# The Role of Toll-like Receptor Ligands and Fatty Acids in the Recruitment of Monocytes Across Liver Sinusoids

By

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# Abstract

The liver is continuously exposed to pathogen associated molecular patterns (PAMPs) such as microbial ligands that are transported from the gut via the portal circulation. In liver disease, microbial translocation increases and patients are at risk of sepsis and poor clinical outcomes. The gut is not only a source of microbial ligands that interact with toll-like receptors (TLRs) in the liver, but also has a role in association with diet in the uptake of fatty acids. Fatty acids also modulate the outcome from disease.

Diseased livers show an increase in TLRs. Under conditions of flow upon hepatic sinusoidal endothelium cells, stimulation of the endothelium by cell surface located TLRs increases adhesion of monocytes but not transmigration in an *in vitro* model of liver sinusoids. To transmigrate across hepatic endothelium efficiently, the endothelium requires priming by pro-inflammatory cytokines. Oleic acid can provide a suppressive effect on inflamed hepatic sinusoidal endothelium by reducing adhesion of monocytes. Treating monocytes with ligands to cell surface TLRs diminishes their ability to migrate across inflamed endothelium.

Tumour necrosis factor α and interferon γ treatment of hepatic sinusoidal endothelial cells followed by treatment with lipopolysaccharides show a synergistic interaction increasing the expression of TLR5 and 7 beyond what is seen treatment individually by the cytokines.

One of the fates of recruited monocytes to the liver is to differentiate into macrophages. Oleic acid is able to increase the expression of CD206, one of the markers of alternative

activation on macrophages. Alternatively differentiated liver derived macrophages show increased expression of TLR5.

# Dedication

To my parents

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# List of Abbreviations

| ABBREVIATION | |
|---|---|
| AA | Amino Acid |
| AFLD | Alcoholic Fatty Liver Disease |
| ALD | Alcoholic Liver Disease |
| ALR | AIM2-Like receptors |
| APC | Antigen Presenting Cells |
| BEC | Biliary Epithelial Cells |
| BSA | Bovine Serum Albumin |
| CARD | Caspase Activation And Recruitment Domains |
| CD | Cluster of Differentiation |
| CLR | C-type Lectin Receptor |
| DAB | 3,3'Diaminobenzidine Tetrahydrochloride |
| DAMP | Damage Associated Molecular Patterns |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | Dimethyl Sulfoxide |
| DPX | Depex |

| | |
|---|---|
| dsDNA | Double Stranded Deoxyribonucleic acid |
| ECM | Extra Cellular Matrix |
| EDTA | Ethylene Diaminotetraacetic Acid |
| EGF | Epidermal Growth Factor |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| FA | Fatty Acid |
| FABP | Fatty Acid Binding Protein |
| FATP | Fatty Acid Transporter Protein |
| FCS | Foetal Calf Serum |
| FFA | Free Fatty Acid |
| HCC | Hepatocellular Carcinoma |
| HDL | High Density Lipoproteins |
| HEA | Human Epithelial Antibody |
| HEPES | 4-(2-hydroxyethyl)-1-Piperazineethanesulfonic Acid |
| HFD | High Fat Diet |
| HFE | Haemochromatosis |
| HGF | Hepatocyte Growth Factor |

| | |
|---|---|
| HKLM | Heat-Killed Listeria Monocytogenes |
| HSC | Hepatic Stellate Cells |
| HSEC | Hepatic Sinusoidal Endothelial Cells |
| ICAM-1 | Intercellular Adhesion Molecule 1 |
| IL-4 | Interleukin 4 |
| IL-6 | Interleukin 6 |
| IL-8 | Interleukin 8 |
| IRAK | IL-1 Receptor Associated Kinase |
| LDL | Low-Density Lipoprotein |
| LGP2 | Laboratory Of Genetics And Physiology 2 |
| LPS | Lipopolysaccharide |
| MAL | Myeloid Adaptor Like |
| MDA5 | Melanoma Differentiation-Associated Protein 5 |
| MHC | Major Histocompatibility Complexes |
| mRNA | Messenger Ribonucleic Acid |
| MyD88 | Myeloid Differentiation Factor 88 |
| NAFLD | Non-Alcoholic Fatty Liver Disease |
| NASH | Non-Alcoholic Steatohepatitis |

| | |
|---|---|
| NF-κB | Nuclear Factor kappa B |
| NK | Natural Killer |
| NK | Natural Killer T-Cells |
| NLR | NOD-Like Receptor |
| NO | Nitrogen Oxide |
| OA | Oleic Acid |
| PA | Palmitic acid |
| PAMP | Pathogen Associated Molecular Patterns |
| PBC | Primary Biliary Cirrhosis |
| PBMC | Peripheral Blood Mono Nuclear Cells |
| PBS | Phosphate Buffered Saline |
| PI3-K | Phosphoinositide 3-Kinase |
| PNALD | Parental nutrition associated liver disease |
| Poly(I:C) | Polyinosine-Polycytidylic Acid |
| PPR | Pattern Recognition Receptors |
| RBC | Red Blood Cells |
| RLR | RIG-I-Like receptors |
| ROS | Reactive Oxygen Species |

| | |
|---|---|
| RTC | Rat Tail Collagen |
| SIGGR | Single Immunoglobulin IL-1R-Related Molecule |
| SOCS | Suppressor of Cytokine Signalling |
| ssRNA | Single Stranded Ribonucleic Acid |
| TAG | Triglyceride |
| TBS | Tris-Buffered Saline |
| TGFβ | Transforming Growth Factor β |
| TIRAP | TIR-Associated Protein |
| TLR | Toll-like receptors |
| TNFα | Tumour Necrosis Factor-Alpha |
| TOLLIP | Toll-Interacting Protein |
| TRAM | TRIF-Related Adaptor Molecule |
| TRIF | TIR Domain-Containing Adaptor Protein Inducing IFN-ß |
| VAP-1 | Vascular Adhesion Protein 1 |
| VEGF | Vascular Endothelial Growth Factor |
| VLDLPs | Very Low Density Lipoproteins |

# Chapter 1

## Introduction

## 1.1 General introduction

The burden of liver disease continues to increase in the United Kingdom and globally. Despite the varying aetiologies of liver diseases, they generally cause damage through a common pathway that leads from fibrosis to cirrhosis. However, the therapeutic options available to treat end staged liver disease remain limited. Currently the only effective treatment that results in a long-term survival advantage in end stage liver disease is transplantation. Liver transplantation however is constrained by the number of available suitable donors. Transplantation involves undergoing a major operation associated with a significant level of morbidity and mortality.

There are also long term risks following successful transplantation that include those related to lifelong immunosuppression, rejection, infection, neoplasia and recurrence of primary disease.

Those living with end stage liver disease are at a greater risk of infections and more serious health sequelae than the general population. The greater understanding of the inflammatory mechanisms resulting in end stage liver disease may lead to novel targeted treatments.

There is an intimate relationship between the gut and the liver, the so called “liver-gut axis” (1). This is more than simply transporting ingested nutrients from the intestines to the liver. The gut is able to modulate the liver environment and its inflammatory state. The process of inflammation is central to the development and progression of liver disease with an infiltration of leukocytes from the blood stream, that include monocytes. Monocytes are not only implicated in propagating inflammation but also in the resolution and repair (2).

## 1.2 **Overview of the liver**

The liver is a rich metabolic and glandular organ. The functions of the liver range from metabolism, detoxification of exogenous and endogenous substances, bile secretion, to clearance of microbial products from the blood (3). Weighing typically between 1.2 to 1.8 kg in an adult, the liver is the second largest organ in the body and is located in the upper abdomen, where it receives a unique dual blood supply consisting of 80% venous blood from the portal vein and 20% blood from the hepatic artery (Figure 1-1). This nutrient-rich and low oxygen tension mixture flows at a low perfusion pressure through an enormous network of thin-walled hepatic micro-vessels called sinusoids.

**Figure 1-1. Dual blood supply to the liver.**

The blood supply to the liver is from the hepatic artery providing 20% of the blood and 80% from the portal vein that arises from the gut. Image adapted from: http://biology-diagrams.blogspot.co.uk/2014/01/liver-blood-supply_10.html

The liver is also considered a lymphoid organ (4) as it has a repertoire of immune cells involved in both innate and adaptive immunity and is a site where naïve T-cells can be activated (5).

The liver is adapted to be able to deal efficiently the elimination of pathogenic organisms whilst being tolerant of the numerous gastrointestinal derived antigens. There are multiple components that lead to tolerogenic properties of the liver (Figure 1-2). This includes the continuous exposure of endotoxin and the presence of immunosuppressive factors such as IL-10, prostaglandin E2 and transforming growth factor-β. Individual cell population within the liver have different mechanisms as well, to form a tolerant environment. For example, the interaction of T-cells with sinusoidal endothelial cells leads to tolerant T-cell rather than effector T-cells (6).

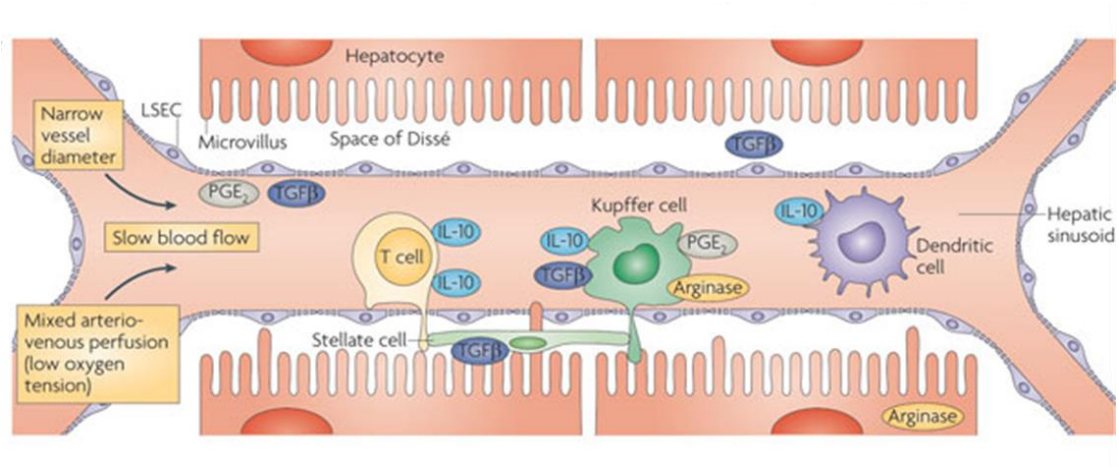



**Figure 1-2. The tolerogenic liver.**

Cells from the liver produce mediators such as IL-10, TGFβ and prostaglandin E2 that regulate the immune environment of liver. T-cells in the liver also interact with these mediators together with the sinusoidal endothelial cells altering to a more tolerant phenotype. Adapted from: Antigen-presenting cell function in the tolerogenic liver environment - Thompson and Knolle, 2010 (3)

## 1.3 The liver

### 1.3.1 Anatomy

Embryologically the liver is derived as a ventral outgrowth of the foregut. The anterior part forms the liver and intrahepatic biliary tree whereas the posterior part forms the extrahepatic ducts and gallbladder (7).

The liver can be divided topographically into two lobes by the falciform ligament, the larger right lobe and the smaller left lobe; however, this division is of limited functional consequence. Of more importance is the division of the liver based upon the division of the portal vein in to left and right branches. This division is used during surgery to transect the liver. Claude Couinaud, the French anatomist, further subdivided the liver based on its vasculature into eight segments; with each segment having their own independent vascular in and out flow in addition to biliary drainage (8).

The microstructure of the liver can be divided into units based on the bile ducts, central veins or blood flow.

The liver has a unique dual blood supply with 80% of the blood to the liver being supplied by the portal vein and the remainder from the hepatic artery. The portal vein is the main vascular drainage trunk from the gastrointestinal tract transporting matter from the gut. Despite providing 80% of the blood supply to the liver the portal vein only provides around 50% of is oxygen requirements.

### 1.3.2 Functional units of the liver

The liver can microscopically be divided into approximately 440000 functional units (9). This microstructure of liver is named units, triads or acini, based on the terminal branches of the blood vessels and bile ducts within the liver.

The classical liver lobule consists of radiating plates of hepatocyte from a central hepatic vein outwards forming hexagonal units (Figure 1-3).

The portal lobule is a triangular region made up of neighbouring central veins and centres on to a bile duct in the portal area. Thus, it contains portions of three adjacent classic liver lobules.

The hepatic acinus of Rappaport consists of two triangular sections of adjacent classic liver lobules forming a diamond shape. It is based on the distribution of blood flow from a single portal area. It is then divided in to three zones based on the proximity of hepatocytes to the portal vessels and thus oxygenated blood. The periportal zone I receives the most oxygenated and nutrient rich blood whilst the centrilobular zone III is relatively poorly oxygenated.

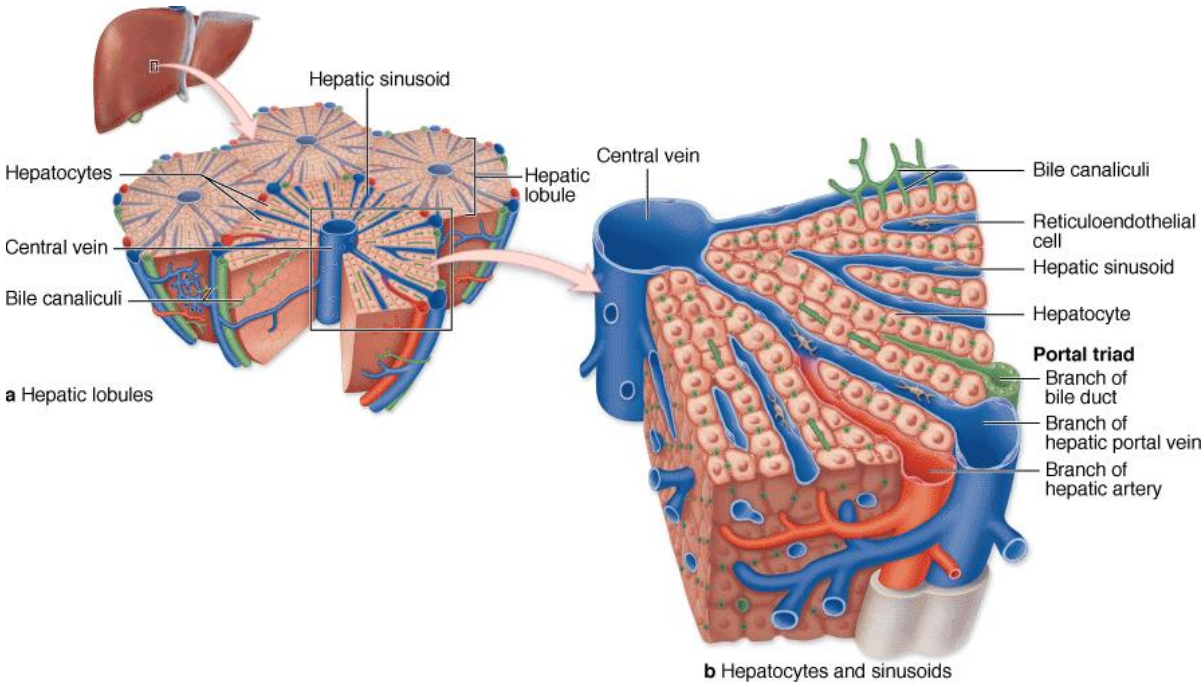



**Figure 1-3. The hepatic lobule.**

The hepatic lobule consists of a central draining vein from which radiates sheets of hepatocytes that are supplied with blood from branches of the portal vein and hepatic artery which form the hepatic sinusoids. Adapted from: Mescher AL. Junqueira's Basic Histology: Text and Atlas. McGraw-Hill Medical; 2013

### 1.3.3 Hepatic sinusoids

Liver sinusoids are a well-connected network lined by hepatic sinusoidal endothelial cells together with Kupffer cells. The liver sinusoids run between plates of hepatocytes, the major cell type of the liver, preventing them from direct contact with the bloodstream. These channels are supplied by both arterial and portal blood. This blood is delivered to the central veins. Liver sinusoids are a unique low shear vascular environment allowing for prolonged interaction with the flowing blood stream and its constituents (10).

### 1.3.4 Spaces of Disse

The space of Disse is the subendothelial matrix that separates the hepatic sinusoidal endothelial cells and the parenchymal hepatocytes. It contains the microvilli of hepatocytes and stellate cells. The space allows for exchange of material from the blood carried through the sinusoids and the hepatocytes without directly contacting the bloodstream.

### 1.3.5 Bile canaliculi

Between hepatocytes lie the bile canaliculi with lumen protruding microvilli. Adjacent bile canaliculi unite to from the bile ducts, which eventually drain bile in to the gut. Bile is involved in the elimination of products such as bilirubin as well as aiding the digestion and absorption of nutrients from the gut.

## 1.4 Capacity to regenerate

One of the most remarkable properties of the liver is its high regenerative capability. This capacity to regenerate was recognised all the way back to the ancient Greeks and mentioned in the myth of Prometheus. Hepatocytes typically do not undergo cell division and are long

lived cells however they do retain the capacity to proliferate in response to insult (11). The size of the liver is determined by the functional and metabolic demands placed upon it.

## 1.5 Cell populations

### 1.5.1 Hepatocytes

Approximately 80% of the mass of the liver is made up by hepatocytes.

They are involved in numerous functions to maintain health including:

Protein synthesis and storage

Carbohydrate and lipid metabolism

Detoxification

Synthesis of bile

Immunity

Hepatocytes are heterogeneous with graded zonation of their functions across the hepatic acinus. As blood flows through an acinus there are gradients in oxygen tension, nutrients and hormones. Periportal hepatocytes are thus exposed to higher concentrations of components carried in the blood compared to those located pericentrally (12).

### 1.5.2 Hepatic sinusoidal endothelial cells (HSEC)

Hepatic sinusoidal endothelial cells (HSEC) are highly specialised endothelial cells whose structure differs from other endothelial cells in that they lack a basement membrane and furthermore contain fenestrae, pore like structures (13) (14). These fenestrae that vary in size between 100 to 200nm in diameter (15) according to their location within the liver as

well as exposure to other stimuli and mediators. They act as filters controlling access to the space of Disse. HSEC also have a high endocytotic capacity taking up substances from the blood by receptor mediated endocytosis (16). The combination of endocytosis and fenestrae make the sinusoids selective filters between the blood and liver parenchyma (15).

Another feature of HSEC is that they can act as antigen presenting cells (APC) expressing major histocompatibility complexes (MHC) class I and II (17).

During disease processes and ageing hepatic sinusoids undergo capillarisation with loss of fenestrae and the formation of tight junctions and Weibel-Palade bodies. Also, a basement forms leading to a more vascular type endothelial (18). This subsequently has adverse effects on the normal liver homeostasis with reduced exchange though the fenestrae and decreased compliance of the sinusoids resulting in portal hypertension.

Hepatic sinusoidal endothelial cells lack significant expression of selectins, the main capture receptors found in other vascular beds yet are still able to capture leukocytes. Due to the lack of selectins in the hepatic sinusoids there is minimal rolling of leukocytes with rapid arrest in the low shear environment, which is at variance with the multistep adhesion cascade found in other vascular beds (19).

### 1.5.3 Kupffer cells

Kupffer cells, first observed by Karl Wilhelm von Kupffer, are the resident macrophages of the liver, forming around 15% of the liver cell population (20). Together with hepatic sinusoidal endothelial cells and stellate cells they form the non-parenchymal sinusoids of the liver. Their location allows them privileged and critical exposure to portal blood. This

key location plays a pivotal role in innate immunity by providing rapid response to dangerous stimuli such as bacterial products providing local and systemic defence to maintain homeostasis (21). Kupffer cells have a diverse role in scavenging particles from the blood, antigen presentation, regeneration of the liver and tumour surveillance to name a few (22). They also are central in mediating hepatic injury directly and indirectly through the release of inflammatory substances and recruitment of other mediators.

Due to the continuous exposure of bacterial products, Kupffer cells express low levels of CD14 during times of wellbeing, limiting the inflammatory response such as the production of TNFα, IL-6, IL-12, IL-18 and other chemokines (23). Furthermore, the anti-inflammatory cytokine IL-10 is secreted by Kupffer cells upon stimulation by LPS which in turns downregulates IL-6 and TNFα (24). Additionally, repeated stimulation by LPS on Kupffer cells results in hyposensitivity to further stimulation by LPS.

### 1.5.4 Hepatic stellate cells

Hepatic stellate cells (HSC) are located in the space of Disse and store vitamin A in lipid droplets of their cytoplasm. Upon activation HSC differentiate in to myofibroblasts and lose their vitamin A containing lipid droplets. In the activated state, they contribute to liver injury to produce fibrosis via the production of extracellular matrix. Activation factors include TGF-β, PDGF and other proinflammatory cytokines produced by Kupffer cells. HSC express TLRs that may enhance the fibrogenic response (25).

### 1.5.5 Liver lymphocytes

The liver has a large resident lymphocyte population. It is estimated that the average human liver contains 1 x $10^{10}$ lymphoid cells (26), approximately 25% of the non-parenchymal cell

population (27). The presence of lymphocytes in the liver is not only associated with recruitment during diseased processes but are also resident in non-diseased livers. These resident lymphoid cells are functionally specialised for their location (28). Studies of lymphocyte populations in the liver show compared to peripheral blood they are enriched in memory T cells, activated T cells; and CD8+ T-cells that outnumber the CD4+ T-cells present (29). There is also a predominance of the innate immune natural killer cells (NK) and natural killer T-cells (NKT) allowing for a swift response to potential threats. NK and NKT cells can make up to 50% of the liver lymphocyte population (4).

### 1.5.6 Neutrophils

Neutrophils are the most abundant white cell type in the blood and readily track to sites of liver injury and stress forming an early response (30). They can clear bacteria and debris as well as initiate healing process (31). They are implicated in the pathogenesis of liver damage through uncontrolled activation. Neutrophils also modulate the hepatic immune environment, by interacting and tempering the function of T-cells and NK cells (32).

### 1.5.7 Monocytes

Human monocytes are highly plastic and heterogenous cells that form approximately 10% of the peripheral circulating leukocytes. Monocytes are derived from a common myeloid progenitor (from which neutrophils also are derived) in the bone marrow which gives rise to promonocytes. Promonocytes under the influence of M-CSF undergo two or three mitotic divisions over two days in the bone marrow upon which they egress into the blood stream. The average half-life of monocytes in the blood stream in humans is short at around 1-3 days. The circulating pool of monocytes acts as a reservoir of the precursors to tissue

macrophages, dendritic cells and osteoclasts. Monocytes are able to patrol blood vessels using CD11a and $CX_3CR1$ and be recruited to sites of injury and inflammation (33).

Monocytes play key roles in both innate and acquired (adaptive) immunity. The roles of monocytes can be broadly divided in to antigen presentation, phagocytosis, immunomodulation and as a source of precursors (34). The purpose of phagocytosis by monocytes includes the killing of pathogens in addition to clearing up debris. Monocytes like neutrophils contain preformed granules which as reservoirs of enzymes that are readily available to take part in inflammatory reactions. However, unlike neutrophils, monocytes retain an ability to produce new granule proteins which is diminished in mature neutrophils.

In the early 1980s it was recognises that monocytes functionally and phenotypically could be divided in to subsets (35, 36). In mice, monocytes form subpopulations and divided based upon their expression of Ly6C and $CX_3CR1$. The two main subpopulations are the inflammatory $Ly6C^{hi}$ $CX_3CR1^{lo}$ $CCR2^{+}$ $CD62L^{+}$ and the resident $Ly6C^{lo}$ $CX_3CR1^{hi}$ $CCR2^{-}$ $CD62L^{-}$. In mice each group forms approximately 50% of the circulating monocytes population.

As in mice, human monocytes are also divided into subpopulations but based upon their expression of CD14 (LPS receptor) and CD16 (FcγRIII) cell surface receptors. These subpopulations of monocytes have differential chemokine expression and functional properties.

The classical resident monocyte $CD14^{++}$ $CD16^{-}$ form approximately 90-95% of human circulating monocytes. The non-classical inflammatory $CD14^{+}CD16^{++}$ monocytes compared to $CD14^{++}$ $CD16^{-}$ have higher expression of MHC II and lower amounts of CD11b

and CD33. They produce significant amounts of TNFα but little of the anti-inflammatory IL-10.

The differential expression of chemokines and increasing literature suggests that the inflammatory monocytes are recruited initially following an insult to the tissue with phagocytic and proteolytic activities. During resolution, the classical monocytes are recruited with more of an anti-inflammatory response that promote repair and remodelling.

A further subdivision includes a group of intermediate monocyte $CD14^{++}$ $CD16^{+}$ which form a small number of the circulating monocytes population however are present with increased frequency in livers and in particular diseased livers with a suggested role in hepatic fibrogensis (2).

#### *1.5.7.1 Monocyte migration to liver*

MCP-1 expression during liver injury and inflammation is upregulated (37). It is a potent chemotactic factor for monocytes. It is particularly more expressed in areas of fibrogenesis and appears to be released from biliary epithelial cells, activated stellate cells as well as macrophages. Additionally, release of CCR2 from the liver also provides a key signal in promoting the release of monocytes from the bone marrow in to the circulation and which then can progress in to the injured liver.

Following adhesion monocytes undergo morphological changes involving rearrangement of their actin based cytoskeletons to allow for polarization and the propulsive forces required for migration as well formation of new adhesive interactions at the leading edge and disengagement at the trailing edge, thus maintaining overall adhesion to resist shear forces. The migration process is regulated by the Rho family of small GTPases (38). The morphological changes of monocytes on some endothelial cells are ICAM-1 and VCAM-1

dependent events (39), and LFA-1 and VLA-4 interaction with ICAM-1 and VCAM-1 respectively modulate diapedesis across endothelium (40).

Monocytes are able to differentiate into macrophages or dendritic cells depending upon the local environment. It was recognised back in the 1920s that monocytes could develop in to macrophages (41). Although macrophages can be derived from monocytes there is also evidence that their population can be maintained through local proliferation (42). Whether this is from local precursors, or proliferation from the existing resident macrophages, is not clear.

Dendritic cells were discovered in the 1970s by Steinman and Cohn. They are specialised migrating antigen presenting cells. *In vitro* both main subtypes of monocytes are able to differentiate into immature dendritic cells by being cultured with IL-4 and granulocyte-macrophage colony stimulating factor. They can then be matured by treatment with TNFα or TLR agonists.

Migration of monocytes across endothelium also influences differentiation either in to macrophages or dendritic cells which will reverse migrate. *In vitro* $CD14^{+}CD16^{+}$ monocytes are more likely to differentiate into dendritic cells (2).

Despite the ability of monocytes *in vitro* to be cultured into dendritic cells they show significant differences to dendritic cells isolated *in vivo* (43, 44). Also, this raises the possibility that dendritic cells *in vivo* do not directly differentiate from monocytes.

## 1.6 **Leukocyte recruitment**

Controlled inflammation is a protective mechanism that eliminates detrimental stimuli and allows for repair of damaged tissue and restoration of homeostasis. In acute inflammation, there is an initial recognition of infection or tissue damage by innate immune cells. This recognition leads to the production and release of inflammatory mediators that selectively recruit leukocytes locally from the vasculature (45). The locally recruited leukocytes eliminate the damaging agent and coordinate repair. If there is a failure to eliminate a persistent chronic inflammatory process develops with a change in the inflammatory recruited leukocytes. Chronic injury to the liver that releases inflammatory cytokines and the recruitment of leukocytes also sets up the conditions for the activation of hepatic stellate cells. Activated HSCs turn into myofibroblasts which produce the extra cellular matrix proteins that include collagen types I, III and IV. Liver fibrosis results from the irreversible deposition of collagen.

To be able to effectively provide immune surveillance between tissues and lymphoid organs leukocytes need to be able to egress from the circulation. The process by which they leave the circulation, crossing vascular barriers, is known as the multi-step adhesion cascade (46). Whether leukocytes leave the circulation to enter a tissue is dependent on its interaction with endothelial cells and overcoming the continuous shear forces within the circulation. This process usually occurs in regions of microvasculature where it is easier for leukocytes to come out of the central flow forces that occur in vessels (47).

In the liver, in contrast to other organs, leukocyte adhesion and migration occurs in the sinusoids as opposed to post-capillary venules (48).

### 1.6.1 Capture and rolling

The classical description of the initial capture of leukocytes out of the continuous blood flow involves reversible tethering interactions that slows the leukocytes and results in a rolling motion. These tethering interactions are mediated by the member of the immunoglobulin superfamily or selectins group of molecules and glycosylated ligands in a "catch–bond" model (49, 50). The resultant velocity reduction provides time for the leukocytes to be exposed to the endothelial microenvironment and any expressed factors on the vasculature which determines if it is activated and thus proceeds to the next step of the adhesion cascade.

Selectins consists of a family of three type I cell surface glycoproteins named according to the cell type they were discovered in. They consist of short cytoplasmic tails, a transmembrane domain, two (L-selectin), six (E-selectin) or nine (P-selectin) consensus repeats that are homologous to complement regulatory proteins, an epidermal growth factor domain and an N-terminal lectin domain (51).

L-selectin (CD62L) is expressed on neutrophils and monocytes, most circulating lymphocytes and subsets of NK cells and memory T-cells (52). E-selectin (CD62E) is expressed primarily on endothelium that has been exposed to inflammatory stimuli. P-selectin (CD62P) is stored in the secretory storage granules namely, α-granules and Weibel-Palade bodies of platelets and endothelium respectively. P-selectin is rapidly released from these granules and expressed on the cell surface membrane following cell activation (53).

### 1.6.2 Activation and arrest

For tethered and rolling leukocytes to be able to cross the endothelial barrier, firm and stable adhesion between the leukocyte and endothelium is required. In this second step of the adhesion cascade, leukocytes are exposed to and stimulated by chemokines presented on

heparin-like glycoaminoglycans on the luminal endothelial surface (54). Chemokine stimulation of leukocytes via G-protein coupled receptors leads to conformational change on the integrins of the leukocyte resulting in a higher affinity state facilitating the binding to their ligands which are members of the immunoglobulin superfamily (55). This firm binding allows leukocytes to withstand the shear forces of the circulation and arrest the flow and rolling.

## 1.6.3 The immunoglobulin superfamily

This family of adhesion molecules are again trans-membrane gylcoproteins that are calcium independent (47). They are expressed on the surface of leukocytes and endothelial cells. Being trans-membrane it allows for linking between the external environment and the internals of the cell whose functions can then be modulated.

This family consists of ICAM-1, ICAM-2, VCAM-1 and MadCAM-1.

### *1.6.3.1 ICAM-1 (CD54) and ICAM-2 (CD102)*

Intercellular adhesion molecules 1 and 2 are either present or inducible on endothelium by inflammatory mediators. ICAM-1 and 2 are also found on some active leukocytes. Both bind to the beta integrin lymphocyte function-associated antigen 1 (LFA-1, αLβ2, CD11a/CD18) (56).

### *1.6.3.2 Vascular cell adhesion molecule-1 (VCAM-1)*

VCAM-1 is not as widely distributed as ICAM-1 but is inducible on endothelium (47) and found on Kupffer cells. It is involved in lymphocyte and monocyte interaction with endothelial cells via the integrin very late antigen 4 (VLA-4). VLA-4 undergoes confirmation change on leukocytes upon stimulation leading to increased binding to VCAM-1 (57).

#### *1.6.3.3 Mucosal addressin cell adhesion molecule-1*

MadCAM-1 is able to interact with lymphocytes that express α4β7 and is expressed in inflammatory liver disease (58).

### 1.6.4 Chemokines

Chemokines are small (eight to twelve kDa long) heparin binding chemotactic cytokines. They are pivotal in conducting leukocytes to the right place, at the right time to orchestrate immune responses in both homeostasis and inflammation.

The classification of the over 50 human chemokines is based on their structure. Four major subsets have been classified based on the sequencing and presence of $NH_2$-terminal cysteine motifs, namely the CXC, CC, $CX_3C$ and XC subsets.

The CC family of chemokines in the largest group to date and consists of the first two terminal cysteine residues being adjacent. The CXC family have a single amino acid residue that separates the cysteine residues. Fractalkine (CX3CL1), the only member has 3 amino acid residues that separate the first two cysteine residues. The two chemokines XCL1 and XCL2 that makeup the XC family, both lack the first two adjacent cysteine residues.

The CXC family can be further subdivided based on whether an ELR amino acid motif (glutamic acid [E]–leucine [L]–arginine [R]) at the N–terminus is present or not. The ELR containing chemokines are strongly involved in acting upon neutrophils. Those lacking the ELR motif mainly act on lymphocytes (59).

There are over 20 human chemokine receptors which can have more than one chemokine ligand. Chemokine receptors are G protein coupled receptors made up of seven transmembrane spanning molecules coupled to heterotrimeric G proteins. Signalling by

chemokine receptor on leukocytes following interaction with endothelial proteoglycan presented chemokines during tethering and rolling, initiates conformational activation of the integrins to form firm adhesion to the endothelium.

### 1.6.5 Integrins

These are a group of transmembrane receptors that mediate cell to cell or extracellular matrix interactions. They are present on leukocytes and upon stimulation by chemokines on endothelial surfaces are upregulated. Integrins are heterodimers consisting of an alpha and beta chains.

Integrins are a family of single pass type I transmembrane receptors of 24 αβ heterodimers. They are made up of non-covalent combinations of 18 alpha and eight beta subunits in human. They provide a link between a cell's motility via its cytoskeleton and the surroundings. To this end, they have large extracellular domains to allow bind to their surroundings. They have short cytoplasmic tails that links to the actin cytoskeleton that less than 75 amino acids long except for β4 tail that is around 1000 amino acids long (60, 61).

Through the cytoskeleton integrins can modulate beyond motility other behaviours such as cell survival, shape, gene expression and haptotaxis. In turn these properties have roles in tumourgenesis, wound healing, atherosclerosis and inflammation (61).

Migration of cells depends on turnover of integrin adhesion with the formation of integrin adhesion at the leading edge of a cell with detachment at the trailing end (62).

The heterodimeric structure of integrins regulates its affinity to ligands. The degree of affinity can determine the motility of a cell during the adhesion cascade from rolling during an intermediate affinity state to arrest when in a high affinity state (63). There are two models

relating to this conformational change namely the “switchblade” (64) and “deadbolt” model (65). The function of integrins on leukocytes as adhesion and signalling molecules is tightly regulated only them to deal with the unique challenges in the face of shear forces allowing the rapid establishment of adhesion as well as for allowing migration across endothelium (66). Low shear forces can stabilise the activated open confirmation of integrins maintaining its adhesiveness (67).

Most leukocytes have their integrins in low affinity states (68) (exceptions include T- and B- cell blasts and some myeloid subsets). They become rapidly activated to bind and adhere to endothelium against the shear forces when they come in to contact with chemokines presented on the endothelial surface.

## 1.7 Liver disease

### 1.7.1 Fibrosis

Liver inflammation and fibrosis is a highly coordinated response to tissue injury with the aim of repairing and restoring homeostasis. The tight control of multiple pathways determines whether the response is appropriate and time-limited. Key to this process are the cells of the immune system. If uncontrolled, excessive irreparable damage occurs through extensive fibrotic scarring altering the function of the liver (69).

Most forms of liver injury result in damage to the epithelial cells, namely the hepatocytes and cholangiocytes, whether this is due to viral hepatitis, fatty liver disease or alcohol. Inflammatory mediators are released that recruit immune cells to site of the injury to clear the damaged cells and debris. In addition, the recruited immune cells release further

mediator to amplify the immune response. Pro-fibrotic factors such as transforming growth factor-β (TGFβ) and IL-13 are released inducing the trans-differentiation of mesenchymal precursors in to myofibroblasts. TGFβ upregulated the production of collagen and α smooth muscle actin (70).

