containing media or remained non-primed in culture media alone for 18 hours. Kupffer Cells were then washed with PBS and fresh media containing adiponectin +/- IL10 peptide or IL10 antibody was applied to each well for 4 hours.
4.2.4 Quantitative Real Time Polymerase Chain Reaction.

Standards were diluted 1 in 50, 1 in 10 (IL10) or 1 in 2 (IL6) for analysis. The Stratagene Mx3000 QPCR System with Platinum SYBR Green qPCR Master Mix (Invitrogen) was used for all samples. GAPDH was the housekeeper gene and primer sequences were as described in table 2.1. All samples were analysed in duplicate. Results were analysed using the Stratagene software program.

4.2.5 ELISA

TNFα, IL6 and IL10 were measured in cell culture media using Rat ELISA Quantikine® Kits (R&D Systems, Minneapolis, MN, USA) according to manufacturers instructions.

4.2.6 Western Blot

Adiponectin or adiponectin-containing media was loaded under non-reducing conditions and without loading buffer to a 10% polyacrylamide gel and proteins were separated by electrophoresis. Proteins were transferred to a PVDF membrane and bands were detected using the conditions described in Table 4.1.

Phospho-AMPKα, T-cadherin and AdipoR1 were detected from cell lysates prepared in a RIPA buffer containing 1:100 phosphatase and protease inhibitors. Lysates were heat and βME reduced prior to loading 20 µg protein per lane. Membranes were stripped then re-blocked to detect total AMPKα. β-actin was used as a loading control. Densitometry determined using the Kodak M1 densitometry software.

4.2.7 Statistical Analysis

All experiments were repeated 3 to 6 times. Statistical analysis was performed using ANOVA to compare data between different treatment groups (Prism 6 for Macintosh, version 6.0e, Graphpad Software Inc, La Jolla, Ca, USA). The probability level of p
≤0.05 was set for statistical significance. Results are expressed as mean values relative to the control group with the standard error of the mean.
Table 4.1 Antibody Conditions for Western Blot

<table>
<thead>
<tr>
<th>Protein</th>
<th>Block</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiponectin</td>
<td>5% SM</td>
<td>1:1000 anti-Adiponectin</td>
<td>1:1000 anti-rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4% SM</td>
<td>4% SM</td>
</tr>
<tr>
<td>P-AMPKα</td>
<td>5% SM</td>
<td>1:1000 anti-P-AMPKα</td>
<td>1:2000 anti-rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5% BSA</td>
<td>5% BSA</td>
</tr>
<tr>
<td>T-AMPKα</td>
<td>5% SM</td>
<td>1:1000 anti-AMPKα</td>
<td>1:2000 anti-mouse</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5% BSA</td>
<td>5% SM</td>
</tr>
<tr>
<td>T-cadherin</td>
<td>5% SM</td>
<td>1:500 anti-T-cadherin</td>
<td>1:2000 anti-rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5% SM</td>
<td>5% SM</td>
</tr>
<tr>
<td>AdipoR1</td>
<td>5% BSA</td>
<td>1:1000 anti-AdipoR1</td>
<td>1:10000 anti-rabbit</td>
</tr>
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<td>5% BSA</td>
<td>3% SM</td>
</tr>
<tr>
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<td>1:7500 anti-β-actin</td>
<td>1:5000 anti-mouse</td>
</tr>
<tr>
<td></td>
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<td>1% SM</td>
<td>5% SM</td>
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</tbody>
</table>
4.3 Results

4.3.1 Adiponectin induces IL10 synthesis and release in Kupffer Cells.

In non-primed Kupffer Cells, 4 hours of adiponectin treatment increased IL10 mRNA synthesis 6.35 fold (p<0.05) compared to adiponectin non-exposed control Kupffer Cells and 5.34 fold (p<0.005) in adiponectin primed Kupffer Cells (Figure 4.2A). There was no significant difference in IL10 mRNA synthesis between primed and non-primed Kupffer Cells.

Media from non-primed Kupffer Cells had elevated levels of IL10 at 216.6 pg/ml (p<0.05) compared to untreated control Kupffer Cells that had a basal media concentration of 18.68 pg/ml IL10 (Figure 4.2B). Primed Kupffer Cells secreted 359.5 pg/ml of IL10 in response to 4 hours of adiponectin (p<0.05 compared to control) and again there was no difference in IL10 between primed and non-primed Kupffer Cells.

4.3.2 Adiponectin induces an initial inflammatory cytokine response in Kupffer Cells, followed by tolerance to further adiponectin exposure.

To confirm the findings observed in Chapter 3 of this thesis that initial adiponectin exposure induced TNFα and IL6 synthesis and secretion in Kupffer Cells, Kupffer Cells were exposed to 4 hours of adiponectin at a concentration of 1 µg/ml. Adiponectin treatment increased Kupffer Cell TNFα mRNA synthesis 149.8 fold compared to non-adiponectin exposed control Kupffer Cells (p<0.0001), increased TNFα media concentration from 71.54 pg/ml in control Cells to 2580 pg/ml (p<0.005), increased IL6 mRNA 23.15 fold compared to control Kupffer Cells (p<0.05) and IL6 media concentration increased from 44.53 pg/ml in control cells to 1836 pg/ml (Figure 4.3).
Figure 4.2 Adiponectin Induces IL10 in Both Primed and Non-Primed Kupffer Cells.

Isolated Kupffer Cells were primed with adiponectin (ADN) 1 µg/ml for 18 hours or culture media alone (non-primed). Fresh media containing 1 µg/ml adiponectin was subsequently applied for 4 hours. Basal, control Kupffer Cells were cultured in media alone for both treatment periods. A: Kupffer Cells IL10 mRNA was determined by qPCR and normalized to GAPDH. B: Media IL10 concentration was determined by ELISA.
As adiponectin was shown to induce tolerance to subsequent LPS exposure in Kupffer Cells, adiponectin-induced tolerance to itself was assessed by pre-treating or “priming” Kupffer Cells with 1 µg/ml adiponectin prior to re-exposing the same Kupffer Cells to fresh media containing 1 µg/ml adiponectin. Adiponectin primed Kupffer Cells had reduced TNFα and IL6 synthesis and release compared to non-primed Kupffer Cells. TNFα mRNA synthesis decreased from 149.8 fold in non-primed, adiponectin-treated Kupffer Cells to 4.67 fold (p<0.05) in adiponectin primed Kupffer Cells with a corresponding decrease in TNFα media concentration from 2580 pg/ml to 1334 pg/ml (p<0.005). IL6 mRNA decreased from 23.15 fold in non-primed Kupffer Cells to 5.11 fold (p<0.05) in primed Kupffer Cells and IL6 media concentration decreased from 1836 pg/ml in non-primed Kupffer Cells to 815.7 pg/ml in primed Kupffer Cells.

**4.3.3 Adiponectin Becomes Predominantly Low-Molecular Weight Form in Kupffer Cell Culture Media.**

The mechanism/s by which adiponectin develops tolerance to itself in Kupffer Cells is unknown. As adiponectin exists in both high and low molecular weight isomers, with several studies suggesting that different adiponectin isomers have different biological activity, a change in adiponectin isomer may potentiate different biological actions; in this case an inflammatory or anti-inflammatory response in Kupffer Cells. To evaluate whether the observed adiponectin-induced inflammatory or anti-inflammatory response in Kupffer Cells is due to either decreased adiponectin concentration or alteration in multimer form, Western Blot was used to assess adiponectin. Kupffer Cells were cultured in adiponectin containing media; media was removed from the Kupffer Cells after 20 minutes, 2 hours, 6 hours, 12 hours and 24 hours of culture. A Western Blot performed under non-reducing conditions is shown in figure 4.4. This shows that adiponectin remained within the media for the entire time period, however adiponectin
Figure 4.3 Initial, but not Ongoing Adiponectin Exposure Induces TNFα and IL6 in Kupffer Cells.

Isolated Rat Kupffer Cells were primed with adiponectin (ADN) 1 µg/ml for 18 hours or culture media alone (non-primed). Fresh media containing 1 µg/ml adiponectin was subsequently applied for 4 hours. Basal, control Kupffer Cells were cultured in media alone for both treatment periods. A: Kupffer Cell TNFα mRNA was determined by qPCR and normalized to GAPDH. B: Media concentration of TNFα was determined by ELISA. C: Kupffer Cell IL6 mRNA was determined by qPCR and normalized to GAPDH. D: Media concentration of IL6 was determined using ELISA.
Figure 4.4 Adiponectin Progressively Becomes Low Molecular Weight Form in Kupffer Cell Culture Media.

Kupffer Cells were cultured in DMEM and adiponectin 5 µg/ml. Media was removed at 20 minutes, 2 hours, 6 hours, 12 hours and 24 hours and adiponectin content of the media was determined using Western Blot under non-reducing conditions (A). Positive controls used were undiluted 0.5 µg, 1 µg and 2 µg of adiponectin. Bands were quantified by Kodak M1 software.
rapidly became low molecular weight under cell culture conditions; by 2 hours the adiponectin was predominantly low molecular weight.

4.3.4 Adiponectin exposure alters AdipoR1 mRNA in Kupffer Cells.

Adiponectin signals through AdipoR1, AdipoR2 and T-cadherin. Alterations in levels of adiponectin receptor expression may be responsible for the observed differing responses to adiponectin in Kupffer Cells. To understand the mechanism by which tolerance to adiponectin occurs in Kupffer Cells, the expression of adiponectin receptors mRNA over time was investigated.

