The Regulation of Inflammatory Liver Responses by the TNF Ligand and Receptor System-TWEAK and Fn14, via Human Intra-hepatic Endothelial Cells

by

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ABSTRACT

TWEAK (TNF-like weak inducer of apoptosis) and Fn14 (FGF-inducible protein 14) are TNF (tumour necrosis factor) superfamily members which have multifunctional capabilities. They have been shown to regulate inflammation, angiogenesis, cell fate, and tumourigenesis. They have recently been shown to contribute to hepatic inflammatory responses and regeneration during liver insult and chronic inflammatory liver disease (CILD). We propose that TWEAK and Fn14 may regulate CILD via human intra-hepatic endothelial cells (HIEC) and subsequently be developed in future as novel therapeutic agents towards CILD. Our investigation showed that TWEAK was predominantly expressed in leukocyte infiltrates in CILD tissue, it was highly regulated by interferon-γ in leukocytes, and contributed directly towards leukocyte recruitment via HIEC. Fn14 expression in CILD tissue was expressed in endothelium and portal vessels, and Fn14 expression was highly regulated by TNF-α and interleukin-1β in HIEC. We showed that TWEAK can directly contribute to angiogenesis in the inflamed liver via HIEC, by orchestrating a specific angiogenic cytokine response, and that TWEAK and Fn14 may contribute to portal-associated lymphoid tissue formation. We further found that TWEAK mediated functions via HIEC were highly regulated via NF-kB and Erk signalling, and that TWEAK can directly mediate HIEC reactive oxygen specie production and responses to necrosis. We can conclude from our findings that TWEAK and Fn14 may regulate CILD by complex signal transduction, and specifically mediate the inflammatory angiogenic responses by adopting paracrine mechanisms; whereby Fn14 is endogenously expressed in HIEC, and TWEAK and Fn14 interactions during CILD are facilitated by TWEAK positive leukocyte infiltration.

In the name of Allah the most gracious, the ever merciful

If anyone travels on a road in search of knowledge, Allah will cause him to travel on one of the roads of paradise. The angels will lower their wings in their great pleasure with one who seeks knowledge, the inhabitants of the heavens and the earth and the fish in deep waters will ask forgiveness for the learned man. The superiority of the learned man over the devout is like that of the moon on the night when it is full, over the rest of the stars. The learned are the heirs of the Prophets, and the Prophets leave neither dinar nor dhiram, leaving only knowledge, and he who takes it takes an abundant portion.

Prophet Muhammed ﷺ (Sunan Abi Dawud 3641).

DEDICATION

This thesis is dedicated to my late mother Naseem Akhtar, my father Hafiz Munir Ahmad Sabir, my husband Shazad Hussain, my son Muhammed Yahya, and my family without whom I am incomplete.

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PUBLICATIONS

Munir M, Humphreys EH, Wilhelm A, Reynolds G, Caamano J, Adams DH, Linda C Burkly and Afford SC (2015), Fn14 and TWEAK promote neovessel formation and organisation of portal associated lymphoid tissue during chronic hepatic inflammation. *Manuscript in preparation.*

Wilhelm A, Amatucci A, **Munir M**, Reynolds G, Humphreys EH, Resheq Y, Adams DH, Hübscher S, Burkly LC, Weston CJ and Afford SC (2015), Fibroblast Growth Factor Inducible 14 and its ligand TWEAK regulate human hepatic stellate cell proliferation and liver fibrogenesis in a murine model. *Manuscript in preparation.*

ABSTRACTS AND PRESENTATIONS

Munir M, Humphreys EH, Willhelm A, Stephenson B, Camaano J, Adams DH, Burkly LC and Afford SC, FGF inducible protein 14 is up-regulated in neovessels during chronic inflammatory liver disease and promotes intrahepatic endothelial cell angiogenesis in vitro following stimulation via TWEAK.

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Willhelm A, Weston CJ, Humphreys EH, Shepherd EL, **Munir M**, Stephenson B, Camaano J, Adams DH, Aldridge VS, Burkly LC and Afford SC, The Fn14/TWEAK receptor-ligand system regulates hepatic stellate cell function in liver fibrosis. *Presented at EASL 2013 as a poster.*

Stephenson B, Humphreys EH, Muirhead G, Willhelm A, **Munir M**, Camaano J, Adams DH, Burkly LC and Afford SC, Fn14 is expressed on cholangiocytes and promotes biliary ductular remodelling via apoptosis and reactive oxygen species after interaction with TWEAK. *Presented at DDF 2012 as a poster. Published in Gut 2012; 61:S2: A118-A119. Presented at EASL 2013 as a poster.*

Willhelm A, **Munir M,** Humphreys EH, Adams DH, Burkly LC, Afford SC and Weston CJ, The TWEAK and Fn14 pathway as a potential mediator of liver fibrosis. *Published in Gut 2014; 63:S2: A16-A17. Presented at EASL 2014 as a poster and oral presentation.*

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LIST OF ABBREVIATIONS

aa	amino acids
Ab	Antibody
ALD	Alcoholic liver disease
Ang-1	Angiopoietin-1
BAFF-R	B cell activating factor-receptor
BEC	Biliary epithelial cell
CCL4	Carbon tetrachloride
CD31	Cluster of differentiation 31
CD40	Cluster of differentiation 40
CD40-L	CD40-ligand
CD68	Cluster of differentiation 68
CD90	Cluster of differentiation 90
CDE	Choline deficient, ethionine supplemented
CK18	Cytokeratin 18
CK19	Cytokeratin 19
CMA	Chaperon mediated autophagy
CXCL16	Chemokine (CXC) ligand 16
DAPI	4', 6 diamidino-2-phenylindole
DCF	2', 7'- dichlorofluorescin
DDC	3, 5- Diethoxycarbonyl-1, 4-dihydrocollidine
DISC	Death inducing signalling complex
DPPIV	Dipeptidyl peptidase IV
EGF	Epidermal growth factor
EGVEGF	Endocrine gland derived VEGF
ER	Endoplasmic reticulum
Erk	Extracellular signal regulated kinases
FADD	Fas activated death domain
Fas-L	Fas-Ligand
FGF	Fibroblast growth factor
Fn14	FGF inducible protein 14
GDNF	Glial cell line derived neurotrophic factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HB-EGF	Heparin binding EGF like growth factor
HBV	Hepatitis B virus
HBx	Hepatitis B protein
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HGF	Hepatocyte growth factor
HIEC	Human intrahepatic endothelial cells

HIF	Hypoxia Inducible Factor
HmVEC	Human micro vascular endothelial cells
HSC	Hepatic stellate cells
HUVEC	Human umbilical vein endothelial cells
IGFBP	Insulin like growth factor binding protein
IF	Immuno-fluorescence
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IHEC	Intra-hepatic endothelial cells
IL-1β	Interleukin 1 beta
IL-8	Interleukin 8
KC	Kupffer Cells
JNK	c-jun N terminal kinases
LN	Lupus nephritis
LPS	LipoPolysaccharide
mAb	monoclonal antibody
MAPK	Mitogen activating protein kinases
MCP-1	Monocyte chemoattractant protein 1
MDC	Monodansylcadaverine
MIF	Macrophage migration inhibitory factor
MIP	Mitochondrial intermediate peptidase
MMP-8	Matrix metalloproteinase 8
MS	Multiple sclerosis
NASH	Non-alcoholic steato hepatitis
NF-kB	Nuclear factor kappa B
PDGF	Platelet derived growth factor
PBC	Primary biliary cirrhosis
PFA	Paraformaldehyde
PHx	Partial hepatectomy
PlGF	Placental growth factor
PMA	Phorbol 12-myristate 13-acetate
PSC	Primary sclerosing cholangitis
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
SE	Standard error
SEM	Standard error of mean
TIMP	Tissue inhibitors of metalloproteinases
TGF-β	Tumour growth factor beta
TNF-α	Tumour necrosis factor alpha
TNFIA	Tumour necrosis factor inhibitor of apoptosis
TNFSF	TNF super family

TRADD	TNF receptor associated death domain
TRAF	TNF receptor associating factor
TWEAK	TNF like weak inducer of apoptosis
VCAM	Vascular cell adhesion molecule 1
VAP-1	Vascular adhesion protein 1
VEGF	Vascular endothelial growth factor
uPA	urokinase type PA

CHAPTER 1

GENERAL OVERVIEW

1.1 Introduction

Liver disease is the 5th largest cause of death in England and Wales, contributing to 16'000 deaths in 2008 alone, and at present contributing to 2% of all deaths in the UK (British liver trust. 2015. *Facts about liver disease*). Each year there is an increase in the number of people suffering from a form of liver disease, and it is the only disease which has shown a year on year rise in incidence (Figure 1.1). Liver disease is presenting in people averaging at 33-55 years of age, and patients dying from liver disease average at 59 years of age in comparison to patients with heart and lung disease, who average at 82-84 years of age. What sets liver disease apart from other diseases is that the main causes of liver disease such as obesity, alcohol abuse, and viral diseases are all preventable. The majority of liver diseases documented have been caused by alcohol abuse, contributing to 37% of all registered liver diseases (British liver trust. 2015. *Facts about liver disease*). Chronic liver diseases can subsequently lead to financial implications and strain on the health care system, as liver disease management can only be carried out in hospitals and currently occupies 8-10% of all UK hospital admissions. The need for liver transplants is increasing, and the available donor livers are insufficient. Therefore, there is a need to raise awareness of liver disease and preventative measures amongst people, and most importantly there is a significantly urgent need to develop novel therapies directed towards promoting liver regeneration, which can ultimately reduce the incidence of developing chronic liver disease requiring transplantation (British liver trust. 2015. *Facts about liver disease*).

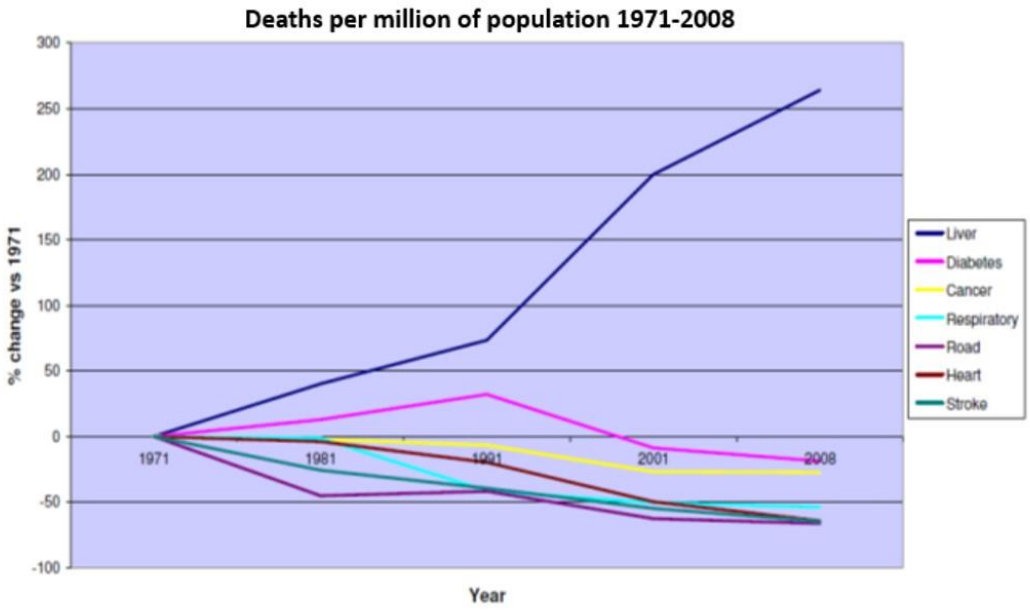


Figure 1.1 Liver disease mortality statistics: mortality rates from 1971 to 2008 caused by liver disease have shown a consistent rise. In contrast, deaths caused by other diseases and road accidents have shown a decline over the same time frame.

Adapted from; www.britishlivertrust.org.uk

1.2 The Immune System

The immune system has developed to protect the host from damage caused by harmful pathogenic organisms. It functions to successfully remove pathogens whilst protecting the host from damage, and retaining the ability to distinguish self from non-self. There are two branches of immunity which work independently, as well as dependently interacting with one another forming efficient protection for the host. The innate immune system consists of the host defence mechanisms biologically in place in the body, leading to rapid responses to foreign organisms. These include physical, chemical, and biological barriers. The innate system consists of neutrophils, monocytes and macrophages for pathogenic removal, whilst

making use of chemokine and cytokine secretion, soluble effector molecules, and the complement system for targeting pathogens. The adaptive immune response targets specific antigens. This response has a delayed, pro-longed onset in comparison to the innate immune system, but retains memory of the invasion thus preventing future attack. B and T lymphocytes are key cells of the adaptive immune response and express specific receptors for antigens present on invading pathogens, which can then be targeted for removal by the innate response (Parkin e Cohen, 2001; Chaplin, 2010).

1.3 The Liver

The liver is the largest internal organ in the developed human body, totalling 2.5% of the total body weight (Juza e Pauli, 2014). It is highly efficient and often described as being the first line of defence to protect the host from external insult, as the liver plays an important role facilitating the host immune response. The liver filters blood from circulation and the gastrointestinal tracts where it comes into direct contact with pathogens. The highly complex structure of the liver facilitates its many functions including metabolism of nutrients for absorption, bile secretion, the clearance of toxins, and immune surveillance (Si-Tayeb, Lemaigre e Duncan, 2010; Jenne e Kubes, 2013).

1.3.1 Liver Anatomy: Anatomically the liver is situated to the right upper quadrant of the abdomen, to the right of the stomach, below the diaphragm, where it is protected by the ribcage. The liver is secured within the abdomen in a connective tissue capsule and the peritoneum. It consists of the left and right lobes (which are separated by the Falciform

ligament), and the caudate and quadrate lobes which extend from the posterior side of the right lobe. The liver is made up of several functional units which consist of hexagonal lobules of hepatocytes. At the periphery of each lobule a portal triad is formed consisting of a portal vein, hepatic artery, and a bile duct (Si-Tayeb, Lemaigre e Duncan, 2010). The portal triad drains into a central vein leading to the inferior vena cava to transport blood back to the heart via sinusoidal capillaries. These capillaries are lined by fenestrated endothelial cells, which facilitate contact of portal blood with hepatocytes (Figure 1.2) (Malarkey *et al.*, 2005).

1.3.2 Liver Blood Supply and Biliary System: The liver has a dual blood supply and can filter 30% of the total volume of circulating blood every minute (Sheth e Bankey, 2001). It receives portal and arterial blood from two main vessels; the hepatic artery which carries oxygenated blood from the aorta, and the portal vein which carries blood from the digestive tract, spleen, and pancreas to the liver (Sheth e Bankey, 2001; Jenne e Kubes, 2013). The blood is carefully filtered through the liver sinusoidal network where blood pressure significantly reduces and the flow of blood is slowed down, this maximises contact between the flowing blood and the endothelium for efficient clearance of pathogens (Oda, Yokomori e Han, 2003). Pathogen removal is achieved by endocytosis by liver endothelial cells, liver resident macrophages called Kupffer cells (KC), NK (Natural Killer) cells and NKT cells (Jenne e Kubes, 2013). An important function of the liver is bile production and transportation. Bile is transported within the liver by bile canaliculi and bile ducts which form a biliary tree in the liver. Bile canaliculi are situated adjacent to hepatocytes and run parallel to hepatic

sinusoids. These ensure that bile is efficiently drained away from the liver to the gall bladder via hepatic ducts where it remains until required for fat digestion (Roskams *et al.*, 2004).

1.3.3 Liver Metabolic Functions: Hepatocytes within the liver have a central role of metabolising complex carbohydrates, proteins, and lipids. Carbohydrate metabolism leads to increased levels of glucose entering the liver via the portal vein. Hepatocytes function to store this excess glucose as glycogen for release when required to maintain blood glucose levels (Klover e Mooney, 2004). Hepatocytes produce energy in the form of ATP by the metabolism of fatty acids and proteins entering the liver. Protein metabolism by hepatocytes requires processing of amino acids into urea which can be excreted in urine. Hepatocytes thus facilitate gluconeogenesis and produce lipids such as cholesterol. Targeted release of enzymes allows hepatocytes to remove harmful toxins from the liver such as drugs and alcohol. The liver can store essential nutrients, vitamins, and minerals obtained from circulation including vitamin A and D, B12, and essential minerals such as iron which can be released and used when required by the body. The liver also functions to produce blood plasma components such as albumin, prothrombin and fibrinogen (Klover e Mooney, 2004; Martini, Ober e Welch, 2006; Lodish, 2008).

1.4 Cells of the liver

1.4.1 Hepatocytes: Hepatocytes are the most abundant cell type in the liver constituting 70-80% of liver mass. They are situated around sinusoidal capillaries organised into bi-hepatocyte cords usually two hepatocytes thick. Hepatocytes are critical for liver function;

including liver regeneration, filtering portal venous blood, liver metabolic processes, bile production, protein synthesis, and toxin neutralisation. Hepatocytes contain a large number of cellular organelles mainly smooth and rough endoplasmic reticulum and mitochondria which facilitate these functions. They also have important roles in pathogen detection and aiding the immune response of the host. Hepatocytes facilitate the innate immune response by secretion of cytokines. Hepatocytes can also initiate the adaptive immune response by antigen presentation to naïve T cells and expression of MHC I and II (major histocompatibility complex) for lymphocyte interactions (Warren *et al.*, 2006; Jenne e Kubes, 2013).

1.4.2 Biliary Epithelial Cells: Biliary epithelial cells (BEC) line bile ducts. BEC comprise 3-5% of total liver cells and function in bile modification and transportation, liver homeostasis, facilitating growth of other resident liver cells, and toxin neutralisation in the biliary system. They also function as inflammatory response mediators to injury and disease by the expression of adhesion molecules, cytokines, chemokines, and pattern recognition receptors to facilitate leukocyte infiltration to the site via portal vessels (Németh *et al.*, 2009; Priester, Wise e Glaser, 2010; Juza e Pauli, 2014). The biliary system is continuous from the GI tract and prone to microbial infection, thus the flow of bile is essential as a preventative measure from infection. In biliary diseases, bile flow is obstructed and as a consequence higher rates of infection within the liver are observed (Björnsson, Kilander e Olsson, 2000). Subsequently biliary cirrhosis is a consequence of persistent inflammatory responses targeting the biliary epithelium which compromises the bile transportation system, and perpetuates

inflammation lead tissue damage via apoptosis and necrosis. This can lead to pathogenesis such as the bile duct vanishing syndrome which is a common feature of diseases of the biliary epithelia (Priester, Wise e Glaser, 2010).

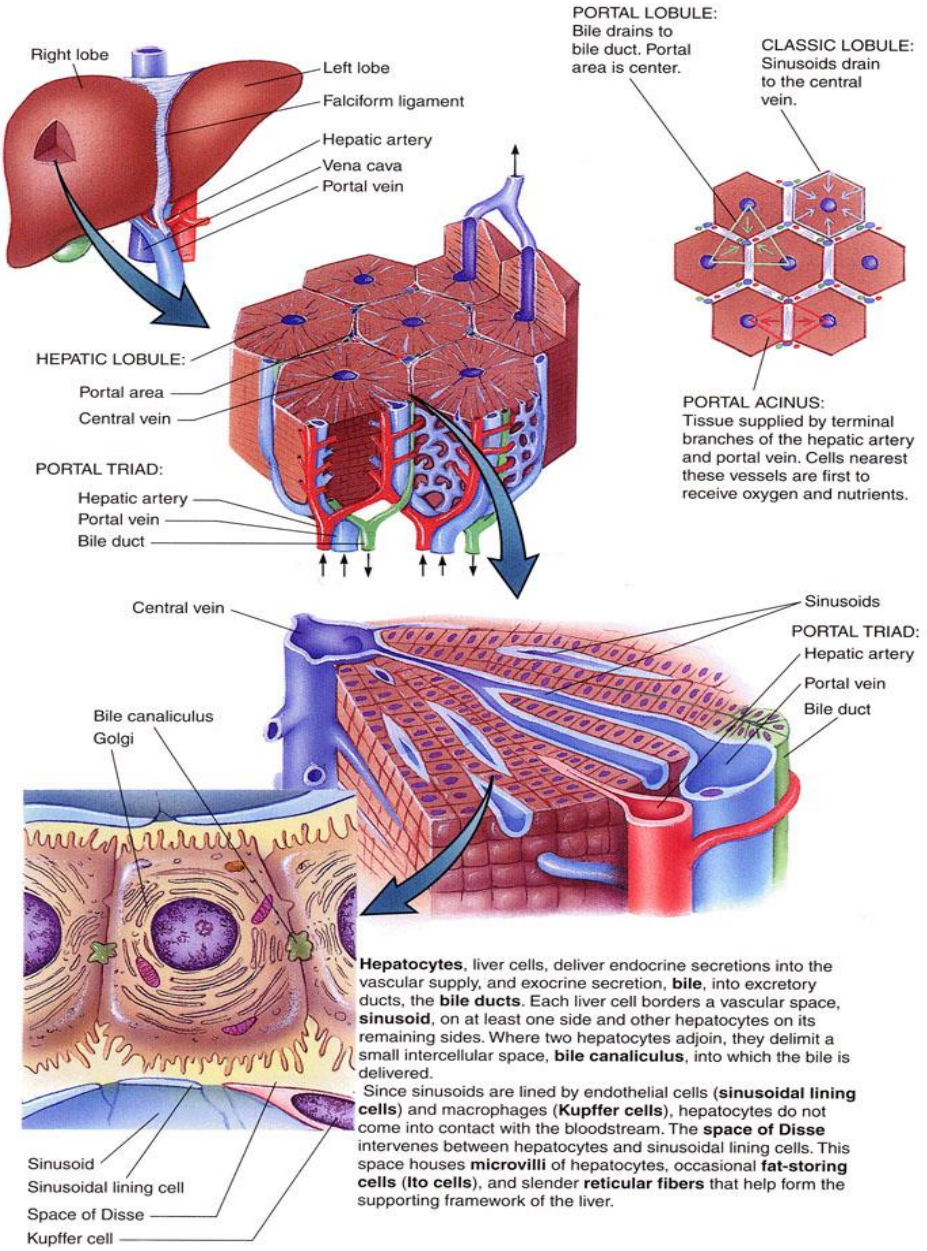


Figure 1.2 The liver architecture: The gross anatomy of the liver consists of the left and right liver lobe separated by the Falciform ligament. The liver functional units consist of hexagonal lobules of hepatocytes. At the periphery of each lobule, a portal triad is formed consisting of a portal vein, hepatic artery, and a bile duct. The portal triad drains in to a central vein leading to the inferior vena cava. Sinusoidal capillaries are lined by fenestrated endothelial cells, which facilitate contact of portal blood with hepatocytes. The biliary tree is formed by bile canaliculi which transport the bile produced by liver cells. Bile canaliculi run parallel to hepatic sinusoids (Si-Tayeb, Lemaigre e Duncan, 2010).
Image: (http://medcell.med.yale.edu/systems_cell_biology_old/liver_and_pancreas.php)

1.4.3 Hepatic Stellate Cells: Hepatic stellate cells (HSC) are located in the space of Disse; the perisinusoidal space between hepatocytes and Intra-hepatic endothelial cells. HSC have a star like conformation which allows them to attach to the IHEC (Intra-hepatic endothelial cells). Normal HSC contain lipid droplets within the cytoplasm which are important for vitamin A storage. They also are regulators of the basement membrane like matrix in hepatic sinusoids and thus regulate blood flow and portal pressure within the liver. Their critical function is the regulation of fibrosis during liver injury and insult, which they facilitate by excessive production of ECM (extra cellular matrix) and collagen deposition (Winau et al., 2008). HSC become activated, adopting a myofibroblastic phenotype during liver insult, subsequently leading to scar formation and loss of liver function, and ultimately chronic cirrhosis (Bartley et al., 2006). During chronic insult to the liver, HSC trans-differentiate, proliferate and facilitate matrix re-modelling. An enhanced inflammatory response is initiated, mediated by the chronically inflamed tissue and myofibroblasts are recruited to the site of injury. Once the liver insult is removed and the liver is repaired, the activated HSC are either returned back to their quiescent state or undergo apoptosis. Subsequently when this reversion process is impaired, chronic fibrosis is observed (Ramm, 2009). HSC also contribute to the liver immune response leading to liver damage by the expression of and responding to several cytokines, chemokines, growth factors, and inflammatory mediators. PDGF (platelet derived growth factor) and TGF-β (transforming growth factor beta) have been shown to promote the proliferative and fibrogenic response of HSC, and subsequently several molecules including MCP-1 (monocyte chemoattractant protein 1) and TNF-α (tumour necrosis factor alpha) contribute to the fibrogenic response by inhibiting HSC apoptosis (Pinzani e Marra, 2001).

1.4.4 Liver Immune Cells: Kupffer cells (KC) are liver resident macrophages making up 20% of non-parenchymal liver cells, with a primary role for the clearance of pathogens, red blood cells, and antigen presentation. It has been suggested that KC originate from bone marrow, and other work has also suggested that they are derived from local liver haematopoietic stem cells. They are stationary cells, situated adjacent to IHEC, where they have contact with flowing blood in the sinusoidal endothelium, and thus can have direct access to pathogens, where they can bind and endocytose them. This distinguishes KC from other monocyte and macrophage populations which crawl in search for potential pathogens (Sheth e Bankey, 2001; Bilzer, Roggel e Gerbes, 2006). KC also have direct contact with hepatocytes and can phagocytose apoptotic hepatocytes. Subsequently, the absence of KC and their receptors has been shown to lead to chronic bacteraemia and even host death (Bilzer, Roggel e Gerbes, 2006; Helmy *et al.*, 2006). KC endocytosis is further facilitated by the presence of various immune receptors on the surface of KC. Under basal conditions, KC poorly activate the adaptive immune response. Inflammatory conditions and pathogens can enhance KC activity promoting T cell activation. Activated KC are further known to express chemokines and cytokines, which can activate other KC (Jenne e Kubes, 2013).

Liver resident lymphocytes consist of approximately 10^{10} cells in the liver; these include innate lymphocytes such as NK cells, and adaptive T and B lymphocytes. NK cells form up to 50% of lymphocytes in the liver (Racanelli e Rehermann, 2006) and have important immuno regulatory roles whereby they respond to ligands present on damaged, infected, and tumour cells. They also express a large number of cytokines when activated which facilitate immune

response functions, and are regulated by MHC expression by hepatocytes and HSC. NK cells control the proliferation and migration of NKT cells (Jenne e Kubes, 2013). NKT cells are highly abundant in the liver in comparison to other organs, 50% of NKT cells express the T cell receptor (TCR) with the majority of CD8+ T cells to CD4+ T cells (classical NKT cells), and the non-classical NKT cells which can co-express TCR-αβ and TCR-δβ. They also have central immuno-regulatory roles, expressing a large number of cytokines, and have anti and pro-inflammation properties. NKT cells actively contribute to immune surveillance in the liver by patrolling the liver vasculature for potential pathogens, and they recognise antigens possessing MHC class 1 CD1d (cluster of differentiation 1d) (Exley e Koziel, 2004; Racanelli e Rehermann, 2006).

Intrahepatic dendritic cells (DC) are derived from the bone marrow and are typically observed in the liver vasculature such as the central veins and portal tracts. Resting dendritic cells have antigen presentation functions and are described as having tolerogenic functions. When DC are activated they carry their antigen products and they migrate to extra-hepatic lymph nodes via the lymphatics in the portal tracts and Space of Disse, and initiate an immune response by activating lymphocytes (Racanelli e Rehermann, 2006; Jenne e Kubes, 2013).

1.4.5 Intra-Hepatic Endothelial Cells: IHEC form 50% of the reticulo-endothelial system which is comprised of IHEC and KC (Juza e Pauli, 2014). The primary function of this system is the removal of waste products, clearance of viruses and lipo-polysacharides (LPS) from blood.

This is achieved by efficient pathogen detection, capture and antigen presentation (Smedsrød, 2004; Racanelli e Rehermann, 2006). They have no organised basement membrane and form the Space of Disse, creating a gap which avoids direct interaction between themselves and hepatocytes (Figure 1.3). IHEC separate hepatocytes from blood flow in the sinusoidal lumen. They cluster into sieve plate structures which form fenestrations of approximately 100nm diameter, this allows passage and exchange of fluids, solutes, and molecules between the sinusoidal lumen and parenchyma, and thus allowing interactions of these with hepatocytes (Wisse *et al.*, 1996; Lalor *et al.*, 2006). This facilitates contact between residing hepatocytes and flowing lymphocytes (Jenne e Kubes, 2013).

IHEC tightly regulate the immune response and inflammation in the liver. This is achieved by an abundance of receptors present on the surface of these cells, including scavenger, mannose, and immunoglobulin receptors. IHEC can express MHC I and II, adhesion molecules, and various immune receptors to initiate receptor mediated endocytosis; internalising antigens, cellular waste products, and immune complexes, and presentation of blood and gut derived pathogens to T cells in circulation (Jenne e Kubes, 2013). During critical disease processes IHEC lose their fenestrations and can form a basement membrane leading to subsequent hepatic dysfunction, as IHEC have a more vascular capillarised morphology with increased expression of CD31 and VCAM (Xu *et al.*, 2003). Loss of fenestrations is linked to disease (Mak e Lieber, 1984), liver infection (Steffan *et al.*, 1995) and ageing (LeCouter *et al.*, 2003). IHEC also have the ability to prevent the activation of HSC, however capillarised IHEC which form in diseased livers, lose this functional

characteristic which can thus contribute to chronic fibrosis (DeLeve, 2013). IHEC are also critical to the angiogenic response (the formation of new blood vessels from pre-existing vessels) of the liver. This is critical to resolve liver injury during tissue damage, wound healing, and remodelling processes (See chapter 4), as IHEC can undergo proliferation and migration, and form vascular structures; tightly regulated by VEGF (vascular endothelial growth factor) (DeLeve, 2013).

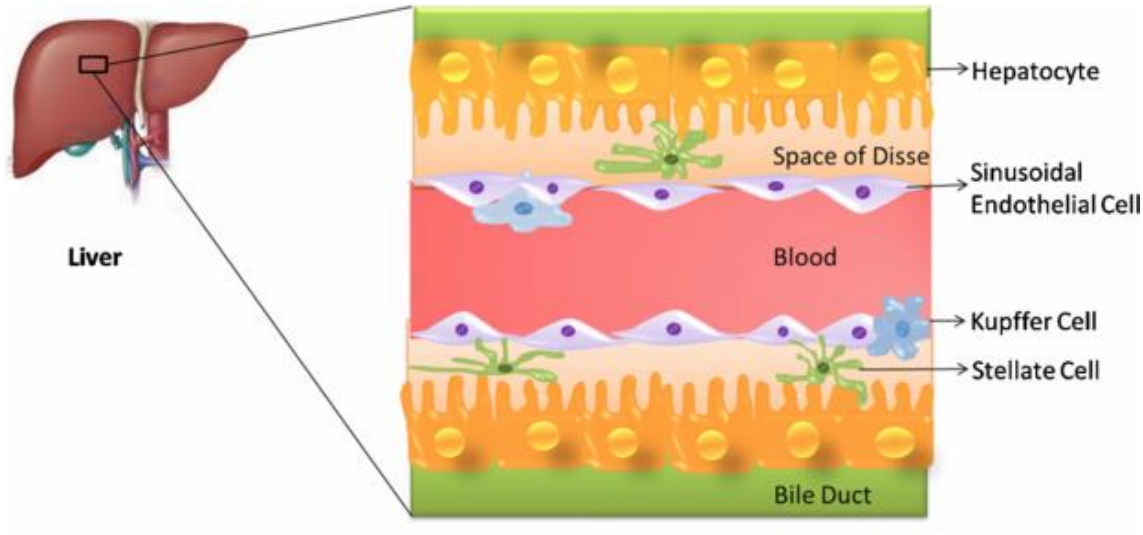


Figure 1.3 Organisation of hepatic cells within the lobule: The sinusoid is comprised of IHEC and hepatocytes. IHEC separate hepatocytes from blood flow in the sinusoidal lumen. IHEC have no organised basement membrane and so form the Space of Disse. They cluster into sieve plate structures which form fenestrations, this allows tightly regulated passage and exchange of fluids, solutes and molecules and thus allowing interactions of these with hepatocytes. Kupffer cells are found situated next to IHEC where they have direct contact with flowing blood and can phagocytose pathogens from flow. HSC are found present in the Space of Disse, they regulate hepatic blood flow and pressure and have critical functions in perpetuating liver fibrosis when activated. Bile ducts are found parallel to hepatic sinusoids, they facilitate transport of bile. *Image:* (Kang *et al.*, 2013).

1.5 Leukocyte – endothelium interactions

Leukocyte – endothelial interactions are vital for the regulation of inflammatory processes in response to immune activation, infection, and injury. The general leukocyte recruitment cascade involves the movement of leukocytes to the vascular endothelial wall as they leave circulation (Figure 1.4). This process requires leukocyte tethering to the endothelium, initiated by selectin mediated capture. Rolling is the process between firm and lack of adhesion, which is initiated by the activation of receptors expressed on endothelial cells such as selectins, integrins, adhesion molecules, and immunoglobulins (Ley *et al.*, 2007). Enhanced shear stress allows firm bonds between selectins and their ligands, and the rolling velocity determines the level of leukocyte exposure to stimuli on the vessel wall. Specifically, rolling requires interactions of L-selectin, P-selectin and E-selectin; which are expressed on leukocytes and endothelial cells, with glycosylated ligands specifically PSGL-1 (p-selectin glycosylated ligand 1) (McEver e Cummings, 1997). Selectin interactions thus allow the binding of leukocytes to endothelium under conditions of blood flow, and are further enhanced by cytokine and chemokine activation of leukocytes via G protein coupled receptors present on endothelial cells in response to selectin mediated rolling, this initiates an intracellular signalling cascade. Subsequent signalling from integrins and chemokines allow firm arrest of leukocytes whilst α4, β2 integrins support rolling and mediate adhesion via adhesion molecules (Shamri *et al.*, 2005). The leukocytes adhere to the endothelium and migrate between and through endothelial cells which requires leukocytes to change shape, this process is mediated via activated integrins, cytoskeletal reorganisation, and cell motility. The leukocytes are then directed to the site of inflammation in the tissue as they transmigrate across the endothelium (Ley *et al.*, 2007; Granger, 2010).

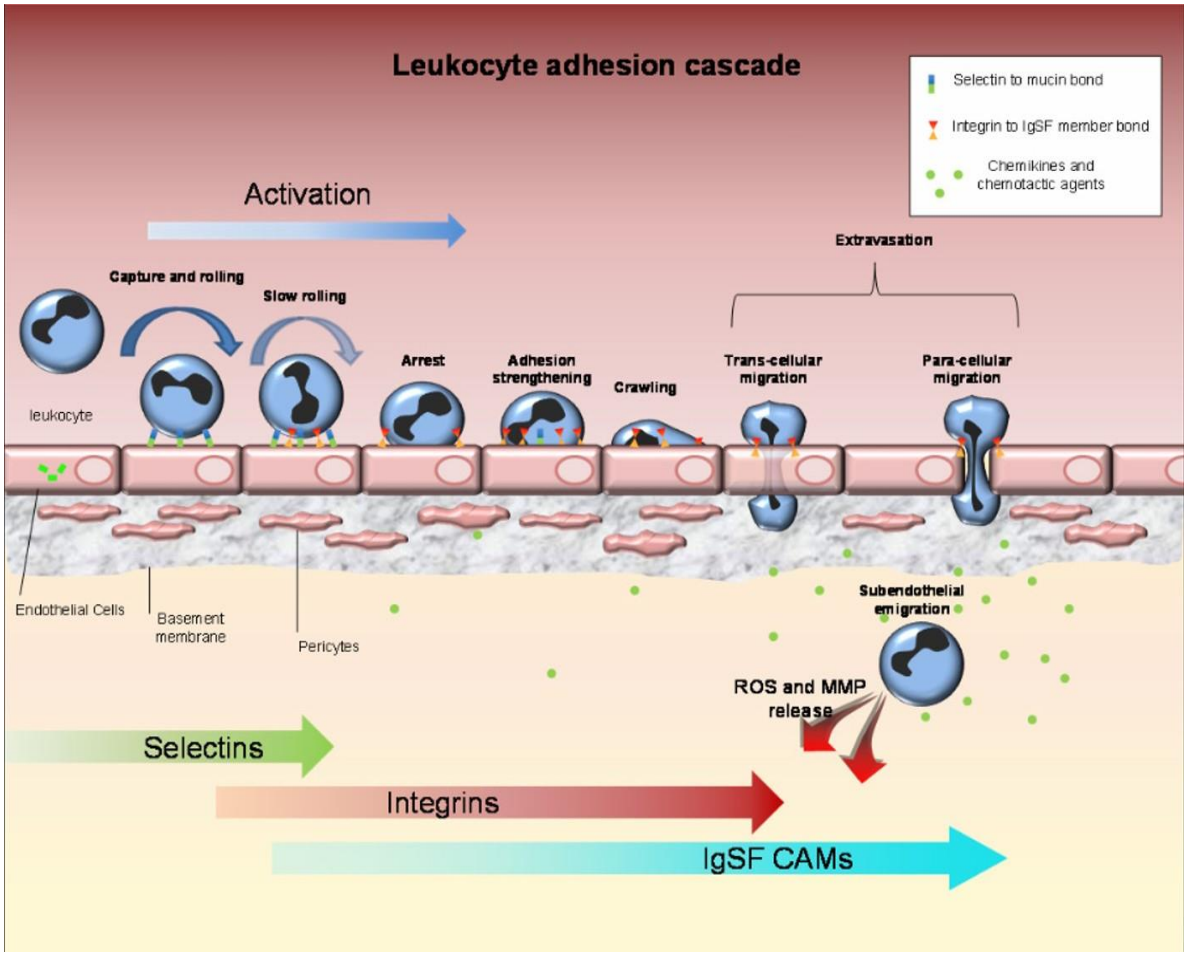


Figure 1.4 leukocyte recruitment cascade: Leukocytes leave circulation and migrate towards the endothelial cell wall. The endothelium then captures the leukocytes, rolling is initiated followed by arrest and firm adhesion. This process is strengthened by G protein coupled receptor interactions between leukocytes and endothelial cells via cytokine and chemokine activation. This initiates intracellular signalling cascades. The leukocytes transmigrate through the endothelium and are directed towards the site of inflammation where they contribute towards resolution of inflammation and tissue repair. All of these processes are tightly regulated by specific molecules such as selectins, integrins and immunoglobulins which mediate each specific process.
http://www1.imperial.ac.uk/resources/A0F93F15-C796-4B19-9631-06877300AE3B/pic3.jpg

1.6 Leukocyte-endothelium interactions in the liver

The liver under normal physiological conditions has large granular lymphocytes called pit cells, present in the sinusoidal endothelium, which are thought to have entered the liver via circulation (Doherty e O'Farrelly, 2000). Lymphocytes are also present in portal tracts and in the parenchyma via circulation, and perform local surveillance activities (Faint *et al.*, 2001). The local distribution of lymphocytes in normal liver and diseased liver varies and is highly dependent on the stimuli leading to subsequent leukocyte recruitment. KC are also present within the liver, and may largely be replenished by monocytes in circulation (Gale, Sparkes e Golde, 1978). Infiltration of monocytes contributes to liver inflammatory and fibrotic responses during liver injury (Imamura *et al.*, 2005; Heymann, Trautwein e Tacke, 2009). These can aid the progression of fibrosis by the subsequent release of enhanced cytokine expression, augmenting the inflammatory response and the subsequent activation of HSC (Imamura *et al.*, 2005). Migration of monocytes into the tissues and differentiation into macrophages and DC is determined by adhesion proteins, chemokines, and pattern recognition receptors (Geissmann *et al.*, 2010). Lymphocyte infiltration within the liver is also a tightly regulated process which is first determined by the port of entry of lymphocytes; either from the gut or circulation, and also contributes to distinctive infiltrate patterns (Figure 1.5). For example in normal liver, ICAM-1 (Intracellular adhesion molecule – 1), VAP-1 (vascular adhesion protein – 1) and low chemokine expression has been observed. In inflammatory diseased liver increased ICAM-1 and chemokine expression has been observed with the addition of VCAM-1 (Vascular cell adhesion molecule), and P and E-selectin expression, whilst VAP-1 expression has been shown to be maintained throughout (Lalor *et al.*, 2002; Jenne e Kubes, 2013).

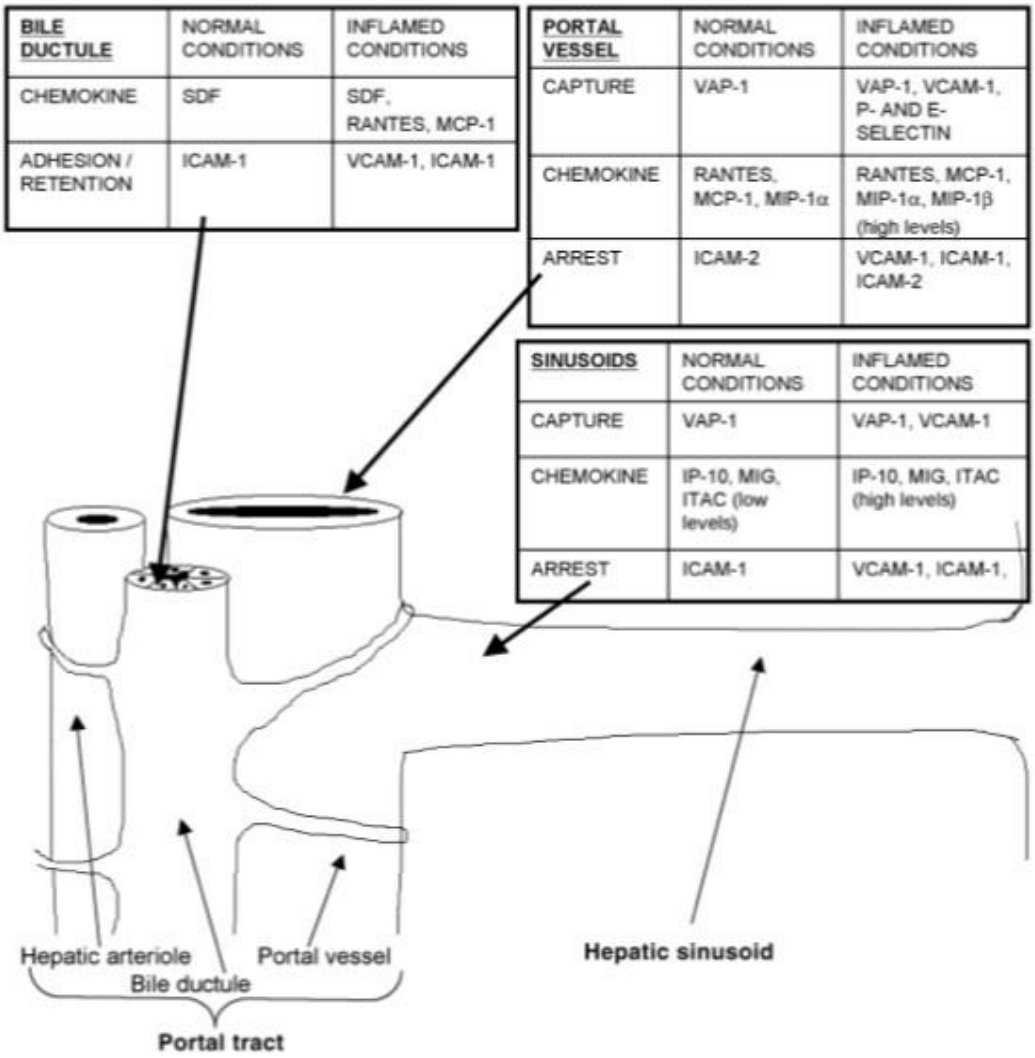


Figure 1.5 Leukocyte endothelium interactions during liver inflammatory disease: this figure illustrates the different molecules which promote leukocyte interactions with endothelial cells. These molecules include adhesion molecules, cytokines, chemokines, and retention molecules. Portal vessels, bile ducts and hepatic sinusoids within the liver under normal and inflammatory conditions can promote differing responses of leukocyte interactions. They achieve this by the expression of specific molecules to initiate specific leukocyte cascade responses (Lalor *et al.*, 2002).

Subsequently, leukocyte recruitment in the liver has also been shown to be co-ordinated by liver sinusoids via adhesion molecule interactions with the sinusoidal endothelium (Jaeschke e Smith, 1997). Blood flow while passing through the liver sinusoids significantly decreases

allowing endothelium and leukocyte maximal interactions. The endothelium has adhesion protein expression including ICAM and VAP-1 which promote lymphocyte adhesion and trans-endothelial migration (McNab *et al.*, 1996; Yoong *et al.*, 1998). In diseased liver, adhesion protein expression has been found up-regulated, and VCAM expression has also been found in the sinusoidal endothelium, followed by neovascularisation processes. During inflammatory conditions these adhesion proteins play important roles during leukocyte recruitment; leukocyte capture in the sinusoidal endothelium has been shown to be modulated by VAP-1, VCAM and α4 integrins, and firm adhesion achieved by β2 integrins and ICAM (Lalor *et al.*, 2002).

It has been reported that in chronic Inflammatory liver disease (CILD) such as PBC, PSC and hepatitis C, leukocyte infiltrates can organise into portal associated lymphoid tissue (PALT) (Adams e Afford, 2002; Grant *et al.*, 2002). PALT are formed to facilitate leukocyte recruitment and retention during CLD. These follicles are formed by B and T lymphocyte accumulation, dendritic cell recruitment, CD34+ vessel formation, and increased number of high endothelial venules. During chronic liver disease, new blood vessels show enhanced expression of adhesion proteins and chemokines, and new vessel formation has been shown to trigger the formation of secondary high endothelial venules, which facilitate the recruitment of lymphocytes (Wanless, Nakashima e Sherman, 2000; Hjelmström, 2001; Grant *et al.*, 2002). Dendritic cells have also been detected in portal tracts which are recruited in response to liver infection and contribute towards PALT (Kudo *et al.*, 1997).

1.7 Chronic inflammatory liver disease

CILD is a leading cause of morbidity and mortality in the world. CILD can be caused by genetic factors, viral infections, toxicity, metabolic disorders, and autoimmunity. The onset, progression and pathogenesis of various CILD is a highly complex process, therefore for the purpose of this thesis some CILD will be discussed briefly.

1.7.1 Alcoholic liver disease: Alcoholic liver disease (ALD) is caused by the excessive consumption of alcohol, leading to several chronic conditions in the liver including; steatosis, steatohepatitis, fibrosis, cirrhosis, and ultimately in some cases leading to hepatocellular carcinoma. The progression of ALD is still speculation lead and thought to be linked to processes such as insulin resistance and metabolic syndromes (Purohit, Gao e Song, 2009). The consequences of ALD are loss of liver function; including hepatocyte death, HSC activation leading to fibrosis, and damage caused by the inflammatory responses of the innate immune system (Byun e Jeong, 2010). It has been found that ALD progresses from a large range of molecular and cellular events. Briefly; steatosis in ALD livers is thought to be derived by HSC derived endocannabinoid and interactions with the CB1 receptor (cannabinoid receptor type 1) (Jeong *et al.*, 2008). Subsequent inhibition of anti-fibrotic measures after exposure to excessive alcohol is thought to be mediated by NK cells and IFN-γ (interferon gamma) against HSC, and further perpetuated by TGF-β expression (Jeong, Park e Gao, 2008). Fat accumulation observed in hepatocytes is thought to be due to imbalanced fat metabolism caused by excessive alcohol intake, such as enhanced triglyceride synthesis and inhibited mitochondrial lipid oxidation (Purohit, Gao e Song, 2009). KC have been shown

to contribute to ALD progression by responding to excessive LPS infiltration from the gut caused by alcohol, subsequent enhanced inflammatory responses are initiated by KC, followed by enhanced reactive oxygen species, collagen, and TGF-β expression (Mandrekar e Szabo, 2009).

1.7.2 Non-alcoholic steatohepatitis: Non-alcoholic steatohepatitis (NASH) resembles ALD but is diagnosed after excluding that the patient has not consumed excessive alcohol. NASH is developed from the progressive pathogenesis of NAFLD (Non-alcoholic fatty liver disease), by subsequent enhanced inflammation, steatohepatitis, fibrosis, and cirrhosis. The onset of NAFLD is usually associated with metabolic disorders such as diabetes, obesity, dyslipidaemia, and nutritional causes. The progression of NASH is thought to be due to several causes such as excessive inflammatory cytokine presence, mitochondrial dysfunction, oxidative stress, insulin resistance lead adipose tissue lipolysis leading to enhanced free fatty acid accumulation (Dowman, Tomlinson e Newsome, 2011).

1.7.3 Cholangiopathies: Cholangiopathies are diseases caused by the destruction of cholangiocytes (BEC). These are characterised by the destruction or loss of BEC by apoptosis and necrosis, and subsequent ductal branches formed in order to try and regain bile duct function by BEC proliferation, and persistent inflammation. This leads to hepatic cirrhosis and portal hypertension (O'Hara *et al.*, 2013). Primary biliary cirrhosis (PBC) is autoimmune mediated liver damage caused to the intrahepatic biliary tree, which is characterised by the presence of automitochondrial antibodies present in serum. This can cause chronic

cholestasis which is the build-up of excess bile, inflammation of the portal tract, and fibrosis, ultimately leading to cirrhosis and liver failure. PBC is also characterised by granuloma formation, and in later stages nodule formation and fibrous tissue. PBC is primarily limited to smaller interlobular and septal bile ducts, and PBC is highly prevalent in women in comparison to men at a ratio of 9 to 1, suggesting possible genetic causative factors (Selmi *et al.*, 2010; Selmi *et al.*, 2011).

Primary sclerosing cholangitis (PSC) is best described as an immune mediated disease rather than an autoimmune mediated liver disease. It is caused by inflamed bile ducts, leading to scar tissue formation, which can narrow the bile ducts preventing bile transport to the gut, and causing a build-up of bile in the liver. PSC is mostly found to effect intra and extra-hepatic bile ducts. PSC is most commonly found in men, and genetic causative factors have been proposed for the onset of PSC. PSC is also often associated with irritable bowel syndrome (IBS), with 70% of PSC patients developing IBS. This is thought to be due to 'the leaky gut' syndrome which allows the influx of microbes and metabolites from the gut to the biliary tree, and there are also links of enhanced lymphocyte infiltrate to the liver from the gut leading to PSC progression via inflammation. PSC is also linked to cholangiocarcinoma formation which is not often seen in PBC patients (Silveira e Lindor, 2008; O'Hara *et al.*, 2013).

1.7.4 Hepatocellular Carcinoma: Hepatocellular carcinoma (HCC) is often the final consequence of pathogenesis from chronic cirrhosis of many inflammatory liver diseases

including; autoimmune disease, hepatitis B and C, alcohol, chronic inflammation, and haemachromatosis. Hepatitis virus infection is a major risk factor for HCC. 70% of patients with HCC have HCV (Hepatitis C virus) in their serum (Nishioka, 1991). It is hypothesised that the cirrhosis associated with disease and viral infection caused by the consistent damage and repair cycle induced by the immune system, enhances the risk of mutations as cells follow the cell cycle. This is also seen in cases of chronic cirrhosis which go on to develop HCC (Gomaa *et al.*, 2008). This has also been seen in patients with HBV (Hepatitis B virus). The HBx (Hepatitis B viral protein) has been associated with encoding oncogenic viral proteins which lead to HCC development, this has been demonstrated in transgenic mice overexpression of HBx protein. It has also been shown that HCC progression may be mediated by p53 suppression (Michielsen, Francque e van Dongen, 2005; Liu e Kao, 2007). Similarly cirrhosis induced by toxic damage by Aflatoxin B1, enhances progression of HBV induced HCC (Bruix *et al.*, 2001), and cirrhosis induced by diabetes related NAFLD and NASH has been shown to enhance the risk of developing HCC by 3 fold (Davila *et al.*, 2005).

1.8 Liver Regeneration

In between the constant exposure to pathogens, toxins, injury, viruses, and disease, the liver has the ability to efficiently regenerate itself. Liver mass regeneration has been studied extensively using models of partial hepatectomy (PHx) of up to 70% (Figure 1.6), often accompanied by other liver injury models such as CCL4 (carbon tetra chloride) to study inflammatory and necrotic responses during regeneration. Hepatocytes are the main driving cells of liver regeneration, whereby their ability to undergo indefinite DNA synthesis allows

the liver to compensate for damage and loss of tissue via hepatocyte proliferation (Fausto e Campbell, 2003). In the normal liver, hepatocytes and IHEC remain in a quiescent state. After PHx, hepatocytes remain uninjured however the hepatic vascular structure significantly changes leading to enhanced sinusoidal blood flow, subsequent portal capillary pressure, and influx of growth factors and hormones. In response to these changes, hepatocyte proliferation is the first to be initiated regulated by cytokine activation, transcription factors, growth factors, cell cycle regulation, matrix remodelling, and various regeneration regulating genes, specifically; urokinase plasminogen activator (uPA) and HGF (hepatocyte growth factor) (Kang, Mars e Michalopoulos, 2012). Hepatocytes undergo DNA synthesis for two cycles within 24-36 hours post PHx followed by a wave of apoptosis to compensate for the excessive proliferation (Sakamoto *et al.*, 1999). Hepatocyte proliferation is followed BEC proliferation by 48-72 hours this is then followed by KC and HSC proliferation, and finally IHEC are activated in response to hypoxia and hepatocyte signalling to proliferate and migrate to formed hepatocyte clusters, leading to separation of hepatocytes, recanalization, and the initiation of vascular sinusoidal structure formation (Wack *et al.*, 2001; Kang, Mars e Michalopoulos, 2012).

Hepatocytes produce mitogenic signals for other non-parenchymal cells in the liver by the expression of growth factors specific to proliferation such as PDGF, VEGF, FGF (fibroblast growth factor), and angiopoietins (Block *et al.*, 1996; Michalopoulos, 2007). Non parenchymal cells in response provide hepatocytes with growth factors such as HGF, EGF (epidermal growth factor) and HB-EGF (heparin binding epidermal growth factor), as well as

KC provide TNF-α and IL-6 (Interleukin-6). HGF is initially activated from inactive stored HGF in the liver ECM, and then subsequently HGF is synthesised by IHEC and HSC. HGF binds and activates its tyrosine kinase receptor MET to activate signalling to promote hepatocyte proliferation and aid hepatocyte survival, to maintain their correct morphology, and promote hepatocyte mitogenesis (LeCouter *et al.*, 2003). HGF has been shown to significantly contribute to *in vivo* and *in vitro* liver regeneration and proliferation, and without the presence of HGF, liver regeneration has been shown to be a redundant process (Liu *et al.*, 1994; Block *et al.*, 1996). TNF-α and IL-6 produced by KC contribute to liver regeneration indirectly by activating other processes which contribute to liver regeneration. TNF has shown to contribute to regeneration via activation of NF-kB (nuclear kappa light chain activator of B cells) and growth factors. IL-6 has been shown to activate STAT3 (signal transducer and activator of transcription 3) and act as a direct mitogen for BEC. KC depletion has subsequently shown inhibitory effects on liver regeneration (Liu *et al.*, 1998; Webber *et al.*, 1998; Michalopoulos, 2007), and antibodies and genetic deletions abrogating the TNF response have also shown inhibited liver regeneration responses (Kirillova, Chaisson e Fausto, 1999; Michalopoulos, 2007).

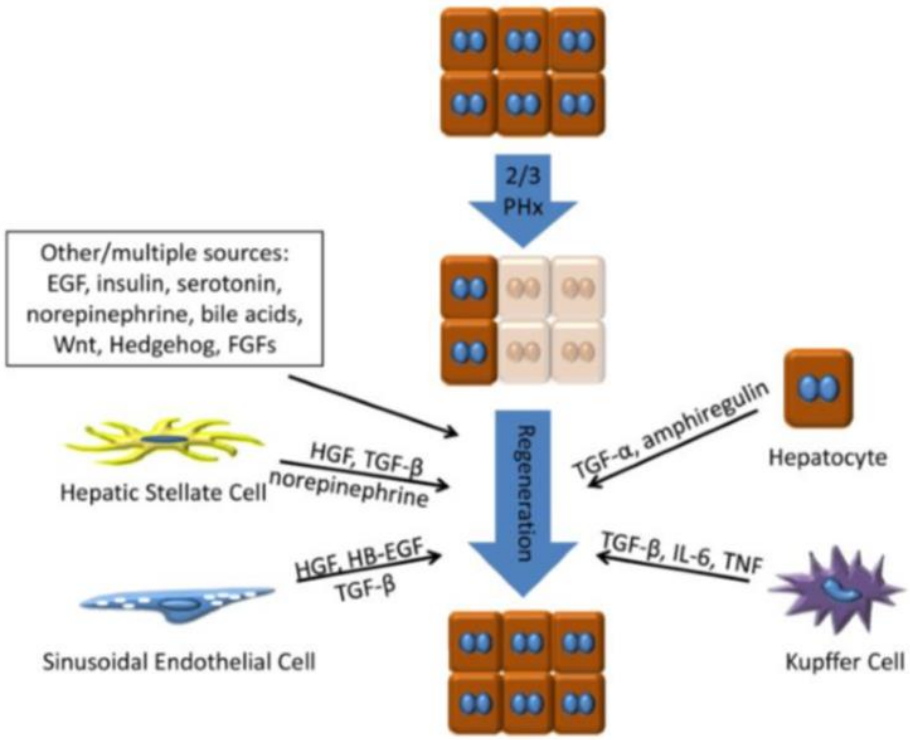


Figure 1.6 Liver regeneration after partial hepatectomy: The liver has the ability to regenerate liver mass after up to 70% PHx. Liver regeneration is a highly regulated process supported by indefinite proliferation of hepatocytes which is facilitated by growth factors, cytokines, chemokines, hormones, and signalling promoted by other resident liver cells including KC, HSC and IHEC. (Kang, Mars e Michalopoulos, 2012)

Remodelling of vascular structures via angiogenesis is crucial to the regeneration process of the liver via endothelial cells. Sato T *et al* showed after PHx in rodent liver models, IHEC proliferation was coupled to VEGF related proteins and Angiopoietin/Tie family proteins expressed simultaneously, suggesting an IHEC response closely related to angiogenesis (Sato *et al.*, 2001). bFGF has been shown to enhance IHEC proliferation, and subsequently bFGF and VEGF are known factors for regulating liver regeneration as well as angiogenesis (LeCouter *et al.*, 2003). VEGF have been shown to promote proliferation of endothelial cells during regeneration and enhancing vascular permeability by maintaining endothelial cell

fenestrations. VEGF also supports the breakdown of the endothelial extracellular matrix by enhanced expression of proteases, specifically uPA, MMP's (Matrix metalloproteinases) and collagenase. Hepatocytes normally express low amounts of VEGF but enhanced VEGF expression has been observed during liver regeneration leading to enhanced cytokine induction and hypoxia. Several studies have shown VEGF induction post PHx which may induce IHEC and hepatocyte proliferation leading to gain of liver mass and hepatic recovery (Fernández *et al.*, 2009). VEGF may induce HGF expression from IHEC to enhance hepatocyte proliferation, contributing to a liver regeneration process driven by IHEC and hepatocyte co-regulation. It has been found in a recent study that the endothelial progenitor cell populations may be adopting paracrine mechanisms with IHEC to facilitate regeneration, where they showed that IHEC progenitor cells enhanced the ability of IHEC to undergo proliferation via an angiogenesis tube formation assay. They found that this was mediated by the up-regulation of angiogenesis inducing factors; VEGF and PDGF (Kaur *et al.*, 2012). The role of IHEC during liver regeneration has become a controversial issue, as more recent publications have made suggestions that bone marrow derived liver progenitor cells of IHEC, may be the main contributors of liver regeneration. They have suggested that these cells are recruited in response to injury, and are expressing far higher amounts of HGF in comparison to IHEC, hence driving hepatocyte proliferation. However, as these cells share cell surface markers it has been challenging to distinguish between cell populations of IHEC and IHEC progenitors (Wang *et al.*, 2012).

1.9 TNF Superfamily

TNF was first chemically isolated in 1984 from macrophage conditioned media and found to have the ability to necrotise tumour cells (Old, 1985), it has since been found to be expressed by many different cells including monocytes, macrophages, and lymphocytes. Since this discovery; the TNF Superfamily (TNFSF) of ligands and receptors has developed and grown to approximately 19 ligands and 32 receptors which can form several ligand receptor pairs (Table 1). TNFSF ligands possess the TNF homology domain, which is a folded anti parallel β sandwich, which assembles to form a trimer and forms a binding site capable of binding multiple receptors (Ware, 2003; Aggarwal, Gupta e Kim, 2012). TNF ligands are typically type II transmembrane proteins with an extracellular domain which can be cleaved to form a functional soluble cytokine (Ware, 2003). Membrane bound and soluble proteins can activate various signalling pathways. TNF receptors are mostly expressed as type I transmembrane proteins and contain one to six cysteine rich domains (CRD) in their extracellular region. TNF receptors can possess a death domain in their carboxyl terminal, this can form death inducing signalling complexes (DISC) with death inducing ligands such as TNF, Fas-L (Fas Ligand), and TRAIL (TNF related apoptosis inducing ligand), and mediate apoptotic cell death. Some receptors do not have the death domain and function via TRAF (TNF receptor activating factor) binding in response to TNF receptor ligand interactions to activate various signalling pathways including NF-kB (Aggarwal, 2003).

TNFSF members are important mediators of the adaptive immune response and can regulate a range of functions from necrosis, apoptosis, proliferation, differentiation, inflammatory

responses, tumourigenesis and viral replication. The range of functions observed from TNF ligand and receptor interactions may be in large due to the ability of each ligand to bind several receptors (Table 1). TNF ligands are predominantly expressed by immune effector cells, whereas TNF receptors (TNFR) have a wider expression pattern. For example, TNF is expressed by several immune cells whereas TNFR1 has shown expression in all types of cells, signifying the diverse functional capabilities of TNF via its receptor interactions (Aggarwal, 2003; Aggarwal, Gupta e Kim, 2012; Bremer, 2013). TNF ligands and receptors have been found to be key regulators during pathological disease progression. In developing a deeper understanding of their interactions, functions, and signalling mechanisms in various diseases, we can improve and develop potential targeted therapies via the immune system towards inflammatory disease and cancer in future.