Fibrosis is the accumulation of excessive extracellular matrix proteins due to chronic inflammation. Fibrosis in its initial stages is beneficial in limiting organ damage and importantly is reversible. However, the longer the damaging process goes on for, the greater the accumulation of avascular extracellular matrix (ECM) scar tissue. Once this ECM is deposited and there is reduced capacity for resolution even after the causative trigger is eliminated. The ability for resolution of the fibrotic scar tissue diminishes as cellular context in extensive scarring is lost, vascular distortion, and extensive cross-linking of ECM components.

If fibrosis continues unabated it progresses towards cirrhosis where there is architectural and vascular distortion together with replacement of the functional parenchyma with fibrotic scar tissue. The consequence of cirrhosis include the functional failure of its homeostatic synthesis and storage functions, failure of detoxification systems, increased susceptibility to infections and carcinoma, and portal hypertension and resultant bleeding risk (71).

## 1.8 **The gut and bacteria in liver disease**

The liver is continually exposed to gut derived antigens and is involved in the clearance of toxic components. Kupffer cells and hepatocytes can recognize microbial components, including through Toll-like receptors leading to the release of proinflammatory cytokines and oxygen free radicals. An exaggerated response can lead to organ damage, hence the liver

has developed a mechanism of immune tolerance to avoid T-cell mediated immune responses against hepatocytes, even if they express antigens which are toxic for the host (29).

In cirrhosis, there is impairment of the reticuloendothelial system with altered phagocytic capacity of cells but also due to portosystemic shunting thus bypassing the liver's resident macrophage pool of Kupffer cells. Hence the circulation is not cleared of microbial products and cytokines. Adding to the load of microbial products in the circulation is the increased bacterial translocation from the intestinal lumen.

Bacterial products include LPS which interacts with CD14-bearing inflammatory cells (which include monocytes-macrophages, neutrophils and other non-immune cells). CD14 acts as a co-receptor with TLR4 for the detection of LPS. CD14 can only bind LPS in the presence of lipopolysaccharide-binding protein (LBP). Upon binding there is a release of pro-inflammatory cytokines, including TNFα, interleukin (IL)-1ß, IL-8, IL-12, cyclic endoperoxides, platelet activating factor (PAF), complement, tissue factor and other harmful mediators, which contribute to the induction of the systemic inflammatory response syndrome (72). These mediatory further contribute to the circulatory disturbances that are common in both sepsis and cirrhosis.

In sepsis, there is an unbalanced pro-inflammatory state in response to tissue damage or infection that can results in further tissue damage, organ dysfunction and death. In sepsis, activation of immune cells by damage-associated molecular patterns results in a cytokine storm that rather than being beneficial in defending the host is excessive resulting in further damage.

## 1.9 **Immune paralysis in sepsis**

Despite the pro-inflammatory response observed in sepsis, therapies aimed at reducing mortality have had limited success for a number of reasons such as targeting only one part of a complex activation cascade with multiple layers of redundancy in the system. However, another concept gaining recognition is immune paralysis of immune cells including that of neutrophils and monocytes (73). Monocytes in the circulation in response to sepsis show attenuated responses to bacterial agonists and reduced expression of class II antigen presenting molecules to propagate the immune response (74, 75).

The complement system becomes activated in sepsis and can further modulate the function of monocytes depending on their state. Monocytes that are adherent to endothelium are primed to produce greater quantities of inflammatory cytokines and upon exposure to complement factors and LPS have enhanced cytokine production (TNFα and IL-1β) (76, 77).

## 1.10 **Pattern Recognition Receptors (PPRs)**

The first line of defence against pathogens and tissue damage is provided by the innate immune system. Acquired immunity is involved in later stages of infection with clonal selection of specific lymphocytes and generation of immunological memory (78). There are a group of receptors that can recognise either pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) as part of the innate immune system whilst discriminating from self (79). This group of receptors include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs). Additionally, there are cytosolic receptors consisting of NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and AIM2-

Like receptors (ALRs). Activation of PRRs can result in the expression of pro-inflammatory and anti-viral molecules or in some cases such as activation of some NLRs and ALRs can result in the formation of inflammasome complexes that cleave and activate cysteine protease Caspase 1 which in turn proteolytically cleaves the precursor form of other proteins such as the inflammatory cytokines interleukin 1β and 18 thus amplifying the inflammatory response (80).

Single pathogens can activate multiple PRRs to modulate the immune response. Microbial pathogens usually consist of multiple PAMPs and thus activate multiple PRRs. PRRs may detect the same PAMP.

### 1.10.1 RIG-I-like receptors (RLRs)

There are three RIG-I-like receptors based in the cellular cytoplasm, namely retinoic acid-inducible gene 1 (RIG1), melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). This group of intracellular pattern recognition receptors are RNA helicases that are involved in the recognition of viral replication by interacting with dsRNA. dsDNA is an intermediary for the replication of RNA viruses. RIG-1 and MDA5 contain caspase activation and recruitment domains (CARDs) that are able signal for a cellular response to viruses. LGP2 is required for effective RIG-1 and MDA5 antiviral responses. RIG-1 binds preferentially to short dsRNA whilst MDA-5 to long dsRNA. RLRs are expressed in most tissues at low levels, however upon IFNγ or viral exposure this is vastly increased (81-83).

### 1.10.2 Nucleotide-binding, oligomerization domain-like receptors (NLRs)

NLRs are cytoplasmically expressed. They are made up of three domains and depending on their N-terminal structure subdivided into subfamilies. They are intracellular PAMP receptors (84).

### 1.10.3 C-type Lectin Receptors (CLRs)

These are a family of soluble and transmembrane receptors that contain C-type lectin-like domains (CTLD). C-type lectins are carbohydrate binding domains that are dependent on calcium for binding. They recognise fungal and bacterial PAMPs (85).

## 1.11 Toll-Like Receptors

In 1985 Christiane Nüsslein-Volhard of the Max Planck Institute in Tübingen, identified the Toll gene in the fruit fly, Drosophila and its protein product is involved in embryogenesis (86) and immune responses (87). In 1991 the sequence homology between the cytoplasmic domain of toll and the human IL-1 receptor was reported (88). Human toll-like receptors were first described in 1997 when Ruslan Medzhitov and Charles Janeway at Yale University cloned a mammalian homologue (now called TLR4) and demonstrated it as having a role as an innate immune system receptor (89). Subsequent discoveries demonstrated that these toll-like receptors form a family of pattern recognition receptors.

Toll-like receptors are germline-encoded type I transmembrane molecules with cytoplasmic domain that is similar to the IL-1 receptor and is known as the Toll/IL-1 receptor (TIR) domain. The extracellular domains of TLRs consist of leucine rich repeat (LRR) motifs (90).

They are expressed on a variety of cells, both of immune and non-immune origins. They recognise PAMPs on their leucine-rich ectodomain. Downstream signalling pathways are activated by their cytosolic Toll/interleukin (IL)-1 tails (91).

To date, in humans, 10 TLRs have been identified. Each TLR recognises specific PAMPs from a variety of sources. They however share many of the same signalling molecules and activation pathways to mediate proinflammatory, antibacterial and antiviral responses. Expression levels of TLRs in healthy adults varies from site to site and is affected by many factors.

Upon recognition of a PAMP by a TLR, adaptor molecules that have TIR domains such as MyD88 and TRIF are recruited and initiate further downstream signals that result in the secretion of inflammatory cytokines, chemokines and interferons aimed at clearing the pathogen and recruiting leukocytes. Additionally, TLR signalling mature dendritic cells thus linking into the adaptive immune system (92).

TLRs can be divided into two groups based on their compartmentalisation at the cellular level. One group, TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the cell surface and can detect microbial membrane components. The others, TLR3, TLR7, TLR8 and TLR9 are located within intracellular compartment. They are expressed on the endoplasmic reticulum and trafficked to endosomes and lysosomes upon stimulation (93). By being located intracellularly these TLRs detect nucleic acids from viruses and other pathogens that have been taken up. This avoids them encountering self-nucleic acids in the extracellular environment and initiating autoimmunity. The extracellular nucleic acids are degraded by nucleases in the extracellular environment.

### 1.11.1 TLR Signalling Pathways

Upon ligand stimulation, a downstream signalling pathway is initiated mediated by one or more adaptor molecules activating NF-kB and interferon regulatory factors that produce cytokines that act upon other defence cells.

The response of a TLR and the binding of its PAMPs, is dependent on the recruitment of TIR-domain-containing-adaptor proteins. These adaptor proteins include myeloid differentiation factor 88 (MyD88), TIR-associated protein (TIRAP) (also known as myeloid adaptor like (MAL), TIR domain-containing adaptor protein inducing IFN-ß (TRIF) and TRIF-related adaptor molecule (TRAM). MyD88 and TIRAP produced proinflammatory cytokines whilst TRIF and TRAM induce IFNs. TLRs share a common TIR domain that can signal via one of two main pathways. One depends on the adaptor protein myeloid differentiation factor 88 (MyD88) and the other does not (MyD88 independent pathway). The different responses mediated by distinct TLR ligands are in part explained by the selective use of these adaptor molecules (94).

#### *1.11.1.1 MyD88-dependent signalling*

All TLRs bar TLR3 signal through MyD88. Following recruitment of MyD88 to the TIR domain, IL-1 receptor associated kinase 4 (IRAK-4) is activated and phosphorylates IRAK-1 and IRAK4 which associates with tumour necrosis factor- receptor associated factor 6 (TRAF6). This leads to phosphorylation of TAK1, IκB kinase complex and MAP kinase 6. Phosphorylation of IκB and releases NF-κB. The activated NF-κB translocates to the cell nucleus and binds to response elements to the DNA of the proinflammatory genes. MyD88 also activates the transcription factor IRF5 which to induces the synthesis of IL-6, IL-12 and TNF-α. IRF7 is activated by MyD88 upon activation of TLR7 and TLR9 to upregulate IFNα

and IFNβ to provide an antiviral response. TRIF mediated NF-κB activation is a later and weaker form of activation compared to direct MyD88 NF-κB activation (95).

#### *1.11.1.2 MyD88-independent signalling*

TLR3 and TLR4 can signal independently of MyD88. For this to occur both TLRs are dependent on TRIF and in addition TLR4 also requires TRAM. TLR3 activation of TRIF not only leads to the activation of NF-κB and inflammatory cytokine production but also of IRF3 and production of type I IFN (96).

#### *1.11.1.3 Regulation of TLR signalling*

TLR signalling is tightly regulated to prevent unnecessary activation and the subsequent collateral damage that may result from excessive or prolonged inflammation through these pathways. TLR signalling can be regulated along the signalling pathway (through protein phosphorylation, degradation, interaction with inhibitory adaptor molecules or sequestration) as well as at the receptor level (97).

#### *1.11.1.4 TLR2*

It is involved in recognition of lipoproteins and peptidoglycans that are present in the cell walls of bacteria. It also is involved in responding to mycobacteria, fungi, hepatitis C and cytomegalovirus. It also recognises endogenous ligands such as heat shock proteins. TLR2 forms heterodimers with TLR1, TLR6 and non TLR molecules such as CD36 to provide its range of sensing abilities.

#### *1.11.1.5 TLR2 heterodimers: TLR1/TLR2 and TLR2/TLR6*

TLR2 is able to recognise a variety of PAMPs from both gram negative and positive bacteria through forming multiple heterodimers.

The TLR1/TLR2 complex recognises bacterial triacyclated lipopeptides, such as Neisseria meningitides. TLR2/TLR6 recognises diacylated lipopeptides such as found in Staphylococcus aureus (98).

#### *1.11.1.6 TLR2 and TLR6 association with CD36*

The association of TLR2/TLR6 with CD36 are not preformed as is the case with TLR1/TLR2 but instead induced upon ligand binding (99). Before stimulation CD36 is present in lipid rafts but TLR6 is not, until stimulated. This complex may be involved in sterile inflammation (100).

#### *1.11.1.7 TLR3*

TLR3 recognises double stranded (ds)DNA that is produced during the replication of ssRNA viruses such as West Nile virus and respiratory syncytial virus. It also recognises the synthetic dsDNA, polyinosinic-polycytidylic acid (poly IC) (101).

#### *1.11.1.8 TLR4*

TLR4 recognises LPS from gram negative bacteria. LPS is a component of the outer membrane of gram negative bacteria. LPS consists of three parts, O antigen, core oligosaccharide and lipid A. The O antigen is a repetitive glycan polymer that forms the outer most part of LPS. The presence of full length O antigen chains results in a form of smooth LPS where as those with reduced O antigen result in rough LPS. The core oligosaccharide component links to lipid A. Lipid A is a phosphorylated glucosamine disaccharide with multiple fatty acids. It is responsible for much of the toxicity associated with gram negative bacteria (102). For a robust response to LPS, LPS binds to the soluble plasma protein LPS binding protein (LBP). This LPS-LPB complex binds to CD14 which delivers it to TLR4-MD2 complexes. Without CD14 cells are unresponsive to smooth and low dose LPS but still respond to rough LPS and lipid A. Additionally in the absence of CD14 lipid A only signals using the MyD88 dependent pathway. MD2 is involved in the release of TLR4 from the endoplasmic reticulum and its cell surface expression in addition to its LPS responsiveness (103, 104). In mice alcohol induced liver injury is reduced by intestinal sterilisation and the subsequent reduced endotoxin levels. Also mice lacking CD14 are protected. In humans the role of endotoxin in liver disease has been described. In the liver Kupffer cells in humans express CD14 (105). CD14 positive cells are increased in liver disease.

#### *1.11.1.9 TLR5*

TLR5 detects the conserved central part of flagellin from the flagella of bacteria (106). Although many non-pathogenic commensals express flagellin only pathogenic organisms release the monomeric form. In mice models of liver disease the activation of TLR5 by

flagellin can be detrimental or protective against diet induced liver injury (107) in contrast to the injury that occurs by TLR4 activation in the liver.

#### *1.11.1.10 TLR7 and TLR8*

These are both similar binding to single stranded RNA (ssRNA) from viruses such as influenza and human immunodeficiency virus I (HIV-I). TLR8 does not signal in mice. (108)

#### *1.11.1.11 TLR9*

TLR9 detects the unmethylated 2'-deoxyribo (cytidine-phosphate-guanosine) (CpG) DNA motifs that are found in bacteria and viruses like HSV-1 and HSV-2. Vertebrates have highly methylated CpG DNA motifs that allows it to be differentiated (109).

#### *1.11.1.12 TLR10*

TLR10 is expressed in humans but the ligand is unknown (110).

### 1.11.2 TLR tolerance

The chronic exposure of HSEC to low levels of LPS results in relative insensitivity due to the reduction in TLR4 expression thus tolerance to LPS (111). This tolerogenic affect can also occur across TLRs. For example exposure of Kupffer cells to the TLR3 ligand poly I:C results in reduced sensitivity to LPS (112, 113). HSEC like Kupffer cells show tolerance to repetitive LPS stimulation however there is not a change in TLR4 cell surface expression. In addition, repetitive stimulation with LPS results in a decrease in ICAM-1 (CD54) expression and the ability to produce leukocyte adhesion (111).

### 1.11.3 Endogenous PAMPS

The TLRs within the liver can also respond to endogenous signals such as those released be damaged cells (79). Damaged cells can release RNA (TLR3), DNA (TLR9) and high mobility group box protein (TLR4). Thus, there is potential for a self-sustaining state resulting in ongoing tissue damage.

### 1.11.4 TLR and liver disease

Endotoxin (lipopolysaccharides (LPS)) is largely confined to the bowel lumen. A small amount penetrates the intestine and then transported to the liver bound to LPS-binding protein, where they are cleared by Kupffer cells.

In the western world, alcohol is the leading cause of chronic liver disease. Up to 30% of individuals who drink heavily will go on to develop chronic liver injury in the form of alcoholic hepatitis, fibrosis, cirrhosis and hepatocellular carcinoma (HCC) (114).

Alcohol increases intestinal permeability by disrupting the epithelial barrier. This then allows for greater translocation (115-117) of LPS which reaches the liver via the portal system. This greater load activates Kupffer cells contributing to alcohol induced liver injury. Kupffer cells also perpetuate this injury by increasing CD14 expression (118). Interruption of this pathway by either reducing the gut microflora with antibiotics or depleting Kupffer cells or their TLR4 receptors blunts liver injury (119, 120).

Chronic alcohol ingestion not only activates TLR4 through the increased intestinal epithelial permeability but other TLRs are also activated (121).

Hepatitis B is a self-limiting condition in 80-90% of adults however in the remaining population it becomes a chronic condition leading to the common cause of cirrhosis and hepatocellular carcinoma in the world. Activation of TLR4, TLR5, TLR7 and TLR9 ligands inhibit HBV replication in the liver using interferon dependent pathways (122).

Hepatitis C causes chronic infection in 70 to 80% of the population infected which once again can leads to cirrhosis and hepatocellular carcinoma. HCV evade the immune system including evasion of the TLR system. The non structural protein 3 (NS3) of the hepatitis C virus degrades the TLR adaptor molecule TRIF. NS3/4a protein inhibits IRF3, TBK1 and NF-κB activation (123, 124).

### 1.11.5 Sepsis

Sepsis occurs when there is life threating organ dysfunction in due to the inflammatory response to an infection (125). Sepsis causes a significant and increasing burden worldwide in terms of morbidity and mortality (126). Bacteria trigger inflammatory responses not only by the detection of the invading bacteria itself, but also is by the host recognition of released endotoxins and exotoxins. Endotoxins, such as LPS, make up the cell walls of gram negative bacteria are released upon the breakdown of these cells. (Endotoxins also can be secreted (127)). Exotoxins are produced and secreted by both gram positive and negative bacteria.

## 1.12 Non-alcoholic fatty liver disease

Obesity has increased throughout the world and is not confined to only high income western countries. If now affects significant proportion of the population with major health and economic implications.

Obesity has implications for the health and function of the liver. Not only does it result in liver disease in its own right, in the form of non-alcoholic liver disease, but also exacerbates liver disease due to other aetiologies. For example, visceral obesity and liver steatosis accelerate the damage from hepatitis C (128). Also, obesity is considered as creating a state of chronic inflammation.

Non-alcoholic fatty liver disease (NAFLD) is an increasingly common form of liver disease and reportedly affects up to a third of the Western population (129) and up to 90% of morbidly obese individuals (130). It consists of a spectrum of histological changes that consists of simple steatosis that progresses to steatohepatitis with cellular necrosis and injury with an associated inflammatory infiltrate (non-alcoholic steatohepatitis (NASH)). NASH may progress to fibrosis and cirrhosis and their associated complications such as hepatocellualar carcinoma. NAFLD is increasingly being considered as the hepatic manifestation of metabolic syndrome that relates to insulin resistance and dysregulation of energy storage and utilisation. Central obesity in one of the major components of metabolic syndrome. The increased adipose tissue in obesity modulates fat derived factors such as TNFα, adiponectin and free fatty acids that alter inflammatory responses, including those of the liver.

Adiponectin is protective against NAFLD by preventing fatty acid uptake and fat accumulation in hepatocytes. TNFα is an antagonist of adiponectin inhibiting its synthesis and production and increases insulin resistance. Furthermore, increased visceral adipose tissue results in greater lipolysis and thus free fatty acids that in the liver increase TNFα production, insulin resistance and promotion of NAFLD (131).

Additionally, obesity has negative consequences for those undergoing treatments for end-stage liver disease by the means of liver transplantation. All cause morbidity and mortality of patients who are obese compared to non-obese liver transplant recipients is raised (132, 133).

Liver transplantation also necessitates the use of immunosuppression that causes insulin resistance. Calcineurin inhibitors inhibit synthesis and secretion of insulin from the pancreas and steroids increase insulin resistance. They also increase blood pressure and cholesterol levels (134). All these factors add to the metabolic dysfunction observed in such patients. Furthermore, in obesity, sepsis results in increased morbidity, compared to non-obese patients (135).

## 1.13 **Fatty acids**

Fatty acids have crucial roles in making up biological cell membranes together with providing energy. Fatty acids are formed from a carboxylic acid with an aliphatic tail composed of a carbon chain skeleton with attached hydrogen atoms. Fatty acids can be divided in to two groups depending on whether they have carbon-carbon double bonds, the unsaturated group or without, saturated. The carbon-carbon double bonds can be saturated by adding hydrogen atoms.

Free fatty acids are esterified into triglycerides and then enter the circulation as very low density lipoproteins (VLDL) or undergo β oxidation to generate acetyl-coA to produce energy. In NAFLD the livers role in maintaining lipid homeostasis is disrupted by the excessive amounts of free fatty acids delivered to it and synthesised. As a consequence of this exceeded capacity, triglycerides then accumulate in hepatocytes as hepatic vacuoles.

This disruption is further exacerbated by the metabolic syndrome and insulin resistance which increases lipolysis in adipose tissue and thus serum free fatty acid levels.

It has been shown that all fatty acids do not behave same way and that in particular there is a division between saturated and unsaturated fatty acids. Clinically relevant outcomes include:

- Mediterranean diet rich in olive oil rich in oleic acid has beneficial effects on health outcomes compare to diets rich in palmitic acid (136).
- Parental nutrition is associated with parental nutrition associated liver disease (PNALD). Oleic acid rich nutrition has shown improved clinical outcomes in critically ill patients (137).
- Nutrition can modulate the outcome from sepsis and depending on the composition of the nutrition. Critically ill patients often require some form of supplementary nutrition. Nutrition based on oleic acid, reduces the negative effects seen with other lipid based nutrition with regards to bacterial clearance (138).
- Recruitment – Human studies have shown in peripheral mononuclear cells in those consuming diets rich in monounsaturated fatty acids can reduce expression of adhesion molecules (139). Fatty acids also have differential effects on lymphocyte cytokine expression. Saturated fatty acids are more potent in inducing cytokine production than unsaturated fatty acids (140).
- Those with NAFLD have diets with higher fat content. Increasingly it is being recognised the form in which this fat intake is consumed affects liver homeostasis. NAFLD patients have diets high in saturated fatty acids and low in monounsaturated fatty acids (141).

## 1.14 **Aim of thesis**

The liver and gut are intimately related. The gut can influence the development and progression of liver disease. This is possible through dietary intake that includes fatty acids and the presence of a rich flora of bacteria within the gut lumen that can egress into the portal blood stream directly to the liver.

The hypothesis for this thesis was that TLR ligands and fatty acids, two components that link the gut to the liver, have a role in recruiting monocytes out of flow in an *in vitro* model of a hepatic sinusoid.

The specific aims of this project were:

1) Can different TLR agonists cause the recruitment of monocytes out of flow in a model of a hepatic sinusoid
2) Can fatty acids alter the recruitment of monocytes from flow in a model of hepatic sinusoids
3) What is the expression of TLRs in liver sinusoidal endothelial cells and liver derived macrophages and does this change in inflammation
4) To develop the *in vitro* model of hepatic sinusoids currently used to include liver derived macrophages to more accurately represent what occurs *in vivo*

# Chapter 2

## Materials and Methods

## 2.1 Ethics Statement

Human liver tissue from the Queen Elizabeth Hospital, Birmingham, was obtained that was surplus or unsuitable for liver transplantation. Pathological explanted livers were also used with approval from the Local Research Ethics Committee (reference number 06/Q702/61). Blood for isolation of monocytes was obtained from healthy volunteers or patients with haemochromatosis (HFE) attending clinic at the Queen Elizabeth Hospital for venesection.

## 2.2 Isolation of HSEC

To isolate HSEC, a slice of approximate 50g of liver tissue from explanted human livers were finely chopped using sterile scalpels. The processed tissue was then transferred to a sterile beaker containing 20mls of PBS. To this 5mls of 0.2% collagenase solution (Sigma, Poole UK) was added to the beaker, covered and incubated at 37° for 20 minutes to allow enzymatic digestion of the tissue, till it took on a gelatinous consistency.

The digested liver suspension was then passed through a fine mesh to another sterile beaker with PBS till a final volume of 200mls was obtained.

The 200ml cell suspension was divided between 8 universal tubes and centrifuged for 5 minutes at 550g to pellet the cells. Each pellet was re-suspended with fresh PBS, centrifuged, and the supernatants discarded.

Percoll$^{TM}$ (Amersham Biosciences, Bucks UK) gradients were created by layering 3 ml of 33% Percoll$^{TM}$ on top of 3mls 77% Percoll$^{TM}$ in eight conical-bottomed centrifuge tubes. Each cell pellet was re-suspended in 3mls of PBS and layered on top of the Percoll$^{TM}$ gradients and centrifuged at 890g for 25minutes.

The bands of cells (non-parenchymal cells) at the Percoll$^{TM}$ interface were collected and transferred to two universal tubes and washed in PBS and pelleted.

To remove biliary epithelial cells from the cell suspension, 50 µl of Human Epithelial Antibody (HEA) (50µg/ml) (Progen, Biotec, GMBH Germany) was added and incubated at 37°C for 30 minutes. The excess antibody was then washed off with PBS and pan mouse Dynabeads (4 x $10^8$ beads/ml, Dynal, UK) added. The biliary epithelia cells were then removed using magnetic selection. The remaining cell suspension than underwent positive selection with CD31 antibody (10µg/ml, DAKO, Ely UK) and Dynabeads (Dynal,UK), again with magnetic selection. The selected HSEC cells were incubated in Rat Tail Collagen (Sigma, UK) coated T25cm$^2$ flasks (Corning, UK). The HSEC were cultured in Endothelial basal media (Invitrogen, Paisley UK) with 2mM L-Glutamine, 100U/ml Penicillin and 100µg/ml Streptomycin (Sigma, Dorset UK). This was supplemented with 10% human serum (H+D supplies, UK) and 10ng/ml of hepatocyte growth factor (Peprotech, UK) and vascular endothelial growth factor (Peprotech, UK). The cells were maintained in a 5% $CO_2$ humidified incubator at 37°C.

## 2.3 **Isolation of human peripheral blood monocytes**

50mls of peripheral blood was obtained from healthy volunteers or patients attending for venesection for haematochromatosis at the Queen Elizabeth Hospital. This was placed in a 50ml polypropylene tube (Corning, UK) and centrifuged at 900g for 30 minutes to create a buffy coat which was harvested in 10mls of the plasma supernatant. To the buffy coat – plasma supernatant mixture 4mls of Optiprep (60%w/v) (Axis-Shield PoC AS, Oslo,

Norway) was added. This was overlaid with 7.5ml of the 1.084g/ml Optiprep solution followed by 20mls of the 1.068g/ml Optiprep solution and finally with 2 mls of Gibco® RPMI 1640 medium (Sigma-Aldrich, UK). This was centrifuged at 800g for 25minutes. The monocytes were collected from the top of the 1.068g/ml layer.

## 2.4 Isolation of liver derived macrophages

Liver derived macrophages were isolated from by a method adapted from Alabraba et al 2007 (142). Approximately 50 gram slices of liver were finely chopped and washed in RPMI-1640 (Invitrogen, Paisley, UK) to remove any blood. The tissue was then transferred into GentleMACS C tubes (Milteni Biotec, Germany) and suspended in Gey's balanced salt solution (GBSS) (Sigma–Aldrich, Poole, UK) containing 0.2% (w/v) Pronase (Sigma–Aldrich, Poole, UK) and 0.8 ug/ml DNase (Roche, Basel, Switzerland). This was incubated for 20minutes at 37°C then run on GentleMACS Dissociator (Milteni Biotec, Germany). The homogenised tissue was filtered through a fine mesh cloth. The filtrate was layered over a 16% Nycodenz (Axis Shield) gradient and centrifuged at 600 g for 20 min in a 25ml polypropylene tube (Corning, UK).

The cell at the interface was collected and washed in RPMI-1640. The cells were then cultured in RPMI-1640 with 10% v/v heat-inactivated human serum (HD Supplies, Glasgow UK) in a 5% $CO_2$ humidified incubator at 37°C. After 2 hours, the culture vessel was washed to remove non-adherent cells.

## 2.5 **Immunohistochemistry**

Fixed frozen section slides of liver tissue were thawed at room temperature. A wax ring was placed around the liver section. The sections were fixed for 5 minutes with acetone and peroxidise activity blocked with peroxidise block (Dako) in humidified trays. The slides were washed with Tris Buffered Saline pH 7.6 (TBS) with Tween 20 (Sigma, Poole UK). The sections were incubated with 2.5% horse blocking serum (Vector ImmPress Kit, Vector Labs UK) for 20 minutes and then removed. The sections were then incubated with the primary antibody or respective control antibody as shown in Table 2-1. The sections were then washed with TBS pH7.6 and then incubated with 100µl of the secondary antibody (Vector ImmPress Kit, Vector Labs UK) for 30 minutes. This was then removed and washed twice with TBS pH7.6 and incubated for five minutes with peroxidase substrate NovaRed (VECTOR NovaRED Peroxidase (HRP) Substrate Kit, Vector Labs UK) and then washed with water. Counterstain was with Mayers haematoxylin for 30 seconds (Leica, Biosystems, Peterborough). The sections were mounted with Depex (DPX) (Shandon, UK).

| Primary Antibody | Supplier | Concentration used | Antibody Isotype |
|---|---|---|---|
| TLR1 | IMGENEX Corporation | 5µg/ml | Rabbit Polyclonal |
| TLR2 | IMGENEX Corporation | 5µg/ml | Mouse monoclonal IgG2a |
| TLR3 | IMGENEX Corporation | 5µg/ml | Mouse monoclonal IgG1 |
| TLR4 | IMGENEX Corporation | 5µg/ml | Mouse monoclonal IgG2a |
| TLR5 | IMGENEX Corporation | 5µg/ml | Rabbit polyclonal |
| TLR6 | IMGENEX Corporation | 5µg/ml | Mouse monoclonal IgG1 |
| TLR7 | IMGENEX Corporation | 5µg/ml | Rabbit polyclonal |
| TLR8 | IMGENEX Corporation | 5µg/ml | Rabbit polyclonal |
| TLR9 | IMGENEX Corporation | 5µg/ml | Mouse monoclonal IgG1 |
| IgG1 | Invitrogen | 5µg/ml | Mouse monoclonal IgG1 |
| IgG2a | Invitrogen | 5µg/ml | Mouse monoclonal IgG2a |
| Rabbit | Invitrogen | 5µg/ml | |

**Table 2-1. Antibodies used for immunohistochemistry staining of TLRs.**

## 2.6 Relative quantification PCR

RNA was isolated from cultured cells using RNeasy kit (Qiagen, Crawley UK) as per the manufactures instructions. The concentration and purity of RNA was measured on a NanoDrop spectrometer (ThermoFisher Scientific, UK). cDNA was synthesised using Superscript III Reverse Transcriptase kit (Invitrogen, UK). Using a NanoDrop spectrometer (ThermoFisher Scientific, UK) the quality and concentration of cDNA was determined.

Relative quantification PCR was performed using QuantiFast SYBR Green PCR Kit (Qiagen, Crawley UK) with Quatitect Primer Assays (Qiagen, Crawley UK) on a LightCycler 480 system (Roche Life Sciences) as per Qiagen's protocols and settings. GAPDH was used as the house keeping gene. The results were analysed using LightCycler 480 SW 1.5 software.

## 2.7 Enzyme-linked immunosorbent assay (ELISA)

HSEC were plated in 96 well plates and cultured in media for 24 hours till confluent. The media was then replaced with media containing cytokines or fatty acids for a further 24 hours. Upon completion of the incubation period, media was removed from the wells which were then fixed with methanol for 5 minutes which was rinsed off with PBS. The cells were then blocked with goat serum. After washing off the blocking serum the primary antibodies were added at the concentrations shown in Table 2-2 for 45 minutes. CD31 was used as a control. The secondary antibody after washing the cells was then added. The ELISA was developed using *O*-phenylenediamine substrate (OPD, Dako). Absorbance values at 490 nm was measured using a Dynatech Laboratories MRX plate reader.

| Antibody | Supplier | Concentration |
|---|---|---|
| IMC Mouse IgG1 | Invitrogen | 5µg/ml |
| ICAM-1 | Dako | 5µg/ml |
| ICAM-2 | Dako | 5µg/ml |
| E-Selectin | Dako | 5µg/ml |
| VCAM | Dako | 5µg/ml |
| CD31 | Dako | 5µg/ml |
| Secondary Goat and mouse HRP | Dako | |

**Table 2-2. Antibodies used for ELISAs.**

## 2.8 Staining of CD68 in single cell cultures

HSEC and or liver derived macrophages were cultured in 96 well plates. After completion of the culturing period the media from the wells was removed and the plated cells washed with PBS. The cells were fixed with ice cold methanol for 5 minutes. They were then blocked with 2% goat serum in PBS for 30 minutes which was then removed. CD68 (Mouse IgG3a) (Dako) was then incubated on the cells for 30 minutes before being washed on and the secondary RPE antibody (Goat anti mouse) (Dako) being added. After washing off the secondary antibody the cells were visualised.

## 2.9 Ibidi Flow Assays of monocytes across HSEC

Ibidi μ-slide VI (Ibidi GmbH, Martinsried, Germany), shown in Figure 2-1, were coated with rat tail collagen prior to use. HSEC, from a confluent T75 flask, was re-suspended in 500μl of media and loaded into the six individual chambers and allowed to become confluent prior to flow assays.

The Ibidi slides were mounted on top a microscope (Olympus IX50; Olympus, Southend-on-Sea, UK) within a heated 37°C chamber and connected to a DVD recorder to allow for off-line analysis (Figure 2-2). The slides were connected to a syringe drive (Harvard PHD 2000, USA) set to produce a shear stress of 0.5 dynes/$cm^2$. Monocytes were prepared at a concentration of 1 million cells per ml of media.

Initially media was pumped across an individual chamber of a slide for 5 minutes to wash away any debris. Then the pump was switched to allow monocytes to flow across the HSEC for 5 minutes. After the monocytes flow was stopped, cell free media was flowed a second time for a further 5 minutes.

The recordings were analysed to determine the number of adherent monocytes. Monocytes that are adherent to the surface of the HSEC appear phase bright whilst those that have migrated through the HSEC monolayer appear phase dark (Figure 2-3) (143). For each individual chamber, 10 different fields of view were analysed to determine the average number of adherent cells. This together with the flow rate was used to determine the number of adherent cells/$mm^2$/$10^6$ cells perfused. Each experiment was performed a minimum of three times with different batches of HSEC and monocytes.