AdipoR1 mRNA expression was significantly down regulated from baseline values to 0.51 of baseline ($p<0.0005$) after 18 hours of adiponectin exposure and 0.62 after 18 hours plus a further 4 hours of adiponectin exposure ($p<0.0005$) (Figure 4.5A). There was a non-significant increase in AdipoR1 mRNA expression at 18 hours (1.14 fold) and 18 plus 4 hours in control media (1.58 fold). There was a significant difference ($p<0.05$) difference in AdipoR1 expression at 18 plus 4 hours between control and adiponectin-exposed Kupffer Cells.

AdipoR2 mRNA expression was decreased from baseline values to 0.64 of baseline ($p<0.05$) and unchanged from baseline at 1.02 fold after 18 hours plus a further 4 hours of adiponectin exposure (Figure 4.5B). There was a non-significant increase in AdipoR2 mRNA expression at 18 hours (1.14 fold) and 18 plus 4 hours in control media (1.18 fold). There was no statistically significant difference in AdipoR2 expression at 18 hours or 18 plus 4 hours between control and adiponectin-exposed Kupffer Cells.

T-cadherin mRNA expression was significantly decreased from baseline values to 0.21 of baseline after 18 hours of adiponectin exposure ($p<0.0005$) and 0.21 of baseline after 18 hours plus a further 4 hours of adiponectin exposure ($p<0.0001$) (Figure 4.5C). There was no significant change in T-cadherin mRNA expression at 18 hours (0.94
fold) and 18 plus 4 hours in control media (0.77 fold). There was no statistically significant difference in T-cadherin expression at 18 hours or 18 plus 4 hours between control and adiponectin-exposed Kupffer Cells.

As it has been reported that T-cadherin is not expressed on macrophages, T-cadherin expression was assessed using Western Blot. Bands corresponding to T-cadherin were detected in both control and adiponectin-exposed Kupffer Cells membrane-protein fractions (Figure 4.6), thus providing a novel insight into adiponectin receptors on Kupffer Cells.

To assess for alterations in adiponectin receptor-signalling efficiency, the phosphorylation of AMPKα, the downstream mediator of the adiponectin receptors was investigated using Western Blot. No differences in AMPKα phosphorylation occurred between Kupffer Cells that were exposed on unexposed to adiponectin (Figure 4.7).

4.3.5 IL10 Mediates Kupffer Cell tolerance to Adiponectin

As demonstrated above, IL10 a cytokine with well-recognized anti-inflammatory and immunomodulatory effects is induced by adiponectin in Kupffer Cells. Unlike the inflammatory cytokines TNFα or IL6, IL10 expression is not down regulated by adiponectin pre-exposure in Kupffer Cells. It is therefore likely that IL10 that is synthesized and secreted during adiponectin pre-exposure may contribute towards TNFα and IL6 down-regulation in adiponectin primed Kupffer Cells. To assess this hypothesis in these experiments, recombinant IL10 or neutralizing IL10 antibodies were used in conjunction with adiponectin to assess the role of IL10 in development of Kupffer Cell tolerance to adiponectin.

In non-primed Kupffer Cells that were exposed to adiponectin and recombinant IL10 for 4 hours, TNFα mRNA decreased from 149.8 fold in Kupffer Cells exposed to adiponectin alone to 15.5 fold (p<0.05) and TNFα media concentration decreased from
Figure 4.5 Adiponectin Receptor mRNA Expression is Altered By Adiponectin Exposure.

Day 1 cultured rat Kupffer Cells were exposed to adiponectin 1 µg/ml (Adiponectin group) or media alone (Control group) for 18 hours. Media was then removed and replaced with fresh media containing adiponectin 1 µg/ml (Adiponectin group) or vehicle (Control group) for 4 hours. AdipoR1 (A), AdipoR2 (B) and T-cadherin (C) mRNA expression was determined at 3 time-points; baseline, 18 hours and 18 plus 4 hours. GAPDH was used as the housekeeper gene in all experiments.
Figure 4.6 T-cadherin is expressed on Kupffer Cells.

Kupffer Cells were cultured in media alone or adiponectin 1 μg/ml for 18 hours. The protein membrane fraction was assessed for T-cadherin expression using Western Blot. B-actin was used as a loading control.

Figure 4.7 AMPKα signalling is not altered by Adiponectin in Kupffer Cells.

In the Control group, Kupffer Cells were cultured in media alone for 18 hours prior to media being exchanged for fresh media for 4 hours. In the Adiponectin Treatment group, Kupffer Cells were cultured in 1 μg/ml adiponectin for 18 hours prior to being replaced with fresh adiponectin-containing media for 4 hours. Phosphorylated AMPKα, total AMPKα and β-actin as a loading control were determined at baseline, 18 hours and 18 + 4 hours in both groups.
2580 pg/ml in Kupffer Cells exposed to adiponectin alone to 180.2 pg/ml (p<0.0005) (Figure 4.8).

IL6 mRNA decreased from 23.15 fold in non-primed Kupffer Cells to 5.13 fold (p<0.05) in non-primed Kupffer Cells exposed to adiponectin and IL10 peptide (Figure 4.9A). Similarly, IL6 peptide decreased from 1836 pg/ml in non-primed, Kupffer Cells to 175.4 pg/ml (p<0.05) in non-primed Kupffer Cells exposed to adiponectin and IL10 peptide (Figure 4.9B).

In primed Kupffer Cells that were exposed to adiponectin and soluble IL10 antibody for 4 hours, TNFα mRNA increased from 4.67 fold to 6.41 fold and TNFα peptide increased non significantly from 1334 pg/ml in Kupffer Cells treated with adiponectin alone to 1862 pg/ml in Kupffer Cells exposed to adiponectin and soluble IL10 antibody (Figure 4.10).

IL6 mRNA increased from 5.11 fold in non-primed Kupffer Cells treated with adiponectin alone to 27.29 (p<0.05) fold in non-primed Kupffer Cells treated with adiponectin and IL10 peptide (Figure 4.11A). IL6 peptide however increased non-significantly from 815.7 pg/ml in non-primed, adiponectin treated Kupffer Cells to 1035 pg/ml in non-primed Kupffer Cells treated with adiponectin and IL10 peptide (Figure 4.11B).
**Figure 4.8 Recombinant IL10 abrogates adiponectin-induced TNFα in Kupffer Cells.**

Day 1 primary rat Kupffer Cells were cultured for 18 hours in media alone. Fresh media containing 1 μg/ml (ADN) adiponectin +/- 20 ng/ml mouse IL10 was then applied for 4 hours. Control Kupffer Cells were cultured in media alone for 18 hours, then fresh media was applied for 4 hours. A: TNFα mRNA was determined by qPCR with GAPDH as the housekeeper gene. B: Media concentration of TNFα was determined with ELISA.
Figure 4.9 Recombinant IL10 abrogates adiponectin-induced IL6 in Kupffer Cells.

Day 1 primary rat Kupffer Cells were cultured for 18 hours in media alone. Fresh media containing 1 µg/ml (ADN) adiponectin +/- 20 ng/ml mouse IL10 was then applied for 4 hours. Control Kupffer Cells were cultured in media alone for 18 hours, then fresh media was applied for 4 hours. A: IL6 mRNA was determined by qPCR with GAPDH as the housekeeper gene. B: Media concentration of IL6 was determined with ELISA.
Figure 4.10 IL10 neutralisation increases adiponectin-induced TNFα in Kupffer Cells.

Day 1 primary rats Kupffer Cells were cultured for 18 hours in media alone. Fresh media containing 1 μg/ml (ADN) adiponectin +/- 5 μg/ml soluble mouse IL10 antibody was then applied for 4 hours. Control Kupffer Cells were cultured in media alone for 18 hours, then fresh media was applied for 4 hours. A: TNFα mRNA was determined by qPCR with GAPDH as the housekeeper gene. B: Media concentration of TNFα was determined with ELISA.
Figure 4.11 IL10 neutralisation increases adiponectin-induced IL6 mRNA in Kupffer Cells.

Day 1 primary rat Kupffer Cells was cultured for 18 hours in media alone. Fresh media containing 1 µg/ml (ADN) adiponectin +/- 5 µg/ml soluble mouse IL10 antibody was then applied for 4 hours. Control Kupffer Cells were cultured in media alone for 18 hours, and then fresh media was applied for 4 hours. A: IL6 mRNA was determined by qPCR with GAPDH as the housekeeper gene. B: Media concentration of IL6 was determined with ELISA.
4.4 Discussion

Adiponectin has multiple and often contradictory, anti-inflammatory and immunomodulatory affects [252]. In particular these contrasting roles of adiponectin have been observed in macrophages, an important cellular component of the innate immune system [263]. In order to understand the apparent dual pro-inflammatory and anti-inflammatory effects of adiponectin, Kupffer Cells were treated with adiponectin and tested for the development of tolerance. Once it was ascertained that Kupffer Cells did develop tolerance to adiponectin I then proceeded to identify mechanisms of tolerance by assessing for changes in adiponectin, adiponectin receptors and the effects of IL10.

In the studies described in this chapter, I found that four hours of adiponectin exposure resulted in up regulation of TNFα, IL6 and IL10 in Kupffer Cells. However, this observed TNFα and IL6 up regulation decreased when Kupffer Cells were primed in adiponectin-containing media prior re-exposure to adiponectin. IL10 levels were unaltered in adiponectin primed Kupffer Cells. Potential mechanisms for this tolerance that was identified in this chapter include AdipoR1 down regulation, a change from predominantly high molecular weight to low molecular weight adiponectin and IL10 forming an autocrine feedback loop with resultant inhibition of further inflammatory response.