1.10 TNFSF during inflammatory liver disease

Many TNFSF members are known to contribute to the inflammatory response mediated by the immune system which is often seen during the progression of inflammatory liver disease. For the purpose of this thesis, some TNF ligands which have been highly characterised and studied extensively for their receptor interactions will be discussed briefly:

1.10.1 TNF-α: TNF-α was the first identified member of TNFSF of ligands and found to be expressed mostly by cells of the immune system including macrophages and lymphoid cells, as well as a variety other cells including endothelial cells and fibroblasts. It has a membrane bound 26 kDa (kilo dalton) form which can be cleaved by TNF-α converting enzyme (TACE) to

release a soluble functional 17kDa form. TNF functions via interactions with two receptors, TNFR1 and TNFR2, and can subsequently regulate a whole host of biological functions. Notably it has been known to be a key ligand mediating the host pro-inflammatory response specifically via NF-kB signalling (Bradley, 2008) TNF has been reported in several studies to contribute to liver disease. Briefly; TNF-α has been found up-regulated in hepatic tissue and serum from ALD samples obtained from humans and mice, suggesting TNF induced cytotoxicity may have a role in metabolic dysfunction. It was further speculated that TNF induced cytotoxity was mediated by NF-kB which promotes hepatic protection, as down-regulation of NF-kB up-regulated TNF expression, leading to TNF induced hepatocyte death (McClain *et al.*, 1998). Similarly serum TNF-α has been described as a potential marker for liver disease, as serum levels of TNF-α have been found to correspond to the severity of liver damage induced in patients with a history of acute pancreatitis (Liang *et al.*, 2010). TNFR1 mediated fibrosis in hepatic damage has also been observed (Tarrats *et al.*, 2011). HSC isolated from TNFR1 knockout mice showed decreased pro-collagen expression, HSC proliferation, and PDGF dependent mitotic signalling. It was further found that TNFR1 mediated MMP-9 expression. Subsequent *in vivo* studies in TNFR knockout mice showed attenuated fibrosis during liver injury. These results indicated a critical role of TNF in mediating liver fibrosis during liver injury via HSC, and demonstrated that TNFR1 may be a potential target for therapeutic development in future (Tarrats *et al.*, 2011). Furthermore, TNF-α has been found to regulate LPS induced cholestatic sepsis in the liver via aquaporin-8; a hepatocyte canalicular protein. A TNF fusion protein TNF75:Fc was shown to inhibit LPS induced aquaporin-8 expression and bile flow impairment, and subsequent TNF-α recombinant protein down-regulated aquaporin-8 expression (Lehmann *et al.*, 2008). TNF

has been shown to also mediate hepatic growth hormone resistance during sepsis in rat cultured hepatocytes via interfering with the Jnk (c-jun N terminal kinase) 2/STAT 5 signalling pathway, and growth hormone has been shown to contribute to muscle protein catabolism during sepsis (Yumet *et al.*, 2002).

1.10.2 CD40-Ligand: CD40-Ligand (CD40-L) has been found expressed on activated T cells, B cells, monocytes, and several other cell types (Kawabe *et al.*, 2011). Its receptor CD40 was initially characterised to be expressed on B cells, but has also since been found expressed on a wide variety of cell types including DC, monocytes and non-haematopoietic cells. CD40 ligand-receptor interactions have known functions in regulating cellular adaptive immune and inflammatory responses including specific antigen presentation functions (Elgueta *et al.*, 2009; Kawabe *et al.*, 2011). CD40 receptor ligand interactions have been shown to modulate cell fate and inflammation during CLD in several studies. Afford *et al* 1999 showed CD40 induction can promote apoptosis in cultured human hepatocytes in CLD, they showed that this process was induced via Fas activation as enhanced Fas-L was observed after CD40 ligation. They subsequently showed that CD40 mediated hepatocyte apoptosis was down-regulated in response to anti Fas-L mAb (monoclonal antibody) (Afford *et al.*, 1999). CD40 expression has been observed in liver disease tissue as a marker of apoptosis, with enhanced expression observed in viral hepatitis tissue (Schmilovitz-Weiss *et al.*, 2004). CD40 has been described as a functional receptor during HCV associated liver disease. It has been shown to be an indicator of hepatocyte cell fate; as CD40 positive hepatocytes have been found in HCV liver tissue, localised to periportal and lobular areas, and in areas of increased

inflammatory infiltrates (Shiraki *et al.*, 2006). CD40 expression was found to correlate to disease severity indicated by histology grading. Enhanced CD40 mediated apoptosis has been observed in hepatocytes in combination with Actinomycin D, and anti-CD40 was shown to activate the pro-inflammatory NF-kB signalling pathway in HepG2 cells (Shiraki *et al.*, 2006). Anti CD40 therapy in inbred mice has shown B cell and macrophage mediated inflammatory liver disease progression which was suppressed by B cell deficient mice, and by macrophage depletion. Disease progression by anti-CD40 was mediated by inflammatory cytokines IFN-γ and TNF-α via inflammatory cell recruitment (Kimura *et al.*, 2006).

Table 1 The TNF superfamily: The TNF superfamily of ligands and receptors and their expression (MacEwan, 2002; Aggarwal, 2003; Ware, 2003; Aggarwal, Gupta e Kim, 2012)

	TNF Ligands	Cell Expression	TNF Receptors	Cell Expression
TNFSF1	TNF-β	NK, T and B cells	TNFR1, TNFR2	Most normal and transformed cells, immune and endothelial cells
TNFSF2	TNF-α	Macrophages, NK, T and B cells	TNFR1, TNFR2, HVEM	Immune and endothelial cells
TNFSF3	LT-α/β	DC's. T and B cells	LT-βR	T cells and NK cells
TNFSF4	OX40L	T and B cells, DC's, endothelial cells and smooth muscle cells	OX-40	T cells and neutrophils
TNFSF5	CD40L	T cells, NK cells, mast cells, basophils and eosinophils	CD40	B cells, monocytes, DC's, thymic epithelium, Reed-Sternberg cells
TNFSF6	FasL/ CD95L	splenocytes, thymocytes, non-lymphoid tissues and NK cells	Fas, DcR3	Epithelial, hepatocytes, lymphocytes and transformed cells, lung and colon cells
TNFSF7	CD27L	T and B cells, NK cells, mast cells, smooth muscles and thymic epithelial cells	CD27	haematopoietic progenitor and T cells
TNFSF8	CD30L	T and C cells, monocytes, granulocytes, thymic epithelial cells	CD30	Reed-Sternberg cells
TNFSF9	4-1BBL	B cells, macrophages, DC's and mast cells	4-1BB	T, NK, mast and neutrophils
TNFSF10	TRAIL	NK, T cells and DC's	TRAILR1, TRAILR2, TRAILR3, TRAILR4, OPG	normal, transformed cells
TNFSF11	RANKL	T cells, thymus and lymph nodes	RANK, OPG	osteoclast, osteoblasts, T cells, endothelial cells
TNFSF12	TWEAK	monocytes, macrophages, transformed cells, tumour cells, mesenchymal stem cells	Fn14	endothelial cells, tumour cells, epithelial cells, fibroblasts
TNFSF13	APRIL	macrophages, lymphoid cells and tumour cells	BCMA, TACI	B cells, lymphocytes, spleen, thymus, lymph nodes, liver and adrenal tissue.
TNFSF13B	BAFF	T cells, monocytes, macrophages and DC's	TACI, BAFFR, BCMA	T and B cells, lymphocytes, spleen, thymus, small intestine, lymph tissue
TNFSF14	LIGHT	T cells, granulocytes, monocytes and DC's	LIGHTR, LT-βR, HVEM, DR3	T and B cells, monocytes and lymphoid cells, haematopoietic and stromal cells
TNFSF15	VEGI	Endothelial cells, B cells, macrophages and DC's	DR3 DcR3	NK cells and T cells
TNFSF18	GITRL/ EDA-A1/ EDA-A2	HUVECs, skin	GITR, EDAR2, XEDAR	T cells, ectodermal, embryonic hair follicles
NI			TROY	embryonic skin, epithelium, hair follicles and brain
NI			RELT	lymphoid tissue and haematopoietic tissue
NI	APP	Expressed in most tissues	Dr6	T cells, most tissues
NI	NGF	Immune cells	NGFR	neuronal axons, Schwann cells and perineural cells

1.10.3 Fas-Ligand: Fas-L also known as CD95-L is found expressed on immune cells. Fas is an apoptosis signalling receptor to Fas-L which has an intrinsic death domain. Fas expression has been found on lymphocytes, hepatocytes, and transformed cells. As well as regulating apoptosis by DISC formation via interactions with its ligand, Fas has been shown to mediate cellular differentiation, proliferation and activation, as well as co-ordinating the immune response, and pro-inflammatory signalling via NF-kB (Strasser, Jost e Nagata, 2009; Bremer, 2013). Initially Fas was described as having a specific role in clonal deletion of mature T cells in Fas knock out mice (Adachi *et al.*, 1996). However Fas mediated apoptosis has also been implicated in the progression of liver disease and pathogenesis in several studies. Fas and its ligand have been associated with liver cirrhosis derived HCC by the regulation of apoptosis. Fas was detected in inflammatory infiltrate in chronic hepatitis C patients using an immunohistochemistry (IHC) approach, and as this progressed in patients displaying advanced cirrhosis, this directly correlated with enhanced Fas expression showing enhanced expression in fibrotic and necro-inflammatory areas of tissue. Enhanced soluble Fas was subsequently detected in patients with cirrhosis and HCC in comparison to normal healthy patients (Hammam *et al.*, 2012). T cells overexpressing Fas-L function in inducing liver damage in an autoimmune Fas mutation mouse model causing lymphocyte infiltration, apoptosis of hepatocytes, and endothelialitis. The mouse model displayed defective activation induced death of T cells via a Fas mediated pathway, and subsequently lead to over expression of the Fas-L which induced cytotoxicity when spleen cells were engrafted into the host liver. This study showed that Fas-L can directly influence liver damage via lymphocyte activation (Bobé *et al.*, 1997).

1.10.4 TRAIL: TRAIL is a TNFSF ligand which can bind 5 different receptors of the TNFSF (Aggarwal, Gupta e Kim, 2012). It is expressed on NK and T cells and DC. TRAIL has been shown to mediate liver injury via the innate immune response. In a bile duct ligation model, enhanced TRAIL hepatic mRNA expression in NK and NKT cells was observed in comparison to wild type controls. TRAIL knock out bile duct ligation models showed overall reduced liver injury by a decrease in fibrosis and enhanced survival (Kahraman *et al.*, 2008). TRAIL is particularly intriguing in liver disease as normal hepatocytes do not express this ligand (Hao *et al.*, 2004), TRAIL receptors however have been shown expressed in sensitized hepatocytes (Malhi *et al.*, 2007), indicating possible paracrine mechanisms of TRAIL ligand-receptor interactions. TRAIL has been shown to contribute to tumourigenesis. It has been shown to induce receptor mediated apoptosis in tumour cells while healthy cells show no apoptotic cell death induced by TRAIL (Shin *et al.*, 2002), making TRAIL an attractive target for tumour therapy. Subsequently HCC resistance to TRAIL has been seen and studied extensively and the search for novel factors which may overcome this resistance has been the main focus of this research. TRAIL receptors have been found expressed on HCC cells and therefore attempts to induce apoptosis in HCC cells have been employed via TRAIL activation. It has been shown that TRAIL in combination with kinase inhibitors and chemotherapy drugs can induce apoptosis in HCC cells by overcoming BCL-2 induced apoptosis resistance (Koehler *et al.*, 2009). Similarly TRAIL resistance by HCC cells has been shown to be inhibited by EGFR (epidermal growth factor receptor) targeted TRAIL in combination with proteasome inhibitors, where EGFR has been shown to promote HCC proliferation (Wahl *et al.*, 2013).

1.11 TWEAK

1.11.1 TWEAK discovery: TWEAK is a member of the TNF superfamily of ligands found by Chicheportiche *et al* in 1997 (Chicheportiche *et al.*, 1997), and was described as a TNF like weak inducer of apoptosis (TWEAK). It was found to weakly induce apoptosis in a transformed adenocarcinoma cell line HT29; when these cells were activated with IFN-γ. TWEAK was identified from a mouse macrophage cDNA library which was screened for TNF sequence motifs, and found to have β-sandwich structures which is a common feature of TNF ligands, and found to be highly conserved between mice and humans (Chicheportiche *et al.*, 1997). Soon after, it was reported by Marsters et al that a cDNA related to TNF encoding an Apo3 receptor binding site was cloned and subsequently named Apo3 ligand (Marsters *et al.*, 1998); this cloned cDNA was identical to TWEAK. TWEAK is a type II transmembrane protein like most TNFSF ligands. The TWEAK gene is located at chromosomal position 17p13.1. Full length TWEAK protein is synthesised as a 249 amino acid (aa) protein, with a 206 aa C terminal extracellular region which contains the TNFSF homology domain, a 25 aa transmembrane region, and an 18 aa N terminal intracellular region. TWEAK can be cleaved at the C terminal domain to form a functionally active soluble protein. Soluble TWEAK forms a homotrimer, and has a highly conserved C terminal receptor binding sequence (Figure 1.7) (Chicheportiche *et al.*, 1997; Marsters *et al.*, 1998).

1.11.2 TWEAK expression: The expression of TWEAK was found in a wide range of tissues including the brain, pancreas, heart, skeletal muscle, and cell lines (Chicheportiche *et al.*, 1997). Liver TWEAK expression was generally found to be low in comparison to tissue from

the heart and kidney. It was also found that TWEAK had the ability to induce excretion of chemokines, notably IL-8 (Chicheportiche *et al.*, 1997). TWEAK expression has also been reported in a wide range of cells including macrophages (Kim *et al.*, 2004), monocytes (Chicheportiche *et al.*, 1997; Desplat-Jégo *et al.*, 2009), DC, NK cells (Maecker *et al.*, 2005), fibroblasts (Semov *et al.*, 2002), and B and T lymphocytes (Burkly, Michaelson e Zheng, 2011; Dharmapatni *et al.*, 2011; Michaelson *et al.*, 2011). TWEAK expression has also been observed in non-lymphoid type cells such as endothelial cell lines (Stephan *et al.*, 2013), tumour cells (Kawakita *et al.*, 2005) and astrocytes (Desplat-Jégo *et al.*, 2002). This is not consistent with other TNFSF ligands which usually have more specific and well defined cell type expression which is mostly limited to cells of immune, lymphoid and haematopoietic lineage (Aggarwal, Gupta e Kim, 2012). The expression of TWEAK has been reported to be mediated by IFN-γ and PMA (phorbol myristate acetate), where increased TWEAK expression was observed in response to these cytokines (Maecker *et al.*, 2005). LPS activation on the other hand, has shown differential regulation of TWEAK mRNA expression depending on the cell type; where up-regulated mRNA expression was observed in monocytes (Chacón *et al.*, 2006) and down-regulated expression was observed in macrophages (Chicheportiche *et al.*, 2000).

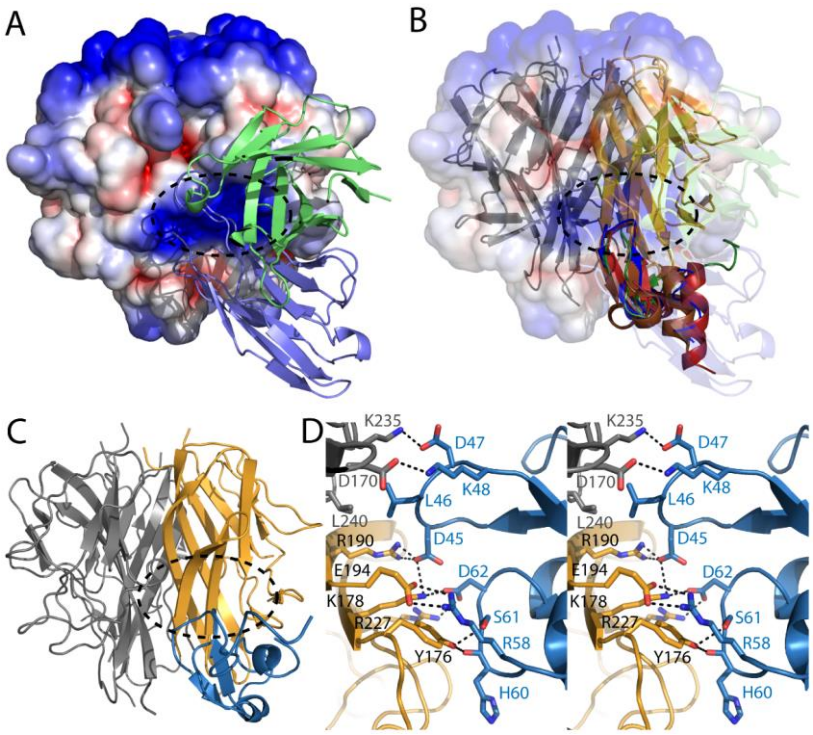


Figure 1.7 TWEAK and Fn14 human crystal structure: A) Shows a side view cartoon crystal structure of the human TWEAK trimer and indicated receptor binding site within the dashed elipse. B) Shows superimposed CRD (cysteine rich domain) co-localisation of other TNF receptors (coloured ribbons) to map the potential binding site of Fn14. The TWEAK surface is set as transparent cartoon. C) Shows an NMR model of receptor binding site (dashed elipse) of TWEAK with Fn14 CRD (blue). Only one receptor is shown. D) Shows a stereo view of TWEAK-Fn14 CRD binding site interphase with hydrogen bond regions (Lammens *et al.*, 2013).

1.12 Fn14

1.12.1 Fn14 discovery: The discovery of the correct functional TWEAK receptor was a complex process which took a few years after discovering TWEAK. The following year after TWEAK was originally characterised, Marsters *et al* 1998 suggested that the receptor for TWEAK was DR3 (death receptor 3), proposing that DR3 was the death receptor which regulated TWEAK apoptotic activity (Marsters *et al.*, 1998). This finding was quickly

disproved, as subsequent studies showed that TWEAK can bind to cells which do not have the DR3 receptor (Kaptein *et al.*, 2000; Nakayama *et al.*, 2002). In 2001 Wiley *et al* successfully identified Fn14 (FGF inducible protein 14) as the functional receptor to TWEAK. They used an expression cloning strategy to identify the receptor from human umbilical vein endothelial cells (HUVEC) (Wiley *et al.*, 2001). The TWEAK receptor was identical to a receptor identified previously, which had induced expression from NIH 3T3 fibroblasts in response to FGF 1 and 2. The receptor had a predicted molecular mass of 14 kDa; and so was named Fn14 (Meighan-Mantha *et al.*, 1999; Wiley e Winkles, 2003). Competitive inhibitor and binding studies were employed to determine the interaction affinity constant of TWEAK and Fn14, which was found to be similar to other TNFSF receptor ligand pairs (Wiley *et al.*, 2001).

The Fn14 gene is located on chromosome 16p13.3. Fn14 is expressed as a type 1 transmembrane 129 aa protein and has one cysteine rich domain (CRD). This is atypical of other TNFSF receptors which usually possess multiple CRD's, however BCMA (B cell maturation antigen) and BAFF-R (B cell activating factor receptor) have also been documented as possessing one CRD. Fn14 has a 27 aa N terminal signalling peptide sequence, and so Fn14 is proteolytically cleaved into a 102 aa mature protein. Fn14 like CD40 does not have an 80 aa intrinsic death domain which is typical of many TNF receptors (Wiley e Winkles, 2003; Brown *et al.*, 2006). However, it has a 28 amino acid TRAF binding sequence which can bind TRAF 1, 2, 3, and 5 leading to specific signalling transduction cascades (Figure 1.8) (Brown *et al.*, 2003).

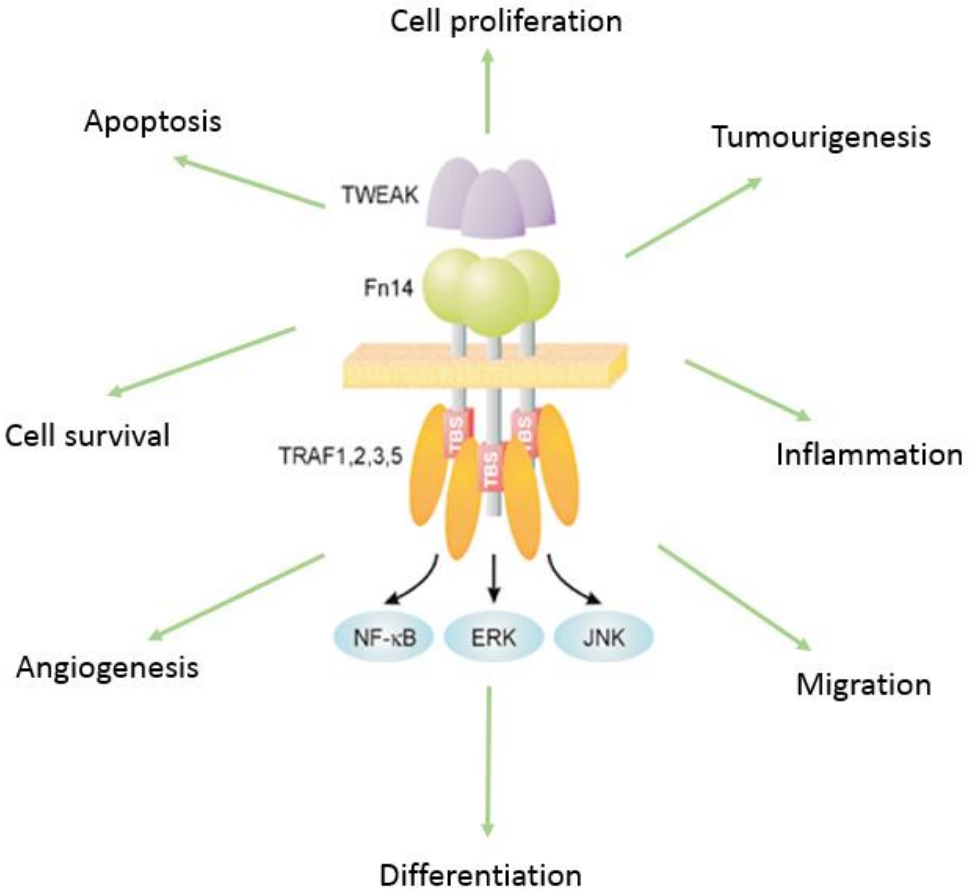


Figure 1.8: TWEAK and Fn14 mediated functions: Soluble TWEAK forms a homotrimer which has a receptor binding domain where it can bind Fn14. Fn14 can present on the cell surface, it has a 28 amino acid TRAF binding domain and can bind TRAF 1, 2, 3 and 5, these can activate signal transduction via specific pathways, notably NF-kB, and mediate several functions including inflammation, cell fate and angiogenesis dependent upon the cell type, context and environment.

1.12.2 Fn14 expression: Fn14 is expressed in a variety of cells and tissues including, endothelial (Harada *et al.*, 2002) and epithelial cells (Ebihara *et al.*, 2009), embryonic stem cells, immature erythrocytes (Felli *et al.*, 2005) progenitor cells (Jakubowski, Ambrose, Parr, Lincecum, Wang, Zheng, Browning, Michaelson, Baetscher, Baestcher*, et al.*, 2005), and tumour cells (Tran *et al.*, 2003). Fn14 expression has not been found in B and T cells so far

(Maecker *et al.*, 2005), there has however been expression detected in monocytes (Maecker *et al.*, 2005) and macrophages (Schapira *et al.*, 2009). Fn14 tissue expression has been observed in regenerating liver tissue post PHx (Feng *et al.*, 2000) and inflammatory brain tissue (Serafini *et al.*, 2008). Expression of Fn14 has been found to be regulated by growth factors such as FGF (Meighan-Mantha *et al.*, 1999), VEGF (Donohue *et al.*, 2003), EGF and PDGF (Wiley *et al.*, 2001), and its expression has been found to be regulated by various cytokines, such as TNF-α (Justo *et al.*, 2006), IFN-γ (Maecker *et al.*, 2005), TGF-β, and IL-1β (Interleukin -1 beta) (Hosokawa *et al.*, 2006). Fn14 expression has generally found to be low in normal tissue, however in diseased tissue this expression has been found induced; most often in response to growth factors and cytokines released in these pathological environments, for instance in normal brain tissue low Fn14 expression was found, but enhanced in brain tumour samples (Brown *et al.*, 2003; Wiley e Winkles, 2003; Burkly e Dohi, 2011).

1.13 TWEAK-Fn14 expression inducible factors

Several TWEAK and Fn14 activating factors have been found in studies investigating the expression patterns and functions of this ligand-receptor system in normal and pathophysiological environments. These will be discussed briefly:

1.13.1 IFN-γ: IFN-γ is a key regulator of the immune system and it has known expression from a range of cells such as macrophages, T cells, and NK cells. IFN-γ was initially described as a macrophage activating factor as it was shown to tightly regulate the macrophage

response in the innate immune system, mediating anti-pathogen, anti-tumour, and antigen presentation and processing functions (Schroder *et al.*, 2004; Schoenborn e Wilson, 2007). Enhanced TWEAK activation has been described in cells stimulated with IFN-γ. Chicheportiche reported TWEAK activated apoptosis in HT29 cells in response to IFN-γ stimulation (Chicheportiche *et al.*, 1997). It was also reported that TWEAK potentiated anti-tumour activity exerted by IFN-γ activated monocytes (Nakayama *et al.*, 2000). IFN-γ was also found to regulate Fn14 expression in cells of the immune system including monocytes, NK cells and dendritic cells (Maecker *et al.*, 2005).

1.13.2 TNF-α: TNF-α has been shown to be a significant regulator of TWEAK and Fn14 activity, as TWEAK and TNF-α in combination have been shown to regulate various functions. Soluble TWEAK has been shown to protect human adipocytes from insulin resistance activated by TNF-α (Vázquez-Carballo *et al.*, 2013). TWEAK and TNF-α have also been shown to regulate osteoblast functions via the MAPK (mitogen activated protein kinases) pathway (Vincent *et al.*, 2009). Fn14 expression has been found to be regulated by TNF-α and IFN-γ, and potentiating TWEAK induced apoptosis in murine cortical tubular cell lines (Justo *et al.*, 2006).

1.13.3 FGF: Fibroblast growth factors 1 and 2 were first described as mitogenic regulators in fibroblasts. Now they are also described to be key cell fate mediators; regulating cell survival, proliferation, differentiation, and *in vivo* development (Dorey e Amaya, 2010). Meighan-Mantha et al first showed that FGF activated fibroblasts release Fn14 (Meighan-

Mantha *et al.*, 1999). Subsequently, FGF induced fibroblasts and aortic smooth muscle cells, have been found to show enhanced Fn14 expression in other studies, and Fn14 has been shown to inhibit angiogenesis induced by FGF suggesting that Fn14 and TWEAK are key regulators of FGF induced angiogenesis (Feng *et al.*, 2000; Wiley *et al.*, 2001).

1.13.4 IL-1β: interleukin was first discovered in 1977 and found to facilitate leukocyte interactions. Interleukins are expressed by cells of the immune system including lymphocytes, macrophages, monocytes, dendritic cells, and NK cells. They regulate immune system and inflammatory functions. IL-1β is a member of the interleukin family and has been implicated in various inflammatory and immune mediated diseases such as RA, Psoriasis and irritable bowel syndrome (Akdis *et al.*, 2011). IL-1β activated aortic smooth muscle cells have shown enhanced Fn14 expression (Muñoz-García *et al.*, 2006). IL-1β in combination with TWEAK and TGF-β have been shown to induce a pro-inflammatory response in human gingival fibroblasts (Hosokawa *et al.*, 2006).

1.13.5 TGF-β: transforming growth factor-β is a regulator of cell fate, *in vivo* development, and tumour biology. It is expressed by a wide variety of cells and is thus implicated in a wide variety of functions (Kubiczkova *et al.*, 2012). TGF-β has been described as mediating inflammatory responses of TWEAK and Fn14. In combination with TWEAK, TGF-β has been shown to induce RANTES production by human keratinocytes inducing an inflammatory response (Jin *et al.*, 2004). TGF-β in combination with Fn14 has been shown to reverse intervertebral disc degeneration caused by TWEAK (Huh *et al.*, 2010). Meighan-Mantha *et al*

showed enhanced Fn14 mRNA expression in response to TGF-β activation in murine fibroblasts (Meighan-Mantha *et al.*, 1999).

1.14 TWEAK and Fn14 functional interactions

1.14.1 TWEAK and Fn14 signalling: TWEAK with its receptor Fn14 has been described as a multifunctional ligand and receptor, with functions from cell fate, proliferation, angiogenesis, inflammatory mediation and tumour cell biology (Figure 1.8) (Winkles, 2008). This multifunctional ability is most likely due to TWEAK and Fn14 interactions which activate various signalling pathways, such as NF-kB (Roos *et al.*, 2010) and MAPK (Donohue *et al.*, 2003). This activation of various signalling pathways via TWEAK and Fn14, over time and subsequent investigations, has proven to be a cell specific and context dependent mechanism (Burkly, Michaelson e Zheng, 2011). The complex signalling regulation via TWEAK and Fn14 interactions will be discussed in detail in Chapter 5. However briefly; TWEAK and Fn14 signalling is mediated via interactions of this ligand and receptor and the subsequent binding of TRAFs which lead to the activation of various transcription factors. TNFSF ligand and receptor mediated signalling is often seen to be via the activation of the classical NF-kB signalling pathway. TWEAK activated functions have also been extensively studied to signal via NF-kB as well as several other signalling pathways including p38 MAPK, Erk 1/2, Jnk and NF-kB Rel B (Winkles, 2008).

TWEAK and Fn14 expression in normal tissue is generally found to be low. Enhanced TWEAK and Fn14 expression can lead to tissue damage or can limit damage depending on the

environment and tissue type; for example in the normal liver, TWEAK overexpression has been shown during liver regeneration (Karaca *et al.*, 2014). However in induced fibrosis models, liver regeneration fibrosis was reduced by blocking TWEAK activation using anti TWEAK mAb, indicating a damaging effect with the presence of this ligand (Kuramitsu *et al.*, 2013). There have been no abnormalities discovered in normal development or growth in TWEAK and/or Fn14 knock out mouse models, however enhanced innate and adaptive immune responses have been observed. TWEAK -/- mice showed significant accumulation of NK cells in secondary haematopoietic tissues compared to WT mice. The TWEAK -/- mice further showed hypersensitivity to bacterial endotoxin increasing IFN-γ and IL-12 expression from innate cells, oversized spleens, and enhanced memory and helper T cell response to ageing and tumour formation (Maecker *et al.*, 2005); suggesting a crucial role of TWEAK and Fn14 in mediating innate and adaptive immune responses during disease and injury.

1.14.2 TWEAK and Fn14 interactions with other TNFSF members: TWEAK and Fn14 interactions with other members of the TNFSF have been characterised extensively highlighting the complexity of signal transduction via this ligand and receptor. TWEAK and Fn14 have been found to down-regulate CD40 ligand and receptor signalling complex formation, which has been found to compromise TRAF-2 mediated signalling which regulates CD40 functions. TWEAK activation has been found to inhibit signalling via NF-kB and MAPK's induced by CD40 activation, which was found to be impaired by defective signalling complex formation of CD40 ligand and receptor via TWEAK (Salzmann, Lang*, et al.*, 2013). TWEAK mediated apoptosis induced in Kym-1 cells has been found to be dependent on TNF-TNFR1

activation, as subsequent inhibition of apoptosis was observed when antibodies to TNF and TNFR1, and TNFR1 Fab fragments were used (Schneider *et al.*, 1999). The use of TRAIL and Fn14 fusion proteins have been shown to down-regulate disease severity in induced autoimmune encephalomyelitis mouse models. This was indicated by a reduced clinical score, inflammation, cytokine response and incidence. This fusion protein was developed on the basis of TWEAK and Fn14 association with pathological inflammation and the ability of Fn14 to block TWEAK activity, and the ability of TRAIL to inhibit the pathogenic response of activated T cells (Razmara *et al.*, 2009). Fn14:TRAIL fusion proteins have also been shown to inhibit HCC growth. It is thought that TWEAK signalling potentiates TRAIL resistance in HCC, therefore blocking TWEAK signalling with Fn14 will reverse TRAIL resistance and induce apoptosis in HCC. *In vitro* it was shown that this fusion protein induced apoptosis in HCC cell lines, and normal hepatocytes were unaffected by this induced apoptosis. In *in vivo* mouse models, subcutaneously injected Fn14:TRAIL stunted tumour growth, and it was shown that Fn14, TRAIL, and both in combination in their soluble forms did not show the same apoptosis induction in HCC cell lines (Aronin *et al.*, 2013).

1.14.3 TWEAK and CD163 receptor: There have been reports of TWEAK activity present in Fn14 negative cell populations. The ability of TWEAK to induce apoptosis without the presence of a death domain in Fn14, is an intriguing aspect to the functional capabilities of TWEAK. Therefore it has been speculated that there may be other functional unidentified receptors present for TWEAK. CD163 is from the scavenger receptor cysteine rich superfamily, and has been predominantly found expressed on monocytes and macrophages

(Pulford *et al.*, 1992). Bover *et al* 2007 adopted a peptide screening strategy to identify new TWEAK binding proteins. They found CD163 as a potential binding receptor for TWEAK, and found that CD163 was present on Fn14 negative monocyte populations which bound TWEAK in a dose dependent manner. They also found that TWEAK and IFN-γ mediated HT29 apoptosis can be blocked by this receptor . Since then Moreno *et al* have found that TWEAK and CD163 ratios correlate to disease severity in atherosclerotic plaques and athero-thrombosis (Moreno *et al.*, 2009; Moreno *et al.*, 2010). LLaurado *et al* showed TWEAK and CD163 ratios in serum may indicate cardiovascular disease severity (Llauradó *et al.*, 2012).

1.15 TWEAK mediated leukocyte recruitment

Over the years, TWEAK activated inflammatory responses have been a focus of TWEAK/Fn14 functional activities. TWEAK mediated inflammatory responses have been extensively characterised by leukocyte infiltration and recruitment via cellular interactions, cell surface adhesion proteins, and chemokine expression. TWEAK activated human gingival fibroblasts showed enhanced ICAM and VCAM expression which could subsequently be regulated by TGF-β, which enhanced ICAM expression but had suppressive influences on VCAM, leading to an enhanced inflammatory response, contributing to periodontal gingival disease (Hosokawa *et al.*, 2006). Subsequently TGF-β activating kinase -1 knock out mouse models showed diminished MMP-9, CCL-2 and VCAM expression in response to TWEAK activation in cultured myoblasts and fibroblasts (Kumar *et al.*, 2009). In human dermal microvascular endothelial cells and *in vivo* cutaneous vasculitis mouse models, E-selectin and ICAM expression were enhanced in response to TWEAK activation, which directly contributed to

cutaneous vasculitis progression. TWEAK activated endothelial cells also enhanced leukocyte recruitment which was shown using adhesion assays (Chen *et al.*, 2013). In other investigations, ICAM and VCAM expression regulation by TWEAK were closely modulated by other cytokines in inflammatory disease progression. Such as ICAM and VCAM enhanced expression in response to TWEAK and IFN-γ activation in fibroblast like synoviocytes, contributing to rheumatoid arthritis inflammatory progression (Mo Kang 2008). TWEAK activated endothelial cells also showed enhanced ICAM expression accompanied by inflammation mediating cytokines CCL2, IL-8 and IL-6 (Stephan *et al.*, 2013).

TWEAK activation mediated leukocyte recruitment has been characterised in a number of inflammatory diseases and in pathological settings. TWEAK has been found to be a tumourigenic cytokine contributing to enhanced tumour growth and decreasing the anti-tumour immune response. Detection of leukocytes in the blood, spleen and tumours showed that TWEAK blockade by RG7212 (TWEAK mAb) lead to monocyte and macrophage number decrease in circulation, but enhanced expression was observed in tumours. CD3+ T cells were subsequently found enhanced in blood and tumours. It was also found that TWEAK activation lead to enhancement of leukocyte recruitment protein expression such as CD274, CCL2, CXCL10 and CXCL11. TWEAK was also found to mediate T cell activation and macrophage differentiation. Depleting CD8+ T cells and NK cells showed a loss of tumour inhibition by RG7212, but CD4+ T cell depletion lead to enhanced tumourigenic properties, indicating specific leukocyte tumourigenic responses co-ordinated by TWEAK (Yin *et al.*, 2013). TWEAK activation has also been shown to lead to T cell infiltration in the kidney (Gao

et al., 2009). TWEAK activated renal tubular cells showed the non-canonical pathway activation accompanied by CCL21A expression. *In vivo* acute kidney injury models showed TWEAK knockout depleted CD3+ lymphocytes which are usually found highly expressed (Sanz *et al.*, 2010). TWEAK administration in a peritoneal inflammatory mouse model showed enhanced expression of MCP-1, Fn14 expression and GR1+ macrophage recruitment. TWEAK activation also lead to enhanced CD8+ T cells and neutrophils contributing to peritonitis (Sanz, Aroeira*, et al.*, 2014). In periodontal gingival disease, TWEAK and Fn14 expression was observed in monocytes and fibroblasts. TWEAK expression was associated with increased renal and vascular damage, and enhancing the pro-inflammatory responses seen in atherosclerotic lesions and kidney disease (Muñoz-García *et al.*, 2009). These studies all indicate a significant role of TWEAK and Fn14 in contributing to local inflammatory responses in disease settings via regulating leukocyte recruitment and direct leukocyte interference, facilitated by expression of selectins, adhesion molecules and cytokines.

1.16 TWEAK and Fn14 regulation of inflammatory disease

Inflammation is often in response to infection, trauma, toxicity, and autoimmunity. Inflammation is a highly regulated process, however when this is de-regulated leading to excessive or prolonged inflammatory responses, pathogenesis of disease is often observed (Medzhitov, 2008). During inflammatory disease, leukocytes can facilitate actions of resident non haematopoietic cells. TWEAK is highly expressed on cells of heamatopoietic origin and activated during acute disease processes which can have beneficial and detrimental effects

(Burkly, Michaelson e Zheng, 2011). It has been hypothesised based on several experimental models and findings that during acute tissue injury and disease, TWEAK can facilitate tissue repair by initiating an inflammatory response, inducing cellular proliferation, angiogenesis, and progenitor differentiation, which can lead to tissue repair. During chronic inflammatory disease however TWEAK and Fn14 may facilitate pathological tissue destruction, whereby TWEAK and Fn14 up-regulated expression may sustain the inflammatory response, progenitor expansion may be reduced, and chronic angiogenesis and proliferation eventually may lead to detrimental effects of pathological remodelling and tissue degeneration (Figure 1.9) (Burkly *et al.*, 2007). This hypothesis has been proven by a number of studies investigating the functional roles of TWEAK and Fn14 in chronic inflammatory diseases.

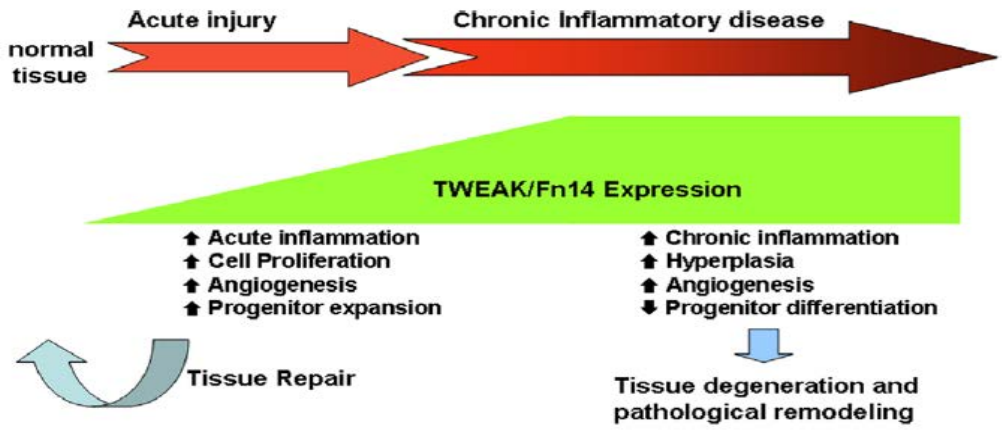


Figure 1.9 TWEAK mediated chronic inflammatory disease regulation: In normal tissue TWEAK and Fn14 expression is low. During acute injury, TWEAK and Fn14 expression is up-regulated promoting inflammation, cellular proliferation, angiogenesis, and progenitor expansion leading to tissue repair. During chronic inflammatory disease, it is hypothesised that TWEAK and Fn14 sustained up-regulation deregulates these processes leading to tissue degeneration and pathological remodelling (Burkly *et al.*, 2007)

1.16.1 Rheumatoid Arthritis: Rheumatoid arthritis (RA) is a debilitating chronic inflammatory disease, characterised by joint swelling, and progressive cartilage and bone destruction (Pincus e Callahan, 1993). This involves severe inflammation caused by the induction of inflammatory cytokines, chronic angiogenesis, synovial cell proliferation, and changes in vascular permeability which leads to fibrosis of the synovium (Fava *et al.*, 1994; Paleolog, 2002; McInnes e Schett, 2007). TWEAK is a known mediator of RA. There is growing *in vitro* and *in vivo* evidence of TWEAK and Fn14 expression present in synovial fluid, and anti-TWEAK mAb has been shown to alleviate joint swelling caused by RA (Kamata *et al.*, 2006). Subsequently, TWEAK and Fn14 expression has been found up-regulated on synovial cells, and recombinant TWEAK protein has been shown to enhance cytokine induction and ICAM expression on these cells. This process was reversed with TWEAK mAb treatment (Kamijo *et al.*, 2008). *In vivo* collagen induced arthritis (CIA) mouse models have been used to determine the functional regulation of TWEAK and Fn14 in RA disease progression. In CIA mouse models, anti-TWEAK mAb treatment has shown a significant decrease in disease severity, indicated by decreased paw swelling, synovial cell hyperplasia, inflammatory cell infiltration, and inhibited angiogenic responses in synovial tissue indicated by fewer vessel formation (Kamata *et al.*, 2006). Subsequently Fn14 expression has been found up-regulated in synovial tissue from CIA models (Park *et al.*, 2012). Phase I clinical trials using TWEAK mAb BIIB023 in RA patients has shown reduced serum levels of TWEAK 6 hours post dosage (0.03-20mg/Kg) which lasted for up to a month, which also showed a decrease in inflammatory markers, including MCP-1, macrophage inhibitory protein-1, tissue inhibitor of metalloproteinase-1 and inducible protein-10 in comparison to placebo controls. The use of BIIB023 was found to be safe and well tolerated by RA patients (Wisniacki *et al.*, 2013).

1.16.2 Systemic Lupus Erythematosus: Systemic Lupus Erythematosus is an autoimmune disease characterised by autoantibody production. It can lead to immune complex deposits forming in organs, and can cause disease such as Lupus Nephritis (LN). TWEAK has been described as being directly involved in the inflammatory response in LN progression, and can promote renal cell proliferation, apoptosis and fibrosis in tissue (Michaelson *et al.*, 2012). TWEAK has been found highly up-regulated in the urine in LN patients, and it has been proposed that TWEAK can serve as a clinical biomarker for LN, as no urinary expression of TWEAK was subsequently observed in healthy patients and other disease controls (Schwartz *et al.*, 2009). *In vivo* graft vs host induced LN mouse models showed reduced kidney disease severity once treated with anti-TWEAK antibodies, which ameliorated the damaging inflammatory response to LN. Fn14 knock out models with induced LN also showed decreased kidney disease severity coupled to an inhibited inflammatory response, indicating that TWEAK and Fn14 contribute to kidney disease induced by LN (Zhao *et al.*, 2007). A similar response of TWEAK induced kidney disease was observed in nephrotoxic serum nephritis models, however the response was not mediated by the systemic inflammatory immune response, rather it was regulated directly in the local kidney immune response promoting fibrosis and inflammatory infiltration via TWEAK (Xia, Y. *et al.*, 2012). A phase II clinical randomised placebo controlled trial using BIIB023 in patients with LN in underway, to observe the efficiency of using this antibody as an add-on therapy in patients who have failed to undergo disease remission using standard steroid and mycophenolate mofetil therapy (Sanz, Izquierdo*, et al.*, 2014).

1.16.3 Neuro-inflammation: TWEAK also has been shown to directly mediate inflammatory diseases of the brain. The blood brain barrier (BBB) is made up of microvascular endothelial cells, pericytes, astrocytes and tight junctions, and required to separate the circulatory system from the central nervous system, whilst facilitating the passage of required molecules by active permeabilisation. When this permeabilisation process is disrupted, this can lead to inflammatory brain disease including Multiple Sclerosis (MS), ischemic stroke and brain edema. TWEAK has been shown to be key in disrupting the BBB leading to an increased production of inflammatory cytokines, and increased permeability of the BBB. Anti-TWEAK mAb therapy has shown to alleviate symptoms in ischaemic stroke and brain edema models (Winkles, 2008; Yepes, 2013). MS models have shown TWEAK expression up-regulated on monocytic fractions in comparison to other haematopoietic populations, defining a specific inflammatory response via TWEAK during MS (Desplat-Jégo *et al.*, 2009).

1.16.4 Cancer Progression: TWEAK has been described as a key regulator of pro-tumorigenic functions which range from promoting angiogenesis, inflammation, cell invasion and proliferation; and can thus support and facilitate ideal environments for survival and metastasis of tumour cells (Figure 1.10) (Winkles, Tran e Berens, 2006). TWEAK mediated angiogenic functions have been extensively investigated since the initial discovery of this ligand and receptor system. These will be discussed in detail in Chapter 4. However briefly; TWEAK was shown to promote angiogenesis and proliferation *in vitro* in HUVEC, and in an *in vivo* rat cornea model. TWEAK was shown to directly induce angiogenesis comparable to well characterised angiogenesis mediators FGF and VEGF (Lynch *et al.*, 1999). It was

subsequently found that Fn14 mediates TWEAK activated angiogenesis (Wiley *et al.*, 2001). Further studies have since characterised and confirmed the potential TWEAK and Fn14 have in promoting pro-angiogenic functions (Kawakita *et al.*, 2004; Shimada *et al.*, 2012). TWEAK has been shown to promote the invasive and migratory capacity of tumourigenic cells, this was demonstrated in human glioma cells where Fn14 over expression promoted glial cell invasion via the activation of Rac1 (Ras related C3 botulinum substrate 1) and NF-kB, and Fn14 expression was found highly up-regulated in glial tumours (Tran *et al.*, 2003; Tran *et al.*, 2005). Fn14 has been found highly expressed in Her2+ breast tumours (Willis *et al.*, 2008). It has subsequently been shown that Her2 over expression in MCF7 (Michigan cancer foundation 7) cells induced Fn14 protein expression, which was found to be reduced in response to Her2 kinase inhibitors. Fn14 inhibition in Her2+ cells down-regulated migration and invasion capacity and MMP-9 expression, indicating the importance of TWEAK and Fn14 signalling in tumour progression (Asrani *et al.*, 2013). Fn14 expression has subsequently been shown to correlate directly with poor prognosis and patient outcome in cancer patients (Huang *et al.*, 2011; Kwon *et al.*, 2012).

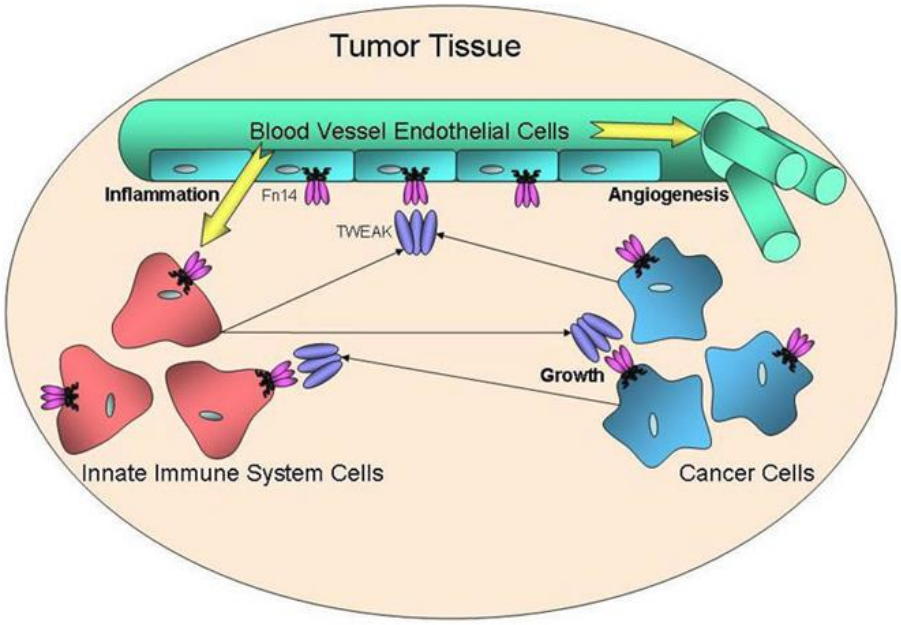


Figure 1.10 TWEAK regulation of tumour progression: TWEAK and Fn14 may regulate critical functions such as inflammation, angiogenesis, proliferation and migration, which may aid the TWEAK pro-tumourigenic response. This diagram illustrates how TWEAK activation by tumour cells may drive endothelial cell proliferation and angiogenesis. TWEAK activation by the inflammatory response may subsequently be driven by the immune response to the tumour and may lead to the enhanced growth of the tumour cells by indirect activation of growth factors, cytokines and chemokines favouring the tumour environment. In turn the immune response may favour endothelial cell leukocyte recruitment and angiogenic response leading to tumour progression (Winkles *et al.*, 2007).

Subsequently, TWEAK and Fn14 expression has been found in a range of tumour types and tumour cell lines. TWEAK mRNA and protein expression has been observed to be up-regulated in HCC tissue and cell lines in comparison to normal liver (Feng *et al.*, 2000). Ho *et al* described TWEAK mRNA expression found in 11 tumour tissue types from 68 tumour specimens indicating the range of expression observed, their findings included kidney, prostate, cervical cancer and colon cancer. They also described large highly vascularised tumours developed when TWEAK over-expressing cells were grafted into nude mice (Ho *et al.*, 2004). Fn14 expression was subsequently found up-regulated in HCC tissue and TWEAK

was found to promote cellular proliferation and NF-kB activation in HCC cells (Kawakita *et al.*, 2004). Further investigations found up-regulated Fn14 expression in a range of tumours, and when tumour cell lines were treated with anti-Fn14 antibodies the proliferation of tumour cell lines was down-regulated, indicating tumour progression functions via Fn14 (Culp *et al.*, 2010). Similarly up-regulated TWEAK and Fn14 expression was found in a range of esophageal and pancreatic cancer tissue and cell lines. Blocking TWEAK and Fn14 signalling using anti TWEAK antibodies showed decreased cell line growth (Yoriki *et al.*, 2011).

1.17 TWEAK and Fn14 regulation of inflammatory liver disease and regeneration

There are very limited studies which have investigated the functional roles of TWEAK and Fn14 in the liver. Data has indicated that TWEAK and Fn14 regulate proliferative, inflammatory and regenerative responses in the liver. Feng *et al* 2000 initially described low Fn14 expression in normal human liver tissue, and subsequent up-regulation of Fn14 mRNA in human HCC tissue and transformed cell lines. They also showed in mouse models of liver regeneration using PHx and induced hepato-carcinogenesis that Fn14 expression was highly up-regulated in early liver regeneration, and sustained up-regulation was observed throughout the regeneration process, indicating a critical role for Fn14 during tissue repair by controlling hepatocyte proliferation and remodelling (Feng *et al.*, 2000). This was followed by a study by Kawakita et al 2004 who showed Fn14 expression found in 4 cell lines of HCC; HepG2, Huh7, SkHep1 and HLE. TWEAK expression was shown to promote cellular proliferation of HCC cells in a dose dependent manner; and by activating the NF-kB pathway.

They found TWEAK and Fn14 co-expressed on HCC tissue, indicating a possible autocrine and/or paracrine influence on cellular proliferation. IHC revealed metastatic HCC showed enhanced TWEAK expression in comparison to HCC tissue, and interestingly they found non tumour diseased tissue generally negative for TWEAK with exception to areas of lymphocyte infiltration. This study confirmed the potential roles of TWEAK and Fn14 signalling during HCC progression and metastasis and indicated towards a role for TWEAK mediated inflammatory responses during liver disease. They went on to find that TWEAK induced proliferation of endothelial cells (HUVEC) and found an enhanced proliferative response and expression of IL-8 and MCP-1, further confirming previous studies looking at TWEAK regulation of inflammatory mediated angiogenesis, and speculated that TWEAK mediated tumourigenesis may be promoted via inflammatory angiogenesis (Kawakita *et al.*, 2004).

The focus then shifted to liver progenitor cell expansion via TWEAK activation. Jakubowski *et al* 2005 showed that TWEAK has the ability to promote the proliferation of hepatic progenitor cells and BEC when overexpressed in TWEAK transgenic mice. They showed that this was mediated through Fn14 interactions as Fn14 null mice did not show the same proliferative effect. They also found Fn14 expression on BEC and in periportal regions of liver tissue sections from these transgenic mouse models. They subsequently found no proliferative effect on hepatocytes, suggesting a progenitor cell specific response. In a DDC (3, 5- diethoxycarbonyl-1, 4-dihydrocollidine) liver injury model they found Fn14 expression up-regulated in reactive portal areas, they also found TWEAK expression highly elevated in comparison to controls in the parenchyma of the DDC diet mice, and TWEAK mAb inhibited

progenitor cell expansion in these models which was found to be regulated by Fn14, as Fn14 null mice exhibited no progenitor cell hyperplasia. They further found that TWEAK enhanced proliferation *in vitro* in rat BEC, and enhanced Fn14 expression was observed in NASH, ALD, HCV and HCC human liver tissue concentrated in areas of bile ducts and fibrotic regions in comparison to normal liver, suggesting a direct role of TWEAK and Fn14 signalling during liver disease and pathogenesis (Jakubowski, Ambrose, Parr, Lincecum, Wang, Zheng, Browning, Michaelson, Baetscher, Baestcher*, et al.*, 2005). Tirnitz-Parker *et al* 2010 also confirmed that TWEAK promoted the expansion of liver progenitor cells, in a CDE (choline deficient, ethionine supplemented) induced liver injury model; which was mediated through Fn14. They found enhanced Fn14 expression in CDE induced injury models, and showed that Fn14 null mice had decreased liver progenitor cell numbers, decreased inflammatory cells (CD45+ leukocytes and F4/80+ macrophages), inhibited cytokine production of TNF, IFN-γ, IL-6 and LT-β (lymphotoxin-beta), and decreased fibrosis indicated by down-regulated collagen deposition and TIMP (tissue inhibitor of metalloproteinase) expression, in comparison to wild type mice. They also found that TWEAK promoted proliferation of a liver progenitor cell line in a dose dependent manner via NF-kB *in vitro* and *in vivo*, with enhanced CD45+ leukocyte expression. (Tirnitz-Parker *et al.*, 2010; Tirnitz-Parker, Olynyk e Ramm, 2014). Subsequently, their findings demonstrated the important functions of TWEAK and Fn14 during liver injury and regeneration via liver progenitor cells and how TWEAK and Fn14 regulate the inflammatory and fibrogenic responses during liver injury via possible paracrine interactions of inflammatory cells and resident liver cells.

Modulation of the TWEAK mediated inflammatory response has been observed in all studies investigating the potential of TWEAK and Fn14 regulating liver disease and injury. Tirnitz-Parker et al found TWEAK expression in macrophages and NK cells in the CDE injury model and no Fn14 expression by these inflammatory cells (Tirnitz-Parker *et al.*, 2010). This indicated a specific regulation of the inflammatory response via this ligand and receptor in liver disease. A follow up study revealed that macrophages in close proximity to ducts in the liver produce TWEAK in the CDE liver injury model (Viebahn *et al.*, 2010). The TWEAK dependent inflammatory response of liver progenitor cells and BEC was also demonstrated in a study where bone marrow derived macrophages were engrafted into normal livers. These macrophages were shown to subsequently express elevated TWEAK which initiated ductular reactions (Bird *et al.*, 2013). These studies demonstrate how TWEAK is fundamental to the autocrine regulation of the inflammatory response during liver mediated injury and disease.

Recent studies have further demonstrated that TWEAK and Fn14 are mediators of the liver regenerative response by using models of PHx. Fn14 positive cells were shown to drive the proliferative response in the regenerating liver. After PHx in wild type mice, Fn14 positive cell accumulation was observed, this was demonstrated by Fn14 mRNA and protein expression, and IHC analysis. IHC revealed the presence of Fn14 positive cells surrounding portal tract regions, it was suggested that these cells resembled hepatocytes. Fn14 was shown to drive the proliferative response shortly after PHx which was dependent upon TWEAK. Fn14 knockout significantly reduced the ability of the liver to regenerate, as liver

progenitor, BEC, and hepatocyte expansion was significantly inhibited (Karaca *et al.*, 2014). It has also been shown that post PHx in mouse models of induced fibrosis by CCL4 (carbon tetra chloride) injury, the regenerative response was driven by TWEAK and Fn14 signalling. They showed that TWEAK neutralising antibodies reduced the pro-fibrotic response, reduced progenitor cell proliferation, normalised serum ALT levels, and enhanced liver regeneration. Alternatively, TWEAK protein administration enhanced liver progenitor cell proliferation and the pro-fibrotic liver response (Kuramitsu *et al.*, 2013).

Studies so far have indicated that TWEAK and Fn14 drive proliferation and regenerative responses, and mediate pathogenesis during liver disease via inflammatory regulation. It is of high importance to understand this further at a molecular level and to understand the interactions, functions, and signalling crosstalk, of TWEAK and Fn14 in the different liver cell populations. Studies investigating the role of TWEAK in liver disease regulation have observed that progenitor cells drive many of the functional responses observed. Therefore it is equally important to further examine the role of differentiated progenitor cells to further dissect how TWEAK and Fn14 mediate these responses. The liver angiogenesis mediated regenerative response is of prime importance for the development of therapies targeting inflammatory liver disease for regeneration. Therefore it is essential to understand TWEAK and Fn14 interactions in HIEC (Human Intra-hepatic Endothelial Cells) which are the driving feature of liver regeneration via angiogenic responses of the liver.

1.18 General Aims

We believe that TWEAK and Fn14 may regulate hepatic inflammatory and angiogenic responses during chronic inflammatory liver disease via human intra-hepatic endothelial cells, thus facilitating the regulation of hepatic regeneration. The overall aims of this investigation were:

- To determine the expression of TWEAK and Fn14 in liver tissue sections and isolated HIEC.
- To determine the role of TWEAK and Fn14 during regulation of HIEC functional responses, angiogenesis, cell fate, and leukocyte recruitment.
- To determine intracellular signalling pathways activated via Fn14 in HIEC.

CHAPTER 2

MATERIALS AND METHODS

2.1 Human Tissue

Human liver tissue consented for scientific research, was donated by the Queen Elizabeth Hospital, Birmingham UK, to the Centre for Liver Research. Donated livers included explant diseased livers, resected marginal tumour liver tissue, and normal donor liver tissue surplus to surgical requirement. Ethical approval was obtained from The Local Research Ethics Committee (reference: 06/Q702/61). Blood was donated by the Queen Elizabeth Hospital, Birmingham, UK, from patients with Haemochromatosis. All tissue was used after obtaining written consent from all participants.

2.2 Primary HIEC Isolation

HIEC were isolated according to a previously described method (Lalor *et al.*, 2006). To describe briefly; approximately 30g of liver tissue was finely sliced and digested in 20ml PBS (phosphate buffered saline; Oxoid, Basingstoke, UK) with 5ml Collagenase type 1A (Sigma-Aldrich, UK). This was incubated at 37°C in a humidified incubator with 5% CO_2, for 25-45 minutes, depending on how fibrotic the tissue was. The digest was sieved through a fine mesh, and washed with sterile PBS into a sterile beaker. The digest was subsequently washed and combined by centrifuging at 2000rpm for 5 minutes with a brake. This was continued until the supernatant was clear. The final pellet was re-suspended in PBS. A Percoll™ (GE Healthcare, UK) gradient 70%:30% was used to separate the cells. The cell mixture was layered on each gradient and centrifuged at 2000rpm for 25 minutes, with no brake. The layer of cells at the interphase of the gradient was transferred to universals, washed, centrifuged and pelleted as previously described. The final pellet was re-suspended in 500µl PBS and 50µl anti HEA-125 Antibody (Ab) (Progen, Biotechnic, Germany), and

incubated at 37°C in a humidified 5% CO_2 incubator for 30 minutes, with agitation every 10 minutes. The suspension was washed in PBS and pelleted by centrifuging at 2000rpm for 5 minutes. The pellet was re-suspended in 500µl ice cold PBS and 10µl goat anti-mouse IgG (immunoglobulin G) secondary Ab, conjugated to magnetic Dynabeads™ (Invitrogen, UK) for 30 minutes on ice with constant agitation. The suspension was diluted in 5ml cold PBS, and negative magnetic selection was used to remove BEC, as they conjugated to the HEA-125 beads. The supernatant was retained and washed in PBS and pelleted by centrifugation, re-suspended in 500µl ice cold PBS and 10µl CD31 conjugated Dynabeads™ (Invitrogen) for 30 minutes on ice, with constant agitation. The suspension was made up to 5ml with ice cold PBS and positive magnetic selection was used to obtain the HIEC. The cells with beads attached were suspended in complete HIEC media, and transferred to a rat tail collagen (Sigma-Aldrich, UK) coated 25cm^2 (Corning, Costar Incorporated, Bucks, UK) coated flask; allowing the cells to adhere for 24 hours before changing the media.

2.3 Complete HIEC Media

Human endothelial serum free media (Invitrogen) was supplemented with 10% heat inactivated human serum (HD Supplies, UK), and 200units/ml Penicillin, 200µg/ml Streptomycin, 600µg/ml Glutamine (Invitrogen), 10ng/ml VEGF (Peprotech EC, UK), and 10ng/ml HGF (Peprotech EC).