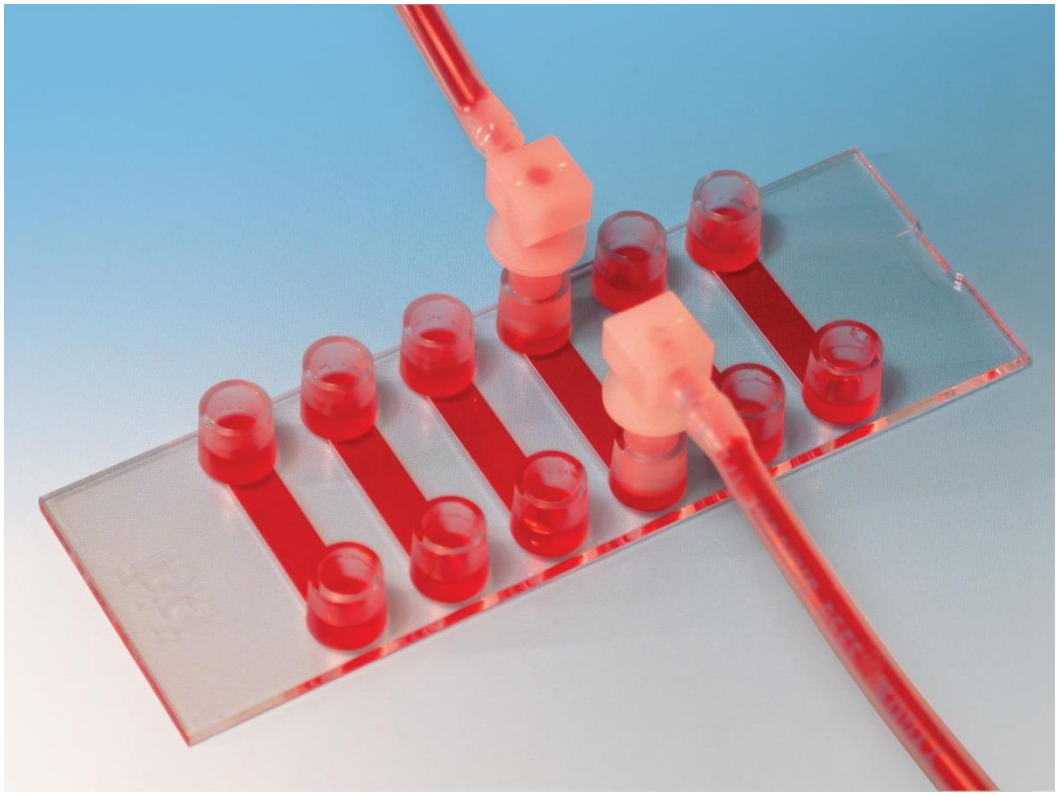


**Figure 2-1. Ibidi µ-slide VI (Ibidi GmbH, Martinsried, Germany) with 6 channels.**

Ibidi slides were coated with rat tail collagen prior to use. HSEC were loaded in to the individual chambers and allowed to become confluent within 24 hours prior to stimulation and use in flow assays. Shown are the connectors to one of the channels. Image from: http://www.ibidi.com

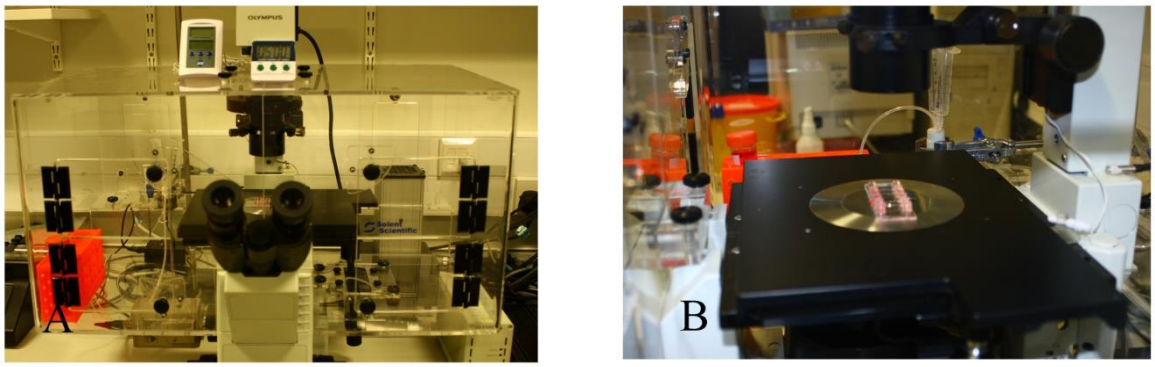



**Figure 2-2. Flow assay apparatus.**

The flow assay apparatus consisted of a heated incubator with a microscope (A). Shown (B a Ibidi slide mounted on the microscope. Image from http://www.jove.com/files/ftp_upload/51330/51330fig2highres.jpg

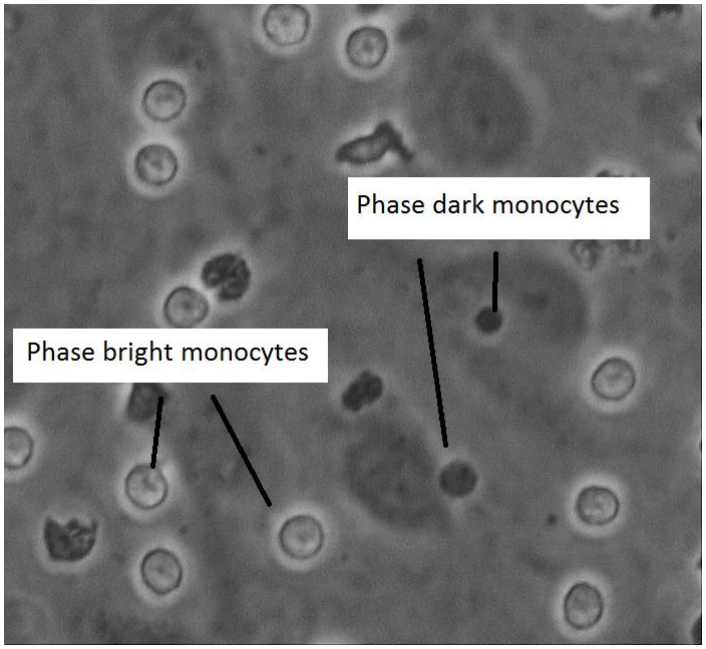



**Figure 2-3. Sample still from a flow assay.**

Monocytes that are adherent to the surface of HSEC appeared as phase bright whilst those that had migrated through the endothelial layer became phase dark.

## 2.10 **Parallel chamber flow assays**

To flow across liver derived macrophages the Ibidi slides were replaced with parallel flow chambers (Figure 2-4) and the liver derived macrophages were cultured on TC25mm Coverslips (Cole-Palmer Instruments Company Ltd, UK) due to the inability to culture these cells with in Ibidi slides reliably.

**Figure 2-4. Parallel flow chambers**

## 2.11 **Crystal Violet Proliferation Assay**

After 24 hours of treatment media was removed from HSEC and washed with PBS twice and fixed with ice cold methanol. To each well 100µl of 0.1% aqueous crystal violet was stained with crystal violet 0.1% in PBS and 25% methanol for 1 hour, then removed with distilled water. The plates were then read on a Dynatech Laboratories MRX plate reader.

## 2.12 **Human Chemokine Proteome Assay**

Proteome Profiler™ Human Chemokine Array Kit (R&D systems) was used to analyse the cell culture supernatants of treated HSEC. After following the manufacturer's instructions the captured proteins were visualised on Hyperfilm-ECL (Amersham Biosciences). Average pixel density was determined with ImageJ software (National Institutes of Health) and background signal subtracted.

## 2.13 **Stimulations**

HSEC, liver derived macrophages or monocytes were stimulated with cytokines, TLR agonists or fatty acids as shown in Table 2-3.

The concentrations used where all in the range as recommended by the suppliers. Peripheral blood mononuclear cells have been shown to be responsive at the concentrations chosen for TLR agonists through the release of cytokines (144, 145) and in the range in what has been used to stimulate endothelial cell lines (146). HSEC were stimulated with the TLR agonists for 24 hours, a time point with which other groups have shown a responses to different TLR stimulations with regards to cytokine production in human lung endothelial and murine liver sinusoidal cells (146, 147). Work within our group has shown that HSEC change they ability to recruit leukocytes after 24 hours of stimulation with cytokines under flow conditions (19, 148).

| Treatment | Supplier | Concentration |
|---|---|---|
| TLR1/2 Pam3CSK4 | InvivoGen | 10ng/ml |
| TLR2 HKLM | InvivoGen | $10^8$ cells/ml |
| TLR3 Poly (I:C) | InvivoGen | 25μg/ml |
| TLR4 LPS | InvivoGen | 10ng/ml |
| TLR5 Flagellin | InvivoGen | 0.1μg/ml |
| TLR6/2 FSL1 | InvivoGen | 0.1μg/ml |
| TLR7 Imiquimod | InvivoGen | 2.5μg/ml |
| TLR8 ssRNA40 | InvivoGen | 10μg/ml |
| TLR9 ODN2006 | InvivoGen | 5μM |
| TNFα | PeproTech | 10ng/ml |
| IFNγ | PeproTech | 10ng/ml |
| IL-4 | PeproTech | 10ng/ml |
| Oleic acid | Sigma, UK | 100μM |
| Palmitic acid | Sigma, UK | 100μM |

**Table 2-3. Treatment for primary cells.**

## 2.14 **Statistical analysis**

Statistical analysis was performed with Graphpad Prism version 5. Data are presented as mean values ± standard error of the mean (SEM). Comparison between groups was performed by Student's t test. Where more than 2 treatment conditions have been compared repeated measures ANOVA was used. Differences were considered statistically significant at a value of $p<0.05$.

# Chapter 3

## Recruitment of Monocytes to Hepatic Sinusoidal Cells in Response to Toll Like Receptor Agonists

## 3.1 Introduction

We hypothesised that activation of toll-like receptors (TLR) on hepatic sinusoidal endothelium by microbial ligands would play a role in modulating endothelial activation leading to enhanced monocyte recruitment and the potential to exacerbate inflammation. Because monocytes recruited from the blood are pivotal in determining the balance between persistent hepatic inflammation and resolution we investigated the effect of different TLR ligands on the recruitment of monocytes via hepatic sinusoidal endothelium.

### 3.1.1 Modelling monocyte recruitment

Though mechanism of recruitment of leukocytes has been well documented it is well recognised that the liver is an unique environment compared to other vascular beds resulting in distinctive differences. Thus data from the use of non-liver specific cells is not necessarily generalisable to the hepatic environment. Even the use of animal models has limitations with significant differences including metabolic rates and immune system (149).

The lining of the liver sinusoids, HSEC, differ from other vascular beds firstly in their morphology with characteristic fenestrae and lack of basement membrane. They also have a specialised phenotype with a lack of or low expression of certain classical adhesion molecules such as P-selectin, E-selectin and CD31.

In this chapter, I have used an *in vitro* model of hepatic sinusoids to investigate monocytes recruitment allowing the complex interactions that occur *in vivo* to be broken down.

Previous work modelling the flow in hepatic sinusoids has used glass capillary microslides (150). However, several disadvantages exist, that include the challenges of preparing and

setting up the glass microslides prior to use. In particular ensuring the formation of a secure continuous endothelial monolayer is grown and not disrupted during exchanging media and avoiding the introduction of air bubbles during experiments. Hence commercially available multichannel gas permeable plastic Ibidi slides were used that have shown comparable results in flow assays. The use of Ibidi slides allows for modelling of vasculature beds and can be used to model the low shear environment of the hepatic sinusoids (143).

## 3.2 Results

### 3.2.1 Ibidi slides can be used to flow monocytes without nonspecific adhesion

The ability of monocytes to securely adhere to plastic is well known and this feature is exploited in their isolation and differentiation.

To ensure any monocyte adhesion in the planned experiments was not simply due to adhesion to the plastic construct of the Ibidi slides, monocytes were flowed across the uncoated Ibidi slides, after washing with the basal media. Flowing monocytes across the uncoated Ibidi slides resulted in minimal adhesion of monocytes to the slides (Figure 3-1).

To ensure monocytes could be pulled out of flow the slides were coated in the classical adhesion molecules, ICAM-1 and VCAM-1, and compared.

There was no additional adherence of monocytes to ICAM-1 whilst being exposed to shear stress compared to flowing over uncoated Ibidi slides. This is in keeping with was has been reported in other vascular systems (151). Monocytes express the integrin LFA-1 that binds to ICAM-1 but in other previous studies it has been shown that to create high affinity bonds LFA-1 requires an activation signal (152, 153). Additionally, inflamed endothelium can cause reverse signalling such as by release of CD137 part of the tumour necrosis factor family which increases the clustering and affinity of LFA-1 to ICAM-1 (154). Furthermore static conditions may be a prerequisite for adhesion to occur initially between monocytes on only immobilised ICAM-1 (154, 155).

Coating of Ibidi slides with VCAM-1 did result in a significant increase in the adherence of monocytes under flow conditions (Figure 3-1).

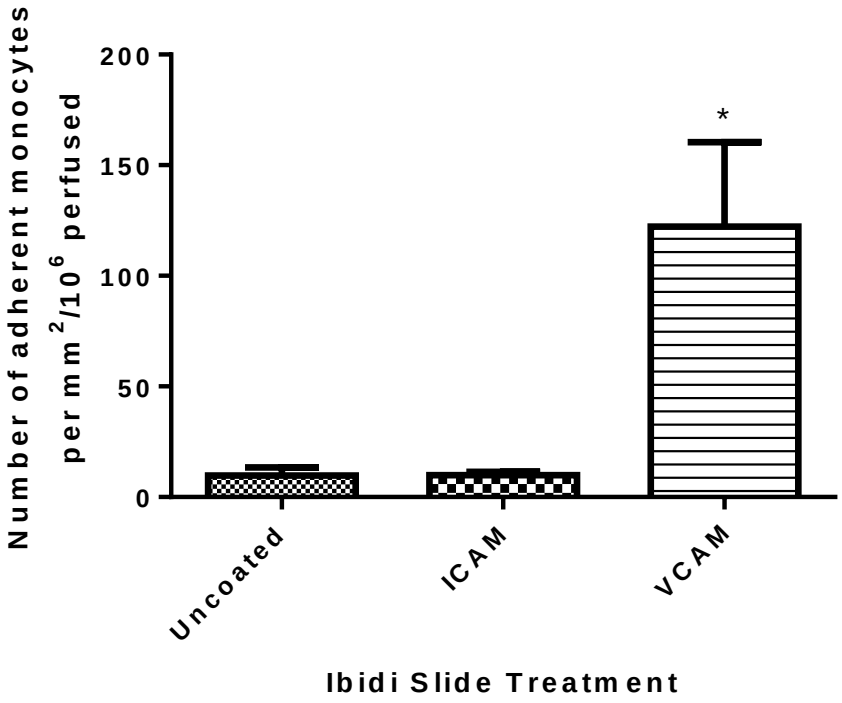



**Figure 3-1. Adhesion of monocytes flowed across immobilised adhesion molecules on Ibidi slides.**

Ibidi slides were coated with either the classical adhesion molecules VCAM-1 or ICAM-1 at a concentration of 10μg/ml in basal media or left uncoated (basal media alone) prior to being rinsed and then exposed to the flow of peripheral blood monocytes at a concentration of $1x10^6$/ml. The data represents the mean ± SEM of three experiments. * $p<0.05$

### 3.2.2 On VCAM-1 coated Ibidi slides LPS stimulated monocytes do not show altered recruitment

LPS is a potent stimulator of the immune system and of monocytes resulting in an activated state. This can lead to a pro-adhesive state and morphological changes of monocytes into a more spread state (156). Continued exposure to LPS is also able to result in a state of immune paralysis. To see if any of these scenarios would occur on VCAM-1 treated Ibidi slides, monocytes were treated with 10ng/ml LPS for one hour and then washed with fresh basal media. Stimulated monocytes were flown either in basal media or basal media containing LPS.

Unstimulated monocytes adhered to immobilised VCAM-1 and the stimulation of monocytes with LPS prior to flowing did not alter the amount adhering (Figure 3-2).

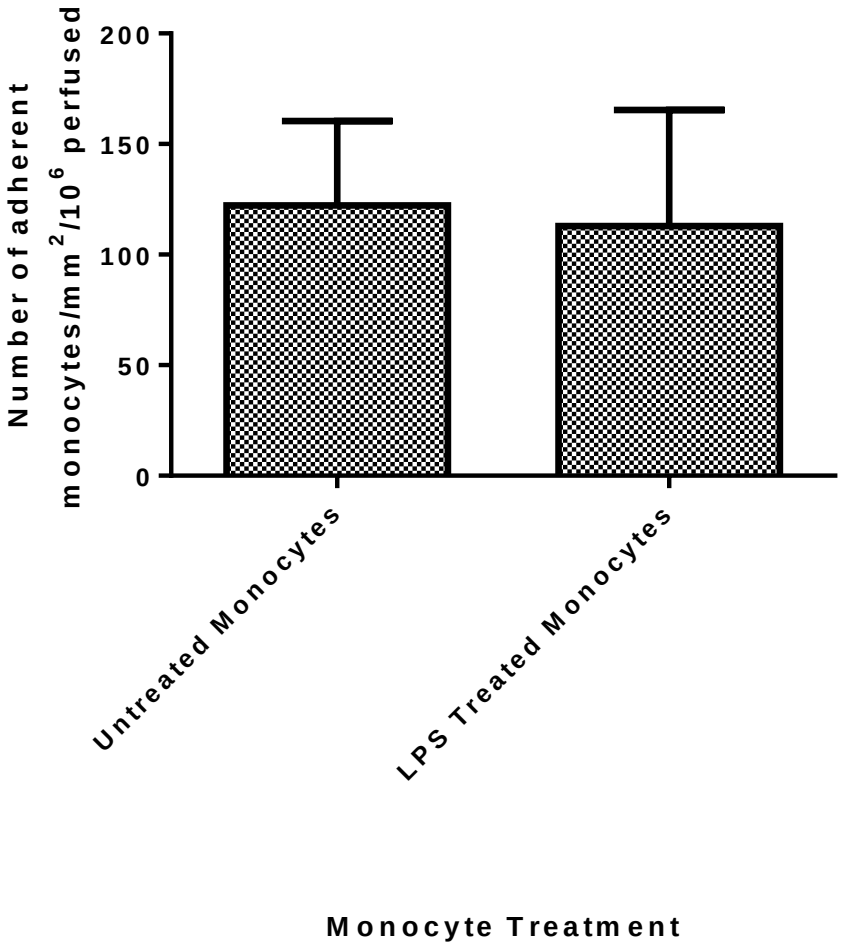



**Figure 3-2. Freshly isolated monocytes treated with LPS flowed over immobilised VCAM-1 in Ibidi slides.**

Freshly isolated peripheral blood monocytes were either flowed in basal media or after stimulation with LPS (10ng/ml) which was then washed off replaced with fresh media. The data represents the mean ± SEM of three experiments.

### 3.2.3 Recruitment of monocytes in an *in vitro* model of hepatic sinusoids in response to endotoxin

To model the sinusoids of the liver to allow investigation of monocytes recruitment out of flow, HSEC were cultured within Ibidi slides to create a uniform monolayer. Previous work has demonstrated that in response to proinflammatory cytokines TNFα, the expression of adhesion molecules is upregulated in cultured HSEC cells and results in increased adherence of lymphocytes out of flow (19). To see if monocytes behave in the same way as peripheral blood lymphocytes, isolated monocytes were flowed over either unstimulated HSEC or after HSEC that had been stimulated for 24 hours with TLR4 agonist LPS, TNFα and or IFNγ.

IFNγ did not cause any appreciable change in adhesion of monocytes to the endothelium above that found in unstimulated endothelium. TNFα with or without IFNγ caused the maximal adhesion of monocytes. LPS also causes increased adhesion of monocytes, but of note fewer monocytes transmigrated through the HSEC monolayer to become phase dark in comparison to TNFα stimulated HSEC (Figure 3-3).

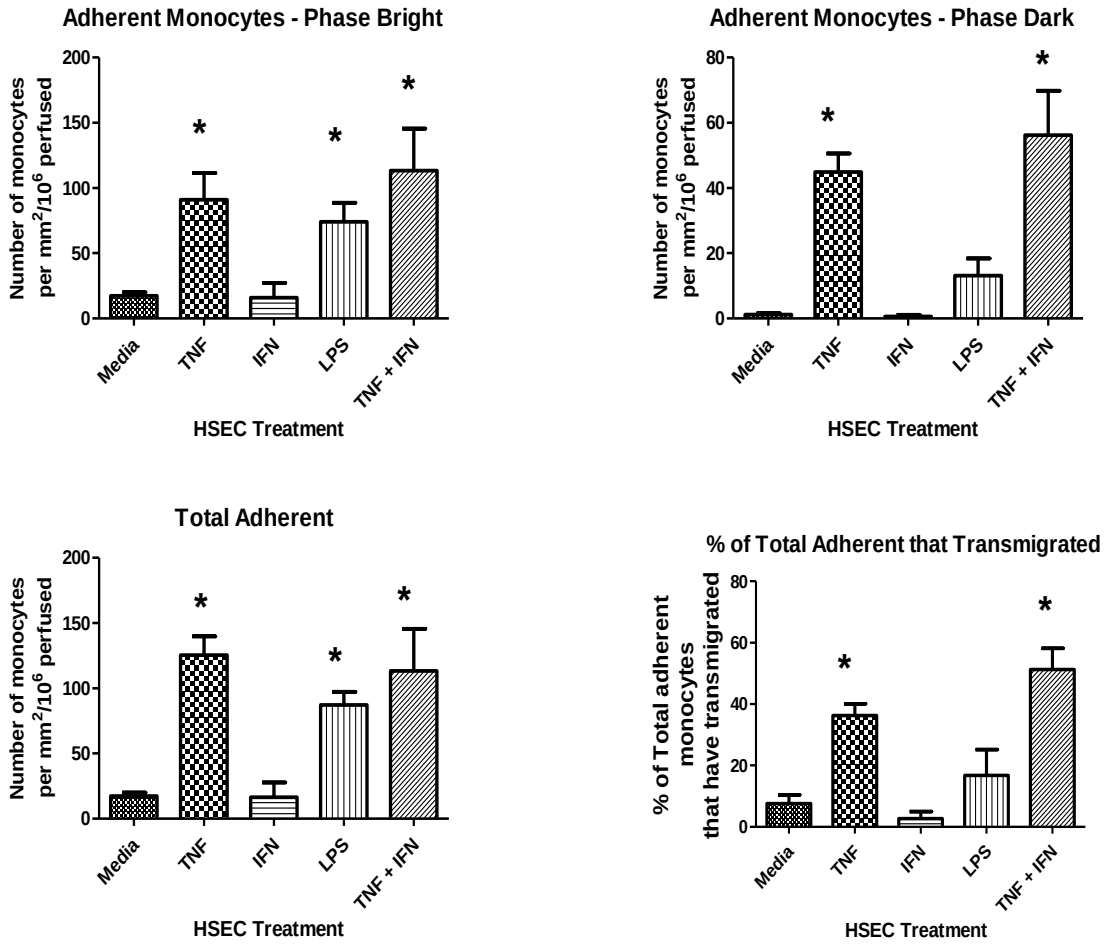



**Figure 3-3. Adhesion of monocytes upon stimulated HSEC.**

HSEC cultured within Ibidi slides were treated for 24hours with the TNFα and or IFNγ and compared to the effect of the TLR4 agonist LPS at 10ng/ml. Stimulation with TNFα and IFNγ was at 10ng/ml. The total adherent monocytes is made up of the phase bright monocytes adherent to the surface of HSEC and those that had transmigrated through the endothelial layer to become phase dark. Data represent mean ± SEM of 4 experiments. * $p<0.05$

### 3.2.4 LPS is a more potent stimulator of monocytes recruitment to HSEC than Monophosphyoryl lipid A

Monophosphyoryl lipid A (MPL) which is derived from the lipid A part of LPS is considered a non-toxic TLR4 ligand. To see if the different effect of LPS compared to TNFα is a unique effect of LPS or the stimulation through TLR4 pathways the experiments were repeated with MPL.

MPL stimulated HSEC did result in recruitment of monocytes but to a lesser extent than observed with LPS. Monophosphoryl Lipid A (MPL) is a TLR4 agonist formed by hydrolysis the diphosphoryl Lipid A component of LPS. These changes results in a decrease in the toxicity compared to unaltered lipid A so that there is a reduction in proinflammatory cytokines released after exposure (157, 158). This reduction in toxicity is to such an extent that it can be used in clinical use as a vaccine adjuvant in humans (159, 160). The reduction in recruitment of monocytes is in keeping with this reduced toxicity compared to LPS. Other work has shown MPL and LPS are able to differentially activate the anti-inflammatory genes such as IL-10 (158). Though the total number of adherent monocytes on MPL stimulated HSEC was less than upon LPS stimulation the same lower proportion of transmigration was seen (Figure 3-4).

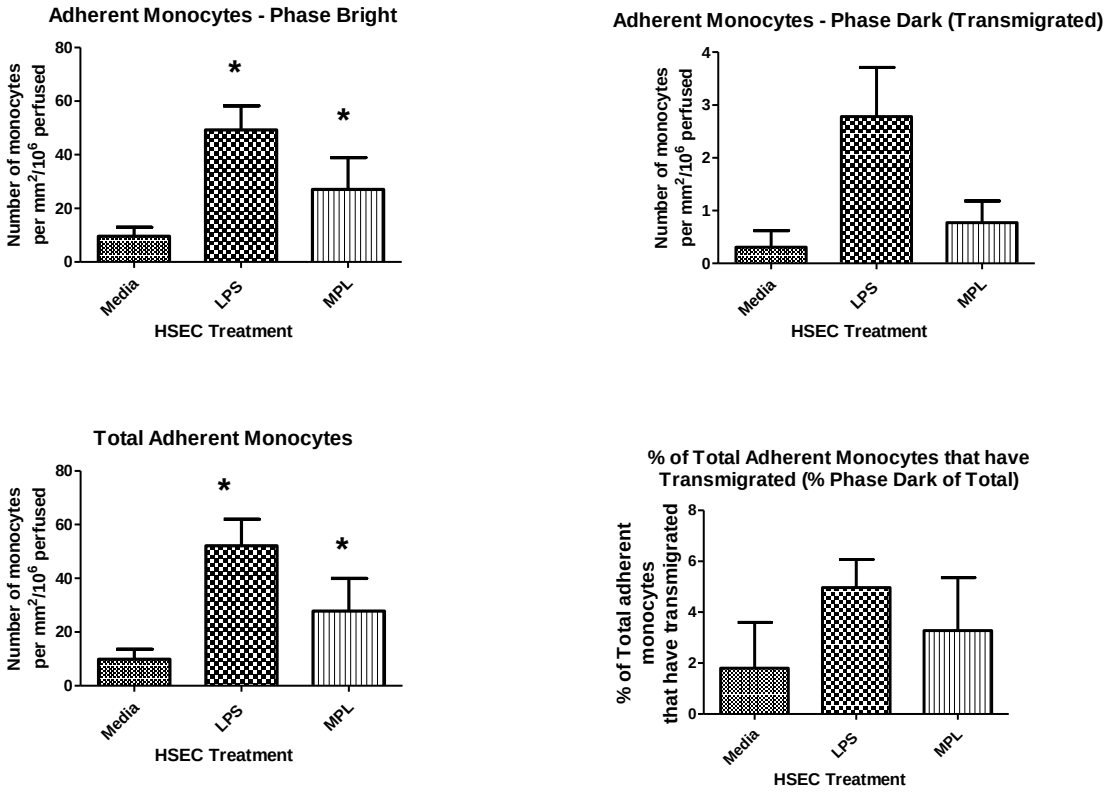



**Figure 3-4. Adhesion of monocytes upon MPL versus LPS treated of HSEC**

HSEC cultured within Ibidi slides were treated for 24 hours with either the TLR4 agonist LPS at 10ng/ml or MPL at 1μg/ml. Freshly isolated peripheral blood monocytes were then flowed across. Data represent mean ± SEM of 4 experiments. * $p<0.05$

### 3.2.5 Tolerance: Continued prolonged exposure of HSEC *in vitro* to LPS results in reduced monocyte adhesion however they are still responsive to repeated stimulation.

Because the liver is continuously exposed to bacterial products via the portal vein I checked whether HSEC are still responsive despite continued stimulation. Murine work has shown hypo-responsiveness of monocytes on repeated exposure to LPS due to reduced expression of CD14 and TLR4 on repeated exposure whereas sinusoidal endothelial cells retain their expression of these receptors (111). As a result, pre-treatment of mice with TLR4 agonist in caecal ligation and puncture model of septic shock is able to induce endotoxin tolerance by reducing subsequent cytokine and chemokine release (161) and consequently neutrophil recruitment in to the liver, potentially limiting host damage.

To assess whether a shorter exposure to LPS rather than the 24 hours used in the flow assay experiments would result in a different amount of adhesion of monocytes a 4 hour time point was used. Confluent monolayers of HSEC within Ibidi slices were treated with LPS for either 4 or 24 hours prior to flowing monocytes across. Freshly isolated peripheral monocytes were then flowed over the HSEC. HSEC that had been stimulated for 4 hours produced the maximal amount of adhesion that reduced by 44% on HSEC that had been stimulated for 24 hours (Figure 3-5).

Re-stimulating HSEC that had been treated with LPS for 24 hours with fresh media containing LPS resulted in boosting monocytes recruitment, showing they are still responsive, however not back to levels seen on short stimulation of naive HSEC (Figure 3-5).

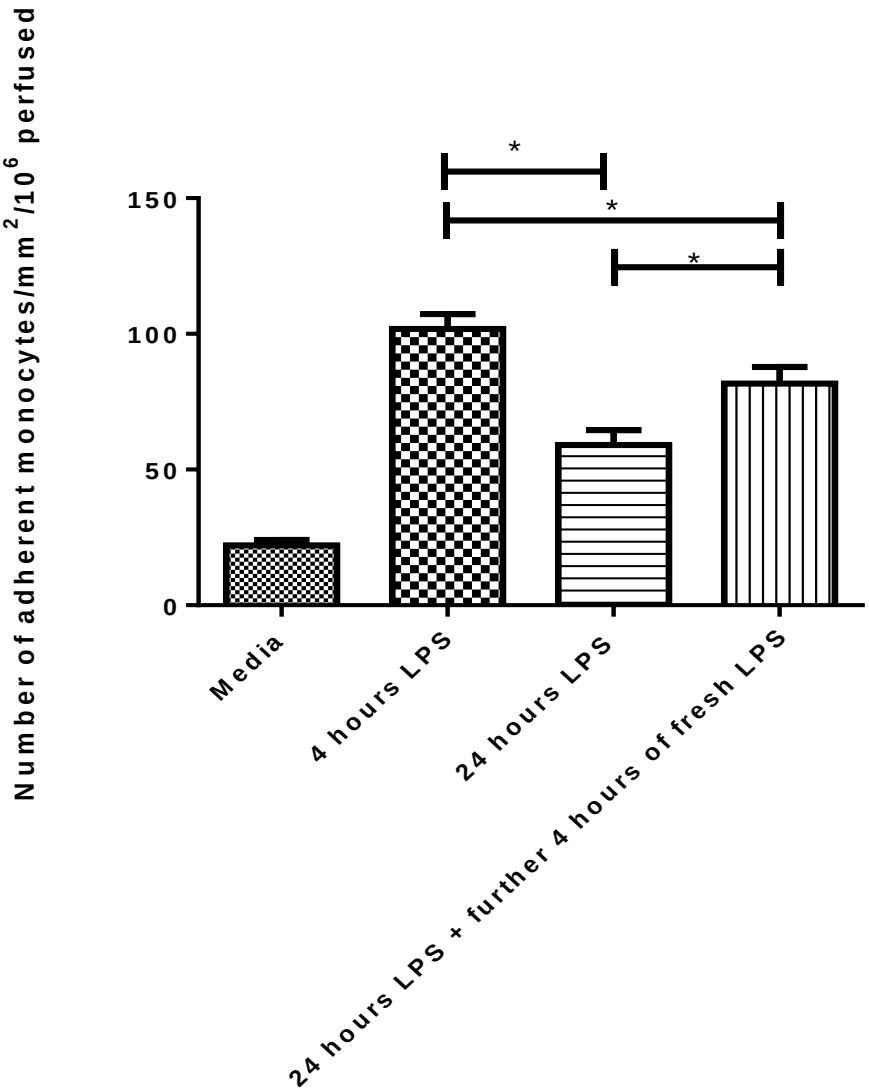



**Figure 3-5. The effect on monocytes recruitment upon different length of LPS stimulation of HSEC.**

HSEC were cultured within Ibidi slides and were treated for either media or the TLR4 agonist LPS at a concentration of 10ng/ml. LPS treatment was 4 or 24 hours immediately prior to flowing freshly isolated monocytes. A further group of HSEC that had been stimulated for 24 hours were re-stimulated for a further 4 hours with fresh LPS containing media. Data represent mean ± SEM of 4 experiments. $P<0.05$

### 3.2.6 Pertussis toxin treated monocytes reduces the proportion of transmigrating across TNFα but not LPS activated HSEC

To investigate the role of G-coupled proteins in the role of adhesion and transmigration of monocytes on activated HSEC, monocytes were incubated with pertussis toxin. Pertussis toxin is a protein exotoxin from the bacterium *Bordetella pertussis*. This exotoxin is taken up by cells and prevents heterotrimeric Gαi proteins from interacting with G protein coupled receptors and their subsequent signalling pathways which affects the production of chemokines, which are important in leukocyte recruitment.

Lymphocytes blocked with pertussis toxin then flowed over chemokine activated HSEC results in reduced adhesion (162). This is a similar finding to what happens with the pre-treatment of $CD16^+$ monocytes with pertussis toxin, with reduction in both the number of adherent monocytes and the number transmigrated, though with a much more profound effect on transmigration (163).

In these experiments using unselected monocytes treated with pertussis toxin to inhibit G-protein coupled receptors reduced the number of monocytes transmigrating and becoming phase dark only when flown over HSEC that had been stimulated with TNFα with or without IFNγ but not IFNγ alone. Pertussis toxin did not reduce the total number of adherent monocytes whatever the cytokine stimulation of the HSEC. No effect was seen when pertussis toxin treated monocytes were flown over LPS stimulated HSEC. This suggests that the initial adhesion of unselected peripheral blood monocytes is G-couple protein independent whilst a significant proportion of transmigration on TNFα activated HSEC is partially dependent on G-couple proteins (Figure 3-6).

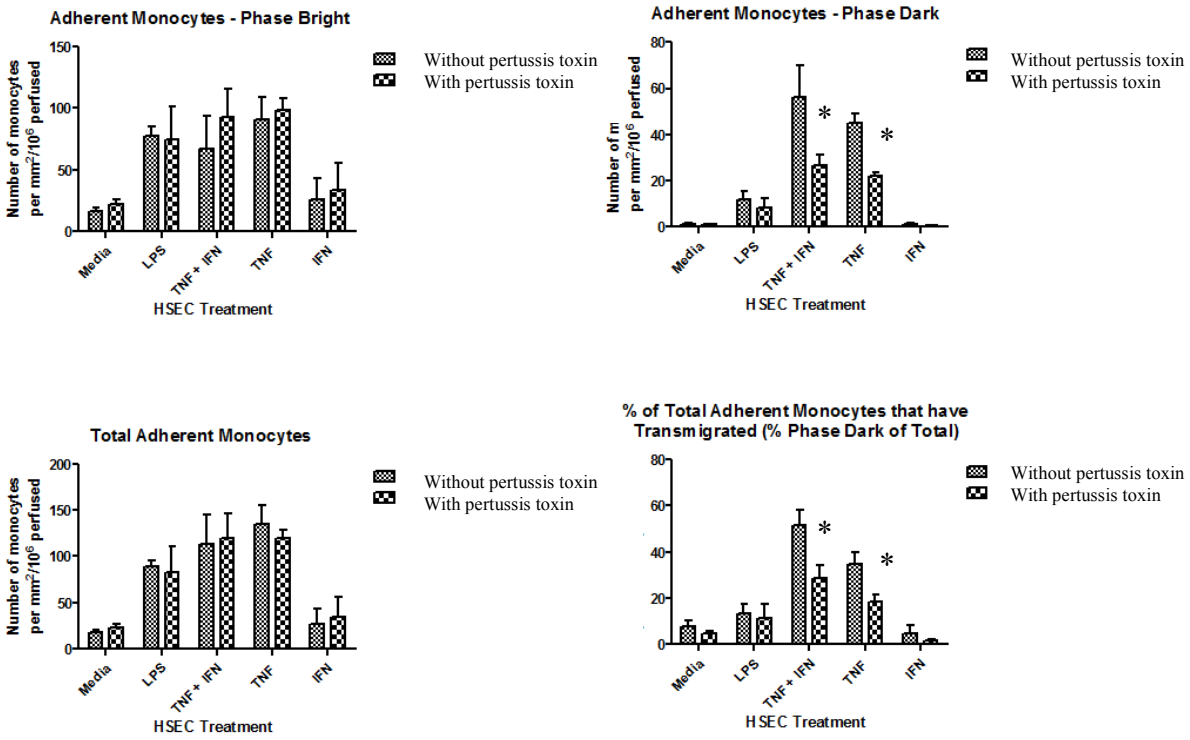



**Figure 3-6. Pertussis toxin treatment on monocytes recruitment to stimulated HSEC**

Monocytes were treated with pertussis toxin at a concentration of 100ng/ml to inhibit G-coupled protein signalling. They were then flowed across HSEC cultured within Ibidi slides that were treated for 24 hours with the LPS, TNFα and or IFNγ. Data represent mean ± SEM of 4 experiments. *$p<0.05$

### 3.2.7 Primed endothelium increases transendothelial migration

Liver disease is often considered as two or multiple hit process (164). The second or subsequent hit includes the exposure to gut derived LPS. It has been repeatedly shown that germ free raised mice have attenuated liver disease progression (165).