The results presented in this chapter are consistent with the outcome of the previous chapter of this thesis and add to the growing field of literature supporting inflammatory effects of adiponectin; as well as 18 hours of adiponectin resulting in an inflammatory response, 4 hours of adiponectin has now been shown to exert an inflammatory response in Kupffer Cells. The observed initial up regulation in Kupffer Cell TNFα and IL6 is consistent with the described inflammatory response to adiponectin in monocytes and macrophages. In particular, when the RAW 264.7 macrophages cell line was
exposed to globular adiponectin, TNFα mRNA synthesis peaked following 2 hours of treatment and TNFα peptide peaked after 8 hours of treatment [230]. In this study using full length adiponectin, both TNFα mRNA and IL6 mRNA and peptide were significantly increased from basal levels at 4 hours, the duration of adiponectin exposure to obtain peak mRNA synthesis and peptide secretion was not ascertained. Further studies confirm an initial adiponectin induced inflammatory response in cell culture systems. Globular adiponectin induced TNFα and IL6 secretion in the THP-1 differentiated macrophage cell line, isolated human peripheral monocytes and mouse thioglycollate-induced peritoneal macrophages [261]. This inflammatory response has been shown to extend out to 24 hours, with TNFα and IL6 both up regulated in human macrophages cultured for 24 hours with full-length adiponectin [262]. Tolerance to adiponectin was demonstrated in this chapter with a decrease in TNFα and IL6 mRNA and peptide observed in Kupffer Cells that were pre-exposed to adiponectin. IL6 peptide returned to basal levels in adiponectin primed Kupffer Cells, however TNFα mRNA, TNFα peptide and IL6 mRNA failed to return to basal levels. Despite a failure to reach basal levels, there was significant decrease in TNFα mRNA synthesis and peptide secretion, and IL6 mRNA from non-adiponectin primed Kupffer Cells to primed Kupffer Cells. As previously mentioned, an inflammatory response to adiponectin was observed after 24 hours in human macrophages [262], and certainly a return to basal levels of TNFα and IL6 may have been observed if either the pre-exposure or treatment periods were prolonged.

This study is the first to demonstrate the development of tolerance to adiponectin in Kupffer Cells. Tsatsanis and colleagues describe adiponectin-induced tolerance in macrophages [261]. Isolated human macrophages, THP-1 human macrophages and mouse peritoneal macrophages initially secreted TNFα and IL6 in response to adiponectin, however on repeated adiponectin exposure, the authors found that cells
failed to elicit a TNFα or IL6 response. In addition the authors also determined that adiponectin tolerance was dose-dependent; pre-treating with a low dose of adiponectin did not induce tolerance in cells re-exposed to a high dose of adiponectin. In the Tsatsanis study, the protocol was slightly different; cells were pre-exposed to 10 µg/ml of globular adiponectin and tolerance was tested at time points from 2 to 8 hours in the re-exposure period. Interestingly, tolerance was not observed until after 8 hours for TNFα secretion in both THP-1 macrophages and human macrophages, and IL6 was suppressed at 8 hours in human macrophages and 12 hours in THP-1 macrophages. Overall this is suggestive that Kupffer Cells are particular sensitive to developing tolerance in that they demonstrated tolerance to a greatly lower adiponectin dose of 1 µg/ml versus the 10 µg/ml used in the Tsatsanis study, and both the pre-exposure period was decreased (18 hours versus 24 hours) and the re-challenge time to response was less at 4 hours compared to 8 to 2 hours. This is likely to be an inherent characteristic of the Kupffer Cell; the Kupffer Cell is continually exposed to high levels of enteric-derived toxins due to it’s sinusoidal location within the liver, and as such has developed the ability to rapidly tolerise itself to these insults.

Adiponectin-induced tolerance to LPS in macrophages has been well described and may offer further insight into the mechanisms by which adiponectin induces tolerance to itself in Kupffer Cells [227, 232, 296, 297, 299, 305]. In particular, adiponectin has been demonstrated to induce the anti-inflammatory cytokine IL10 in human monocytes and isolated rat Kupffer cells, with HO-1 being identified as the pathway required to mediate the effects of adiponectin-induced IL10 in rat Kupffer Cells [229, 232]. Similarly, the studies in this thesis confirmed adiponectin-stimulated IL10 up regulation in Kupffer Cells, and unlike the pro-inflammatory cytokines TNFα and IL6, IL10 was not suppressed with repeated adiponectin treatment. Adiponectin-induced IL10 mediates down regulation of LPS-induced inflammatory cytokines in rat Kupffer Cells
and RAW264.7 macrophages [230, 232]. The mechanism by which IL10 mediates LPS-induced inflammatory cytokine release has been attributed to IL10 decreasing the stability of TNFα mRNA which contains an AU-rich cluster at the 3’ untranslated region [243, 244]. As IL10 modulates adiponectin-induced and LPS-induced tolerance to LPS in macrophages [230, 235], the role of IL10 in modulating adiponectin-induced tolerance to self was examined. Addition of exogenous IL10 resulted in a significant decrease in TNFα and IL6 mRNA and peptide in non-adiponectin primed Kupffer Cells, IL10 blockage with a soluble antibody did not consistently lead to an increase in TNFα and IL6 in adiponectin primed Kupffer Cells. This suggests that although IL10 does contribute towards adiponectin tolerance in Kupffer Cells, there may be other cellular mediators that are contributing towards this eventual tolerance. Folco and colleagues similarly found that IL10 neutralisation failed to abrogate adiponectin-mediated suppression of LPS-induced TNFα in human macrophages [299]. Folco and colleagues attributed this disparity from the findings of Park et al [230] to the use of full-length adiponectin expressed in HEK293 (Folco et al) as opposed to E. coli derived globular adiponectin which fails to form HMW structures. Alternatively, the IL10 antibody concentration used in this thesis may have been insufficient to obtain complete IL10 neutralisation or IL10 may be acting, at least partially, intra-cellularly. Repeating these experiments in an IL10 knock out model or with si-RNA against IL10 and directly comparing globular and full-length adiponectin may be useful to further define the role of IL10 in the development of adiponectin tolerance.

Both AdipoR1 and AdipoR2 are expressed on the monocyte-macrophage cell line [321], and consistent with this, both receptors were identified on Kupffer Cells. In this study, the T-cadherin receptor was present on Kupffer Cells as determined by qPCR and Western Blot. This is in contrast to previous studies where by mRNA expression of T-cadherin was not detected in RAW264.7 cells [192] and T-cadherin expression by
immunoblotting was absent in vascular monocytes, macrophages and foam cells [202]. Certainly this finding warrants further confirmation with an alternative detection method such as in situ immunofluorescence or an alternative qPCR primer sequence and may be a reflection of the Kupffer Cell as a tissue macrophage having developed specific and distinct characteristics from the monocyte macrophage pool.

Recent evidence suggests that the anti-inflammatory actions of adiponectin are mediated through the AdipoR1 receptor [322] with globular adiponectin induced IL10 in Kupffer Cells being dependent on AdipoR1 [232]. Furthermore, the inhibitory effects of adiponectin upon LPS-mediated cytokines in RAW264.7 macrophage are mediated via AdipoR1 with globular adiponectin and AdipoR2 with full-length adiponectin [306]. Pro-inflammatory effects of adiponectin however, are mediated through both AdipoR1 and AdipoR2 in human macrophages [262].

In animal models of fatty liver, AdipoR2 was reduced in mice with fatty liver compared to control animals however both AdipoR1 and AdipoR2 were reduced in mice following bile-duct ligation [280]. The functional roles of each receptor in vivo remains unclear with AdipoR2 having anti-inflammatory and anti-fibrotic effects in the MCD diet mouse model of NASH [281] and both AdipoR1 and AdipoR2 contributing towards resolution of steatosis and insulin resistance in a diabetic mouse model [282].

The studies described in this chapter are not designed to ascertain a functional role for each of the adiponectin receptors; rather they evaluate changes in expression following adiponectin exposure. The results demonstrate that AdipoR1 mRNA is down regulated in response to adiponectin exposure however no down regulation in protein expression is detected and no alteration in down stream AMPKα is detected. This suggests that a down regulation in AdipoR1 mRNA has little effect on the expression or function of the membrane receptor. In addition to this, the conflicting observations of maintained IL10 synthesis and release and inhibition of TNFα and IL6 in response to ongoing
adiponectin exposure as found in this study, suggests the mechanism of tolerance is more involved than receptor down regulation alone. Awazawa and colleagues recently reported NFκB activation and IL6 production in response to adiponectin and independent of AdipoR1 and AdipoR2 [192], suggesting a further, as yet unidentified adiponectin receptor may be responsible for these observed inflammatory effects.