2.4 Maintenance and Storage of HIEC

2.4.1 Passaging HIEC: The media was removed and the cells were then washed with 10ml PBS before adding 2ml Tryp-LE™ (Invitrogen). The cells were incubated at 37°C for 5 minutes to release the cells from the flask. These were then suspended in 10ml PBS before being transferred to a universal with 2ml FCS (Foetal calf serum- heat inactivated, Invitrogen) to neutralise the Tryp-LE™. The cell suspension was centrifuged at 2000rpm for 5 minutes with brake 3 to pellet the cells. The supernatant was discarded, and the cells were re-suspended in complete HIEC media at the required density, and then transferred to a T75 flask (Corning 75cm2).

2.4.2 Counting Cells: After trypsinising and pelleting the cells, the pellet was re-suspended in 1ml of media. 10µl of this suspension was diluted in 90µl of Trypan Blue (Sigma-Aldrich) and viable cells were counted using a haematocytometer chamber.

2.4.3 Freezing and Bringing Cells Up From Frozen: The protocol for passaging cells (Section 2.4.1) was followed to pellet the cells which were then suspended in FCS with 5% DMSO (Dimethyl Sulphoxide, Sigma-Aldrich) at the required density, then transferred to Cryovials, and frozen in a Mr Frosty™ isopropanol chamber at -80°C overnight. The Mr Frosty™ Chambers allow gradual freezing of cells which prevents ice crystals forming in the cell. The vials were then stored in liquid nitrogen until required. To bring up cells from frozen; 1ml of warm HIEC media was added to each vial and incubated at 37°C until defrosted. The suspension was transferred to a universal and washed with PBS, and centrifuged to pellet

the cells. The cell pellet was re-suspended in complete HIEC media and transferred to a rat tail collagen coated T75 flask.

2.5 Immunohistochemistry

2.5.1 Frozen Liver Tissue Sections: Liver tissue blocks 1cm^3 were snap frozen in liquid nitrogen and 5-7µM thick liver tissue sections were cut using a cryostat. Sections were stored at -20°C until required. Frozen liver tissue sections were thawed at room temperature for 30 minutes before waxing around the section and fixing in acetone for 5 minutes. The sections were then washed in TBS (Tris-Buffered Saline, 25mM Tris (Sigma-Aldrich) and 50mM NaCl (BDH Supplies, UK) pH 7.6 for 5 minutes. Endogenous peroxidase activity was eliminated by incubating the sections for 5 minutes in 0.3% H_2O_2 in methanol. The sections were then washed in TBS pH 7.6 for 5 minutes, before incubating in 2.5% horse serum blocking solution (Vector Immpress Kit; MP-7500) for 20 minutes, or the sections were blocked using 2% Caesin (Leica Microsystems, Germany) for 10 minutes, to inhibit non-specific binding. The primary antibodies (Table 2.1) were diluted in the horse serum blocking solution or in TBS pH 7.6 and added to the sections for 1 hour on a rocking platform, before washing in TBS-Tween pH 7.6 for 5 minutes. Immpress secondary reagent (Vector Immpress Kit; MP-7500) was added for 30 minutes and the sections washed again in TBS-Tween pH 7.6.

2.5.2 Paraffin Liver Tissue Sections: The paraffin sections were de-waxed and hydrated by incubating the sections two times in Clearene (Leica Microsystems, Germany) for 5 minutes

at room temperature, then incubating the sections in alcohol (Leica) twice for 5 minutes, and then re-hydrating the sections in water for 5 minutes. Antigen retrieval was performed by incubating the sections for 20 minutes in low pH antigen retrieval solution (Leica) in a microwave at full power. The sections were then returned back to room temperature, before circling the section with a hydrophobic pen and resuming staining as for frozen liver tissue sections.

2.5.3 AEC Substrate: The AEC (3-amino 9-ethylcarbazole) substrate solution (Vector AEC peroxidise substrate kit; SK-4200) was added to the sections for approximately 30 minutes, or until the desired stain intensity developed. The sections were washed in distilled water to stop the reaction, and counter-stained with Mayers Haematoxylin (Signet) for 2 minutes. The sections were developed with tap water and mounted using an aqueous mounting solution (ThermoScientific, UK) and left to dry overnight.

2.5.4 DAB Substrate: The DAB (3, 3' diaminobenzidine) substrate solution (Vector) was added to the sections for 5 minutes at room temperature, before washing with distilled water for 5 minutes. The sections were counterstained with Mayers Haematoxylin for 2 minutes and developed using tap water. The sections were dehydrated by incubating in alcohol twice for 5 minutes, and then Clearene twice for 5 minutes, before mounting with DPX mounting media (Leica).

2.5.5 Haematoxylin and Eosin stain: All Hematoxylin and Eosin (H and E) staining was carried out to a standard protocol (Bancroft e Gamble, 2008). All reagents were obtained from Leica Microsystems.

2.5.6 Analysis: Sections were visualised using a light microscope (Nikon) and images were processed using Axiovision v 4.4 software.

2.5.7 HIEC Cytospin™ preparations: HIEC were stimulated with and without TWEAK (10ng/ml) conditioned media for 24 hours. The HIEC were detached using Tryp-LE, pelleted and re-suspended in PBS after counting the cells using a haematocytometer at 1 x 10^6 cells/ml. HIEC preparations were subsequently centrifuged on to Poly-L-lysine coated glass slides for 5 minutes 550rpm. The slides were allowed to air dry before fixing them in acetone for 2 minutes, and wrapping the slides in foil and storing at -20°C until required.

2.5.8 Ki-67 Proliferation Stain: cytospin preparations were thawed until they reached room temperature, and subsequently antigen retrieval was carried out for 5 minutes, and staining was carried out following protocol from section 2.5.2 and 2.5.3.

2.5.9 Analysis of Nuclear Proliferation Marker: Images were taken from 5 fields of view from each section using a light microscope and camera (Nikon), and images were processed using

Axiovision v 4.4. Percentage of cells undergoing proliferation was determined by comparing ki-67 positive cells to the total number of cells in each image.

2.6 Immuno-fluorescence

2.6.1 HIEC: HIEC were cultured on glass coverslips until a confluent HIEC monolayer was formed, and fixed for 10 minutes using methanol -20°C. After aspirating the excess methanol, the coverslips were subsequently frozen at -20°C until required.

2.6.2 Liver Tissue Sections: Liver tissue sections were fixed using acetone, and washed using PBS. The Immuno-fluorescence (IF) protocol was identical for fixed cells and sections. The cells/ sections were blocked using TBS pH 7.6, 3% BSA (Albumin from bovine serum; Sigma-Aldrich) and 0.1% Triton X-100 (Sigma-Aldrich) for 1 hour. This was followed by incubating them with the primary antibody overnight at 4°C (Table 2.1). The slides were washed extensively with PBS for 45 minutes followed by incubation of the fluorescently conjugated secondary antibody (Table 2.2) at 37°C for 1-2 hours. The slides were washed again for 30 minutes using PBS followed by a DAPI (4', 6 diamidino-2-phenylindole) counterstain, before mounting using IF mounting media (DAKO).

2.6.3 Dual Immuno-fluorescence: Liver tissue sections were fixed using acetone and washed using TBS pH 7.6 + 0.1% NaN_3 + 0.3% Triton X-100 for 5 minutes. The sections were blocked using TBS + 0.1% NaN_3 + 0.1% Triton X-100 + 10% goat serum (blocking buffer) for 1 hour at

room temperature. The primary antibody was diluted in the blocking buffer, and added to the sections for 1 hour at room temperature or overnight at -4°C. The sections were washed in TBS + 0.1% NaN_3 + 0.1% Triton X-100 (wash buffer) 3 x 10 minutes, before adding the fluorescently conjugated secondary antibody (Table 2.2) diluted in the blocking buffer for 1 hour, and washed again using the wash buffer for 3 x 10 minutes. This process was repeated for the second primary and secondary antibody. A DAPI nuclear stain was added for 5 minutes, before mounting the sections using IF mounting media.

2.6.4 Golgi Plug and Stop Immuno-fluorescence: HIEC were seeded into Ibidi μ-Slide VI 0.4 ibiTreat microslides at $3.5x10^4$, and left for the HIEC to form a monolayer for two hours, before changing the media and incubating the cells overnight at 37°C. The HIEC were stimulated with TNF-α, TNF-α + Golgi Plug, TNF-α + Golgi Stop (Table 2.3), for 16 hours before removing the media, and fixing the cells using 2% PFA (paraformaldehyde) for 10 minutes at room temperature, and proceeding with IF staining as described for HIEC.

Table 2.1 Primary Antibodies: Primary antibodies used during flow cytometry, IHC and IF, their clone, concentrations and supplier.

Antibody	Clone	Concentration used µg/ml		Source
		Flow Cytometry	IHC and IF	
IgG1	Mouse (MOPC21)	1.62	13	Biogen Idec
IgG2a	Mouse (P1.17)	1.85	13.6	Biogen Idec
IgG1	Rabbit		10	AbCam
Fn14	Mouse (MP4A8)	1.62	13	Biogen Idec
TWEAK	Mouse (MP2D10)	1.85	13.6	Biogen Idec
CD31	Mouse (JC70A)	25	10	DAKO
CD68	Mouse (Y1/82A)	25	10	eBioscience
CD90	Mouse (5E10)	25	10	eBioscience
CK18	Mouse (CD10)	30	12	DAKO
CK19	Mouse (RCK108)	25	10	DAKO
CD31 (Polyclonal)	Rabbit	-	10	AbCam
CD20	Mouse (B-Ly1)	-	10	DAKO
CD3	Mouse (F72.38)	-	10	DAKO
FGF R1	Rabbit (D8E4)	-	1:400	Cell Signalling

Table 2.2 Secondary and Conjugated Antibodies: secondary and conjugated antibodies, their clone and supplier.

Antibody	Clone	Source
CD14 FITC	mouse α human	Miltenyi MACS
CD14 PE	mouse α human	Miltenyi MACS
CD14 APC	mouse α human	Miltenyi MACS
CD66b PE	mouse α human	Miltenyi MACS
CD56 PE	mouse α human	Miltenyi MACS
IgG2a APC	mouse α human	Miltenyi MACS
IgM FITC	mouse α human	Miltenyi MACS
IgG1 PE	mouse α human	Miltenyi MACS
VCAM PE	mouse α human	BD Biosciences
ICAM APC	mouse α human	BD Biosciences
IgG1 PE	mouse α human	BD Biosciences
IgG1 APC	mouse α human	BD Biosciences
Alexa Fluor 488	goat α mouse	Invitrogen
Alexa Fluor 546	goat α mouse	Invitrogen
FITC	goat α mouse	Southern Biotech
TRITC	goat α rabbit	Southern Biotech
Texas Red	goat α mouse	Southern Biotech
Alexa Fluor 610	goat α mouse	Invitrogen
Alexa Fluor 405	goat α mouse	Invitrogen
Pacific Orange	goat α mouse	Invitrogen

2.7 Flow Cytometry

2.7.1 Cell Surface Staining: Cells were stimulated with the appropriate cytokines for 24 hours (Table 2.3). The cells were then washed with PBS, and detached using Tryp-LE™ as previously described. The cells were subsequently washed in PBS again and centrifuged at 2000rpm for 5 minutes. The pelleted cells were then suspended in PBS serum buffer containing PBS and 10% FCS at $1x10^6$ cells/ 100µl buffer. Primary antibodies (Table 2.1) were added at the required concentration diluted in PBS serum buffer at 4°C for 30 minutes with agitation. The cells were washed with 0.5ml PBS and centrifuged at 2000rpm for 5 minutes. The secondary fluorescently conjugated antibodies (Table 2.2) were diluted in PBS serum buffer, and added to each tube in a volume of 100µl, and incubated at 4°C for 30 minutes with agitation. The cells were washed again in PBS before fixing in 100µl of 2% PFA per tube for 20 minutes on ice. The cells were then washed in PBS and re-suspended in 0.5ml PBS before analysis.

2.7.2 Intracellular Staining: The cells were fixed using 2% PFA on ice for 20 minutes, before staining with antibodies. The antibodies were diluted in permeablisation buffer (BD Biosciences) and left to incubate for 30 minutes at 4°C to permeablise the cell membrane. The cells were washed in permeablisation buffer, and re-suspended in 100µl permeablisation buffer with the secondary fluorescent conjugated antibody, and left to incubate at 4°C for 30 minutes. The samples were washed again, and re-suspended in 500µl PBS prior to analysis.

2.7.3 Flow Cytometry Analysis: Analysis was carried out on a Dako Cyan ADP flow cytometer and Summit 4.3 for analysis of data.

Table 2.3 HIEC stimulations: Cytokines used to stimulate HIEC, their concentrations and supplier.

Stimulations	Concentration used (ng/ml)	Source
TNF-α	10	Peprotech
TWEAK	100	Biogen IDEC
IFN-γ	20	Peprotech
FGF	0.5	Peprotech
IL-1β	10	Peprotech
IL-2	10	Peprotech
IL-4	100	Peprotech
IL-6	20	Peprotech
IL-8	20	Peprotech
IL-10	50	Peprotech
IL-17A	50	Peprotech
IL-18	100	Peprotech
IL-21	50	Peprotech
IL-22	50	Peprotech
MCP-1	20	Peprotech
TGF-β	10	Peprotech
Golgi Plug	1:1000	BD Biosciences
Golgi Stop	0.7:1000	BD Biosciences

Table 2.4 Functional Inhibitors: Inhibitors used, their concentrations and supplier.

Inhibitor	Concentration	Source
TWEAK Inhibitor (Fn14 mAb)	74 ng/ml	Biogen IDEC
NF-kB Rel A Inhibitor	10 µg/ml	Calbiochem
Erk 1/2 Inhibitor	20µM	Cell Signalling

2.8 Quantitative Real Time PCR

2.8.1 RNA Isolation: Total RNA was isolated from HIEC and whole liver blocks using the RNeasy® Mini Kit (Qiagen) according to the manufacturer's instructions. Liver blocks, approximately 30mg in weight were submerged in RNA later (Sigma-Aldrich) prior to RNA (ribonucleic acid) isolation to preserve RNA. The HIEC and PBMC (peripheral blood mononuclear cells) were stimulated for 24 hours prior to RNA isolation (Table 2.3), and the cells were detached from their flasks and pelleted by centrifugation prior to RNA isolation. If RNA was not isolated immediately, then the cell pellets were suspended in RNA later. DNA (deoxyribonucleic acid) contamination was removed from the isolated RNA, using the RNase Free DNase Set (Qiagen).

2.8.2 cDNA Synthesis: cDNA (complementary DNA) was synthesised from 1µg of isolated total RNA using the 1st strand cDNA synthesis kit for RT-PCR (AMV) (Roche).

2.8.3 Real Time Polymerase Chain Reaction: Primers specific to TWEAK and Fn14 were designed using the Primer-BLAST program using the NCBI databases. The Primers were purchased from Alta Bioscience (Birmingham, UK; Table 2.5). Primer probes specific to

TWEAK (04689097001) and Fn14 (04687574001) were designed and purchased from Roche, and relative expression levels were calculated and compared to the housekeeping gene GUSB (Roche). The lightcycler 480 master-mix and system (Roche) was used for the real time PCR (Table 2.6).

Table 2.5 TWEAK and Fn14 primer sequence: Primers used for qPCR to detect TWEAK and Fn14 mRNA.

Primers	**Fn14**
Forward	5'-CAC-GAA-GGT-CAG-GCT-CAG-A-3'
Reverse	5'-GAC-CGC-ACA-GCG-ACT-TCT-3'
	TWEAK
Forward	5'-CTC-ACT-GTC-CCG-TCC-ACA-C-3'
Reverse	5'-ATC-GCA-GCC-CAT-TAT-GAA-GT-3'

Table 2.6 Thermal profile for real time PCR: qPCR programme.

Number of Cycle: 65	**Temperature °C**	**Time (minutes)**
Denaturing	95	1
Annealing	60	1
Extension	72	1
Hold	4	5

2.9 MTT to assess cell viability

HIEC were seeded into a 24 well plate at a density of 5.0 x 10^4 per well, and were stimulated for 24 hours in HIEC complete media. Thereafter, the media was removed and cells were treated with Yellow MTT (Thiazolyl Blue Tetrazolium Blue; Sigma-Aldrich) to assess cell

viability. Each well was treated with 300µl of 0.4mg/ml MTT, made up in complete HIEC media, and incubated for 1 hour until the development of a purple insoluble formazan product was visualised. The media was removed and 600µl of DMSO was added to each well to solubilise the purple formazan product. The plate was incubated at room temperature for 5 minutes on a plate shaker, and then read at an absorbance of 490nm. The percentage viability was calculated using the following formula:

$$\% \text{ viability} = \frac{\text{mean absorbance of sample X 100}}{\text{Mean absorbance of control}}$$

2.10 Angiogenesis Assay

2.10.1 Angiogenesis Assay: BD Natural Matrigel ™ (VWR) was thawed on ice at 4°C overnight. A 12 well plate was coated with the natural Matrigel ™ after wetting the plates with PBS. The Matrigel ™ was allowed to set at 37°C prior to seeding harvested HIEC at 1.4 x 10^5 cells/ well in complete HIEC media, with the appropriate stimulations. The cells were then incubated at 37°C for 24 hours.

2.10.2 Analysis: Images were taken using a Zeiss Axiovert 40C microscope and Canon DS126171 camera, at 0, 4, 8 and 24 hours post stimulation. Images were taken from five fields of view of each well. The assay was quantified by counting the average number of nodes with 1 or more branch points, from five fields of view of each condition at 8 hours.

2.10.3 Angiogenesis and Cytokine Array: Supernatants from the Angiogenesis Assay were analysed using the Proteome Profiler™ Array; Human Angiogenesis Array Kit (R&D Systems;

ARY007), and Human Cytokine Array Panel A (R&D Systems; ARY005) according to the manufacturer's instructions.

2.11 Scratch Wound Assay

2.11.1 Scratch Wound Assay: HIEC were seeded at 1.75 x 10^5 cells/well in complete HIEC media in a 6 well plate coated with 0.1% (w/v) gelatin in PBS, and left overnight. The media was then aspirated, and pen marks were made at the bottom of the wells before a scratch was made perpendicular to the pen marks. Fresh media was made containing 25µg/ml mitomycin C (to prevent cell mitosis) (Sigma-Aldrich), and appropriate cytokines were added. The media in each well was replaced by the conditioned media and incubated at 37°C.

2.11.2 Analysis: Cell migration was observed by taking images at 0, 4, 8, 24 and 48 hours. Images were taken using a Zeiss Axiovert 40C microscope and Canon DS126171 camera.

2.12 Determination of ROS accumulation, apoptosis, necrosis and autophagy

HIEC were stimulated for 24 hours with the appropriate conditioned media. The media was then aspirated and kept in a separate tube and replaced with HBSS (Hanks Balanced Salt Solution; Sigma-Aldrich), an ROS accumulation marker: 30µM 2', 7'- dichlorofluorescin (DCF) (Gibco) and an autophagy marker: 1µM monodansylcadaverine (MDC) (Gibco) were added to the media, and incubated at 37°C for 20 minutes with constant agitation. The cells were harvested using Tryp-LE™, and placed into the aspirated media from the initial step and washed extensively in PBS and 10% FCS. The cells were then re-suspended in Annexin-V

staining buffer and labelled with Annexin-V (Molecular Probes, Paisley, UK); an apoptosis marker, and 7-AAD (Molecular Probes, Paisley, UK); a necrosis marker, and incubated on ice for 15 minutes before analysis using flow cytometry. Appropriate compensation tubes were used for each channel the antibodies were analysed at.

2.13 Peripheral Blood Mononuclear Cell Isolation

Peripheral blood was layered upon a lympholyte (Cedarlane) gradient at a ratio of 2:1 and centrifuged at 2000rpm for 20 minutes with brake 0. The mononuclear layer was removed and washed with PBS, before centrifuging for 5 minutes at 2000rpm. The pellet was re-suspended and washed again in PBS, before centrifuging for 10 minutes at 800rpm to remove platelets.

2.14 Monocyte Subset Sorting

PBMC were isolated and counted using a haematocytometer, before labelling the sample using fluorescently conjugated antibodies (Table 2.2). CD56 and CD66 were used for negative selection of neutrophils and NK cells. Separate tubes of PBMC were also set up with the relevant isotype controls and compensation tubes for APC, FITC and PE. The samples were incubated for 30 minutes at 4°C, then washed with PBS serum buffer and centrifuged at 2000rpm for 5 minutes, brake 3. The cell pellet was re-suspended in PBS serum buffer at 1 x 10^6 cells/ml. The sample for sorting was transferred into sterile tubes using a filter (PARTEC Cell Trics®) to separate the preparation into a single cell suspension.

The MoFlow high speed cell sorter was set up using the isotype controls, and all three fluorescent channels were compensated. The sample was then sorted into Classical; CD14++CD16-, Non-classical; CD14+CD16++ and Intermediate; CD14++CD16+ subsets into sterile tubes containing RPMI (Sigma-Aldrich) + 10% FCS. The monocyte cloud was isolated, and the PE negative cloud was further sorted into the monocyte subsets (Figure 2.1). Once cell sorting was complete the subsets were centrifuged at 2000rpm for 5 minutes brake 3; re-suspended in RPMI + 10% FCS, and incubated overnight at 37°C with and without IFN-γ 50ng/ml activation. RNA was isolated from each subset, converted to cDNA, and RT-PCR was used to determine TWEAK mRNA expression.

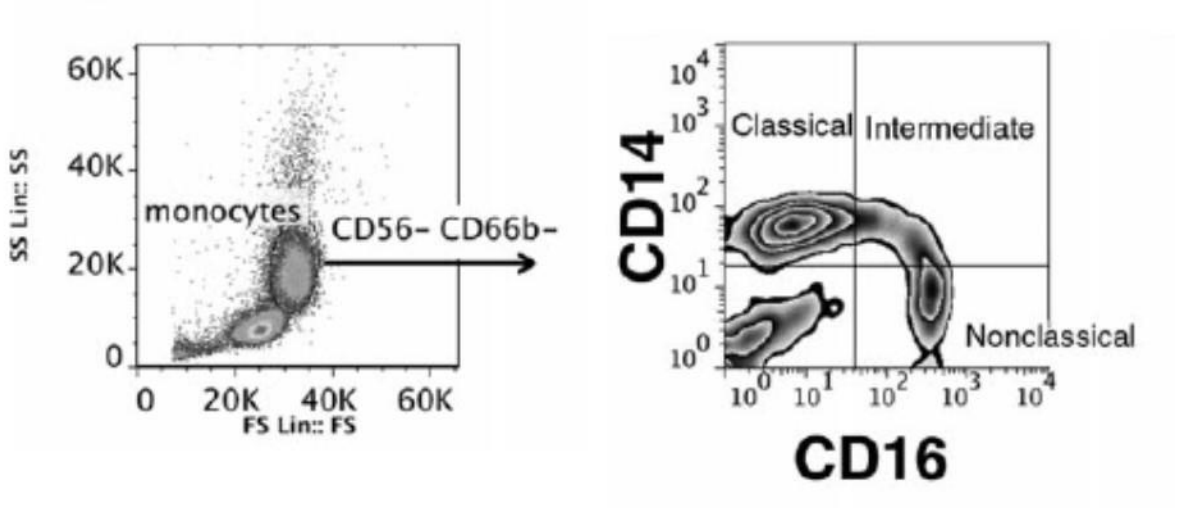


Figure 2.1 Monocyte subset sorting: Monocytes were isolated by selecting the correct cell gate and subsequently negatively selecting for neutrophils and NK cells (CD56 and CD66). The monocytes were separated in to their subsets by selective markers for CD14 and CD16. These were classical monocytes; CD14++CD16-, Intermediate monocytes; CD14++CD16+, and non-classical monocytes; CD14+CD16++. (Liaskou *et al.*, 2013)

2.15.1 Ibidi µ-Slide preparation: Ibidi µ-Slide VI 0.4 (ibi-Treat microslides) were prepared by incubating the slides for 30 minutes after adding rat tail collagen into each lane, ensuring the

entire lane was covered. The rat tail collagen was removed and the slides were then washed through with sterile PBS, and then washed with HIEC complete media to ensure no residual rat tail collagen was remaining.

2.15.2 HIEC monolayer preparation: HIEC were seeded into the coated Ibidi µ-Slides at $3.5x10^4$ cells/lane, and incubated for two hours to allow an HIEC monolayer to adhere, before changing the media and incubating overnight at 37°C. The HIEC were then stimulated for 24 hours with the appropriate conditions.

2.15.3 Flow based adhesion assay: PBMC were isolated (Section 2.13) and counted, and re-suspended at 10^6 cells/ml in endothelial media + 10% FCS. The Ibidi microslide was connected to a Harvard pump (Harvard Apparatus, South Natic, USA), a syringe to remove waste, and an electronic valve which allowed alternating perfusion of the PBMC cell suspension and the wash buffer (endothelial serum free media + 0.1% BSA) (Figure 2.2). The microslide, PBMC suspension, and wash buffer were kept incubated in a temperature controlled chamber to ensure optimal conditions for the duration of the assay. The PBMC were perfused over the stimulated HIEC at 0.05Pa for 5 minutes, and then the wash buffer was perfused over the HIEC layer for 3 minutes. The assay was recorded for 2 minutes by a microscope and camera unit, whilst changing the field of view 10 times.

2.15.4 Analysis: the data was analysed by counting PBMC which have adhered and migrated to the HIEC monolayer, in 10 different fields per condition of a known dimension. Monocytes and lymphocytes were counted and easily distinguishable by their size difference, with

monocytes being much larger in size than lymphocytes. PBMC shape change was analysed by counting all PBMC which have changed shape and comparing them to PBMC which have remained round. All data was normalised to the total number of cells perfused per condition, and data was shown as cells/$mm^2/10^6$ cells perfused.

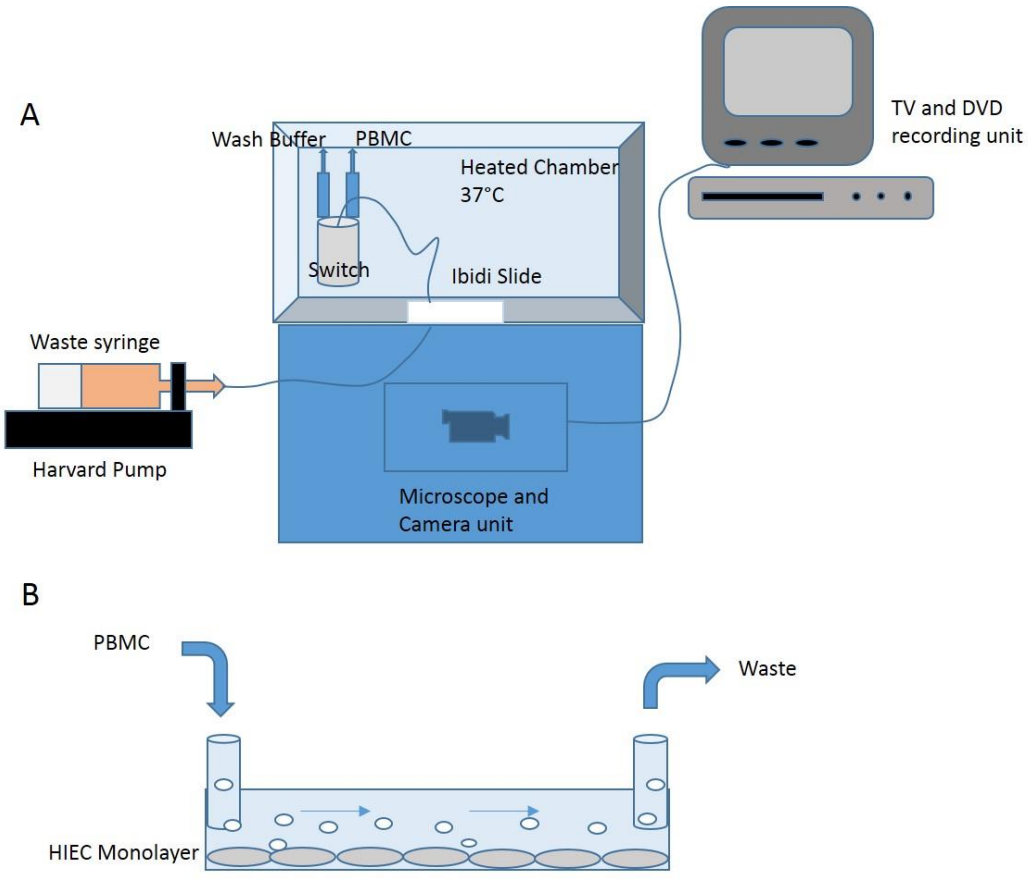


Figure 2.2 Flow based adhesion assay: A) The Ibidi microslide with the HIEC monolayer was connected to a Harvard pump, a syringe to remove waste, and an electronic valve which alternated between PBMC suspension and wash buffer. The whole assay was carried out in a heated chamber to ensure optimum experimental conditions. The slide was mounted on to a microscope with an internal camera which was attached to a TV and DVD recording unit. B) The PBMC were perfused over an HIEC monolayer for 5 minutes at 0.05PA shear stress before subsequent analysis.

2.16.1 Protein Lysate Preparation: HIEC were stimulated and collected into universal tubes. The cells were washed with PBS and centrifuged for 5 minutes at 2000rpm brake 3. The cell pellet was re-suspended in Cell lytic MT™ (Sigma-Aldrich), + phosphatase and protease inhibitor cocktail (1:100; Cell Signalling), + DNase (Sigma-Aldrich), and incubated for 15 minutes on a shaking platform. The lysed cells were sonicated briefly to further disrupt the cell membrane, and centrifuged at 13'000 rpm for 30 minutes. The supernatant containing the protein lysate was transferred to a chilled tube and kept at -80°C until required.

2.16.2 Protein Determination Assay: A BSA calibration curve was made from a concentration range of 1-0.016mg/ml. The samples were diluted 1:10 in 0.85% NaCl solution, which was also used as a blank. 20µl of each sample and standard was loaded on to a 96 well plate in duplicates, and 220µl of Biuret reagent was added to each well and incubated at room temperature for 10 minutes. 10 µl of Folin and Ciocaulteaus (Sigma-Aldrich) reagent was added to each well, mixed and incubated at room temperature for 30 minutes. The plate was then read at 750nm absorbance.

Table 2.7 Western Blotting reagents: Western blotting reagents, molecular weight and supplier.

Reagent	Molecular Weight (FW)	Source
Tris Base	121.14	Sigma
Tris HCl	157.6	Sigma
Glycine	75.07	Sigma
Sodium Dodecyle Sulphate	288.38	Sigma
NaCl (Sodium Chloride)	58.44	Sigma
Non Fat Milk	-	Marvel
BSA (Bovine Albumin Serum)	-	Sigma
APS (Ammonium Persulphate)	228	Sigma

2.16.3 SDS PAGE: 40-50µg of protein was diluted in the appropriate sample buffer (200mM Tris pH 6.8, 20% Glycerol, 10% SDS and 0.05% bromophenol blue), and boiled at 100°C for 10 minutes. The resolving gel was made (Table 2.9) and poured between glass plates, and a layer of butanol was used to prevent oxidisation of the gel. Once the gel had set, the butanol was removed and the stacking gel was layered over the resolving gel, and a comb was inserted to create wells. Once set, the gel was placed into an electrophoresis tank, and the samples and molecular weight marker was loaded before running the gel at 200V, until the dye front reached the bottom of the plate. The separated protein was transferred to nitrocellulose membrane using transfer apparatus, at 100V for 60 minutes.

Table 2.8 Western Blotting buffers: Buffers used for Western blotting and their constituents.

Western Blotting Buffers	Constituents
Stacking Gel Buffer	1.M Tris Base pH 6.8
Resolving Gel Buffer	1.5M Tris Base pH 8.8
Electrophoresis Buffer	2.5mM Tris Base, 200mM Glycine, 1g SDS, 1L Distilled H2O
Transfer Buffer	2.5mM Tris Base, 200mM Glycine, 200ml Methanol, 800ml Distilled H2O
Stripping Buffer	100mM β-2-mercaptoethanol, 2% w/v SDS, 62.5mM Tris-HCL pH 6.7 and Distilled H2O
10x TBS (Tris Buffered Saline)	24.2g Tris Base, 80g NaCl, pH 7.6
Wash Buffer	TBS, 0.1% (v/v)Tween
Blocking Buffer	100 ml TBS, 0.1% (v/v)Tween, 5g non-fat milk
Antibody Dilution Buffer	100 ml TBS, 0.1% (v/v)Tween, 5g BSA

Table 2.9 SDS PAGE: SDS PAGE components.

	Stacking Gel (ml)		Resolving Gel (ml)		
	4%	**5%**	**6%**	**8%**	**10%**
Resolving Gel Buffer	0	0	2.5	2.5	2.5
Stacking Gel Buffer	0.63	0.63	0	0	0
Distilled H20	3.6	3.4	5.3	4.6	4
30% acrylamide (BioRad)	0.64	0.83	2	2.7	3.3
10% w/v SDS	0.1	0.1	0.1	0.1	0.1
10% w/v APS	0.1	0.1	0.1	0.1	0.1
TEMED	0.02	0.02	0.02	0.02	0.02

2.16.4 Ponceau Stain: To ensure successful transfer of the protein, the nitrocellulose membrane was placed in Ponceau S solution (Sigma), and immediately washed with tap water until the bands could be visualised. The membrane was then washed in tap water until the Ponceau stain was completely removed. The membrane was then blocked in 5% milk solution or 5% BSA solution for 60 minutes.

2.16.5 Blotting: The primary antibody was diluted in BSA (Table 2.10), and the nitrocellulose membrane (Hy-bond Nitrocellulose, Amersham) was incubated overnight at -4°C in the solution. The membrane was then washed in TBS-Tween (0.1%) for 3 x 5 minutes on a shaking platform, before adding β-actin (as a positive control) diluted in BSA for 1 hour. The membrane was washed again in TBS-Tween (0.1%) 3 x 5 minutes before adding the secondary HRP conjugated antibodies (Table 2.10). The membrane was then washed finally in TBS-Tween (0.1%) 3 x 5 minutes, before adding the ECL reagent according to manufacturer's instructions. The membrane was then placed in a cassette with X-ray film and developed at various time points in the dark.

2.16.6 Stripping and Re-probing the Nitrocellulose Membrane: The membrane was incubated in stripping buffer for 30 minutes at 50°C. It was then washed 2 x 10 minutes in TBS Tween, before blocking the membrane and re-probing as before.

Table 2.10 Western Blotting antibodies: Western blotting antibodies used, their clone, concentration and supplier.

	Clone	Concentration used	Source
NF-kB Rel A Phosphorylated	Rabbit (93H1)	1:100	Cell Signalling
NF-kB Rel A Total	Rabbit (D14E12)	1:100	Cell Signalling
NF-kB Rel B Phosphorylated	Rabbit	1:100	Cell Signalling
NF-kB Rel B Total	Rabbit (C1E4)	1:100	Cell Signalling
Erk 1/2 Phosphorylated	Rabbit (D13.14.4E)	1:100	Cell Signalling
Erk 1/2 Total	Rabbit (137F5)	1:100	Cell Signalling
Src Phosphorylated	Rabbit (D49G4)	1:100	Cell Signalling
Akt Phosphorylated	Rabbit (D9E)	1:100	Cell Signalling
FAK Phosphorylated	Rabbit (D20B1)	1:100	Cell Signalling
PLCγ1 Phosphorylated	Rabbit (D25A9)	1:100	Cell Signalling
VEGFR2 Phosphorylated	Rabbit (19A10)	1:100	Cell Signalling
P38 MAPK Phosphorylated	Rabbit (D3F9)	1:100	Cell Signalling
β-actin	Mouse (AC-15)	1ug/ml	Sigma Aldrich
Goat α Rabbit HRP conjugated	P0448	1:200	DAKO
Goat α Mouse HRP conjugated	P0260	1:200	DAKO

2.17 Statistical analysis

Statistical analysis of data from numerical variables between two groups was carried out using a paired Student's t test, when the data followed normal distribution. Mann-Whitney U test was performed when the data followed non-normal distribution. p-values of ≤ 0.05 were considered statistically significant, and data was presented as *≤0.05, **≤0.01 and ***≤0.001. All statistical analysis was carried out using Microsoft Excel and GraphPad Prism software.

CHAPTER 3

TWEAK and Fn14 may regulate specific inflammatory responses in the liver via paracrine interactions of leukocytes and HIEC

3.1 INTRODUCTION

Cells of the innate and adaptive immune system highly express TWEAK, indicating the critical role TWEAK may play in mediating inflammatory responses. Over the years, TWEAK activated inflammatory responses have been a focus of TWEAK and Fn14 functional activities. These have been extensively characterised by TWEAK and Fn14 regulated leukocyte infiltration and recruitment via cellular interactions, cell surface adhesion proteins, and chemokine and cytokine expression. TWEAK and Fn14 expression has subsequently been observed in several studies of inflammatory diseases such as MS, RA and LN (Winkles, 2008). The expression of TWEAK and Fn14 mRNA and protein has also been found in a variety of tumours including pancreatic, ovarian and colorectal tumours, and often a variable response to malignancy by TWEAK and Fn14 has been observed (Feng *et al.*, 2000; Ho *et al.*, 2004; Winkles, 2008). Fn14 and TWEAK protein and mRNA expression has been detected in normal and diseased liver in several investigations, where their expression has been found to be key during liver inflammatory processes and regeneration (Feng *et al.*, 2000; Jakubowski, Ambrose, Parr, Lincecum, Wang, Zheng, Browning, Michaelson, Baetscher, Baestcher*, et al.*, 2005).

The general TWEAK and Fn14 expression pattern in normal tissue is found to be low, however in inflamed tissue TWEAK and Fn14 expression is found to be up-regulated. This expression has been found to be highly regulated by many growth factors and cytokines, and the expression patterns of TWEAK and Fn14 always vary and are dependent on the tissue, conditions, and context they are present in. Thus, TWEAK and Fn14 interactions have been

found to be highly complex and multifunctional capabilities even within the same type of tissue have been described (Chicheportiche *et al.*, 1997; Burkly *et al.*, 2007; Winkles, 2008; Burkly e Dohi, 2011).

This investigation aimed to understand functional regulation of TWEAK and Fn14 in chronic inflammatory liver disease by further characterising the expression and regulation patterns of TWEAK and Fn14 in HIEC. TWEAK and Fn14 expression has previously been detected in liver tissue in several studies but not characterised to individual liver cell populations; and specifically in HIEC. It is first important to understand the expression patterns of TWEAK and Fn14 in HIEC and in liver tissue. It is then highly essential to further characterise leukocyte recruitment via TWEAK and Fn14 by HIEC, as these cells mediate a host of physiological, immune, and pathogenic functions within the liver; and understanding these processes are essential for regulating these during pathological settings as an aim to develop novel therapeutics in future.

3.1.2 Aims

The aims of this chapter were:

- To determine TWEAK and Fn14 expression in liver tissue and in isolated HIEC.
- To determine conditions which may activate TWEAK and Fn14 expression in HIEC.
- To determine TWEAK expression in PBMC and in monocyte subsets, to further understand Fn14 and TWEAK interactions with HIEC during inflammation.
- To determine if TWEAK stimulated HIEC express ICAM and VCAM adhesion proteins on their cell surface which may facilitate leukocyte recruitment.
- To determine if TWEAK stimulated HIEC, contribute to leukocyte recruitment by TWEAK, and TWEAK in combination with other cytokines.

3.2 MATERIALS AND METHODS

Materials and Methods for this chapter; refer to sections: 2.1-2.8, 2.13 - 2.15 and Tables: 2.1-2.6.

3.3 RESULTS

3.3.1 Liver tissue architecture

We wanted to identify the different cell types and structures within the liver we were analysing, to do this we used IHC immunohistochemistry (IHC). Liver tissue sections were stained using IHC and antibodies of phenotypic markers of typically identified liver cells and structures, and compared these to the IgG control for all antibodies used (Figure 3.3.1a A). Figure 3.3.1a B shows a representative CD31 stained liver tissue section. CD31 is a marker of cells of an endothelial phenotype, staining in this section was observed in portal veins, hepatic arteries, neovessels, and surrounding sinusoidal endothelium. Figure 3.3.1a B is also a representative image of a portal triad which consists of a bile duct, portal vein and hepatic artery. CK18 is a marker for hepatocytes and biliary epithelial cells. Figure 3.3.1a C is a representative image of stained bile ducts and hepatocytes. CK19 is a marker for biliary epithelium, Figure 3.3.1b D shows staining present in epithelia and bile ducts. CD90 is a marker for fibroblasts which is represented in Figure 3.3.1b E. CD68 is a marker for monocytic cells including monocytes, macrophages and KC, Figure 3.3.1b F shows representative staining of this marker.

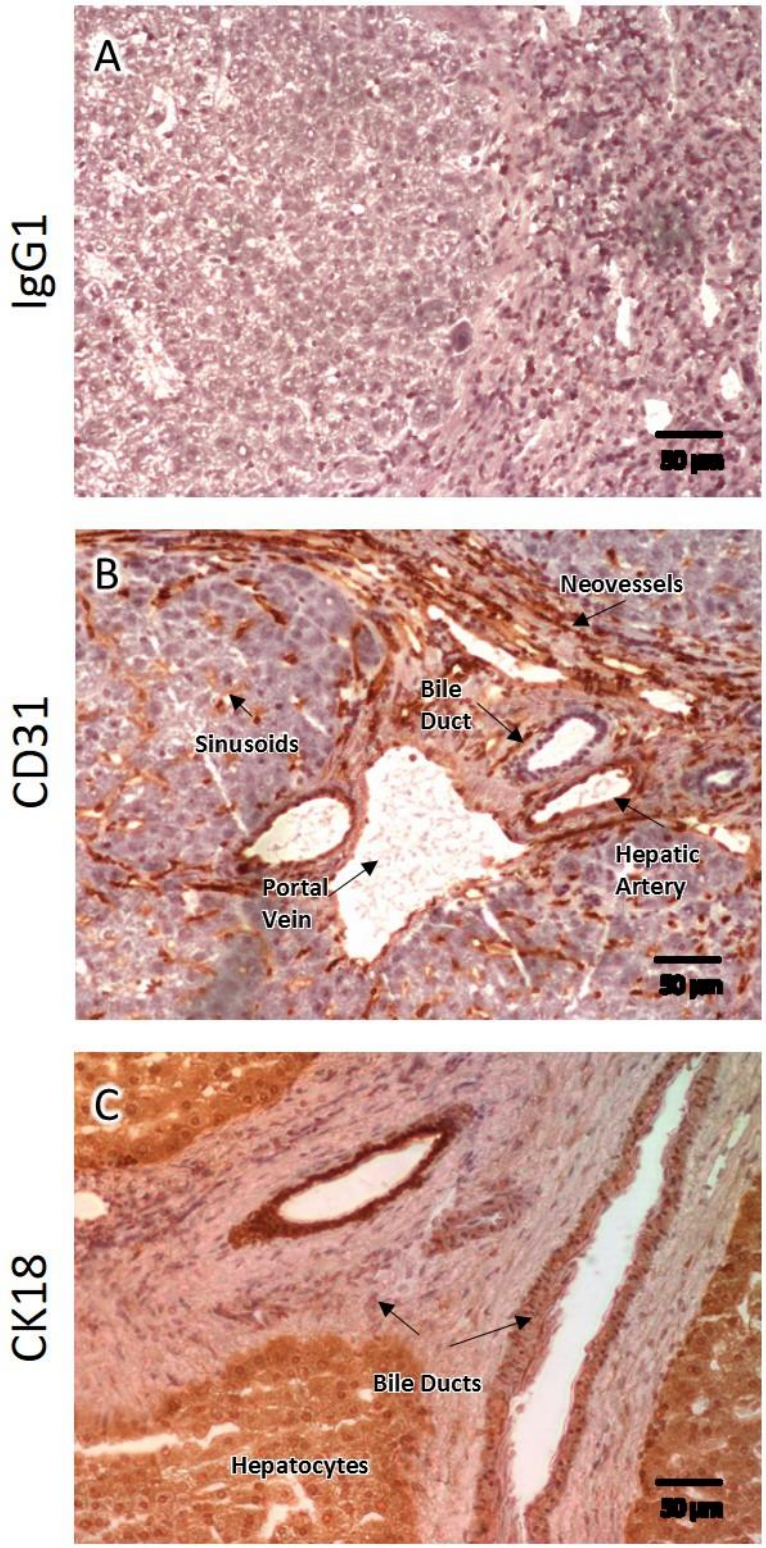


Figure 3.3.1a Phenotyping liver tissue: frozen liver tissue sections were stained for phenotypic markers of liver cells and structures. A) Shows the IgG1 control for the antibodies used. B) Shows CD31 staining which is an endothelium marker, staining was present in liver sinusoidal endothelium, neovessels, portal veins and hepatic arteries. C) Shows CK18 staining which was present in biliary epithelial cells and hepatocytes. All antibodies were developed with a DAB substrate. Images were taken with a Zeiss microscope and axiovision v4.4 software 200x magnification.

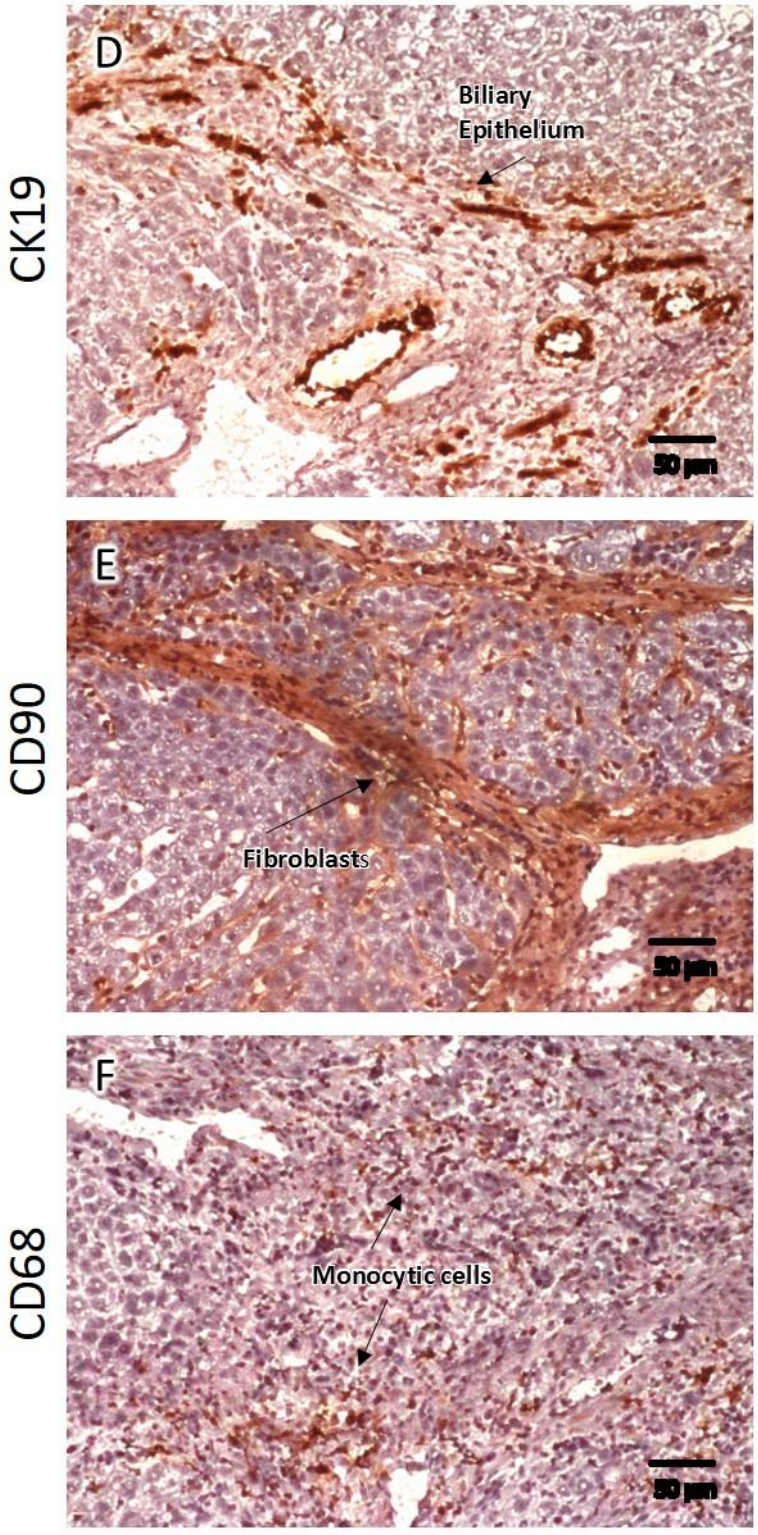


Figure 3.3.1b Phenotyping liver tissue: frozen liver tissue sections were stained for phenotypic markers of liver cells and structures. D) Shows CK19 staining which was present in biliary epithelium. E) Shows CD90 staining which was present in fibroblasts. F) Shows CD68 staining which was present in monocytic cells. All antibodies were developed with a DAB substrate. Images were taken with a Zeiss microscope and axiovision v4.4 software 200x magnification.

3.3.2 TWEAK and Fn14 expression was up-regulated in inflammatory liver disease tissue

To determine the mRNA expression difference of TWEAK and Fn14 between normal liver tissue and inflammatory diseased liver tissue. We used PBC and PSC diseased liver tissue as models of CILD and normal liver tissue, and analysed these for mRNA expression of TWEAK and Fn14 using quantitative real time PCR. TWEAK mRNA expression was significantly (*p=0.01) up-regulated 3.5 fold in diseased liver tissue (n=6), in comparison to normal liver controls (n=3). Fn14 mRNA expression was also significantly (*p=0.03) up-regulated 17 fold in diseased liver tissue (n=6) (Figure 3.3.2 A and B respectively).

Chronic inflammatory liver disease sections from explanted liver tissue were stained for TWEAK using an anti-TWEAK mAb (Figure 3.3.3). The sections stained included NASH, PBC, and ALD tissue. In the liver tissue sections analysed, TWEAK expression was observed in monocytic cells present in inflammatory infiltrates in close proximity to portal vessels (Figure 3.3.3 A, E and F). Representative magnified images of positive TWEAK staining is shown in Figure 3.3.3 C and G. All TWEAK staining was compared to the relevant IgG2a control (Figure 3.3.3 D and H).

Fn14 expression was determined using IHC in liver tissue sections and this was compared to normal donor tissue (Figure 3.3.4). Liver tissue sections from normal donor (A and B) and diseased liver tissues including PBC (C and F) and ALD (D and E) were analysed for Fn14 expression, this was compared to the relevant IgG1 control (Figure 3.3.4 G-I) representative images are shown. Fn14 expression was predominantly seen surrounding portal vessels,

neovessels, sinusoidal endothelium in close proximity to portal vessels, and in hepatocytes. Fn14 expression was also observed in ductular reactive cells (Figure 3.3.4 F). Fn14 expression did not appear in the same regions consistently; it was present in some neovessels and in certain portal vessels. One normal donor case showed a very light Fn14 stain present in the hepatocytes (Figure 3.3.4 A), however in the second normal donor case Fn14 was expressed highly in portal vessels (Figure 3.3.4 B). The staining pattern in the different diseased liver tissue types all showed an individual staining pattern.

Fn14 distribution and expression patterns indicated that Fn14 may be involved in ductular and portal vessel neogenesis. It was important to further establish whether the Fn14 expression observed, was actually present on endothelial regions of diseased liver tissue. Therefore dual immuno-fluorescence staining was used to co-stain diseased liver tissue sections with Fn14 (stained green) and CD31 (stained red) (Figure 3.3.5), ALD (Figure 3.3.5 A) and NASH (Figure 3.3.5 F) cases were stained. Fn14 expression was confirmed to be present in areas where portal vessels were present, and in hepatocytes (Figures 3.3.5 A and F), re-confirming observations made in the IHC staining. Fn14 co-localisation with CD31 was present in the sections; however this co-localisation was present in select areas and not entirely throughout the liver tissue section as was observed with IHC staining (Figures 3.3.5 D and I).

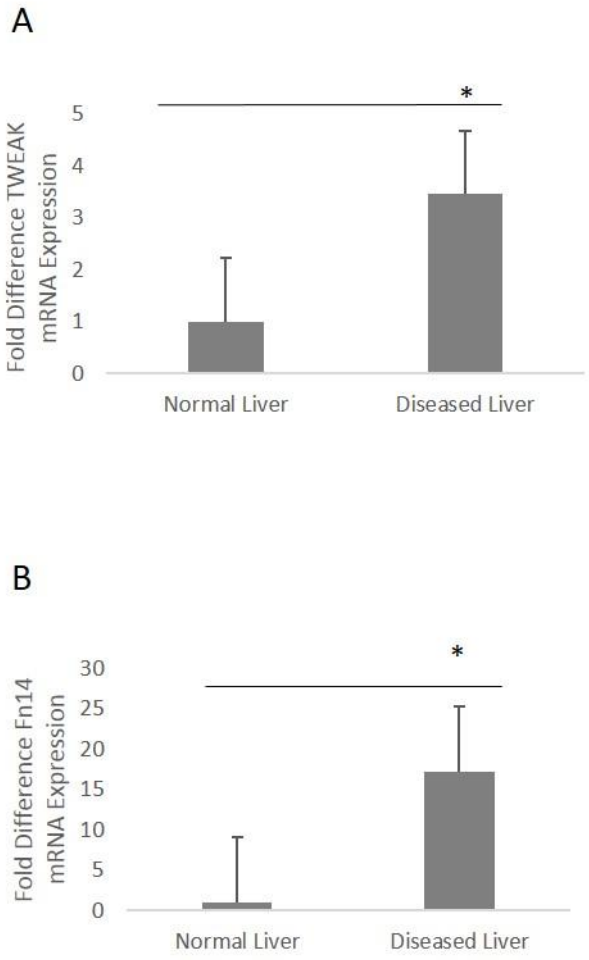


Figure 3.3.2 TWEAK and Fn14 mRNA expression was elevated in PBC and PSC diseased liver tissue in comparison to normal liver tissue: total RNA was isolated from normal liver blocks (n=3) and diseased liver blocks (PBC and PSC; n=5-6). qPCR was performed to detect TWEAK and Fn14 mRNA expression. TWEAK and Fn14 mRNA expression was significantly higher in diseased liver tissue in comparison to normal donor liver (*p=0.01 and *p=0.03 respectively). All samples have been analysed as duplicates against a GUSB reference gene. Fn14 and TWEAK forward and reverse primer concentration 1µM/ well. Data was shown as fold difference with the normal donor normalised to 1. Data is shown as ± standard error. Statistical analysis was performed using a Mann Whitney U test.

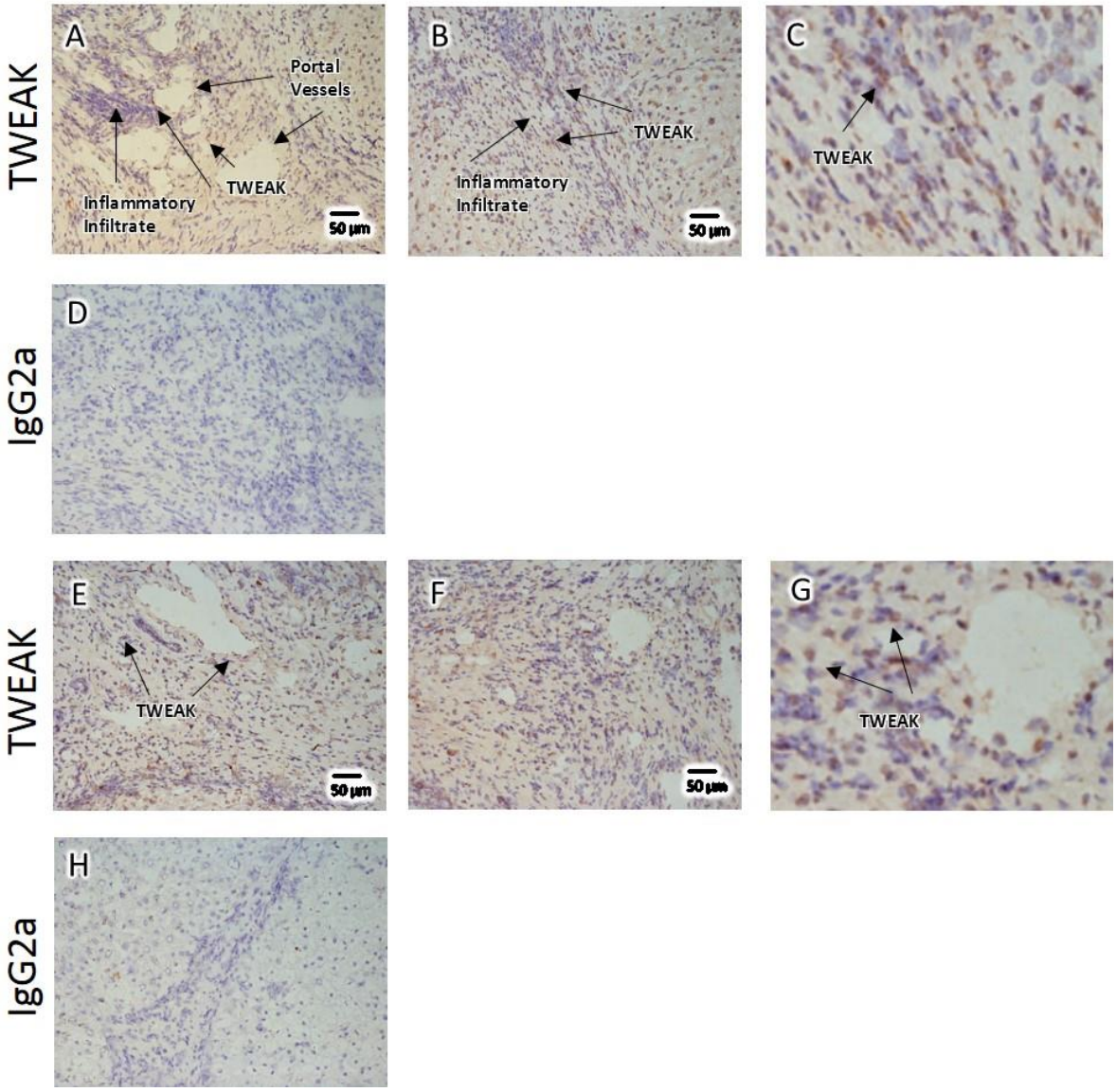


Figure 3.3.3 TWEAK localises to monocytic cells in close proximity to portal vessels in chronic inflammatory liver diseased tissue: PBC, NASH and ALD liver tissue sections were stained for TWEAK. A, B, E, and F) Show corresponding TWEAK IHC staining of diseased liver tissue. Magnified images (x3) of TWEAK stained tissue is shown in C and G. TWEAK expression was confirmed within monocytic infiltrates near portal vessels. Staining was compared to the corresponding IgG2a controls (D and H). All antibodies were developed with a DAB substrate. Images were taken with Zeiss microscope and axiovision v4.4 software 200x magnification.

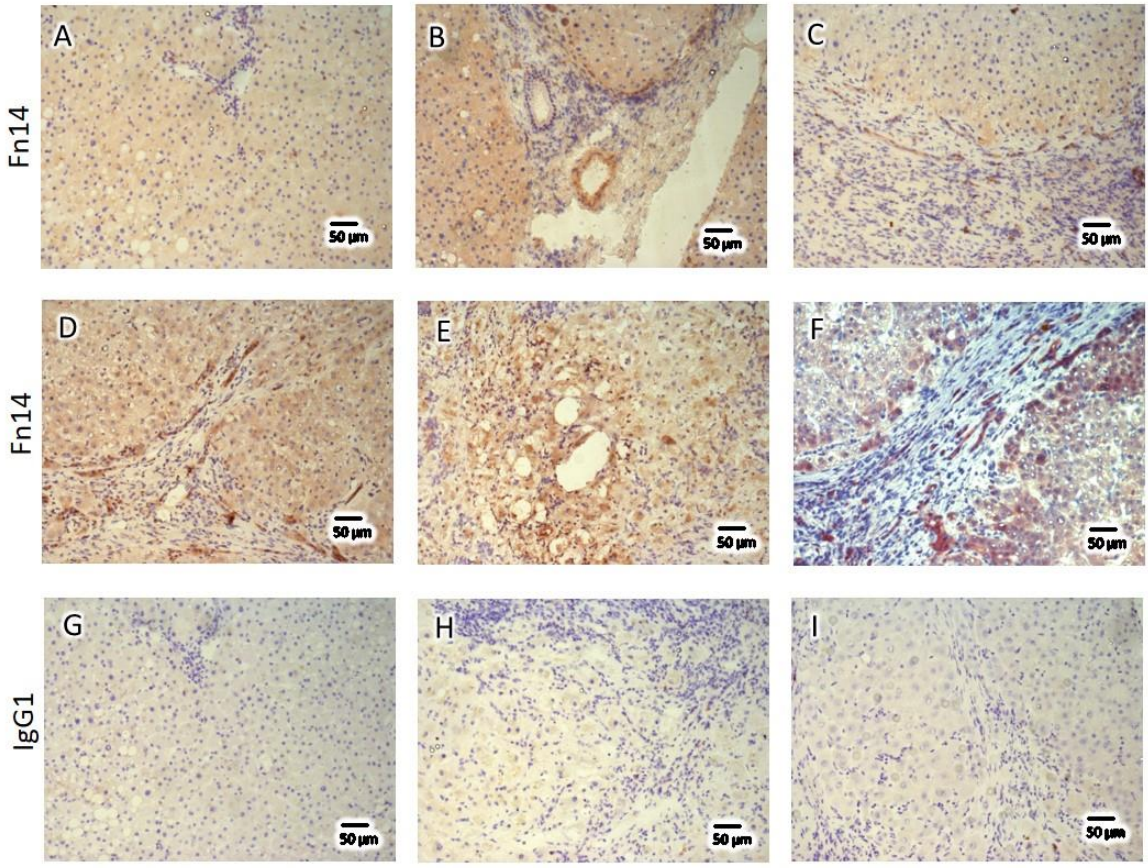


Figure 3.3.4 Fn14 localises to portal vessels and neovessels in inflamed and normal liver tissue: Normal liver (A and B), PBC (C and F) and ALD (D and E) liver tissue were stained for Fn14. G-I Shows corresponding IgG1 controls. Fn14 expression was confirmed to be present on hepatocytes, portal vessels, neovessels and liver sinusoidal endothelium. All antibodies were developed with a DAB substrate. Images were taken with Zeiss microscope and axiovision v4.4 software 200x magnification.