The previous experiments have shown that HSEC treated with LPS alone prior to the flow of monocytes does not result in as much transmigration compared to TNFα treated HSEC. As liver disease progresses the serum levels of TNFα of affected individuals increases (166, 167).

Therefore, to assess if priming with inflammatory cytokines prior to exposure of LPS would alter the pattern of recruitment of monocytes, HSEC were treated with the proinflammatory cytokines TNFα and IFNγ prior to stimulation with LPS within Ibidi slides.

The prior treatment of HSEC with TNFα and IFNγ followed by LPS stimulation increased significantly the number of transmigrated monocytes compared to only treating with LPS (Figure 3-7).

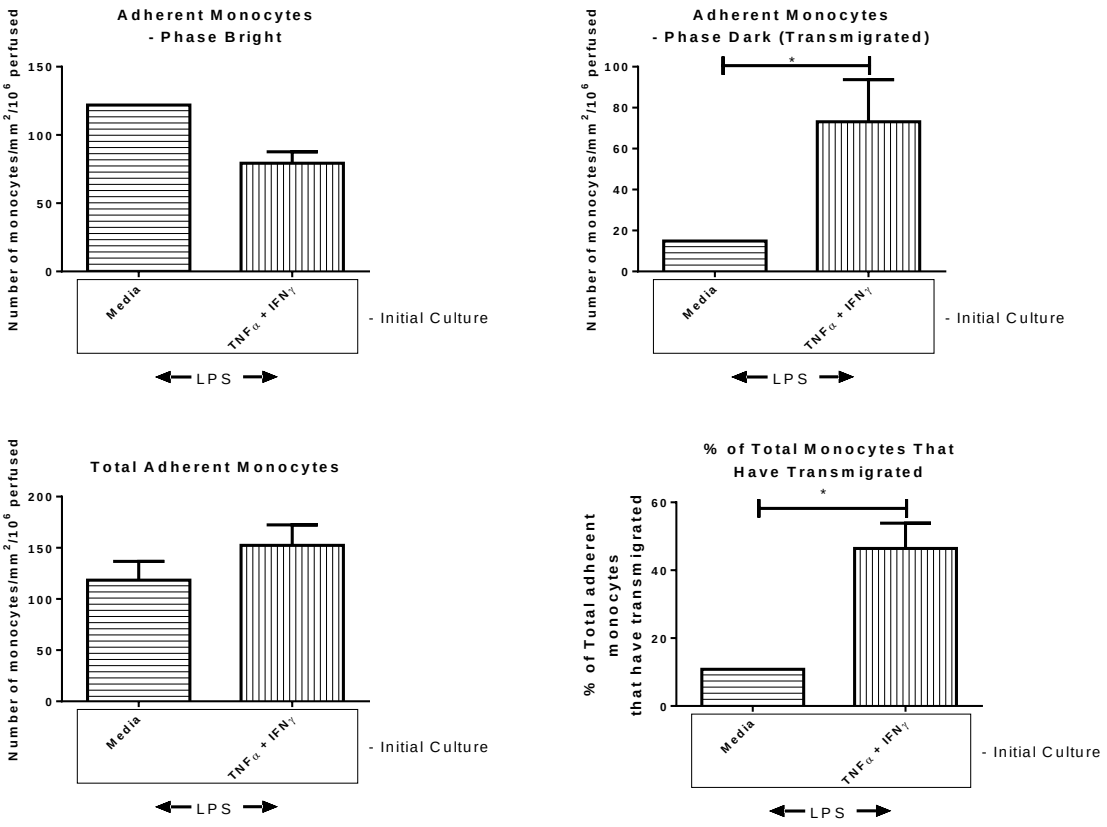



**Figure 3-7. Priming of HSEC prior to LPS stimulation**

HSEC cultured within Ibidi slides were primed for 24 hours with TNFα and IFNγ or media only. The media after 24 hours in the Ibidi slides was then exchanged with LPS containing media. Freshly isolated peripheral blood monocytes were then flowed across. Data represent mean ± SEM of 4 experiments. *$p<0.05$

### 3.2.8 Pertussis toxin reduces transmigration of monocytes on TNFα and IFNγ primed HSEC treated with LPS

Previous experiments have demonstrated that priming HSEC with TNFα with IFNγ results in around half of peripheral blood monocytes transmigrating under conditions of flow. Furthermore, the low proportion of monocytes transmigration across LPS treated HSEC is increased by first priming with TNFα and IFNγ. Pertussis toxin treated monocytes showed reduced transmigration across TNFα and IFNγ activated HSEC. To investigate the effect of G-coupled signalling on LPS treated HSEC that had been primed by TNFα and IFNγ was tested.

When HSEC had been primed with TNFα and IFNγ prior to stimulation with LPS the reduced monocyte transmigration of pertussis toxin treated monocytes was maintained without affecting the total number of adherent monocytes (Figure 3-8).

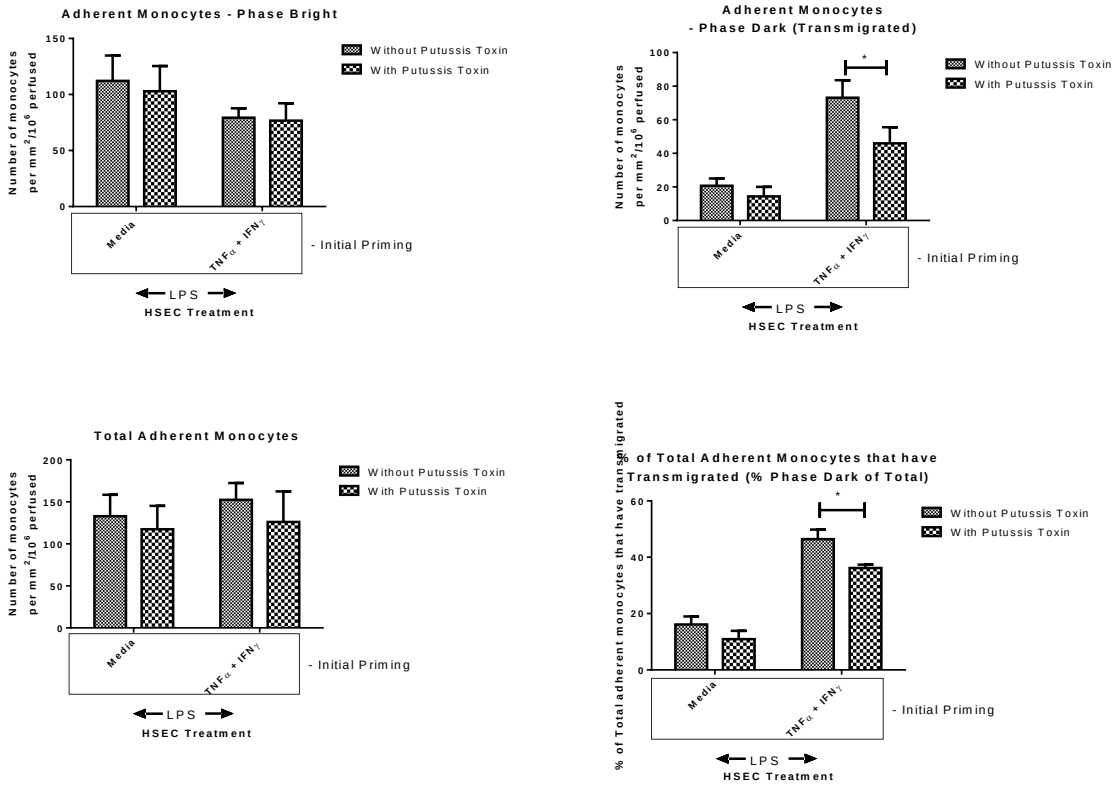



**Figure 3-8. Adhesion of pertussis toxin treated monocytes on primed HSEC treated with LPS.**

Monocytes were treated with pertussis toxin at a concentration of 100ng/ml to inhibit G-coupled protein signalling. They were then flowed across HSEC cultured within Ibidi slides that were stimulated with LPS after first being primed TNFα and IFNγ (compared to no priming). Data represent mean ± SEM of 3 experiments. *$p<0.05$

### 3.2.9 Cell surface located TLRs increase monocyte adhesion

So far, the experiments showed that the TLR4 agonist LPS as well as MPL are able to induce adhesion of flowing monocytes on to HSEC. In humans, there are, to date 9 well described TLRs. (TLR10 does not have a known ligand specificity) (168). To investigate the effect of stimulation with other TLR agonist, HSEC was stimulated with known TLR agonists prior to flowing peripheral blood monocytes.

Treatment of resting endothelium with ligands to TLRs 1, 2, 4, 5 or 6 for 24 hours resulted in increased adhesion of monocytes under flow. Activation of TLRs 3, 7, 8 or 9 with specific ligands had no significant effect on adhesion or migration (Figure 3-9). Of note TLRs 1, 2, 4, 5 or 6 are all located on cell surfaces and all signal down MyD88 pathway. TLRs 3, 7, 8 and 9 are compartmentalised intracellularly to detect nucleic acids.

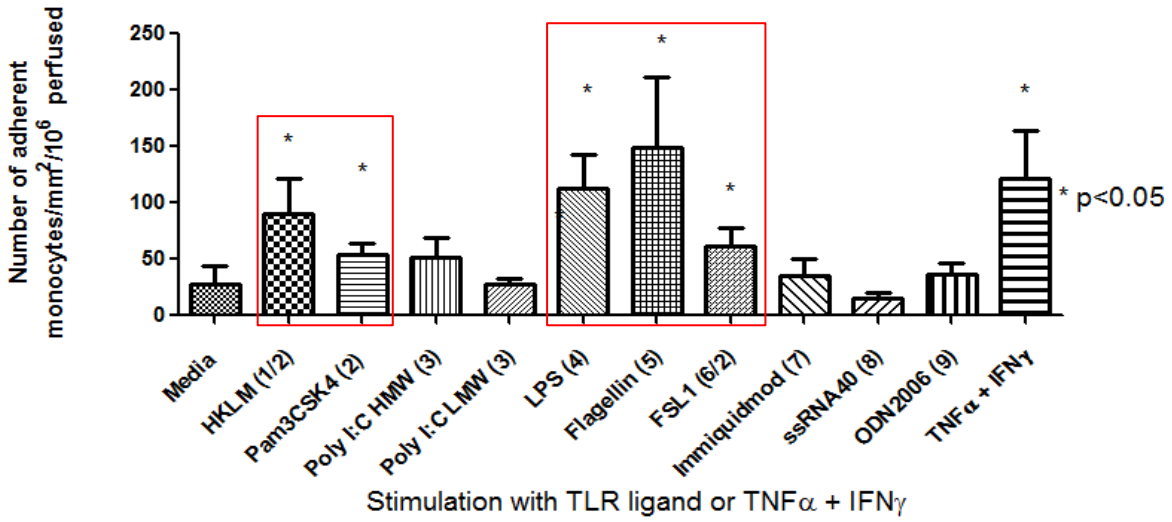



**Figure 3-9. Adhesion of monocytes to HSEC stimulated with TLR ligands.**

HSEC were stimulated with TLR ligands for 24 hours and then freshly isolated monocytes were flowed across. The data represent the mean ± SEM of 4 experiments. P<0.05

### 3.2.10 Inflammatory priming of HSEC increases transendothelial migration in response to bacterial sensing TLRs

As the previous experiment showed, stimulation of HSEC with microbial components that interact with cell surface TLRs supports adhesion out of flow of monocytes. However, few of the adherent monocytes transmigrated as previously seen with HSEC treated with LPS. To see if priming HSEC with proinflammatory cytokines beforehand would alter this state, HSEC was treated with TNFα and IFNγ before further stimulation with TLR ligands. The priming of HSEC with the proinflammatory cytokines TNFα and IFNγ resulted in allowing adhered monocytes to transmigrate compared to HSEC treated with only TLR ligands. The proportion of monocytes transmigrating on TNFα and IFNγ pre-treated HSEC was similar no matter which cell surface TLR agonist was used subsequently. The priming of HSEC with TNFα and IFNγ prior to stimulation with bacterial ligands of cell surface TLRs resulted in increased transmigration as seen previously with LPS and thus the effect was not confined only to TLR4 stimulation (Figure 3-10).

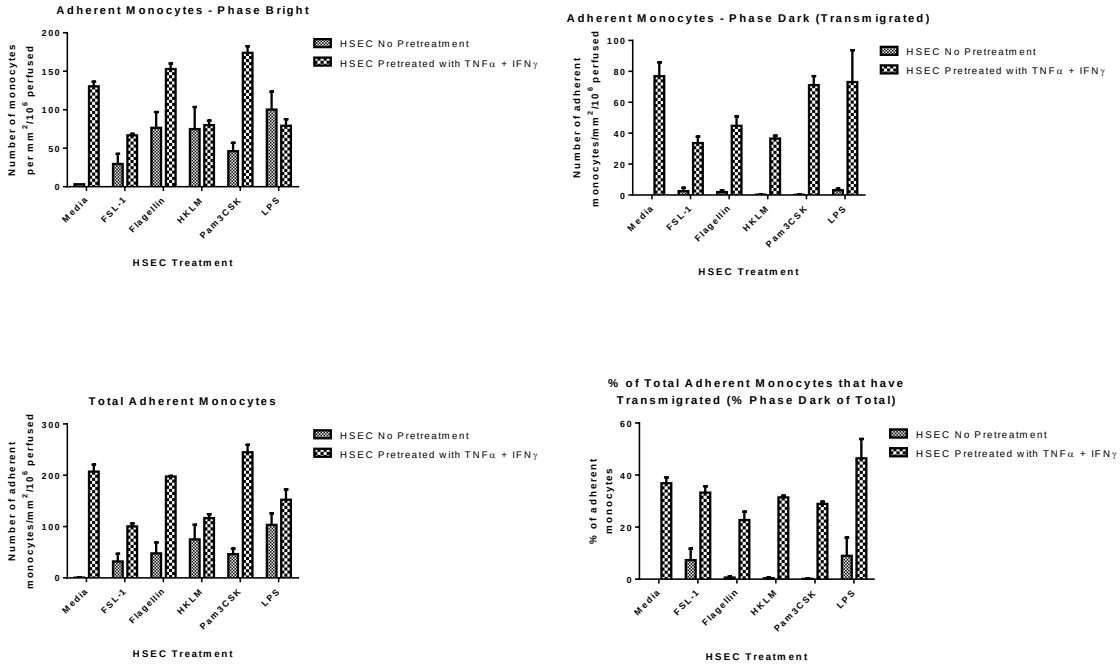



**Figure 3-10. Adhesion of monocytes to primed HSEC stimulated with TLR ligands**

HSEC were primed with TNFα and IFNγ prior to stimulation with TLR ligands within Ibidi slides. Freshly isolated monocytes were flowed across the HSEC and monocyte adhesion assessed. The data represent the mean ± SEM of 3 experiments

### 3.2.11 LPS stimulation of monocytes diminishes their ability to transmigrate across inflamed endothelium

We also looked at what occurred when monocytes were treated with LPS as opposed to the endothelium. On unstimulated endothelium, LPS treated monocytes were no more adhesive than untreated monocytes. However, when the experiment was repeated upon TNFα and IFNγ treated HSEC, the treatment of monocytes diminished the migration seen of adhering monocytes by almost 70% (Figure 3-11).

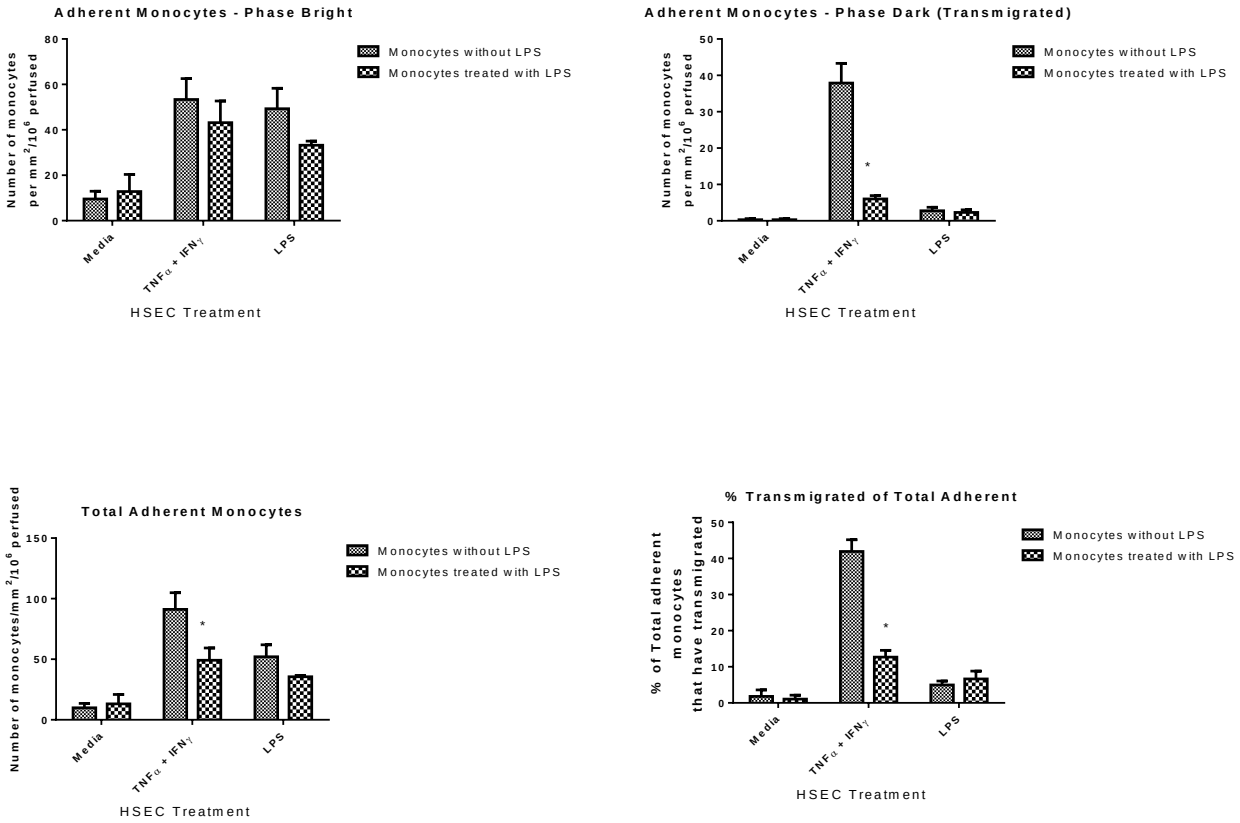



**Figure 3-11. Recruitment of LPS treated monocytes upon inflamed endothelium.**

Monocytes were treated with LPS for 1 hour after isolation and then either flowed across untreated HSEC or after stimulation with TNFα with IFNγ or LPS in Ibidi slides. The data represents the mean ± SEM of three experiments. * $p<0.05$

### 3.2.12 Cell surface TLR stimulation of monocytes reduces transmigration of monocytes across activated endothelium

Having seen the effect upon LPS treated monocytes recruitment upon inflamed HSEC we went on to investigate the effect of other cell surface TLR stimulation of monocytes upon TNFα and IFNγ treated HSEC modelling inflamed endothelium.

When unstimulated monocytes were flowed over TNFα and IFNγ stimulated HSEC as expected resulted in an increase in total adhesion of monocytes and this was unaffected by treating the monocytes with used TLR agonists. However, the proportion of monocytes transmigrating across the endothelium reduced by approximately on average by around 50% and not by only LPS (Figure 3-12).

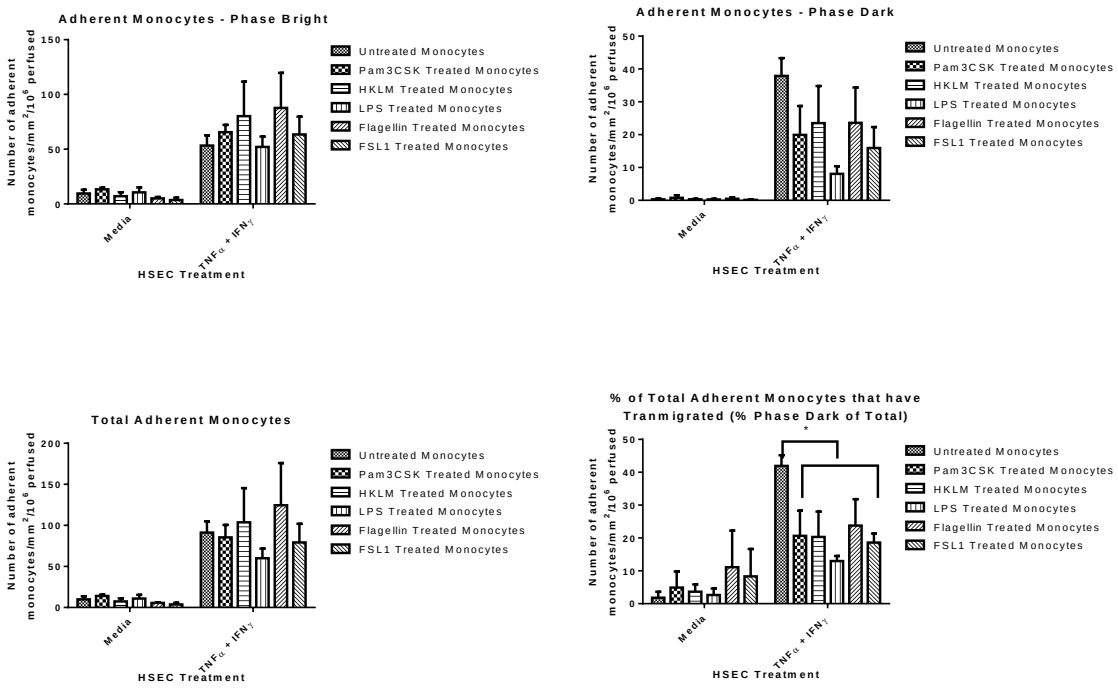



**Figure 3-12. Treatment of monocytes with bacterial ligands prior to flowing across HSEC.**

Freshly isolated peripheral blood monocytes were treated with the cell surface TLR agonists Pam3CSK, HKLM, LPS, Flagellin or FSL-1. They were then flowed across HSEC or TNFα and IFNγ treated HSEC in Ibidi slides. The data represent the mean ± SEM of 3 experiments.

## 3.3 **Discussion**

A defining feature of inflammation is the recruitment of innate immune cells. This includes the recruitment of monocytes. Studies have shown that the recruitment of monocytes in response to liver damage perpetuate the inflammation as well as expanding the macrophage pool within the liver (169).

To investigate the mechanisms of leukocytes recruitment in the liver, our laboratory has developed specialised techniques. The vascular beds within the liver are unique and specialised allowing it to perform its multitude of functions. Thus, it is not possible to generalise the results from studies of other vascular beds. The use of animal studies though helpful, there remains major differences when compared to humans. The ability to isolate human HSEC and the ability to flow leukocytes across them at low shear pressure to mimic the conditions within hepatic sinusoids, allows breaking down the complex interactions involved in multicellular interactions. The importance of shear stresses is well recognised to alter recruitment of leukocytes out of flow. Previous work in our laboratory has demonstrated the usefulness of the use of Ibidi slides in investigating recruitment of lymphocytes (143). In this thesis we have gone on to look at monocytes recruitment. Monocytes are well known to adhere to plastic, a fact exploited in some methods of isolation. The observation of the lack of adhesion whilst flowing monocytes across the Ibidi slides but when given the appropriate signals could be captured out of flow and change phase allowed the model to be used for further experiments.

We have shown that LPS stimulation of HSEC is able to recruit monocytes out of flow and for them to become adherent. This same finding was also seen with stimulation with other agonists of cell surface located TLRs. However, the same was not true with the TLRs 3, 7,

8 and 9 that are intracellular and involved in detecting nucleic acids. Though the cell surface group of TLRs resulted in increased adhesive properties to HSEC this did not translate to increased transmigration across.

The importance of this finding is that the liver is continuously exposed to microbial products from the gut, yet efficiently clears them without producing an overwhelming local inflammation within the liver and prevents systemic spread and subsequent disseminated inflammation in the body. So, in this model of hepatic sinusoids where there is stimulation by microbial components that interact with cell surface TLR ligands, HSEC support the adhesion but not the transmigration of flowing monocytes. This may give an insight to what occurs physiologically. In humans, this may be a mechanism that prevents accumulation of monocytes into the liver parenchyma and potentially causing unnecessary harm particularly when the portal blood stream contains microbial products derived from the gut are continuously in contact with the hepatic sinusoids.

In keeping with previously published data, monocytes, similar to leukocytes flowing over HSEC did not require a rolling step to be captured out of flow in keeping with the lack of E-selectin in hepatic sinusoids (170).

Work in our laboratory, has demonstrated TNFα to significantly activate HSEC to result in a pro-adhesive state. Further work from the laboratory has shown the addition of IFNγ to stimulate HSEC increases the amount of adhesion molecules expressed but not the pattern of expression (162). IFNγ together with TNFα is able to increase the secretion of the CXCR3 ligands CXCL9, 10 and 11. Despite the ability of IFNγ to increase the expression of adhesion molecules and secretion of CXCR3 ligands the lack of any major increase in adhesion and transmigration when compared to TNFα alone may be partly due to the experiment being

performed under flow conditions. It has previously been shown that flow can attenuate the effects of IFNγ through inhibition of STAT1 activating the target chemokine genes (171).

In this model when HSEC were primed by TNFα and IFNγ simulating the presence of pre-existing inflammation, further stimulation with bacterial products was able to promote transmigration. This may reflect the hepatic infiltration by monocytes that occurs in liver disease as part of a two/multi hit process exacerbating and perpetuating hepatic inflammation.

A feature of LPS stimulation is its ability to induce a state of tolerance resulting in diminished responses upon repeated stimulation. Studies have shown the liver sinusoids made up of HSEC with Kupffer cells gain tolerance to endotoxin and go into a refractory state (172). Monocytes also have the ability to become tolerant. The experiments performed here showed that this ability remained in this flow system, in that repeated stimulation of HSEC by LPS had diminished adhesion compared to the initial stimulation, but nevertheless still retain the ability to respond.

The mechanisms for the tolerance vary by cell type. Monocytes upon exposure to LPS are known to down regulate TLR4 expression. Unlike as described in monocytes and macrophages there is no down-regulation of surface expression of TLR4 in HSEC upon exposure to LPS (111). However, HSEC gain LPS tolerance by reduced nuclear localization of NFkB (111).

*In vivo,* the tolerance effect of LPS is likely to be a result of multiple interactions between cell types. HSEC is likely to be modified by the resident Kupffer cells which also are tolerised by LPS and release IL-10 that can suppress inflammation.

Within a matter of a few hours following stimulation with LPS, monocytes produce significant pro-inflammatory cytokines. The anti-inflammatory cytokine IL-10 production peaks after 24 to 48 hours(173). IL-10 together with TGFβ and prostaglandin E2 desensitise monocytes to further LPS stimulation (174). The effects of IL-10 on monocytes includes the suppression of pro-inflammatory cytokine release, antigen presentation and production of free oxygen radicals.

Though in these assays stimulation of HSEC or monocytes with LPS reduced transmigration there is still the potential of harm. Activated adhered monocytes on endothelium can damage the endothelium by releasing pro-inflammatory mediators that include IL-1 and IL-6, eicosanoids, reactive oxygen species (ROS), platelet activating factor, and nitric oxide (NO) (175).

The use of the flow system with Ibidi slides has a number of draw backs. Firstly, the HSEC and monocytes used were from different donors and thus incompatibility interactions could have unknown effects in this system. This system was also used in normoxic atmospheric oxygen levels. However the liver has a lower oxygen tension and this has been demonstrated to alter HSEC structure and adhesion molecule expression (176). Also flowing across a monolayer of HSEC removes these cell from the influences of the liver environment and the interaction with other hepatic cells that modulate milieu.

For the timings of stimulating cells times of 4 and 24 hours were uses for practicalities of running the flow assays. However, this potentially could be missing the maximal effects of the treatments. LPS in health can be eliminated from the circulation in minutes by the liver, though its effects can last for a longer time (177). Using the same length of exposure for all the different treatments ignores the kinetics of the different stimulations.

The concentrations used for stimulations were chosen on the basis of what is known from other studies to be able to stimulate monocytes and endothelial cells, but this does not necessarily corollate with what is possible physiologically. Though levels of LPS in humans during liver disease can reach high levels they are generally lower than used in these experiments (178). However, the concentration of flagellin used was at a levels that occurs physiologically but can increase in disease (179). The importance of different concentrations has been demonstrated with flagellin's ability to potentially be beneficial at low doses in aiding bacterial clearance by neutrophils in a burns sepsis model (180). However high levels of flagellin result in liver injury (181).

# Chapter 4

## The Expression Of TLRs, Adhesion Molecules And Cytokines In HSEC In Response To Inflammation

## 4.1 **Introduction**

Having seen the functional consequences of TLR ligands stimulation upon monocyte recruitment we have gone on to look at some of the changes that occur in response to inflammation and disease. TLRs are pivotal in the development and perpetuation of liver disease. It has been increasingly recognised that it is not only TLR4 that is involved but also the other members of the TLR family are increasingly implicated. In the previous chapter we have shown that any of the TLR ligands that interact with cell surface TLRs are able to cause the adhesion of monocytes on to HSEC.

In this chapter, we have looked for expression of TLRs in the liver and HSEC. We have also looked at changes in cell surface HSEC adhesion molecules and released cytokines upon activation that may help explain the lack of monocyte transmigration when treated with cell surface TLR ligands.

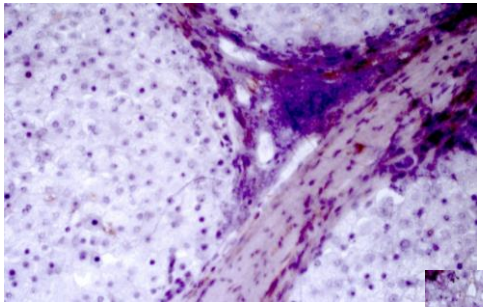
### 4.1.1 Diseased liver tissue from explanted livers shows increased expression of TLR2, TLR4, TLR5, TLR6 and TLR9

Though current published literature report the presence of the different TLRs within the liver, there is variance as to which TLRs are expressed. To see if we could demonstrate the presence of TLRs in livers immunohistochemisty staining was performed. Sections from normal donor livers and those from livers diseased by either alcoholic liver disease, autoimmune hepatitis, primary biliary cirrhosis or primary sclerosing cholangitis were stained. (TLR1, 3, 7 and 8 staining was attempted but did not produce reproducible staining. Attempts were made to use different antibody concentrations and staining on lymph nodes that express these TLRs.)

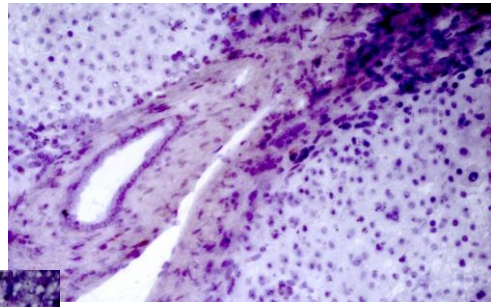
The diseased liver sections showed visibly higher staining of TLRs in particular of TLR2, TLR4, TLR5, TLR6 and TLR9. Most of the increased staining appeared to be related to the fibrotic scars (Figure 4-1).

Though there was positive staining in diseased livers with certain antibodies for TLRs great care must be taken in interpreting the results. Staining with antibodies for TLRs often fail to recognise their designated TLR proteins in their native forms on cells (182).