The adiponectin used within this thesis is full-length adiponectin sourced from human HEK293 cells (Human embryonic kidney cells) and as such is subject to post-translational modification, which contributes towards multimer formation. As demonstrated this adiponectin is predominantly high molecular weight under storage conditions but rapidly became low molecular weight in cell culture conditions. Alterations in the multimer structures may contribute towards the observed differences in TNFα and IL6 between primed and non-primed Kupffer Cells. In THP-1 cells low weight but not high molecular weight adiponectin abrogated LPS-induced IL6 with low molecular weight potentiating LPS-induced IL10 and high molecular weight adiponectin alone resulting in increased IL6 [194]. In C2C12 myocytes, NFκB activation via IκB phosphorylation was observed when cells were exposed to hexameric forms of adiponectin; trimeric and globular adiponectin failed to elicit NFκB activation [313]. These findings are in keeping with a change from high molecular weight adiponectin to low molecular weight adiponectin and a parallel shift from inflammatory response to anti-inflammatory response that was seen in this chapter. Any biological relevance would be difficult to assess as in a normal homeostatic state adiponectin is continually produced. In addition, monocytes, and perhaps macrophages, secrete leukocyte elastase, a protease capable of cleaving globular adiponectin from full-length adiponectin [323]. The presence and potential role of this enzyme is not well described in Kupffer Cells and again may negate any observed changes in adiponectin multimer form.
The data presented within this chapter supports the hypothesis that maintaining a stable, high circulating adiponectin level is important in maintaining an anti-inflammatory effect on Kupffer Cells. These findings can be paralleled with the clinical situation of obesity with associated low circulating adiponectin levels [179] where this tonic, inhibitory effect of adiponectin upon Kupffer Cells would be lost. A low basal adiponectin level would lead to the development of hepatic inflammation in response to fluctuations in adiponectin levels which may be bought about by sudden weight loss. Indeed rapid weight loss through bariatric surgery has been associated with liver-related morbidity and mortality [324, 325] and reports of acute liver failure in individuals with anorexia nervosa [326] may actually be a result of a rapid fluctuation in serum adiponectin. This theory is supported by a review of 142 patients undergoing bariatric surgery; post-operatively BMI decreased 22%, adiponectin increased 93% and TNFα increased 120% despite metabolic improvements [327]. In a study of 69 patients who had liver biopsies at the time of bariatric surgery and 27 +/- 15 months after surgery, those who had marked weight loss had a significant decrease in hepatic steatosis grade but had significant increase in inflammatory lobular hepatitis [328]. A further publication however, demonstrated that rapid weight loss did not decrease pre-existing elevated TNFα levels, nor alter adiponectin levels [329]. This study somewhat rejects this hypothesis, however alterations in functional adiponectin signalling were not measured therefore this cannot be entirely dismissed.

The results from the above study demonstrate not only the pro-inflammatory effects of adiponectin, but also the tolerance-promoting effects of adiponectin upon itself in Kupffer Cells. Potential mechanisms by which this tolerance occurs include the development of an autocrine IL10 feedback loop with adiponectin receptor down regulation and alterations in adiponectin multimer form being less likely to contribute towards this tolerance. This reinforces the multiple positive clinical findings of a steady
lean body mass being associated with high circulating levels of adiponectin and low incidence of NAFLD, insulin resistance and metabolic complications. This also adds support to the current first line treatment of steady, gradual weight loss for individuals with NAFLD and NASH. The mechanisms underlying the inflammatory effects of adiponectin are not well described and will be further delineated in the following thesis chapter.
Chapter 5 Mechanisms of Adiponectin-Induced Inflammatory Response in Rat Kupffer Cells.
5.1 Introduction

Adiponectin has multiple anti-inflammatory, anti-fibrotic and insulin sensitizing properties and certainly in animal models of NAFLD and NASH, has therapeutic potential. As documented in the previous two chapters of this thesis, Adiponectin exerts an initial inflammatory response in Rat Kupffer Cells. Although temporary and short-lived, this initial inflammatory response has the potential to cause further morbidity and mortality in individuals with pre-existing liver damage. Understanding the underlying mechanism and identifying downstream molecular targets by which the initial adiponectin-induced inflammatory response occurs in Kupffer Cells has not been described; this is important to enable future therapy designed around the positive anti-inflammatory actions of adiponectin whilst avoiding potential harmful inflammatory effects.

Several inflammatory pathways have been shown to be important in the generation of TNFα. These include the NFκB pathway that plays an important role in obesity related inflammation [330], and the MAPK pathways [137]. Similarly to adiponectin associated inflammatory and anti-inflammatory response, both adiponectin induced MAPK activation and adiponectin induced suppression of MAPK pathways have been described. Studies of RAW 264.7 macrophages have shown that globular adiponectin induces ERK1/2 phosphorylation and subsequent NFκB nuclear translocation prior to TNFα synthesis [230, 331]. Similarly, exposure of human placental tissue to adiponectin was shown to increase IL6 and TNFα, both of which were abrogated by ERK1/2 and NFκ-B inhibitors [332]. However other studies have suggested that adiponectin decreases LPS-induced TNFα and IL6 via ERK1/2 suppression in porcine blood macrophages [227] and inhibits LPS-induced ERK1/2 activation in RAW264.7 Macrophages [296]. Tsatsanis and colleagues have suggested that adiponectin tolerance
occurs via NFκB as adiponectin pre-treatment prevented NFκB translocation on further adiponectin exposure in THP-1 macrophages [261].

The aim of the studies presented in this chapter is to determine the role of the MAPK pathways in adiponectin induced inflammatory and anti-inflammatory cytokine changes in Kupffer Cells and to determine possible differential effect of adiponectin upon the MAPK and pathways between adiponectin primed and non-primed Kupffer Cells.
5.2 Methods

5.2.1 Isolation and Culture of Primary Rat Kupffer Cells.

Primary Kupffer Cells were isolated from male Sprague-Dawley Rats using the previously described two-stage collagenase and pronase digestion followed by centrifugal elutriation.

5.2.2 Cell Culture Technique

Kupffer Cells were plated into plastic 6 or 12 well cell culture plates at a density of 3 x $10^6$ or 2 x $10^6$ cells per well with 2 ml of DMEM media containing 10% FCS and P/S per well. Kupffer Cells from different rats were not combined with Kupffer Cells from a single rat plated to enable paired comparative treatments. Cells were incubated overnight at 37 degrees with 5% CO$_2$.

The first protocol is similar to that used in Chapters 3 and 4; day 1 cultured rat Kupffer Cells were left in culture media alone for 18 hours (non-primed). Media was removed and Kupffer Cells were washed prior to new media containing 1 µg/ml adiponectin and 20 nM JNK Inhibitor and/or 7 µM SB203580 and/or 10 µM PD98059 MAPK inhibitors being applied for 4 hours prior to Kupffer Cell lysis for mRNA extraction.

In the time course experimental protocol (Figure 5.1), day 1 Kupffer Cells were treated with vehicle (control group) or adiponectin (1 µg/ml) for 18 hours. After the incubation period, Kupffer Cell media was aspirated, and 1 ml of fresh media containing Adiponectin (1 µg/ml) +/- recombinant IL10 peptide or IL10 antibody was added. Kupffer Cells were left in media from 0 minutes to 60 minutes, with Kupffer Cells being periodically lysed at time points; 0 minutes, 5 minutes, 15 minutes, 30 minutes and 60 minutes for assessment of MAPK phosphorylation by Western Blot.
Figure 5.1 Experimental Protocol: Adiponectin Primed or Non-Primed Kupffer Cells Time Course Study.

Kupffer Cells were primed in adiponectin containing media or remained non-primed in culture media for 18 hours. Kupffer Cells were then washed and fresh media containing adiponectin +/- IL10 peptide or IL10 antibody, was applied to each well. Kupffer Cells lysed for protein analysis at the indicated time points throughout the protocol.
5.2.3 Molecular Techniques

mRNA was extracted from Kupffer Cells using the Quigen RNeasy kit according to the manufacturers instructions. RNA yield and purity was determined using the Thermo Scientific Nanodrop. 200 ng of RNA was reverse transcribed to cDNA using the Invitrogen Superscript First Strand Synthesis System using the manufacturers instructions.

5.2.4 Quantitative Real Time Polymerase Chain Reaction.

Standards were diluted 1 in 50 for analysis. The Stratagene Mx3000 QPCR System with Platinum SYBR Green qPCR Master Mix (Invitrogen) was used for all samples. GAPDH was the housekeeper gene and primer sequences were as described in table 2.1. All samples were analysed in duplicate. Results were analysed using the Stratagene software program.

5.2.5 Western Blot

Kupffer Cells were lysed in RIPA buffer containing 1:100 phosphatase and protease inhibitors. 20 µg of protein from each sample was heat denatured and loaded with a βME containing loading buffer and resolved using a large 7.5%polyacrylamide gel. Proteins were all transferred to a PVDF membrane. Blocking and antibody conditions as per table 5.1. The phosphorylated form of each protein were detected first, membranes were then stripped and re-probed for total protein. β-actin was used as a loading control. Densitometry was determined using the Kodak M1 densitometry software.