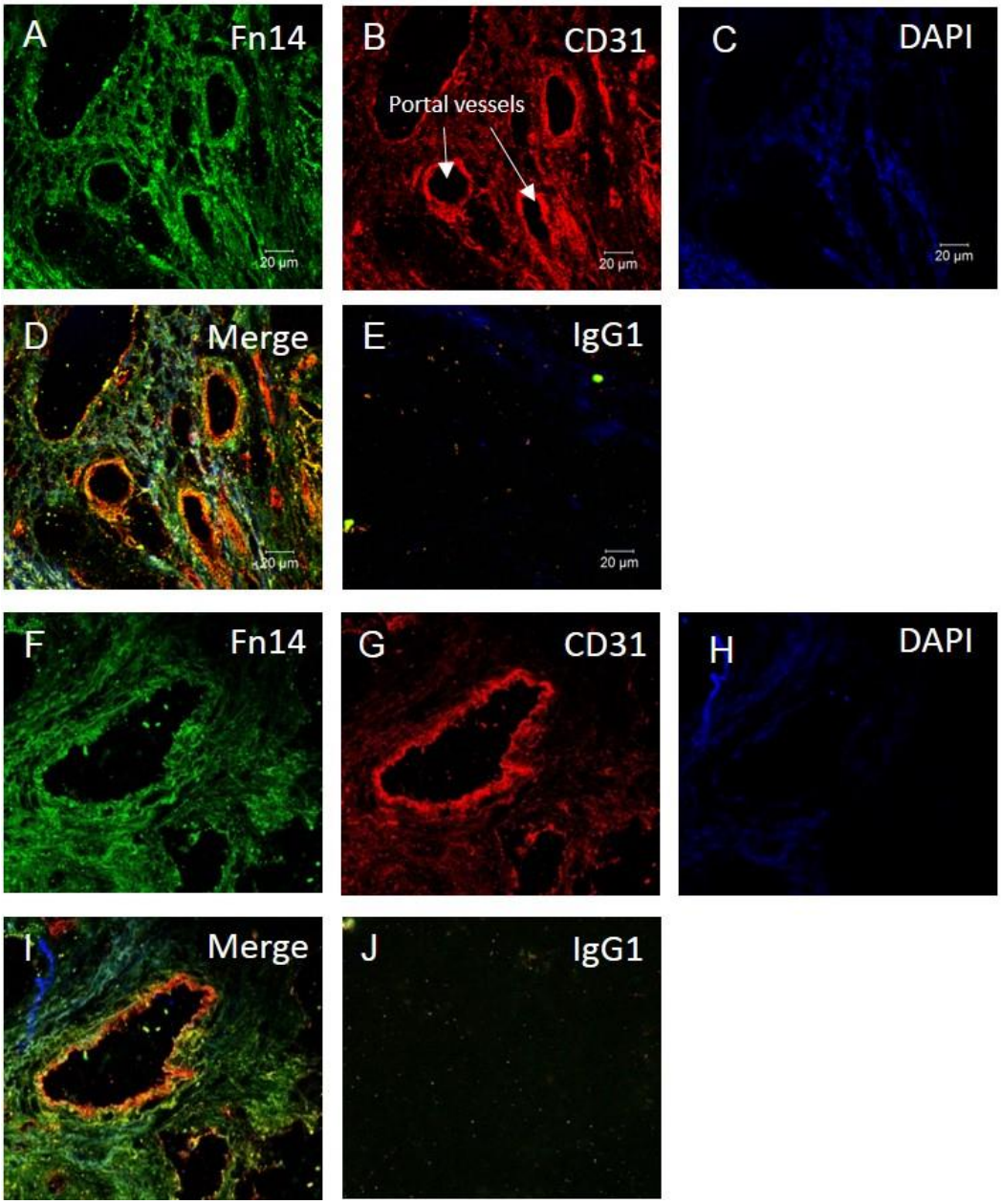


Figure 3.3.5 Fn14 was expressed in portal vessels in inflamed liver tissue: A and F) show dual immuno-fluorescent staining of portal vessels in inflamed liver tissue (ALD and NASH respectively) for Fn14 (FITC; green); B and G) shows the same section stained for the endothelial cell marker CD31 (TRITC; red); C and H) shows DAPI nuclear staining (Blue). D and I) shows the merged image confirming co-localisation of Fn14 to portal vessels. E and J) Show the control with primary antibodies substituted for isotype matched control IgG1. Images were taken with a Zeiss LSM 510 UV confocal microscope magnification 200x.

3.3.3 Phenotyping isolated primary HIEC

To establish the purity of HIEC isolated for the experiments in this study, phenotypic markers were used. IHC was used to stain isolated HIEC preparations for the following markers: CD31, CK18, CK19, CD90 and CD68 (Figure 3.3.6). All staining was compared to IgG1 isotype matched control (Figures 3.3.6 A). Isolated HIEC stained positive for CD31 which was the specific marker used to isolate cells of an endothelial phenotype from whole liver preparations (Figures 3.3.6 B). CK18 stained predominantly negative, however some contamination was observed (Figures 3.3.6 C). All other markers stained HIEC negatively (Figures 3.3.6 D, E, F).

HIEC were isolated and analysed for purity using flow cytometry. Cells were gated around the region where HIEC appear on the FACS plot to ensure no other cell type was included in the analysis (Figure 3.3.7 A). A further gate was used to isolate the region where only single cells were included in the analysis, to ensure accuracy of data obtained (Figure 3.3.7 B). CD31 was used as a positive endothelial cell marker for HIEC and only cells exhibiting ≥ 97-100 percent positivity were used in this analysis (Figure 3.3.7 C (shown as a solid red graph) and G). HIEC displayed typical morphological and phenotypical characteristics of HIEC; this included a very low, negligible percentage of contamination from hepatocytes, BEC (CK18 and CK19) and fibroblasts (CD90) (Figure 3.3.7 D, E, F, G). Any contamination was eliminated during the analysis by ensuring only CD31 positive populations were included. This further demonstrated that the isolation technique used to obtain HIEC, yielded cells of high purity with minimal contamination from other cells from the liver tissue. All data obtained for this

analysis was quantified against the correct isotype control for each antibody used; IgG1 (shown as a black line). Data is shown as percentage positive HIEC and median fluoresence intensity (Figure 3.3.7 G) ± SEM.

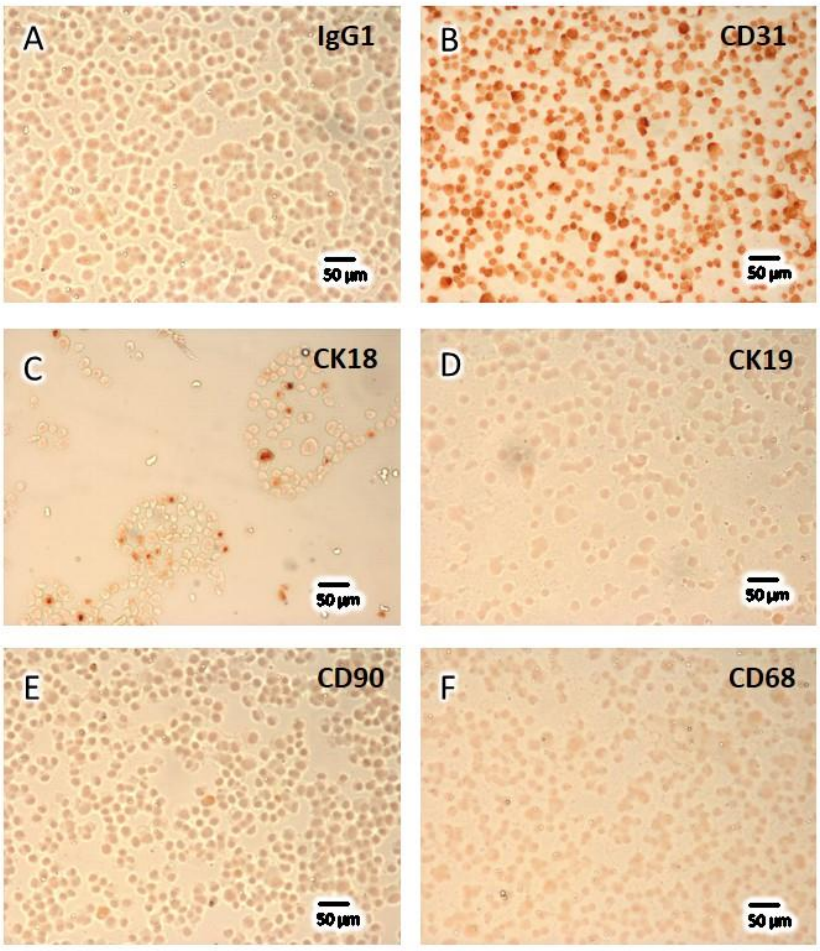


Figure 3.3.6 Phenotyping isolated HIEC: Isolated HIEC from normal and diseased liver were stained using different liver cell phenotypic markers to determine the purity of HIEC. Representative images are shown. A) Shows the IgG1 control for the antibodies used. B) Shows CD31 staining which was present in all cells. C) Shows CK18 staining which was present in a small population of HIEC. D) Shows CK19 staining which was negative. E) Shows CD90 staining which was negative. E) Shows CD90 staining which was negative. F) Shows CD68 staining which was negative. All antibodies were developed with a DAB substrate. Images were taken with Zeiss microscope and axiovision v4.4 software 200x magnification.

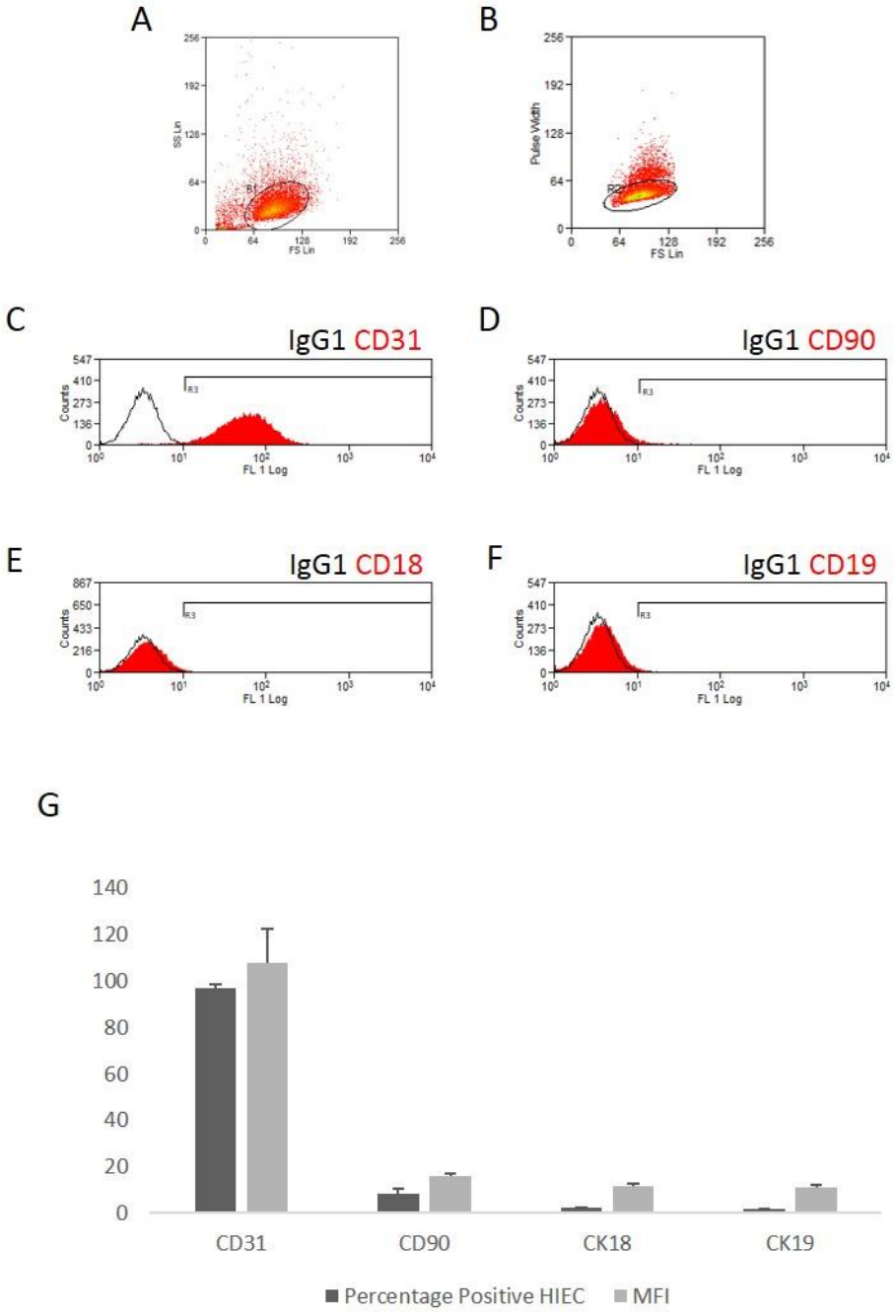


Figure 3.3.7 Phenotypic analysis of HIEC to determine purity of isolation: HIEC were analysed using flow cytometry. A) Shows the gate used to isolate HIEC to include in the analysis. B) Shows the gate isolating the single cell scatter of HIEC to include in the analysis. The HIEC were phenotypically analysed for cell surface markers CD31 (C), CD90 (D), CK18 (E) and CK19 (F) to ensure that the cells used in all experiments were of an endothelial lineage. CD31 (Red) was used to phenotype HIEC used in this study to determine purity of isolation. The histograms show the IgG control (Black line) and the respective markers (red solid histogram). G) Shows a representative graph of the phenotypic analysis done on HIEC prior to use. HIEC showed almost 97.22% percentage positivity for CD31, 8.49% CD90, 2.15% CK18 and 1.77% CK19. This was also confirmed with MFI data (n=3). All data is shown as mean ± SEM, representative of 3 independent experiments. No statistical analysis was performed on this data.

3.3.4 Fn14 expression on HIEC was up-regulated in response to TNF-α

Flow cytometry was used to determine basic cell surface and intracellular protein expression of Fn14 and TWEAK from HIEC, in response to various inflammatory cytokines. Cell surface Fn14 protein expression was significantly up-regulated by TNF-α, increasing the average percentage positive HIEC for Fn14 from 27 to 51.8 ± SEM (***P=0.0002) (Figure 3.3.8 C and E). This was also shown in the median fluorescence intensity (MFI) values which significantly increased from 9.1 to 16.2 ± SEM (*p=0.04) (Figure 3.3.8 F). To determine mRNA Fn14 expression; total RNA was isolated from TNF-α stimulated HIEC, and analysed for Fn14 mRNA expression using qPCR. Fn14 expression significantly increased in reponse to TNF-α (**p=0.006), where relative Fn14 expression increased almost 2-fold (Figure 3.3.8 G).

FGF stimulated HIEC, marginally increased Fn14 cell surface protein expression on HIEC from 27 to 31.4 ± SEM percentage positive HIEC (*p=0.03) (Figure 3.3.8 D and E). This however, was not reflected in the MFI data where expression values remained the same (Figure 3.3.8 F). IFN-γ stimulated HIEC, did not significantly effect Fn14 cell surface protein expression. HIEC were analysed for presence of the FGF receptor on the cell surface and and to determine if this presence was altered in response to TWEAK and TNF-α activation. Flow cytometry data did not show the presence of an FGF receptor on HIEC cell surface (Figure 3.3.9).

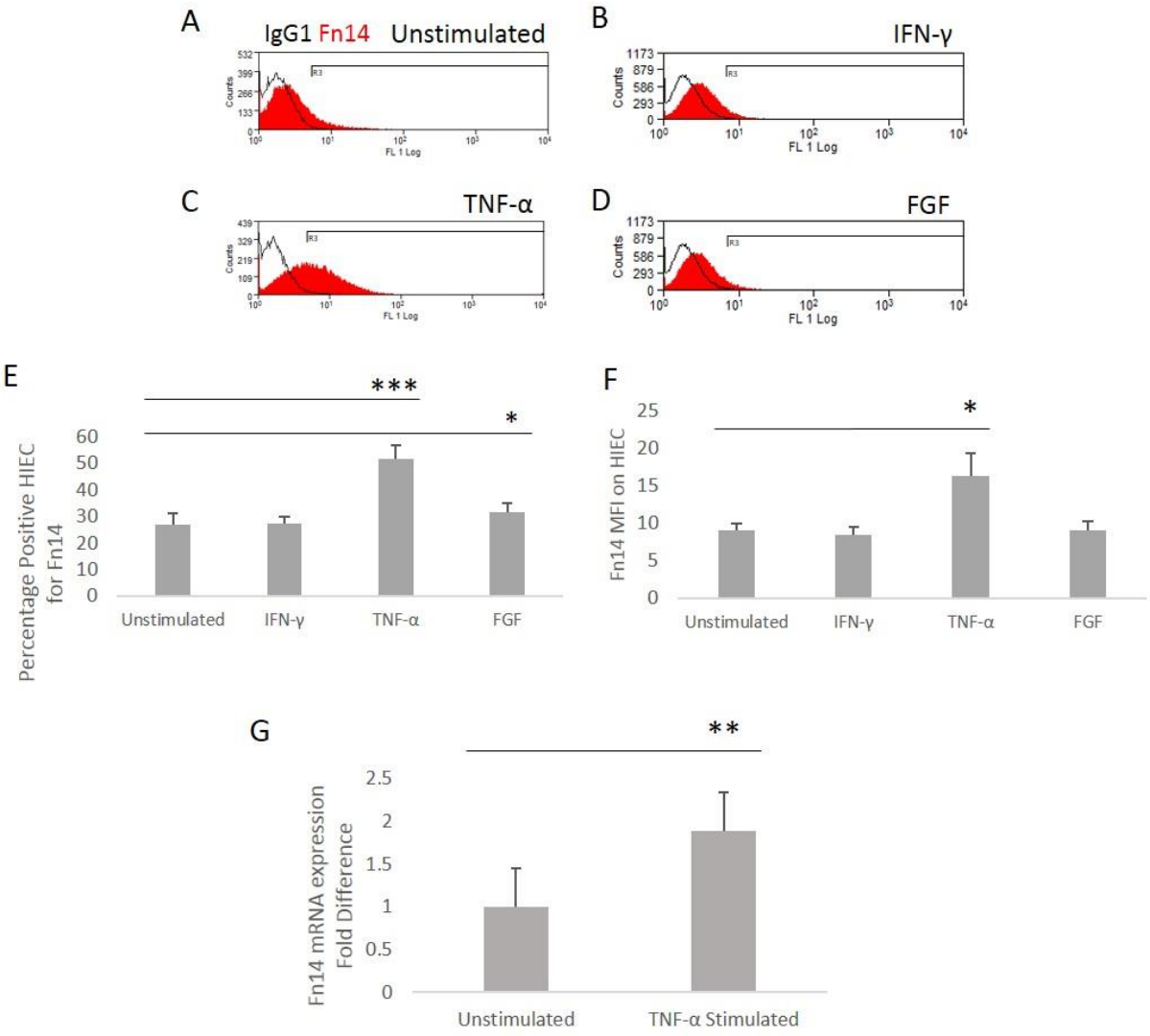


Figure 3.3.8 Increased Fn14 protein and mRNA expression was observed when HIEC were stimulated by TNF-α: HIEC were stimulated with IFN-γ 50ng/ml, TNF-α 10ng/ml and FGF 0.5ng/ml for 24 hours prior to analysis. A) Shows Fn14 cell surface expression (Red) with its IgG1 isotype control (Black). B) Shows HIEC stimulated with IFN-g, C) HIEC stimulated with TNF-α, D) HIEC stimulated with FGF. E) A significant up-regulation of Fn14 expression was seen when HIEC were stimulated using TNF-α in comparison to the un-stimulated control (***p=0.0002 n=13). A marginal increase in Fn14 expression was observed when HIEC were stimulated with FGF (* p=0.03, n=9). IFN-γ did not affect Fn14 expression in comparison to the un-stimulated HIEC control (n=9). F) Median fluorescence intensity data showed an increase in Fn14 expression in TNF-α stimulated HIEC (*p=0.04, n=10). FGF and IFN-γ stimulation did not show a difference in Fn14 MFI expression (n=6). Data is shown as ± SEM. G) A significant up-regulation of Fn14 mRNA expression was observed using qPCR analysis, when HIEC were stimulated using TNF-α in comparison to the un-stimulated control (n=5 **p=0.006 ± SE). All samples have been analysed as triplicates against a GUSB reference gene. Data is shown as fold difference with the unstimulated HIEC normalised to 1. Statistical analysis was carried out using a paired Student's t test.

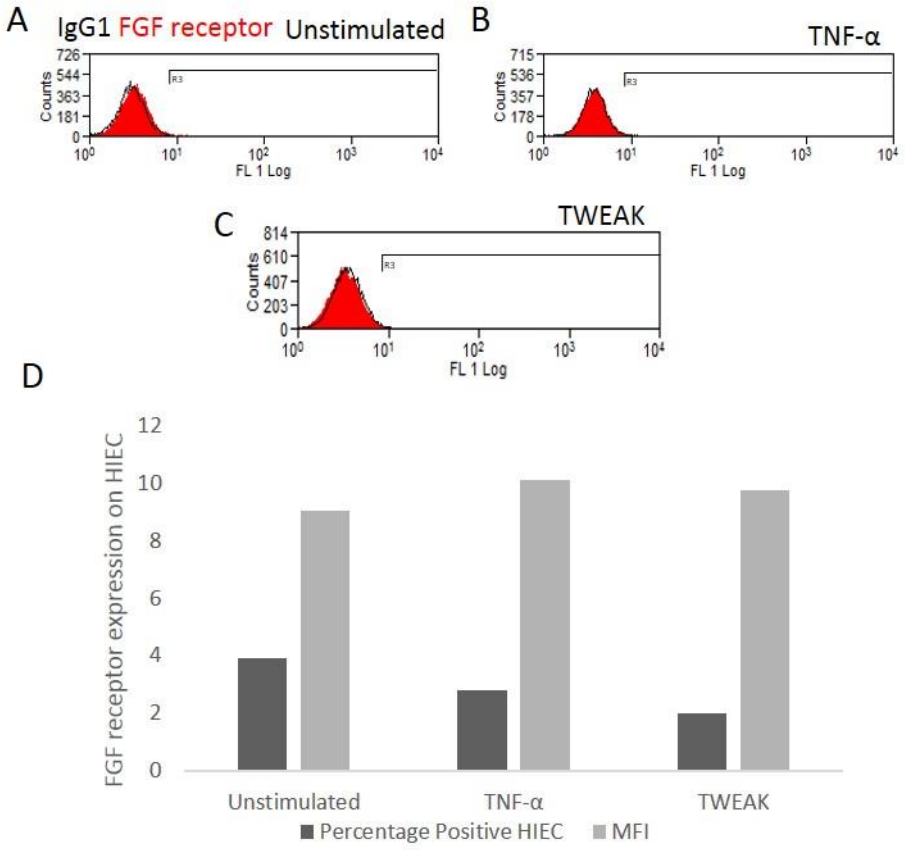


Figure 3.3.9 FGF receptor 2 was not present on HIEC cell surface: HIEC were left unstimulated (A) and stimulated with TWEAK (100ng/ml) (B) and TNF-α (10ng/ml) (C) for 24 hours prior to flow cytometry analysis to detect the presence of FGF receptor 2. D) Analysis of percentage positive HIEC showed that FGF receptor 2 was not expressed on HIEC. The median fluorescence intensity was also negative (n=2). Histograms of cell surface FGF receptor expression are shown in red, and their IgG controls have been shown in black. No statistical analysis was performed on these experiments due to a small sample number.

3.3.5 Passage of HIEC does not alter Fn14 expression

Flow cytometry was used to determine Fn14 expression from HIEC across different passages; from passage 2 to 7. HIEC were stimulated with IFN-γ, TNF-α and FGF for 24 hours prior to analysis. Passage of HIEC showed no significant change in Fn14 expression across the majority of passages (Figure 3.3.10). Significant changes were observed in unstimulated HIEC Fn14 expression from passage 2 to passage 3, where a decrease of Fn14 expression percentage positive HIEC from 38 to 17 ± SEM was observed (*p=0.04). Significant changes were also observed in FGF stimulated HIEC from passage 6 to passage 7 where Fn14 expression levels percentage positive HIEC were decreased from 27 to 21.8 ± SEM (*p=0.05). The general expression pattern of Fn14 from HIEC in response to IFN-γ, TNF-α and FGF stimulations remained the same; where TNF-α stimulation promoted Fn14 expression. Based on this data, all HIEC isolations were used for experiments between passage 3 to passage 6 for consistency.

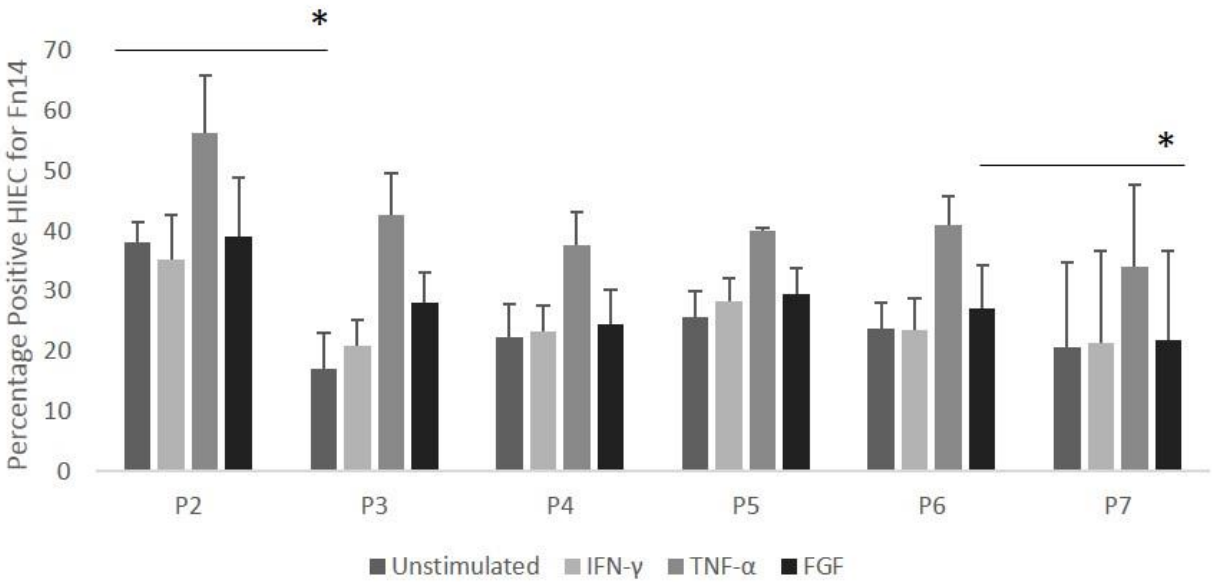


Figure 3.3.10 Fn14 expression did not change with passage of HIEC: HIEC were stimulated with IFN-γ 50ng/ml, TNF-α 10ng/ml and FGF 0.5ng/ml for 24 hours prior to analysis. HIEC were analysed for Fn14 expression at different passages. Significant decreases in expression were observed in unstimulated HIEC from passage 2 to 3, and FGF stimulated HIEC passage 6 to 7. No significant change was observed for Fn14 percentage positive HIEC from passage 3 to 6. The trend of enhanced Fn14 expression in response to TNF-α activation continued through all experiments. Data is shown as mean ± SEM (Passage 2: n=2, Passage 3: n=3, Passage 4: n=5, Passage 5: n=5, Passage 6: n=4 and Passage 7: n=2). Statistical analysis was carried out using a paired Student's t test.

3.3.6 IL-1β stimulates HIEC increase cell surface Fn14 expression

To further assess the inflammatory response of Fn14 on HIEC; a panel of inflammatory cytokines were used to stimulate HIEC, and to detect changes in Fn14 protein expression patterns using flow cytometry (Figure 3.3.11 and 3.3.12). HIEC were stimulated with recommended manufacturers concentrations of IL-1β (10ng/ml), IL-2 (10ng/ml), IL-4 (100ng/ml), IL-6 (20ng/ml), IL-8 (20ng/ml), IL-10 (50ng/ml), IL-17A (50ng/ml), IL-18 (100ng/ml), IL-21 (50ng/ml), IL-22 (50ng/ml), IL-23 (50ng/ml), TGF-β (50ng/ml) and MCP-1 (20ng/ml) for 24 hours prior to analysis. IL-1β stimulation significantly promoted Fn14 cell surface protein expression from 48 to 70.8 percent positive HIEC ± SEM (n=6) *p=0.04. MFI data also showed a significant increase in Fn14 expression in response to IL-1β from 15.1 to 21.9 ± SEM (n=6) **p=0.0007 (Figure 3.3.11). Interestingly, IL-17A and TGF-β significantly decreased cell surface Fn14 expression from 39 to 26 and 29.3 mean percent positive HIEC respectively ± SEM (n=3) (*p=0.02 and *p=0.05) (Figure 3.3.12). MFI data showed a similar trend but the data was not statistically significant. All other stimulations did not significantly alter Fn14 protein expression on HIEC from this panel of inflammatory cytokines.

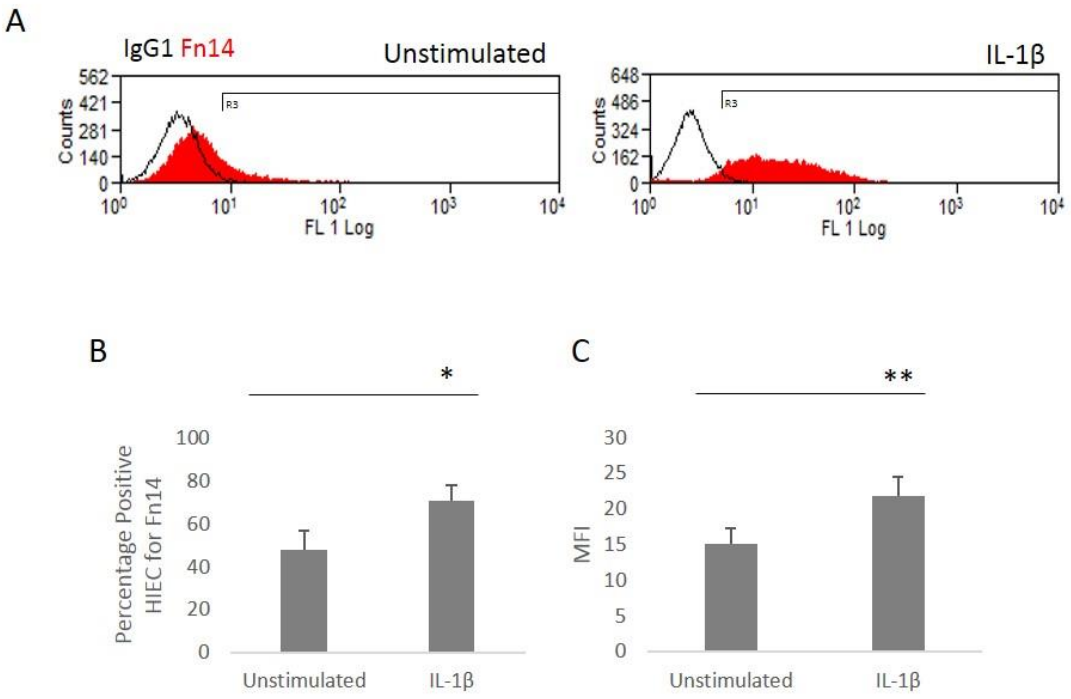


Figure 3.3.11 Increased Fn14 cell surface protein expression was observed when HIEC were stimulated with IL-1β: A) Histogram shows IgG1 control in black, Fn14 unstimulated in red and IL-1β stimulated HIEC in green. HIEC were stimulated with IL-1β (10ng/ml) for 24 hours prior to analysis. B) Flow cytometry data showed a significant up-regulation of Fn14 expression percent positive HIEC when stimulated using IL-1β in comparison to the un-stimulated control *p=0.04 (n=6). C) A similar pattern to the percentage positive expression of Fn14 was observed for the median fluorescence intensity data, where a significant increase in Fn14 expression was observed in IL-1β stimulated HIEC **p=0.007 (n=6). Statistical analysis was carried out using a paired Student's t test. All data is shown as mean ± SEM.

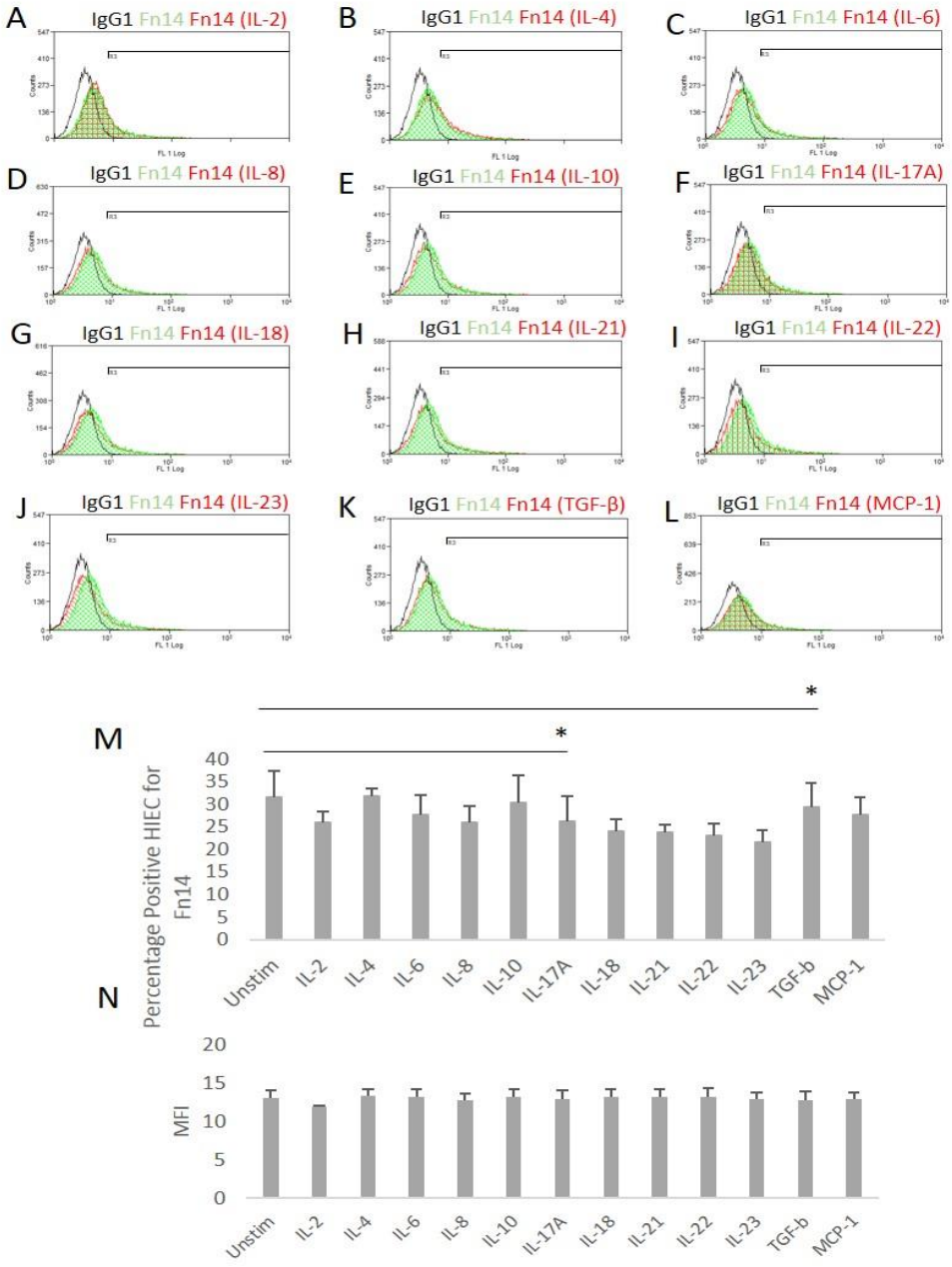


Figure 3.3.12 Decreased cell surface Fn14 protein expression was observed when HIEC were stimulated by IL-17A and TGF-β: Histograms show IgG1 control in black, Fn14 unstimulated in red and all stimulations in green. HIEC were stimulated with A) IL-2 (10ng/ml), B) IL-4 (100ng/ml), C) IL-6 (20ng/ml), D) IL-8 (20ng/ml), E) IL-10 (50ng/ml), F) IL-17A (50ng/ml), G) IL-18 (100ng/ml), H) IL-21 (50ng/ml), I) IL-22 (50ng/ml), J) IL-23 (50ng/ml) and K) TGF-β (10ng/ml) and L) MCP-1 (20ng/ml) for 24 hours prior to analysis. M) A significant decrease in Fn14 expression was observed in IL-17A and TGF-β activated HIEC *p=0.02 and *p=0.05 respectively in comparison to the unstimulated control (n=3). N) A similar pattern to the percentage positive expression of Fn14 was observed for the median fluorescence intensity data. All other stimulations did not significantly alter Fn14 cell surface HIEC expression. Statistical analysis was carried out using a paired Student's t test. All data is shown as mean ± SEM.

3.3.7 Enhanced Fn14 expression in HIEC was observed in the cytoplasm, in response to TNF-α, FGF, and IL-1β activation

Protein and mRNA data showed an increase in Fn14 expression in response to TNF-α, in comparison to un-stimulated HIEC. Therefore, IF was used to observe cellular changes in HIEC when they were stimulated with TNF-α. HIEC were cultured on glass coverslips and stained using a mouse anti human Fn14 mAb coupled to a FITC (green) secondary. DAPI (blue) was used as a nuclear stain. HIEC were stimulated overnight using TNF-α prior to analysis. Results show two HIEC isolates represented with A, B and C from the first isolate and D and E fom the second (Figure 3.3.13). In unstimulated HIEC; Fn14 expression appeared predominantly in the cytoplasm and a punctate staining pattern was observed (Figure 3.3.13 B and D) in comparison to the isotype control (Figure 3.3.13 A). When HIEC were stimulated with TNF-α, increased intensity of staining was observed in the cytoplasm (Figure 3.3.13 C and E). Z-stack images were taken of HIEC unstimulated and stimulated with TNF-α to confirm up-regulated Fn14 expression. The collated 3D images from the Z-stacks confirmed a significant increase in Fn14 expression in HIEC (Figure 3.3.13 B and C).

HIEC were stimulated with FGF (0.5 ng/ml), and IL-1β (10ng/ml) for 24 hours prior to staining (Figure 3.3.14). Fn14 expression was detected in the cytoplasm of HIEC analysed, in comparison to the isotype IgG1 control (Figure 3.3.14 A). Fn14 cytoplasmic expression was slightly enhanced in response to FGF and largly enhanced with IL-1β activation (Figure 3.3.14 C and D), in comparison to the unstimulated control (Figure 3.3.14 B).

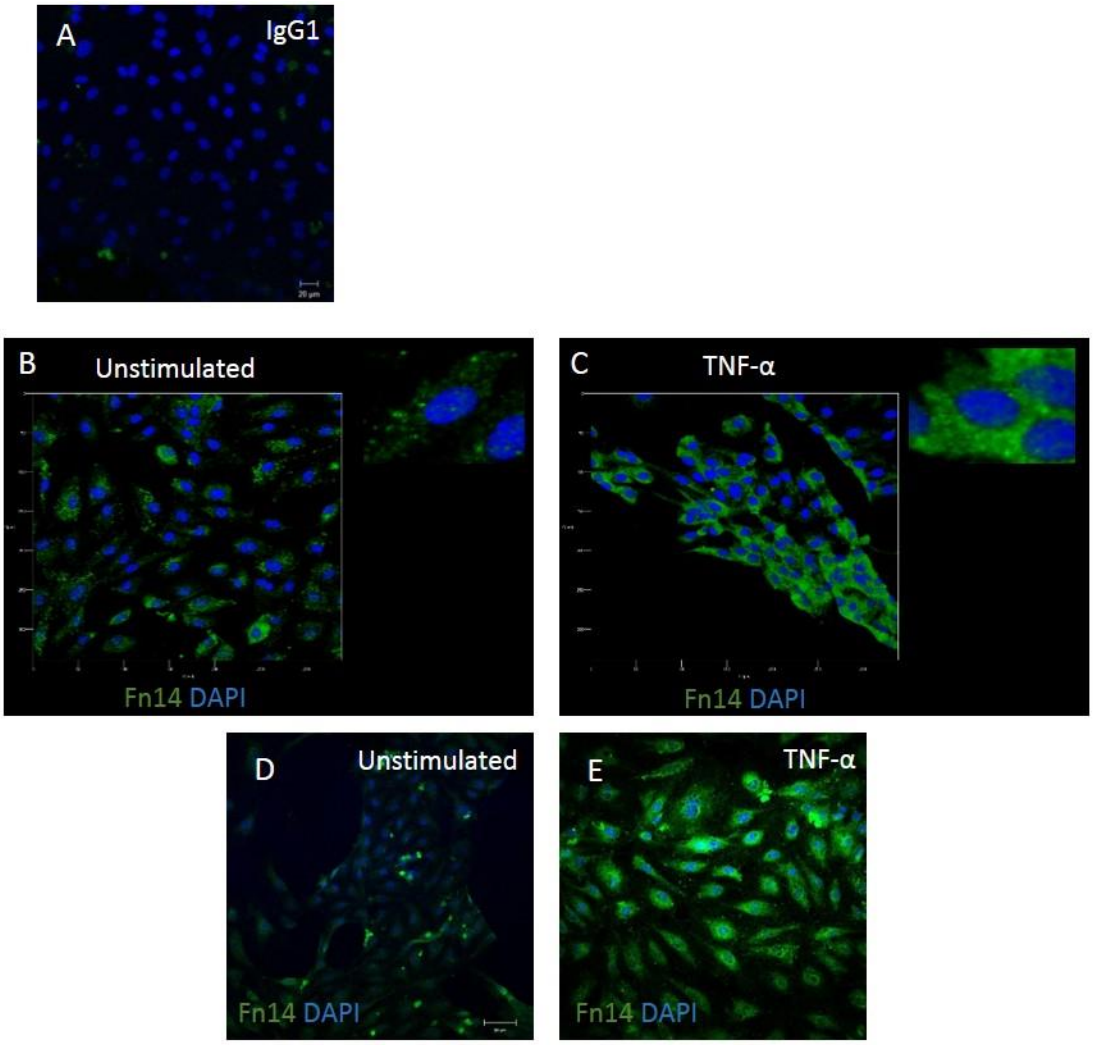


Figure 3.3.13 Increased Fn14 expression was observed in the cytoplasm when HIEC were stimulated by TNF-α: Confocal immuno-fluorescent imaging was used to visualise Fn14 expression in HIEC. Two HIEC isolates are represented with B and C from the first isolate and D and E from the second. An anti Fn14 antibody (3.7µg/ml) was used to detect Fn14 expression in HIEC, this was conjugated to a FITC (green) secondary, DAPI (blue) was used as a nuclear stain. Cytoplasmic fluorescent staining was observed in B, C, D, and E in comparison to the IgG1 Isotype control (A). 3D representative image shown in B and C with a subsequent magnified view. Fn14 expression significantly increased when HIEC were stimulated with TNF-α (10ng/ml) for 24 hours (C and E) in comparison to the unstimulated control (B and D). A punctate staining pattern was visible in unstimulated and TNF- α stimulated HIEC. Magnification 200x Zeiss LSM 780 Zen confocal microscope.

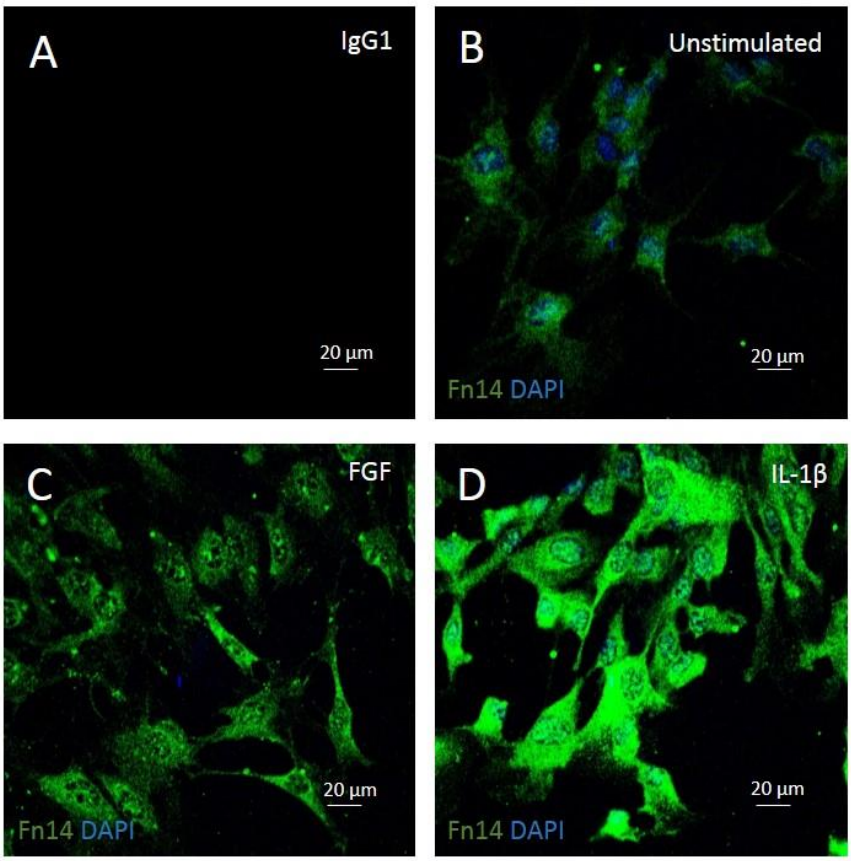


Figure 3.3.14 Increased Fn14 expression was observed in the cytoplasm when HIEC were stimulated with FGF and IL-1β: Confocal immuno-fluorescent imaging was used to visualise Fn14 expression in HIEC, this was conjugated to a FITC (green) secondary, DAPI (blue) was used as a nuclear stain. An anti Fn14 antibody (3.7µg/ml) was used to detect Fn14 expression in HIEC. Cytoplasmic fluorescent staining was observed in B, C, and D in comparison to the IgG1 Isotype control, A. Fn14 expression increased when HIEC were stimulated with FGF (0.5 ng/ml), and a larger increase in Fn14 expression was observed when HIEC were activated with IL-1β (10ng/ml) for 24 hours (C and D respectively) in comparison to the unstimulated control (B). Magnification 200x Zeiss LSM 510 UV confocal microscope.

3.3.8 HIEC store intracellular Fn14

We wanted to confirm if the increased Fn14 expression in response to stimulation, was from intracellularly stored Fn14. To do this HIEC were stimulated with IFN-γ, TNF-α and FGF for 24 hours prior to analysis. Fn14 protein expression percentage positivity was analysed after permeablising the cells to detect intracellular Fn14. Significant up-regulation of Fn14 expression was observed when cell surface Fn14 was compared to intracellular Fn14 (Figure 3.3.15), in all stimulations and unstimulated HIEC (n=6); Unstimulated Fn14 expression mean percentage positivity increased from 26 to 85 ± SEM (***p≤0.001), IFN-γ stimulated HIEC mean percentage positive HIEC increased from 27 to 98 ± SEM (***p≤0.001), TNF-α stimulation increased percentage positivity from 49 to 97 ± SEM (***p≤0.001), and FGF increased percentage positivity from 33 to 87 ± SEM (**p≤0.01). All data was quantified against their individual IgG1 controls.

3.3.9 HIEC transports Fn14 via the Golgi apparatus

Our data showed significant elevation of Fn14 expression in response to TNF-α, FGF and IL-1β activation. This was determined using flow cytometry detecting cell surface protein expression and observed using IF. Fn14 expression was detected in the cytoplasm and a granular punctate stain was observed. This was followed by data showing significant elevation of Fn14 protein expression when HIEC were permeablised, indicating intracellular stores of Fn14 protein. To determine if there are Fn14 present in the Golgi apparatus, and if the Golgi is involved in transport of this protein, a Golgi plug (Golgi to endoplasmic reticulum (ER) transport) and Golgi stop (trans-Golgi transport) was used together with TNF-α

activation before staining for Fn14. All staining was confirmed against an IgG1 negative control and a CD31 positive control (Figure 3.3.16 A and B). Enhanced Fn14 expression was observed in response to TNF-α activation (Figure 3.3.16 D) in comparison to the unstimulated control (Figure 3.3.16 C). The staining after applying the Golgi plug showed large accumulations of Fn14 in the HIEC analysed (Figure 3.3.16 E). The HIEC analysed after using the Golgi stop showed no difference in comparison to the HIEC activated with TNF-α without a Golgi stop (Figure 3.3.16 F). This data showed that the Golgi apparatus may play a significant role in storing intracellular Fn14, and aiding its release after stimulation and transporting Fn14 via the ER.

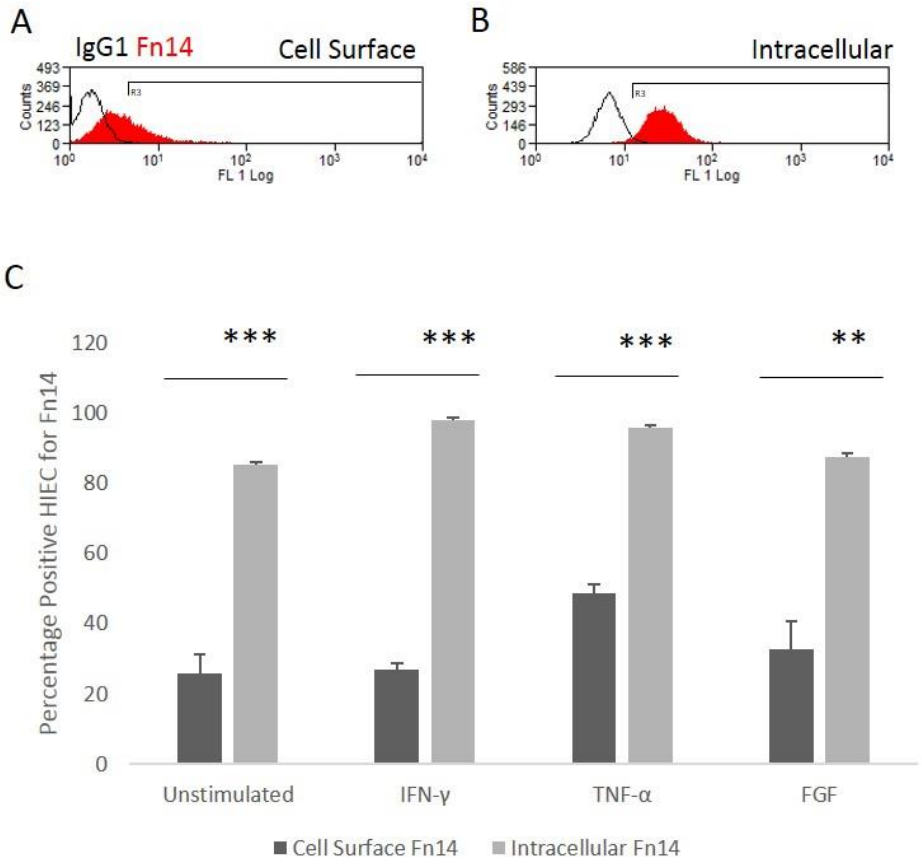


Figure 3.3.15 Increased intracellular Fn14 protein expression was observed in HIEC: A and B) show histograms of cell surface and intracellular Fn14 expression (unstimulated) respectively in red. Their IgG1 controls have been shown in black. C) HIEC were stimulated with IFN-γ 50ng/ml, TNF-α 10ng/ml and FGF 0.5ng/ml for 24 hours prior to analysis. Flow cytometry data showed significant up-regulation of Fn14 expression percentage positive HIEC when HIEC were permeablised to release intracellular Fn14 in comparison to cell surface Fn14 expression. Data was shown as mean ± SEM (n=6) (Unstimulated; ***p=0.0005, IFN-γ; ***p=0.000007, TNF-α ***p=0.00015 and FGF **p=0.0017). Statistical analysis was carried out using a paired Student's t test. All data is shown as mean ± SEM.

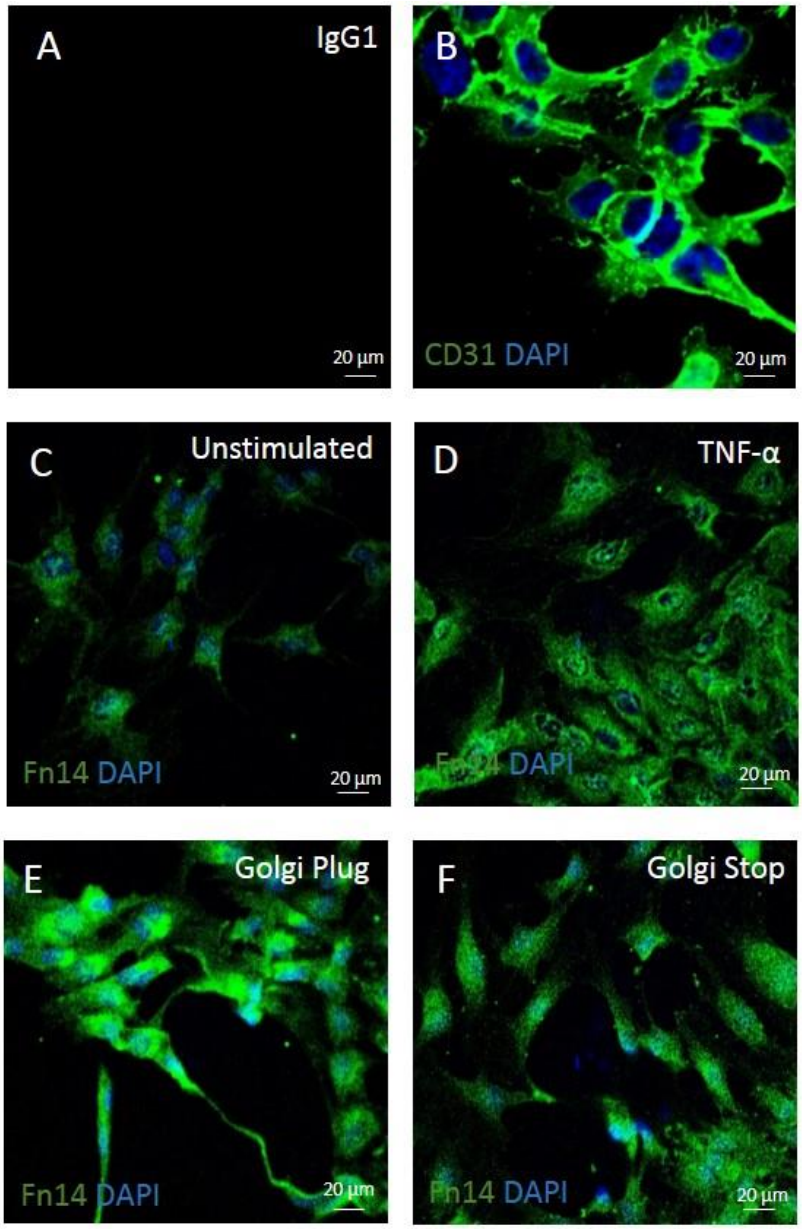


Figure 3.3.16 Fn14 is transported from the Golgi to the ER when activated by TNFα: HIEC were activated with and without TNF-α and confocal imaging and Immuno-fluorescent staining using an anti Fn14 antibody (3.7µg/ml) was used to determine Fn14 expression. TNF-α plus a Golgi plug (E), and TNF-α plus a Golgi Stop (F) were used prior to staining with Fn14, to determine if Fn14 transport may be regulated by the Golgi apparatus. IgG1 was used as a negative control (A) and CD31 was used as a positive control (B). Fn14 expression was up-regulated in response to TNF-α (D) in comparison to the unstimulated control (C). Fn14 protein appears accumulated in E where a Golgi plug was used with TNF-α stimulation. No change in Fn14 expression pattern was observed where a Golgi stop was used. Magnification 200x Zeiss LSM 510 UV confocal microscope.

3.3.10 TWEAK stimulated HIEC decreased cell surface Fn14 protein and Fn14 mRNA expression

We wanted to determine the HIEC response to TWEAK activation and how this alters Fn14 expression. HIEC were stimulated with TWEAK (100ng/ml) for 24 hours prior to analysis. Flow cytometry data revealed that TWEAK activation significantly decreased Fn14 cell surface expression from 32 to 22 percent positive HIEC ± SEM (n=5 *p=0.05) (Figure 3.3.17 A). This decrease was also observed at an mRNA level where Fn14 mRNA expression decreased 3 fold in HIEC in response to TWEAK activation (Figure 3.3.17 B).

HIEC were stimulated with TWEAK (10ng/ml) overnight prior to IF staining to detect Fn14 expression. Unstimulated HIEC showed cytoplasmic Fn14 present. The staining pattern showed a punctate Fn14 stain distributed in the cytoplasm (Figure 3.3.18 B). In TWEAK activated HIEC, Fn14 expression was still observed in the cytoplasm, however the intensity decreased in particular in the previous punctately stained regions (Figure 3.3.18 C). Staining was compared to the relevant IgG1 control for Fn14 (Figure 3.3.18 A).

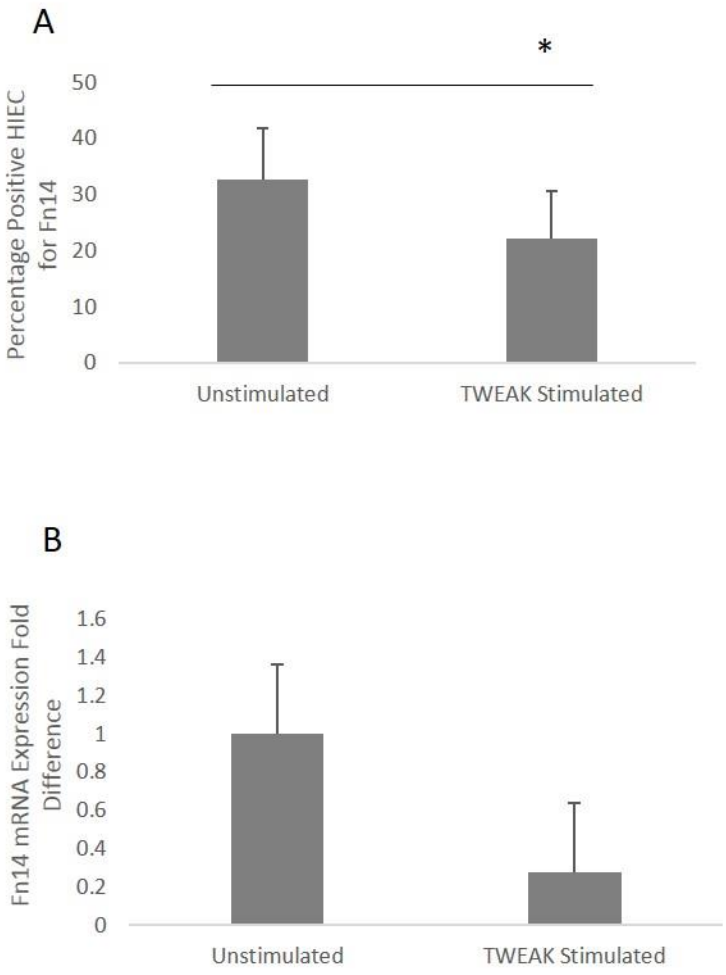


Figure 3.3.17 TWEAK stimulated HIEC showed a decrease in Fn14 protein and mRNA expression: HIEC were stimulated with TWEAK 100ng/ml for 24 hours prior to flow cytometry and qPCR analysis. A) Flow cytometry data showed Fn14 expression percentage positivity significantly decreased with TWEAK stimulation. Data is shown as mean ± SEM (* P=0.05, n=5). Statistical analysis was carried out using a paired Student's t test. B) qPCR data showed Fn14 mRNA expression also decreased with TWEAK stimulation. All qPCR samples were analysed as triplicates against a GUSB reference gene. Fn14 forward and reverse primer concentration 1µM/ well. Data is shown as mean ± SE (n=3) as fold difference with unstimulated HIEC normalised to 1. This data was not statistically significant.