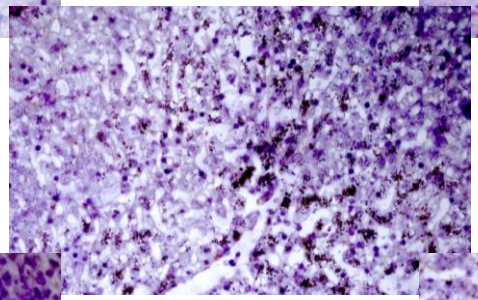
# TLR2 – 20x



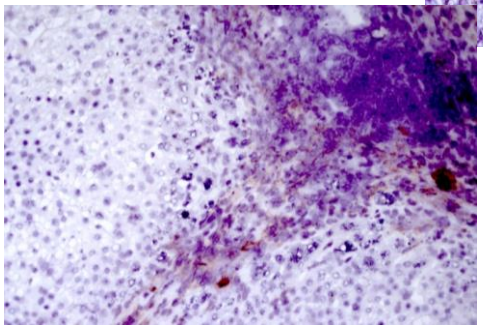
Autoimmune Hepatitis



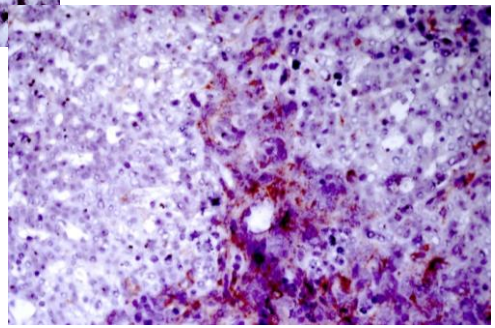
Alcoholic liver disease



Normal Liver

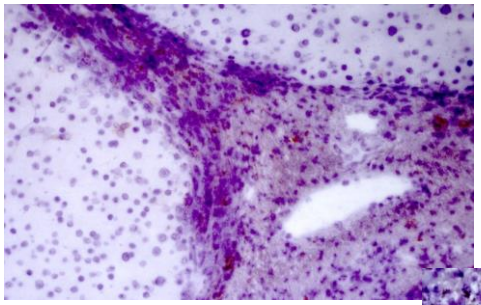


Primary Biliary Cirrhosis

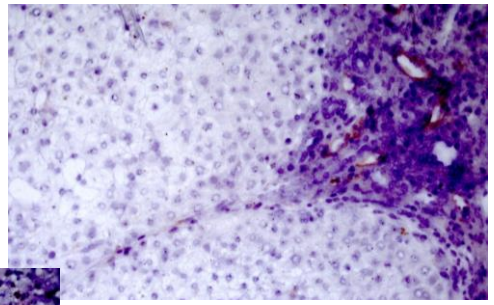


Primary sclerosing cholangitis

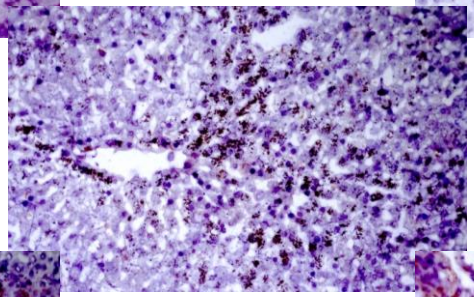
# TLR4 – 20x



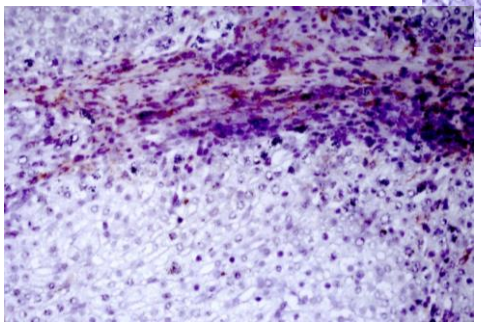
Autoimmune Hepatitis



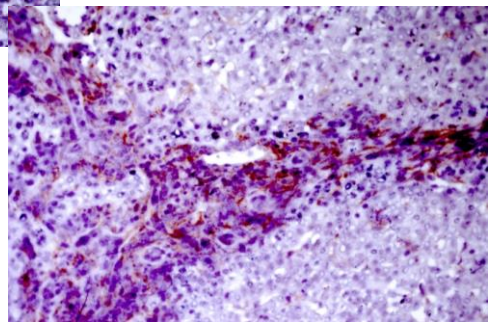
Alcoholic Liver Disease



Normal liver

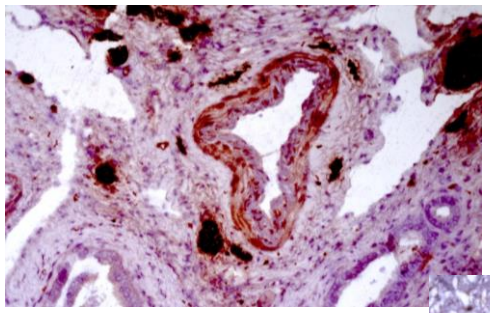


Primary Biliary Cirrhosis

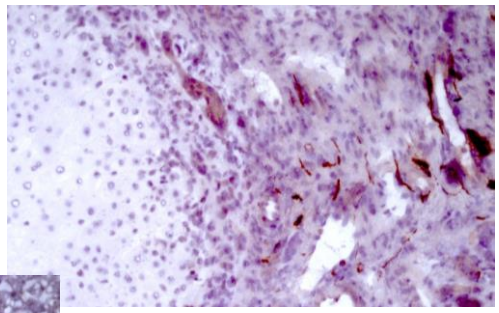


Primary Sclerosing Cholangitis

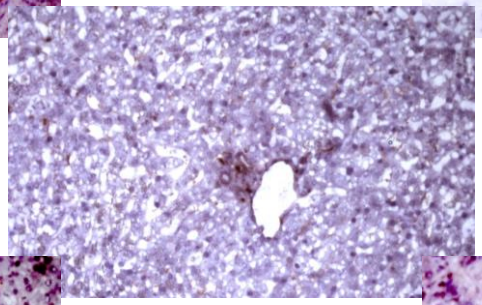
# TLR5 – 20x



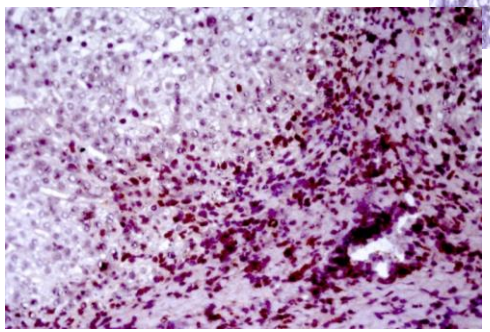
Autoimmune Hepatitis



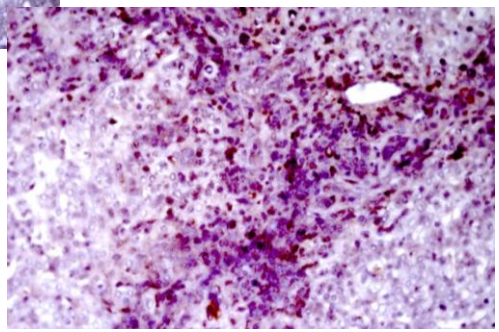
Alcoholic Liver Disease



Normal Liver

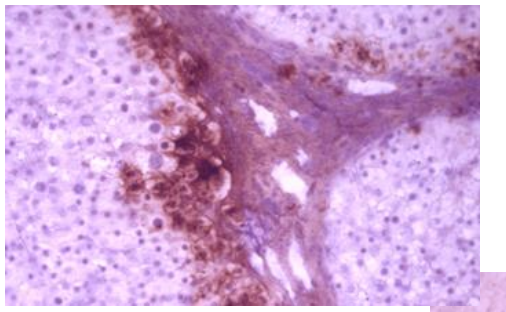


Primary Biliary Cirrhosis

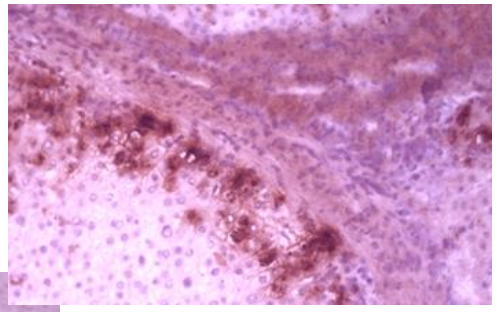


Primary Sclerosiing Cholangitis

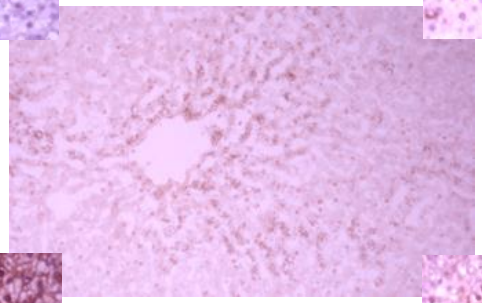
# TLR6 – 20x



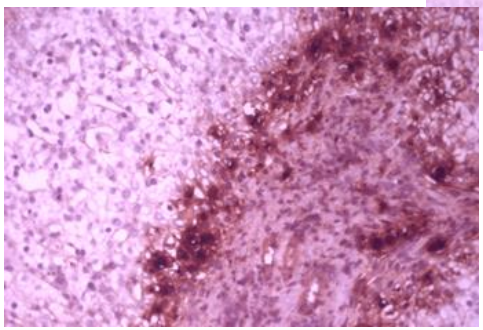
Autoimmune Hepatitis



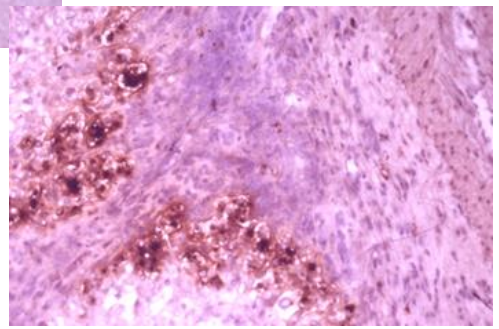
Alcoholic Liver Disease



Normal Liver



Primary Biliary Cirrhosis



Primary Sclerosing Cholangitis

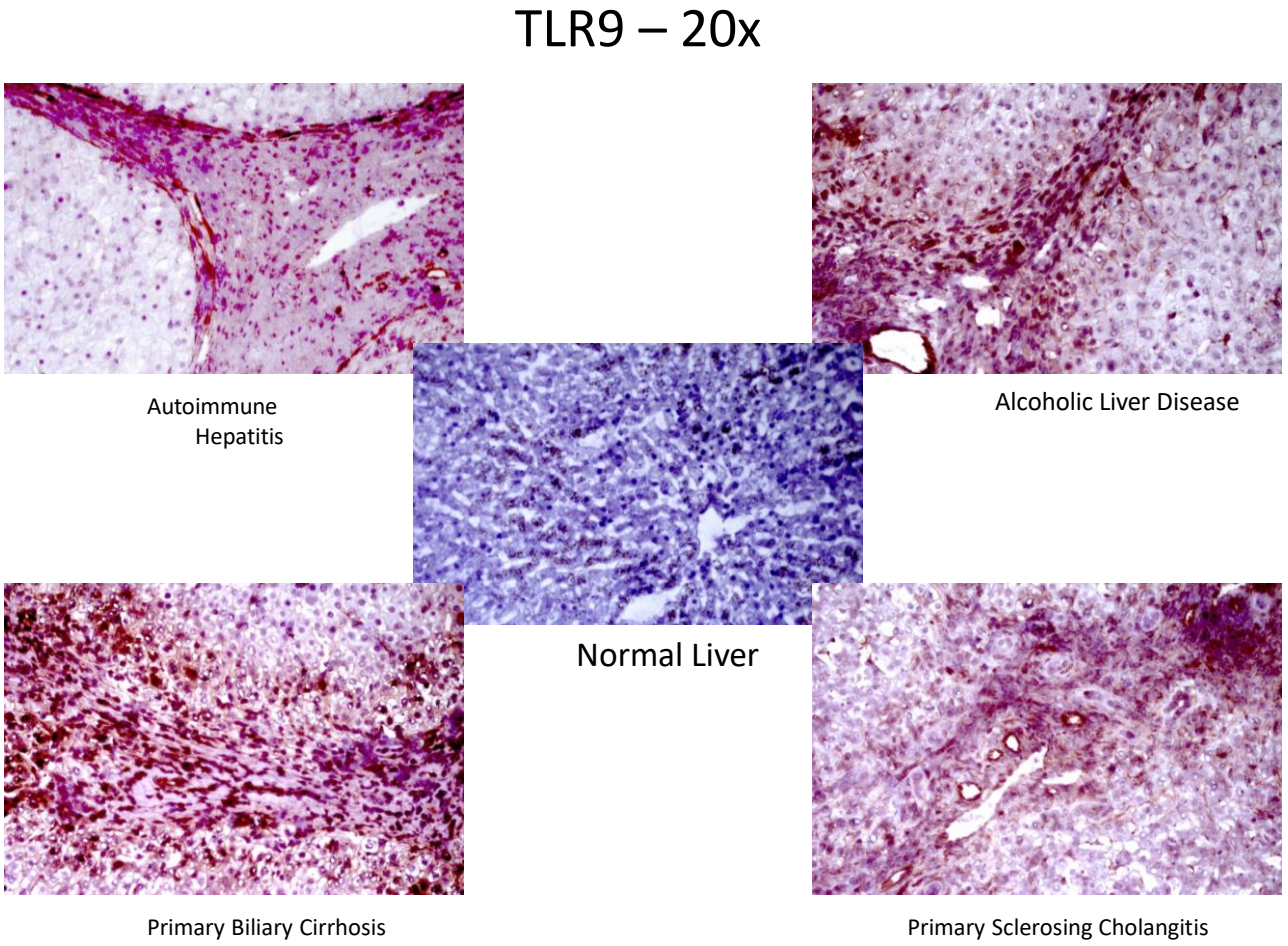



**Figure 4-1. Staining of normal and disease liver section for expression of TLRs.**

Frozen sections of liver from normal or diseased livers were stained for TLRs. Isotope control staining was performed to look for non-specific staining (images not shown).

## 4.2 Inflammatory cytokines alter the expression of TLRs on HSEC at a mRNA level

To assess what occurs in the sinusoids to HSEC when exposed to inflammatory cytokines, relative quantification PCR was used to determine what would happened to relative TLR mRNA levels following exposure to classical inflammatory cytokine TNFα and IFNγ.

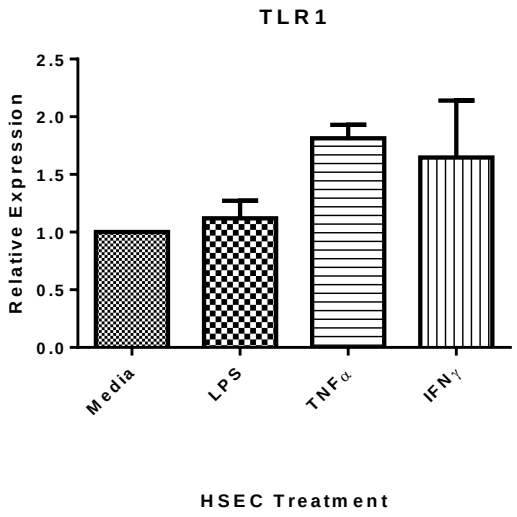
The role of LPS was also investigated as though TLRs respond to specific ligands, a number of studies have shown that LPS (as well as other TLR ligands) are able to alter the expression of other TLRs through cross talking (183). This means that TLR ligands can modulate unrelated TLR receptors. To see if HSEC would show altered mRNA expression of unrelated TLRs to LPS, PCR was performed on LPS treated HSEC.

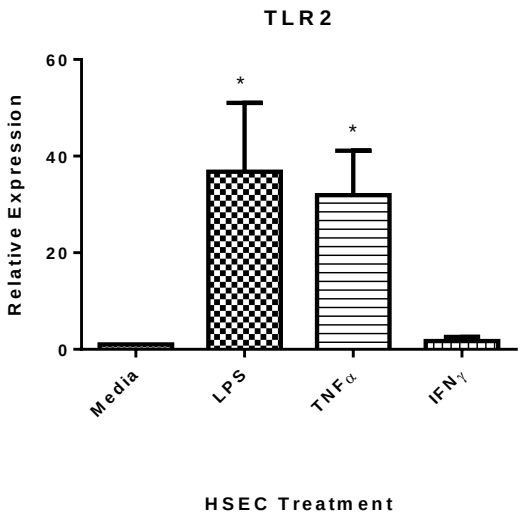
IFNγ upregulated the expression of TLR3 and downregulated TLR2 and 9. It did not increase the expression of any of the cell surface bacterial sensing TLRs.

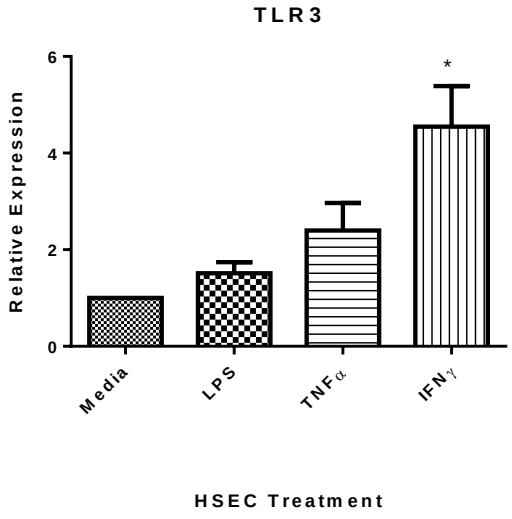
TLR2 had the greatest increase in expression in response to TNFα and LPS. Interestingly the effect of TNFα and LPS on the levels of TLR1 and TLR6, which associate with TLR2 to form heterodimers, were far more modest.

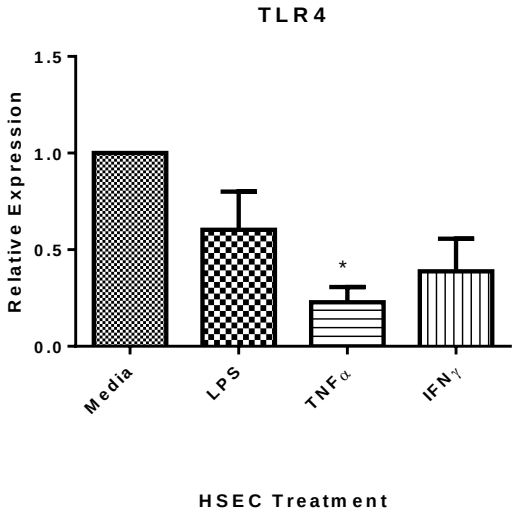
TLR4 expression in HSEC was reduced by stimulation with TNFα, IFNγ and LPS, individually.

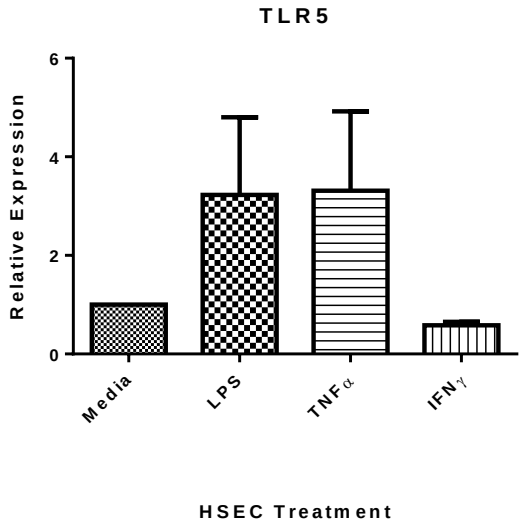
The expression of TOLLIP, a negative regulator of TLR signalling, was unaffected by the presence of inflammatory cytokines (Figure 4-2).

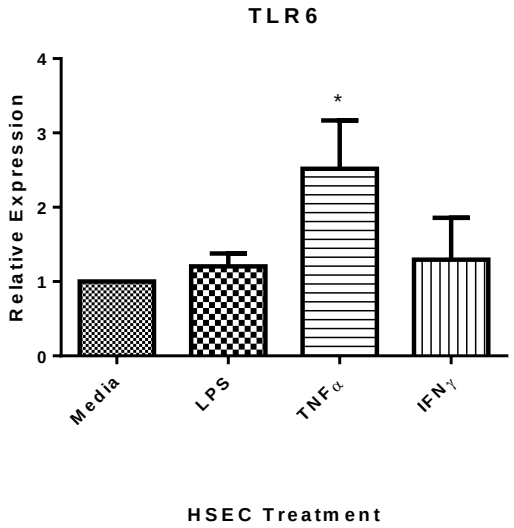

TLR1
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
Media
LPS
TNFα
IFNγ
HSEC Treatment

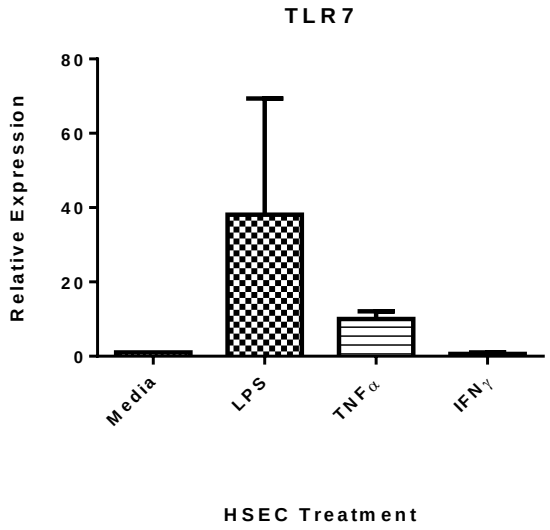

TLR2
Relative Expression
60
40
20
0
*
*
Media
LPS
TNFα
IFNγ
HSEC Treatment

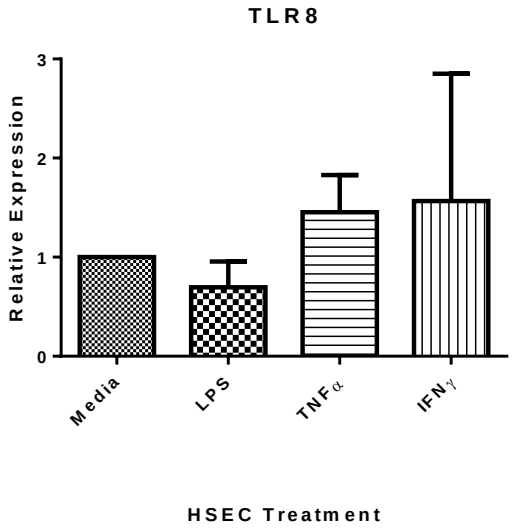

TLR3
Relative Expression
6
4
2
0
*
Media
LPS
TNFα
IFNγ
HSEC Treatment


TLR4
Relative Expression
1.5
1.0
0.5
0.0
*
Media
LPS
TNFα
IFNγ
HSEC Treatment


TLR5
Relative Expression
6
4
2
0
Media
LPS
TNFα
IFNγ
HSEC Treatment


TLR6
Relative Expression
4
3
2
1
0
*
Media
LPS
TNFα
IFNγ
HSEC Treatment


TLR7
Relative Expression
80
60
40
20
0
Media
LPS
TNFα
IFNγ
HSEC Treatment


TLR8
Relative Expression
3
2
1
0
Media
LPS
TNFα
IFNγ
HSEC Treatment

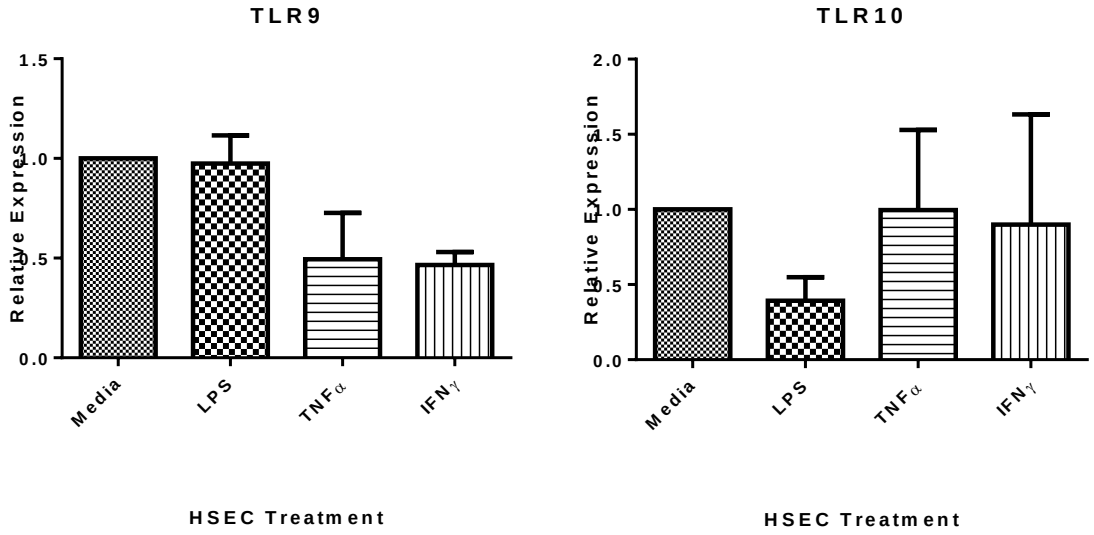
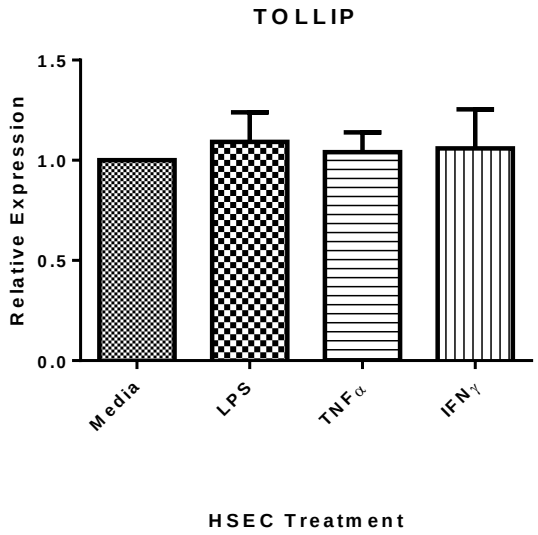



**Figure 4-2. Relative Real Time PCR on cytokine stimulated HSEC.**

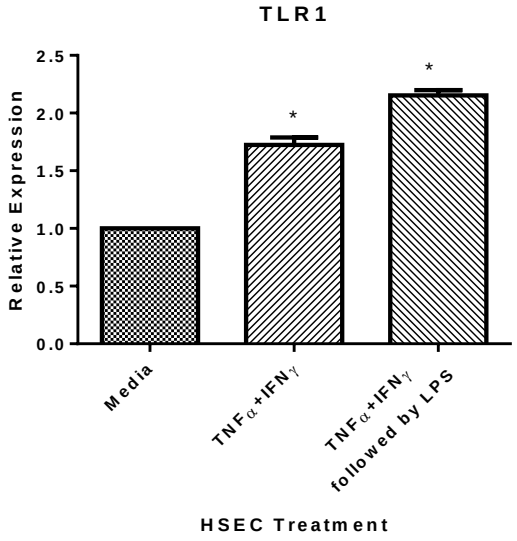


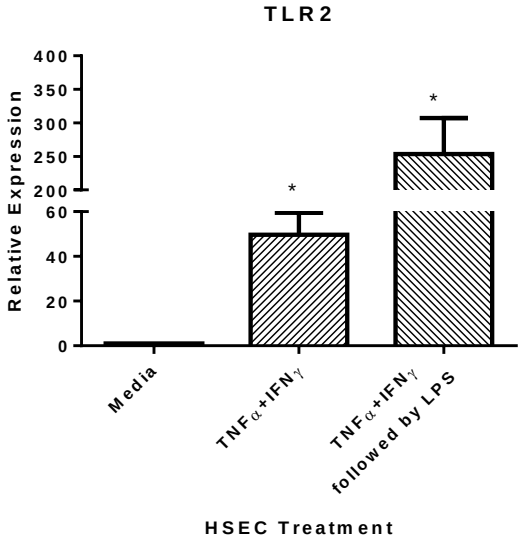

HSEC were stimulated with TNFα, IFNγ, or LPS. Relative Real Time PCR was carried out for mRNA expression of TLRs and TOLLIP using SYBR green and GPADH as the house keeping gene with all performed in triplicate. The relative expression is presented against unstimulated HSEC. The data represents the mean ± SEM of 3 experiments each with a different donor HSEC. *$p<0.05$

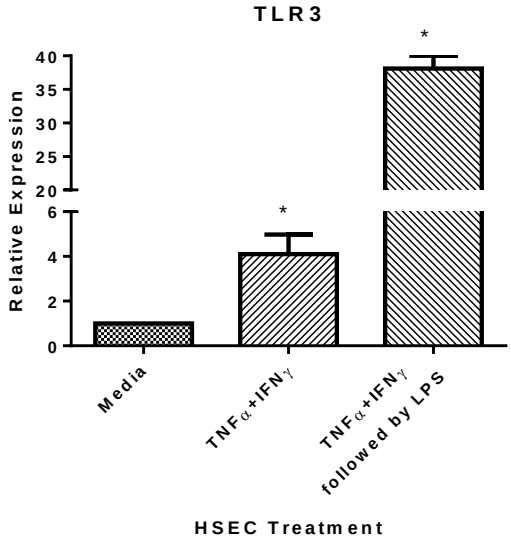
### 4.2.1 Treatment with combination of cytokines has a greater effect on TLR mRNA expression in HSEC

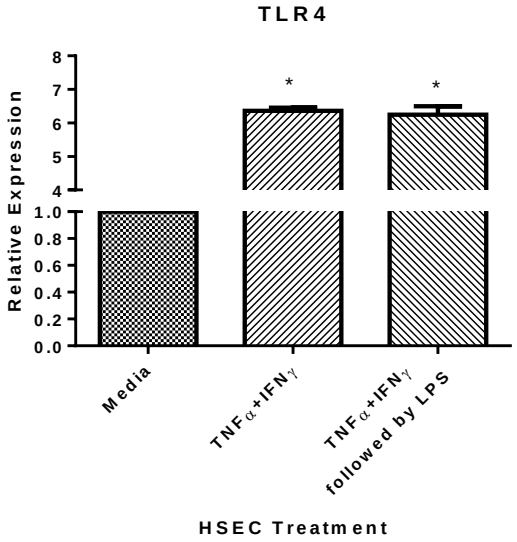
Real time PCR was also performed on HSEC after treating with the combination of TNFα with IFNγ for 24 hours with or without further LPS stimulation afterwards.

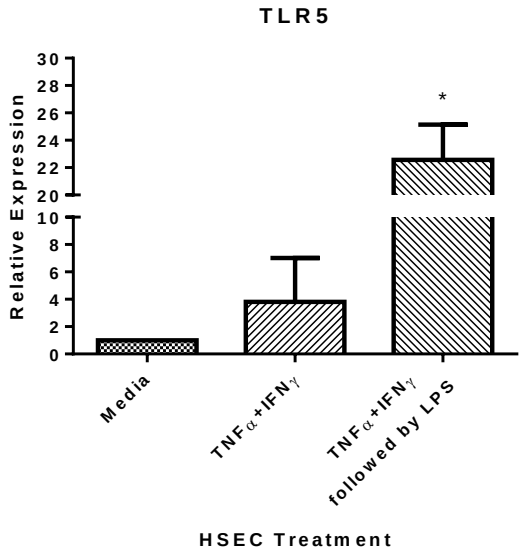
The combination of TNFα with IFNγ resulted in a dramatic rise in the expression of TLR7 compared to the individual cytokines alone. The priming of HSEC with TNFα and IFNγ prior to LPS stimulation caused a large increase in the expression of TLR3 and TLR5 beyond what was seen without the additional LPS stimulation, demonstrating some sort of synergistic effect (Figure 4-3).

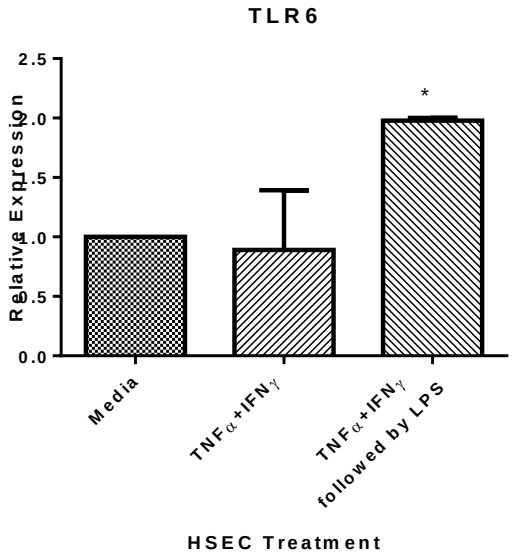

TLR1
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Media
TNFα+IFNγ
TNFα+IFNγ followed by LPS
HSEC Treatment

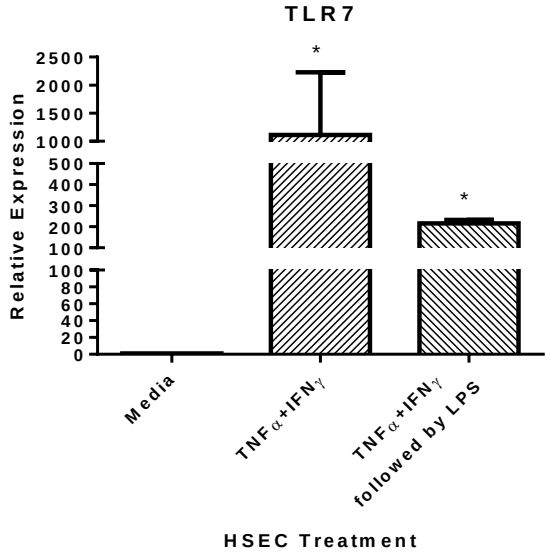

TLR2
Relative Expression
400
350
300
250
200
60
40
20
0
*
*
Media
TNFα+IFNγ
TNFα+IFNγ followed by LPS
HSEC Treatment

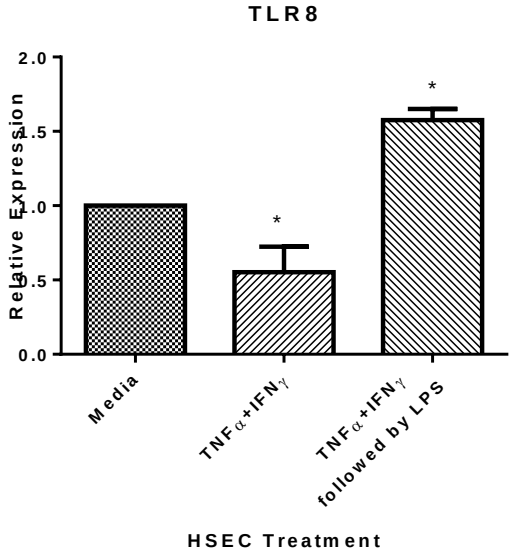

TLR3
Relative Expression
40
35
30
25
20
6
4
2
0
*
*
Media
TNFα+IFNγ
TNFα+IFNγ followed by LPS
HSEC Treatment

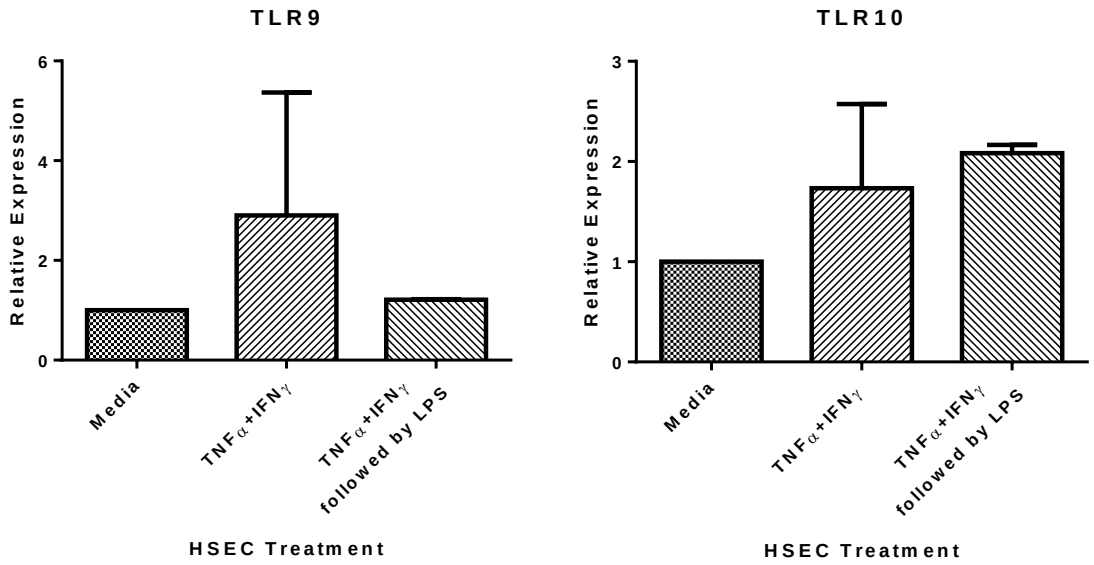

TLR4
Relative Expression
8
7
6
5
4
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Media
TNFα+IFNγ
TNFα+IFNγ followed by LPS
HSEC Treatment


TLR5
Relative Expression
30
28
26
24
22
20
10
8
6
4
2
0
*
Media
TNFα+IFNγ
TNFα+IFNγ followed by LPS
HSEC Treatment


TLR6
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
*
Media
TNFα+IFNγ
TNFα+IFNγ followed by LPS
HSEC Treatment


TLR7
Relative Expression
2500
2000
1500
1000
500
400
300
200
100
100
80
60
40
20
0
*
*
Media
TNFα+IFNγ
TNFα+IFNγ followed by LPS
HSEC Treatment


TLR8
Relative Expression
2.0
1.5
1.0
0.5
0.0
*
*
Media
TNFα+IFNγ
TNFα+IFNγ followed by LPS
HSEC Treatment



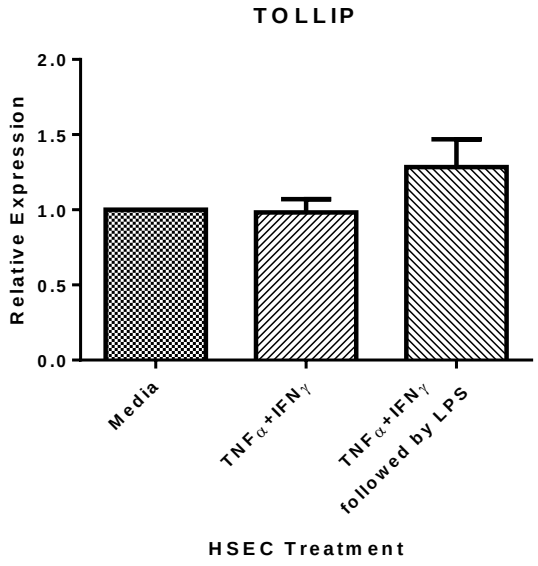



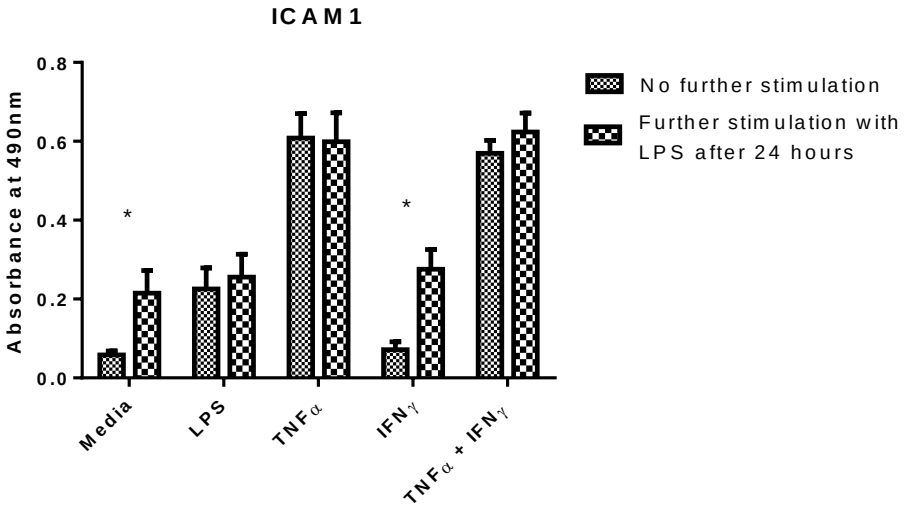

**Figure 4-3. Relative Real Time PCR on primed HSEC in response to LPS.**

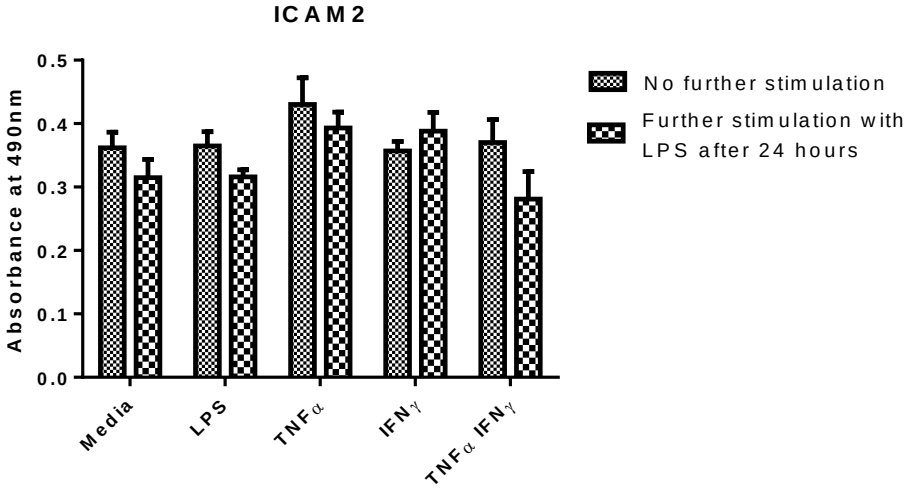
HSEC were stimulated with TNFα and IFNγ, with then media with or without LPS. Relative Real Time PCR was carried out for mRNA expression of TLRs and TOLLIP using SYBR green and GPADH as the house keeping gene with all performed in triplicate. The relative expression is presented against unstimulated HSEC. The data represents the mean ± SEM of 3 experiments each with a different donor HSEC. *$p<0.05$

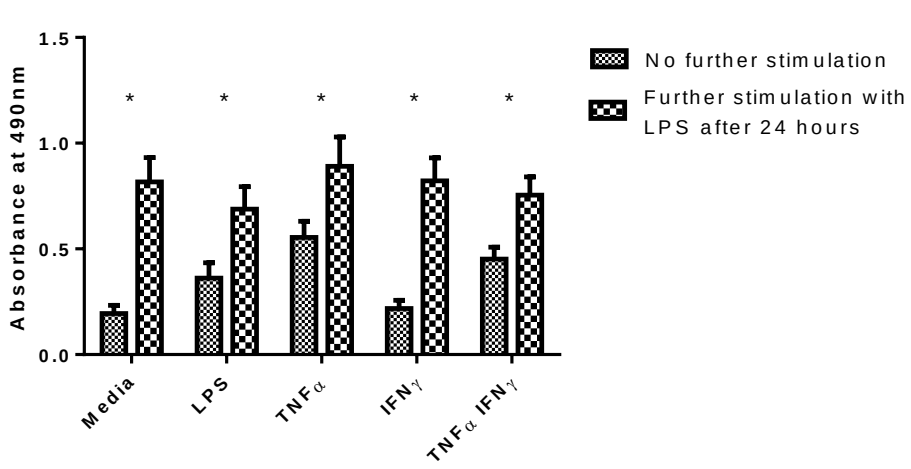
### 4.2.2 Priming HSEC with TNFα and IFNγ prior to LPS stimulation does not alter the expression of classical adhesion molecules

To assess the effect of the inflammatory mediators on adhesion molecule expression, Cell based ELISAs were performed on HSEC that had been stimulated with TNFα, IFNγ or LPS. IFNγ did not alter the expression of any of the adhesion molecules tested. Levels of CD31 were also assessed as a control as previously shown stimulation with inflammatory cytokines showed no increase (19, 148). TNFα and LPS increased the expression of ICAM-1, E-selectin and VCAM-1 but not ICAM-2.