5.2.6 Statistical Analysis

All experiments were repeated 3 times. Statistical analysis was performed using the ANOVA to compare data between different treatment groups and Student’s t-test to compare treatment a single treatment group to the control (Prism 6 for Macintosh, version 6.0e, Graphpad Software Inc, La Jolla, Ca, USA). The probability level of p
≤0.05 was set for statistical significance. Results are expressed as mean values relative to the control non-primed group with the standard error of the mean.
Table 5.1 Antibody Conditions for Western Blot Analysis.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Block</th>
<th>Primary</th>
<th>Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-ERK1/2</td>
<td>5% BSA</td>
<td>1:2000 anti P-ERK1/2</td>
<td>1:5000 anti rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5% BSA</td>
<td>5% BSA</td>
</tr>
<tr>
<td>T-ERK1/2</td>
<td>5% SM</td>
<td>1:1000 anti ERK1/2</td>
<td>1:5000 anti mouse</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5% SM</td>
<td>5% SM</td>
</tr>
<tr>
<td>P-IkB</td>
<td>5% BSA</td>
<td>1:1000 anti P-IkB</td>
<td>1:2000 anti mouse</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5% BSA</td>
<td>3% SM</td>
</tr>
<tr>
<td>T-IkB</td>
<td>5% BSA</td>
<td>1:1000 anti IκB</td>
<td>1:2000 anti rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5% BSA</td>
<td>3% SM</td>
</tr>
<tr>
<td>P-P38</td>
<td>5% BSA</td>
<td>1:1000 anti ERK1/2</td>
<td>1:5000 anti rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5% BSA</td>
<td>5% BSA</td>
</tr>
<tr>
<td>T-P38</td>
<td>5% SM</td>
<td>1:1000 anti P38</td>
<td>1:5000 anti mouse</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5% SM</td>
<td>5% SM</td>
</tr>
<tr>
<td>P-SAPK/JNK</td>
<td>5% BSA</td>
<td>1:1000 anti P-SAPK/JNK</td>
<td>1:2000 anti rabbit</td>
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<tr>
<td></td>
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<td>5% BSA</td>
</tr>
<tr>
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<td>1:1000 anti SAPK/JNK</td>
<td>1:2000 anti rabbit</td>
</tr>
<tr>
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<td>5% BSA</td>
</tr>
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<td>β-Actin</td>
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<td>1:7500 anti β-actin</td>
<td>1:5000 anti mouse</td>
</tr>
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<td></td>
<td></td>
<td>1% SM</td>
<td>5% SM</td>
</tr>
</tbody>
</table>
5.3 Results

5.3.1 Adiponectin-induced TNFα occurs via MAPK activation.

There are previous reports of adiponectin-induced MAPK phosphorylation in monocytes and macrophage cell lines being required to enable TNFα and IL6 generation [230, 231]. To assess the role of adiponectin induced ERK1/2, P38 and JNK phosphorylation, in the acute inflammatory response to adiponectin, non-primed Kupffer Cells were cultured for 4 hours with adiponectin and MAPK inhibitors. TNFα mRNA was then determined by qPCR. Treatment of non-primed Kupffer Cells with adiponectin and the P38 inhibitor SB203580, ERK1/2 inhibitor PD98059 and the JNK inhibitor either alone or in combination, resulted in a significant decrease in TNFα mRNA when compared to non-primed Kupffer Cells (Figure 5.2) with TNFα mRNA decreasing from 145.6 fold from basal to 12.99 fold (p<0.05) when P38 alone was inhibited, 16.8 fold (p<0.005) when ERK1/2 was inhibited, 17.0 fold (p<0.005) when JNK was inhibited, 13.12 fold (p<0.005) when P38 and ERK1/2 were inhibited together, 12.55 fold (p<0.005) when P38 and JNK were inhibited together, 11.47 fold (p<0.005) when ER1/2 and JNK were inhibited together and 8.22 (p<0.005) when all three MAPK kinases were inhibited. There were no observed differences in TNFα mRNA between inhibitors and the co-treatment with two or more inhibitors did not cause any additional down-regulation in TNFα mRNA.

5.3.2 Adiponectin Induced IL10 occurs via P38 activation in Kupffer Cells.

To assess whether the adiponectin-induced anti-inflammatory and immuno-modulatory cytokine IL10 requires activation of similar cellular pathways to TNFα in Kupffer Cells, IL10 mRNA was determined following 4 hours of adiponectin treatment in non-primed Kupffer Cells in the presence of MAPK inhibitors SB203580, PD98059 and JNK inhibitor.
There was a statistically significant decrease in Kupffer Cell derived IL10 mRNA when non-primed Kupffer Cells were cultured in adiponectin and the P38 inhibitor SB203580 from 6.35 fold to 0.74 fold (p<0.05), SB203580 and the ERK1/2 inhibitor PD98059 in combination decreased IL10 mRNA expression to 0.77 fold (p<0.05) and combined SB203580, PD98059 and JNK inhibitor decreased IL10 mRNA expression to 0.51 fold (p<0.05) (Figure 5.3). There was a non-significant decrease in IL10 mRNA in Kupffer Cells that were cultured with adiponectin, SB203580 and the JNK inhibitor.

5.3.3 Adiponectin-induced MAPK Inflammatory Pathways in Kupffer Cells are inhibited by Adiponectin Pre-Exposure.

To assess whether there was a difference in MAPK activation between primed (non-inflammatory) Kupffer Cells and non-primed (non-inflammatory) Kupffer Cells, time course experiments were carried out. As MAPK activation typically occurs rapidly, a one-hour time period was assessed for any differences in signalling pathways between adiponectin primed and non-primed Kupffer Cells. All three MAPK pathways had observed differences between primed and non-primed Kupffer Cells.

In non-primed Kupffer Cells there was a statistically significant (p<0.0005) increase in ERK1/2 phosphorylation after 60 minutes of adiponectin exposure when compared to primed Kupffer Cells (Figure 5.4). In addition, there was a significant (p<0.0005) increase in ERK1/2 phosphorylation in non-primed Kupffer Cells at 60 minutes compared to non-primed Kupffer Cells at 0, 5 and 15 minutes of adiponectin exposure. Non-primed Kupffer Cells had a significant increase in P38 phosphorylation at 30 minutes (p<0.005) and 60 minutes (p<0.05) compared to primed Kupffer Cells (Figure 5.5). 30 and 60 minutes of adiponectin exposure in non-primed Kupffer Cells caused a significant increase in P38 phosphorylation compared to non-primed Kupffer Cells at time points 0, 5 and 15 minutes (p<0.05). Total P38 levels were found to decrease over
n=3

Figure 5.2 ERK1/2, P38 and JNK Inhibition Prevents Adiponectin-Induced TNFα Synthesis in Non-Primed Kupffer Cells.

Day 1 cultured rat Kupffer Cells were primed with DMEM containing adiponectin 1 µg/ml for 18 hours or left in culture media alone (non-primed). Media was removed and Kupffer Cells were washed prior to new DMEM containing 1 µg/ml adiponectin and 20 nM JNK Inhibitor, 7 µM SB203580 P38 inhibitor and/or 10 µM PD98059 ERK1/2 inhibitor being applied for 4 hours. Kupffer Cells were lysed for mRNA extraction and TNFα mRNA was determined using qPCR. GAPDH was used as the housekeeper gene.
Figure 5.3 P38 Inhibition Prevents Adiponectin-Induced IL10 mRNA in Kupffer Cells

Day 1 cultured rat Kupffer Cells were primed with adiponectin (ADN) 1 µg/ml for 18 hours or left in culture media alone. Fresh media was applied containing 1 µg/ml adiponectin and 20 nM JNK Inhibitor and/or 7 µM P38 inhibitor SB203580 and/or 10 µM ERK1/2 inhibitor PD98059 for 4 hours. Kupffer Cells were lysed for mRNA extraction and IL10 mRNA was determined using qPCR. GAPDH was used as the housekeeper gene. Results are expressed as fold change from basal IL10 mRNA expression in non-adiponectin exposed control Kupffer Cells.
time in both non-primed Kupffer Cells and primed Kupffer Cells suggesting that adiponectin reduces total P38 levels.

JNK phosphorylation was significantly (p<0.05) increased in non-primed Kupffer Cells after 30 minutes of adiponectin treatment (Figure 5.6). There was significant increase in JNK phosphorylation in non-primed Kupffer Cells after 5 minutes of adiponectin exposure compared to time 0 (p<0.005), after 15 minutes of adiponectin exposure compared to time 0 (p<0.05) and after 30 minutes of adiponectin exposure compared to time 0 (p<0.05), 5 minutes (p<0.05) and 15 minutes (p<0.005). In primed Kupffer Cells there was significant increase in JNK phosphorylation in Kupffer Cells exposed to adiponectin for 60 minutes compared to those exposed for 5 minutes (p<0.05).
Figure 5.4 Adiponectin-Induced ERK1/2 Phosphorylation occurs maximally at 60 minutes in non-primed Kupffer Cells.

Kupffer Cells (KC) were primed in media containing 1 μg/ml adiponectin for 18 hours or cultured in media alone (Non-Primed KC). After 18 hours, Kupffer Cells were washed and fresh DMEM containing 1 μg/ml adiponectin was applied. Kupffer Cells were lysed at 0, 5, 15, 30 and 60 minutes and total and phosphorylated ERK1/2 was determined by Western Blot to give a phosphorylated to total ERK1/2 ratio (adjusted to non-primed, time=0 value).
Figure 5.5 Adiponectin-Induced P38 Phosphorylation occurs maximally at 30 to 60 minutes in non-primed Kupffer Cells.

Kupffer Cells (KC) were primed in media containing 1 μg/ml adiponectin for 18 hours or cultured in media alone (Non-primed KC). After 18 hours, Kupffer Cells were washed and fresh DMEM containing 1 μg/ml adiponectin was applied. Kupffer Cells were lysed at 0, 5, 15, 30 and 60 minutes and total and phosphorylated P38 was determined by Western Blot to give a phosphorylated to total P38 ratio (adjusted to non-primed, T=0 value).
Figure 5.6 Adiponectin-Induced JNK Phosphorylation occurs maximally at 30 minutes in non-primed Kupffer Cells.

Kupffer Cells (KC) were primed in media containing 1 µg/ml adiponectin for 18 hours or cultured in media alone (Non-Primed KC). After 18 hours, Kupffer Cells were washed and fresh DMEM containing 1 µg/ml adiponectin was applied. Kupffer Cells were lysed at 0, 5, 15, 30 and 60 minutes and total and phosphorylated SAPK/JNK was determined by Western Blot to give a phosphorylated to total SAPK/JNK ratio (adjusted to non-primed, T=0 value).
5.3.4 Adiponectin Rapidly Induces IκB phosphorylation in Kupffer Cells.