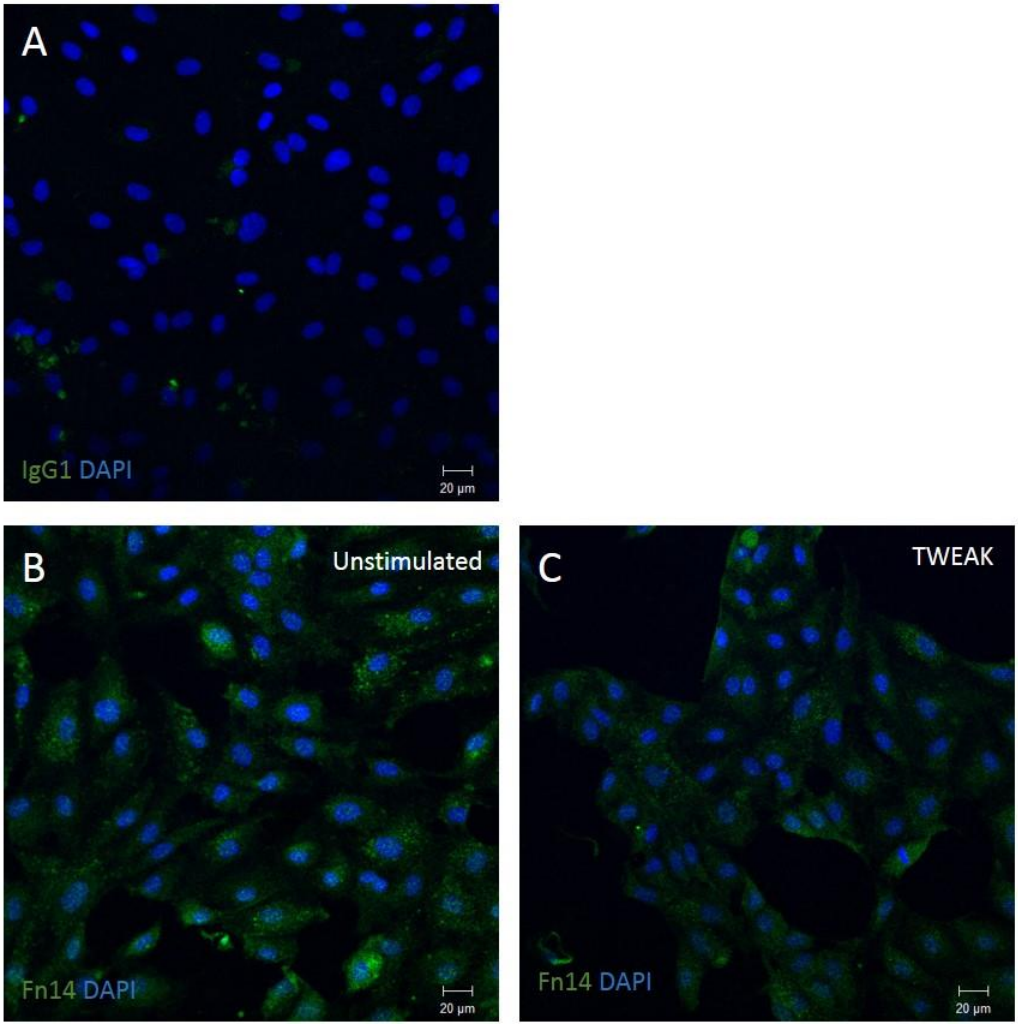


Figure 3.3.18 Decreased Fn14 expression was observed in the cytoplasm when HIEC were stimulated by TWEAK: Confocal immuno-fluorescent imaging was used to observe Fn14 expression in HIEC, this was conjugated to a FITC (green) secondary, DAPI (blue) was used as a nuclear stain. An anti Fn14 antibody (3.7µg/ml) was used to detect Fn14 expression in HIEC. Cytoplasmic fluorescent staining was observed in B and C in comparison to the IgG1 Isotype control, A. Fn14 expression decreased when HIEC were stimulated with TWEAK (100 ng/ml) for 24 hours (C) in comparison to the unstimulated control (B). Magnification 200x Zeiss LSM 510 UV confocal microscope.

3.3.11 PBMC and monocytes expressed TWEAK mRNA which was regulated by IFN-γ

HIEC were consistently negative for TWEAK cell surface protein expression in comparison to its isotype control IgG2a. This was in un-stimulated HIEC and HIEC stimulated with TNF-α, IFN-γ and FGF (Figure 3.3.19 E). This was also confirmed in corresponding MFI data (Figure 3.3.19 F). PBMC stimulated with IFN-γ were used as a positive control for TWEAK mRNA expression. A significant increase in TWEAK mRNA expression was observed when IFN-γ stimulated PBMC were compared to unstimulated PBMC *p=0.01. Data was shown as TWEAK mRNA fold difference with un-stimulated PBMC normalised to 1. HIEC showed little to no expression of TWEAK mRNA in comparison to un-stimulated PBMC (Figure 3.3.20). This mRNA expression data was consistent with the TWEAK protein expression data from HIEC, obtained using flow cytometry. Intracellular TWEAK expression was also assessed using flow cytometry. Increased TWEAK expression was observed when HIEC were permeablised, however this increase was insignificant (Figure 3.3.21), and below 10% percentage positive HIEC.

TWEAK has been found to be expressed on monocytes, macrophages and PBMC in a number of studies. The data obtained from this investigation was also suggestive that TWEAK was not present on HIEC but may be acting in a paracrine manner with Fn14 which is constitutively expressed on HIEC, and TWEAK interacting via mononuclear cells, as we have found TWEAK mRNA present in PBMC, and this expression was elevated in response to inflammatory cytokine IFN-γ activation. We thus wanted to determine the presence of TWEAK mRNA on monocytes, in particular in different subsets of monocytes, and to

determine how this expression was altered in response to IFN-γ activation. This would enable us to beginto understand how possible TWEAK paracrine interactions with HIEC may be regulated. TWEAK mRNA expression was analysed in CD14+ (CD14++CD16-), CD16+ (CD14+CD16++) and dual positive monocyte populations (CD14++CD16+). Relative expression analysis showed that TWEAK mRNA was expressed the highest in CD14+ monocyte populations and IFN-γ activation increased TWEAK mRNA expression (Figure 3.3.22). Dual positive monocytes and CD16+ monocytes expressed relatively low amount of TWEAK mRNA to CD14+ populations; however TWEAK mRNA expression was doubled in IFN-γ activated dual positive HIEC populations, and CD16+ monocytes showed 3.5 fold decreased TWEAK mRNA expression when this population of monocytes was activated with IFN-γ.

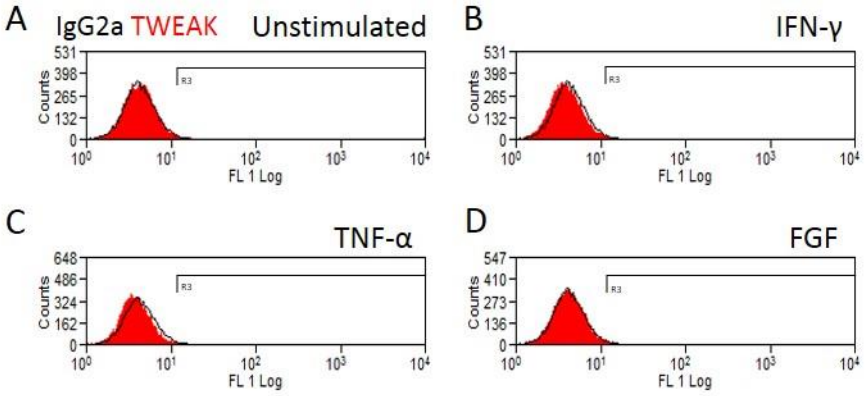


Figure 3.3.19 HIEC do not express TWEAK protein on the cell surface: HIEC were unstimulated (A) and stimulated with IFN-γ 50ng/ml (B), TNF-α 10ng/ml (C) and FGF 0.5ng/ml (D) for 24 hours prior to flow cytometry analysis. Histograms show the IgG2a control in black and the TWEAK cell surface expression in red. HIEC were negative for TWEAK expression showing less than 2% percentage positivity. Data is shown as mean ± SEM; Unstimulated and TNF-α (n=8), IFN-γ and FGF (n=4). MFI data also confirmed this observation. Data is shown as mean ± SEM (n=3). No statistical analysis was performed on this data.

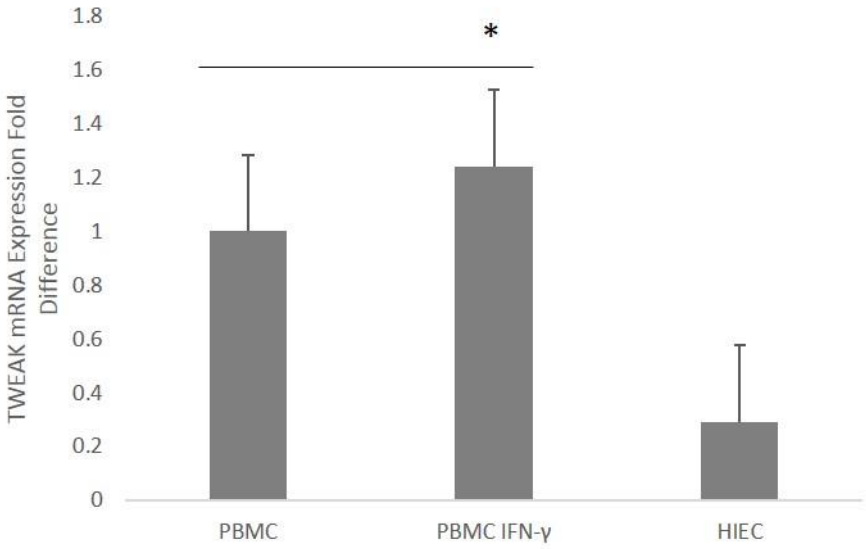


Figure 3.3.20 TWEAK mRNA expression was enhanced by IFN-γ activation in PBMC: qPCR data showed a significant increase in TWEAK mRNA expression in cDNA obtained from PBMC stimulated with IFN-γ *p=0.01. HIEC showed a low amount of TWEAK mRNA in comparison to PBMC. All samples were analysed as triplicates against a GUSB reference gene. TWEAK forward and reverse primer concentration 1µM/ well. Data has been shown as fold difference of mean (n=3 ± SE) with the un-stimulated PBMC control normalised to 1. Statistical analysis was carried out using a paired Student's t test.

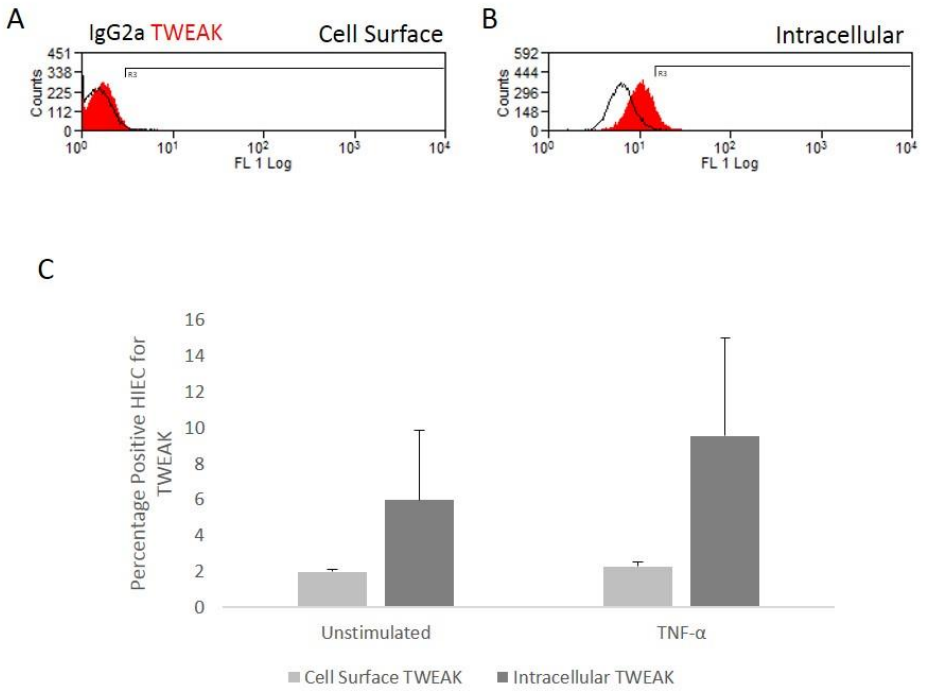


Figure 3.3.21 Enhanced intracellular TWEAK protein expression was observed in HIEC: C) HIEC were stimulated with TNF-α 10ng/ml for 24 hours prior to analysis. Flow cytometry data showed a marginal up-regulation of TWEAK protein expression percentage positive HIEC when HIEC were permeablised to detect intracellular TWEAK in comparison to cell surface TWEAK expression. Data was shown as mean ± SEM (n=4). Histograms show IgG2a control in black and TWEAK expression in red. A) Shows cell surface expression and B) shows intracellular expression. This data was not statistically significant.

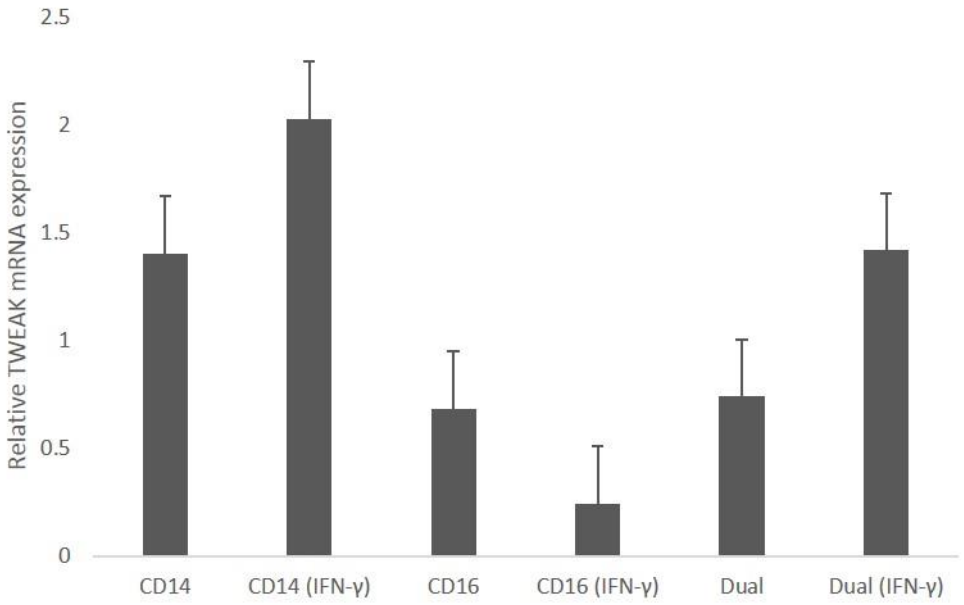


Figure 3.3.22 IFN-γ promoted TWEAK mRNA expression on CD14+ and dual positive monocytes: Total PBMC were isolated and sorted using a high speed cell sorter in to CD14+, CD16+ and dual positive monocyte populations. Monocyte fractions were cultured for 24 hours with IFN-γ and total RNA was isolated. qPCR was used to determine TWEAK mRNA expression from the different monocyte subsets. Enhanced TWEAK mRNA expression was observed in CD14+ monocyte and dual positive monocyte populations with IFN-γ activation in comparison to unstimulated controls. CD16+ monocyte populations showed decreased TWEAK mRNA expression in response to IFN-γ activation. Data is shown as relative expression from 3 different experiments ± SE. This data was not statistically significant.

3.3.12 TWEAK activated HIEC facilitate leukocyte recruitment to HIEC

We have found TWEAK and Fn14 expression present in areas surrounding leukocyte infiltration and portal and neovessels in diseased liver tissue, suggesting a potential role in regulating inflammation and angiogenesis. Therefore we wanted to determine the role of TWEAK and Fn14 in leukocyte recruitment to HIEC. Initially we wanted to determine expression of VCAM and ICAM on TWEAK activated HIEC to understand if leukocyte recruitment was mediated via these adhesion proteins. TNF-α was used as a positive control, Fn14 expression in response to TWEAK and TNF-α stimulation was also analysed in parallel to ensure the data obtained was consistent with previous findings. TWEAK activated HIEC did not promote Fn14, VCAM and ICAM protein expression on HIEC cell surface (Figure 3.3.23). TNF-α activated HIEC significantly increased Fn14, VCAM and ICAM expression on HIEC cell surface; from 28.7 to 66.2, 19.5 to 89 and 13.8 to 61.8 percentage positive HIEC respectively.

Following this, we wanted to determine if TWEAK activated HIEC enhanced leukocyte recruitment, and further analysed differences between monocyte and lymphocyte adhesion (Figure 3.3.24 and 3.3.25). We used flow based *in vitro* assays to determine if TWEAK influenced leukocyte adhesion (Figure 3.3.25 A), migration (Figure 3.3.25 C), and morphological changes (Figure 3.3.25 B) of leukocytes in response to activated HIEC. TWEAK activated HIEC showed enhanced monocyte, lymphocyte and total PBMC adherence in comparison to the unstimulated control. A significant increase in monocyte adherence was observed in TWEAK activated HIEC from 6 to 11.5 ± SE adherent cells to HIEC/mm^2/10^6 cells

perfused *p=0.04. HIEC stimulated with TWEAK and TNF-α in combination also significantly enhanced PBMC adherence from 15.7 to 51.4 ± SE adherent cells to HIEC/$mm^2/10^6$ cells perfused *p=0.03. TNF-α and IFN-γ activated HIEC were used as a positive control and showed the largest number of adherent cells in comparison to all stimulations 64.8 ± SE adherent cells to HIEC/$mm^2/10^6$ cells perfused. TNF-α, IFN-γ and TWEAK were used in combination and showed a large increase in adherent cells to HIEC, 60.3 ± SE adherent cells to HIEC/$mm^2/10^6$ cells perfused in comparison to the unstimulated control and TWEAK stimulation. These increases in adherence were observed in all conditions, however this data was not statistically significant. Blocking TWEAK activation using Fn14 mAb showed no differences to TWEAK activation in all assays.

Enhanced shape changing PBMC were observed when HIEC were activated with TWEAK in comparison to unstimulated controls from 6 to 14.2 ± SE shape changing cells to HIEC/$mm^2/10^6$ cells perfused (Figure 3.3.25 B). TWEAK, TNF-α, and IFN-γ activation in combination showed the largest amount of shape changing PBMC observed 61.3 ± SE. Followed by TNF-α and IFN-γ 54.2 ± SE and then TWEAK and TNF-α in combination 46 ± SE. TWEAK activated HIEC appeared to enhance migration in response to TWEAK but only marginally and this finding was not statistically significant (Figure 3.3.25 C). The positive control of TNF-α and IFN-γ activated HIEC showed an increase in migration in comparison to the unstimulated HIEC from 2.6 to 22.1 ± SE migrated cells to HIEC/$mm^2/10^6$ cells perfused. This was further enhanced in HIEC activated by TWEAK and TNF-α. The largest increase in migration was observed in HIEC activated with TWEAK, TNF-α and IFN-γ in combination from

2.6 to 24.6 ± SE migrated cells to HIEC/mm^2/10^6 cells perfused in comparison to the unstimulated control.

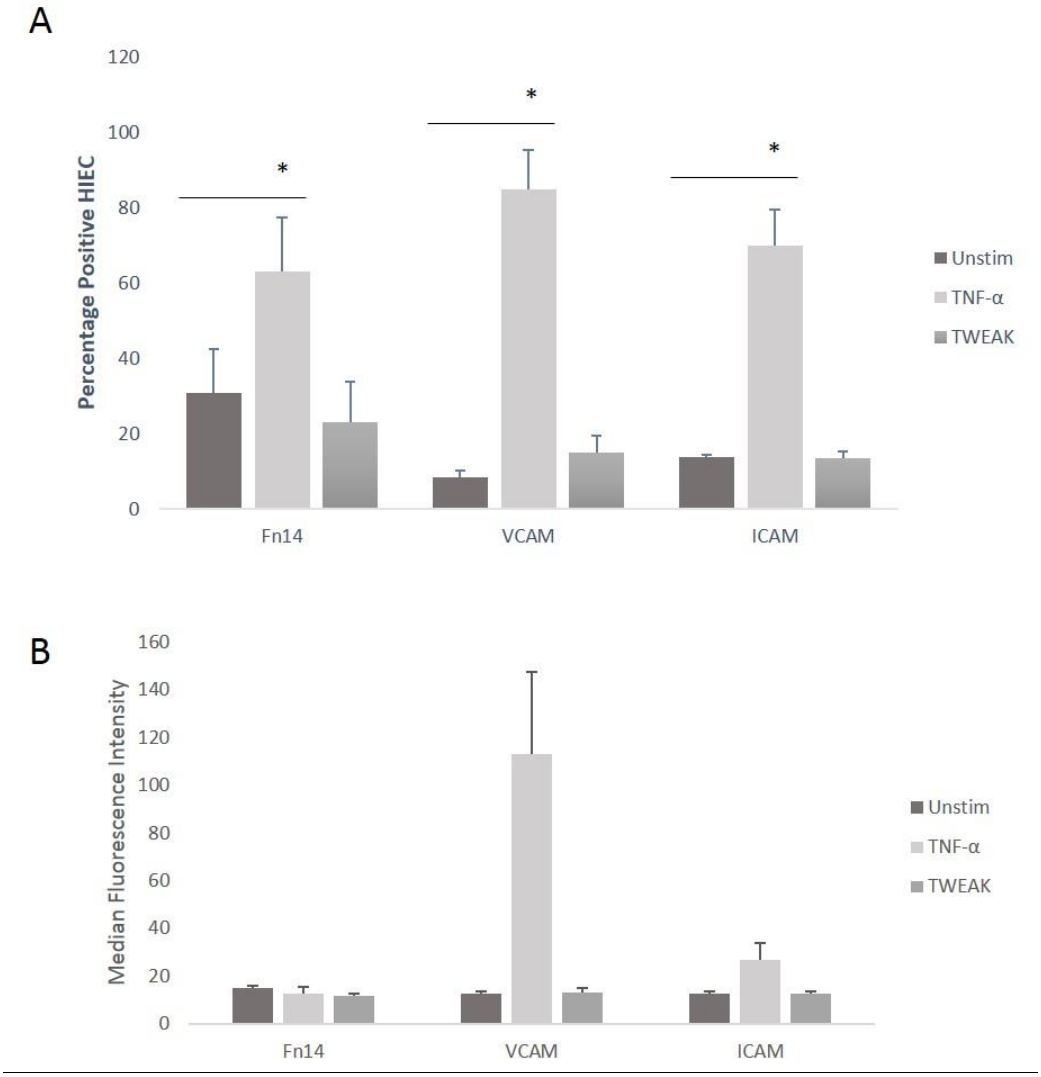


Figure 3.3.23 - TWEAK does not promote the expression of VCAM and ICAM on the cell surface of HIEC: HIEC were stimulated with TNF-α (10ng/ml) and TWEAK (100ng/ml) for 24 hours prior to FACS analysis. TNF-α was used as a positive control and unstimulated HIEC were used as a negative control. A) The percentage positive population of HIEC expressing Fn14, VCAM and ICAM. B) Median Fluorescence Intensity from HIEC expressing Fn14, VCAM and ICAM, this data was not statistically significant. TWEAK did not promote the expression of Fn14, VCAM and ICAM. TNF-α significantly enhanced Fn14, VCAM and ICAM expression on HIEC cell surface p=0.03* p=0.03* and p=0.04* percentage positive HIEC respectively. Data is shown as mean ± SEM n=4. Statistical analysis was carried out using a paired Student's t test.

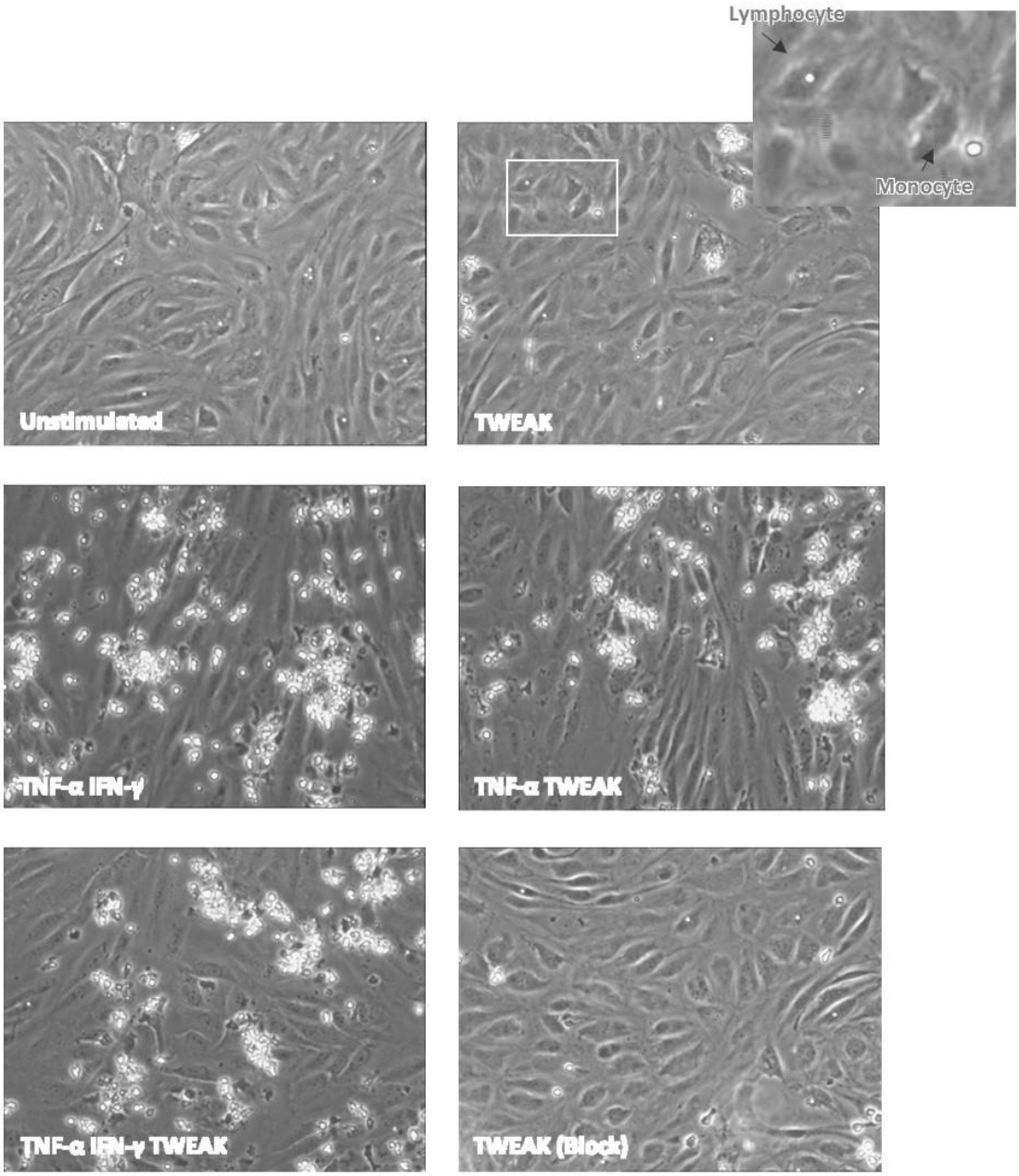


Figure 3.3.24 - TWEAK activated HIEC leukocyte recruitment in a flow based adhesion assay: HIEC were stimulated for 24 hours with combinations of IFN-γ (50ng/ml), TNF-α (10ng/ml) and TWEAK (100ng/ml). Total PBMC were perfused over a monolayer of HIEC with a shear stress of 0.05Pa. Adherent monocytes and lymphocytes (zoomed in image shows size difference between monocytes and lymphocytes) were counted from 10 fields of view and presented as cells/mm^2/10^6 cells perfused. Unstimulated HIEC were used as a negative control and TNF-α and IFN-γ in combination were used as a positive control.

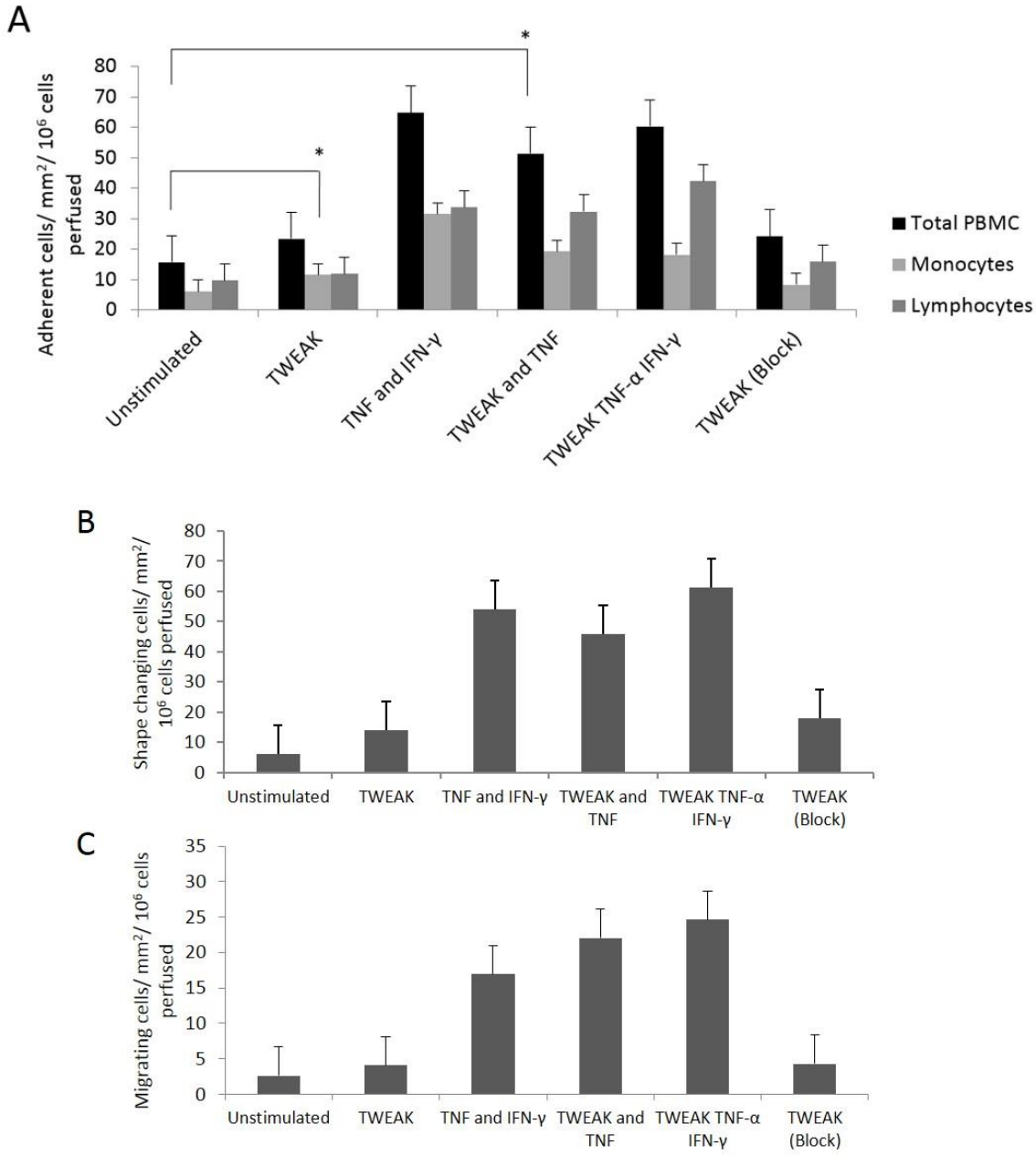


Figure 3.3.25 TWEAK supports the adhesion of PBMC to HIEC in a flow based adhesion assay: HIEC were stimulated for 24 hours with combinations of IFN-γ (50ng/ml), TNF-α (10ng/ml) and TWEAK (100ng/ml). Total PBMC were perfused over a monolayer of HIEC with a shear stress of 0.05Pa. A) Adherent, B) shape changing and C) migrating monocytes and lymphocytes were counted from 10 fields of view and presented as cells/mm^2/10^6 cells perfused. Unstimulated HIEC were used as a negative control, and TNF-α and IFN-γ in combination were used as a positive control. Data is presented as mean ± SEM from 4 experiments. TWEAK significantly enhanced monocyte adherence to HIEC (*p=0.037). TWEAK and TNF-α stimulated HIEC significantly enhanced adherence of total PBMC in comparison to their unstimulated control (*p=0.0286). Data is shown as mean ± SE n=4. Statistical analysis was carried out using a paired Student's t test. Data from experiment B and C was not statistically significant.

3.3.13 Summary of Results

- TWEAK and Fn14 mRNA expression significantly enhanced in diseased liver tissue in comparison to normal liver tissue.
- TWEAK was found expressed in inflammatory infiltrates in human liver tissue sections using IHC.
- Fn14 was found expressed on sinusoidal endothelium, neovessels, portal vessels, and in ductular reactive cells in human liver tissue sections using IHC.
- Fn14 was expressed on HIEC cell surface and enhanced in response to TNF-α, IL-1β and FGF stimulation. Fn14 expression was significantly decreased in response to TWEAK, IL-17A and TGF-β stimulation.
- Fn14 was found stored in the Golgi apparatus in HIEC and subsequently released in response to cytokine stimulation to the cytoplasm, endoplasmic reticulum and cell surface.
- No TWEAK was found expressed on HIEC, TWEAK mRNA was detected in total PBMC and significantly enhanced when stimulated with IFN-γ.
- TWEAK activated HIEC promoted leukocyte recruitment in combination with TNF-α and enhanced monocyte recruitment to HIEC.

3.4 DISCUSSION

Previous literature has shown low Fn14 and TWEAK expression in normal tissue, and enhanced expression in diseased or injured tissue. Our observations have confirmed enhanced TWEAK and Fn14 mRNA expression in diseased liver, in comparison to normal donor tissue (Figure 3.3.2). This was also consistent with previous unpublished findings from our lab, where IHC revealed TWEAK and Fn14 expression in normal donor liver tissue to be very low in comparison to diseased liver tissue. The observed low expression of TWEAK in diseased liver tissue analysed using IHC, was consistent with mRNA fold difference of expression which revealed a 3 fold increase of TWEAK in diseased liver tissue in comparison to the normal liver samples (Figure 3.3.2). IHC data revealed the presence of TWEAK in inflammatory infiltrates, highly localised to areas near portal vessels (Figure 3.3.3). A faint stain was also observed in hepatocytes surrounding portal vessels. Kawakita *et al* extensively studied TWEAK expression in liver tissue using IHC. They showed increased TWEAK expression in HCC tissue, and a higher expression observed in metastatic HCC tissue. They subsequently found hepatitis and cirrhotic liver tissue to be generally negative for TWEAK expression, but they found TWEAK to be present in lymphocyte infiltrates surrounding portal vessels, suggesting an inflammation mediating role (Kawakita *et al.*, 2004). Our study confirmed TWEAK expression consistently found in inflammatory infiltrates, and studies have further shown that TWEAK can initiate secretion of other pro-inflammatory chemokines, cytokines and MMP's in response to injury (Winkles, 2008). This is suggestive that TWEAK may be involved in co-ordinating the inflammatory response, and further perpetuating this response in inflammatory liver disease by enhanced TWEAK expression leading to further inflammatory cell recruitment.

Fn14 expression was found to be localised to hepatocytes, ductular reactive cells, neovessels, and highly localised to portal vessels (Figure 3.3.4). The expression of Fn14 varied on a case by case basis. The first normal liver sample (Figure 3.3.4 A) showed very low Fn14 expression, with a faint stain throughout hepatocytes. The second normal liver sample (Figure 3.3.4 B) showed high expression of Fn14 and localisation to portal vessels. The liver disease samples all showed high expression of Fn14. The differences observed between the two normal donor samples may be dependent on the origin of the 'normal' tissue. In this study, normal donor tissue has been classed as tissue obtained from a normal donor surplus to surgical requirement, reasons including; too much fat accumulation in the tissue, and/or scar tissue from previously undiagnosed liver trauma. Normal tissue can also be obtained from normal tissue adjacent to surgically resected tumour liver samples, which are retrieved from patients who have undergone extensive radio and chemotherapy, which can cause damage to otherwise healthy liver. Enhanced Fn14 expression is usually associated with detrimental consequences (Burkly *et al.*, 2007), therefore, the high expression of Fn14 in IHC stained liver samples in generally described normal tissue, is not surprising. The mRNA data however, showed a significant difference of Fn14 mRNA expression between normal and diseased liver tissue, showing a 17 fold increase of Fn14 mRNA in diseased liver tissue in comparison to the normal liver samples (Figure 3.3.2).

Fn14 expression has previously been described to be present in hepatocytes surrounding portal vessels in wild type murine liver tissue, in comparison to Fn14 knock out mouse models after PHx (Karaca *et al.*, 2014), our data confirms these Fn14 expression patterns in

human liver tissue, and we also observed Fn14 expression in areas of neovessels and ductular reactive cells. Dual IF staining showed co-localisation of Fn14 with CD31+ endothelium in liver tissue sections, on portal vessels and where the liver tissue was particularly fibrotic (Figure 3.3.5). This co-localisation was seen only in some of these areas and not consistently throughout the tissue section. This was a similar staining pattern observed by using IHC, and was suggestive of a more specific localised TWEAK and Fn14 response in inflammatory liver conditions. There was also some auto-fluorescence observed in our IF data with the CD31 stain and Fn14 stain. This was present but at a lower level than our specific stains of CD31 and Fn14. It could also be that this was not auto-fluorescence in the case of Fn14 and was in fact hepatocytes which also stain for Fn14, and Fn14 staining of fibrotic areas as was observed using IHC. Fn14 activity in endothelial areas of the liver suggests that Fn14 may facilitate neovascularisation indicated by Fn14 presence in areas of neovessels, and Fn14 expression found highly localised to portal vessels indicates that Fn14 may influence the influx of TWEAK positive inflammatory infiltrates leading to possible fibrogenic responses in the liver. This further supports current literature indicative of TWEAK and Fn14 involvement in the hepatic inflammatory response, repair and regeneration.

We further characterised expression of TWEAK and Fn14 on HIEC isolated from liver tissue, and observed responses to stimulating HIEC by various cytokines and growth factors which have known functions in inflammatory and regenerative responses. Previous literature has suggested that this ligand and receptor are highly inducible. A large part of our study confirmed this, we showed increased expression of Fn14 at a protein and mRNA level in HIEC

when stimulated with TNF-α, in comparison to un-stimulated HIEC (Figure 3.3.8). This up-regulated Fn14 expression was observed in HIEC cytoplasm using IF staining and confocal microscopy (Figure 3.3.13). We also showed significantly enhanced Fn14 cell surface protein expression in response to FGF (Figure 3.3.8) and IL-1β (Figure 3.3.11), and observed enhanced Fn14 expression in the cytoplasm of HIEC in response to these cytokines (Figure 3.3.14); although the enhanced FGF response was limited. Data from this study revealed a significant decrease in Fn14 expression in response to TGF-β, TWEAK, and IL-17A activation (Figure 3.3.12 and 3.3.17). This investigation revealed no significant changes in Fn14 expression in HIEC in response to IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, IL-21, IL-22, IL-23, and MCP-1 (Figure 3.3.12).

Previous literature has shown enhanced Fn14 mRNA and protein expression in human gingival fibroblasts in response to IL-1β and TGF-β activation. In combination with TWEAK, they have shown an up-regulation of IL-8 and VEGF, facilitating inflammation (Hosokawa *et al.*, 2006). Justo *et al* 2006 showed enhanced Fn14 expression in TNF-α and IFN-γ induced murine cortical tubular cell lines, which induced TWEAK dependent apoptosis (Justo *et al.*, 2006). These studies were in agreement with our findings of TNF-α and IL-1β activated HIEC inducing Fn14 expression, however our observed HIEC responses to TGF-β induction did not agree with these findings. In contrast, Jin *et al* 2004 found that TGF-β and IFN-γ had no effect on Fn14 mRNA and protein expression on human keratinocytes (Jin *et al.*, 2004). This was consistent with our findings of IFN-γ activated HIEC which showed no effect on Fn14 expression, however it was in contrast with our findings of TGF-β, where we showed a

significant down-regulation of Fn14 expression in HIEC. These varying cellular responses may be explained by the specific cell types and environment during detection of Fn14 responses.

TWEAK activation has shown a pro-inflammatory effect in female lower genital tract endothelial cells in combination with IL-1β activation (Han, Mekasha e Ingalls, 2010). This pro-inflammatory effect was also seen with TWEAK and IL-1 activation in combination with TNF-α, where an enhanced expression of inflammatory cytokines was observed in chronic RA samples (Chicheportiche *et al.*, 2002). IL-1β and TNF-α have been shown to have a synergistic effect on TWEAK in rheumatoid arthritis fibroblast-like synoviocytes enhancing inflammation, and have shown that they can enhance Fn14 expression (Xia *et al.*, 2010). This was in agreement with our findings, where activation with IL-1β and TNF-α in HIEC, enhanced the Fn14 response. IL-17 has been shown to amplify inflammation observed in rheumatoid arthritis patients, and IL-17 induction has been observed after TWEAK stimulation (Park *et al.*, 2012). There have been no studies however, investigating Fn14 expression in response to IL-17A at present.

The FGF response to Fn14 expression in HIEC was the most surprising. FGF is a known growth factor for endothelial cells, and Fn14 is a known FGF inducible protein. Therefore, we were expecting a large up-regulation of Fn14 in response to FGF activation. Our data showed a significant up-regulation of FGF induced Fn14 expression, however the response was very small, and our data only showed significant percentage positive HIEC for Fn14, and the MFI data was not statistically significant. IF data also showed a limited FGF response in Fn14

expression which was mildly up-regulated in the cytoplasm (Figure 3.3.14). Our data further showed the lack of an active FGF receptor present on HIEC (Figure 3.3.9), which would explain the limited response to FGF stimulation in these cells. This could possibly be due to FGF using alternative FGF receptors to induce Fn14 activation in HIEC. This will need to be further investigated to understand the limited FGF response on Fn14 in HIEC.

Enhanced Fn14 expression observed in HIEC cytoplasm when they were stimulated, was suggestive of Fn14 being secreted once HIEC have been exposed to the appropriate stimuli such as TNF-α, FGF, and IL-β in this case. Unstimulated HIEC still showed basal Fn14 expression on HIEC cell surface which may function to mediate normal physiological liver functions. Unstimulated HIEC showed a punctate staining pattern, with TNF-α stimulation, the staining pattern evened out and enhanced in the cytoplasm with some punctate staining remaining. This indicated that Fn14 may be held in compartmentalised cell structures and then released. We then showed that Fn14 was stored in the Golgi apparatus and released in response to activation, and transported via the Golgi and endoplasmic reticulum (Figure 3.3.16). Previous reports have suggested that Fn14 is expressed in the Golgi, as well as cell membrane in several cells including normal human keratinocytes, HUVEC, HEK293 cells, and HaCat cell line. They also reported TWEAK expression present in the cytoplasm (Sabour Alaoui *et al.*, 2012; Gurunathan *et al.*, 2014). This was consistent with our observations of Fn14 present in the HIEC cytoplasm using IF and on HIEC cell surface using flow cytometry. Our study did not however reveal any TWEAK expression in HIEC.

Increase in fluorescence intensity for Fn14 expression was consistent with the increase of Fn14 protein expression observed at the cell surface of HIEC using flow cytometry, and mRNA expression using real time PCR post TNF-α stimulation of HIEC. Enhanced Fn14 cell surface expression was detected using flow cytometry, which may indicate the potential of Fn14 to be involved in protein-protein interactions on the cell surface. This is potentially where Fn14 may interact with its ligand TWEAK, which may become activated after an inflammatory response. A large increase in Fn14 protein expression was observed when HIEC were permeablised. This intracellular expression of Fn14 was further increased when HIEC were stimulated overnight with TNF-α prior to analysis (Figure 3.3.15). This data was consistent with the observations made of HIEC using IF where compartmentalised Fn14 migrated out into the cytoplasm, and flow cytometry data which showed enhanced Fn14 expression on the cell surface.

Our data showed a significant down-regulation of Fn14 expression on HIEC cell surface and mRNA in response to TWEAK activation. We also observed down-regulated Fn14 expression using IF in response to TWEAK activation (Figure 3.3.17 and 3.3.18). In unstimulated HIEC, cytoplasmic Fn14 was present with a punctate staining pattern (Figure 3.3.18 B). In TWEAK activated HIEC, Fn14 expression remained in the cytoplasm but the punctate staining pattern was not present (Figure 3.3.18 C), and an overall slight down-regulated expression of Fn14 observed. It has been seen in other TNFSFR's where ligand binding has resulted in down-regulated receptor expression, such as in TNFR1 (Higuchi e Aggarwal, 1994), and Fas (Algeciras-Schimnich *et al.*, 2002); these have been shown to include receptor

internalisation, shedding, and DISC formation mechanisms. A previous study has described up-regulated expression of Fn14 in response to TWEAK activation in fibroblast like synoviocytes (Xia *et al.*, 2010). Another study showed no effect of TWEAK activation on cell surface Fn14 expression in an endothelial cell line (Stephan *et al.*, 2013). Our findings are in contrast with these studies.

A recent publication has characterised the regulation of Fn14 expression in TWEAK dependent and independent assays (Gurunathan *et al.*, 2014). They have found that Fn14 expression is a tightly controlled process from synthesis and trafficking from the Golgi to the plasma membrane, and lysosomal mediated degradation within the cell, dependent on the extracellular domain of Fn14. They found that Fn14 can undergo this receptor degradation and turnover in a TWEAK independent manner, but the process is accelerated with the addition of TWEAK and further perpetuated in a dose dependent manner. This process was seen in all cells that they tested indicating an endogenous Fn14 process. They also highlighted the importance of the Golgi in synthesising and continuous trafficking of Fn14 to the cell surface (Gurunathan *et al.*, 2014). Our findings are in agreement with these, as we also found Fn14 transport via the Golgi (Figure 3.3.16), and we found Fn14 expression on the cell surface using flow cytometry, and subsequent loss of Fn14 expression with TWEAK activation, indicating possible TWEAK accelerated Fn14 degradation within HIEC. We also observed down-regulated Fn14 expression in response to IL-17A and TGF-β activation in HIEC. It may be that these cytokines enhance TWEAK expression to the HIEC microenvironment and thus indirectly enhance Fn14 degradation via TWEAK interactions.

This will need experimental validation and we will need to determine if IL-17A and TGF-β activation in HIEC induce TWEAK expression.

It is apparent that TWEAK and Fn14 interactions on HIEC are highly regulated by all of these cytokines and may be initiating a complex signalling response leading to various functions during inflammatory conditions in the liver. To further understand the regulation of TNF-α, IL-1β, FGF, IL-17A, and TGF-β of TWEAK and Fn14 responses during inflammatory liver disease in comparison to normal donor samples, it would be essential to observe changes in TWEAK and Fn14 mRNA from normal and diseased liver tissue in response to activation, and to observe and characterise these changes using IHC, before making further conclusions. Our observations of TWEAK and Fn14 regulation have been made in HIEC, which have not been characterised before. The potential implications these stimulations will have on Fn14 activity in HIEC may be determined by functional assays, which will help to develop a better understanding of the roles that Fn14 may play in endothelial cells of the liver.

To further characterise TWEAK and Fn14 interactions on HIEC it was important to understand which various cell types of PBMC were expressing TWEAK. TWEAK expression was first discovered on macrophages, and subsequent studies have confirmed TWEAK expression on haematopoietic and immune cells. Monocytes are precursors of macrophages, and monocyte subsets have been shown to differentiate into macrophages and dendritic cells, depending on the physiological environment. Recruited monocytes from blood have been shown to contribute to the fibrogenic response in chronic liver inflammation, whilst KC

have been shown to play only a minor role in the fibrogenic response (Karlmark *et al.*, 2009). Previous unpublished data in our lab had shown 11% TWEAK percentage positive expression in isolated CD14+ve PBMC. Other work in our lab has also shown high percentage positivity of TWEAK protein expression in mesenchymal stem cells and activated hepatic stellate cells (Annika Wilhelm; Personal Communication). Throughout this study no TWEAK protein expression was observed on HIEC cell surface (Figure 3.3.19). This was shown with and without known stimuli which have previously been found to activate TWEAK expression; such as IFN-γ. In confirmation of our TWEAK protein expression data on HIEC, little to no expression of TWEAK mRNA was observed in HIEC in comparison to PBMC TWEAK mRNA expression (Figure 3.3.20). An increase in TWEAK expression was however observed when HIEC were permeablised; this further increased when HIEC were permeablised after TNF-α stimulation of the HIEC overnight (Figure 3.3.21). This increase in TWEAK expression was large in comparison to cell surface TWEAK protein expression which was negative. However enhanced TWEAK expression was only 8% of HIEC analysed, which was still relatively very low expression to Fn14 expression. There have been previous studies indicating the expression of TWEAK in endothelial cell lines (Stephan *et al.*, 2013), but no data has suggested TWEAK expression observed in endothelial cells of a primary lineage, specifically HIEC.

To further understand the regulation of TWEAK expressing monocytes and macrophages, we determined the expression of TWEAK in the classical (CD14+), and non-classical (CD16+ and dual positive) monocyte populations isolated from total PBMC. Our data showed that

TWEAK may be responsive during inflammatory cytokine stimulation of CD14+ and dual positive monocytes (Figure 3.3.22). We found that classical CD14+ monocytes expressed the highest amounts of TWEAK, and when these were stimulated with IFN-γ this expression further enhanced. This was followed by dual positive and CD16+ monocyte populations which showed equal amounts of basal TWEAK expression. When these were activated with IFN-γ; CD16+ monocytes showed decreased TWEAK expression and dual positive monocytes showed enhanced TWEAK expression. Liaskou *et al* 2013 showed that CD14+ monocytes were recruited in response to chronic inflammation in the liver, and they differentiated into dual positive subsets, enhancing the immune response, inflammation, and fibrosis (Liaskou *et al.*, 2013). Our data would support this as TWEAK mRNA expression was up-regulated in response to inflammatory cytokine activation, and has been previously shown to promote the fibrogenic response during chronic inflammation. The data obtained from this experiment showed a trend in TWEAK expression patterns in the different monocyte subsets. However we cannot draw conclusions from this data as further functional assays would be required to determine the responses of TWEAK activation in the different monocyte sub-populations.

Leukocyte recruitment in response to liver insult and injury has been observed in inflammatory liver disease tissue, and TWEAK expression has been found highly localised to these areas. Subsequently the inflammatory responses of TWEAK and its putative receptor Fn14 has been shown to be manipulated by the expression of adhesion proteins during inflammatory disease (Hosokawa *et al.*, 2006; Stephan *et al.*, 2013). Our data showed no

enhanced expression of ICAM and VCAM on HIEC cell surface in response to TWEAK activation in comparison to the unstimulated control (Figure 3.3.23). There was however, basal expression of ICAM and VCAM present on HIEC, which may be sufficient to support the TWEAK activated enhanced leukocyte adhesion observed. Our TWEAK data was not in agreement with several studies which have characterised the enhanced expression of ICAM and VCAM on endothelial cells in response to TWEAK activation (Chen *et al.*, 2013; Stephan *et al.*, 2013).

We characterised TWEAK activated HIEC responses to leukocyte recruitment using a flow based adhesion assay and found that TWEAK activated HIEC significantly promoted monocyte adhesion (Figure 3.3.25), however the response was small in comparison to positive controls. Lymphocyte and total PBMC were un-responsive to TWEAK activated HIEC, however when HIEC were activated with TWEAK in combination with TNF-α, significantly enhanced total PBMC adhesion was observed. We subsequently observed no significantly enhanced responses to total PBMC shape change or migration in response to TWEAK activation of HIEC (Figure 3.3.25). Our data which showed that TWEAK alone or in combination with TNF-α may enhance leukocyte adhesion to HIEC, is in agreement with previous findings from this investigation which found the presence of TWEAK localised to areas of leukocyte infiltrates in chronic inflammatory liver disease tissue (Figure 3.3.3). This data although limited, was also in agreement with several studies which have investigated and found that TWEAK can promote the recruitment of leukocytes (Yin *et al.*, 2013; Sanz, Aroeira*, et al.*, 2014). Our previous data also showed that monocyte subsets isolated from

PBMC showed enhanced TWEAK expression in response to inflammatory cytokine IFN-γ (Figure 3.3.22). This data indicated that inflammatory conditions can promote the expression of TWEAK and subsequently TWEAK activation can promote leukocyte infiltration which enhances the inflammatory response, and TWEAK further enhances inflammatory responses of other pro-inflammatory cytokines including TNF-α and IFN-γ; indicating a positive inflammatory mediating TWEAK dependent pathway. This data was in agreement with our hypothesis which highlighted the potential of TWEAK to promote and sustain acute and chronic inflammation by utilising key processes. Our data further showed that blocking TWEAK activity using Fn14 mAb had no effect on leukocyte adhesion and migration to HIEC. The rate of adherence remained consistently the same as with TWEAK activation alone. This may be a signalling pathway specific response and will need to be further investigated before making any more conclusions. A recent study by Salzmann *et al* 2013, showed that Fn14 mAb's can regulate NF-kB mediated responses, but cannot fully mimick activities of natural Fn14 in response to its ligand TWEAK in its soluble and membrane bound form. Therefore the responses of Fn14 mAb to TWEAK activation may vary in a signalling dependent manner, and may explain the lack of response to blocking TWEAK using Fn14 mAb that we observed. Salzmann *et al* showed that Fn14 bound to FcγR is a more efficient mechanism of mimicking natural Fn14, as this mAb activated the full range of activities observed of natural Fn14 (Salzmann, Seher*, et al.*, 2013). Therefore in future to determine If TWEAK activated responses are indeed mediated by Fn14, Fn14 mAb bound to FcγR will be a superior way of determining this, as it is not dependent on NF-kB signalling.

It has been reported that in cases of chronic inflammatory liver diseases, lymphoid follicles can form from portal tract infiltrate organisation. These are formed from B and T lymphocytes, DC, and neovessels which have a high endothelial venule phenotype (Grant *et al.*, 2002). These follicles function to sustain lymphocyte recruitment and retain them using the high endothelial vessels. Lymphoid follicles develop in response to chronic immune stimulation such as inflammatory cytokines like TNF-a. The presence of lymphoid follicles have been observed in chronic inflammatory liver diseases such as PBC and PSC, and other chronic inflammatory diseases such as SLE and RA (Grant *et al.*, 2002; Neyt *et al.*, 2012). Our findings show that there may be a significant role for TWEAK and Fn14 in the possible maintenance, organisation and development, of portal lymphoid aggregation during inflammation and liver disease. We showed this by the presence of TWEAK in inflammatory infiltrates in close proximity to portal vessels. We further found that TWEAK activated HIEC may promote the adhesion of leukocytes in particular monocytes to HIEC which can mimic specific inflammatory responses in settings of chronic inflammatory liver disease. Fn14 expression was highly up-regulated surrounding portal vessels and neovessels. We further showed that Fn14 cell surface expression in HIEC was highly variable in response to pro-inflammatory cytokines and growth factors, and no significant TWEAK expression was present on HIEC. Therefore we can suggest that TWEAK and Fn14 may be facilitating PALT formation in inflammatory liver disease, and paracrine mechanisms of action by TWEAK and Fn14 may be regulating cellular responses in the liver endothelium. This may be by transportation of TWEAK by certain immune cells such as IFN-γ activated monocytes from circulation, to the site of liver injury or disease, and interactions with Fn14 present on the cell surface of HIEC. It would seem that TWEAK may further promote inflammation by

facilitating the recruitment of leukocytes to the site of liver injury and disease and can help sustain this inflammation via interactions with specific cytokines and chemokines.

CHAPTER 4

TWEAK Activated HIEC Promote Endothelial New Vessel Formation and Stimulate a Tailored Immune and Angiogenic Cytokine Response to Inflammation

4.1 INTRODUCTION

4.1.1 Angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing vessels. It is a critical process required to regulate normal biological function such as embryonic development, wound healing, regeneration of the uterus during menstruation, regulating blood pressure, the immune system, and inflammation (Otrock et al., 2007). Angiogenesis is initiated in response to ischaemic conditions, and responds by enhanced vasodilation which increases vascular permeability, allowing angiogenic proteins free passage for functional interactions (Figure 4.1). This process is followed by degradation of the extracellular matrix surrounding existing vessels, this is carried out by activated proteins such as plasminogen and MMP9. Endothelial cells then proliferate and migrate towards activated pro-angiogenic stimuli such as VEGF, and position into the stroma formed. The endothelial cells then form a lumen, and the basement membrane reforms. Endothelial progenitor cells then undergo maturation and differentiation, and recruitment of vascular smooth muscle cells and pericytes occurs. These cells adhere to form tubular structures, and blood flow is resumed (Rundhaug, 2003; Ma et al., 2007). All of these processes are tightly regulated by growth factors and angiogenic cytokines such as plasma proteins and integrins, which act in processes such as cellular interactions, and positioning and scaffolding of endothelial cells. For normal angiogenesis, the correct balance of pro and anti angiogenic factors is essential. (Brooks, 1996; Coulon *et al.*, 2011).

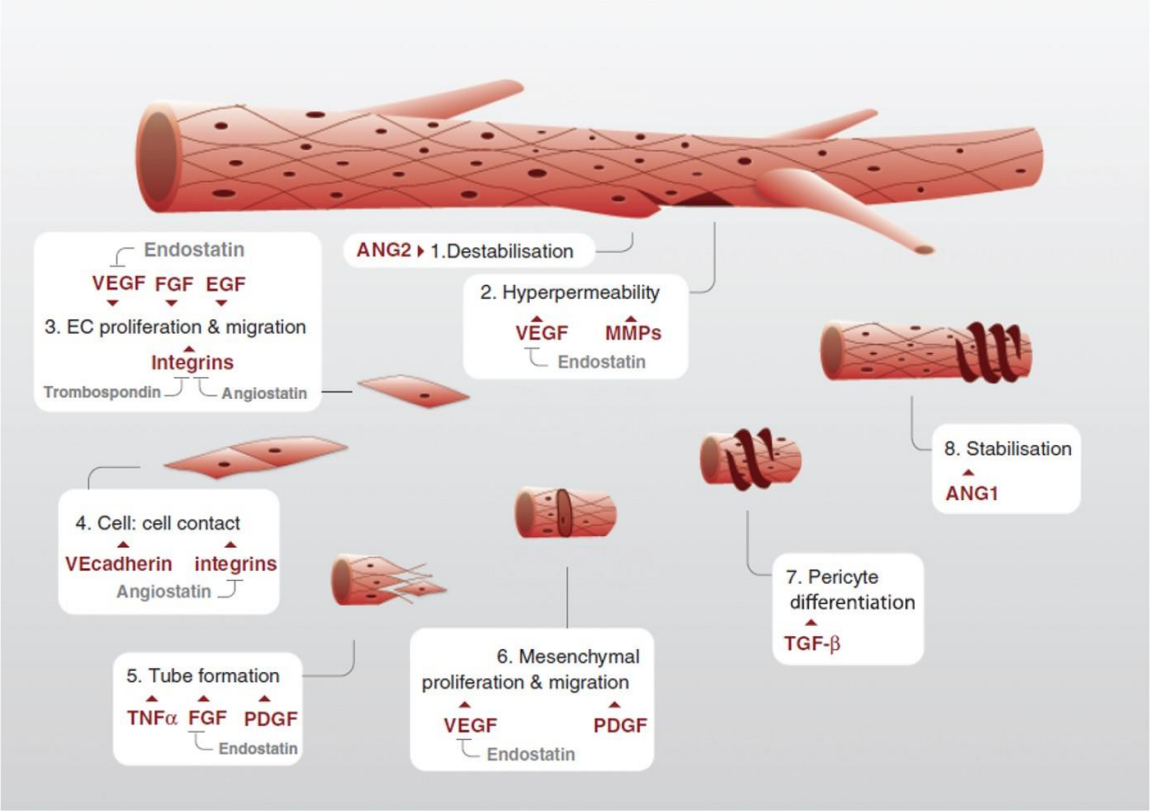


Figure 4.1 Angiogenesis: Angiogenesis is initiated by vessel destabilisation followed by permeablisation of the vessel walls to allow passage of angiogenesis regulating factors, and followed by breakdown of the ECM. Vessel formation is progressed by endothelial cell proliferation and migration to the stroma formed, and initiation of cell-cell contact and tube formation. Vessel formation is then completed by mesenchymal proliferation and migration, pericyte differentiation and stabilisation. All of these processes are regulated by angiogenesis promoting and inhibiting factors at different stages of vessel formation, as highlighted in the image (Coulon *et al.*, 2011).

HIF (hypoxia inducible factor) accumulation is a response to the ischaemic conditions which initiate angiogenesis and further stimulates angiogenic growth factors such as VEGF and FGF (De Spiegelaere *et al.*, 2010). The VEGF family are key mediators of angiogenesis and vasculogenesis, and induce angiogenic functions via interactions with VEGFR2 in a nitrox

oxide signalling pathway dependent manner (Coulon *et al.*, 2011). FGF family members are also key promoters of the angiogenic response, as well as promoting mitogenesis they respond to arterial formation more so than VEGF which respond to capillary formation (Klein, Roghani e Rifkin, 1997). PDGF and angiopoietins also positively regulate angiogenesis by aiding promotion of endothelial cell migration and proliferation (Risau *et al.*, 1992; Kim *et al.*, 1999). Inhibitors of angiogenesis are also critical for the regulation of normal physiological conditions. These include; thrombospondins which regulate endothelial cell migration and apoptosis. They are responsible for decreasing MMP expression which leads to inhibition of VEGF expression to regulate angiogenesis (Dawson *et al.*, 1997). Endostatin also inhibits growth factors and regulates endothelial cell apoptosis during angiogenesis (Dhanabal *et al.*, 1999). Angiostatins decrease the activation of the Erk signalling pathway which is activated by FGF and VEGF, and induce endothelial cell apoptosis (Claesson-Welsh *et al.*, 1998; Coulon *et al.*, 2011). As well as these mediators; integrins play key roles in positively and negatively regulating angiogenesis, and cadherins which are responsible for endothelial cell interactions and the passage of molecules across endothelium during angiogenesis (Lampugnani *et al.*, 1992; Serini, Valdembri e Bussolino, 2006; Coulon *et al.*, 2011).

Pathological implications can occur when angiogenesis is deregulated, these often occur during diabetes, psoriasis, rheumatic diseases, and tumour growth and metastasis (Paleolog, 2002; Martin, Komada e Sane, 2003; Otrock *et al.*, 2007), for example; when there is insufficient angiogenesis in a diabetic patient during wound healing, impaired or limited new

vessel formation can occur. This can lead to an inadequate inflammatory response to the inflicted wound, and a poor oxygen and nutrient supply; leading to impaired wound healing processes. In contrast, when there is excessive angiogenesis in diabetic patients, this can lead to other complications such as retinopathy, and kidney disease (Tahergorabi e Khazaei, 2012). There have been associations made between diabetes lead cardiovascular disease where de-regulated vessel formation is a key target of pathogenesis; for example, during coronary atherosclerosis, collateral vascular formation is required, this process in diabetic patients who have de-regulated angiogenesis is severely impeded (Sasso *et al.*, 2005). Angiogenesis is also vital for the progression of tumour growth. Small dormant tumours when activated have the ability to recruit mature blood vessels to initiate angiogenic processes allowing infiltration of vessels to the tumour. This provides blood flow and all the required components for the tumour to grow, for example VEGF which is highly expressed in tumours (Coulon *et al.*, 2011).