Next we went on to see if priming the HSEC for 24 hours with TNFα, IFNγ and LPS before further stimulation with LPS had an effect upon the adhesion molecules. E-Selectin was increased upon exposure to LPS with any of the priming inflammatory cytokines. Expression ICAM-1 and VCAM-1 was increased on stimulation with LPS, only if the HSEC had been primed with IFNγ. Priming with the TNFα and IFNγ combination did not alter adhesion molecule expression (Figure 4-4).


ICAM1
Absorbance at 490nm
0.8
0.6
0.4
0.2
0.0
*
*
Media
LPS
TNFα
IFNγ
TNFα + IFNγ
No further stimulation
Further stimulation with LPS after 24 hours


ICAM2
Absorbance at 490nm
0.5
0.4
0.3
0.2
0.1
0.0
Media
LPS
TNFα
IFNγ
TNFα IFNγ
No further stimulation
Further stimulation with LPS after 24 hours


E-Selectin
Absorbance at 490nm
1.5
1.0
0.5
0.0
*
*
*
*
*
Media
LPS
TNFα
IFNγ
TNFα IFNγ
No further stimulation
Further stimulation with LPS after 24 hours

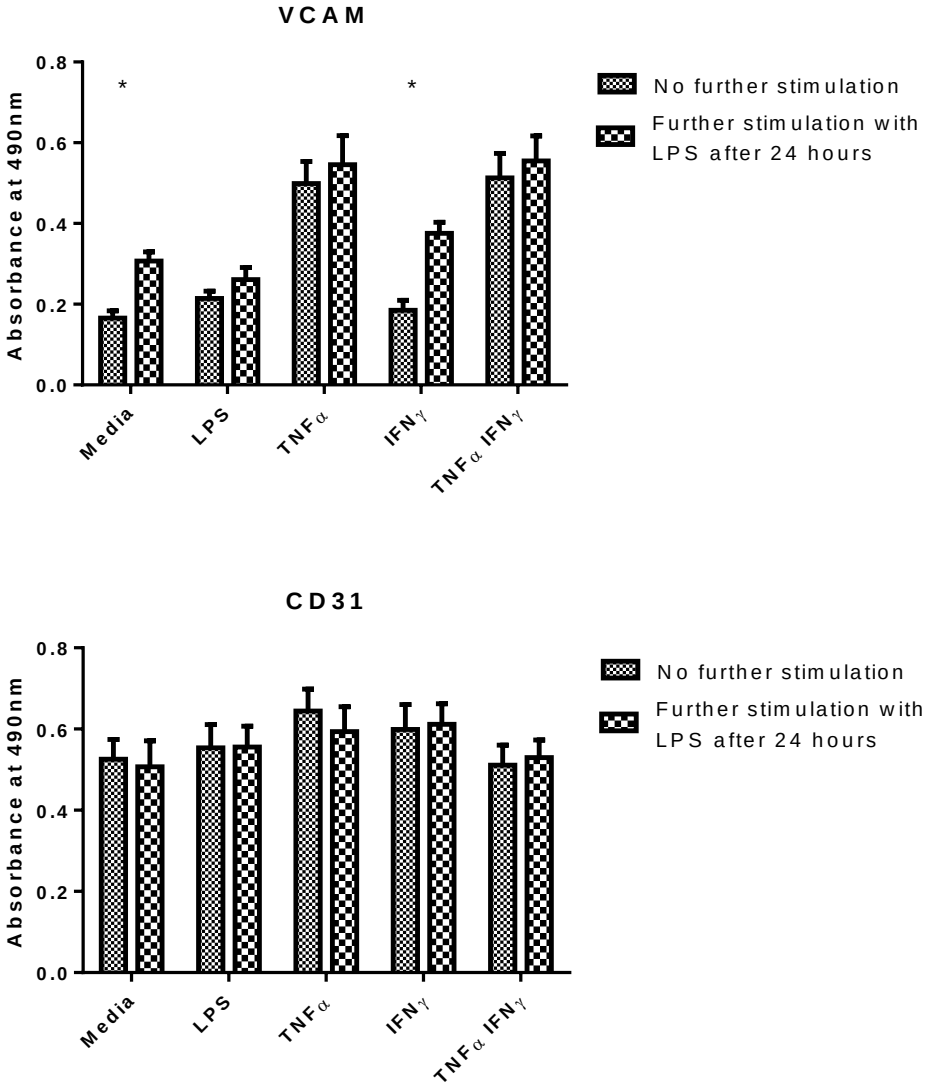



**Figure 4-4. Expression of adhesion molecules on HSEC in response to LPS following priming.**

HSEC were treated with basal media alone or supplemented with LPS, TNFα and/or IFNγ. They were then stimulated with LPS. Adhesion molecules were assessed by cell based ELISA. Data represents mean ± SEM of the absorbance of three experiments on different batches of HSEC performed in triplicate. *$p<0.05$

### 4.2.3 HSEC release distinctly different chemokine signals in response to different proinflammatory stimuli

Chemokines, are a family of small secreted molecules, that are involved in a wide variety of immune functions including leukocyte trafficking and activation. Chemokines are classically considered chemo-attractant molecules. In the previous experiments, different proinflammatory chemokines demonstrated different effects on flowing monocytes across HSEC, namely in response to LPS monocytes became adherent but did not transmigrate whilst they did migrate across HSEC in response to TNFα. To further assess the differential migration of monocytes across hepatic sinusoidal endothelial cells in response to proinflammatory stimuli, the chemokines released by the HSEC was assessed following proinflammatory stimulation with LPS, TNFα and IFNγ. In the previous flow studies IFNγ did not produce any significant adhesion of monocytes in flow compared to TNFα and LPS. The factors released was analysed using a cytokine proteome profiler array (Figure 4-5).

A number of chemokines were raised in TNF and LPS treated HSEC compared to HSEC treated with IFNγ or media only. The chemokines that were raised were in TNFα and LPS stimulated HSEC compared to IFNγ or media treated HSEC included:

ENA 78 (CXCL5)

Fractalkine (CX3CL1)

GROα (CXCL1)

I-309

MIP-3α

MCP-1

GROα and MCP-1 have well described roles in the recruitment of monocytes, and both are upregulated on inflamed endothelium. GROα is immobilised by heparin proteoglycans where as MCP-1 is released and present predominantly in a soluble form. GROα has previously been shown to be involved in the arrest of monocytes from flow acting on CXCR2 on monocytes. MCP-1 acting on CCR2 and CXCR2 (184) present on monocytes has also been shown to be able to cause arrest of flowing monocytes on vascular endothelium (185) and support spreading and migration (184). Elevated levels of MCP-1 have been found in hepatitis with an associated accumulation of an inflammatory infiltrate (186). Fractalkine has been shown to be upregulated on other endothelium in response to inflammatory cytokines again with the ability to capture monocytes (187). MIP3α (CCL20) that acts through CCR6 on monocytes has also been revealed to be chemoattractant to monocytes (188).

Epithelial cell-derived neutrophil-activating peptide 78 (ENA-78), a CXC chemokine, CXCL5 is usually not considered a monocytes chemoattractant, however upon citrullination it does acquire the ability to recruit monocytes (189).

CCR8 a receptor on monocytes (190) to the ligandI309 (CCL1) has been shown to be important in the accumulation of monocytes and subsequently macrophages into injured livers (191).

Though no single chemokine was markedly raised in TNFα treated HSEC compared to treatment with LPS there were a number raised with LPS treatment over the levels seen with TNFα. Those raised in LPS more than TNFα include:

ENA 78

Eotaxin-3

I-309

IL-16

IP-10 chemotactic for monocytes (192)

MIP-3β

TARC

(VCC-1)

MCP-3

MIP-3α

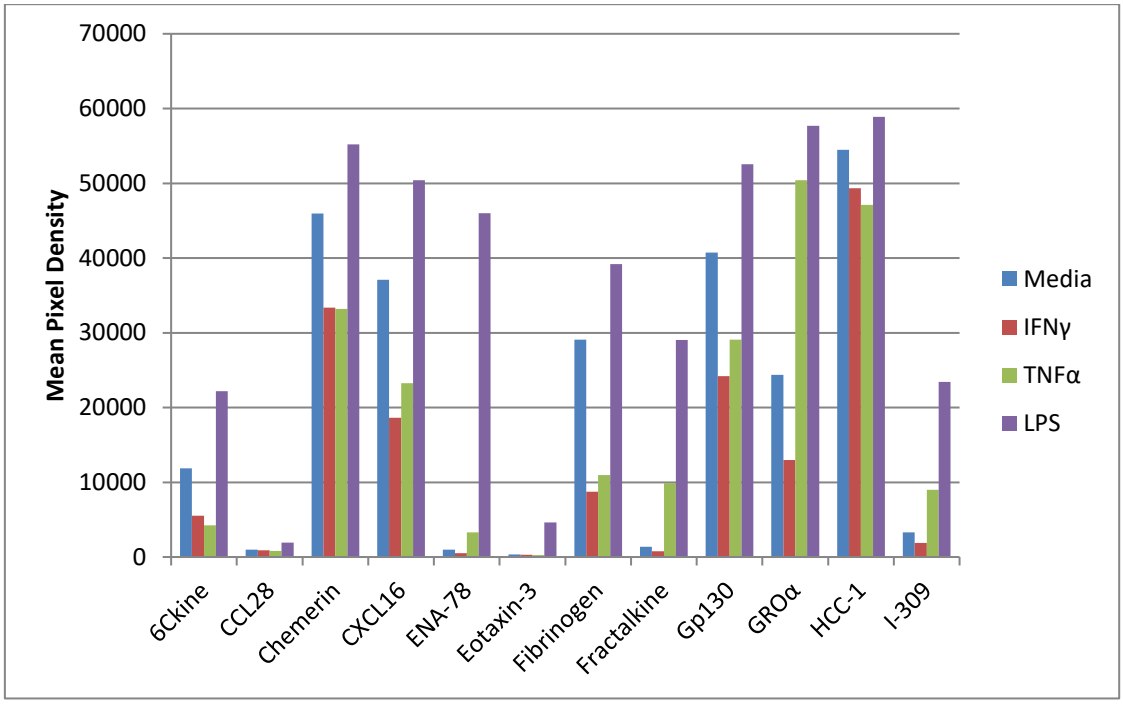
Lymphotaxin

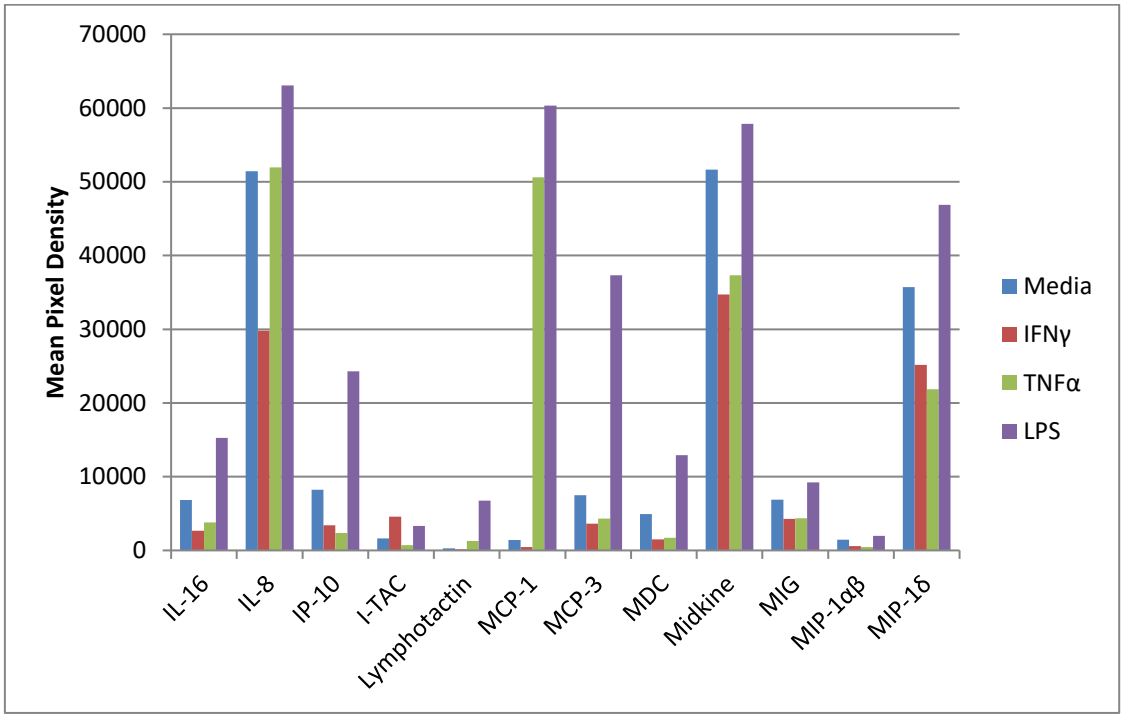
MDC (CCL22)

6Ckine

TARC/CCL17 is a ligand for CCR4 that is found on T-cells selectively and not usually on B-cells, natural killer cells or monocytes (193). However in certain inflammatory conditions, such as rheumatoid arthritis, monocytes upregulate the expression of CCR4 (194). IL16 and 6Ckine have been demonstrated not to be chemotactic for monocytes (195, 196).

Of particular interest is eotaxin-3/CCL26 which is a member of the CC chemokines and is a chemoattractant through CCR3 on eosinophils, basophils and Th2 Lymphocytes. A feature of eotaxin-3 is its ability to also bind to CCR2 on monocytes however with differential affects to other CCR2 agonists such as monocyte chemotactic protein 1 (MCP-1/CCL2). Eotaxin-3 acts as an antagonist for CCR2 inhibiting the typical MCP-1 response of intracellular calcium mobilization and activation of mitogen-activated protein (MAP) kinase ERK. Furthermore in vitro studies show that monocytes move away from eotaxin-3 gradients which is amplified by the presence of MCP-1 gradients (197).


Mean Pixel Density
70000
60000
50000
40000
30000
20000
10000
0
6Ckine
CCL28
Chemerin
CXCL16
ENA-78
Eotaxin-3
Fibrinogen
Fractalkine
Gp130
GROα
HCC-1
I-309
Media
IFNγ
TNFα
LPS


Mean Pixel Density
70000
60000
50000
40000
30000
20000
10000
0
IL-16
IL-8
IP-10
I-TAC
Lymphotactin
MCP-1
MCP-3
MDC
Midkine
MIG
MIP-1αβ
MIP-1δ
Media
IFNγ
TNFα
LPS

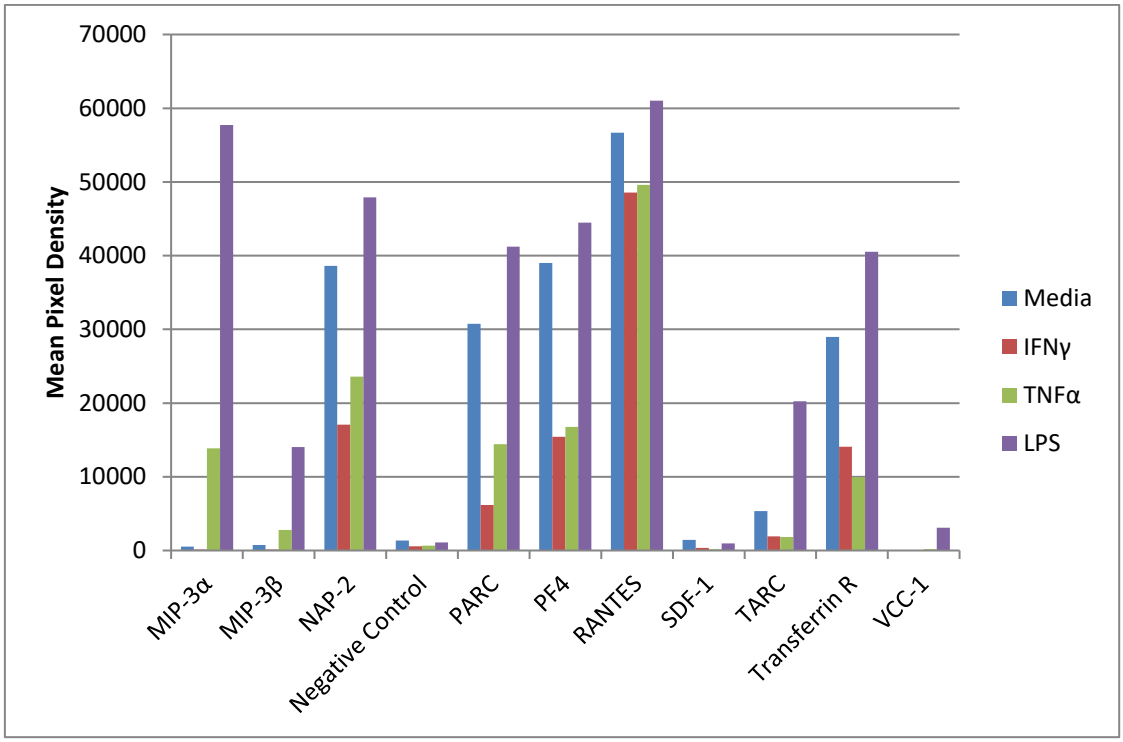



**Figure 4-5. Proteome profiler array of cytokines produced by HSEC.**

HSEC were cultured in media alone or with IFNγ, TNFα or LPS. Supernatants were collected and tested on the proteome profiler array in duplicate. The mean pixel density was calculated using Image J software.

## 4.3 **Discussion**

Toll like receptors are implicated in many chronic liver diseases which are characterised by chronic inflammation. There are a number of mechanisms in the liver that prevent excessive inflammation from perpetuation of TLR signalling. These include:

- Cross tolerance between TLRs altering the expression or activation state of downstream signals (198)
- Local cytokines such as IL-10 are able to modulate TLR pathways (199)
- The expression of TLRs or its downstream molecules compared to other organs is reduced (200)
- Increased expression of negative regulators of TLR signalling (97)

In this chapter, we have looked at immunohistochemical staining of TLRs in liver sections from normal and diseased livers as the expression reported in the literature varies. Murine studies have shown expression of TLR1-9 at the messenger RNA level and at the protein level with generally lower levels of TLR5 compared to the other TLRs (201) (147). Studies on human liver tissue have reported inconsistent TLR expression amongst the cell types of the liver (Table 4-1).

| | Szabo, *Hepatology* 2006 (200) | Mencin, *Gut* 2009 (202) | Testro, *J Gastro Hep* 2009 (121) | Wu, *Immunology* 2010 (201) |
|---|---|---|---|---|
| Hepatocytes | TLR1-9 | TLR2 and 4 | TLR2-5 | |
| Kupffer | TLR2 and 4 | TLR2, 3 and 9 | TLR2-4 and 9 | TLR2, 7 and 9 |
| Biliary Epithelium | TLR2,3,4 and 5 | TLR1-10 | TLR2-5 | |
| Stellate cells | TLR2,3 and 4 | | TLR4 and 9 | |
| Endothelial Cells | | TLR1-9 | TLR4 | TLR1-4 and 6-8 |

**Table 4-1. Literature reported liver TLR expression**

In liver tissue from normal donors the levels of TLRs are lower compared to that of other organs (203) adding to the tolerogenic hepatic environment. In samples of normal liver tissue, we did not see any staining. However, in samples from diseased livers we have shown there is an increase immunohistochemical staining of cell surface expressed TLRs. This was the case for diseased livers from PBC, PSC, ALD or AIH. Furthermore, the expression of intracellularly expressed TLR9 was also increased in liver disease. This fits with animal studies were TLR2, 4 and 9 have been demonstrated to be increased by liver injury (204). In the previous chapter we showed that cell surface TLRs could respond to ligands to increase the recruitment of monocytes, this was not the case for TLR9. Care however is needed, as mentioned earlier TLR immunohistochemistry has a history of being unreliable.

We then went on to focus on HSEC expression of TLRs by looking at mRNA expression and in particular to see the role of stimulation by inflammatory cytokines and LPS. LPS did

not alter the expression of all the TLRs in HSEC but did increase TLR2 expression and decrease that of TLR4, suggesting some cross-talk. Though the other TLRs were not altered that is the possibility there are still functional consequences through downstream signalling pathways which are shared by the different TLRs. Alternatively there may be inhibition of forming responsive heterodimers. TOLLIP a negative regulator of TLR signalling was not altered in these experiments.

Interestingly the combination of TNFα and IFNγ caused a large rise in TLRs 4 and 7 than seen with treatment by these cytokines individually. There was a dramatic increase in expression of TLR2, 3 and 5 on HSEC primed initially with TNFα and IFNγ followed by LPS, showing a marked synergistic effect of these mediators of inflammation. The synergy between TNFα and IFNγ is well appreciated (205, 206). TLR2 usually functions as a dimer with TLR1 or 6 but there was no increase in these. The role of TLR3 and the possible reason for such an increase if translated to a protein level, is interesting. TLR3 though in out studies did not show increased recruitment of monocytes in animal studies have shown the recruitment of NK cells and so could be a mechanism by which there is coordination of different cell types recruited. TLR3 is also reported to inhibit liver fibrosis by stellate cells (207). TLR5 activation in animal models of liver disease also show a recruitment of NK cells different to what is seen with other cell surface TLR agonists (208). TLR5 expression on hepatocytes appears to provide some protective effect in fatty liver disease mice models (107). It may be that TLR5 expression on HSEC is also a protective response.

In this chapter, we also looked for reasons other than altered TLR expression to explain the differences seen in the migratory ability of monocytes depending on HSEC stimulation. Adhesion molecules have functions beyond simply mediating leukocyte adhesion to endothelial in the adhesion cascade. Adhesion molecules have the ability to induce signals

that influence migration (209). ICAM-1 and VCAM-1 interact with CD11a/CD18, CD11b/CD18 and CD49a/CD29 to create "docking structures" involved in migration of monocytes (210) (211). Another CD11a/CD18 ligand is ICAM-2 that is expressed on endothelial cells. There is some evidence with neutrophils that ICAM-2 also supports monocyte migration across HUVEC (56, 212).

Migration of monocytes is a highly co-ordinated event involving multiple signals and interactions between HSEC and monocytes. Studies investigating exogenous chemotactic signals on adherent neutrophils to HUVEC have shown that they can disrupt the transmigration process without preventing adhesion and accumulation (213). Using ELISA of classical adhesion molecules we were unable to note any differences that may explain the observations from the previous chapter.

E-selectin expression on HSEC was increased by LPS and beyond levels seen by pre-treatment with TNFα together with IFNγ. E-selectin is known to be expressed *in vitro* by HSEC but not *in vivo* in normal livers but induced in diseased livers(214).

ICAM-1 and VCAM-1 were upregulated upon LPS stimulation on HSEC. Consistent with findings in mice liver sinusoidal endothelial cells (111). TNFα pre-treatment also was able to tolerise HSEC to further increase in ICAM-1 expression on exposure to LPS but IFNγ was unable to provide cross-tolerance. Pre-treated HSEC also failed to show any increase in VCAM-1 expression except if pre-treated with IFNγ alone.

The constitutively expressed ICAM-2 on *in vitro* HSEC is not affected by stimulation with LPS nor by pre-treatment with TNFα, IFNγ or both in combination.

The maximal time course expression of ICAM-1 and VCAM-1 varies depending on whether the stimuli is shear stress or an alternative stimulus such as LPS. In HUVEC, ICAM-1 is

maximally induced by LPS after 18 hours. Shear stress on the other hand maximally induces the expression of ICAM-1 within 12 hours which then falls back to basal levels within 24 hours (215). Our experiments were carried over time courses of 24 hours and thus some of the effects that occur may be lost by the time the assays were performed.

Also the cell based ELISAs were performed under static conditions. However, shear stress can alter the expression of ICAM-1 and VCAM-1(151).

We went on to use a proteome profiler assay to see whether other factors that are differentially regulated between TNFα and LPS stimulation that may explain why despite both being potent factors in causing adhesion of monocytes to HSEC out of flow only TNFα stimulation results in a significant proportion of monocytes transmigrating. The importance of this accumulation and subsequent failure of migration on LPS stimulated HSEC could have implication in sepsis. The accumulation may result in a number of possible outcomes that include disruption of vascular flow, possible activation and degranulation of toxic mediators into the vascular system.

TNFα stimulated HSEC in this assay did not release any soluble chemokines at levels above that were produced by LPS stimulation to account for the increased transmigration seen in the flow assays performed in the TNFα stimulated HSEC. Other factors that may be at play include immobilisation of the chemokines on activated endothelium, which this assay did not assess. For example MCP-1 is not secreted by endothelium in a polarised manner thus under conditions of flow the concentration at the luminal side is diminished thus establishing a gradient for directing the migration of monocytes (216).

Studies with HUVEC have shown in particular ENA-78 or GRO-α have the ability to prevent efficient transmigration across endothelial monolayers (213). In the studies

performed here ENA-78 was markedly increased on LPS treated HSEC and may be of significance in partly explaining the differences we have seen in transmigration with monocytes.

# Chapter 5

## The Modulation of Monocyte Recruitment on HSEC by Fatty Acids

## 5.1 Introduction

For the purpose of the studies carried out in this work, the two fatty acids used were oleic and palmitic acid. Oleic acid is an example of an (mono) unsaturated fatty acid that occurs naturally in animals and vegetables including olives as an ester. It is found in higher concentrations in Mediterranean diets that are rich in olive oil. Numerous studies have demonstrated its health benefit. Oleic acid appears to result in greater steatosis in hepatocytes but this triglyceride accumulation appears to be a protective mechanism from the lipotoxic effects of free fatty acids (217). Hence oleic acid was chosen in these studies to see if the beneficial effects ascribed to it would be evident with an *in vitro* model of hepatic sinusoids.

Palmitic acid is the most common naturally occurring saturated fatty acid and is found in dairy and meat products. It has been ascribed detrimental effects in cardiovascular and liver disease (218, 219) through creating a proinflammatory state, and thus was used as an example of a saturated fatty acid.

The aim of this chapter was to see if the fatty acids chosen would alter the recruitment of monocytes to HSEC from flow and if there would be a difference between the two fatty acids.

## 5.2 Results

### 5.2.1 HSEC viability was not reduced by culturing with palmitic acid or oleic acid

The most abundant free fatty acids in humans are oleic and palmitic acid, and thus were selected for the following experiments (220). The reported concentration of oleic and palmitic acid varies around 144 to 400 μg/ml (equivalent to 50 to 150 μM) (221) to much higher levels physiologically dependent upon the population (222, 223). Despite the levels of fatty acids that occur physiologically, fatty acids are potentially toxic to cultured cells, a phenomenon termed lipotoxicity. Palmitic acid at concentrations as low as 150μM can negatively affect the viability of cell lines and endothelial cells (224, 225). Thus, to ensure that cultured HSEC could survive dosing with fatty acids, their viability and cell number was assessed prior to forward studies.

Viability was assessed by trypan blue exclusion and cell number by crystal violet blue staining after treating HSEC with 100μM of either oleic or palmitic acid. The presence of supplementary oleic or palmitic acid at 100μM resulted in no alteration in survival of HSEC or their cell numbers (Figure 5-1Figure 5-1. Measurement of HSEC cell number as assessed by crystal violet staining in response to treatment with fatty acids.).

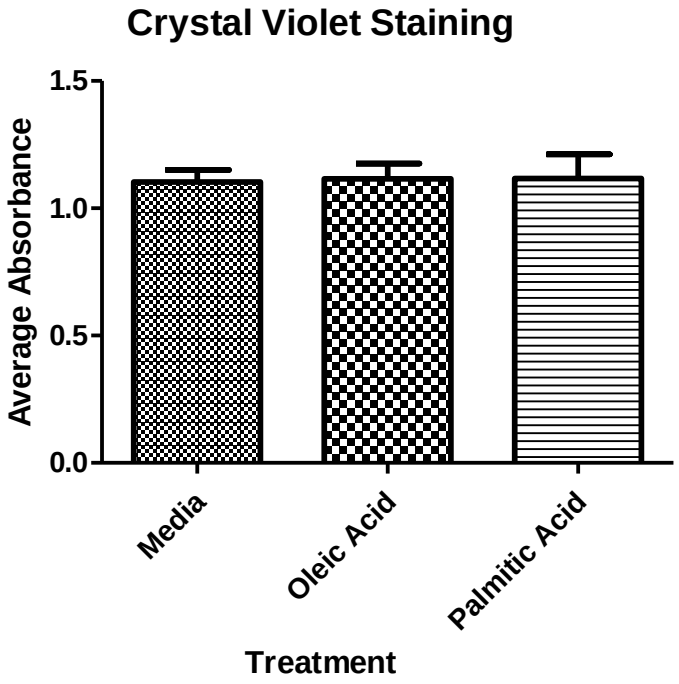



**Figure 5-1. Measurement of HSEC cell number as assessed by crystal violet staining in response to treatment with fatty acids.**

HSEC were treated for 24 hours with 100μM of either palmitic acid or oleic acid. Cell number was then assessed by crystal violet staining. Absorbance was measured on an automated plate reader (Dynax laboratories, MRX). No difference was seen in cell number. Trypan blue staining showed no reduction in cell viability. The data represent the mean ± SEM of 4 experiments

### 5.2.2 HSEC treated with Oleic or Palmitic Acid alone do not alter monocytes recruitment from flow

In static adhesion assays of monocyte adhesion on endothelial cell lines, treatment with fatty acids leads to increased adhesion (226). To see if HSEC could be independently stimulated by fatty acids to modulate recruitment of monocytes out of flow, HSEC were treated for 24 hours with either oleic or palmitic acid prior to flowing freshly isolated peripheral blood monocytes. There was no alteration in adhesion as compared to untreated HSEC (Figure 5-2).