To confirm existing data that adiponectin activates the NFκB pathway in Macrophages [230, 231, 312], IκB phosphorylation in response to adiponectin was assessed (Figure 5.7). When adjusted to baseline basal levels of IκB phosphorylation, IκB phosphorylation was significantly increased after 18 and 4 hours of adiponectin exposure. Compared to non-adiponectin exposed Kupffer Cells, IκB phosphorylation was significantly increased at 18 hours (p<0.05) and 18 and 4 hours of adiponectin exposure (p<0.005).

5.3.5 IL10 Modulates Adiponectin Induced MAPK Phosphorylation in Kupffer Cells.

In the previous chapter of this thesis, IL10 was demonstrated to abrogate adiponectin induced TNFα and IL6 in Kupffer Cells; recombinant IL10 down-regulated Kupffer Cell-derived TNFα and IL6 in non-primed Kupffer Cells and soluble-IL10 antibody up-regulated Kupffer Cell-derived TNFα and IL6 in primed Kupffer Cells. ERK1/2, P38 and SAPK/JNK phosphorylation were demonstrated earlier in this chapter to be activated by adiponectin in non-primed Kupffer Cells and their activation was shown to be necessary to enable adiponectin-induced TNFα, but not ERK1/2 or JNK induced IL10 in Kupffer Cells.

To determine whether IL10 mediated the down-regulation of TNFα via suppression of the MAPK pathways, the previously performed time course experiment was repeated in the presence of recombinant IL10 peptide or soluble IL10 antibody. When non-primed Kupffer Cells were exposed to adiponectin in the presence of recombinant IL10 peptide there was no observed increase in ERK1/2 phosphorylation over time such that occurred in non-primed Kupffer Cells treated with adiponectin alone. ERK1/2 phosphorylation was significantly (p<0.005) decreased at 60 minutes and non-significantly decreased at all other time points (Figure 5.8).
compared to non-primed Kupffer Cells treated with adiponectin alone. IL10 neutralisation by use of a soluble IL10 antibody in primed Kupffer Cells increased ERK1/2 phosphorylation at 60 minutes compared to primed Kupffer Cells exposed to adiponectin without IL10 antibody (p<0.005) (Figure 5.9).

Non-primed Kupffer Cells exposed to adiponectin and recombinant IL10 peptide had a significant decrease in P38 phosphorylation when compared to non-primed, adiponectin exposed Kupffer Cells alone at time points 30 minutes (p<0.005) and 60 minutes (p<0.05) (Figure 5.10). Exposing adiponectin-primed Kupffer Cells to adiponectin and soluble IL10 antibody had no effect on P38 phosphorylation compared to adiponectin exposed primed Kupffer Cells (Figure 5.11).

Adiponectin and recombinant IL10 peptide decreased SAPK/JNK phosphorylation in non-primed Kupffer Cells at 30 minutes compared to adiponectin exposed cells alone (Figure 5.12). However, primed Kupffer Cells that were exposed to adiponectin and soluble IL10 antibody had a significant increase in SAPK/JNK phosphorylation at time points 5 minutes (p<0.05) (Figure 5.13).
Figure 5.7. Adiponectin activates the NFκB pathway in Kupffer Cells.

Kupffer Cells were cultured in media containing 1 µg/ml adiponectin (ADN) for 18 hours or cultured in media alone. After 18 hours, Kupffer Cells were washed and fresh media with or without 1 µg/ml adiponectin was applied for a further 4 hours. Kupffer Cells were lysed at baseline or after 18 hours or 18 + 4 hours of treatment. Phospho and total IκB was determined by Western Blot to give a phosphorylated to total IκB ratio (adjusted to baseline value) with β-actin used as a loading control.
Figure 5.8 Exogenous IL10 abrogates Adiponectin-Induced ERK1/2 phosphorylation in Kupffer Cells

Kupffer Cells were primed in media alone for 18 hours (Non-Primed KC). After 18 hours, Kupffer Cells were washed and fresh DMEM media was applied. Non-primed Kupffer Cells were exposed to 1 µg/ml adiponectin and 20 ng/ml recombinant IL10 peptide. Kupffer Cells were lysed at 0, 5, 15, 30 and 60 minutes and total and phosphorylated ERK1/2 was determined by Western Blot and quantified by densitometry to give a phosphorylated to total ERK1/2 ratio. Results were adjusted to non-primed Kupffer Cells at time=0.

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ERK1/2 Phosphorylation

**p<0.005

n=3
Figure 5.9 IL10 neutralisation increases adiponectin-induced ERK1/2 phosphorylation in Kupffer Cells at 60 minutes.

Kupffer Cells were primed in media containing 1 µg/ml adiponectin for 18 hours (Primed KC). Primed Kupffer Cells were then exposed to 1 µg/ml adiponectin and 10 µg/ml IL10 antibody. Cells were lysed at 0, 5, 15, 30 and 60 minutes and total and phosphorylated ERK1/2 was determined by Western Blot and quantified by densitometry to give a phosphorylated to total ERK1/2 with values normalized to non-primed Kupffer Cells at time point 0.
Figure 5.10 Exogenous IL10 abrogates adiponectin-induced P38 phosphorylation in Kupffer Cells

Kupffer Cells were primed in media alone for 18 hours (Non-primed KC). Non-primed Kupffer Cells were then exposed to 1 µg/ml adiponectin and 20 ng/ml recombinant IL10 peptide. Kupffer Cells were lysed at 0, 5, 15, 30 and 60 minutes and total and phosphorylated P38 was determined by Western Blot and quantified by densitometry to give a phosphorylated to total P38 ratio. Results were adjusted to non-primed Kupffer Cells at time=0.
Figure 5.11 IL10 neutralisation increases adiponectin-induced P38 phosphorylation in Kupffer Cells at 60 minutes.

Kupffer Cells were primed in media containing 1 µg/ml adiponectin for 18 hours (Primed KC). Primed Kupffer Cells were then exposed to 1 µg/ml adiponectin and 10 µg/ml IL10 antibody. Cells were lysed at 0, 5, 15, 30 and 60 minutes and total and phosphorylated P38 was determined by Western Blot and quantified by densitometry to give a phosphorylated to total P38 with values normalized to non-primed Kupffer Cells at time point 0.
n=3

Figure 5.12 Exogenous IL10 abrogates adiponectin-induced JNK phosphorylation in Kupffer Cells at 30 minutes.

Kupffer Cells were primed in media alone for 18 hours (Non-Primed KC). Non-primed Kupffer Cells were then exposed to 1 µg/ml adiponectin and 20 ng/ml recombinant IL10 peptide. Kupffer Cells were lysed at 0, 5, 15, 30 and 60 minutes and total and phosphorylated JNK was determined by Western Blot and quantified by densitometry to give a phosphorylated to total JNK ratio. Results were adjusted to non-primed Kupffer Cells at t=0.
Figure 5.13 IL10 neutralisation only alters adiponectin-induced JNK phosphorylation in Kupffer Cells at 5 minutes.

Kupffer Cells were primed in media containing 1 µg/ml adiponectin for 18 hours (Primed KC). Primed Kupffer Cells were then exposed to 1 µg/ml adiponectin and 10 µg/ml IL10 antibody. Cells were lysed at 0, 5, 15, 30 and 60 minutes and total and phosphorylated JNK was determined by Western Blot and quantified by densitometry to give a phosphorylated to total JNK with values normalized to non-primed Kupffer Cells at time point 0.
5.4 Discussion

The inflammatory effects of adiponectin upon macrophages are now well established; in particular this thesis demonstrates the inflammatory effects of acute adiponectin upon Kupffer Cells. Conflicting potential mechanisms of this adiponectin-induced inflammation have been reported in the literature and in view of this, the experiments described within this chapter were designed to identify the mechanisms by which adiponectin acutely up regulates TNFα in Kupffer Cells.

The findings described in this chapter show that adiponectin induced TNFα occurs via activation of the MAPK pathways ERK1/2, P38 and JNK. In addition adiponectin was found to stimulate the NFκB pathway. As the previous chapter demonstrated that tolerance to adiponectin occurs on repeated adiponectin exposure, MAPK phosphorylation was compared between adiponectin naïve, non-primed Kupffer Cells and adiponectin primed Cells. Non-primed Kupffer Cells demonstrated significant increases in ERK1/2, P38 and JNK phosphorylation at 30 to 60 minutes where as adiponectin primed Kupffer Cells did not have any alteration in MAPK phosphorylation over 60 minutes.

As IL10 was shown to mediate adiponectin-induced tolerance in the Kupffer Cell in the previous chapter, and its up-regulation occurs independently of ERK1/2 and JNK, the effect of IL10 on adiponectin-induced phosphorylation was studied. Exogenous IL10 abrogated adiponectin-induced ERK1/2, P38 and JNK phosphorylation in non-adiponectin primed Kupffer Cells, however IL10 neutralisation via soluble IL10 antibody inconsistently increased ERK1/2, P38 and JNK phosphorylation in primed Kupffer Cells.

The role of MAPK activation in Kupffer Cell derived TNFα generation is not well understood. This thesis demonstrates the novel finding of TNFα mRNA synthesis inhibition when non-primed Kupffer Cells were exposed to adiponectin in the presence
of the ERK1/2 inhibitor PD98059, the P38 inhibitor SB203580 or the JNK inhibitor, either alone or in combination. Using combinations of inhibitors did not further decrease TNFα mRNA when compared to any single inhibitor used alone. Adiponectin led to a rapid phosphorylation of ERK1/2, P38 and JNK with maximal phosphorylation occurring at 30 to 60 minutes. Adiponectin also led to an increase in IkB phosphorylation in Kupffer Cells. Adiponectin primed Cells failed to phosphorylate ERK1/2, P38 or JNK on repeated adiponectin exposure, which remains in keeping with their anti-inflammatory phenotype as described in the previous chapter.