4.1.2 Liver regeneration and angiogenesis

During the pathogenesis of liver disease, excessive inflammation is the initiation of a host of complications such as liver fibrosis, cirrhosis, steatosis, and occasionally tumour formation. When liver damage occurs, inflammatory cells and mediators regulate liver recovery, and the liver inflammatory process is also key to the regulation of angiogenesis. VEGF, HGF, and other pro-angiogenic factors as discussed have been found up-regulated during liver regeneration (Fernández *et al.*, 2009; Coulon *et al.*, 2011). Basic FGF is required for HIEC to proliferate, and bFGF and VEGF together are known to facilitate the increase of liver weight

post resection, and both of these growth factors are essential for angiogenesis . Hepatic stellate cells are closely linked to HIEC and have been known to regulate vessel stabilisation, maturation, and sinusoidal remodelling via interactions with HIEC and release of growth factors, such as VEGF (Lee *et al.*, 2007). Hepatocytes and HIEC have been found to induce HSC proliferation during PHx, and HSC in their active form can release angiogenic chemokines (Michalopoulos, 2007)(Michalopoulos, 2007). Progenitor cells have also been found to expand and differentiate into HSC and IHEC at the neovascularisation site, in response to growth factors and chemokine activation during liver regeneration (Beaudry *et al.*, 2007). Other resident liver cells can contribute to angiogenesis in the liver. KC release cytokines, reactive oxygen species, and platelet activating factors which can regulate angiogenesis (Horie *et al.*, 1997). Mast cells can release angiogenesis inducing mediators such as VEGF, and they contribute to endothelial cell proliferation to form liver sinusoids (Levi-Schaffer e Pe'er, 2001)(Levi-Schaffer e Pe'er, 2001).

4.1.3 Liver disease and angiogenesis

Cirrhosis of the liver in response to inflammation induced fibrosis is strongly associated with abnormal angiogenic processes in the liver, and neoangiogenesis is often associated with liver disease progression (Coulon *et al.*, 2011). Briefly; cirrhotic scar formation can lead to the compression and loss of function of hepatic vessels, accompanied by fibrotic septa formation, and sinusoidal capillarisation. This leads to impaired blood flow and insufficient oxygen delivery around the liver which requires neo angiogenesis (Le Couteur *et al.*, 1999). Specifically HSC migration towards the basement membrane of HIEC cause the narrowing

and distortion of vessels by collagen deposit formation leading to fibrosis mediated sinusoidal dysfunction (DeLeve *et al.*, 2006). Several studies have characterised the pro-angiogenic role of HSC during pro fibrosis and angiogenesis, for example during hypoxic conditions, enhanced VEGF and angiopoietin 1 expression via HIF-1α have been observed in human and rat HSC (Aleffi *et al.*, 2005; Novo *et al.*, 2007). Wound healing during fibrogenic CLD leads to the expression of fibrosis inducing factors which also have pro-angiogenic functions including growth factors, cytokines and MMP's including VEGF, FGF and TGF-β. Enhanced gene expression of ECM remodelling factors and angiogenesis are also enhanced in CLD including integrins and adhesion molecules (Fernández *et al.*, 2009). Portal hypertension can cause severe complications during liver disease, as enhanced collateral vessel formation can be observed in these cases which can route portal blood flow into systemic circulation. This can cause abnormal liver metabolic functions and abnormal clearance of toxins, leading to complications such as sepsis and portosystemic encephalopathy (Hoofring, Boitnott e Torbenson, 2003). Subsequently enhanced VEGF expression has been observed in splanchnic organs of portal hypertensive animals linking portal hypertention and angiogenic processes (Fernandez *et al.*, 2004). New vessel formation within the liver is also key to promoting the inflammatory response during inflammatory liver disease. This is achieved by enhanced expression of inflammatory cytokines and adhesion molecules, which contribute to angiogenesis mediated acute to chronic liver inflammation (Jackson *et al.*, 1997).

4.1.4 TNFSF ligands and receptors and angiogenesis

TNFSF ligands and receptors have been implicated in pro and anti angiogenic function and regulation in several studies, in particular during chronic inflammatory liver disease. TNF-a has been shown to induce angiogenesis in an *in vivo* rat cornea model (Leibovich *et al.*, 1987). Alternatively, Fajardo *et al* 1992 showed TNF-α to have pro and anti-angiogenic responses, where TNF-a inhibited proliferation of endothelial cells *in vitro*, whereas TNF-α promoted *in vivo* vessel formation in mice corneas. This effect was thought to be concentration, species and conditions dependent, where low doses induced angiogenesis and higher doses induced a cytotoxic effect (Fajardo *et al.*, 1992). TNF-α has been shown to induce endothelial cell angiogenesis via other angiogenesis mediators in a VEGFR2, PDGF, and NF-kB dependent mechanism (Sainson *et al.*, 2008). TNF-α has previously shown tumour suppressive activities via targeting of adhesion receptor Integrin alpha V beta 3. Integrin alpha V beta 3 normally funtions to promote tumour progression, however when targeted by TNF-α, this was shown to disrupt angiogenesis (Ruegg *et al.*, 1998). TNF-α expressed in macrophages has also been shown to activate the MAPK-Erk signalling pathway, which has been implicated in angiogenesis regulation as well as cell migration and apoptosis (Reddy, Nabha e Atanaskova, 2003).

Fas-L and its receptor have been also been found to have both anti and pro-angiogenic activities. In RA, Fas has shown anti-angiogenic functions where Fas induced apoptosis of endothelial cells which expressed VEGF165. Subsequently, migration of endothelial cells was also inhibited in response to Fas activation (Kim *et al.*, 2007). Excised neovascular

membranes from patients of age related macular degeneration, showed Fas positive neovessels surrounded by Fas-L positive retinal epithelial cells. Fas and Fas-L inhibition showed enhanced *in vivo* murine corneal neovascularisation, suggesting that Fas and Fas-L regulate retinal neovascularisation (Kaplan *et al.*, 1999). Fas with interactions with Fas-L has been shown to induce inflammatory angiogenesis in an *in vivo* murine model, where subcutaneous delivery of anti Fas mAb resulted in infiltration of endothelial cells and monocytes and macrophages, followed by neutrophils and mast cells after neovascularisation. This was not observed in subsequent Fas mutant mice (Biancone *et al.*, 1997).

CD40-L has shown potential to induce angiogenesis by promoting the expression of pro-angiogenic cytokines such as IL-8, VEGF and HGF from human intestinal microvascular endothelial cells (Danese *et al.*, 2007). A similar study showed that VEGF mediated CD40 regulated promotion of inflammation induced angiogenesis in a study on SCID (severe combined immuno-deficiency) mice with skin grafts. They showed CD40 expression on endothelial cells and monocyte/macrophages, and CD40-L expression induced expression of key angiogenesis proteins (Reinders *et al.*, 2003). CD40-CD40-L interactions have also been shown to promote *in vitro* tube formation coupled to MMP expression, which are both key components of neovascularisation (Mach *et al.*, 1999). CD40 has been shown to enhance expression of platelet activating factor on HUVEC. Expressed platelet activating factor mediated *in vitro* endothelial cell migration and vessel formation. CD40-CD40L also contributed to interactions between endothelial and smooth muscle cells during

angiogenesis (Russo *et al.*, 2003). Furthermore, CD40 was shown to promote neovascularisation and Kaposi's sarcoma tumour survival and growth, and an *in vivo* neovascularisation model showed inhibited tumour neo-angiogenesis in response to CD40 inhibition (Biancone *et al.*, 1999).

4.1.5 TWEAK and angiogenesis

TWEAK and Fn14 have both been described as pro-angiogenic in a number of studies. Lynch *et al* 1999 were the first to describe TWEAK as angiogenic, as they found TWEAK induced endothelial cell angiogenesis and proliferation. They showed that TWEAK induced HUVEC and several microvascular endothelial cell, and smooth muscle cell proliferation which was found to be independent of VEGF and other angiogenesis inducing genes and receptors; shown by PCR quantification and VEGF neutralising antibodies. TWEAK angiogenic function was further characterised in an *in vivo* rat cornea model, where induced neovascularisation by TWEAK was found to be comparable to known angiogenesis inducing growth factors; VEGF and FGF2 (Lynch *et al.*, 1999). Wiley *et al* 2001 described the TWEAK receptor (Fn14) as being a key regulator of TWEAK mediated angiogenesis in several *in vitro* and *in vivo* assays. They found Fn14 can initiate a proliferation inducing signal in HUVEC, and Fn14 was found to be highly regulated by growth factor stimulation including FGF2 in smooth muscle cells. In *in vivo* rat balloon angioplasty models, Fn14 mRNA was found up-regulated at the site of arterial injury, whereas in uninjured rat arteries Fn14 expression was found to be low. Endothelial migration assays revealed Fn14-Fc fusion proteins could inhibit endothelial cell

migration induced by PMA and EGF, and Fn14-Fc fusion protein inhibited FGF induced *in vivo* corneal angiogenesis by 50%, determined by vascular area and density (Wiley *et al.*, 2001).

TWEAK has also been described as having mitogenic activities during cases of liver injury. Progenitor cell expansion during liver regeneration is a key process to facilitate liver growth and neovessel formation in the liver. In Fn14 knock out mouse models and by using a TWEAK mAb, progenitor cell and BEC proliferation in TWEAK transgenic mice, was significantly reduced (Jakubowski, Ambrose, Parr, Lincecum, Wang, Zheng, Browning, Michaelson, Baetscher, Wang*, et al.*, 2005). A similar response was observed in a CDE liver injury model where TWEAK acted as a mitogen for liver progenitor cells, suggesting TWEAK has involvement during hepatic injury and regeneration (Tirnitz-Parker *et al.*, 2010).

Unlike other TNFSF members, TWEAK has been described to be a direct inducer of angiogenesis. This is unlike TNF itself which has only been known to indirectly stimulate angiogenesis through other angiogenesis inducing factors such as EGF, and has been known to degrade tumours by necrosis of blood vessels, rather than promoting vascularisation of tumours. TWEAK was found to directly induce endothelial cell proliferation and angiogenesis in an *in vivo* rat cornea model without the activation of other angiogenesis or proliferation inducing factors (Lynch *et al.*, 1999). Alternatively other investigations showed TWEAK regulated other angiogenesis inducing factors such as FGF, as was demonstrated by Wiley *et al* in their *in vivo* mice cornea model, which showed that FGF-2 induced angiogenesis could be partially reduced by blocking Fn14 activity (Wiley *et al.*, 2001). This has been further

confirmed *in vitro* where TWEAK was shown to promote mitogenic activities of VEGF and FGF in HUVEC which was inhibited by Fn14-Fc fusion protein (Donohue *et al.*, 2003). It has also been shown that TWEAK activated HUVEC also contributed to the enhanced expression of ICAM, E-selectin, and inflammatory cytokines IL-8 and MCP-1, indicating that TWEAK may functionally mediate inflammation and angiogenesis via endothelial cells (Harada *et al.*, 2002).

It has also been suggested that TWEAK promoted angiogenesis may be achieved solely by the regulation of angiogenesis promoting growth factors; bFGF and VEGF. TWEAK was shown to promote VEGF induced HUVEC survival, bFGF induced proliferation, wound repair, and capillary sprout formation. It was shown that TWEAK alone could not significantly promote these processes in endothelial cells, and further suggested that TWEAK may have a dual role in angiogenesis as has been described for TNF-α. They showed that TWEAK can promote or suppress angiogenesis dependent upon experimental conditions and other angiogenic mediators present; such as TWEAK promoting endothelial proliferation induced by bFGF, and on the other hand suppression of morphogenic responses induced by VEGF by TWEAK (Jakubowski *et al.*, 2002)(Jakubowski *et al.*, 2002)(Jakubowski *et al.*, 2002). This study was in contrast to the findings of Lynch *et al* 2001, however there were suggestions that the differences in TWEAK response was dependent upon the environment during experiments, as Lynch *et al* were detecting TWEAK responses during inflammatory conditions post surgery in the rat cornea model, which were not present during the study of Jakubowski *et al* 2002, but injury conditions were present in the other studys which showed indirect TWEAK

activated angiogenesis. This further demonstrates the highly regulated range of functions that TWEAK can mediate which are context and environment dependent.

As well as TWEAK being described as a direct inducer of angiogenesis, the expression of TWEAK has been found in a number of human primary tumours indicating TWEAK may directly regulate tumour angiogenesis. It was found that TWEAK over-expression in nude athymic mice certainly enhanced tumour growth; producing highly vascularised tumours. The expression of Fn14 was found on vascular endothelial cells (HEK293) indicating that TWEAK may indeed regulate tumour angiogenesis by a paracrine mechanism (Ho *et al.*, 2004). TWEAK mRNA and protein expression has also been found up-regulated in hepatocellular carcinoma where TWEAK was found to promote HCC cellular proliferation in an NF-kB dependent manner. Increased Fn14 expression was also found on HCC cell surface and in normal cells analysed, in normal cells Fn14 expression was found to be low (Kawakita *et al.*, 2004).

TWEAK and Fn14 interactions have been found to regulate inflammatory mediated angiogenesis in a number of investigations, comparable to known growth factors such as VEGF and FGF which highly induce and regulate angiogenic functions and have been characterised extensively. There is a need to find novel and innovative mechanisms of regulating angiogenesis for therapeutic purposes in normal and diseased liver as the liver utilises angiogenic processes to regenerate itself, and is an ideal target to further investigate in relation to TWEAK mediated angiogenic regulation for future therapeutic intervention, in

particular to disect the mechanisms of angiogenesis regulation at a cellular level in the liver via TWEAK and Fn14.

4.1.6 Aims

The aims of this chapter were:

- To determine if TWEAK promoted HIEC proliferation, migration and tube formation.
- To determine if TWEAK mediated angiogenesis in HIEC was dependent on Fn14.
- To determine if TWEAK activated HIEC promoted angiogenic cytokine responses during chronic inflammatory liver disease.
- To determine if TWEAK and Fn14 responses during liver disease contributed to PALT formation.

4.2 MATERIALS AND METHODS

Materials and methods for this section; refer to: 2.1-2.5, 2.10, 2.11, and Tables: 2.1, 2.3 and 2.4.

4.3 RESULTS

4.3.1 TWEAK does not enhance HIEC proliferation and cell motility

We wanted to establish functional relationships between TWEAK and HIEC and if TWEAK stimulation would enhance HIEC angiogenic potential. To do this, it was important to establish whether TWEAK promotes HIEC proliferation, chemokinetic cell motility, and tubule formation. Ki-67 staining was used to observe HIEC proliferation. HIEC were cultured with and without the presence of recombinant TWEAK protein. Cell preparations were fixed on to Poly-L lysine coated slides, and subsequently stained for Ki-67; a nuclear cell proliferation marker. Positive cells and negative cells were counted and the percentage of Ki-67 positive cells was calculated which gave an indication of the proliferative capacity of HIEC. Relative proliferation between unstimulated and TWEAK stimulated samples was determined. TWEAK activated HIEC showed 1.3 fold increase in proliferation in comparison to unstimulated controls (Figure 4.3.1), but the data was not statistically significant.

To determine the role of TWEAK in driving HIEC motility, scratch wound assays were performed. A scratch was made on confluent HIEC cultured on gelatine. At 0 hours the wound was considered 100% open and then the wound was analysed at 4, 8, and 24 hours; until it was completely closed. At 4 hours, TWEAK alone marginally enhanced cell motility in comparison to the un-stimulated control, as TWEAK decreased the wound to 72.6%, and the unstimulated control decreased the wound to 77.4% (Figure 4.3.2 B). This was further improved in combination with TNF-α, as the wound decreased to 70.5%. TNF-α alone promoted the most cell motility by decreasing the wound to 67.5%. At 8 hours the wound

closure for all treatments was approximately the same. By 24 hours a difference was observed between treatments, as TWEAK, and TWEAK in combination with TNF-α, completely closed the wound. The unstimulated control had 2.3% wound remaining, and TNF-α alone had 4.5% wound remaining (Figure 4.3.2 B). This data was not statistically significant, therefore no enhancement of TWEAK or TNF-α stimulated HIEC motility was observed.

We wanted to determine if any TWEAK stimulated wound closure seen in HIEC was mediated by Fn14. To do this, scratch wound assays were performed with TWEAK with the presence of anti-Fn14 mAb, and TWEAK and IgG1 control for the Fn14 mAb (Figure 4.3.2 C). Interestingly, using an Fn14 mAb enhanced TWEAK activated wound closure rather than inhibiting it. At 4 hours the unstimulated control closed the wound to 77.4%, TWEAK activated HIEC had closed the wound marginally faster at 72.6%, TWEAK (+ Fn14 mAb) closed the wound much faster at 48.6% wound remaining, and the TWEAK (IgG1 control for Fn14 mAb) had closed the wound to 56.9%. This trend continued at 8 hours post activation where the unstimulated control closed the wound to 51.6%, TWEAK activated HIEC had closed the wound faster at 47%, TWEAK (Fn14 mAb) closed the wound much faster at 14% wound remaining, and the TWEAK (IgG1) had closed the wound to 39.7%. By 24 hours all wounds had closed apart from the unstimulated control which had 2.3% remaining open. Statistical analysis performed on this data showed it was not significant.

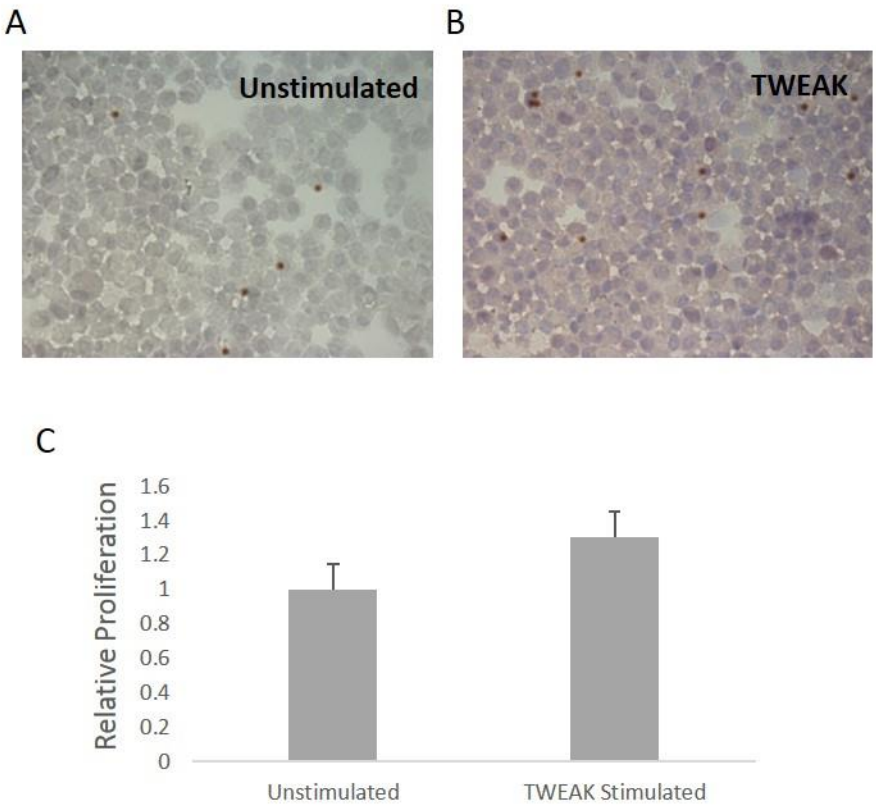


Figure 4.3.1 - TWEAK stimulation did not significantly enhance HIEC proliferation: HIEC were cultured with (B) and without TWEAK (A) (100ng/ml), and cell preparations were fixed on to slides. Unstimulated HIEC were used as a negative control. HIEC were stained for Ki-67 (a nuclear proliferation marker) to determine the proliferative capacity of HIEC after TWEAK stimulation. C) TWEAK stimulated HIEC activated cell proliferation 1.3 fold in comparison to the unstimulated control. Data is shown as relative proliferation of unstimulated HIEC and TWEAK stimulated HIEC (n=4 ± SE). This data was not statistically significant.

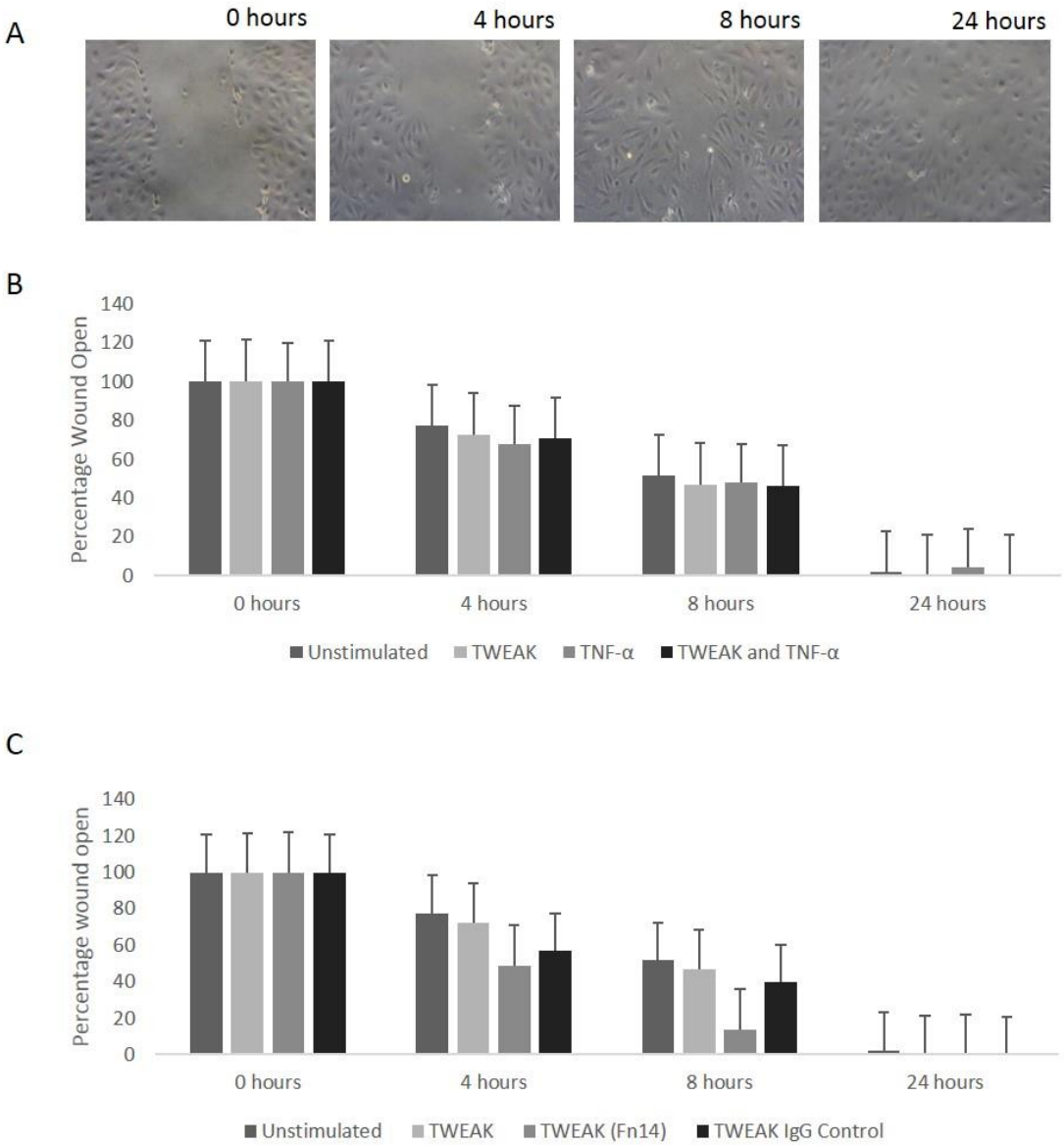


Figure 4.3.2 - TWEAK stimulation did not promote HIEC cell motility: A) HIEC were cultured on gelatine coated plates and scratch wound analysis was performed. HIEC were stimulated with TNF-α (10ng/ml) and TWEAK (100ng/ml) (Image shows TWEAK activated HIEC motility). Unstimulated HIEC were used as a negative control and TNF-α stimulated HIEC were used as a positive control. Images were taken after initialising the scratch at 0, 4, 8, and 24 hours. B) TWEAK activated and TWEAK and TNF-α activated HIEC were most active at closing the wound. Data is shown as mean percentage (n=5 ± SE). C) Scratch wound analysis was performed in the presence of TWEAK, TWEAK (Fn14 mAb to block Fn14 activation) and TWEAK (IgG control) to determine if TWEAK enhanced wound closure is dependent on Fn14 activation. Images were taken after initialising the scratch at 0, 4, 8, and 24 hours. Data is shown as mean percentage (n=3 ± SE). Blocking TWEAK activation using Fn14 mAb comparatively enhanced wound closure. This data was not statistically significant.

4.3.2 TWEAK promoted HIEC *in vitro* tube formation via Fn14

To determine whether TWEAK plays an active role in promoting HIEC angiogenesis; Matrigel™ tube formation assays were performed. HIEC were cultured on natural Matrigel™ for 8 hours and activated with TWEAK, TNF-α, TWEAK and TNF-α in combination, TWEAK + Fn14 mAb (to block Fn14 activation), TWEAK + IgG1 control for Fn14 mAb, and FGF as a positive control. Images for analysis were taken from five fields of view at 8 hours and assessed for node formation. A node was counted as having a central point with ≥ 1 branch (Figure 4.3.3 B; indicated with red mark). TWEAK activated HIEC significantly enhanced angiogenic tube formation in comparison to unstimulated HIEC; from 39 nodes in unstimulated HIEC to 52 nodes in TWEAK stimulated HIEC. Data was shown as mean of 16 experiments ± SEM ***p=0.00011 (Figure 4.3.3 C). The tube formation observed in HIEC stimulated by TWEAK was comparable to FGF which showed an average number of 49.8 nodes in 5 experiments *p=0.02. TNF-α, and TWEAK and TNF-α in combination did not alter tube forming potential in HIEC significantly (n=6).

To determine if the TWEAK promoted tube formation in HIEC was true and mediated by Fn14; an anti Fn14 mAb was used to block Fn14 activity in the angiogenesis assay in TWEAK stimulated HIEC. The anti Fn14 mAb significantly reduced node formation to lower than basal levels, with an average number of 35 nodes in 10 experiments. This data in comparison to TWEAK stimulated HIEC (52 average nodes) was significantly lower **p=0.002. This confirmed that TWEAK was indeed responsible for the increased angiogenic potential observed *in vitro* and that this may be mediated in an Fn14 dependent manner. TWEAK in

addition to the IgG1 control showed a decrease in node formation in comparison to the TWEAK activated samples. Node formation in TWEAK (IgG) was higher than TWEAK (Fn14) samples averaging at 40.2 and 35.3 nodes respectively (n=5).

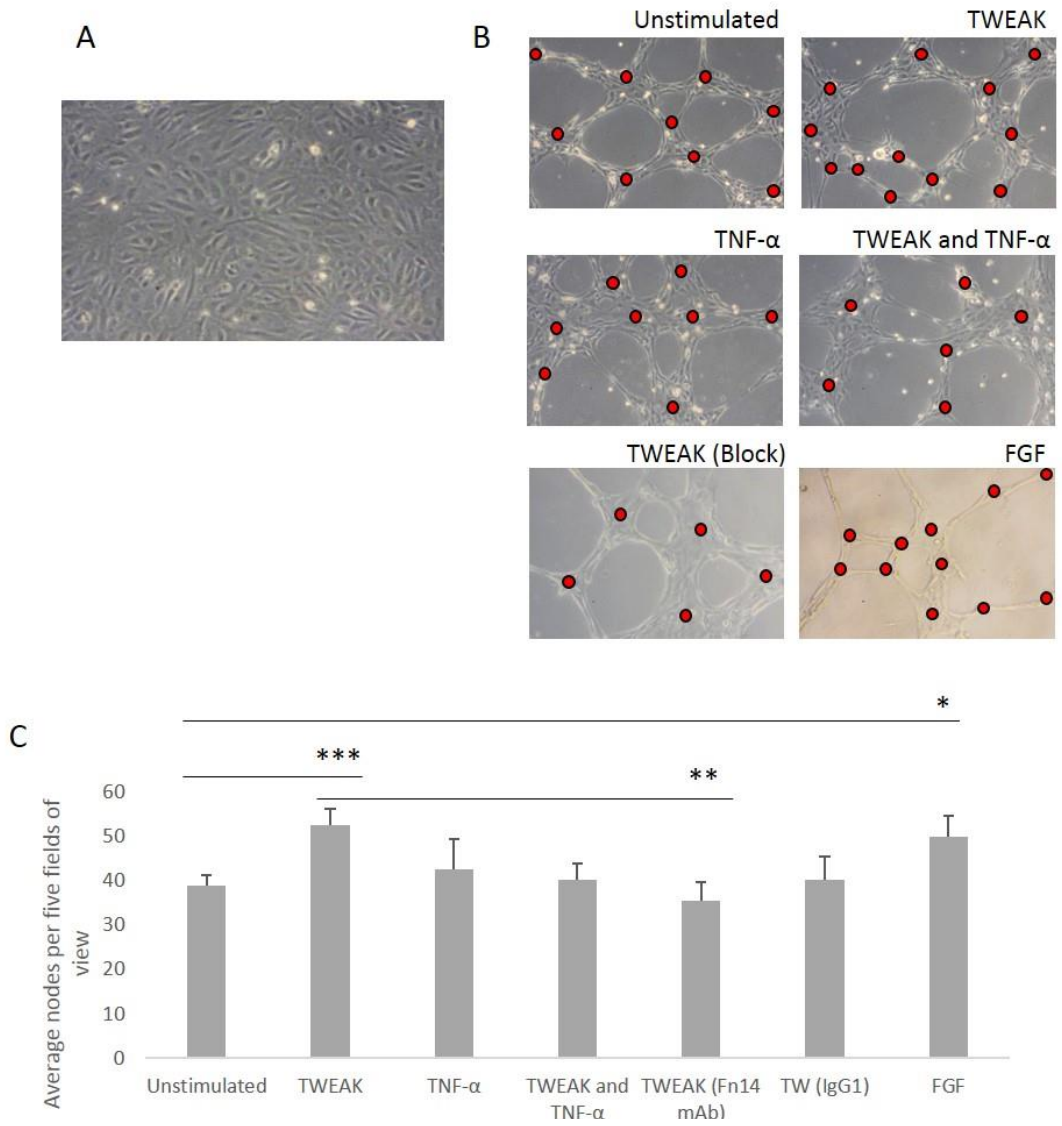


Figure 4.3.3 - TWEAK stimulated HIEC promote endothelial tube formation comparable to the tube formation potential of FGF: A) Representative image of HIEC in culture. B) HIEC were cultured on Matrigel™ for 8 hours and stimulated with TWEAK (100ng/ml), TNF-α (10ng/ml), TWEAK with Fn14 mAb to block TWEAK activity (74ng/ml), and FGF (0.5ng/ml). Node formation was used to calculate angiogenic potential by counting the number of nodes (Red mark) with each condition from five fields of view. Unstimulated HIEC were used as a negative control, TWEAK (IgG1) was used as a negative control for TWEAK (Fn14), and FGF was used as a positive control (n=5). C) HIEC cultured on matrigel showed a significant increase in node formation when stimulated with soluble TWEAK, and FGF for 8 hours (***p=0.000011 (n=16), and *p=0.02 respectively (n=5). No significant increase in node formation was observed when cells were stimulated with TNF-α (10ng/ml) and TWEAK and TNF-α co-stimulation (n=6). A significant decrease in node formation was observed when Fn14 activity was blocked (**p= 0.002385) indicating TWEAK stimulated tube formation was dependent on Fn14 (n=10). Data is shown as mean ± SEM. Statistical analysis was carried out using a paired Students t test.

4.3.3 TWEAK may initiate specific angiogenic and inflammatory responses during inflammatory liver disease

Supernatants collected from the angiogenesis assay conducted on HIEC isolated from normal donor liver tissue (Case 1), was assessed for angiogenic and inflammatory cytokine production. TWEAK activation mildly elevated a small range of inflammatory and angiogenic cytokines including MCP-1, TGF-β1, PDGF, MIF, Serpin E1 and FGF acidic in comparison to the unstimulated control. Some cytokines were down-regulated in response to TWEAK activation including Activin A, MMP-8, CXCL16 and EGF (Figure 4.3.4 C and D). Liver tissue sections from the same donor liver were haematoxylin and eosin stained to assess the histology of the tissue. The histology was grossly normal with mild inflammation in the portal tract which is a common feature in donor tissue (Figure 4.3.4 A and B). Liver tissue sections from this case were stained for Fn14 expression. Fn14 was expressed generally in hepatocytes and a distinctive expression pattern was observed surrounding portal vessels (Figure 4.3.4 F and G) in comparison to its isotype control IgG1 (Figure 4.3.4 E).

Assessment of angiogenic and inflammatory cytokines expressed from TWEAK stimulated HIEC, isolated from a case of chronic inflammatory diseased PBC liver tissue (Case 2) showed a more marked elevation of cytokines. MCP-1 increased 12 fold with TWEAK stimulation. An increase was also observed in VEGF, Prolactin, IL-8, Angiopoietin-1 and CXCL16 expression. TWEAK activation down-regulated PDGF (which was up-regulated in the normal donor sample) and TIMP-4 (Figure 4.3.5 C). The matched liver tissue histology showed heavy inflammatory infiltrates present in the portal tract surrounding portal vessels and granuloma

formation (Figure 4.3.5 A and B). Fn14 expression in corresponding liver tissue sections showed Fn14 highly expressed in areas surrounding inflammatory infiltrates. Staining was observed in hepatocytes, ductular reactive cells, and specifically surrounding portal vessels (Figure 4.3.5 D and E). This staining was compared to the corresponding isotype control IgG1 (Figure 4.3.5 F).

A second case of HIEC isolated from PBC liver presented a wide range of angiogenic and inflammatory cytokines elevated in response to TWEAK activation (Case 3). In contrast to other cases this case did not show a presence or elevation of MCP-1. A decrease was observed in Endothelin, Vasohibin, MIF and Persephin, with TWEAK activation (Figure 4.3.6 C and D). The corresponding liver tissue showed a large inflammatory infiltrate and disorganised vessel formation (Figure 4.3.6 A and B). The liver tissue also showed scattered B and T cells with no cellular organisation (Figure 4.3.8 A and B). Fn14 expression was examined in liver tissue sections from this case and Fn14 expression patterns also showed disorganised un-specific staining. Staining was observed in hepatocytes, and scattered staining around portal vessels (Figure 4.3.6 F and G). This staining was compared to the corresponding isotype control (Figure 4.3.6 E).

Another case of HIEC isolated from PBC liver (Case 4) showed specific increases in angiogenic and inflammatory cytokines. MCP-1 was up-regulated 6 fold when the HIEC were stimulated with TWEAK; and also PDGF, MMP-8, CXCL16, EGF and IL-8 were up-regulated in comparison to the unstimulated control (Figure 4.3.7 C). Marked decrease was observed in Endoglin,

Serpin F1, Prolactin, Platelet factor 4 and Thrombospondin 1 in response to TWEAK activation. The corresponding liver tissue showed a high degree of organisation where well defined granulomas were present in close proximity to portal vessels (Figure 4.3.7 A and B). These showed highly organised PALT with marginated T and B cells (Figure 4.3.8 C and D). Fn14 expression was examined in liver tissue sections from this case. They showed distinctive Fn14 staining in portal and neo vessels in close proximity to the well defined granulomas (Figure 4.3.7 D and E, Figure 4.3.8 E and F) in comparison to their isotype control (Figure 4.3.7 F).

Expression patterns were observed when data from the individual cytokine arrays was pooled together. The expression patterns of a panel of cytokines remained relatively unchanged between normal donor and chronic liver disease samples, with and without TWEAK activation in isolated HIEC (Figure 4.3.9). These included angiogenesis promoting and inhibiting cytokines; Angiogenin, DPPIV, EGF, Endoglin, HGF, IGFBP1, 2 and 3, MMP-9, PDGF-AA, Pentraxin 3, Platelet factor 4, Thrombospondin 1, and TIMP-1. A panel of cytokines in response to TWEAK activation in liver diseased samples increased in expression in comparison to the normal donor with and without TWEAK activation (Figure 4.3.10). This included; Angiopoietin 1 and 2, Angiostatin, CXCL16, EGVEGF, IL-8, MCP-1, MMP-8, PDGF-AB/BB, Prolactin, TIMP-4, uPA and VEGF. MCP-1 expression was enhanced 5 fold when HIEC were stimulated with TWEAK in comparison to the unstimulated HIEC. CXCL16 in the normal donor was down-regulated when stimulated with TWEAK. In contrast TWEAK stimulation in diseased HIEC samples significantly enhanced CXCL16 expression *p=0.04. Angiopoietin-1

expression was unchanged in the normal donor HIEC sample, however when HIEC from the diseased liver samples were stimulated with TWEAK, a large significant increase in Angiopoietin-1 expression was observed *p=0.04. MMP-8 expression in the normal donor sample was decreased in response to TWEAK activation. This expression was increased 2 fold in response to TWEAK activation in chronic inflammatory liver disease samples, although the response was much milder in 2/3 samples. IL-8 and VEGF expression in the normal donor remained relatively unchanged in response to TWEAK activation. In the chronic liver disease samples, TWEAK activation enhanced IL-8 and VEGF expression 2 fold and 1.3 fold respectively. Other cytokines which were enhanced in response to TWEAK activation in diseased liver tissue HIEC, were expressed only in certain cases and not in all cases (Table 4.1).

Expression of cytokines was observed to be down-regulated in response to TWEAK activation in diseased liver samples, in comparison to the normal donor sample with and without TWEAK activation (Figure 4.3.11 A). These included Endothelin, FGF acidic, LAP TGFβ1, Persephin, Thrombospondin 2 and Vasohibin. Expression of Artemin and Activin A was only observed in the normal donor (Case 1) and down-regulated with TWEAK activation (Figure 4.3.11 B). Descriptions of each of these angiogenic cytokines are given in Appendix 1.

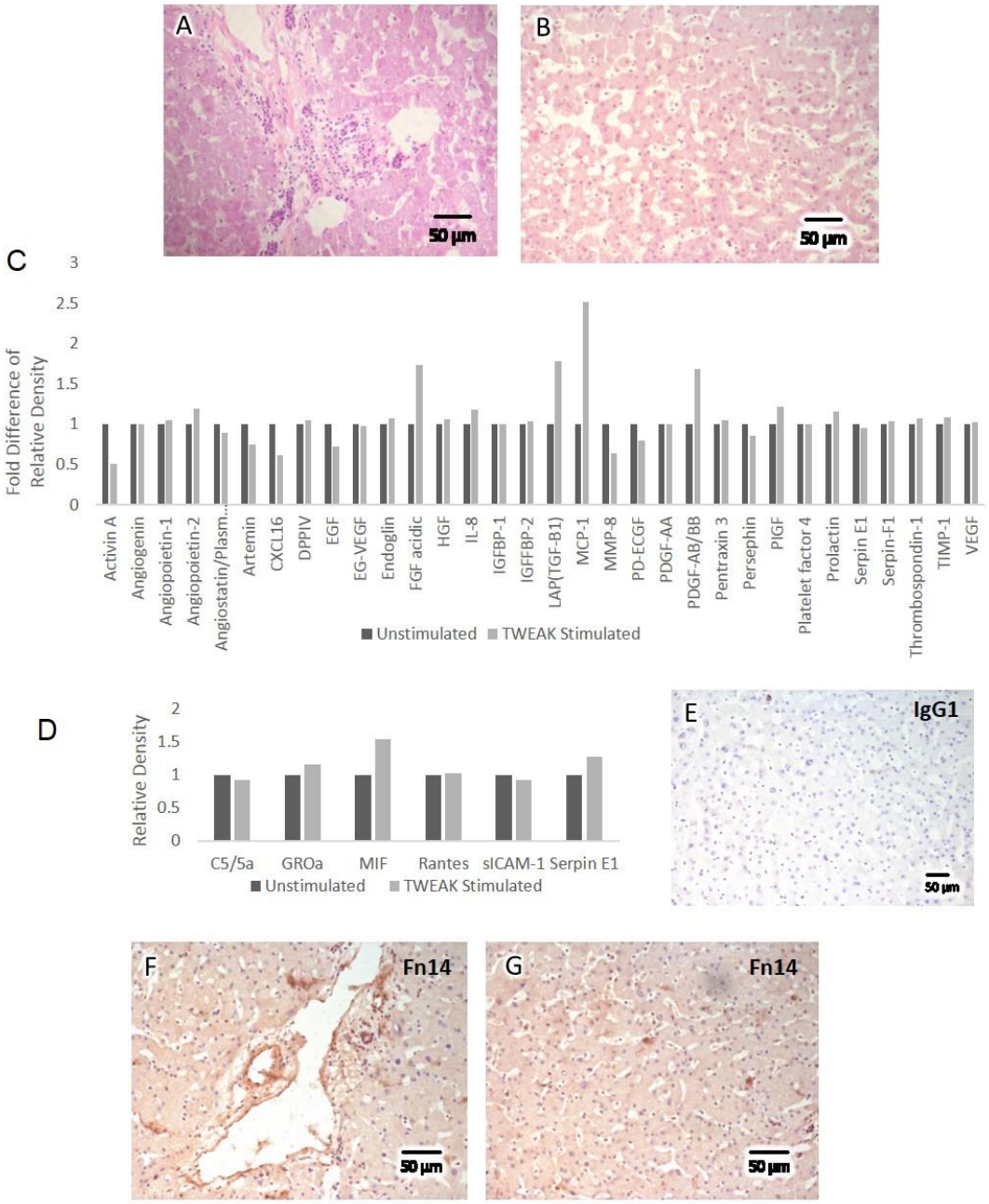


Figure 4.3.4 - Angiogenic and cytokine array profiles of isolated normal donor HIEC stimulated with TWEAK, correlate with the histopathological features of hepatic inflammation present in the tissue from which they were isolated (Case 1): A and B) Shows tissue sections from donor liver with mild inflammation represented by a moderately elevated presence of inflammatory cells within the portal tract. C) Shows an angiogenic cytokine array of HIEC isolated from the corresponding tissue. 8 hour TWEAK stimulation of HIEC moderately elevated some angiogenic cytokines in comparison to the unstimulated control. D) An inflammatory cytokine array from the same supernatant revealed up-regulation of macrophage inhibitory factor and Serpin E1 in response to TWEAK activation. F and G) Shows Fn14 IHC staining of corresponding liver tissue. Fn14 expression was confirmed in the liver tissue, mild staining was observed throughout the hepatocytes and localised heavily surrounding portal vessels. E) Shows the IgG1 control for the Fn14 antibody. Antibodies were developed with a DAB substrate. Images were taken with Zeiss microscope and axiovision v4.4 software 200x magnification

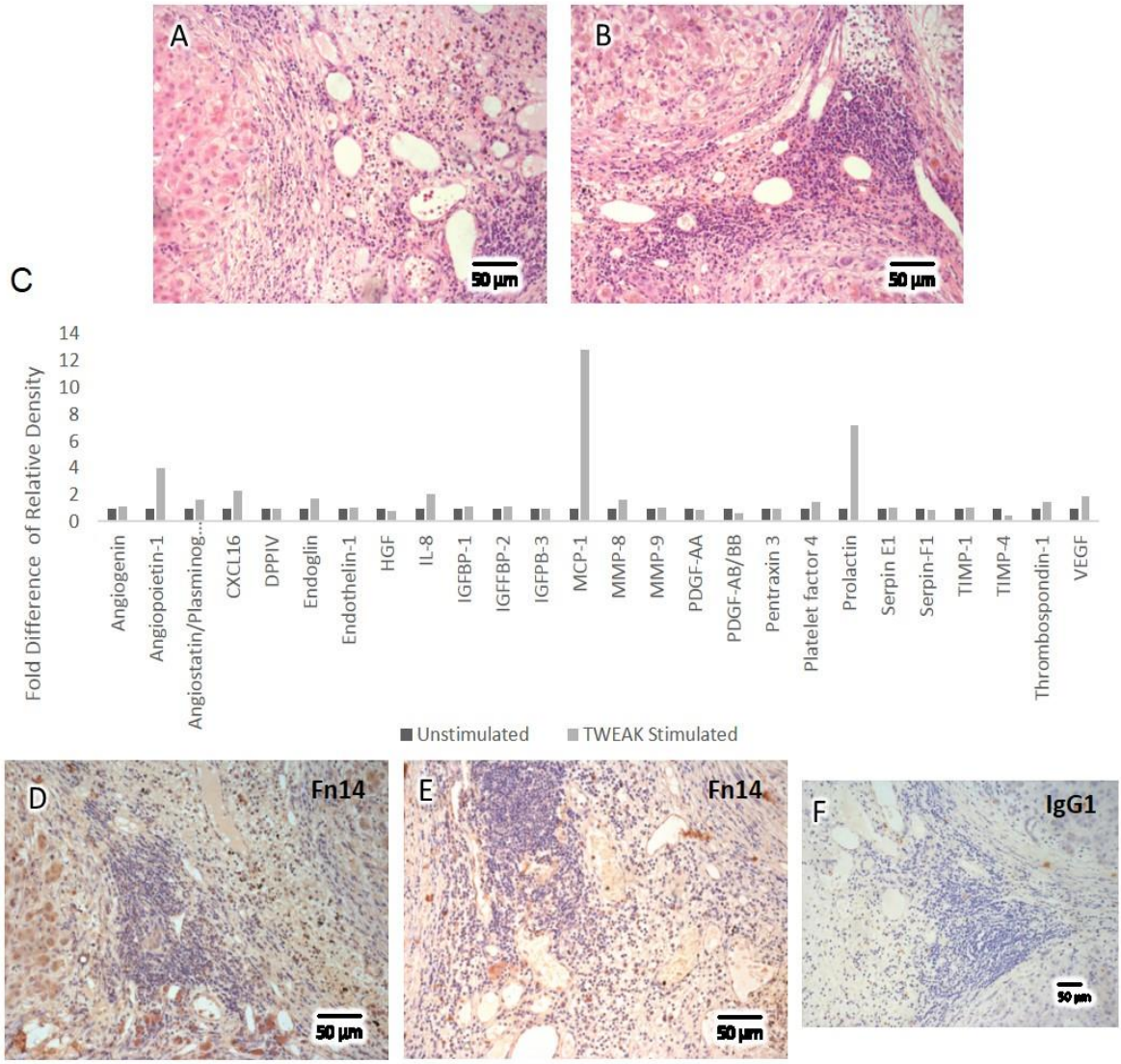


Figure 4.3.5 - Angiogenic cytokine array profile of isolated PBC HIEC stimulated with TWEAK, correlate with the histopathological features of hepatic inflammation present in the tissue from which they were isolated (Case 2): A and B) A degree of organisation was observed in tissue from this liver where large inflammatory infiltrates were observed in close proximity to portal vessels and neovessels. C) Shows the angiogenic cytokine profile of HIEC isolated from tissue A and B. The angiogenic cytokine array showed specific up-regulation of MCP-1, Prolactin, IL-8, angiopoietin 1 and CXCL16. This showed that there was a well-defined array of angiogenic cytokine induction following TWEAK stimulation in HIEC isolated from the corresponding tissue. D and E) The Fn14 staining pattern was observed to be present in portal vessels, neovessels, and hepatocytes. The staining also appeared to be focused on areas near inflammatory aggregates in close proximity to portal vessels. F) Shows the IgG1 control for the Fn14 antibody. All antibodies were developed with a DAB substrate. Images were taken with Zeiss microscope and axiovision v4.4 software 200x magnification

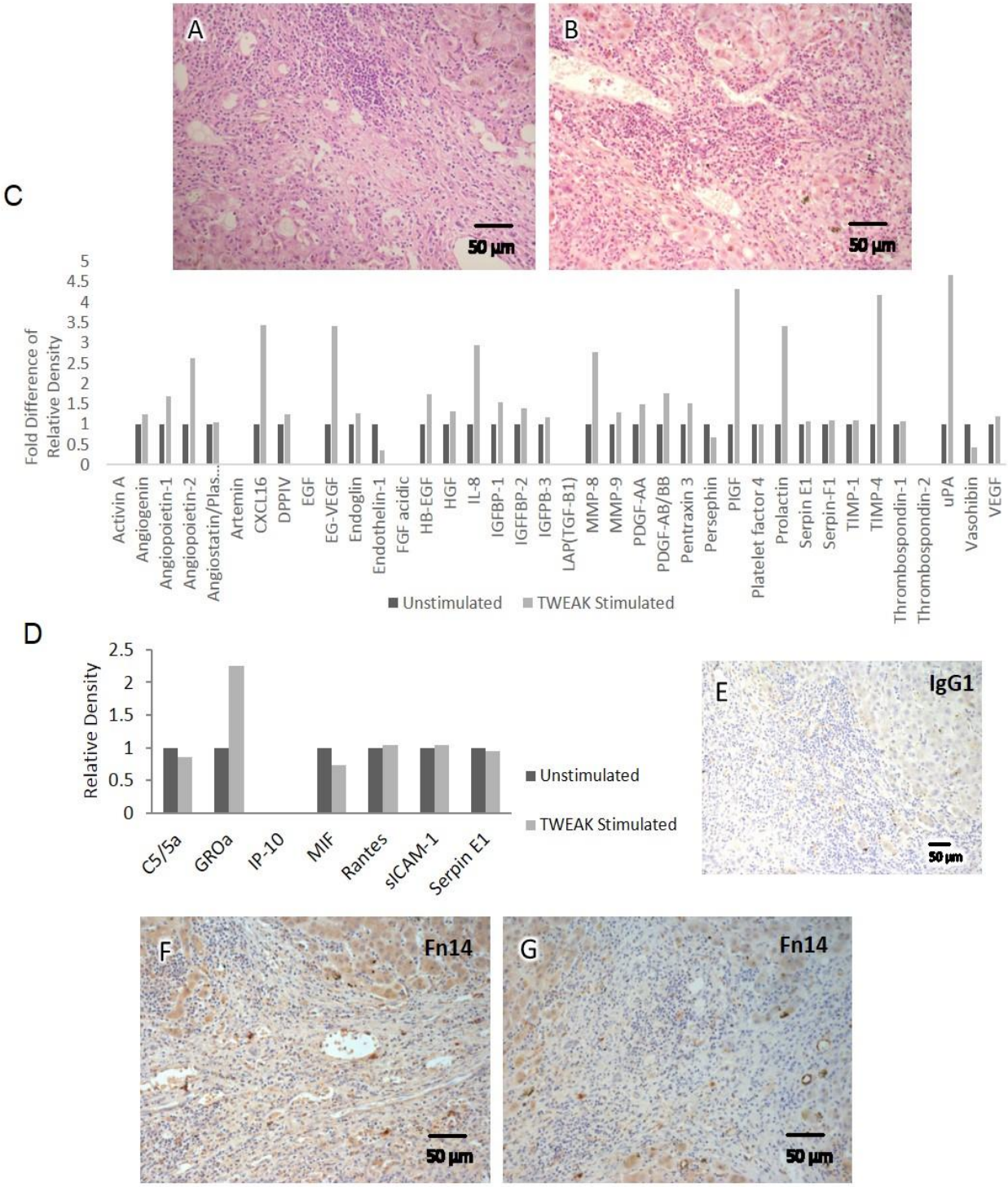


Figure 4.3.6 - Angiogenic cytokine array profile of isolated PBC HIEC stimulated with TWEAK, correlate with the histopathological features of hepatic inflammation present in the tissue from which they were isolated (Case 3): A and B) Shows tissue from a case of PBC liver with severe inflammation and disorganised vessel formation. C and D) The corresponding angiogenic and inflammatory cytokine arrays from supernatants from HIEC stimulated with TWEAK after 8 hours shows a higher expression and range of inflammatory and angiogenic cytokines. The array expression was broader in the diseased liver tissue sample than the normal donor. F and G) Shows Fn14 IHC staining of corresponding liver tissue. Fn14 expression was confirmed in the liver tissue but without a degree of organisation. E) Shows the IgG1 control for the Fn14 antibody. All antibodies were developed with a DAB substrate. Images were taken with Zeiss microscope and axiovision v4.4 software 200x magnification.

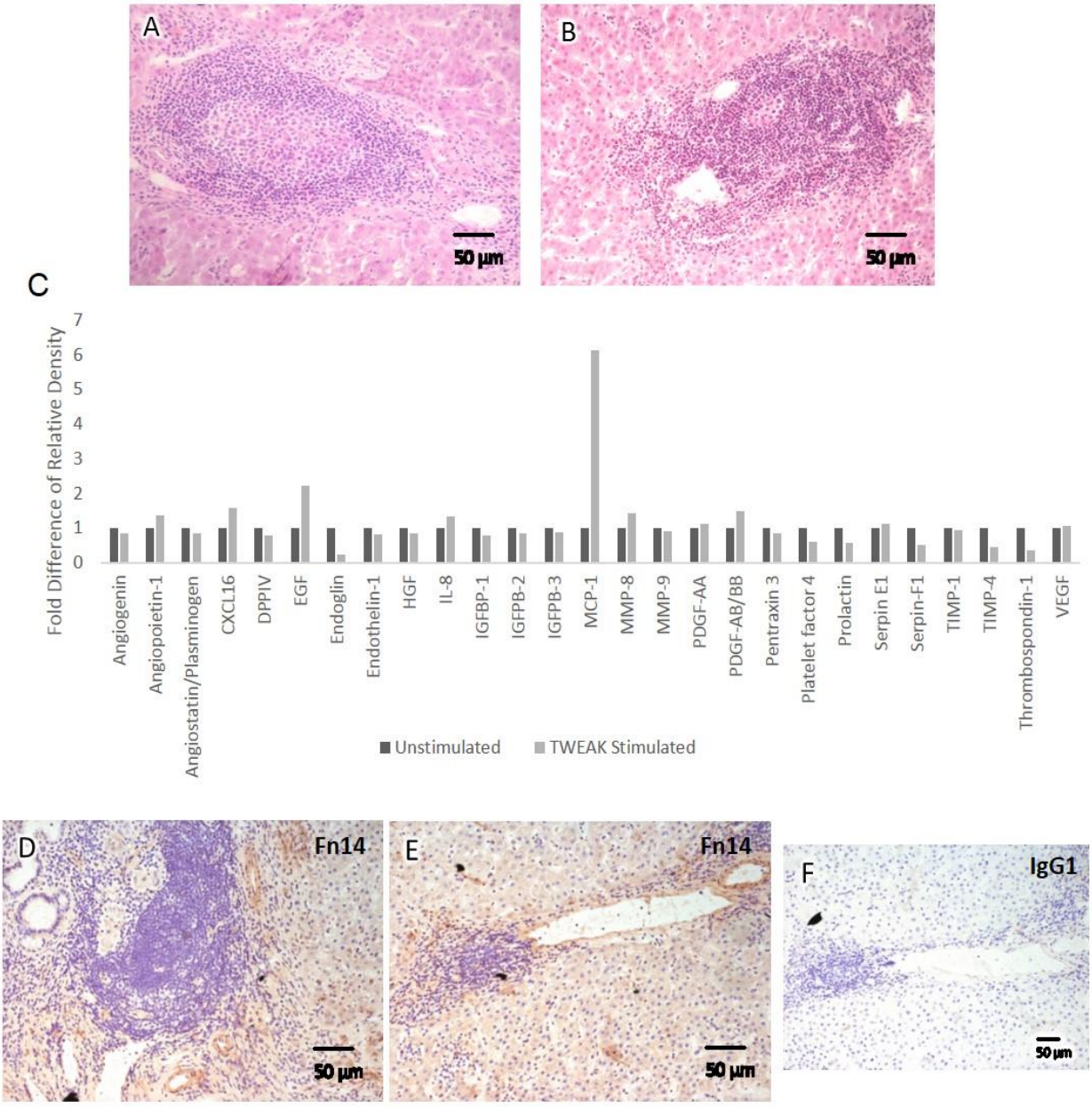


Figure 4.3.7 - Angiogenic cytokine array profile of isolated PBC HIEC stimulated with TWEAK, correlate with the histopathological features of hepatic inflammation present in the tissue from which they were isolated (Case 3): C) Shows the angiogenic cytokine profile of HIEC isolated from tissue A and B. A high degree of organisation was observed in tissue from this liver where well defined granulomas were present in close proximity to portal and neovessels. This showed highly organised PALT with marginated T and B cells (see Figure 4.3.8) with vessels in close proximity. The angiogenic cytokine array also showed well defined specific angiogenic cytokine induction of MCP-1, PDGF, EGF and CXCL16 following TWEAK stimulation in HIEC isolated from the corresponding tissue. D and E) Shows Fn14 IHC staining in corresponding liver tissue. The Fn14 staining pattern was observed to be present in portal and neovessels, and hepatocytes. The staining was heavily localised on areas near lymphoid aggregates in close proximity to portal vessels. F) Shows the IgG1 control for the Fn14 antibody. All antibodies were developed with a DAB substrate. Images were taken with Zeiss microscope and axiovision v4.4 software 200x magnification.

Table 4.1 Angiogenesis cytokine profiles: This table summarises TWEAK stimulation responses of HIEC in the angiogenesis cytokine array. The + represents up-regulation and the – represents down-regulation of cytokine. Data is shown as a HIEC response to TWEAK in comparison to unstimulated HIEC.

Angiogenesis Protein	Normal Donor Case 1	PBC Case 2	PBC Case 3	PBC Case 4	Angiogenesis Protein	Normal Donor Case 1	PBC Case 2	PBC Case 3	PBC Case 4
Activin A	+	-	-	-	IL-8	+	+	+	+
ADAMTS-1	-	-	-	-	LAP(TGF-B1)	+	-	+	-
Angiogenin	+	+	+	+	Leptin	-	-	-	-
Angiopoietin-1	+	+	+	+	MCP-1 (CCL2)	+	+	-	+
Angiopoietin-2	+	-	+	-	MIP-1a	-	-	-	-
Angiostatin/Plasminogen	+	+	+	+	MMP-8	+	+	+	+
Amphiregulin	-	-	-	-	MMP-9	+	+	+	+
Artemin	+	-	+	-	NRG1-β	-	-	-	-
Coagulation Factor III	-	-	-	-	Pentraxin 3 (PTX3)	+	+	+	+
CXCL16	+	+	+	+	PD-ECGF	+	-	-	-
DPPIV	+	+	+	+	PDGF-AA	+	+	+	+
EGF	+	-	+	+	PDGF-AB/BB	+	+	+	+
EG-VEGF	+	-	+	+	Persephin	+	-	+	-
Endoglin	+	+	+	+	Platelet factor 4	+	+	+	+
Endostatin/ Collagen XVIII	-	-	-	-	PIGF	+	-	+	-
Endothelin-1	-	+	+	+	Prolactin	+	+	+	+
FGF acidic	+	-	+	-	Serpin B5	-	-	-	-
FGF basic	-	-	-	-	Serpin E1	+	+	+	+
FGF-4	-	-	-	-	Serpin-F1	+	+	+	+
FGF-7	-	-	-	-	TIMP-1	+	+	+	+
GDNF	-	-	-	-	TIMP-4	-	+	+	+
GM-CSF	-	-	-	-	Thrombospondin-1	+	+	+	+
HB-EGF	-	-	+	-	Thrombospondin-2	-	-	+	-
HGF	+	+	+	+	uPA	-	-	+	-
IGFBP-1	+	+	+	+	Vasohibin	-	-	+	-
IGFBP-2	+	+	+	+	VEGF	+	+	+	+
IGFPB-3	-	+	+	+	VEGF-C	-	-	-	-
IL-1β	-	-	-	-					

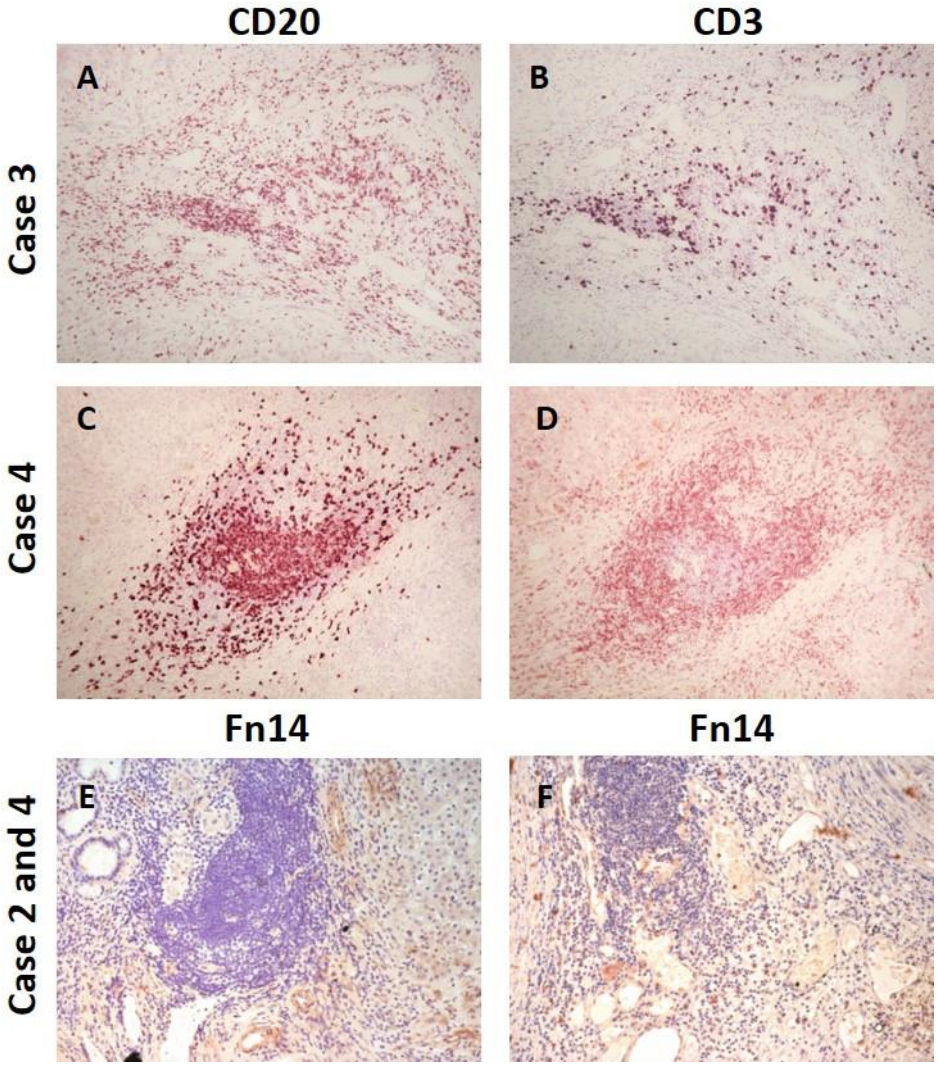


Figure 4.3.8 - Portal associated lymphoid aggregates show zonal organisation of CD3 positive T cells around CD20 positive B cells, Fn14 expression is found surrounding these areas: A and B show B and T cell infiltrates respectively scattered throughout the inflamed port tract without cellular organisation. These sections were taken from the PBC liver with HIEC angiogenic cytokine profile Figure 4.3.6; Case 3. C and D show highly organised marginated B and T cell lymphoid aggregates. These sections were taken from a PBC liver with HIEC angiogenic profile Figure 4.3.7; Case 4. Comparatively Fn14 is expressed surrounding granulomas within inflamed liver tissue taken from case 2 and 4.