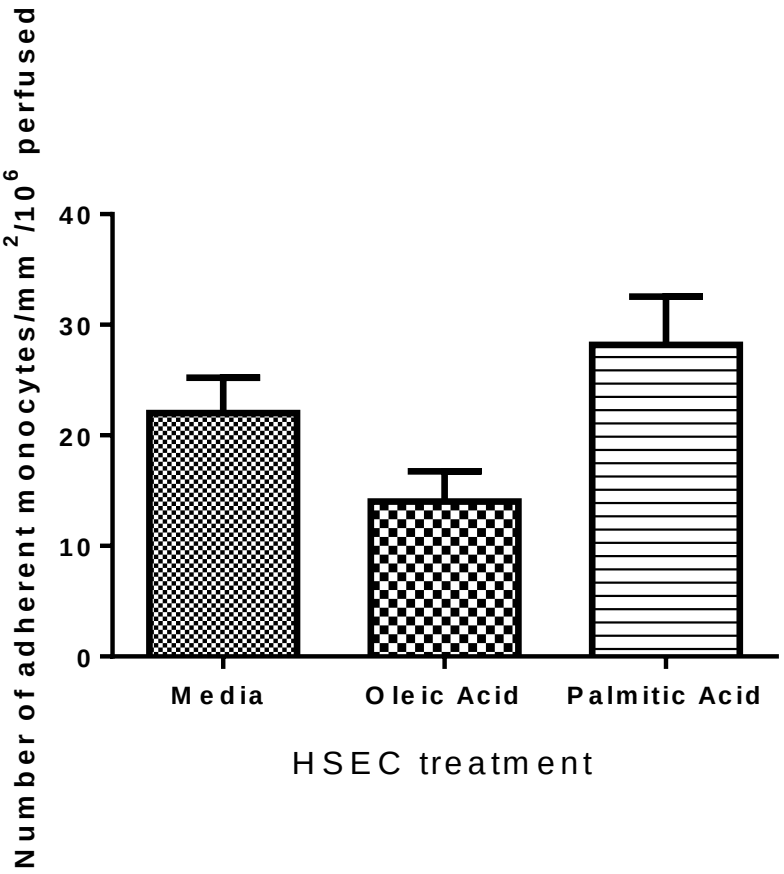



**Figure 5-2. Adhesion of monocytes upon HSEC treated with fatty acids.**

HSEC were stimulated for 24 hours with oleic acid (100μM) or palmitic acid (100μM). Freshly isolated peripheral blood monocytes were then flown across. The experiment was then repeated but with stimulation with LPS (10ng/ml) after pre-treatment with the fatty acids. The data represent the mean ± SEM of 4 experiments. There was no significant difference seen.

### 5.2.3 Pre-treatment of HSEC with Oleic acid prior to stimulation with LPS reduces monocytes adhesion

Though in themselves the fatty acids did not alter monocytes recruitment in flow assays we went on to see if pre-treatment with fatty acids prior to stimulation with LPS on HSEC would have an effect, with LPS being a second insult upon the HSEC. Following exposure of HSEC with media containing free fatty acid supplementation, the media was changed with further media with free fatty acid together with LPS for either 4 hours or 24 hours. HSEC that were pre-treated with oleic acid prior to stimulation with LPS resulted in 45% fewer adherent monocytes whereas palmitic acid treatment did not change significantly the amount of adhering monocytes (Figure 5-3).

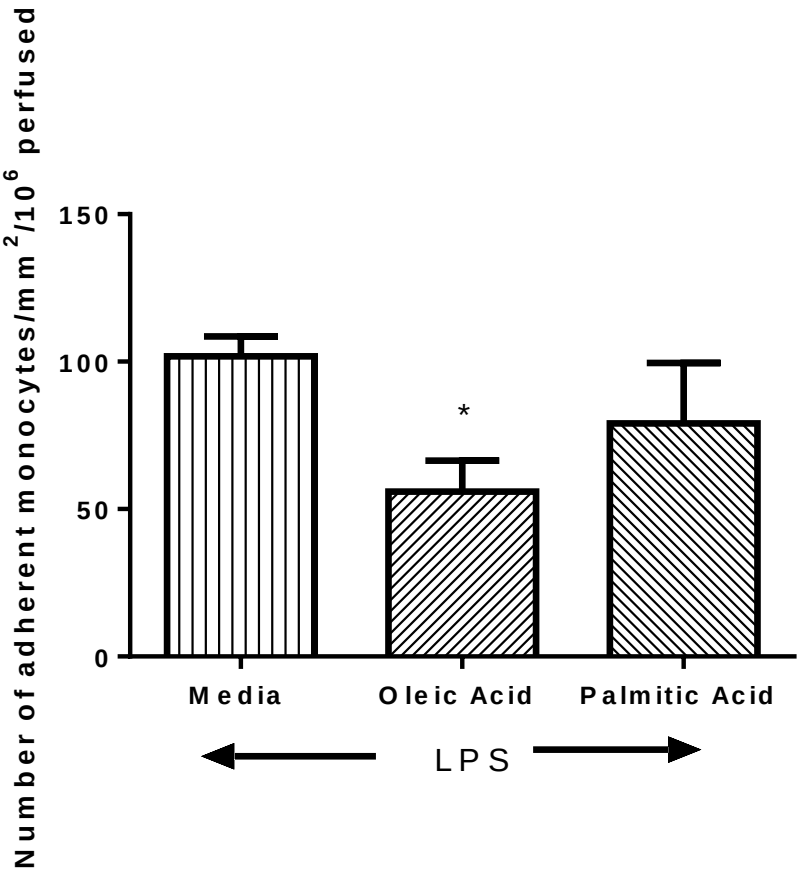



**Figure 5-3. Adhesion of monocytes upon LPS treatment of HSEC pre-treated with fatty acids.**

HSEC were stimulated for 24hours with oleic acid (100μM) or palmitic acid (100μM). They were then stimulated with LPS (10ng/ml) containing media. Freshly isolated peripheral blood monocytes were then flown across. The data represent the mean ± SEM of 4 experiments. *$p<0.05$

### 5.2.4 Repeated exposure to HSEC to LPS was not able to recapitulate the number of monocytes adhering to it

In the previous chapter, we demonstrated that HSEC showed reduced capacity to capture monocytes out of flow after stimulation for 24 hours compared to 4 hours. However further stimuli with LPS after 24 hours could increase adhesion though not back to the levels seen with a single stimulus for 4 hours. We went on to assess what effect the presence of fatty acids would have on this system. Pre-treatment of HSEC with oleic acid suppressed any additional recruitment previously seen with repeated LPS stimulation. Palmitic acid did not alter the pattern previously seen, in that a further bolus of LPS after 24 hours increased adhesion (Figure 5-4).

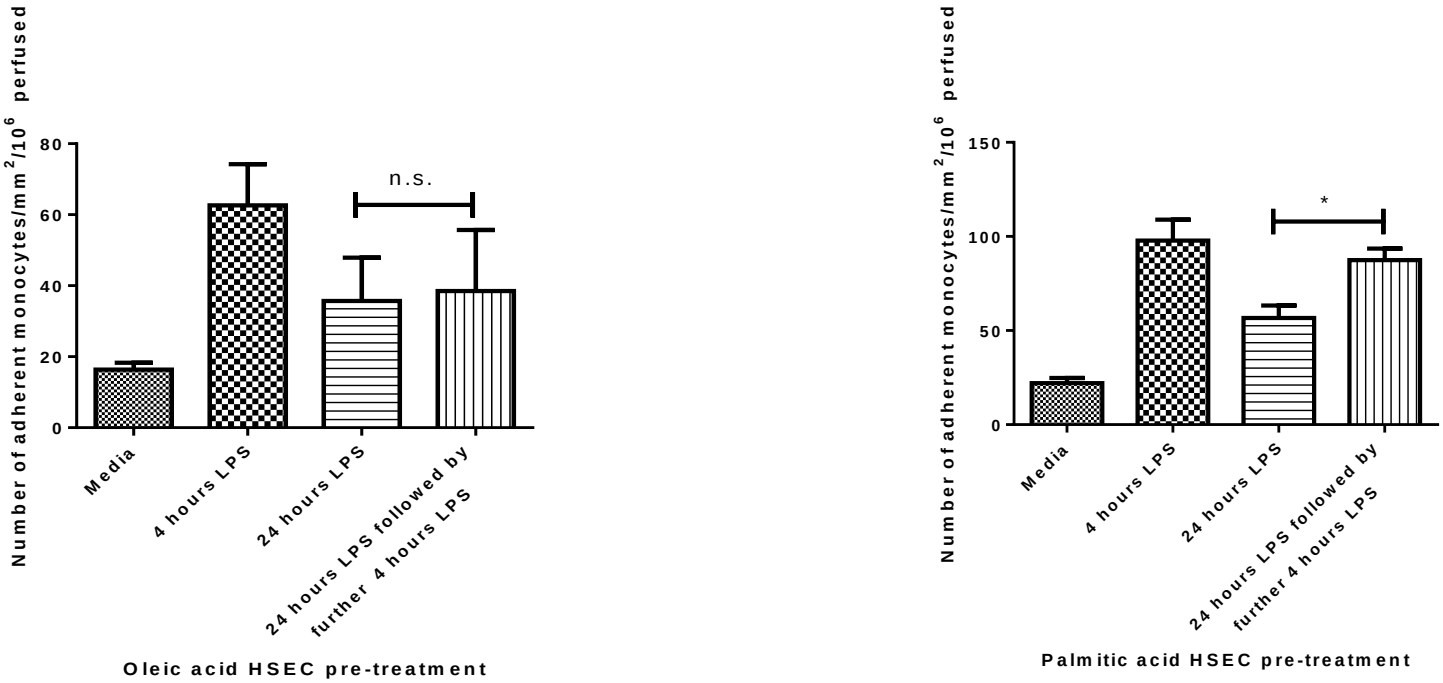



**Figure 5-4. HSEC treated with fatty acids response to repeated exposure to LPS upon recruitment of monocytes.**

HSEC were stimulated for 24 hours with oleic acid or palmitic acid. The HSEC were then treated with LPS for 4 hours, 24 hours or 24 hours with a further treatment with fresh LPS containing media for an additional 4 hours. Freshly isolated peripheral blood monocytes were then flown across. The experiment was then repeated but with stimulation with LPS after pre-treatment with the fatty acids. The data represent the mean ± SEM of 3 experiments. *$p<0.05$

### 5.2.5 Flagellin stimulation of fatty acid treated HSEC show similar effects with regards to monocytes recruitment as LPS

Having seen the effect of oleic acid being able to reduce the adherence of monocytes upon LPS stimulation of HSEC we went on to see if this effect was maintained by flagellin, which signals via TLR5. Oleic acid once again showed an ability to reduce monocytes adhesion (Figure 5-5).

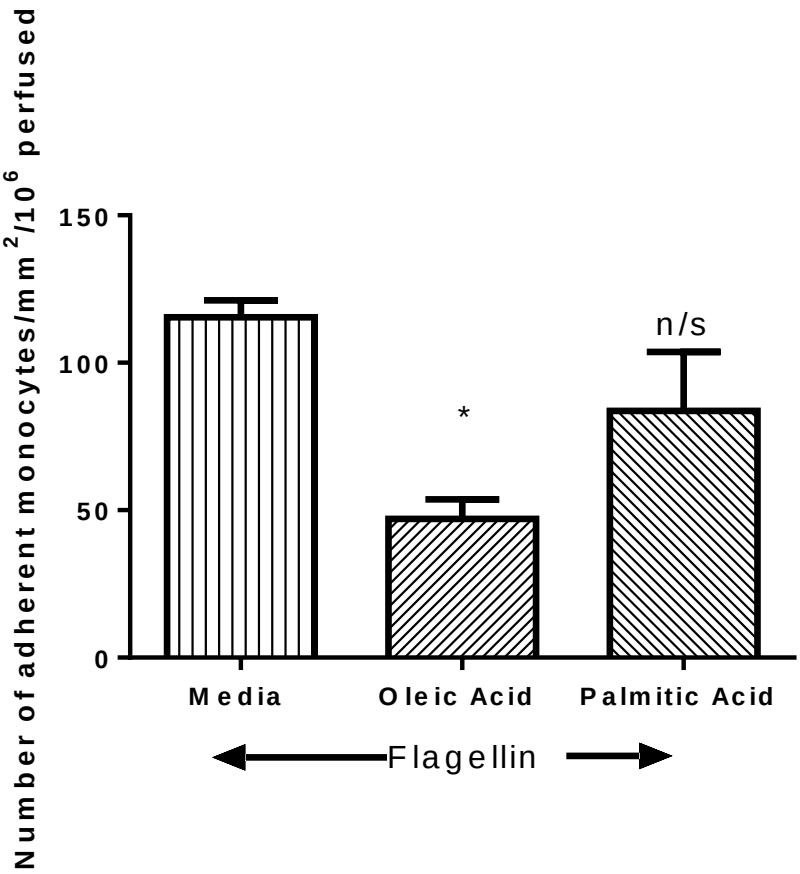



**Figure 5-5. Adhesion of monocytes upon flagellin treatment of HSEC pre-treated with fatty acids.**

HSEC were stimulated for 24 hours with oleic acid (100μM) or palmitic acid (100μM). They were then stimulated with flagellin after pre-treatment with the fatty acids Freshly isolated peripheral blood monocytes were then flown across. The data represent the mean ± SEM of 3 experiments. *$p<0.05$

### 5.2.6 Oleic acid and Palmitic acid do not alter the expression of adhesion molecules on HSEC or alter the ability of LPS to induce them.

To see if fatty acids are modulating monocyte adhesion through alterations in adhesion molecules ELISAs we looked at the adhesion molecules ICAM-1, ICAM-2, VCAM-1 and E-selectin using CD31 as a control. Neither oleic acid or palmitic acid altered the expression of the adhesion molecules tested nor the ability of LPS to induce the expression of ICAM-1, VCAM-1 and E-selectin (Figure 5-6).

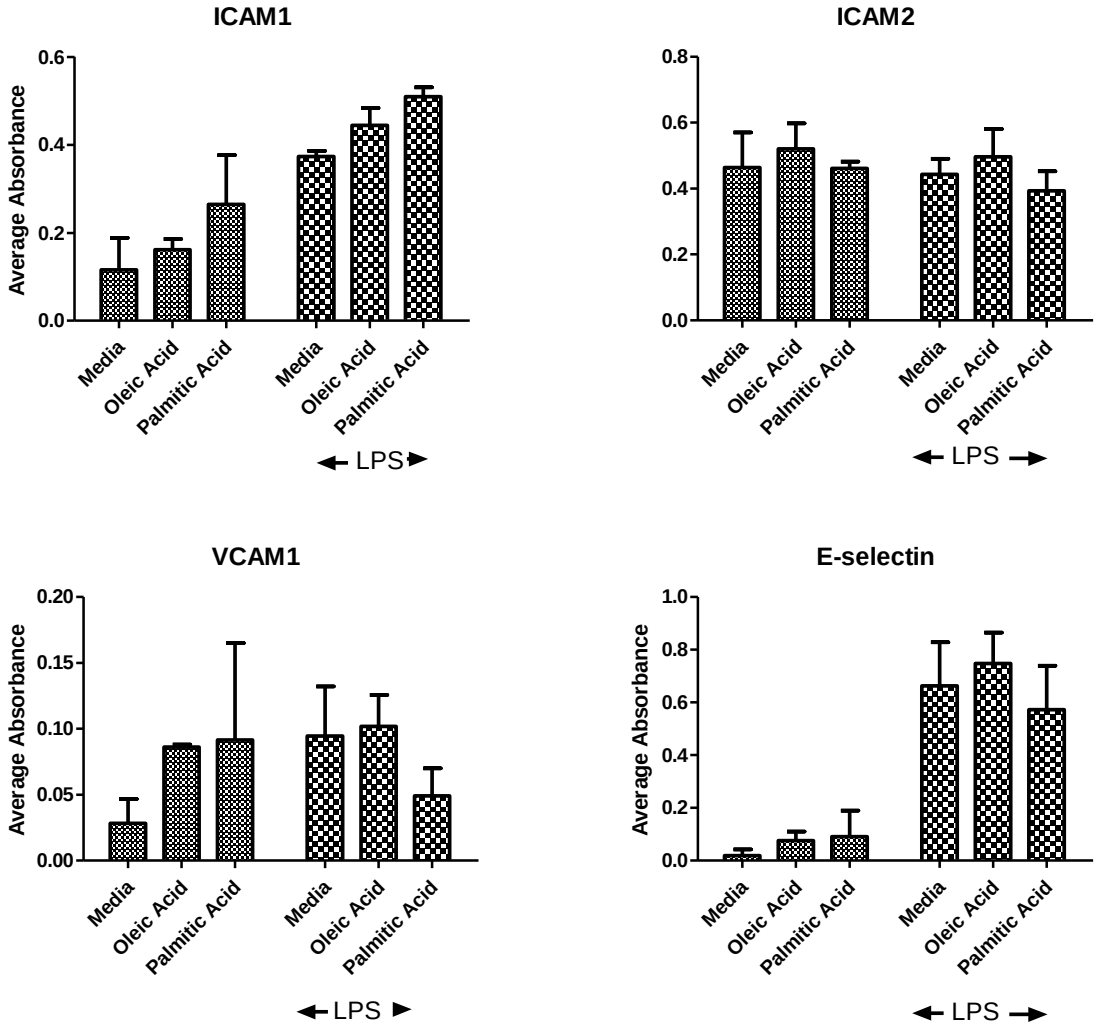



**Figure 5-6. Expression of adhesion molecules on HSEC by ELISA in response to fatty acid treatment.**

HSEC were stimulated for 24 hours with oleic acid (100μM) or palmitic acid (100μM). The HSEC media was then refreshed with or without were LPS (10ng/ml) for a further 24 hours. Absorbance was measured on a plate reader. The data represent the mean ± SEM of 3 experiments.

## 5.3 Discussion

The role of fats in liver disease is complex. The accumulation of fat within the liver does not necessarily result in hepatocellular damage. For example, the accumulation of triglycerides in the liver is not damaging *per se* and provides protection from the effects of some free fatty acids that are deleterious (227).

Free fatty acid levels are related to dietary intake including of triglycerides and release from adipose tissue. The Mediterranean diet rich in olive oil decreases fat accumulation in the liver (230). The benefit of olive oil is related to the high content of mono unsaturated fatty acids principally oleic acid. Mono unsaturated fatty acids decrease serum triglycerides by activating peroxisome proliferator-activated receptor (PPAR)α which increases fatty acid oxidation and inhibits lipogenesis (230). In animal models of liver cirrhosis, dietary modulation with diet rich in oleic acid decreases fibrosis (228). These models have also shown reduced insulin resistance and decreased release of fatty acids from adipose tissue (229).

Generally unsaturated fatty acids such as oleic acid are considered to be anti-inflammatory, suppressing NF-kB signalling whereas saturated fatty acids such as palmitic acid are considered proinflammatory. Importantly fatty acids have been shown to have effects through TLR signalling, and in particular with the cell surface TLRs 2, 6 and 4 which is not unexpected given that these detect molecular patterns in the cell walls of bacteria in particular the lipid components of the cell walls. Palmitic acid has been shown in some systems to be capable of activating TLR signalling pathways that result in a pro-inflammatory state. Not only can fatty acids signalling through the TLR receptors but they

may also prevent dimerisation of the receptors which is required before they can signal and alterations of the lipid rafts of cells. Saturated fatty acids have been shown to activate TLR4, though not directly (231), but possibly through altering receptor numbers (232) or formation of dimmers, increasing inflammatory molecules in adipocytes and macrophages (233). On the other hand, unsaturated fatty acids may limit TLR activation (234). The effects of fatty acids are not limited to TLR4. Dimerisation of TLR2 with either TLR1 or TLR6 to form receptors to detect bacterial products, can be enhanced by saturated fatty acids and inhibited by polyunsaturated fatty acids (235).

Previous published work have demonstrated the modulation of the inflammatory response of fatty acids on endothelial cells (236). It has been shown that different fatty acids depending on whether they are saturated or unsaturated can exert pro- or anti- inflammatory effects through modulation of leukocyte adhesion on human saphenous endothelial cells (237). In this chapter, we looked at role of fatty acids on monocyte adhesion on HSEC. Hepatic sinusoidal endothelial cells are unique with their exposure to portal blood, presence of fenestrations, lack of basement membrane and presence of large number of endocytic vesicles. The composition of the phospholipid layer of HSEC can also be modified by the intake of fatty acids (238). This can have implications for the formation of lipid rafts and presentation of receptors on cell surfaces (239).

Using oleic acid as an example of an unsaturated fatty acid and palmitic acid as a saturated fatty acid at physiological doses HSEC in our studies maintained their viability. The culture of HSEC with either of these fatty acids for 24 hours did not alter the adhesion of monocytes. However only a single concentration was used in these experiments and there is a wide variability in the concentration of these fatty acids in the population. It is possible if different concentrations were used, differences may have become apparent.

Previously the role of endotoxin and fatty acids in the role of liver inflammation and damage has been described through the two hit hypothesis of non-alcoholic steatohepatitis (240). This consists on an initial hit from excessive fatty acids and the accumulation of fat in the liver. This fat accumulation sensitises the liver to further insults that include that of endotoxin. In animal models inhibition of TLR4 signalling in animal models of fatty liver disease accentuates the inflammation (241). Adding to this, fatty livers are poor at clearing LPS thus allowing it to enter into the systemic circulation in greater quantities, having implication for sepsis (242). Peripheral adipose tissue exposure to LPS stimulates further TNFα and free fatty acid release which feedbacks to liver causing more damage. Fatty livers are also more sensitive to the effects of LPS (242).

Flow assays were performed to see if an additional insult in the form of exposure to LPS would modify monocytes recruitment on fatty acid pre-treated HSEC. Only the presence of oleic acid in the HSEC culture medium prior to stimulation with LPS altered the recruitment of monocytes by decreasing the number of adherent monocytes captured from flow. Furthermore, pre-treatment with oleic acid reduced the responsiveness of HSEC that had already been cultured with LPS for 24 hours which were then treated with a further 4 hours of fresh LPS containing media unlike in Chapter 3 were there was an increase when there was no oleic acid treatment. Palmitic acid did not have a significant effect.

Looking at adhesion molecules, studies using HUVEC, preincubation with palmitic acid does not result in altered expression of the adhesion molecule VCAM-1 upon exposure to LPS. Whereas oleic acid preincubation, suppressed expression upon LPS stimulation, but only after prolonged treatment (236). In this study the expression of adhesion molecules on HSEC cultured in fatty acids was not altered. It is possible as seen in other studies that this

was because there was not sufficient time to allow for changes to occur and if a longer time point had been used, changes may have been seen.

The saturated fatty acid palmitic acid has been shown to be pro-inflammatory in different cell lines (235, 239, 243) through the activation of TLR2 and 4 pathways. However, the proinflammatory effects using palmitic acid were not seen in the experiments performed on HSEC in this chapter. HSEC cultured in palmitic acid did not demonstrate increased adhesion molecule expression nor increased monocyte adhesion in flow. Nor was there alteration in adhesion following stimulation with LPS or flagellin.

Although these studies have not shown the pro-inflammatory potential of palmitic acid in this model of monocyte recruitment it does not exclude their inflammatory potential through other mechanisms including potentially recruiting other leukocytes. This study does add to the documented beneficial effects of oleic acid, by reducing, but not abolishing recruitment of monocytes in response to inflammatory stimuli from both TLR4 and 5 agonists.

# Chapter 6

## Phenotypic and Functional Changes of Kupffer Cells in Response to Inflammation and Fatty Acids

## 6.1 Introduction

So far, this thesis has concentrated on the recruitment of monocytes by HSEC. One of the fates of recruited is the differentiation into macrophages. Macrophages are highly plastic cells and depending on environment have different phenotypes with different roles.

The aim of this chapter is to see the if the stimulation of human liver derived macrophages with inflammatory cytokines and fatty acids change the expression of phenotypic markers and TLR expression. We also attempted to build a more representative model of hepatic sinusoids with co-culturing of HSEC with liver derived macrophages.

## 6.2 Phenotype of liver derived macrophages

To determine if human liver derived macrophages retain the plasticity to change phenotype in response to external stimuli they were cultured for 24 hours with media containing LPS, TNFα, IFNγ or IL-4. IL-4 has the ability to result in alternatively activate macrophages. Mannose receptor (CD206) is a marker of M2 alternative activation of macrophages and consistent with IL-4 stimulation resulted in increased expression of CD206 on IL-4 stimulated liver derived macrophages. CD163, a haptoglobin-haemoglobin scavenger receptor, is also a marker of alternative activation. Previous studies have shown that on macrophages derived from human peripheral monocytes express high basal levels of CD163; however exogenous IL-4 stimulation suppresses its expression. Liver derived macrophages treated with IL-4 demonstrated a similar behaviour to monocytes derived macrophages with suppression of CD163 expression (Figure 6-1). TNFα and LPS stimulation also suppress CD163 expression in liver derived macrophages (Figure 6-1) as seen with monocytes derived macrophages (244).

LPS stimulation of liver derived macrophages resulted in upregulation of CD16, a low affinity Fc receptor involved in binding antibodies. CD16 has been shown to have the ability to regulate TRIF dependent TLR4 signalling pathway and enhance the response to gram negative sepsis (245).

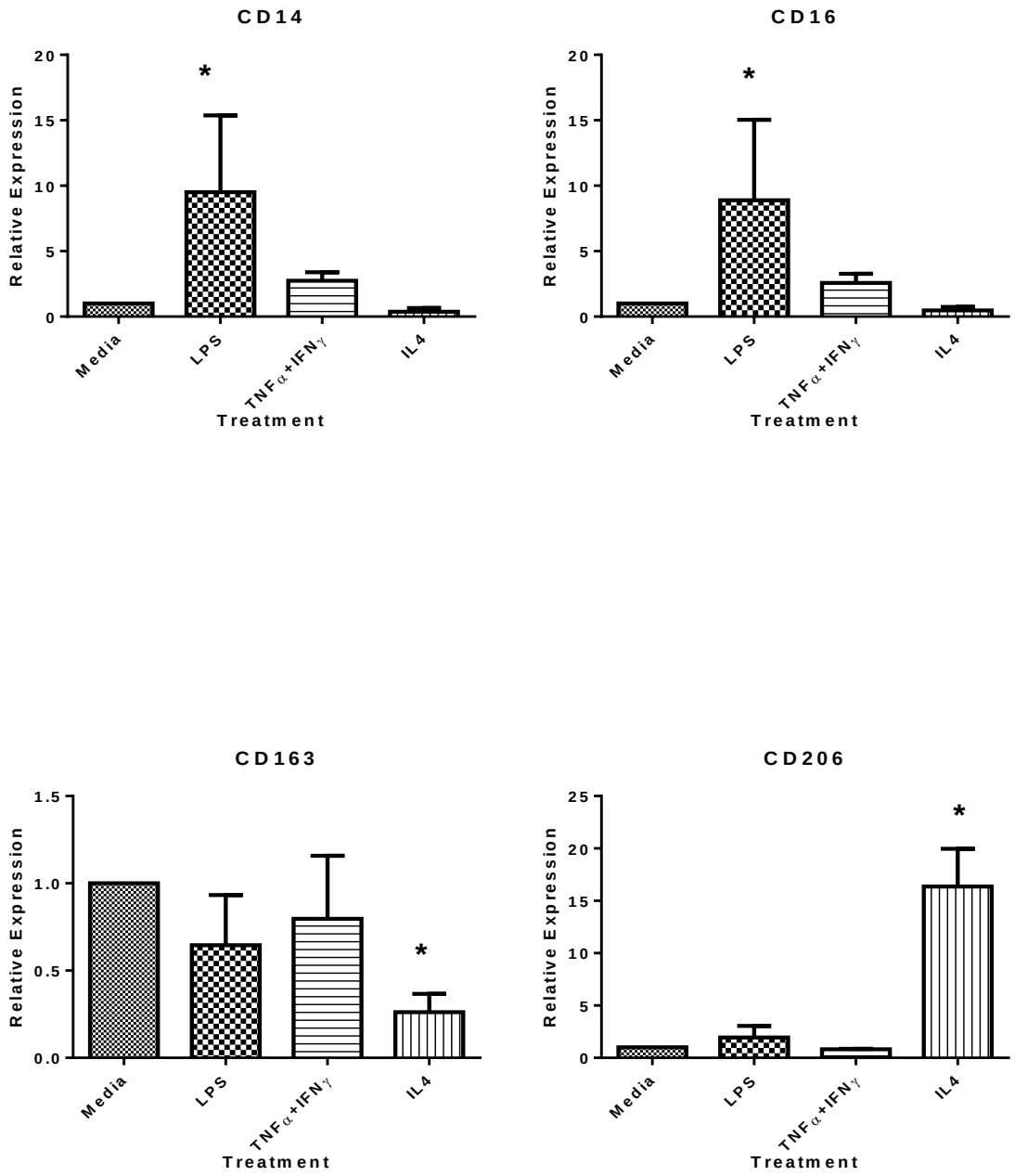



**Figure 6-1. Phenotype of liver derived macrophages in response to external stimuli.**

Liver derived macrophages were treated with either LPS, TNFα, IFNγ or IL-4. Phenotype markers were assessed by relative real time PCR and compared to untreated liver derived macrophages. The results represent the mean ± SEM of three experiments with different donors performed in triplicate. *$p<0.05$

### 6.2.1 Oleic acid is able to increase CD206 expression on liver derived macrophages, a marker of alternative activation

Kupffer cells are involved in lipid metabolism. Alternatively activated Kupffer cells are postulated to have a beneficial effect on metabolic syndrome and type II diabetes (246). Thus to see if human liver derived macrophages would respond to fatty acids they were treated with palmitic or oleic acid for 24 hours. Oleic acid was able to up regulate CD206 consistent with taking on a more alternatively activated phenotype (Figure 6-2).

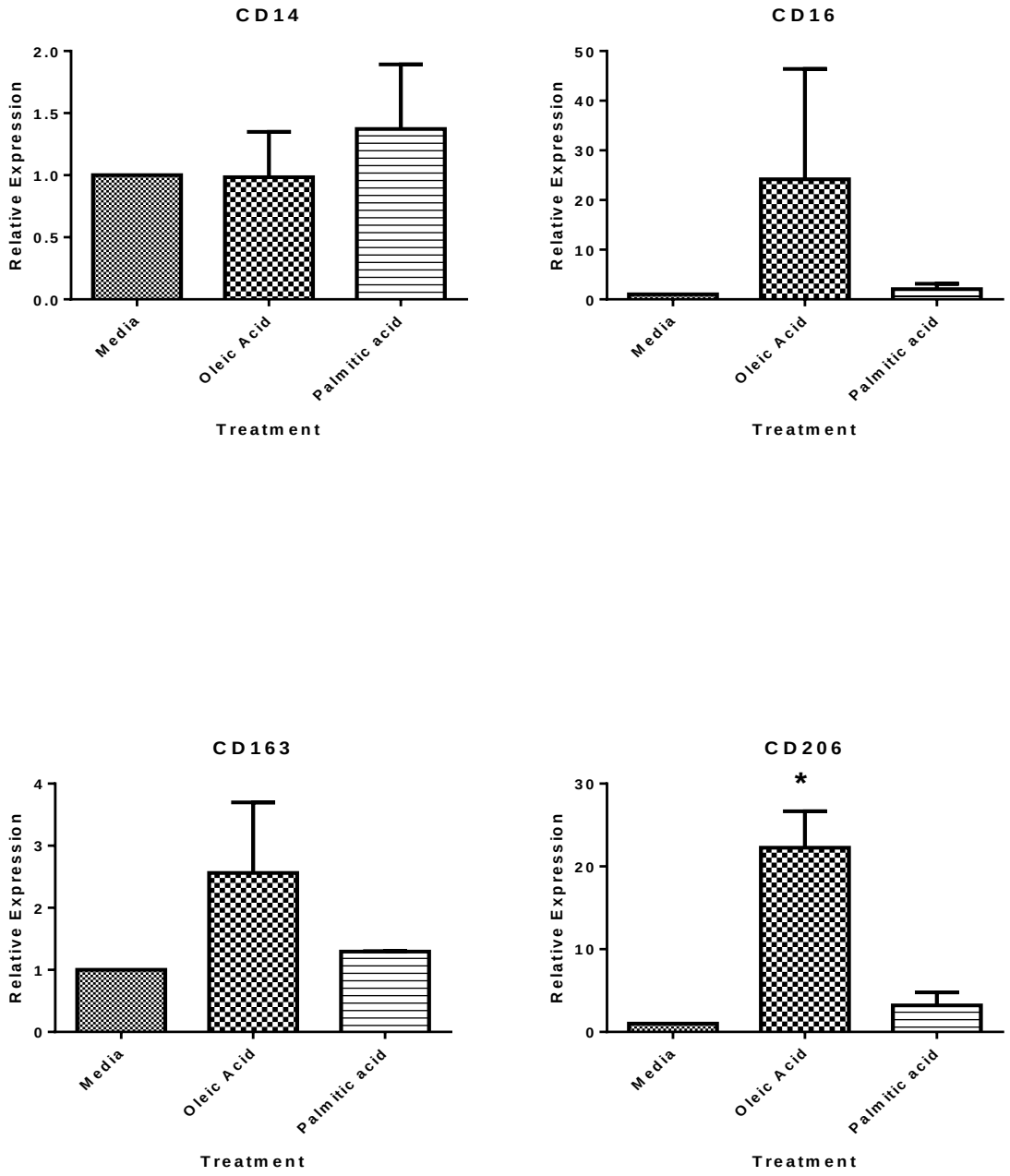
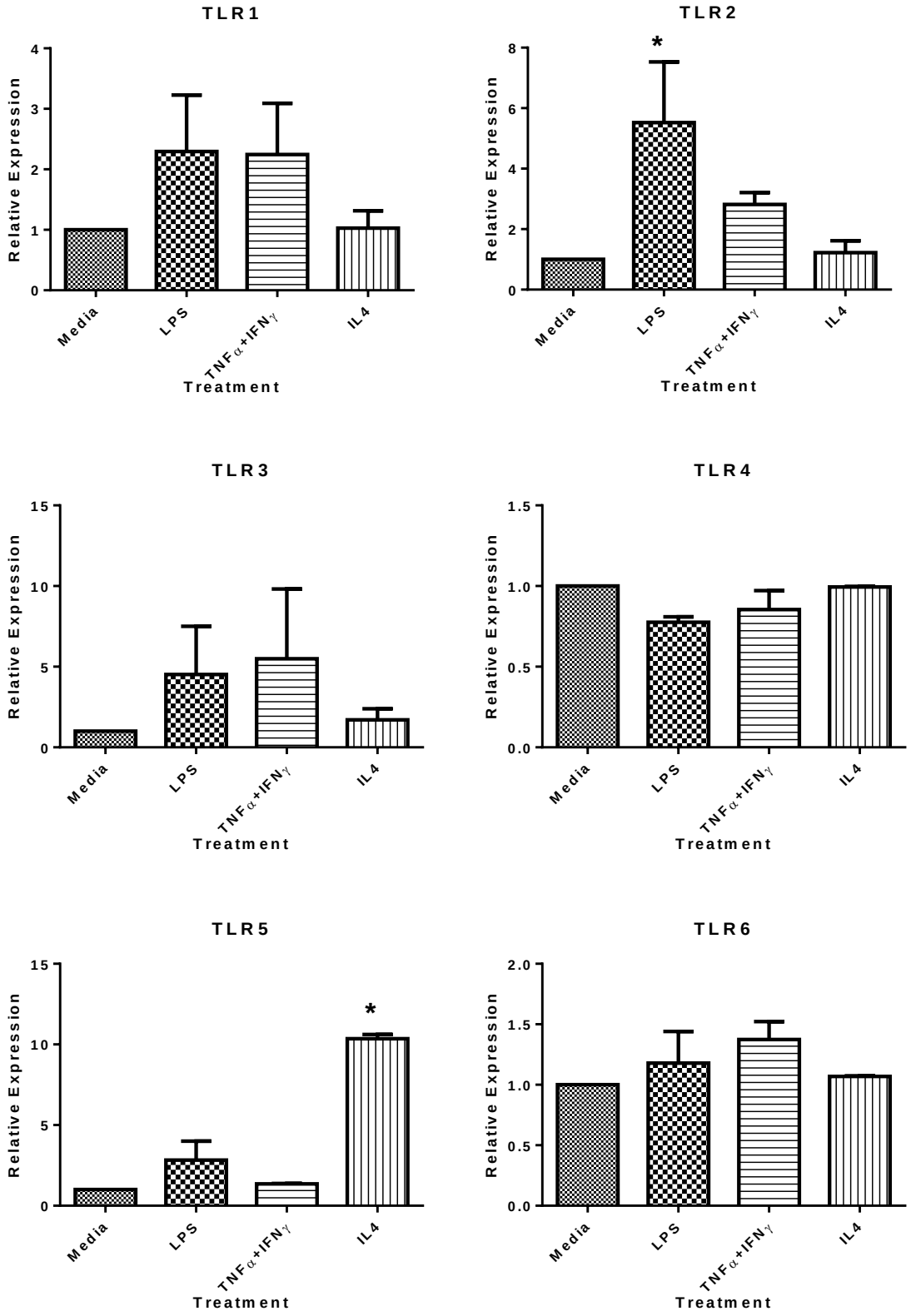



**Figure 6-2. Phenotype of liver derived macrophages in response to fatty acids.**

Liver derived macrophages were treated with either oleic (100µM) or palmitic acid (100µM). Phenotype markers were assessed by relative real time PCR and compared to untreated liver derived macrophages. The results represent the mean ± SEM of three experiments with different donors performed in triplicate. *$p<0.05$

### 6.2.2 TLR expression of liver derived macrophages after cytokine stimulation

To see if treatment of liver derived macrophages would have a subsequent effect upon TLR expression, once again stimulation was performed with LPS, TNFα, IFNγ and IL-4. TLR2 expression was upregulated in response to LPS; however LPS (nor the other cytokines) effected the expression of TLR4 at an RNA level. IL-4 was able to upregulate TLR5 expression. TNFα and IFNγ increased the expression of TLR 7 and 8. The inhibitory protein TOLLIP was not altered by any of the treatments (Figure 6-3).