Studies of other cell types have demonstrated variable inflammatory pathway activation. Species-related and cellular differences are associated with variations within the MAPK family, which is likely to contribute towards differing responses to adiponectin between studies. In addition, different adiponectin forms ie globular compared to HMW or LMW adiponectin, and cellular sources, ie Eukaryocyte HUVEC or E. coli derived, with associated differing post-translational modifications, are used in different studies leading to heterogeneous findings. Globular adiponectin rapidly phosphorylates ERK1/2, P38 and JNK in RAW264.7 macrophages [231] with observed peaks in phosphorylation occurring from 10 to 30 minutes rather than the 30 to 60 minutes observed in Kupffer Cells. In RAW264.7 macrophages, adiponectin induced TNFα occurs via Egr-1 expression and associated NFκB translocation with ERK1/2 but not P38 or JNK required for increased Egr-1 binding activity [230]. Further studies demonstrating adiponectin induced MAPK activation include globular adiponectin modulating microRNA-21 and programmed cell death 4 expression via activation of ERK1/2, JNK and NFκB in RAW264.7 macrophages [333], adiponectin-induced IL1β, IL6, TNFα and PGE₂ in human placental and adipose cells was abrogated with the ERK1/2 inhibitor U0126 and the NFκB inhibitor BAY 11-7082 [332] and adiponectin
increased P38 phosphorylation in CD4⁺ T-cells with the P38 inhibitor SB203580 abrogating adiponectin induced IFNγ [262].

NFκB activation was observed in U937 macrophages treated with high molecular weight and globular adiponectin, however treatment with mutated adiponectin that was unable to form multimers failed to elicit an NFκB response [312]. The authors further demonstrated that inhibition of P38, phosphatidylinositol 3-kinase and protein kinase C abrogated this observed NFκB activation. Globular adiponectin has also been demonstrated to activate NFκB activity after 1 to 4 hours in RAW 264.7 macrophages [230, 296]. NFκB activation was also observed when hexameric adiponectin was applied to C2C12 myocytes with no activation occurring when cells were alternatively treated with trimeric or globular adiponectin [313]. In porcine macrophages, adiponectin pre-treatment prevented subsequent LPS-induced NFκB nuclear translocation however adiponectin alone did not increase NFκB translocation [227]. These described findings, though often conflicting, are certainly consistent and supportive of the observations described in this thesis in which primary Kupffer Cells are treated with HUVEC-derived full-length adiponectin.

Adiponectin tolerance to itself has not been well described, and certainly not previously in Kupffer Cells. Instead the modulatory effects of adiponectin upon inflammatory triggers, in particular LPS has been better described. LPS induces multiple macrophage MAPK pathways including ERK1/2, p38 and JNK [334], and consistent with the findings in this thesis, adiponectin pre-exposure is likely to abrogate these adiponectin-induced MAPK pathways. In the only published study to explore the effects of adiponectin upon Kupffer Cell MAPK pathways, globular adiponectin pre-exposure suppressed LPS-induced ERK1/2 and P38 phosphorylation and prevented IκB degradation [305]. Similarly, in RAW264.7 macrophages, globular adiponectin pre-exposure decreased LPS-induced ERK1/2 and P38 phosphorylation [296] and in human
macrophages, adiponectin pre-exposure suppressed LPS and TNFα-induced IkB, JNK and P38 phosphorylation and IL6-induced STAT3 [299]. However, in human aortic endothelial cells, adiponectin suppressed TNFα-induced IkB phosphorylation without affecting JNK, p38 or Akt kinase phosphorylation [335]. The findings described by Ouchi and colleagues may simply be a consequence of the pleiotropic effects of adiponectin upon endothelial cells rather than the monocyte/macrophage cell line.

In addition to the role of the MAPK pathway in mediating an inflammatory response in adiponectin-exposed Kupffer Cells, this chapter also demonstrates that P38 though not ERK1/2 or JNK, is required for adiponectin-induced IL10 generation. Again, the role of P38 in mediating adiponectin-induced IL10 has not been described in Kupffer Cells.

In the THP-1 cell line, the P38 inhibitor SB203580 non-significantly decreased adiponectin-induced IL10 synthesis with ERK1/2 inhibition also failing to alter adiponectin-induced IL10 [229]. Further conflicting reports from a macrophage cell line demonstrate that cyclic-AMP response element binding protein (CREB) activation is required for globular adiponectin induced IL10 in RAW264.7 macrophages with inhibition of ERK1/2 preventing CREB phosphorylation and subsequent IL10 promoter activity [231]. The role of CREB binding to the IL10 promoter was also found to be required for LPS-induced IL10 in bone marrow derived macrophages, however phosphorylation of CREB was dependent upon P38 phosphorylation and activation of its downstream targets MSK1 and MSK 2 rather than ERK1/2 phosphorylation [139]. P38 inhibition leads to decreased LPS-induced IL10 production in macrophages, primary dendritic cells and human peripheral blood monocytes with ERK1/2 also contributing towards its production [236]. This regulatory role of P38 in mediating LPS-induced IL10 is consistent with recent theories that adiponectin may in fact act through TLR4 to mediate some of its effects. Assessment of adiponectin-induced P38 phosphorylation and IL10 generation in a P38 knock out mouse model would be useful.
to further define the mechanism of adiponectin-induced IL10, however MAPK knockout mice are known to have high lethality preventing their widespread use. More evidence for the important role of P38 in the generation of IL10 comes from a mouse model with a myeloid cell-specific P38 deletion that develops a more severe arthritis than the wild-type mouse with this finding being attributed to decreased IL10 transcription [137]. IL10 expression following TLR-activation occurs in an ERK1/2 dependent fashion, with differences in IL10 production being attributed to the strength of the ER1/2 activation in each cell type [238]. As a result of these differences ERK activation and associated IL10 production is highest in macrophages, less in myeloid dendritic cells and lowest in plasmacytoid dendritic cells, thereby demonstrating inherent differences between immune cells. As IL10 has so many important immunomodulatory effects, it is a major modulator of adiponectin, and acts to dampen immune response and therefore damage to the host, further understanding into the regulation of its production is required, particularly in different immune cells.

The direct modulatory effects of IL10 upon the MAPK pathways are not well described in the literature, and certainly there are no published reports that describe the effect of IL10 on MAPK pathways in Kupffer Cells, or on adiponectin-induced MAPK phosphorylation. The studies in this chapter demonstrate IL10-induced abrogation of ERK1/2 phosphorylation in non-primed Kupffer Cells, and stimulation of ERK1/2 in adiponectin primed Kupffer Cells. Similarly IL10 abrogated adiponectin-induced P38 phosphorylation in non-primed Kupffer Cells, however IL10 neutralisation did not significantly or consistently increase P38 phosphorylation in primed Kupffer Cells. Again findings in the JNK pathway were slightly inconsistent, with exogenous IL10 abrogating adiponectin induced JNK phosphorylation in non-primed Kupffer Cells only at 30 minutes and IL10 neutralisation only increasing JNK phosphorylation at 5 minutes in primed cells. At 5 minutes JNK phosphorylation in both primed and non-primed
Kupffer Cells was unchanged from baseline and as such, is likely to have no clinical relevance. Certainly for a more comprehensive assessment of the impact of IL10 on P38 and JNK MAPK pathways, repeating these studies with either Kupffer Cells from an IL10 knockout mouse or with IL10 knock-down with siRNA would be useful. Current literature reports of IL10 modulation upon MAPK pathways are scarce and do not either support or refute the findings described in my thesis. IL10 neutralisation did not alter adiponectin-mediated suppression of LPS or TNFα-induced IκB, JNK and P38 or IL6-induced STAT3 in human macrophages [299]. This study however, also demonstrated that IL10 neutralisation did not alter adiponectin-mediated down regulation in LPS-stimulated TNFα that is in contrast to the findings by other groups. In mouse bone marrow-derived macrophages, exogenous IL10 was demonstrated to inhibit LPS-induced P38 phosphorylation [336]. This is consistent with another report of IL10 inhibiting P38 with resultant inhibition of TNFα translation in a mouse model of colitis [337]. In a further study, LPS induced cyclooxygenase-2 in monocytes was found to be dependent upon ERK1/2 and P38 activation with IL10 subsequently found to inhibit both ERK1/2 and P38 [338]. Furthermore, IL10 pre-treated monocytes failed to elicit a CD40-mediated activation of ERK1/2 [339]. A potential role of IL10 modulating MAPK pathways was identified in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis mice, where treatment with an IL10- producing *Lactobacillus brevis* G-101 strain inhibited TNBS-induced IRAK-1, p65, P38, ERK, JNK and AKT phosphorylation [340]. In *Trypanosoma cruzi* infected cardiomyocytes, exogenous IL10 inhibited ERK1/2 phosphorylation and NFκB activation, and reduced TNFα and IL6 expression [245]. The authors found this inhibition to be dependent upon IL10-induced STAT3 phosphorylation and up-regulation of SOCS-3.

In summary, this chapter demonstrates that adiponectin acutely induces ERK1/2, P38 and JNK in Kupffer Cells. ERK1/2, P38 and JNK phosphorylation is required to enable...
adiponectin-induced TNFα, with adiponectin-induced IL10 requiring P38 activation. This chapter supports the hypothesis that adiponectin-induced IL10 subsequently inhibits further MAPK phosphorylation with an associated decrease in TNFα. This inhibition of MAPK phosphorylation and TNFα synthesis is consistent with the adiponectin tolerant state.