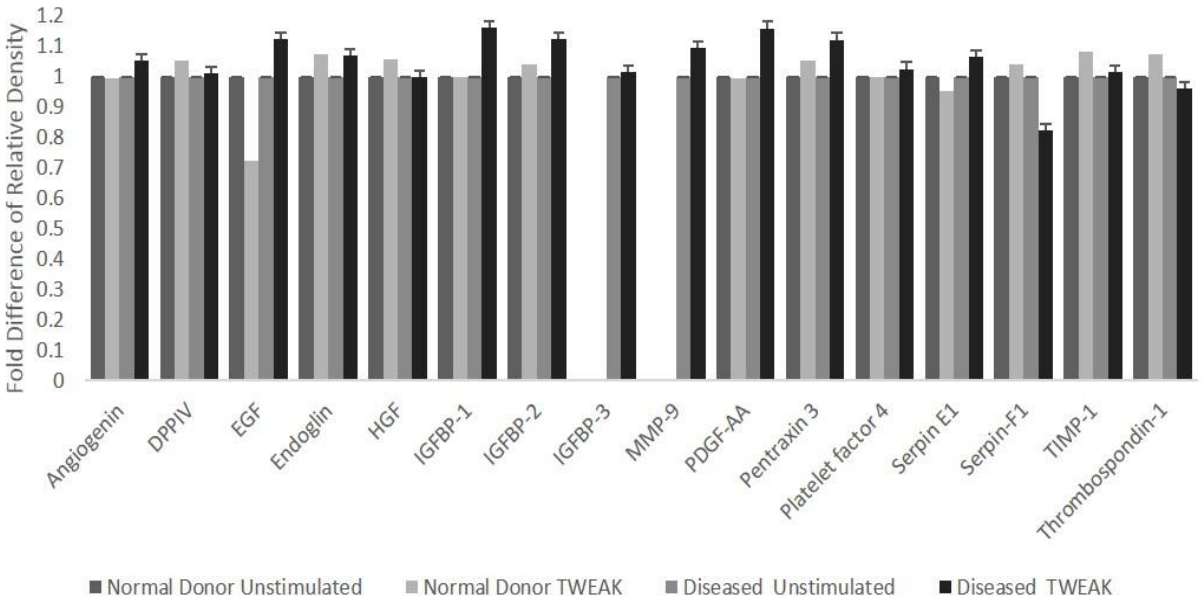


Figure 4.3.9 – Angiogenesis cytokine expression remained unchanged in response to TWEAK activation in inflammatory liver disease: This figure shows pooled data from the 4 angiogenesis arrays. Angiogenesis proteins expressed in the normal donor sample with and without TWEAK activation, and in chronic inflammatory diseased livers with and without TWEAK activation remain relatively unchanged. IGFBP-3 and MMP-9 were not expressed in the normal donor sample, they were only expressed in the diseased liver samples. Data is shown as mean ± SE from 4 experiments; n=1 normal donor, n=3 diseased liver. No statistical analysis was performed on this data.

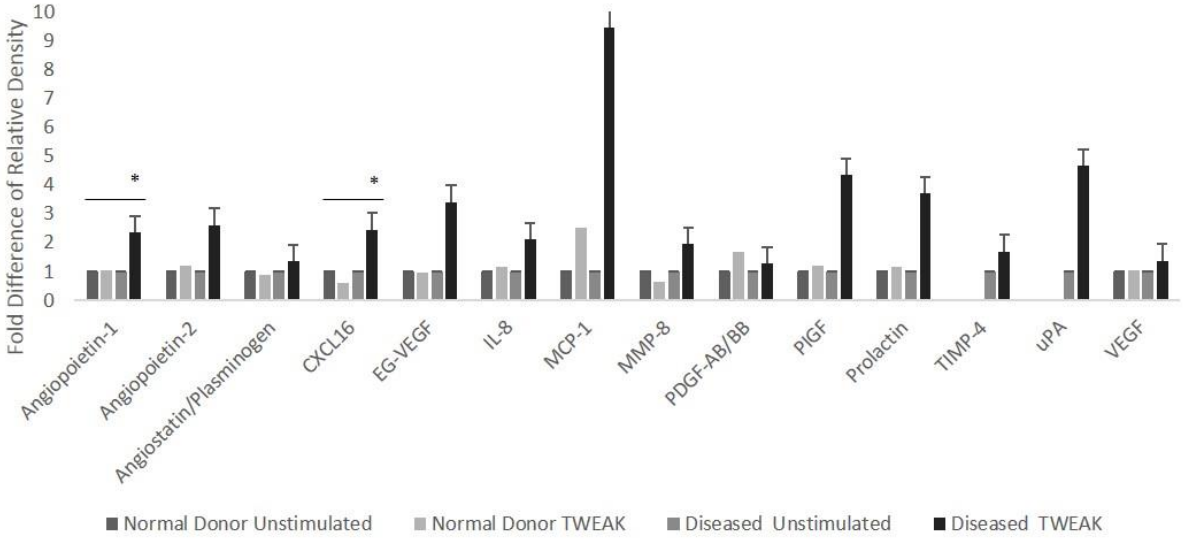


Figure 4.3.10 - Angiopoietin-1, CXCL16 and MCP-1 were enhanced in response to TWEAK activation in chronic inflammatory liver disease: This figure shows pooled data from the 4 angiogenesis arrays. Angiopoietin-1 expression was significantly enhanced in response to TWEAK in Chronic inflammatory disease liver in comparison to unstimulated HIEC *p=0.04. CXCL16 was down-regulated in normal donor tissue in response to TWEAK. This was then significantly up-regulated in response to TWEAK activation in diseased liver samples *p=0.03. C) MCP-1 expression was enhanced 5 fold in response to TWEAK from 3 out of the 4 arrays analysed. IL-8, MMP-8 and VEGF were also enhanced in response to TWEAK activation in HIEC isolated from diseased tissue. This data was not statistically significant. Data is shown as mean ± SE from 4 experiments; n=1 normal donor, n=3 diseased liver. Statistical analysis was carried out using a paired Students t test.

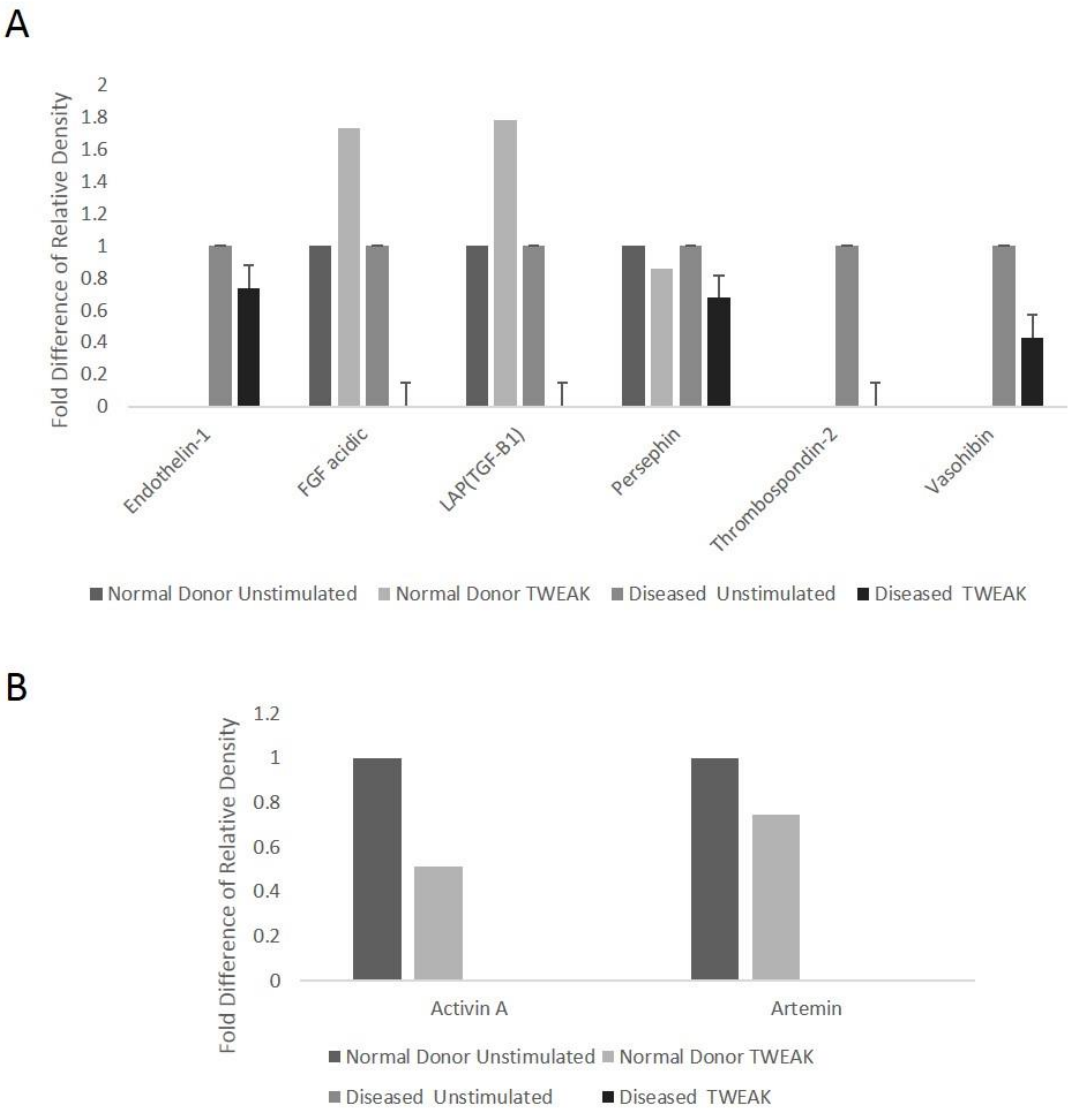


Figure 4.3.11 – Angiogenesis protein expression decreased in response to TWEAK activation in chronic inflammatory liver disease: A) Figure shows pooled data from the 4 angiogenesis arrays. Angiogenesis proteins – FGF acidic and LAP (TGF-β1) expression increased in the normal donor sample with TWEAK activation. Endothelin-1, Thrombospondin-2 and Vasohibin were not expressed in the normal donor sample. Persephin 1 only mildly decreased in response to TWEAK activation in the normal donor tissue. In chronic inflammatory diseased livers TWEAK activation showed decreased angiogenic protein expression. B) Angiogenesis proteins – Activin A and Artemin expression decreased in response to TWEAK activation in the normal donor tissue. In chronic inflammatory diseased livers no angiogenesis protein expression was observed. Data is shown as mean ± SE from 4 experiments; n=1 normal donor, n=3 diseased liver. This data was not statistically significant.

4.3.4 Summary of Results

- TWEAK stimulated HIEC significantly enhanced angiogenic node formation in a tube formation assay. This was regulated via Fn14 which was determined by using Fn14 mAb to block TWEAK activation.
- TWEAK activated HIEC showed specific angiogenic cytokine responses which were isolated from inflamed liver tissue. Notably CXCL16, angiopoietin-1, MCP-1, IL-8 and VEGF were found enhanced as a TWEAK stimulated HIEC angiogenic response.

4.4 DISCUSSION

Our data has shown that TWEAK activated HIEC via Fn14 may play a functional role in directing liver inflammation induced angiogenesis, which may lead to promoting liver regeneration. These findings are in agreement with several studies where TWEAK has been described as pro-angiogenic (Lynch *et al.*, 1999; Wiley *et al.*, 2001; Ho *et al.*, 2004). We showed that TWEAK could directly mediate tube formation, as inhibition of TWEAK by Fn14 mAb reduced angiogenic potential in the presence of VEGF and HGF supplemented media (Figure 4.3.3). The angiogenic potential observed was comparable to FGF induced tubule formation in HIEC, which has previously been shown by Lynch *et al* 1999 in a TWEAK induced *in vivo* rat cornea model comparable to VEGF and bFGF (Lynch *et al.*, 1999).

TNF-α activated HIEC have shown potential to induce Fn14 expression (Chapter 3), therefore we wanted to determine if TNF-α would also regulate TWEAK and Fn14 HIEC angiogenic responses. TNF-α and co-stimulation with TWEAK was used to observe tube formation responses. We showed no increase in angiogenic tube formation potential of HIEC, although a response of TNF-α in combination with TWEAK was expected. TNF-α has been previously described as both an indirect inducer of angiogenesis, and in some cases has been shown to inhibit endothelial cell proliferation and angiogenesis (Fajardo *et al.*, 1992). From our data it can be assumed that TNF-α does not facilitate or promote angiogenesis through TWEAK in HIEC, or there may be a possible cytotoxic effect by using these two cytokines in parallel, which suppresses the ability of TWEAK to induce tubule formation. Our data is in agreement with Lynch et al 1999 where it has been shown that TWEAK can independently induce an

angiogenic effect without the influence of other growth factors and angiogeneis inducing cytokines (Lynch *et al.*, 1999).

To understand the mechanisms by which TWEAK can promote angiogenesis for future therapeutic intervention via HIEC, it would be desirable to further characterise this function by determining the involvement of other growth factors such as VEGF. To do this, TWEAK activated HIEC may be blocked with an anti-VEGF mAb, or growth factor free Matrigel™ and serum starved media would be used in these assays to determine if TWEAK induced tubule formation is indeed mediated by VEGF. Similarly other pro-angiogenic factors and mediators in combination with TNF-α will need to be investigated to observe any angiogenic potential in HIEC using TNF-α.

Our data also showed non responsive proliferative and migratory capacity of TWEAK activated HIEC (Figure 4.3.1 and 4.3.2). An interesting observation was made during the cell motility assays, they showed that TWEAK activated HIEC blocked using an Fn14 mAb, showed a large enhancement of wound closure in comparison to TWEAK activated HIEC. In contrast, the tube formation assays in response to Fn14 inhibition of HIEC activated with TWEAK, showed significantly down-regulated node formation (Figure 4.3.3). As discussed in Chapter 3, this may be a HIEC signalling dependent response.

A key observation from this study was that TWEAK appeared to generate a tailored immune response to varying liver tissue histology. The sample number for these experiments were small, but preliminary observations were made in response to TWEAK activated HIEC which could indicate the potential of TWEAK activated angiogenesis regulation. All of the inflammatory disease samples used in this study for angiogenesis cytokine array studies, were from PBC livers. The histology of each of these cases revealed that each sample had a distinct tissue histological pattern with varying degrees of inflammatory infiltrate, and cellular and structural organisation. In response to each sample, TWEAK initiated a specific angiogenic and inflammatory cytokine activation cascade. When data from these arrays were pooled together, a specific regulatory pattern of TWEAK activation in PBC diseased tissue could be observed. Some angiogenic factors showed no response to chronic liver disease and TWEAK activation (Figure 4.3.9), these included pro and anti-angiogenesis inducing factors. Other angiogenesis proteins were found to be decreased in response to TWEAK activation (Figure 4.3.11). The most compelling finding was the enhanced expression of angiogenic cytokines in response to TWEAK activation in isolated HIEC from chronic liver disease samples in comparison to normal donor sample, where enhanced expression was observed in specific cases or in all liver disease cases investigated (Table 4.1), as this indicated that TWEAK positively regulated angiogenic responses during liver disease via HIEC (Figure 4.3.10). This has previously been described by Lynch et al 1999 where enhanced TWEAK angiogenic responses were observed in inflammatory conditions and alternatively Jakubowski et al 2002 showed a limited angiogenic response under normal conditions. For the purpose of this discussion I will only discuss angiogenesis proteins which showed a distinctive pattern of expression in all four arrays analysed (Table 4.1).

Generally, the Donor case (Case 1) showed a mild elevation of cytokines in response to TWEAK activation, which was also a reflection of the liver tissue histology of the sample (Figure 4.3.4). The PBC diseased samples all showed a higher degree of cytokine induction in response to TWEAK. There were however variations in the range of cytokines elevated or decreased in response to TWEAK. The two cases where the liver tissue histology showed a higher degree of organisation (Figure 4.3.5 and 4.3.7); specifically distinct inflammatory infiltrates in close proximity to portal and neovessels, and granuloma formation were observed. TWEAK activation elevated MCP-1 expression in these cases. MCP-1 expression was observed in the donor sample, however the response to TWEAK was mild. The sample where no organisation was observed in the histology of the liver tissue, MCP-1 expression was not observed either in the unstimulated supernatant or the TWEAK stimulated supernatant (Figure 4.3.6).

MCP-1 is a CC chemokine which is a key regulator of monocytes and macrophages, memory T cells, and natural killer cell recruitment and migration. MCP-1 has been shown to regulate the inflammatory response in disease and also can act as a direct mediator of angiogenesis independent of its inflammatory response, and directly contribute to tumour progression (Salcedo *et al.*, 2000). Subsequently, MCP-1 expression has been found to be related to the formation of granulomas in settings of chronic inflammation (Flory, Jones e Warren, 1993; Conti e DiGioacchino, 2001). MCP-1 has been shown to be involved in chronic inflammatory liver disease progression via inflammatory cell recruitment. Kanda *et al* 2006 showed MCP-1 expression related to hepatic steatosis (Kanda *et al.*, 2006). Czaja *et al* 1994 showed patients

with chronic inflammatory liver disease had increased expression of MCP-1 in comparison to patients with no liver disease (Czaja *et al.*, 1994). Zamara *et al* 2007 showed a decrease in liver damage caused by inflammatory infiltrates and oxidative stress in MCP-1 knock out mouse models (Zamara *et al.*, 2007).

There have been several studies where TWEAK has activated MCP-1 expression in response to disease and angiogenesis. Angiogenic responses to TWEAK activation have shown an elevation in MCP-1 expression, Harada *et al* showed an increase of MCP-1 expression in response to TNF-a, TWEAK and CD40L in HUVEC. This process was shown to be regulated by Fn14 and NF-kB (Harada *et al.*, 2002). TWEAK was also shown to increase expression of MCP-1 at an mRNA and protein level in a human ovarian cancer cell line (Gu *et al.*, 2013). Subsequently anti-TWEAK Ab has shown a decrease in MCP-1 expression in a lupus nephritis mouse model (Zhao *et al.*, 2007). Our data was in agreement with these findings that TWEAK may be regulating the inflammatory angiogenic response via MCP-1 during chronic liver disease, and in particular MCP-1 may be regulating granuloma formation in the liver tissue. Our finding although was not statistically significant, a 5 fold increase in MCP-1 expression was observed in response to TWEAK in two of the diseased HIEC isolates where granulomas were present.

Other up-regulated angiogenesis inducing cytokines in response to TWEAK activation in all chronic liver disease samples included Angiopoietin 1, CXCL16, MMP-8, IL-8, and VEGF (Figure 4.3.10). Angiopoietin 1 (Ang-1) is a known potent regulator of angiogenesis and has

been shown to be active during liver regeneration. Enhanced expression of VEGF and Ang-1 mRNA post partial hepatectomy were observed, and it was thought that these proteins regulated angiogenesis processes during liver regeneration, as expression of these gradually decreased over time (Sato *et al.*, 2001). Taura *et al* 2008 showed that HSC initiated angiogenesis by releasing Ang-1. Subsequently, they found Ang-1 up-regulated in fibrotic livers, and this up-regulation was mediated by TNF-a in an NF-kB dependent manner (Taura *et al.*, 2008). Ang-1 expression has been found to be up-regulated on pyogenic granulomas which have severe neovascularisation, and are an example of inflammatory induced angiogenesis (Yuan, Jin e Lin, 2000). We have made a novel observation where TWEAK and Ang-1 may regulate angiogenesis dependent on one another in settings of chronic inflammatory liver disease. This regulation was only observed in the inflammatory PBC liver disease samples, as the normal donor sample did not induce Ang1 expression in response to TWEAK activation (Figure 4.3.10).

CXCL16 is the ligand to the CXCR6 chemokine receptor which can be expressed as a transmembrane and soluble protein (Ludwig e Weber, 2007). CXCL16 functions to recruit lymphocytes and has been found to be expressed by B and T lymphocytes, endothelial cells, and hepatocytes (Matloubian *et al.*, 2000; Hofnagel *et al.*, 2002; Shashkin *et al.*, 2003). CXCL16 expression has been found to be regulated by inflammatory cytokines such as TNF-α and IFN-γ (Garcia *et al.*, 2007). CXCL16 has been shown to stimulate proliferation of endothelial cells and smooth muscle cells and cancer cells. CXCL16 has been found to be implicated in liver disease where expression of CXCL16 has been associated with steato-

hepatitis and liver fibrosis. Expression of CXCL16 was observed in regenerative nodules within the liver, and has been shown to play a critical role during lymphocyte migration across liver endothelium (Richard Parker 2013; Personal Communication). CXCL16 has been shown to stimulate HUVEC proliferation and induce tubule formation *in vivo* (Zhuge *et al.*, 2005), it has also been shown to recruit endothelial progenitor cells in the synovium of rheumatoid arthritis and during the formation of blood vessels (Isozaki *et al.*, 2013). TWEAK has been found to regulate the expression of CXCL16 in renal tubular cells in an NF-kB dependent manner where the expression of MCP-1, ICAM and Rantes was also up-regulated (Izquierdo *et al.*, 2012). Our data showed significantly enhanced CXCL16 expression in PBC diseased liver isolated HIEC when they were activated by TWEAK. It appears that TWEAK activation may induce an inflammation mediated angiogenesis response via CXCL16 during CLD.

We also observed up-regulated IL-8 and VEGF expression in chronic liver diseased samples in response to TWEAK activation. IL-8 is a member of the chemokine family which has been shown to regulate tumour growth, organisation and metastasis. It has been shown to be a regulator of angiogenesis in several studies (Heidemann *et al.*, 2003). IL-8 activated endothelial cells showed enhanced capillary tube formation and proliferation. Subsequent inhibition of apoptosis of endothelial cells was observed in IL-8 activated cells with up-regulated anti-apoptosis gene expression and MMP production; all facilitating the angiogenesis process (Li *et al.*, 2003). IL-8 has subsequently been shown to induce angiogenesis via Erk and P13K (phosphoinositide 3 kinase) signalling pathways in intestinal

microvascular endothelial cells (Heidemann *et al.*, 2003), and subsequently mediate angiogenesis via other angiogenesis promoting factors such as EGFR (Kyriakakis *et al.*, 2011). Up-regulated IL-8 expression in response to TWEAK has been observed in several studies. Chicheportiche were the first to describe the ability of TWEAK to induce IL-8 cytokine induction in tumour cell lines (Chicheportiche *et al.*, 1997). They then showed that TWEAK activation may contribute to chronic arthritis pathogenesis by the induction of inflammatory cytokines, including up-regulated IL-8 expression in supernatants from TWEAK activated human dermal fibroblasts and synoviocytes (Chicheportiche *et al.*, 2002). Subsequently it was shown that TWEAK activation may contribute to airway inflammatory responses by the induction of IL-8 in human bronchial epithelial cells in response to TWEAK activation (Xu *et al.*, 2004). Our data showed that IL-8 expression was up-regulated in response to TWEAK activated HIEC from inflammatory liver disease samples, and may specifically contribute to angiogenic and pro-inflammatory responses during chronic liver disease.

VEGF is a highly characterised angiogenesis mediating factor. TWEAK activated angiogenesis positively or negatively regulated via VEGF induction has been observed in several studies. VEGF has been shown to induce Fn14 mRNA expression in endothelial cells, and subsequently VEGF was shown to contribute to pathogenic angiogenesis in combination with TWEAK, suggesting that TWEAK and VEGF may promote endothelial cell responses in combination (Donohue *et al.*, 2003). Alternatively, anti-VEGF antibodies showed no effect or inhibition of proliferation of endothelial cells in response to TWEAK activation (Lynch *et al.*, 1999), and TWEAK activation was shown to inhibit morphogenic responses activated in

endothelial cells by VEGF (Jakubowski *et al.*, 2002), indicating that the TWEAK angiogenic response via VEGF is context dependent. Our data showed that TWEAK activation in inflammatory liver disease may induce angiogenesis and this process may require up-regulated VEGF expression. VEGF up-regulation in response to TWEAK activation in the diseased samples was relatively mild in comparison to other cytokine expression. So it may be that TWEAK activation may trigger the angiogenesis potential of HIEC, and VEGF expression may be induced to complete the process of tubule formation and angiogenesis signalling required during inflammatory liver disease.

A key finding from this study was the distribution patterns of Fn14 expression in the different tissues which were analysed for angiogenic protein expression. The Donor case which showed mild elevation of cytokines in response to TWEAK activation, also showed Fn14 expression mildly elevated throughout the tissue and more concentrated surrounding portal vessels (Figure 4.3.4 E and F). The chronic inflammatory liver disease samples all showed a higher degree of cytokine induction in response to TWEAK activation accompanied by elevated Fn14 expression in corresponding liver tissue, and subsequent variations in expression. The two cases where the liver tissue histology showed a higher degree of organisation (Figure 4.3.5 and 4.3.7), confirmed by B and T cell staining (Figure 4.3.8 C, D, E and F); distinct inflammatory infiltrates in close proximity to portal and neovessels, and granuloma formation, Fn14 expression in these cases was increased in these areas. The sample where no organisation was observed in the histology of the liver tissue (Figure 4.3.6 and 4.3.8 A and B), Fn14 expression in this case also showed no organised pattern (Figure

4.3.6 E and F). These findings correlated with the cytokine responses we observed of HIEC isolates to TWEAK activation, which showed subsequent organised cytokine responses, and disorganised wide-ranging cytokine responses according to the tissue histology and Fn14 distribution patterns. This suggests that TWEAK activated responses may be highly regulated, complex and tailored responses to the requirements of the tissue environment, however as the sample number for these experiments was small and the need for further experiments with regards to each cytokine induction will need to be performed, we can only speculate on the potential of this data.

The individual responses observed in the cases of liver samples, showed that TWEAK initiated a specific cytokine response where structural organisation, and heavy inflammatory infiltrate were observed further enforced by Fn14 expression in liver tissue. In particular the cases where granuloma formation was observed in close proximity to portal and neovessels. Other data has suggested that CD68 positive macrophages and stromal organiser cells are involved in the formation of lymph nodes in mice, and this process may be dependent on the activation of the NF-kB pathway (Jorge Caamano; Personal Communication), which has subsequently been shown to be an important signalling pathway in several studies looking at the functional roles of TWEAK and Fn14. This information together with data obtained from this study may suggest a specific role of TWEAK and Fn14 in tertiary lymph node development. To investigate this hypothesis, TWEAK and Fn14 knock out mouse models will be essential to analyse in comparison to wild type tissue in an *in vivo* granuloma formation

model such as induced p.acnes liver injury model (Nishioji *et al.*, 1999), whilst determining the regulatory signalling pathways.

We have found that TWEAK regulation of chronic inflammatory liver disease via HIEC is a complex and highly regulated process, and to understand this completely a lot of further work will be required. The data from the angiogenesis cytokine arrays and IHC was limited, and to specifically make significant findings further experiments are essential. However we can conclude from the data obtained; that TWEAK may contribute to hepatic angiogenesis via HIEC tubule formation. TWEAK may also regulate these functions via Fn14. TWEAK activation can trigger a specific inflammatory and angiogenic response during chronic liver disease, which makes TWEAK a highly attractive target for future therapeutic purposes of liver injury and disease.

CHAPTER 5

TWEAK and Fn14 signal via Erk and NF-kB Rel A during HIEC functional responses

5.1 INTRODUCTION

5.1.1 TNFSF Signalling

TNFSF members activate complex signalling pathways during functional responses via interactions between TNF ligands and receptors. TNF ligands are known to bind one or more receptor and initiate wide ranging functions such as apoptosis, cellular proliferation, differentiation and survival. The receptors can be sub-classified into two groups; the first group of receptors possess a 45 amino acid death domain required to induce cell death, and the second group of receptors do not possess the death domain; these typically function via TRAFs. TRAFs associate with specific motifs in the TNF receptors, and have been found to activate various signalling pathways including NF-kB and the MAPK pathways. There are six mammalian functional TRAFs described in TNFSF responses. The NF-kB signalling pathway is found largely activated in many TNF ligand-receptor functional responses, and is mostly defined as mediating the pro-inflammatory response of TNF family members (Aggarwal, 2003; Aggarwal, Gupta e Kim, 2012).

5.1.2 TWEAK and Fn14 Signalling

TWEAK ligand with its receptor Fn14 has been described as a multifunctional system linked to the activation of various transcription factors, including NF-kB; Rel A and Rel B, MAPK Erk 1/2, p38 and Jnk pathways, and the AKT signalling pathway (Figure 5.1) (Winkles, 2008; Fortin *et al.*, 2009). Fn14 has a conserved 28 amino acid TRAF binding sequence (P-I-E-E-T) in its cytoplasmic tail, which can bind TRAF 1, 2, 3, and 5, activating signalling cascades (Brown *et al.*, 2003). It has been reported that Fn14 contains many transcription factor binding sites

such as AP-1 and NF-kB in its promoter region (Tran *et al.*, 2006). Zheng *et al* 2008 described unpublished findings of a KAFF sequence situated adjacent to the P-I-E-T-T motif in the Fn14 cytoplasmic tail, to be responsible for NF-kB activation. They described NF-kB activation present in Fn14 P-I-E-T-T deletion mutants, and subsequent abrogation of NF-kB signalling was observed when the KAFF sequence was deleted, indicating that this sequence may be responsible for NF-kB signalling via TWEAK (Zheng e Burkly, 2008).

Whilst numerous publications have reported TWEAK and Fn14 dependent signalling, Fn14 signalling independent of TWEAK has also been documented in cases where Fn14 has been highly up-regulated, and specifically during the activation of NF-kB. Fn14 activation has been shown to induce cellular responses such as cell migration, invasion, and survival. It is thought that Fn14 signalling independent of TWEAK is dependent on the ability of Fn14 to bind TRAFs (Han *et al.*, 2003; Tran *et al.*, 2005). In a study investigating mouse intestinal epithelial cell injury models, Fn14 expression was up-regulated and found to activate signalling pathways leading to reduced inflammation in TWEAK knock out mice (Dohi *et al.*, 2009). In non-small cell lung cancer (NSCLC), Fn14 over expression induced EGFR signalling leading to enhanced NSCLC cell migration and invasive capacity in vitro and metastasis in vivo. (Whitsett *et al.*, 2012). Ligand independent signalling pathway activation via TRAFs, has also been observed in other TNFR's such as CD40 and BAFF-R which have been shown to activate NF-kB via TRAF association (Xu e Shu, 2002; Fotin-Mleczek *et al.*, 2004). It has been suggested that these processes may be due to receptor trimerisation, leading to activated signalling pathways via TRAF association, as this has been observed in other TNFR which do not possess a death

domain (Winkles, 2008). Brown *et al* 2013 showed Fn14 signalling independent of TWEAK by the use of Fn14 deletion mutants which do not have the ability to bind TWEAK, and found that Fn14 can activate NF-kB in transfected cells. They also observed that this process may be regulated by Fn14 dimerisation, which may occur on the cell surface via the Fn14 cytoplasmic domain (Brown *et al.*, 2013).

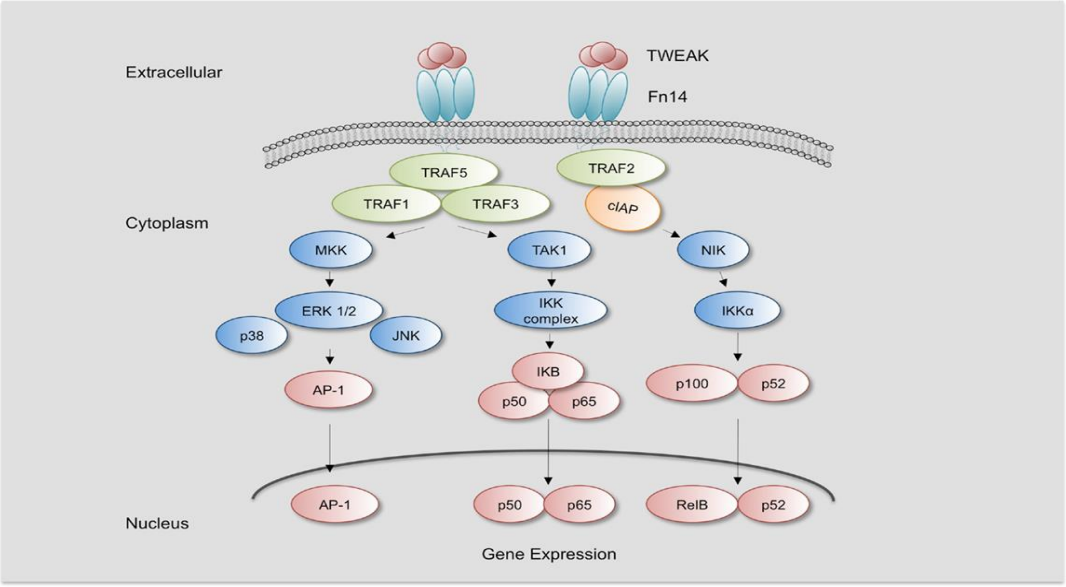


Figure 5.1 TWEAK and Fn14 activated transcription factors: TWEAK and Fn14 interactions on the cell surface can activate various signalling pathways via TRAF binding. TRAF interactions induce kinase phosphorylation to activate signalling pathways leading to gene transcription. Various transcription factors can be activated in the nucleus to induce gene expression which mediate specific functions. This figure illustrates the signalling pathway activation via TWEAK and Fn14 interactions to induce NF-kB and AP-1 regulated functions. (Blanco-Colio, 2014)

5.1.3 TWEAK-Fn14 signalling via NF-kB

TWEAK and Fn14 interactions however lead to the majority of activated signalling pathways observed. The canonical NF-kB signalling pathway (Rel A) is the most common activated pathway during TWEAK induced responses. NF-kB when activated, binds DNA leading to gene expression to regulate normal biological functions, and pathological functions, as well as being an important mediator of the innate and adaptive immune responses, tumourigenesis, cell fate, and lymphoid organ development. The NF-kB signalling pathway is well characterised as a critical immune system regulator as it is highly responsive to external stimuli such as growth factors, cytokines, and functional receptors (Bonizzi e Karin, 2004). There are five NF-kB members which dimerise and function to recognise the kB site; Rel A (p65), Rel B, c-Rel, p50-p105 and p52-p100 (Figure 5.2). The IkB (inhibitor of kB) complex binds to NF-kB dimers and prevents nuclear translocation, keeping the NF-kB complex dormant in the cytoplasm. The canonical pathway is activated by the phosphorylation of the IkB complex; the p50-p65 subunit of IkB is translocated to the nucleus, inducing phosphorylation of the p65 subunit and leading to the degradation of the IkB complex (Hayden e Ghosh, 2004).

The canonical NF-kB pathway is critical during the regulation of liver functions as it regulates normal liver homeostasis, patho-physiological functions, and regulation of inflammation (Sun e Karin, 2008). It is essential for normal embryonic development, as has been shown in *in vivo* knock out mouse models, where NF-kB deletion was shown to trigger fatal liver cell apoptosis (Beg *et al.*, 1995). IkBα phosphorylation in response to TWEAK activation has been

observed in a number of studies, and has been shown to have a rapid onset often within the first hour of TWEAK activation (Harada *et al.*, 2002; Brown *et al.*, 2003).

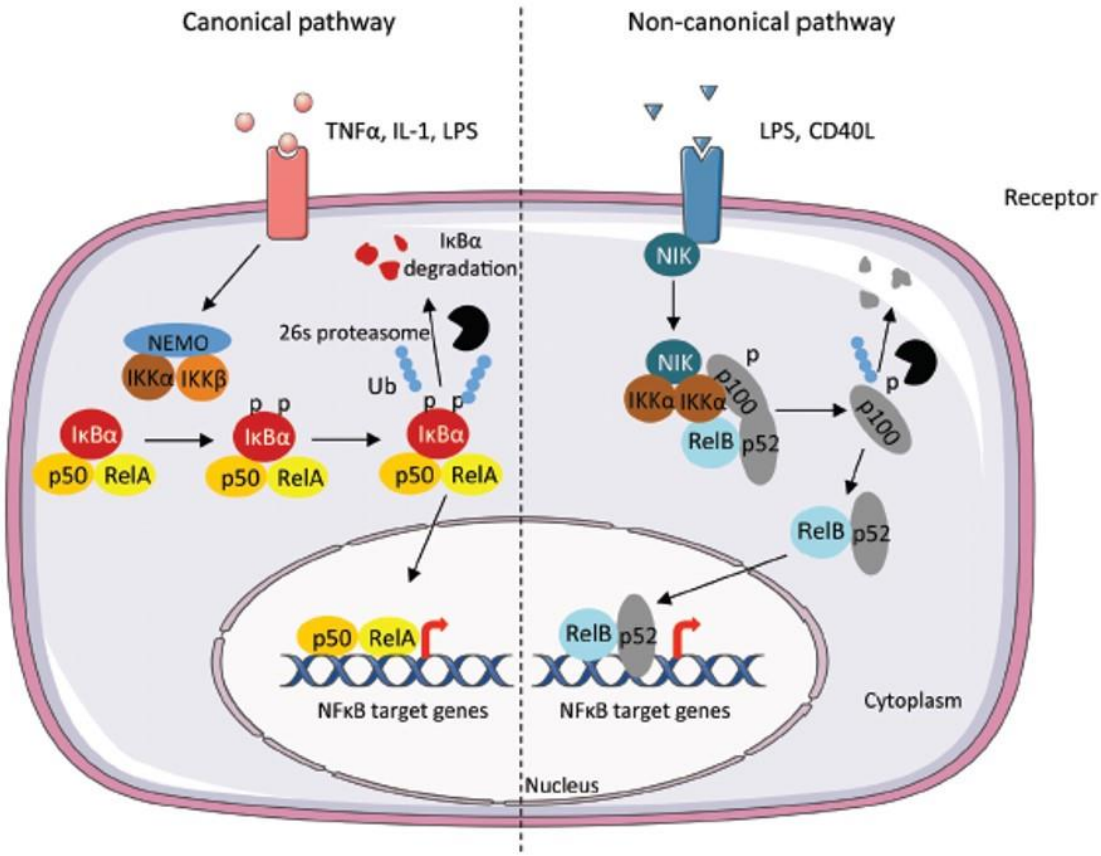


Figure 5.2 The NF-kB signalling pathway: The canonical NF-kB pathway is induced by various stimuli. The activation results in the phosphorylation and degradation of IκBα. The RelA-p50 complex translocates to the nucleus to activate the transcription of target genes. The non-canonical pathway is the activation of IKKα by the NF-κB-inducing kinase (NIK) after stimulation. Phosphorylation of the p100 subunit leads to the processing of the p100 subunit in to p52, which can lead to the activation of p52-RelB. This induces the transcription of target genes (Viennois, Chen e Merlin, 2013).

The non-canonical pathway is activated by the translocation of the p52-RelB complex to the nucleus, via the processing of p100 into p52. This is key to stabilising the NF-kB inducing kinase (NIK) via the activation of the IKKα (IkB kinase alpha) (Hayden e Ghosh, 2004). Studies have found the non-canonical NF-kB pathway functional in response to TWEAK induction, often in combination with Rel A activation. Saito T *et al* 2003 showed in mouse derived fibroblasts activated by a CD8 –TWEAK fusion protein, that the canonical and non-canonical signalling pathways were activated in a biphasic manner. Rel A signalling was seen very early on after TWEAK activation by 15 minutes, and Rel B signalling was activated after 8 hours of TWEAK activation (Saitoh *et al.*, 2003). This time course dependent signalling response of NF-kB Rel A and Rel B has also been seen in TWEAK activated renal tubular cells (Sanz *et al.*, 2010). TNF-α activation in these cells only showed Rel A activation, suggesting that the TWEAK response activating Rel A and Rel B was a specific event of TWEAK induced functional responses. This biphasic activation of NF-kB pathways may be a clue in understanding the role of TWEAK during chronic inflammatory diseases, where for the sustained up-regulation of TWEAK, Rel B may be critical to inducing the prolonged inflammatory response.

There have been reports of variable activation of soluble and membrane TWEAK signalling pathways in the same cell type and conditions as has previously been observed for TNF-α. Both soluble and membrane TWEAK have been found to activate the Rel A signalling cascade, and membrane bound TWEAK has been shown to have a heightened activation of Fn14 mediated Rel A and TRAF 2 degradation, in comparison to soluble TWEAK. In contrast, soluble and membrane bound TWEAK equally activated the non-classical Rel B pathway

(Roos *et al.*, 2010). They suggested that this variable regulation may be important to understanding the wide variety of responses of TWEAK activation during the immune response.

5.1.4 TWEAK-Fn14 signalling via MAPK

MAPK's have been shown to regulate gene expression and cell fate functions, such as proliferation, differentiation, apoptosis and stress responses. The MAPK's can be activated by a range of stimuli such as inflammatory cytokines, mitogens, and osmotic stress (Pearson *et al.*, 2001). The MAPK family members are highly conserved serine-threonine protein kinases which can be regulated by phosphorylation events (Theodosiou e Ashworth, 2002). The classical MAPK include the Erk 1/2 pathway which can activate transcription factors via the Ras-Raf-MEK-Erk cascade. This pathway is predominantly activated by growth and differentiation factors. The MAPKs also consist of the Erk 5, p38 MAPK and the Jnk signalling pathways which are predominantly activated by stress and growth factor signals (Figure 5.3) (Roberts e Der, 2007).

MAPK signalling pathways have been found activated during TWEAK induced responses in a number of studies. These include the p38 regulation of lupus nephritis via PBMC (Zhi-Chun *et al.*, 2012), ERK 1/2 activation in mouse osteoblastic functions and renal inflammation (Ando *et al.*, 2006; Rayego-Mateos *et al.*, 2013), Erk 1/2 activation during TWEAK activation of C2C12 myoblasts (Dogra *et al.*, 2007), and Jnk pathways in endothelial cell activation (Donohue *et al.*, 2003). It has been shown that all three pathways can also be simultaneously

activated upon TWEAK activation in myoblasts and fibroblasts regulating various functions (Kumar *et al.*, 2009).

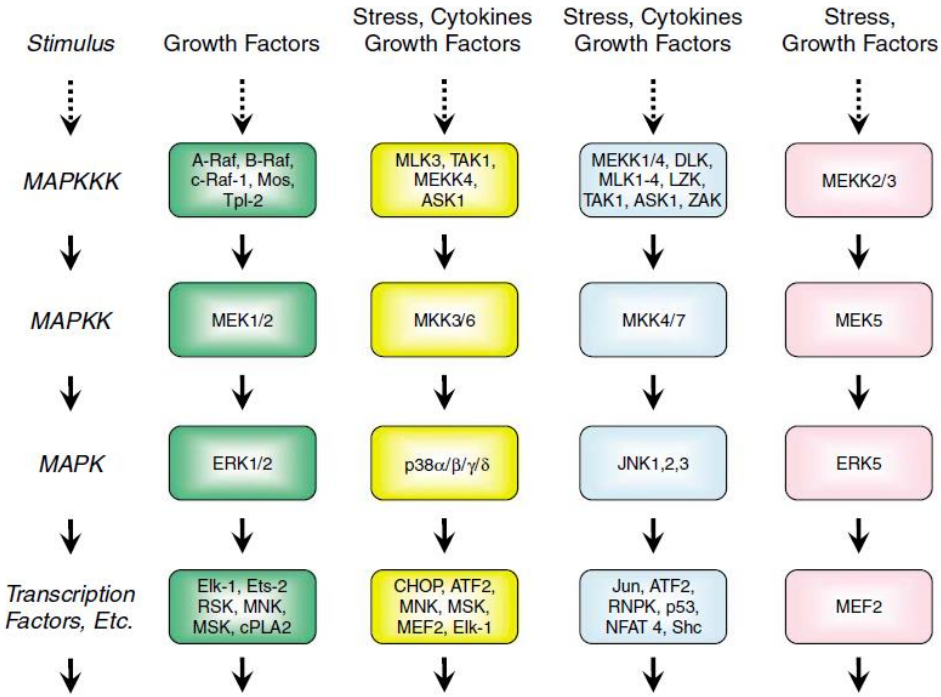


Figure 5.3 The MAPK signalling pathway: The MAPK signalling pathways are activated in response to cytokines, growth factors and stress. There are four major MAPK signalling events; Erk, p38, Jnk, and Erk 5. The MAPKs activate a signalling cascade mediated by phosphorylation of protein kinases leading to the activation of transcription factors (Pearson *et al.*, 2001)

TWEAK activated complex functional responses have been found to induce activation of multiple signalling pathways. Donohue *et al* showed activation of Rel A, Erk and Jnk during TWEAK stimulation of HUVEC during angiogenic functions (Donohue *et al.*, 2003). Similarly a study on mouse osteoblastic cells activated with TWEAK, showed differential signalling dependent functional activity, including AKT which was required for RANTES production and

Fn14 expression, and Erk which was required for bone morphogenic protein 2 induced differentiation of osteoblastic cells (Ando *et al.*, 2006). TWEAK activated myotubes from skeletal muscle tissue showed activation of both classical and non-classical NF-kB and p38, Erk and Jnk MAPK's together functioning to regulate pathological skeletal muscle wasting via MMP9 (Li *et al.*, 2009).

5.1.5 TWEAK-Fn14 other signalling pathways

There have been reports of other TWEAK activated signalling pathways determining functional outcomes. TWEAK and IFN-γ mediated apoptosis was found to be JAK/STAT (janus kinase/ signal transducers and activators of transcription) signalling dependent in tumour cells (Chapman *et al.*, 2013). NF-kB signalling mediated by TWEAK in human neuroblastoma cells were found to be dependent on GSK3β (glycogen synthase kinase 3 β) signalling (De Ketelaere *et al.*, 2004). TGF activated kinase 1 in cultured myoblasts and fibroblasts have been found to regulate NF-kB and p38 MAPK and Jnk signalling pathways. They also showed that TWEAK inhibited the AKT and Erk signalling pathways (Kumar *et al.*, 2009). TWEAK and Fn14 mediated glioma cell migration and invasion has been found to be dependent on the Rho GTPase (guanosine triphosphatase) family member signalling pathways (Fortin *et al.*, 2012). These studies show that TWEAK and Fn14 interactions are highly regulated. The ability of TWEAK to induce a specific signalling cascade during a functional response, is key to the varying functional capabilities observed for TWEAK and Fn14 between cells and cellular environments.

5.1.6 TWEAK-Fn14 signalling with TNFSF members

TWEAK regulated signalling of other TNFSF members has also been documented in several studies. As Fn14 does not have a death domain, pro-apoptotic functions via TWEAK activation have been difficult to explain. There have been suggestions that TWEAK may be indirectly inducing apoptosis via TNF-α, which was found to be stimulated by NF-kB activation (Schneider *et al.*, 1999). It was later found that TWEAK and Fn14 interactions induce degradation of TRAF2-CIAP1 (cellular inhibitor of apoptosis 1) complexes which lead to TNF-α induced apoptosis in immortalised cells (Vince *et al.*, 2008). This was subsequently found to be due to a RIP-1 (receptor interacting protein 1) – FADD (Fas associated death domain) – caspase 8 complex formation in response to TWEAK activation (Ikner e Ashkenazi, 2011). TWEAK has also been found to interfere with CD40-CD40L induced signalling via NF-kB and MAPK. It was found that TWEAK activated cells prevented CD40-CD40L interactions, leading to impaired signalling via TRAF-2, thus indicating that TWEAK has the ability to regulate CD40 related functions (Salzmann, Lang*, et al.*, 2013).

5.1.7 TWEAK-Fn14 VEGFR2 mediated signalling

TWEAK has been described as a potent mediator of angiogenesis *in vitro* and *in vivo* in several studies (Lynch *et al.*, 1999; Ho *et al.*, 2004). There have been subsequent studies investigating the angiogenic potential of TWEAK in endothelial cells, specifically to determine if TWEAK mediated angiogenic functions are VEGF dependent. Some studies have reported VEGF independent angiogenic functions of TWEAK (Lynch *et al.*, 1999), and some studies have reported VEGF mediated angiogenic functions. TWEAK was found to up-regulate VEGF

expression in human ovarian cancer cells to promote tumour metastasis in an NF-kB signalling dependent manner (Dai *et al.*, 2009). TWEAK was also reported to inhibit VEGF mediated morphogenic responses in endothelial cells, and alternatively in the same cells was found to promote FGF induced angiogenic responses (Jakubowski *et al.*, 2002).

We have reported that TWEAK induced angiogenesis via a tube formation assay in HIEC. This angiogenic potential has been found to be more potent than FGF induced tube formation (Chapter 4). It was essential to understand and determine if TWEAK activated HIEC activated VEGFR2 downstream signalling pathways to induce inflammatory mediated angiogenesis. The angiogenic potential in endothelial cells observed via VEGF is a complex and highly regulated process, and a deeper understanding of the downstream activated signals is essential to understand the potential of TWEAK activation contributing to subsequent VEGF signalling. There have been no reports of TWEAK activated responses via this signalling pathway yet. However as previously discussed, certain downstream signalling pathways of VEGR2 have been activated in TWEAK functional responses such as p38 MAPK, Erk 1/2 and AKT.

VEGFR2 is a major receptor which mediates VEGF functions. VEGFR2 mediated signalling has been implicated during endothelial cell proliferation, migration and sprouting of capillarised endothelial cells *in vitro*. *In vivo*, VEGFR2 has functions during development and neovascularisation (Karkkainen e Petrova, 2000). VEGFR2 signalling is controlled during angiogenesis by the activation of other downstream signalling pathways including the

MAPKs; p38 and Erk 1/2, AKT, Src, FAK (focal adhesion kinase), and PLCγ1 (phospholipase C γ 1) (Figure 5.3) (Carmeliet, 1999; Claesson-Welsh, 2003; Olsson *et al.*, 2006). P13K dependent AKT signalling has been found activated by various angiogenic growth factors to regulate blood vessel growth and homeostasis. This signalling pathway is widely activated in response to various stimuli in endothelial cells. It has been found to possess anti-apoptotic and pro-angiogenic activities which have been found to be regulated by VEGF signalling (Shiojima e Walsh, 2002) Erk 1/2 up-regulation has also been described to abrogate apoptosis, and enhance pro-angiogenic functions such as cell proliferation in endothelial cells. It has been described to do this by enhanced VEGF expression via an activator protein 2 complex binding to the promoter region of VEGF (Berra, Pagès e Pouysségur, 2000). Subsequently other studies have also described VEGF induced activation of Erk 1/2 and AKT signalling cascades during *in vivo* angiogenic responses (Zhu, MacIntyre e Nicosia, 2002). P38 MAPK has been found to mediate angiogenesis via VEGF. *In vitro* regulation of VEGF and FGF by p38 in tumour cells was found, and in an *in vivo* neovascularisation mouse model, p38 inhibition reduced VEGF induced angiogenic functions (Tate *et al.*, 2013).

FAK is a focal adhesion kinase which is known to mediate signalling via growth factors and integrin activation, and the regulation of angiogenic processes, for example; regulating endothelial cell migration via matrix metalloproteinase induction. It has also been found to regulate cell growth, survival, migration, and differentiation of endothelial cells (Rizzo, 2004). *In vivo* FAK knock out mouse models have shown decreased tumour growth and angiogenesis. *In vitro* deletion of FAK in endothelial cells have shown decreased VEGF

induced AKT phosphorylation leading to enhanced apoptosis and suppressed proliferation (Tavora *et al.*, 2010). PLCγ1 signalling has been implicated in regulating angiogenesis. During phosphorylation of VEGFR2, the p85 subunit of PLCγ1 binds protein tyrosine phosphatase Tyr1175. It has been shown in HIEC that PLC γ1 has been found to be essential for activating VEGF mediated signalling (Takahashi *et al.*, 2001). In an *in vivo* arterial defect zebra fish model, PLC was found to function downstream to VEGF receptors during embryonic development and arterial formation (Lawson *et al.*, 2003). P13K-PLCγ1 has also been found to regulate FGF2 dependent tubule formation in endothelial cells. P13K-PLCγ1 were also found to mediated FGF activated AKT signalling pathways (Maffucci *et al.*, 2009). There have been no studies yet which have found TWEAK directed regulation of signalling via FAK and PLCγ1 during the mediation of endothelial cell responses.

The Src family of kinases (SFK) has been found to respond to a range of stimuli in the ECM including growth factors and adhesion proteins. The SFK have been shown to activate downstream signalling pathways such as the MAPKs (Courtneidge *et al.*, 1993). Src has been shown to be required for VEGF mediated angiogenesis for embryonic development in *in vivo* mouse and chick models where Src was found to provide protection against apoptosis (Eliceiri *et al.*, 1999). TWEAK and Fn14 activated cell migration and invasion via TRAF2 signalling in gliomas, has been described to be a response mediated by the Src homology 3 domain containing guanine nucleotide exchange factor (Fortin Ensign *et al.*, 2013).

TWEAK and Fn14 interactions lead to diverse functions which is a result of complex signalling pathways governing these responses. To understand TWEAK and Fn14 contributions to chronic liver inflammation and liver regeneration, it is highly important to understand signalling pathways contributing to these functions via HIEC and TWEAK interactions. This will allow us to better understand how we can manipulate this system for future therapeutics towards inflammatory liver disease.

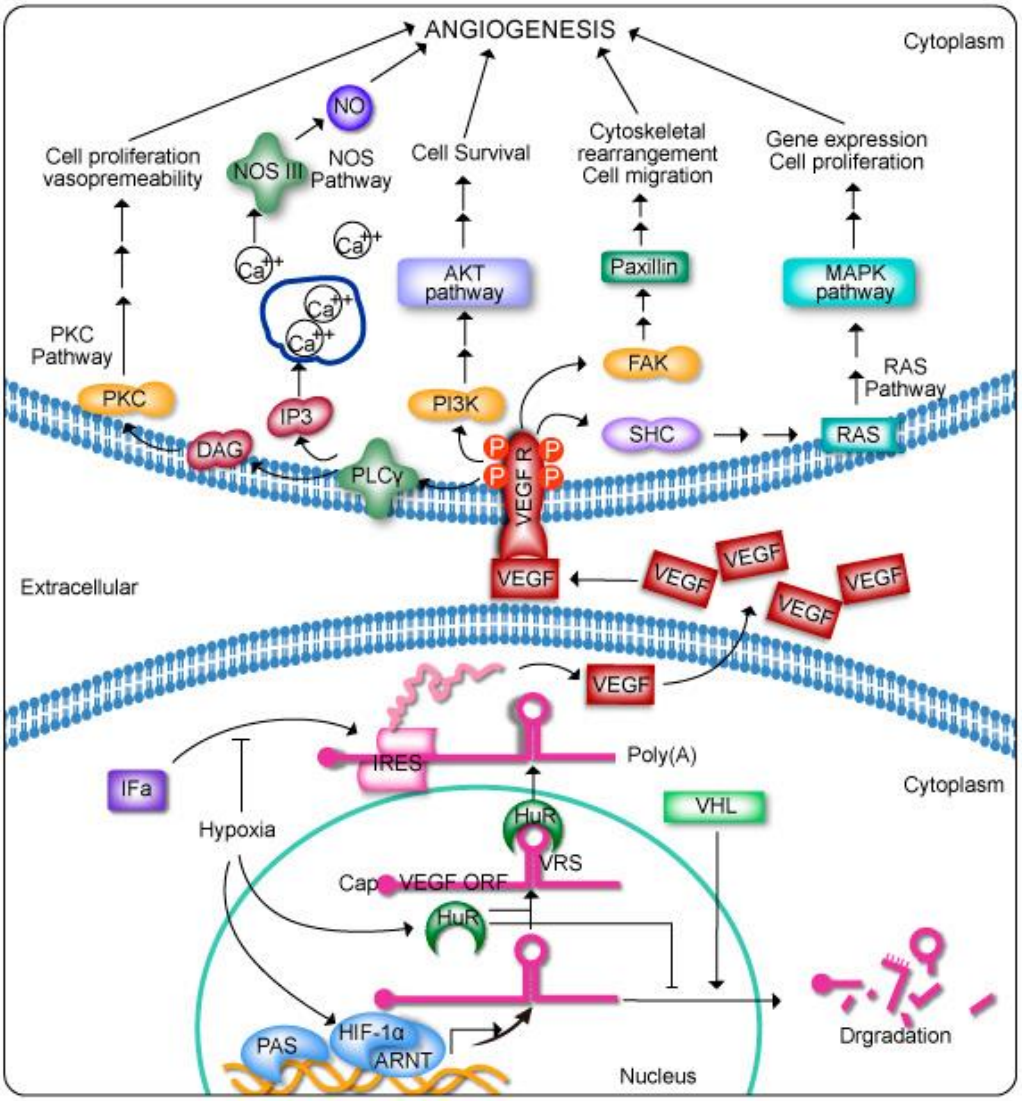


Figure 5.3 The VEGFR2 signalling pathway: VEGFR2 signalling is a common activated transcription pathway for the regulation of angiogenesis. It is required to mediate VEGF functions in endothelial cells including survival, proliferation and migration. This pathway is regulated by downstream signalling events mediated by the recruitment of various adaptor proteins (Olsson *et al.*, 2006)

5.1.8 Aims

The aims of this chapter were:

- To characterise activation of NF-kB Rel A and Rel B signalling pathways in TWEAK activated HIEC.
- To determine if TWEAK activated HIEC function via downstream signalling pathways of VEGFR2 to further understand HIEC angiogenic regulation.
- To determine if TWEAK activated HIEC signalling pathways also mediate HIEC Fn14 expression on HIEC.
- To determine if TWEAK and Fn14 regulation of HIEC angiogenesis and cell migration was regulated by specific cell signalling pathways.

5.2 MATERIALS AND METHODS

Materials and methods for this chapter; please refer to sections: 2.1-2.4, 2.7, 2.10, 2.11, and 2.16, and Tables 2.1-2.4, 2.7 - 2.10.

5.3 RESULTS

5.3.1 TWEAK activated HIEC phosphorylate Erk 1/2

To establish the signalling pathways which may be regulating TWEAK activated HIEC functions, in particular during potential TWEAK activated angiogenesis, we first characterised signalling pathways activated down-stream to VEGFR2, which are common pathways activated during angiogenesis. These include: AKT, FAK, PLC-γ1, p38 MAPK, Erk 1/2 and Src.

Protein lysates were obtained from TWEAK activated HIEC at various time points, and Western blotting analysis was performed. Unstimulated HIEC were used as a negative control, and TNF-α was used as a positive control to compare TWEAK activation. TWEAK activated HIEC did not activate by phosphorylation AKT, FAK, PLC-γ1, p38 MAPK, and Src signalling pathways in comparison to the unstimulated control (Figure 5.3.1). No phosphorylation was observed in TNF-α activated HIEC either. All data was compared to endogenous total β-actin, to ensure correct protein loading.

Initial data obtained, showed that TWEAK activated HIEC, phosphorylated Erk 1/2 at 4 hours after stimulation with TWEAK; showing a 3.5 fold increase of phosphorylation in comparison to the unstimulated HIEC (Figure 5.3.2 A). TWEAK activated HIEC at 2 hours and 24 hours showed no activation of Erk 1/2 in comparison to the unstimulated control. TNF-α on the other hand showed Erk 1/2 activation in HIEC after 2 hours of stimulation, with a 3 fold

increase in phosphorylation in comparison to the unstimulated control. After 24 hour TNF-α activation, HIEC showed an 18 fold increase in Erk phosphorylation.

The general phosphorylation patterns observed across all blots was analysed, and we found that TWEAK activated HIEC at 4 hours showed consistent enhanced phosphorylation of Erk 1/2. However TNF-α activation did vary between liver samples. In accumulated densitometry quantification, TNF-α activated HIEC revealed a large up-regulation of phosphorylated Erk 1/2 at 24 hours post stimulation (Figure 5.3.2 A), which was found to be from one outlier data set (Blot not shown). This large up-regulation was not found in any other Western blot. Therefore in the densitometry quantification graph, TNF-α activated HIEC at 24 hours showed a large up-regulation of phosphorylated Erk 1/2 in comparison to all other conditions. All data was quantified and normalised from the mean densitometry of 3 experiments.

Further Erk 1/2 data was obtained in order to determine if the Erk 1/2 activation was true and a response of TWEAK activation, by the use of a pharmacological inhibitor PD98059 to inhibit Erk activation. Phosphorylated Erk levels were compared to endogenous total Erk levels. Accumulated data confirmed previous findings that TWEAK stimulated HIEC, significantly activated the Erk 1/2 pathway at 4 hours post stimulation, in comparison to the unstimulated control (Figure 5.3.2 B). Accumulated data showed a significant 7.91 fold increase (*p=0.03, n=7). TNF-α was used as a positive control in parallel to TWEAK which showed a 1.9 fold increase in Erk phosphorylation at 4 hours post stimulation n=7.

Phosphorylation of Erk 1/2 decreased two fold when the Erk 1/2 signalling pathway was blocked in TWEAK activated HIEC ± SEM (n=4). TNF-α activated HIEC which showed a 1.9 fold increase in Erk phosphorylation in comparison to the unstimulated control, decreased to 0.5 fold when Erk was inhibited in TNF-α activated HIEC. Unstimulated HIEC remained unchanged with Erk inhibition. Phosphorylated Erk 1/2 was compared to endogenous total Erk 1/2 levels in HIEC. Under the same conditions as for phosphorylated Erk protein lysates, endogenous total Erk levels remained consistent and unchanged across all conditions (n=3) (Figure 5.3.2 B). All data was normalised to its relative total β-actin control and then normalised to the unstimulated HIEC control.