TLR1
Relative Expression
0
1
2
3
4
Media
LPS
TNFα+IFNγ
IL4
Treatment
TLR2
*
Relative Expression
0
2
4
6
8
Media
LPS
TNFα+IFNγ
IL4
Treatment
TLR3
Relative Expression
0
5
10
15
Media
LPS
TNFα+IFNγ
IL4
Treatment
TLR4
Relative Expression
0.0
0.5
1.0
1.5
Media
LPS
TNFα+IFNγ
IL4
Treatment
TLR5
*
Relative Expression
0
5
10
15
Media
LPS
TNFα+IFNγ
IL4
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IL4
Treatment

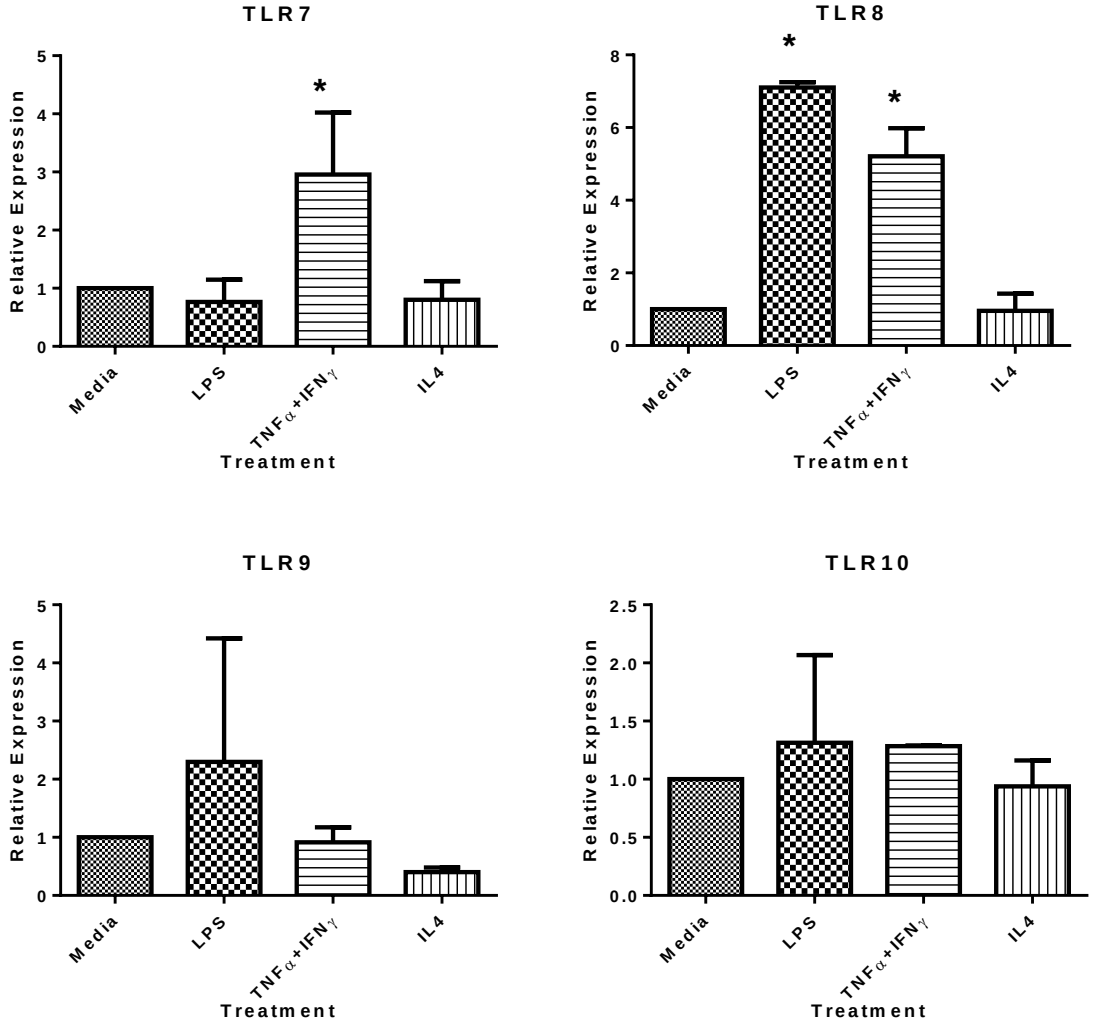
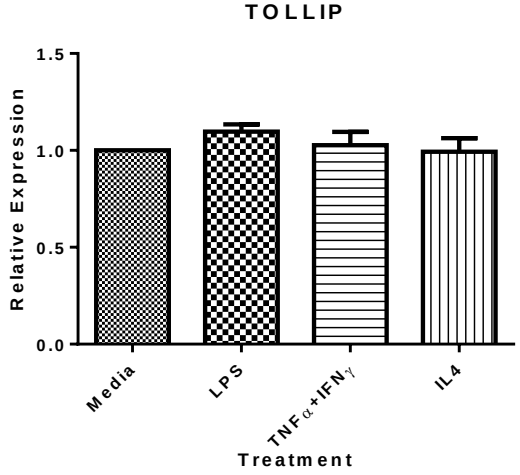



**Figure 6-3. TLR expression in liver derived macrophages.**




Liver derived macrophages were treated with either LPS, TNFα, IFNγ or IL-4. TLR mRNA expression was assessed by relative real time PCR and compared to untreated liver derived macrophages. The results represent the mean ± SEM of three experiments. *$p<0.05$

### 6.2.3 Liver derived macrophages can be co-cultured with HSEC

Liver sinusoids do not only consist of HSEC but also Kupffer cells which are in close proximity to each other and have the ability to alter each other's functionality. To see if we could build up a more representative model of this situation we co-cultured liver derived macrophages with HSEC and demonstrated we were able to maintain the two cell types in culture using endothelial media (Figure 6-4).

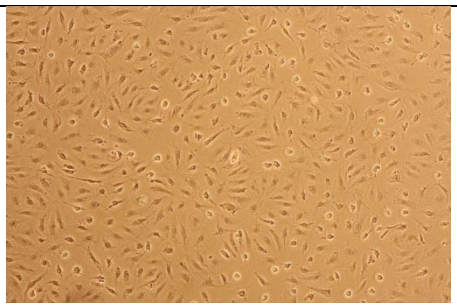
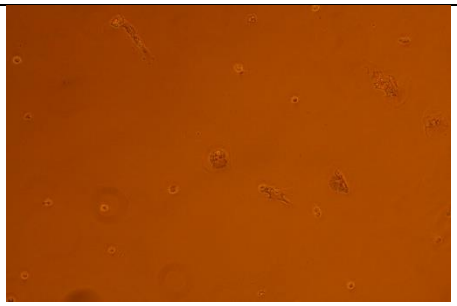
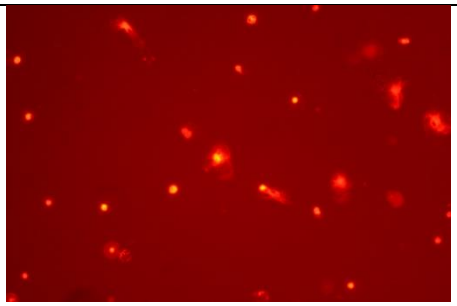
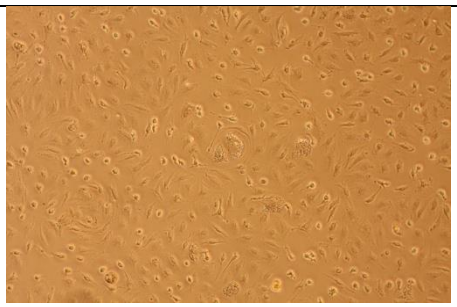
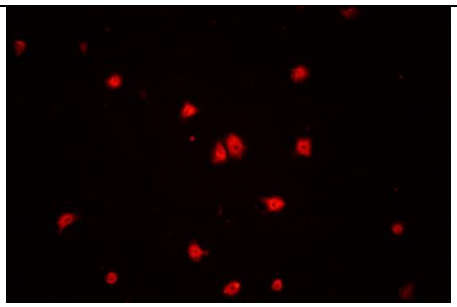
| | Phase contrast image | CD68 staining |
|---|---|---|
| HSEC alone |  |  |
| Liver derived macrophages alone |  |  |
| Co-culture of Liver derived macrophages with HSEC |  |  |

**Figure 6-4. Co-culture of HSEC with liver derived macrophages**

Liver derived macrophages were isolated and cultured in 96 well plates. Once the macrophages had been established in culture after 24 hours, HSEC were plated in to the wells. The images show samples of co-cultures together with CD68 staining to highlight the macrophages, and the cells cultured alone.

### 6.2.4 Co-culturing human liver derived macrophages with HSEC did not alter cell numbers

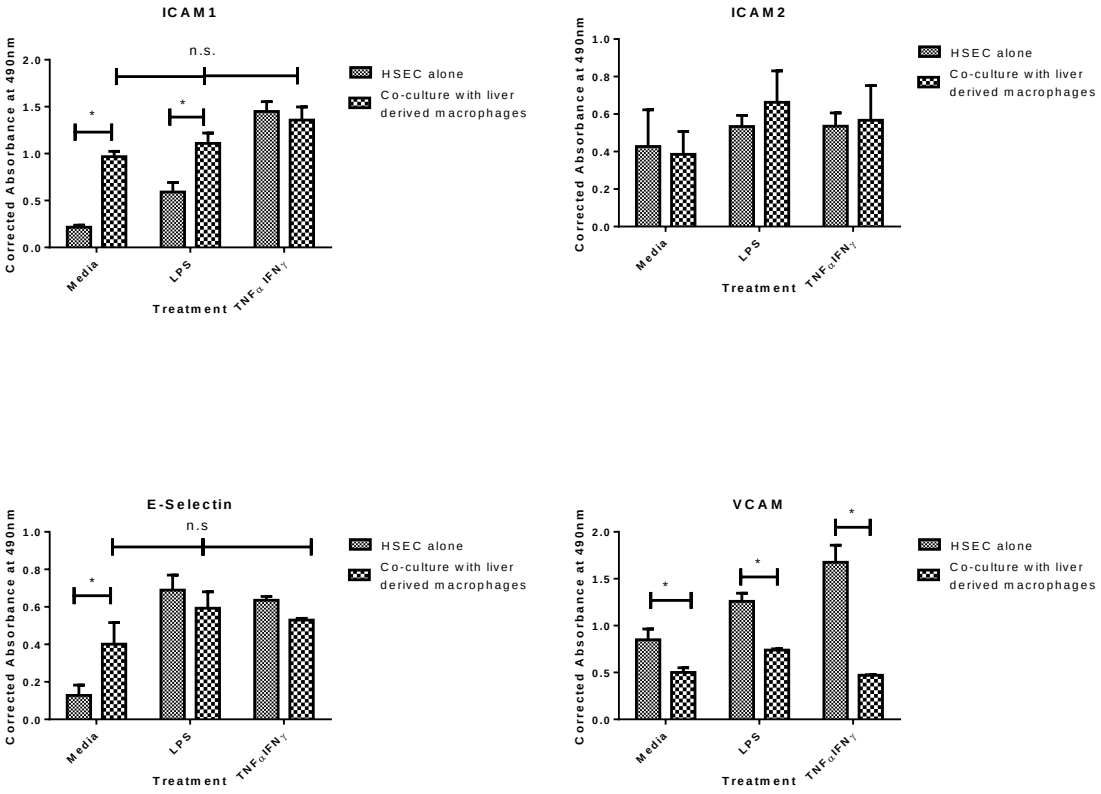
There are studies that have demonstrated the ability of macrophages to alter the proliferation of endothelium and depending on any other exogenous stimuli this can be either proliferative or inhibitive (247, 248). To ensure that there was no alteration in cell numbers that may account for some of the differences seen in the expression of adhesion molecules crystal violet proliferation assay was carried out. The value obtained remained steady though co-culturing and upon cytokine stimulation (Figure 6-5).


**Figure 6-5. Cell number upon co-culturing HSEC with liver derived macrophages.**

Crystal violet staining was used to assess cell number after culturing HSEC with liver derived macrophages. Absorbance was measured on a plate reader and the values represent the mean ± SEM of 3 experiments.

### 6.2.5 ICAM-1 is increased by co-culture of HSEC with liver derived macrophages, whilst VCAM-1 is reduced

As Kupffer cells are key components in the structure of sinusoids, the effect of co-culturing liver derived macrophages with HSEC on the expression of adhesion molecules was investigated. Liver derived macrophages were plated initially in to 96 well plates and after 24 hours HSEC were plated on top. The expression of adhesion molecules was then assessed and including after stimulation with TNFα and IFNγ or LPS. In co-cultures the basal levels of ICAM-1 and E-selectin were raised. However, unlike stimulation with LPS or TNFα and IFNγ on monocultures of HSEC which caused increased expression of ICAM-1 and E-selectin, in the co-cultures no further increase in expression was demonstrated by stimulation with LPS or TNFα and IFNγ. ICAM-2 levels did not alter with co-culturing or stimulation with LPS or TNFα. Interestingly VCAM-1 levels were reduced in co-cultures of HSEC with liver derived macrophages. Once again stimulation of the co-cultures with TNFα and IFNγ or LPS had no effect on the expression of the adhesion molecule VCAM-1 (Figure 6-6).



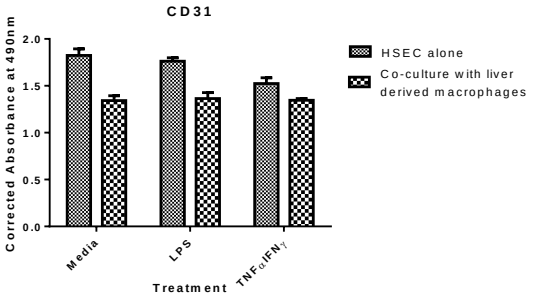




**Figure 6-6. Expression of adhesion molecules in co-cultures of HSEC with liver derived macrophages.**

HSEC were plated on to liver derived macrophages. They were then treated once established with LPS or TNFα. The data represent the mean corrected absorbance ± SEM of three independent experiments.

*p<0.05

### 6.2.6 Human liver derived macrophages are unable to support adhesion out of flow

To determine if Kupffer cells in their own right were able to recruit monocytes out of flow, human liver derived macrophages were cultured in IBDI slides, however they rapidly perished. Eventually we were able to get maintain them on coverslips and mount them in parallel chambers. Then peripheral blood monocytes were flowed across (Figure 6-7). No attachment was seen. Even when the liver derived macrophages were stimulated with TNFα or LPS, monocytes still did not adhere. To investigate whether in co-cultures with HSEC any difference would be seen, given that the previous set of experiments had shown altered expression of adhesion molecules, attempts were made to culture the two cell groups. However, it proved challenging to get the HSEC to grow alongside liver derived macrophages to create a monolayer to perform flow assays on reliably.

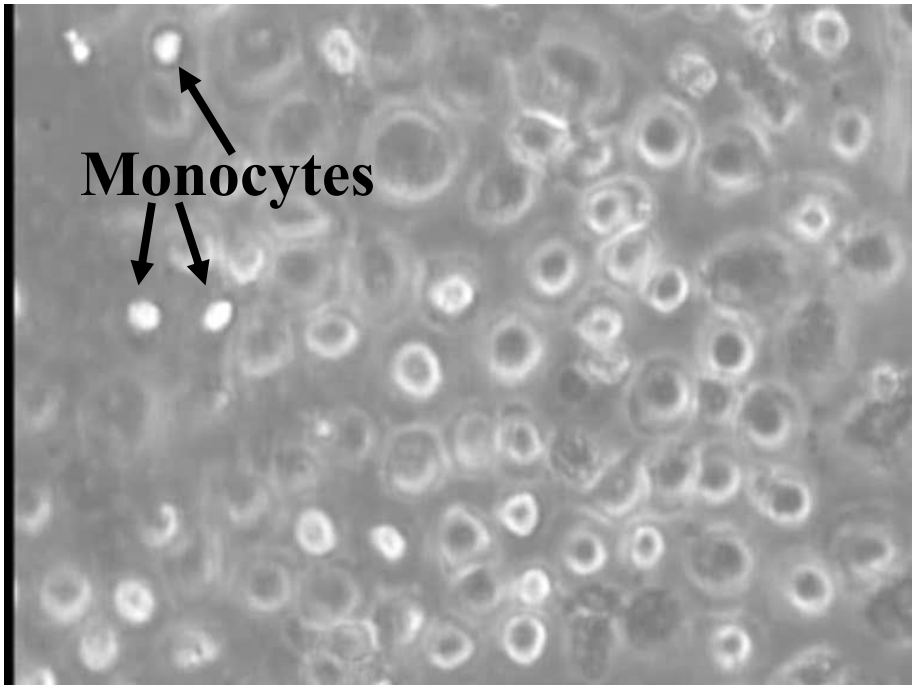



**Figure 6-7. Still from monocytes flowing over liver derived macrophages.**

Liver derived macrophages were cultured on coverslips to form a monolayer. The coverslips were then mounted in a parallel chamber and monocytes were then flowed across.

## 6.3 **Discussion**

Kupffer cells are strategically located and form together with HSEC the liver sinusoids. They are more numerous in the periportal regions of the liver (249). Kupffer cells are well recognised as mediators of liver damage and resolution as well as maintaining homeostasis. Kupffer cells make up 90% of body macrophages (21) and 15-20% of the liver cells (22). Kupffer cells are motile cells characterised by a large nucleus, significant amounts of rough endoplasmic reticulum and cytoplasmic vacuoles, reflecting their functions. They are highly phagocytic cells, able to engulf a range of pathogens, cell and antigens that include bacteria, viruses, red blood cells, tissue debris and certain tumour cells (250).

Given the multitude of roles Kupffer cells play it is not unsurprising that they take on different phenotypes to fulfil each role. The main subdivision is between M1 classically activated forms and M2 alternative activation with further subtypes depending upon the stimuli. Despite classification of macrophages into subtypes, the reality is likely to be the phenotype of macrophages is a spectrum of a highly plastic cell population that are able to differentiate depending on stimuli and back again.

M1 (classical) macrophages occurs in response to microbial products or interferon-gamma and results in high production of IL-12 and IL-23 (and low IL-10) (251). M1 macrophages have a high capacity to present antigen and to produce factors that promote T-cell proliferation and activity. Also there is a high production of toxic intermediates namely nitric oxide (via increased levels of inducible NO synthase) and reactive oxygen intermediates. They express opsonic receptors (e.g. CD16/FcyRIII). M1 macrophages are potent effector cells that kill micro-organisms and tumour cells, present antigens and produce proinflammatory cytokines (252).

On the other end of the spectrum M2 (alternatively activated) macrophages have poor antigen-presenting capability; produce factors that suppress T-cell proliferation and activity. They express preferentially non-opsonic receptors (e.g. mannose receptor) and scavenger receptor. M2 macrophages have a predominant activation of the arginase pathway resulting in the production of ornithine and polyamines. They produce higher levels of IL-10 high. They are thought to be important in debris scavenging, wound healing, and angiogenesis. M2 macrophages may also play key roles in chronic infections, tumourgenesis, and tumour metastasis (253).

Under the influence of IL-4 the population of resident tissue macrophages can expand, and IL-4 is associated with the ability to differentiate macrophages in to alternatively activated macrophages required for resolution and repair of tissue damage (254). The liver derived macrophages we isolated, upon stimulation with IL-4 up regulated the expression of CD206 in keeping with their alternatively activated phenotype. Furthermore, oleic acid also increased CD206 pushing the macrophages towards an alternatively activated phenotype. Palmitic acid had no effect on the expression of CD206.

Macrophages are well described as being particularly plastic cells, readily altering their phenotype according to environmental cues. Additionally following polarisation they are able to upon further stimulation polarise into a different phenotype and back again (264). This plasticity of macrophages allows them to undertake diverse and contrary functions. Macrophages are thus able to dynamically change the polarisation through the course of inflammatory processes without the need to recruit further pre-polarised macrophages (265). We were able to push the expression of phenotype markers of liver derived macrophages including with IL-4 to a more alternatively activated phenotype. We were also able to induce an alternatively activated phenotype using oleic acid. We were unable to induce significant

changes with palmitic acid. However work by others have shown that palmitic acid treated Kupffer cells produce more TNFα compared to media or oleic treated Kupffer cells (266).

Due to the location of Kupffer cells in relation to the hepatic sinusoids and exposure to portal blood containing gut derived pathogens, the expression of TLR was assessed in response to inflammatory stimuli namely TNFα and LPS which causes classical activation and IL-4 which alternatively activates. Of particular note was the change in TLR5 expression with IL-4 treatment which was in line with the findings from other groups on macrophages (255). Why only TLR5 increased in response to IL-4 is open to speculation. A possibility is that is a mechanism by which the innate and adaptive immune system are linked and coordinated. There are recent studies looking at the role of flagellin in vaccines. Flagellin is required to induce activation of adaptive responses (256). Research in inflammatory bowel disease suggest that flagellin may responsible through adaptive response create a state of chronic inflammation (257).

Looking at the functional role of Kupffer cells, it is known that they can interfere with the flow through sinusoids (258) including inducing the temporary stasis of sinusoidal blood flow thus allowing for prolonged contact between the leukocytes and sinusoids. It has been shown that Kupffer cells are capable of directly capturing and recruiting dendritic cells from the circulation (259). It has also previously been documented in flowing systems that Kupffer cells are able to capture neutrophils out of flow (260). There are important direct cell to cell interaction with Kupffer cells and other leukocytes such as NK cells. Kupffer are able in the endotoxic environment to arrest the flow of platelets (261).

We attempted initially to use Ibidi slides to culture liver derived macrophages but they would rapidly perish. Eventually we were able to establish them on coverslips to use in parallel

flow chambers. Monolayers of liver derived macrophages on coverslips could not capture monocytes out of flow even after stimulation with LPS or TNFα.

Macrophages, together with neutrophils and monocytes despite their common origin are complementary in the actions and interactions. Macrophages are long lived and resident in tissues with greater specificity in antigen recognition. Due to their strategic location within tissues they are ideally situated to initiate inflammation in response to attack. Part of the response is the production of chemokines directed at recruiting the potent antimicrobial neutrophils. Additionally the degranulation of neutrophils releases proteins that have a multifactorial effect including the promotion of further chemokine production by macrophages, conversion of chemokines to pro-forms, activation of endothelium and anchoring to endothelial proteoglycans which all work in concert to recruit classical monocytes (262, 263). So though we did not see any direct interaction of Kupffer cells with flowing monocytes additional factors may be involved and this model was too reductionist. Another limitation with these experiments once again was the use of monocytes from one donor and liver derived macrophages from another.

Furthermore, even within the liver, Kupffer cells vary. Kupffer cells within the periportal area of the liver are larger and more phagocytic whereas the smaller Kupffer cells in the centrilobular areas are more avid cytokine producers (20, 249) and in our experiments there were no attempts to isolate different types of Kupffer cells.

# Chapter 7

## Discussion

## 7.1 General Discussion

Liver disease continues to be a significant and increasing socio-economic burden. Liver disease as it progresses share some certain common hallmarks, that includes being in a state inflammation with an associated inflammatory infiltrate resulting in damage. The ability to isolate primary human cells provides the ability to dissect the complex interactions that occur within the liver, to better understand these processes.

The liver has evolved the ability to tolerate numerous and varied insults. However multiple hits can result in progressive and irreversible liver damage. Part of this multi-hit milieu involves microbial products and fatty acids together with interactions involving TLRs. The gut acts as a source of microbial products that are potential sources of inducing a septic inflammatory response, with effects on the liver and further systemically. The gut also is responsible for uptake of nutrients from the diet which includes fatty acids. Through these products, the gut is intimately linked to the liver. Microbial products and fatty acids are able to alter the state of hepatic sinusoids, a key gate keeper to the parenchyma of the liver in particular leukocytes that are crucial in mediating liver injury (as well as resolution)..

One of the leucocyte type involved in mediating inflammation, are monocytes which are highly plastic cells with not only the ability to be effectors in their own right, but also the ability to differentiate into Kupffer and dendritic cells. In this piece of work we have aimed to look at some of the interactions of microbial TLR agonists and fatty acids upon recruitment of monocytes upon HSEC.

Multiple researchers have shown a close relation between bacterial products and in particular LPS in not only being critical in the development of liver disease but also promoting its

progression. LPS acts via TLR4, however is efficiently cleared from the portal blood stream during health without triggering an overwhelming inflammatory response.

The use of flow assays in this studies showed that HSEC exposed to LPS similar to the inflammatory cytokine TNFα is able to result in a pro-adhesive state that is able to recruit monocytes out of flow and similarly as observed previously with other leukocytes, no significant rolling was seen before firm adhesion. However, there is a notable difference in that the adherent monocytes on LPS treated HSEC. These monocytes in this study did not show as extensive transmigration through the LPS treated HSEC monolayers compared to TNFα treated HSEC. The significance of this may reflect what occurs in normal healthy individuals that despite the constant presence of endotoxin from the gut, the liver is not flooded by an influx of monocytes and the potentially deleterious effects. Also noted was that with longer incubation the number of monocytes captured decreased, again adding to the tolerogenic environment.

In these studies, we have been able to demonstrate that the effects seen are not unique to LPS and that another TLR4 agonist, namely MPL is able to produce similar results. We have also been able to expand this finding showing that agonist to other cell surface TLRs including flagellin, are able to stimulate HSEC to be able to recruit monocytes and again with minimal transmigration.

Monocytes have been observed to exhibit a "patrolling" behaviour on the luminal surface of the liver vasculature (33) without infiltrating deeper and this fits in with the observations seen that healthy adults have endotoxin present in the portal blood stream without causing influx of monocytes. We did not look at whether alterations in concentration of LPS or the other TLR ligands have an effect within the modelled sinusoidal system. The relevance of

this lies with the increase presence of microbial products transported to the liver in liver disease due to an increase permeability of the gastrointestinal tract. It would be useful to know if higher concentrations would result in more transmigration of monocytes across HSEC.

Work by others has reported that stimulation of murine TLR5 does not result in TNFα production (201) and taken as meaning liver sinusoidal endothelial cells are unresponsive to TLR5 stimulation (267). Our studies on primary human HSEC has shown that flagellin does have a functional consequence, namely in this set of studies the ability to recruit monocytes out of flow. This is consistent with murine studies with looking at alcoholic liver disease where the administration of flagellin results in an inflammatory infiltrate (268).

TLR5 mRNA expression in HSEC and liver derived macrophages was interesting in the marked increased expression when these cells were stimulated. TLR5 expression was increased on HSEC by stimulating these cells with LPS after initially priming with TNFα and IFNγ showing a synergistic effect. Liver derived macrophages increased TLR5 expression on IL-4 stimulation. Whether this is of functional consequence is dependent on whether the mRNA is translated and if so what is its significance when the mRNA expression of the other TLRs did not change to the same extent.

We also looked at what happened when monocytes were exposed to cell surface TLR ligands rather than hepatic endothelium. Upon stimulated inflamed endothelium, by treating with TNFα and IFNγ, there was a significant reduction in the proportion of adhering monocytes migrating across the endothelium.

There are limitations in the flow assays we performed in particular to the modelling of hepatic environment. The use of static flow rates does not accurately model the true flow

that occurs as liver disease progresses. Haemodynamic changes occur that include diminished portal venous blood flow (269). Also, though certain experiments showed no changes in adhesion they may still have a role through interactions with other cell types not included with these setups. However, these models do provide a mechanism by which complex interactions can be broken down and explored.

A further limitation of the experiments performed is that endothelial cells are outside of their native environment and detached from their usual environmental context and thus undergo phenotypical and physiological changes. Usually they are exposed to a multitude of external stimuli, including flow, neural and hormonal influences. They display spatial and temporal variability. In septic patients further haemodynamic, oxygenation levels and acidity changes occur (270). In an attempt to recreate a more accurate simulated hepatic sinusoid we were able to demonstrate that it is possible to co-culture liver derived macrophages together with HSEC. We were however unable to get the co-culture to establish in Ibidi slides, however to overcome this we eventually used coverslips in parallel flow chambers though this was still unreliable.

Endothelium interaction with monocytes goes beyond recruitment. In co-culture experiments it has been shown that monocyte survival increases with interaction with LPS and other inflammatory cytokine activated endothelial cells. Furthermore CD14 expression is upregulated as well as of chemokines such as MCP-1 potentially recruiting further monocytes (271, 272).

In this work, we have also looked at the role of fatty acids and in particular the differences that have been reported between saturated and unsaturated fatty acids. Though we were unable to demonstrate a pro-inflammatory effect of palmitic acid we were able to show that

oleic acid was able to reduce the recruitment of monocytes upon modelled inflamed hepatic endothelium. Oleic acid was also able to differentiate liver derived macrophages to an alternatively activated phenotype which has been implicated in liver tolerance and repair.

## 7.2 **Summary**

In conclusion, bacterial ligands and fatty acids are critically integrated together being taken up from the gut in the pathogenesis of liver disease. Linking the two are toll-like receptors. Microbial ligands are able to support adhesion to HSEC, but to migrate across the endothelium require priming. Oleic acid is able to suppress monocytes recruitment to inflamed endothelium (Figure 7-1).

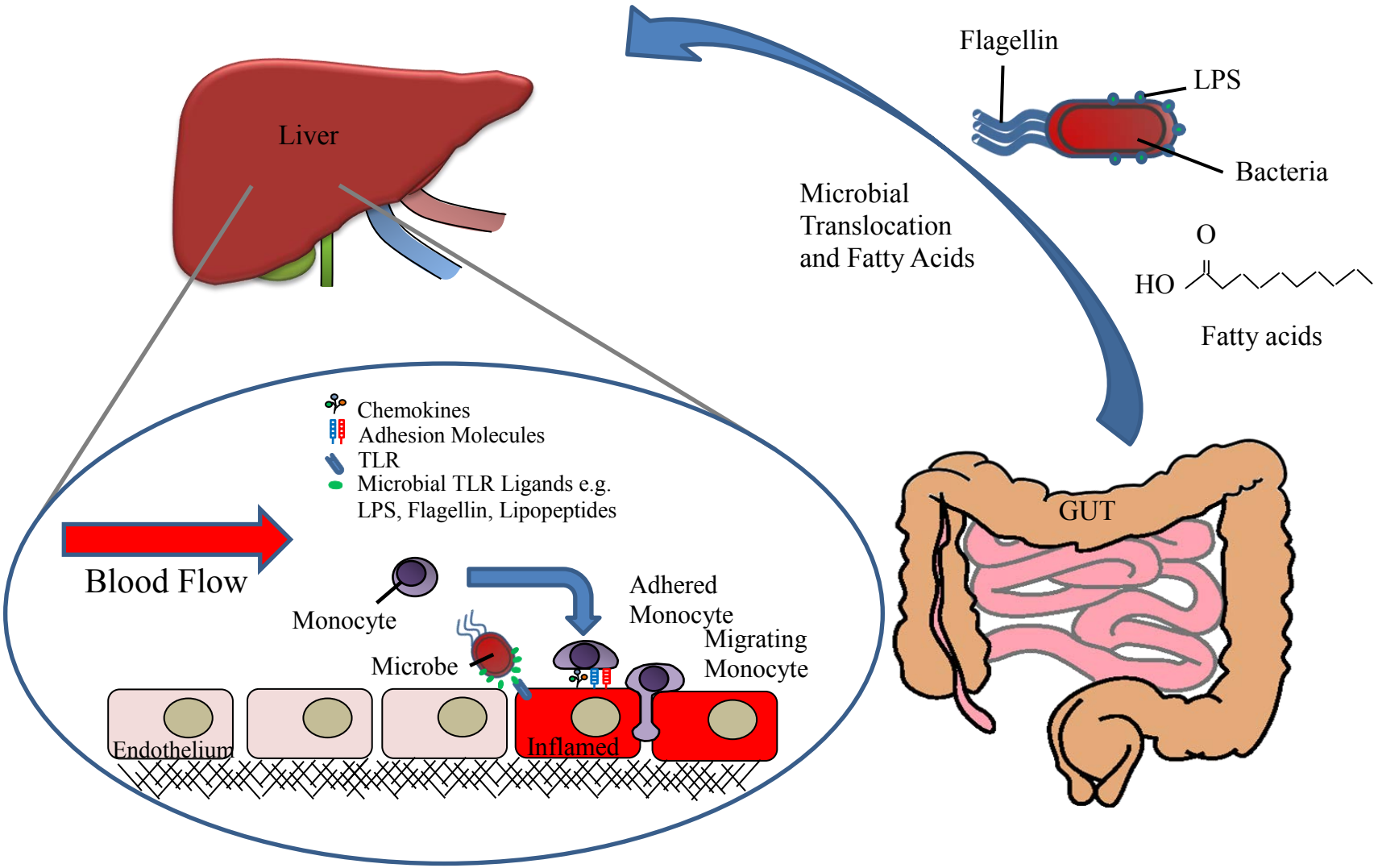



**Figure 7-1. Summary of the liver-gut axis in recruiting monocytes.**

Microbial and fatty acid products from the gut are able to modulate the recruitment of monocytes to the liver out of flow.

## 7.3 Future work

Kupffer cells form an integral part of the hepatic sinusoids, lying on the luminal side and thus exposed to the flow of blood. Due to their size and ability to undergo morphological changes they can alter flow through sinusoids (273, 274). Kupffer cells have been demonstrated to interact with leukocytes, namely neutrophils through complimentary adhesion molecules (260) and that upon depletion reduce the recruitment of neutrophils into the liver (275). Furthermore the depletion of Kupffer cells reduces the recruitment of monocytes into the liver through possibly reduced expression of MCP1 (276). It would be interesting to go on and further develop reliable co-culture models of Kupffer cells and HSEC attempted here to see if different conditions can modulate recruitment of monocytes from flow. Ideally one would isolate all the cell types from a single donor.

Monocytes have been shown to not only migrate across endothelium but reverse transmigrate. Using CELL-IQ an automated cell culture and analysis system, though under static conditions, initial experiments on monocytes demonstrate qualitatively different behaviours depending on the treatment of HSEC. With advances in technology and processing offered through CELL-IQ if would be interesting to track monocytes in continuous flow over prolonged time frames and assess their phenotype over the time course to see if they take on more of dendritic phenotype or that of a macrophage. Work in our lab has already shown some changes that occur with monocytes as they migrate across endothelium (277).

Finally, it would be interesting to explore further the roles of eotaxin and ENA-78 that are produced differentially depending on HSEC stimulation with TNFα or LPS and whether they do have the inability to block migration of monocytes.

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