The MAPK pathways are complex and further work to identify which adiponectin receptor and receptor-mediated signalling pathways are involved in adiponectin-induced inflammation is required. In addition, to fully understand the mechanisms involved in this adiponectin-IL10-induced inhibition of MAPK phosphorylation, further studies particularly assessing downstream targets of IL10 including SOCS-3 or HO-1 is required. This would contribute towards the development of new therapeutic agents that may be useful in treating the burgeoning obesity and metabolic epidemic with associated increasingly high rates of NAFLD and NASH.
Chapter 6 Discussion and Future Directions.
Rates of NAFLD and NASH, with associated complications of cirrhosis, liver failure and HCC are continuing to increase worldwide. Current treatment modalities for NAFLD are sub-optimal and as such further research into more effective management strategies is required. Adiponectin with its well described insulin-sensitising, anti-inflammatory and anti-fibrotic effects, shows promise as a future therapeutic modality for NAFLD. Unfortunately the recently reported pro-inflammatory effects of adiponectin may limit the therapeutic potential of this adipokine and further insight into the mechanisms underlying these inflammatory effects of adiponectin is required. In particular, adiponectin has been shown to induce an inflammatory response in cultured monocytes and macrophages, and macrophage cell lines, however the effects of adiponectin upon they Kupffer Cell, the resident liver macrophage, have not been well described.

In the series of experiments described in this thesis, the novel effects of adiponectin, both pro-inflammatory and anti-inflammatory, on the Kupffer Cell have been described. In the initial experiments, Kupffer Cells that were exposed to adiponectin were found to have up-regulated TNFα, IL6 and IL10 production with adiponectin actually potentiating LPS-induced cytokine production. Despite this observed rise in inflammatory cytokines in response to adiponectin, Kupffer Cells demonstrated a down regulation in pro-fibrotic cytokine (TGFβ, CTGF, PDGF) synthesis. The anti-inflammatory effects of adiponectin became unmasked when Kupffer Cells were pre-treated with adiponectin for 18 hours prior to a sequential exposure to LPS for a further 4 hours. In this setting adiponectin pre-treatment resulted in a down regulation of TNFα and IL6 in response to LPS challenge. These results suggested that during the adiponectin pre-treatment period, a phenotypic change in the Kupffer Cell developed that was manifest as tolerance to further LPS exposure. In view of this potential phenotypic switch and development of tolerance in the Kupffer Cell, the hypothesis that
adiponectin induces tolerance to repeated adiponectin exposure was tested. Adiponectin-primed Kupffer Cells failed to elicit an inflammatory response when re-exposed to a further 4 hours of adiponectin where as non-adiponectin primed Kupffer Cells elicited an inflammatory response characterized by increased TNFα and IL6 on repeated adiponectin exposure. IL10 levels were not altered by adiponectin pre-treatment and remained elevated from basal measurements. As IL10 has multiple immunomodulatory properties and continued to be expressed despite adiponectin pre-treatment, the role of IL10 is modulating the development of tolerance in the Kupffer Cell was explored. In fact, non-adiponectin primed Kupffer Cells failed to elicit an inflammatory response when re-exposed to adiponectin together with exogenous IL10 peptide and adiponectin-primed Kupffer Cells experienced an increase in TNFα and IL6 when repeat treated with adiponectin and soluble IL10 antibody. Together these findings suggest that adiponectin-induced IL10 that is synthesized during the first period of adiponectin exposure forms a feedback loop to prevent an inflammatory response to subsequent adiponectin exposure. To better describe the inflammatory pathways that adiponectin activates in the Kupffer Cell, the role of the MAPK pathways ERK1/2, P38 and JNK were explored. Adiponectin-induced TNFα was abrogated when Kupffer Cells were cultured with adiponectin and PD98059 the ERK1/2 inhibitor, SB203580 the P38 inhibitor and the JNK inhibitor suggesting that all three pathways were required for adiponectin-induced TNFα in Kupffer Cells. In addition, it was found that primed Kupffer Cells failed to phosphorylate the MAPK pathways as was found in non-primed Kupffer Cells. Finally, as IL10 was found to induce tolerance to adiponectin in Kupffer Cells, the effect of IL10 upon the MAPK pathways was explored. Exogenous IL10 decreased adiponectin-induced phosphorylation of the MAPK pathways in non-primed Kupffer Cells and IL10 neutralisation using IL10
antibodies increased adiponectin-induced phosphorylation of the MAPK pathways in adiponectin-primed Kupffer Cells.

Together the results of this thesis suggest that adiponectin initially induces an up regulation of TNFα via ERK1/2, P38 and JNK and IL10 via P38 activation. On repeated adiponectin exposure, IL10 feedbacks to prevent MAPK phosphorylation and further inflammatory response as illustrated in figure 6.1.

The findings in this thesis reinforce the importance of maintaining a stable, lean body mass with a high circulating adiponectin levels. Under these “healthy” physiological conditions Kupffer Cells are likely to remain and tolerant and non-reactive thereby protecting the liver from injury. In those with excess adipose and associated low adiponectin levels however, Kupffer Cells are more likely to mount an inflammatory response to multiple gut derived toxins and therefore cause an initial liver injury or contribute towards pre-existing bland steatosis progressing to NASH and cirrhosis. In addition the findings in this thesis suggest that a rapid flux in adiponectin level, perhaps bought on by starvation or a highly restrictive diet may also bring about an acute hepatitis. Certainly liver failure has been reported in those undergoing rapid weight loss following bariatric surgery and those with anorexia nervosa. This reinforces current recommendations of NAFLD management that individuals aim to obtain a gradual weight loss through a healthy, balanced diet and exercise.

The clinical significance of this observed inflammatory response to a rapid flux in adiponectin level is unlikely to be limited to the liver. Similar inflammatory responses to adiponectin have been found in in vitro studies of monocytes and macrophages though the adiponectin-tolerising properties described here in Kupffer Cells have not been well described in macrophages. Obese individuals with elevated serum levels of TNFα and IL6, low levels of adiponectin with NAFLD and insulin resistance generally
Figure 6.1. Differential effect of adiponectin upon Kupffer Cells.

On initial exposure to adiponectin, ERK1/2, P38 and JNK are activated and increased TNFα synthesis and release occurs. IL10 synthesis occurs via P38 activation. On subsequent adiponectin exposure, IL10 is rapidly released where it acts upon the MAPK pathways to inhibit further TNFα synthesis and release. It is likely that other signaling pathways contribute towards ongoing IL10 synthesis and release as ongoing IL10 production occurs despite IL10 inhibiting P38.
have highly vascular adipose tissue that is heavily infiltrated with macrophages. This inflammatory adipose tissue contributes towards the pathogenesis of type 2 diabetes mellitus, cardiovascular disease, certain cancers and NAFLD/ NASH. An acute flux in adiponectin levels may therefore also contribute towards worsening of diabetes and cardiovascular events.

Despite these described adverse inflammatory findings in Kupffer Cells, adiponectin still has therapeutic potential. Dosing of adiponectin may need to be as a gradual stepped dose increase in order to prevent sudden serum concentration changes and inflammatory response, or may need to occur with dietary modifications. Other barriers to the therapeutic use of adiponectin exist. Adiponectin is a multimeric protein with multiple post-translational modifications that are necessary for its biological actions. Adiponectin also circulates at high serum concentrations, which would make dosing prohibitive currently. In view of these properties, understanding and identifying the downstream mediators of adiponectin, including IL10, their biological effects and mechanism of action would be useful.

To better understand and expand the current findings of this thesis, further research projects are worthwhile. Firstly it would be useful to ascertain whether this inflammatory response as observed in this thesis is limited to Kupffer Cells or extends to monocytes and/or macrophages and systemically. The current hypothesis that we would like to test is that acute adiponectin exposure induces a systemic inflammatory response. In this experiment, both adiponectin and wild type mice will be intravenously injected with a physiological dose of adiponectin following which plasma and liver tissue will be collected for analysis and assessment of inflammatory response. If an inflammatory response is found, this experiment could be replicated in an obese animal model with low adiponectin levels as a more physiological representation of human obesity. Human studies that will also contribute towards our understanding of the
inflammatory effects of adiponectin include the collection of Kupffer Cells (obtained from a liver biopsy sample) and peripheral mononucleocytes from both obese and lean individuals, culturing their cells in adiponectin and assessing inflammatory response. Again this would test the hypothesis that Kupffer cells and mononucleocytes from obese individuals mount an inflammatory response to adiponectin where as cells from a lean individual would fail to elicit an inflammatory response. Furthermore, it would be useful to monitor both systemic and cellular responses following weight loss associated with bariatric surgery to correlate alterations of adiponectin levels with changes in inflammatory markers and markers of liver injury such as CK-19.

To further understand the mechanisms of adiponectin-induced inflammatory response, and to identify the factors associated with causing a phenotypic shift in Kupffer Cells from inflammatory to anti-inflammatory, mouse models including IL10, adiponectin, AdipoR1 and AdipoR2 knockouts would be useful. Injecting knockout mice with adiponectin and assessing inflammatory response can identify the role of IL10, or specific receptors.

In summary, adiponectin has multiple important anti-inflammatory effects. This thesis confirms that adiponectin can promote both an anti-inflammatory and pro-inflammatory response in rat Kupffer Cells, with Kupffer Cells developing tolerance to adiponectin on repeated exposure. These findings contribute towards a better understanding of the mechanism of action of adiponectin, and on how adiponectin impacts on the pathogenesis and treatment of NAFLD.
References