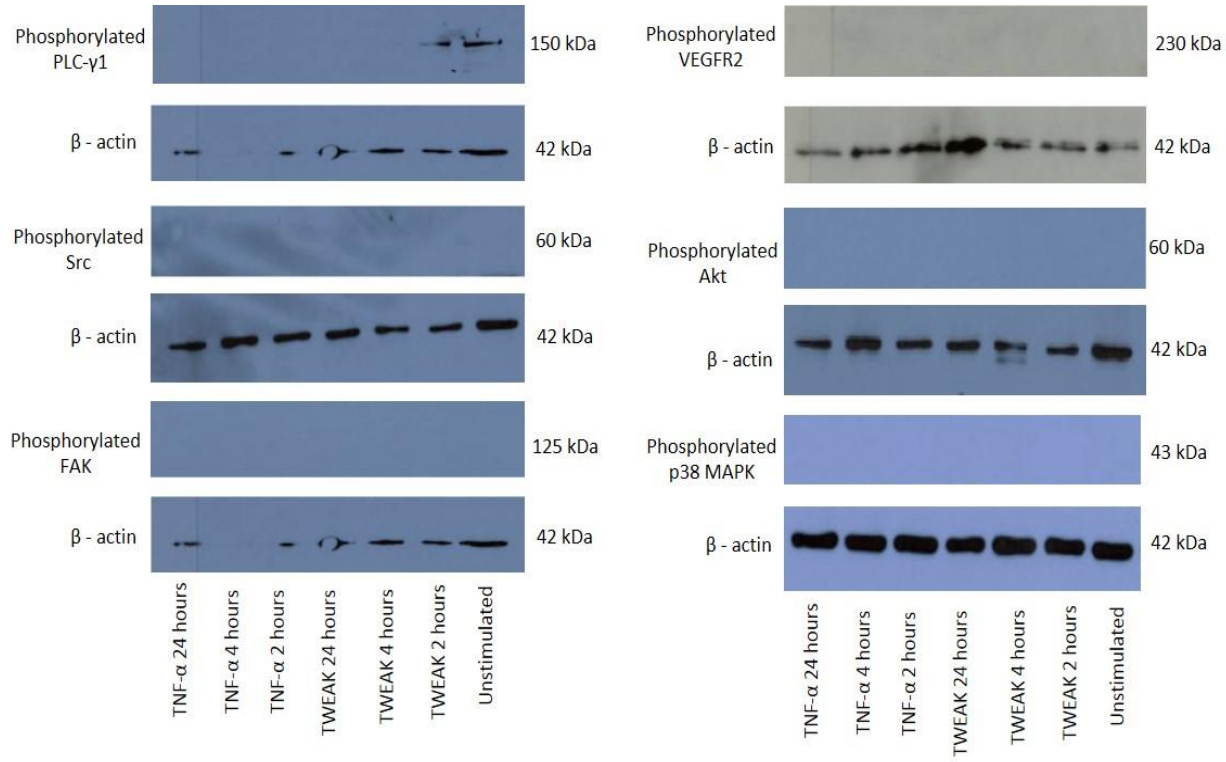


Figure 5.3.1 TWEAK activated HIEC angiogenic transcription factor activation: HIEC were treated with TWEAK and TNF-α for 2, 4, and 24 hours prior to protein lysate extraction and Western blotting analysis. TNF-α was used as a positive control to TWEAK, and unstimulated HIEC were used as a negative control. Western blotting analysis was used to determine if TWEAK activated HIEC, phosphorylate PLC-γ1, VEGFr, Src, AKT, FAK and p38 MAPK. Densitometric analysis detected no TWEAK activated phosphorylation in these transcription factors. All data was normalised to its relative β-actin control and then normalised to the unstimulated control.

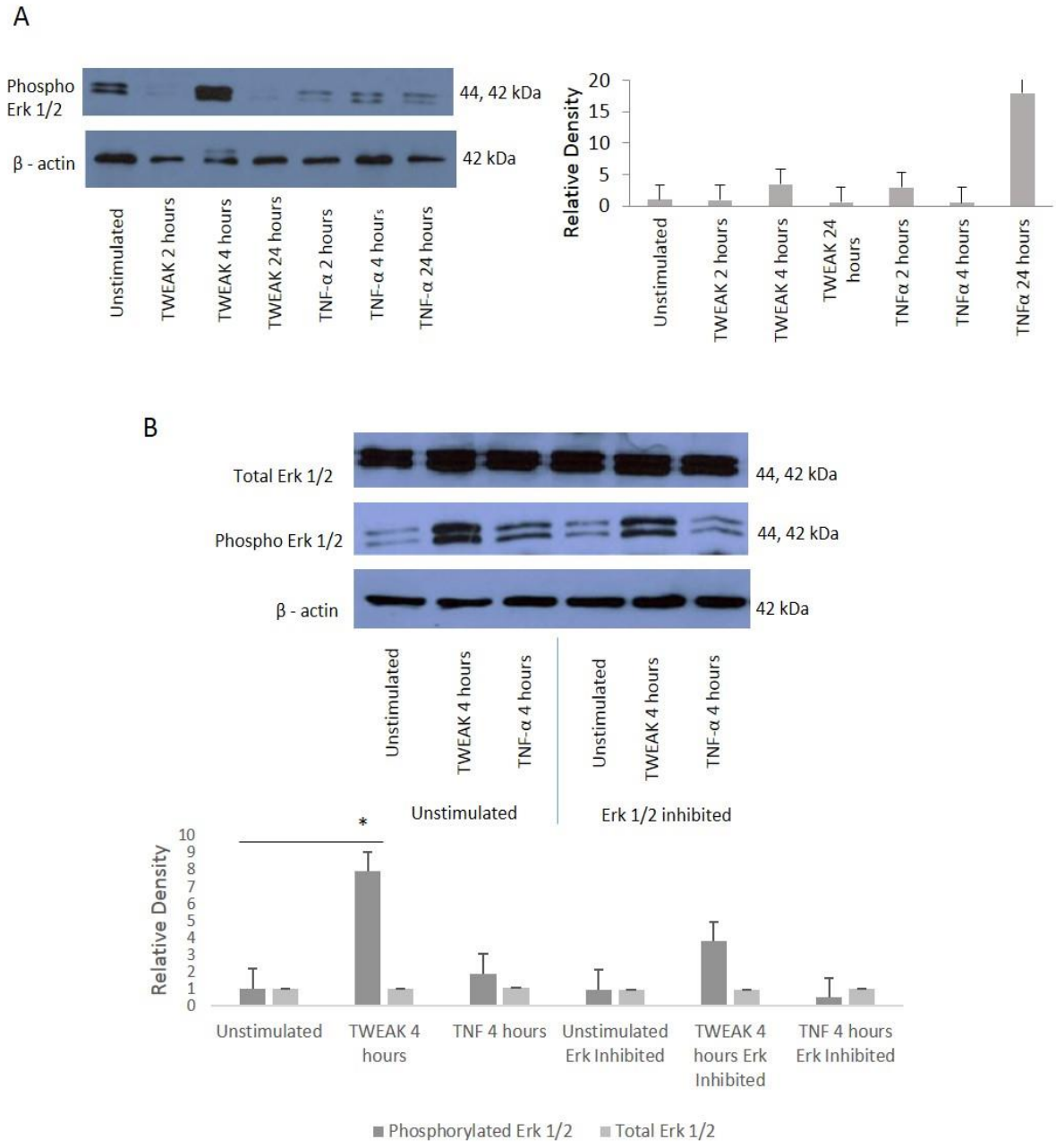


Figure 5.3.2 TWEAK activated HIEC signal via the Erk 1/2 pathway: HIEC were treated with TWEAK and TNF-α for 2, 4 and 24 hours prior to protein lysate extraction and Western blotting analysis. Replicate HIEC were inhibited with PD98059 (an Erk 1/2 inhibitor) prior to stimulation with TWEAK and TNF-α. A) Representative image of Western blot for phosphorylated Erk 1/2 and β-actin control. Graph shows densitometric analysis of replicate Western blots from HIEC protein isolates. Data represents n=3. B) TWEAK activated HIEC significantly phosphorylated Erk 1/2 at 4 hours post stimulation (*p=0.03). Unstimulated, TWEAK and TNF-α stimulated HIEC at 4 hours represents n=7. Total Erk data was n=3. All data was normalised to its relative β-actin control and then normalised to the unstimulated control (represented as the relative density of 1 ± SE). Statistical analysis was carried out using a paired Student's t test.

5.3.2 TWEAK activated HIEC enhance phosphorylation of the NF-kB Rel A pathway

TWEAK activated cells have been shown to activate NF-kB Rel A signalling in a number of studies, in particular TWEAK inflammatory and angiogenic responses. Therefore, we wanted to determine if this signalling pathway is active during TWEAK activated HIEC functional responses. Initially we used TWEAK activated HIEC at time points previously used at 2, 4, and 24 hours. However no response of Rel A activation was detected (data not shown). Previous studies have shown that Rel A activation is a very early response, therefore earlier TWEAK activation time points were used at 15, 30, and 60 minutes. We determined that TWEAK activated this pathway in HIEC comparison to the unstimulated HIEC control; at 30 minutes, showing a 1.7 fold increase in Rel A phosphorylation (Figure 5.3.3 A). This activation was comparable to TNF-α activated Rel A responses which showed a 1.5 fold increase. We also wanted to determine if TWEAK and TNF-α activated HIEC in combination altered Rel A activation responses. The TWEAK and TNF-α activation had a similar response to the TNF-α activation alone, but a slightly lower response of Rel A activation in comparison to the TWEAK response at 30 minutes. This data set showed a slight differential reading of endogenous total Rel A protein across different HIEC samples. It was not consistently expressed in the varying stimulations as was expected.

The raw data from Rel A activation experiments showed higher activation of Rel A at 30 minutes post TWEAK activation, and on occasion at 60 minutes post TWEAK activation (data not shown). Therefore, to test Rel A inhibition of TWEAK activated HIEC, a time point of 45 minutes post TWEAK and TNF-α activation was used (Figure 5.3.3 B). When the NF-kB Rel A

signalling pathway was inhibited using a pharmacological inhibitor, phosphorylation of Rel A in unstimulated HIEC showed an unexpected 2 fold increase. TWEAK activated HIEC at 45 minutes showed a 5.6 fold increase in Rel A phosphorylation in comparison to the unstimulated control. This phosphorylation decreased almost to basal levels when Rel A was inhibited. HIEC activated with TNF-α for 45 minutes, showed an 8.7 fold increase in Rel A phosphorylation in comparison to the unstimulated HIEC. This showed a weaker down-regulation of phosphorylated Rel A (to 6.8 fold NF-kB phosphorylation) when the Rel A inhibitor was used. All Rel A activation data showed a trend in TWEAK activated responses during this investigation, however the data was not statistically significant after densitometric quantification. Data was tested against the total NF-kB Rel A levels for comparison. Endogenous Rel A was consistent and unchanged in response to stimulation and inhibition (Figure 5.3.3 B).

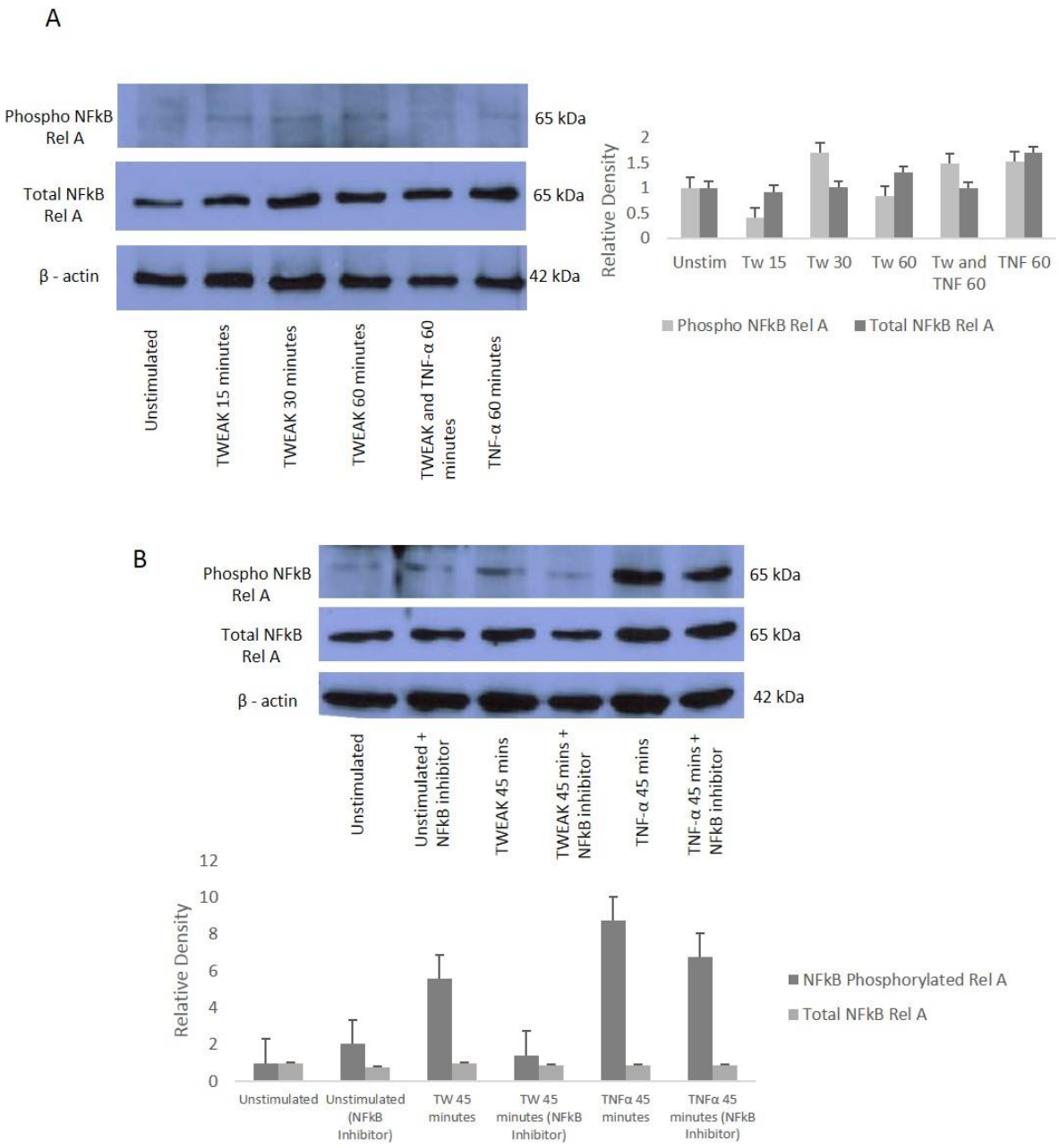


Figure 5.3.3 TWEAK activated HIEC signal via the NF-kB Rel A pathway: HIEC were treated with TWEAK and TNF-α prior to protein lysate extraction and Western blotting analysis. Replicate HIEC were treated with an NF-kB inhibitor 1 hour prior to stimulation with TWEAK and TNF-α. TNF-α was used as a positive control to TWEAK, and unstimulated HIEC were used as a negative control. A) Representative image of Western blot for phosphorylated NF-kB, total NF-kB and β-actin control, and densitometric analysis of replicate Western blots from HIEC protein isolates. B) Representative image of Western blot for phosphorylated NF-kB, total NF-kB and β-actin control with NF-kB inhibitor, and densitometric analysis of replicate Western blots from HIEC protein isolates. Data represents n=3. All data was normalised to its relative β-actin control and then normalised to the unstimulated control (represented as the relative density of 1 ± SE). This data was not statistically significant.

5.3.3 TWEAK activated HIEC do not phosphorylate NF-kB Rel B

It has been reported that TWEAK activates the non-canonical NF-kB pathway during certain functional responses. We wanted to determine if TWEAK also signals via this pathway in HIEC. Previous reports have suggested that TWEAK activation phosphorylates Rel B at later time points and Rel B phosphorylation is sustained over long time points. We had seen a very early signalling response via Rel A and a later response via Erk 1/2, therefore we tested various time points of TWEAK activation on HIEC for signalling responses via Rel B. We analysed 15, 30, and 60 minutes and 2, 4, and 24 hour activation, we also analysed TNF-α activation of HIEC after 60 minutes. We found no activation of Rel B across these time points of TWEAK activated HIEC (n=3) (Figure 5.3.4). In the blots analysed we only detected a single band in one data set which was found in an unstimulated HIEC control. The data was compared to endogenous total Rel B and β-actin. All Western blots were tested against endogenous β-actin protein to ensure even loading and accurate analysis.

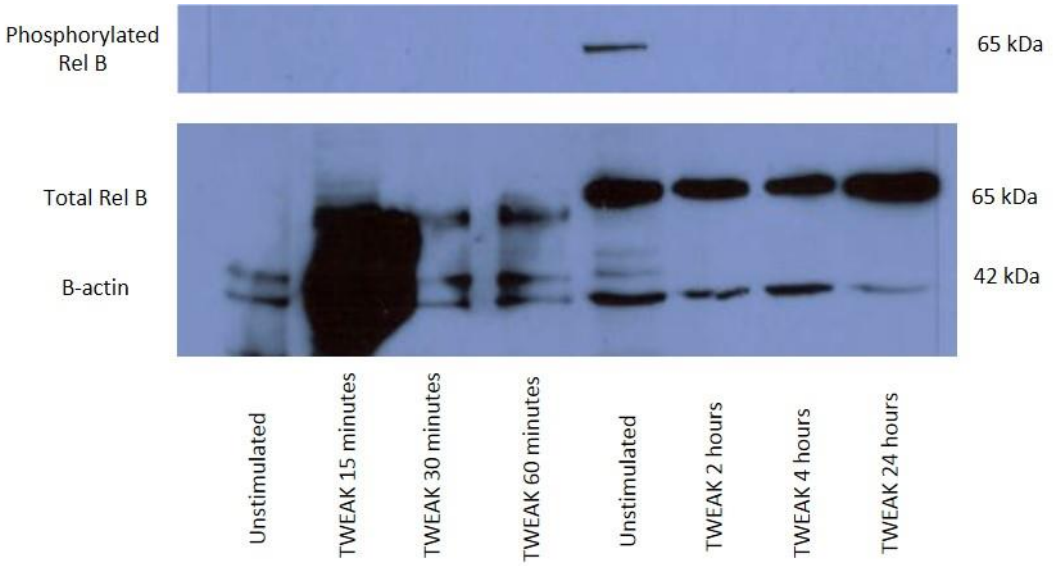


Figure 5.3.4 TWEAK activated HIEC do not signal via the NF-kB Rel B pathway: HIEC were treated with TWEAK (100ng/ml) for the indicated time points, prior to protein lysate extraction and Western blotting analysis. Unstimulated HIEC were used as a negative control. A representative image of Western blot for phosphorylated NF-kB Rel B, total Rel B and β-actin control is shown. Data was normalised to its relative β-actin control and then normalised to the unstimulated control n=4.

5.3.4 Erk and Rel A signalling may determine Fn14 expression patterns on HIEC

Fn14 protein expression has been found on cell curface HIEC and subsequently found to be regulated by various inflammatory and growth factor cytokines. To determine if their expression patterns were mediated by NF-kB Rel A and Erk dependent signalling, these pathways were inhibited using pharmacological inhibitors, prior to detecting cell surface Fn14 expression on HIEC using flow cytometry (Figure 5.3.5 A and B). Erk inhibition significantly reduced Fn14 protein expression on HIEC cell surface from 24.3 to 21.3 percentage positive HIEC ± SEM *p=0.02. NF-kB inhibition on the other hand significantly increased Fn14 protein expression on HIEC cell surface from 24.3 to 35.4 percentage positive HIEC ± SEM **p=0.006. This trend was also refelcted in the MFI data (**p=0.01).

Following this data, we further wanted to assess whether changes in Fn14 expression on HIEC cell surface in response to various stimuli, were also dependent on NF-kB and Erk signalling. HIEC were stimulated with IL-1β, TNF-α, and FGF, and Erk and NF-kB inhibitors prior to Fn14 cell surface expression analysis (Figure 5.3.5 C, D and E). IL-1β, TNF-α and FGF activated HIEC previously had shown a significant increase in Fn14 cell surface expression in comparison to unstimulated controls. This data set confirmed up-regulated Fn14 cell surface protein expression in response to these cytokines. In response to Erk inhibition basal Fn14 expression only marginally reduced, and NF-kB inhibition showed high induction of Fn14 protein expression.

We found that Erk and NF-kB inhibition of IL-1β activated HIEC showed down-regulated Fn14 cell surface expression on HIEC from 42.9% in uninhibited HIEC, to 24.84% in Erk inhibited HIEC, and 29.19% in NF-kB inhibited HIEC ± SEM (Figure 5.3.5 C). Erk inhibition down-regulated Fn14 expression more than NF-kB inhibition. TNF-α activated HIEC also showed down-regulated Fn14 cell surface protein expression from 43.7% in uninhibited HIEC, and 24.1% in response to Erk, and 20.08% in response to NF-kB inhibition ± SEM (Figure 5.3.5 D). In this case both inhibitors showed an equal down-regulation of cell surface Fn14 expression. In response to FGF, HIEC activation up-regulated Fn14 cell surface expression from 20.7% to 41.1% HIEC ± SEM (Figure 5.3.5 E). In response to Erk inhibition the FGF response was blocked, and Fn14 cell surface expression reduced to basal levels showing 18.95%, and NF-kB inhibition reduced to 31.4% Fn14 cell surface expression ± SEM. This data however was not staitically significant.

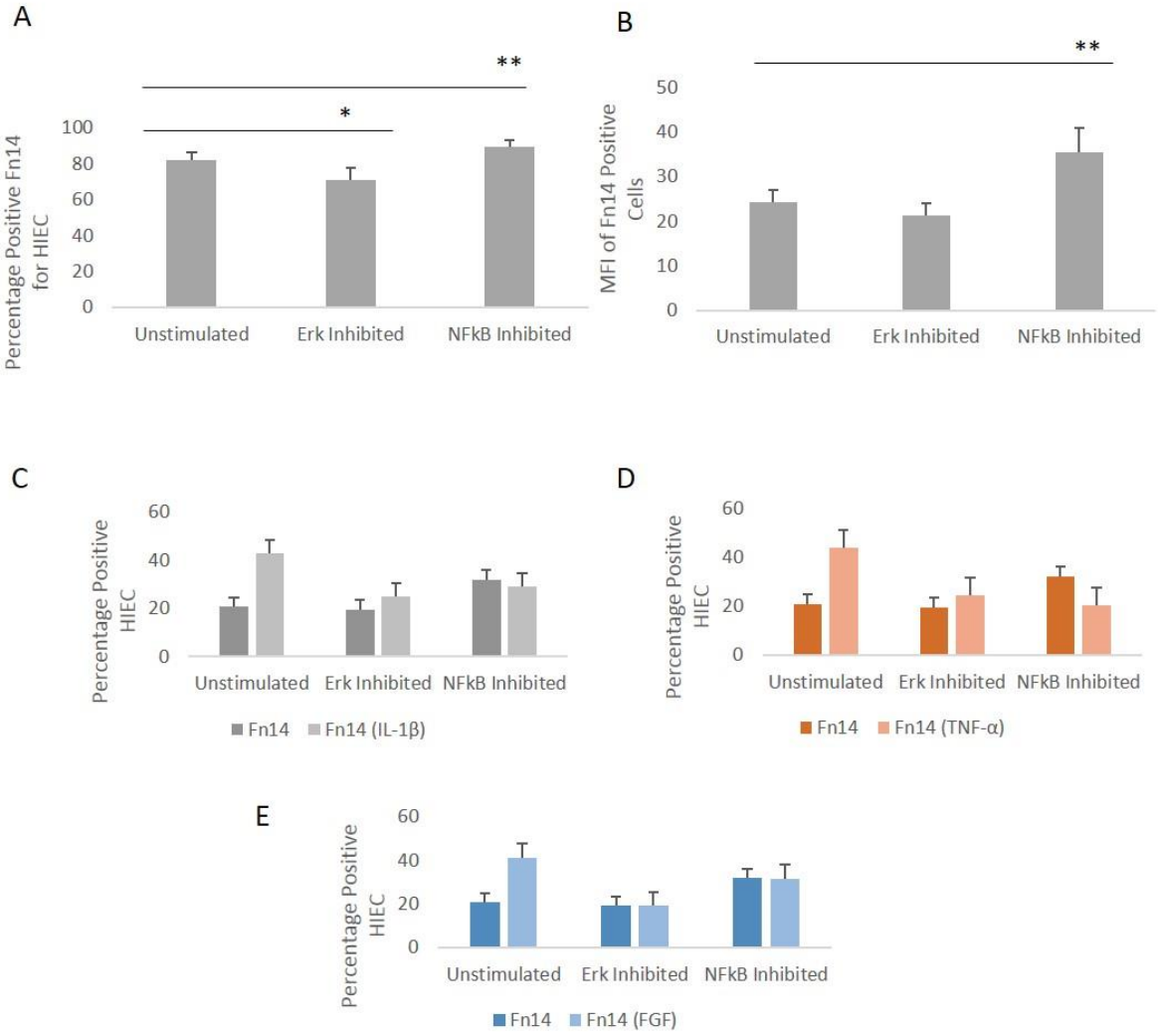


Figure 5.3.5 Fn14 cell surface expression on HIEC may be dependent on Erk and NF-kB signalling: HIEC were cultured in the presence of Erk and NF-kB inhibitors, IL-1β, TNF-α, and FGF for 24 hours prior to analysis. HIEC were analysed for Fn14 expression. A and B) Erk inhibition significantly reduced Fn14 positive HIEC *p=0.02 and significantly increased Fn14 positive HIEC with NF-kB signalling inhibitor **p=0.006. MFI data showed a similar pattern however NF-kB signalling significantly increased Fn14 expression **p=0.01. C, D and E) IL-1β, TNF-α, and FGF activated Fn14 expression was inhibited by Erk and NF-kB inhibitors, this data was not statistically significant. All data is represented as ± SEM n=3. Statistical analysis was carried out using a paired Student's t test.

5.3.5 TWEAK mediated angiogenic potential was regulated via Erk and NF-kB Rel A signalling transduction

In order to further understand the signalling mechanisms mediating TWEAK activated HIEC angiogenesis, we performed the matrigel tube formation assay and scratch wound migration assays with pharmacological inhibitors to NF-kB Rel A and Erk signalling in addition to TWEAK activation.

HIEC cell motility in response to TWEAK activation was tested to see if this was regulated by Erk and NF-kB Rel A dependent mechanisms (Figure 5.3.6). In comparison to TWEAK stimulated HIEC, TWEAK activated HIEC with Erk pathway inhibited showed a slower progress of wound healing at 4 hours (TWEAK alone – 72.6% Erk inhibited -79%) and 8 hours (TWEAK alone- 47% Erk inhibited – 68.2%). By 24 hours both wounds had healed fully. NF-kB inhibition had no marked effect on the speed of wound healing, it appeared to close the wound slightly faster than the TWEAK activated HIEC at 4 hours (TWEAK alone – 72.6% NF-kB inhibited- 68.7%) and 8 hours (TWEAK alone – 72.6% NF-kB inhibited – 43.4%). Erk inhibition alone showed a similar progression of wound closure to the unstimulated control, however failed to completely close the wound, remaining open after 24 hours. NF-kB inhibition alone had no effect on the normal progression of wound healing in comparison to the unstimulated control. All differences observed in these wound healing assays were not statistically significant, but showed a trend across all assays.

Erk and NF-kB Rel A inhibition significantly reduced tube formation in HIEC activated with TWEAK (**p=0.01 and **p=0.007 respectively) (Figure 5.3.7). Erk inhibition reduced TWEAK activated node formation in HIEC from 50.2 to 12.7 nodes ± SEM. NF-kB inhibition reduced TWEAK activated node formation to 34.8 nodes ± SEM. Erk inhibition alone almost completely abrogated node formation (7.2 nodes) in comparison to the unstimulated control **p=0.01, whereas NF-kB inhibition alone reduced node formation in comparison to the unstimulated control (33.8 to 24.2 nodes).

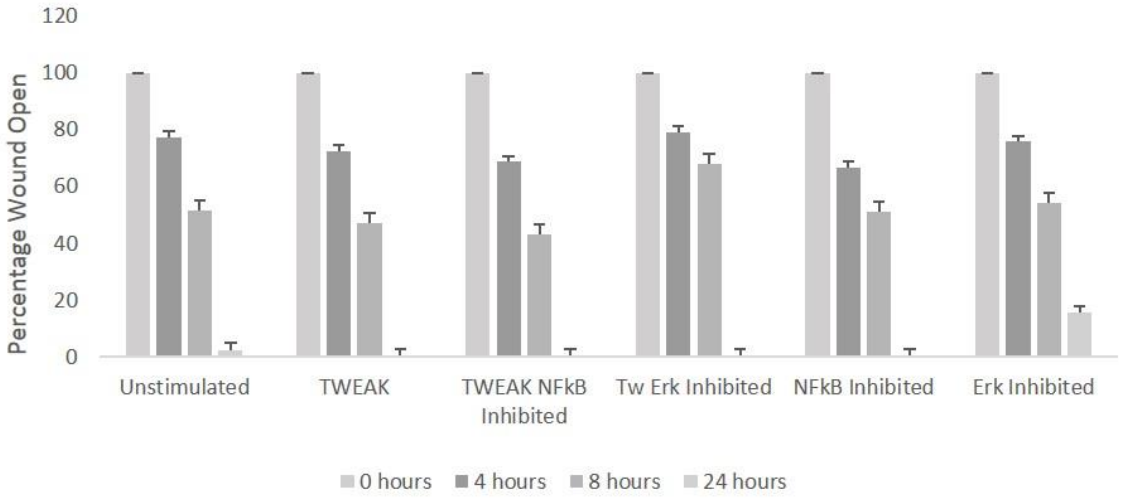


Figure 5.3.6 TWEAK does not significantly promote HIEC cell motility in an NF-kB or Erk mediated pathway: HIEC were cultured on gelatine coated plates. Scratch wound analysis was performed in the presence of TWEAK and inhibitors to Erk and NF-kB, to determine if these signalling pathways regulated HIEC cell motility. Unstimulated HIEC were used as a negative control. Images for analysis were taken after initialising the scratch at 0, 4, 8, and 24 hours. Data is shown as mean percentage ± SE (n=3). Erk inhibition delayed wound healing which was enhanced in response to TWEAK activation. This data was not statistically significant.

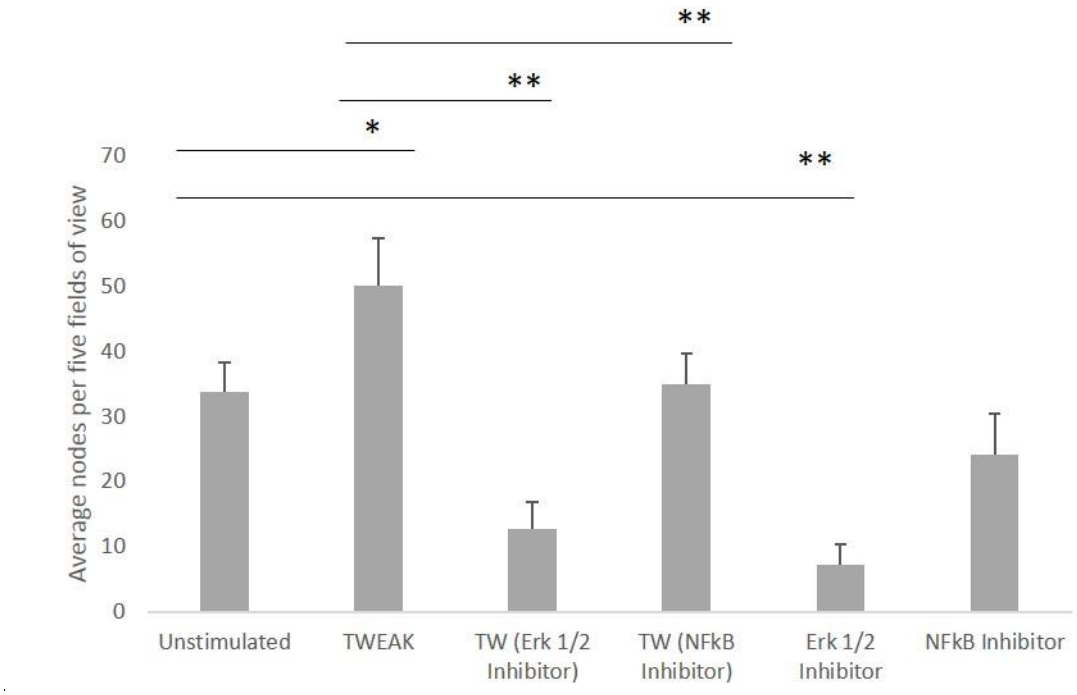


Figure 5.3.7 TWEAK activated tube formation in HIEC may be mediated by signalling via the Erk 1/2 and NF-kB pathways: HIEC were cultured on natural matrigel and stimulated with TWEAK, Erk 1/2 inhibitor and NF-kB inhibitor. Unstimulated HIEC were used as a negative control. Node formation was used to calculate angiogenic potential by counting the number of nodes with each condition from five fields of view. HIEC cultured on matrigel showed a significant increase in node formation when stimulated with soluble TWEAK (100ng/ml) for 8 hours (*p=0.02). This node formation was significantly decreased when the Erk 1/2 (**p=0.01) and NF-kB (**p=0.007) signalling pathways were inhibited. Node formation was also significantly decreased in unstimulated HIEC in response to Erk inhibition (**0.008). All data is represented as ± SEM (n=6). Statistical Analysis was carried out using a paired Student's t test.

5.3.6 Summary of Results

- TWEAK stimulated HIEC induce signal transduction via Erk1/2 and NF-kB Rel A.
- NF-kB Rel A and Erk 1/2 signalling pathways may regulate Fn14 cell surface HIEC expression, and TWEAK stimulated HIEC angiogenesis.

5.4 DISCUSSION

Our study revealed that TWEAK activated HIEC may signal via Erk 1/2 and NF-kB Rel A. Rel A signalling is the most studied transcription factor for TWEAK functions. A common trend observed in studies where TWEAK has activated Rel A, is that signal transduction occurs rapidly, and NF-kB phosphorylation occurs usually within the first hour of TWEAK activation (Donohue *et al.*, 2003; Kumar *et al.*, 2009). Our findings agreed with these investigations and revealed that TWEAK activated HIEC also phosphorylated Rel A within the first hour, and subsequent phosphorylation was not detected after this time point (Figure 5.3.3). TWEAK activated HIEC showed a 5.6 fold increase in Rel A phosphorylation in comparison to the unstimulated HIEC, which was markedly reduced after Rel A inhibition. These findings however were not statistically significant (Figure 5.3.3 B).

The TNF-α activated HIEC showed a more enhanced NF-kB Rel A phosphorylation response than TWEAK activated HIEC, but showed a weaker response to Rel A inhibtion. It has been reported before, that TNF-α can induce a more potent NF-kB response in cancer cell line apoptosis, in comparison to TWEAK activated apoptosis in the same cells (Schneider *et al.*, 1999). Our data is in agreement with these findings that HIEC NF-kB signalling responses are enhanced in response to TNF-α activation than TWEAK activation, however there is potential that TWEAK and TNF-α may co-regulate inflammation via HIEC. The TNF-α response was observed in earlier time points and was sustained through later time points, indicating TNF-α may regulate sustained Rel A mediated inflammation via HIEC.

In this investigation we were determining the potential of TWEAK activation in HIEC functional responses, which are endothelial cells known to function in liver regenerative and pathogen clearance responses in the liver. NF-kB signalling is known to activate a range of immune and inflammatory functions which are observed in a wide range of cells. Subsequently, NF-kB has been described as a critical regulator of the TNFSF inflammatory functions (Aggarwal, 2003). Our data suggests NF-kB signalling may regulate TWEAK activated functions, and is most likely indicative of mediating proinflammatory responses via this signalling pathway as an early response to TWEAK activation in HIEC.

We detected no VEGFR2 activation in HIEC (Figure 5.3.1). We analysed HIEC activated with TWEAK at earlier time points of 15, 30, and 60 minutes (data not shown), and later time points of 2, 4, and 24 hours, all of these time points were negative for VEGFR2 activation. Previous literature has suggested that TWEAK angiogenic responses are independent of VEGF signalling. Donohue et al 2003 showed TWEAK and VEGF activated endothelial cells, and enhanced cell proliferation in vivo, and Erk 1/2 activation was found to be the signalling pathway mediating this response. They further wanted to determine if this response was perpetuated by VEGF and TWEAK co-treatment. TWEAK and VEGF activation in endothelial cells did not show an additive effect on Erk 1/2 phosphorylation (Donohue *et al.*, 2003). Our data is in agreement with these findings that TWEAK does not activate angiogenic functions via VEGF signalling responses, but it does via Erk 1/2 and Rel A signalling pathways. We previously observed up-regulated VEGF expression from TWEAK activated HIEC (Chapter 4). Erk is a downstream activated transcription factor to VEGFR2, therefore Erk activation may

be a secondary response to VEGF activation via TWEAK in HIEC, or VEGF activation by TWEAK may be an Erk mediated response.

We detected phosphorylation of Erk 1/2 signalling during TWEAK activation of HIEC at 4 hours post stimulation (Figure 5.3.2). Other studies have shown that TWEAK activation phosphorylated Erk 1/2 at earlier activation time points; usually within the first hour (Ando *et al.*, 2006; Dogra *et al.*, 2006; Echeverry *et al.*, 2012). The Erk pathway is often activated in response to growth factors and has been shown to induce functions relative to angiogenesis (Roberts e Der, 2007). The late but significantly enhanced onset of this pathway is indicative of key processes preceeding the Erk response in TWEAK activated HIEC, which may trigger endothelial cell regulation in the liver.

We described TWEAK activated HIEC promoting angiogenic functions (Chapter 4), and when NF-kB Rel A and Erk pathways were blocked using pharmacological inhibitors during the angiogenesis tube formation assays, a significant reduction in node formation was observed (Figure 5.3.7), where a larger Erk 1/2 inhibitory response was observed in TWEAK activated HIEC in comparison to NF-kB inhibition. Erk inhibition of unstimulated HIEC showed a large reduction in node formation suggesting that Erk alone may be largely regulating angiogenic tubule formation, therefore TWEAK inhibition data has to be taken with caution from this assay. When we blocked Erk and NF-kB Rel A during scratch wound assays in the presence of TWEAK, we observed that Erk inhibition delayed cell motility which was initially promoted by TWEAK (Figure 5.3.6) but this data was not statistically significant, and NF-kB inhibition in

these assays had no effect on HIEC cell motility activated by TWEAK. This data was suggestive that the overall angiogenic potential observed with TWEAK activation in HIEC was mediated by both the Rel A and Erk signalling pathways, however Erk seemed to have a more specific response to angiogenic functions than NF-kB. This data was in agreement with previous studies observing TWEAK activated Erk endothelial cell angiogenic responses. Erk phosphorylation has previously been observed in HEK293 cell lines and HUVEC during tumour growth, which requires angiogenesis promoting processes (Ho *et al.*, 2004). More specific TWEAK responses in endothelial cells via Erk activation have also been observed in cutaneous vasculitis studies, where human dermal microvascular endothelial cells showed enhanced Erk phosphorylation in response to TWEAK activation (Chen *et al.*, 2013), and HUVEC proliferation was observed to be regulated by TWEAK via Erk phosphorylation (Donohue *et al.*, 2003). Our data indicates that TWEAK may initiate an inflammatory response in HIEC during early activation which triggers signalling pathway activation of NF-kB Rel A. This may be followed by pro-inflammatory mediated angiogenesis which may be co-ordinated by Erk 1/2 phosphorylation later during TWEAK activated HIEC responses.

The TWEAK data for Erk activation in HIEC was consistent in all samples however, the TNF-α activated HIEC showed differing results between samples. TNF-α activated HIEC initially showed Erk phosphorylation upregulated at 2 hours post activation, showing a 2.9 fold increase in comparison to unstimulated controls. The degree of phosphorylation in response to TNF-α was comparable to TWEAK activated HIEC which showed a 3.5 fold increase from unstimulated HIEC. TNF-α activated Erk reduced at 4 hours post stimulation and a large up-

regulation was observed at 24 hours, which we found was due to an outlier data set (Figure 5.3.3 A). In our second data set where we observed Erk responses to TWEAK and TNF-α in the presence of Erk inhibitors in HIEC, we found that TNF-α activated Erk phosphorylation in HIEC at 4 hours post stimulation. This response was 2 fold less than the TWEAK activated Erk phosphorylation. It is apparent that TWEAK and TNF-α may co-regulate complex signalling mechanisms in HIEC and more specifically this may be an isolate specific response, therefore there is a need to further explore the potential of these in mediating specific HIEC functional responses via Erk and NF-kB.

FAK, PLC-γ1, Src, AKT, and p38 MAPK transcription factors were not phosphorylated in response to TWEAK activated HIEC (Figure 5.3.1). This data was in consistency with previous known investigations of TWEAK mediated responses, which have not characterised activation of VEGFR2 and downstream signalling cascades of FAK, Src, and PLCγ1. AKT signalling has been seen activated in a growing number of studies in response to TWEAK activation, including myoblasts, fibroblasts, and renal tubular cells (Kumar *et al.*, 2009; Sanz *et al.*, 2009). p38 MAPK phosphorylation in response to TWEAK has also been observed in a number of studies, including studies observing PBMC interactions with TWEAK in lupus nephritis regulation, and TWEAK enhanced skeletal muscle degeneration (Li *et al.*, 2009; Zhi-Chun *et al.*, 2012). Our investigation in TWEAK activated HIEC did not agree with these findings and were consistently negative for p38 signalling activation. In some of the data intial phosphorylation of PLC-γ1 in unstimulated HIEC protein lysates was observed, this

appeared to disappear post TWEAK activation suggesting that TWEAK might be inhibiting this pathway. Further studies would be requires to confirm this.

During this study we did not find TWEAK phosphorylated Rel B in HIEC at early or later time points (Figure 5.3.6). Previous studies which have shown activation of Rel B signalling pathways during TWEAK induction, have noted that Rel B activation occurs later during TWEAK activation and remains activated for long periods of time (Sanz *et al.*, 2010), and linked to a prolonged inflammatory mediating process by Rel B. From this investigation, we can suggest that TWEAK may be activating HIEC signalling pathways specific to endothelial cell proliferation and angiogenesis in response to rapid inflammatory NF-kB responses. HIEC may not be the critical mediators of a specific prolonged inflammatory response in the liver which would potentially be mediated by Rel B following TWEAK activation, and would explain the lack of activation of this signalling pathway. It will also be beneficial to observe Rel B activation in HIEC with varying TWEAK concentrations, as it has been previously reported that low concentrations of TWEAK have shown induction of Rel B phosphorylation in comparison to high concentrations of TWEAK which activated the Rel A signalling pathway (Roos *et al.*, 2010).

We further found Fn14 cell surface expression from HIEC was regulated by Rel A and Erk. We found that Erk inhibition significantly reduced basal Fn14 expression, and NF-kB inhibition significantly up-regulated Fn14 expression on HIEC cell surface (Figure 5.3.5), indicating positive and negative regulation of TWEAK activation on HIEC via Fn14. Up-regulated TWEAK

and Fn14 expression has often been linked to detrimental pathological consequences. NF-kB is a signalling pathway which is critical to the proinflammatory and immune response, and NF-kB inhibition on HIEC showed up-regulated Fn14 expression. This may be suggestive of NF-kB regulating Fn14 and TWEAK responses during inflammatory and pathological environments, where it may function to neutralise the pathological inflammatory response orchestrated by TWEAK. Erk inhibition reduced Fn14 expression on HIEC. Erk activation is linked to proliferative responses, and it may positively up-regulate Fn14 expression on HIEC to induce angiogenic responses via TWEAK in response to chronic inflammation in the liver.

In response to Erk and NF-kB inhibition we found changes in expression pattern of Fn14 in response to TNF-α, FGF and IL-1β cytokine activation (Figure 5.3.5). We found up-regulated Fn14 expression on HIEC cell surface in response to cytokine activation, and found that Erk and NF-kB inhibition reduced Fn14 expression but showed different effects between each cytokine. TNF-α activated HIEC responded to NF-kB inhibition more than Erk inhibition, this supports the pro-inflammatory functions of TNF-α and NF-kB activation in combination. FGF activated HIEC were the most responsive to Erk inhibition, supporting the pro-angiogenic functions of FGF and Erk. They reduced Fn14 expression two fold in comparison to uninhibited HIEC. Finally IL-1β which showed a higher response to Erk inhibition may also contribute to inflammatory angiogenesis. These effects were not statistically significant therefore we can only comment on the potential this data might have. This data confirmed that TWEAK and Fn14 signalling in HIEC is a highly complex, multi-faceted process which will require a significantly deep understanding of all processes and interactions of this system

and further work to understand how these signalling pathways completely regulate TWEAK activated HIEC. We can conclude that TWEAK activated HIEC, signal via pro-angiogenic and pro-inflammatory Erk 1/2 and NF-kB Rel A signalling pathways to mediate HIEC functions in the liver.

Chapter 6

TWEAK activation regulates HIEC cell fate

6.1 INTRODUCTION

TWEAK and Fn14 have been described as a highly regulated ligand and receptor system and have been implicated in cellular responses including ROS (reactive oxygen species) production, necrosis, apoptosis and autophagy.

6.1.1 Apoptosis

Apoptotic cell suicide is a highly complex process which is designed to remove damaged or infected cells which may be detrimental to normal physiological function. Apoptosis is characterised in the cell as DNA fragmentation, degradation of the cytoskeletal and nuclear proteins, membrane blebbing, and morphological and biochemical changes. There are two major apoptosis inducing pathways; the extrinsic pathway and the intrinsic pathway. The extrinsic apoptosis pathway is regulated by TNFR activation (Figure 6.1). Some TNFR's possess the death domain in their cytoplasmic tail, which mediate a death signal from the cell surface to intracellular signalling pathways. TNF ligand and receptor association can bind functional adaptor proteins FADD, which form a death inducing signalling complex (DISC). This activates initiator caspases (caspase 8, 9 and 10) which leads to the execution phase by the subsequent activation of effector caspases (caspase 3, 6 and 7). This process induces the cleavage of death substrates leading to apoptosis. The intrinsic apoptosis pathway is activated by non-receptor mediated stimulation, leading to an intracellular signalling cascade which directly influences targets within the cell. The intrinsic pathway is regulated by the mitochondria which induces the release of cytochrome C and other apoptosis inducing proteins in response to stress. Apoptosis inducing stimuli can activate Bcl2 family

members which can regulate pro-apoptotic functions such as cytochrome C release. Once the apoptosome is formed, initiator caspases are activated leading to effector caspase activation and subsequent apoptosis. (Elmore, 2007; Portt *et al.*, 2011).

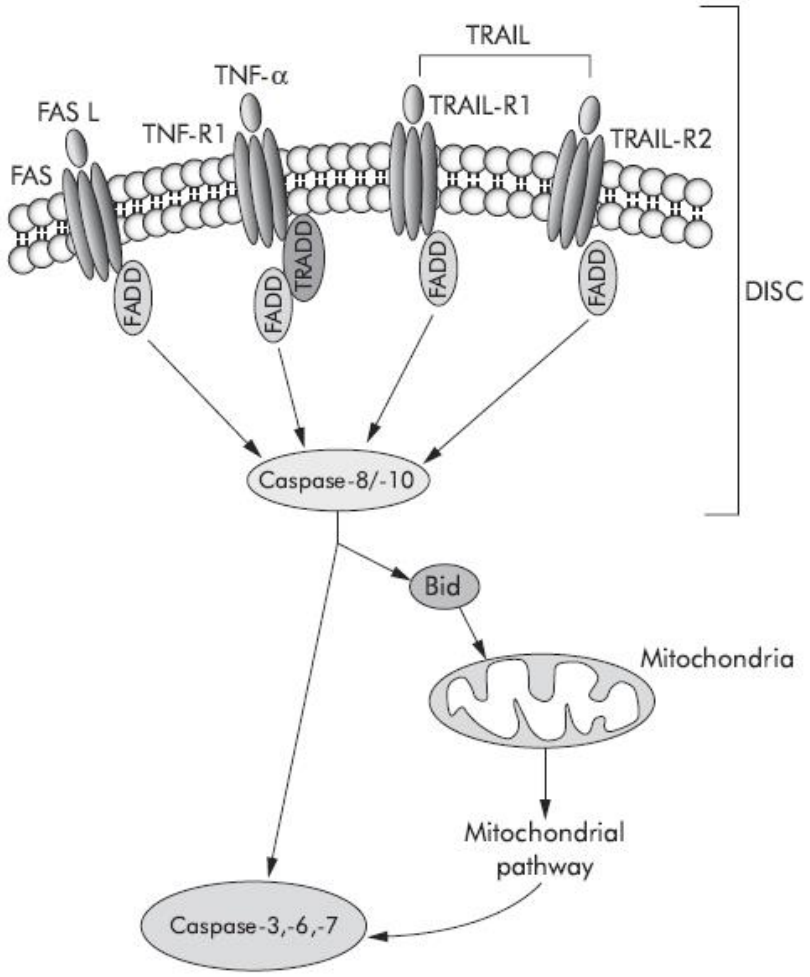


Figure 6.1 The Extrinsic Apoptosis inducing pathway: TNFSF ligands bind their receptors on the cell surface. This interaction induces activation of the death domain via TRAF associated death domain binding (TRADD) and subsequent Fas associated death domain binding (FADD). These interactions lead to the formation of the death inducing signalling complex which activates initiator caspases, including caspase 8 and 10. These caspases can directly lead to the execution phase by activation of effector caspases to induce apoptosis. The initiator caspases can activate Bcl2 family members such as Bid to activate the execution phase via the mitochondrial pathway (Guicciardi e Gores, 2005).

6.1.3 Autophagy

Whilst apoptosis is described as programmed cell death type I. Autophagy is described as programmed cell death type II. Autophagy is the process of lysosomal mediated self-degradation. Autophagy is required to maintain normal function and development, such as apoptotic cell clearance during embryogenesis, and providing cell protection during stress responses. Stress responses include nutrient starvation which results in degradation of un-required proteins to provide amino acids which form essential proteins for survival. Autophagy also provides protection from pathogens and is a regulator of the immune response. Autophagy is critical for the degradation and removal of faulty proteins and damaged organelles, and with the presence of autophagosomes, autophagy can mediate programmed cell death. There are three types of classified autophagy; macroautophagy, microautophagy, and chaperone mediated autophagy (CMA) (Figure 6.2). Macroautophagy is the most widely characterised autophagy function, it involves the processing of target cellular content, by isolation into the double membraned autophagosome. The autophagosome then fuses to the lysosome which initiates degradation. Microautophagy involves the direct engulfment of cytoplasmic components by the lysosome. CMA is the most complex and highly selective form of autophagy, CMA recognises an Hsc70 (heat shock protein 70) binding sequence present in the protein (KFERQ), the protein binds Hsc70 and a suitable chaperone, and forms a complex. This complex then binds lysosomal surface protein LAMP2 (lysosomal associated membrane protein 2), and the target protein then translocates to the lysosome without the Hsc70 and chaperones, and is subsequently degraded (Mizushima, 2007; Glick, Barth e Macleod, 2010).

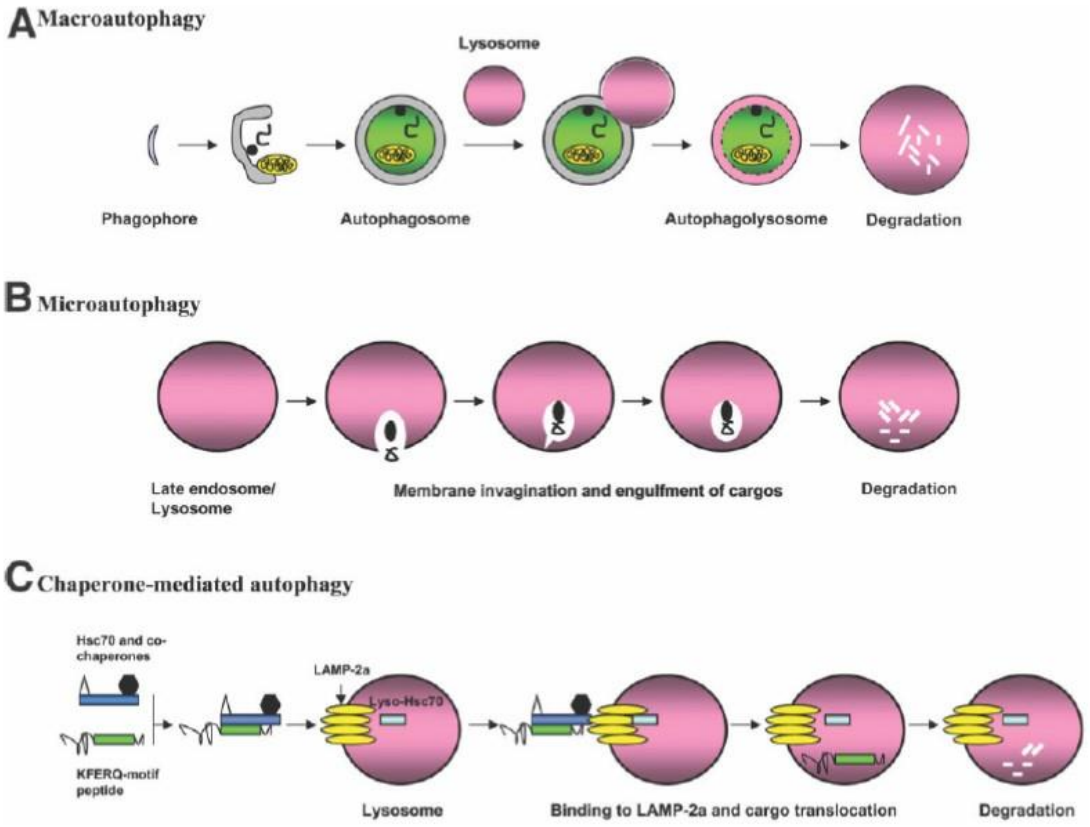


Figure 6.2 Autophagy: There are three classified autophagy pathways. A) Macroautophagy is the most characterised autophagy pathway, it requires the engulfment of target content by the phagophore to form the autophagosome. The autophagosome fuses to the lysosome which leads to subsequent degradation of the target proteins. B) Microautophagy requires the direct engulfment and degradation of the target proteins by the lysosome. C) Chaperone mediated autophagy is a highly specific and complicated autophagy pathway. It requires the specific binding of the Hsc70 protein and a suitable chaperone which binds the LAMP2 protein on the lysosome cell surface. The target protein is translocated in to the lysosome for subsequent degradation (Yin, Ding e Gao, 2008).

Autophagy has been highly characterised in studies of the liver where it was described in rat liver cells which were treated with glucagon. These cells displayed large numbers of lysosomes, some of which contained other organelles such as the mitochondria (De Duve e

Wattiaux, 1966). Since this discovery we now understand that autophagy is an essential process to maintain normal liver homeostasis and a key response during stress activation.

6.1.4 Necrosis

Programmed cell death III is necrosis. Necrosis is a response to extreme stress conditions such as tissue injury or ischemic conditions. Apoptotic cell death has been described as a process which can be beneficial, whereas necrosis is a process which is usually associated with detrimental consequences such as tissue damage and fatality (Figure 6.3) (Festjens, Vanden Berghe e Vandenabeele, 2006). As a more developed understanding of necrosis is emerging, it is found to be a regulated process which can be independent of apoptotic cell death, although both processes share key features of cell death which is mediated by mitochondrial permeablisation (Golstein e Kroemer, 2007). Necrosis and autophagy have been found to be initiated when apoptotic cell death is defective or absent. Necrosis can be activated in response to mitochondrial dysfunction which leads to the production of ROS and cell swelling, as opposed to cell shrinking during apoptosis. Cell swelling is closely followed by the accumulation of membrane blebs (Portt *et al.*, 2011). The mechanistic process involves depletion of ATP in response to metabolic stress. This is followed by Calcium ion up-regulation from external and ER sources which leads to the activation of calpains. Calpains are proteins which lead to lysosomal rupture and the subsequent activation of cathepsins which mediate cellular destruction. Cellular destruction is characterised by the irreversible plasma membrane breakdown which leads to leakage of enzymes from the cell and disrupted ion and electrical exchange. Necrosis can initiate the local inflammatory response

as it may activate the innate immune system by the subsequent release of factors by the necrotising cells (Golstein e Kroemer, 2007; Portt *et al.*, 2011).

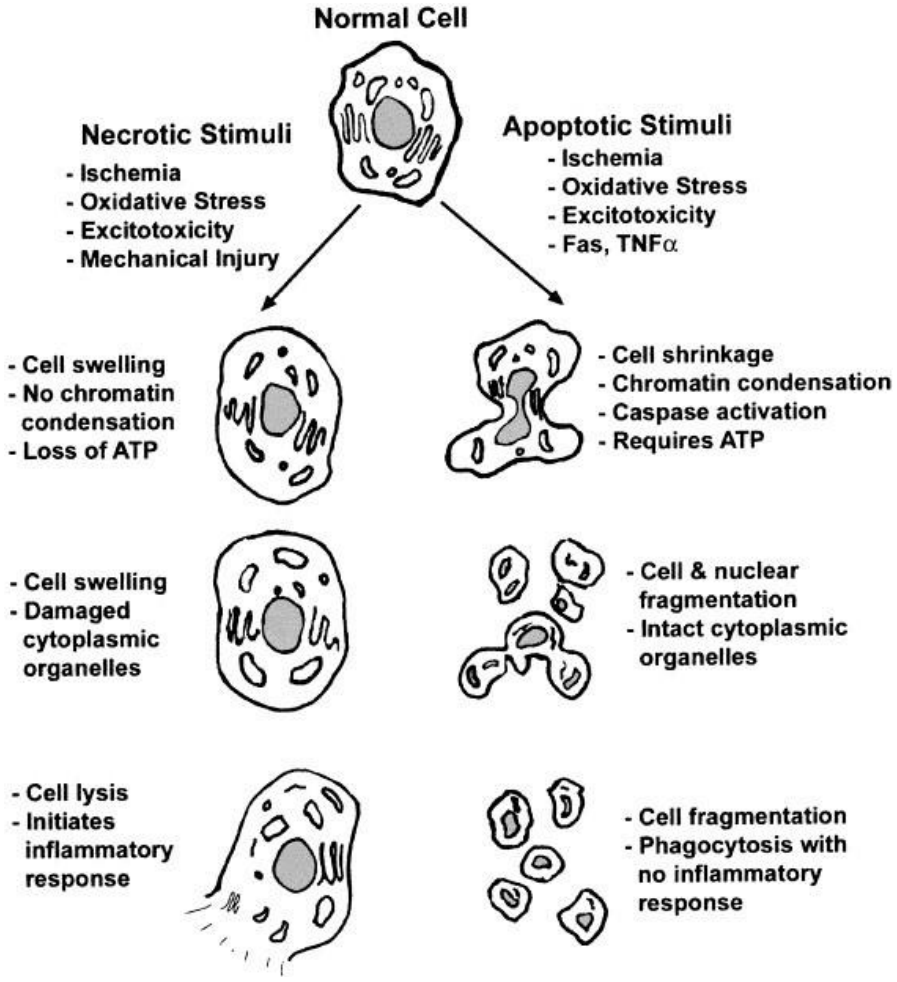


Figure 6.3 Necrosis and Apoptosis: Necrosis and apoptosis are cell death pathways which can be activated by the same stimuli such as ischaemia, oxidative stress and toxicity. Necrosis can also be activated by mechanical injury, and apoptosis can be activated by TNFSF member binding and activation on the cell surface. Necrosis is characterised by the loss of ATP and cell swelling. This leads to damaged cytoplasmic organelles, cell lysis and subsequent initiation of the inflammatory response. Apoptosis on the other hand requires ATP to induce cell death. A cell undergoing apoptosis will require membrane blebbing, cell shrinking, chromatin condensation, and caspase activation. The cell will undergo fragmentation with the cellular content still intact. These apoptotic bodies will be targeted by phagocytosis for clearance and the inflammatory response will not be activated. http://clinicalscienceblogcindy.wordpress.com/

6.1.5 Reactive Oxygen Species

Reactive signalling intermediates include ROS, reactive nitrogen species (RNS), carbon monoxide, and hydrogen sulphide intermediates. ROS are chemically reactive molecules which contain oxygen. The mitochondrion electron transport chain is responsible for producing the most ROS. Oxygen contains two unpaired electrons orbiting the outer electron shell, when oxygen is reduced by the addition of more electrons, reactive oxygen species are formed; these include superoxides, hydrogen peroxide, hydroxyl radicals and ions, and nitric oxides (NO). ROS are complicated molecules which can be expressed in all types of cells where they can influence various functions in different targets. ROS mediate normal physiological functions including cell signalling, apoptosis, gene expression and homeostasis regulation. ROS are also implicated in a number of pathological functions including inflammatory responses, ischaemia, cancer and ageing (Muriel, 2009; Nathan e Cunningham-Bussel, 2013).

During oxidative stress, ROS expression is highly up-regulated whilst antioxidant expression is down-regulated, leading to damage to cell structures. ROS function during inflammation can induce cellular cytotoxicity, generation of pro-inflammatory mediators, and sensitisation of cells towards inflammation. The mitochondria can mediate TNF induced apoptosis signal transduction by the production of ROS; including NF-kB, MAPK and AP1 (Garg e Aggarwal, 2002). Several TNFSF members have been implicated in ROS production in endothelial cells. In cerebral microvascular endothelial cells, NOX4 (a NADPH oxidase) has been shown to regulate cell fate in TNF-α activated cells; in particular during apoptosis and oxidative stress.

NOX4 NADPH derived ROS were shown to adapt to carbon monoxide mediated enhanced cell survival when activated by TNF-α via signalling through AKT, Erk, and p38 signalling pathways (Basuroy *et al.*, 2011). Soluble CD40-L has been shown to regulate endothelial cell dysfuntion via regulation of reactive signalling intermediates. It was shown that soluble CD40-L decreased endothelial nitric oxide synthase, NO, and superoxide production, and enhanced NOX activity in human coronary artery endothelial cells. These processes were induced via p38, Erk, and NF-kB dependent signalling pathways (Chen *et al.*, 2008).

6.1.6 Cell fate responses during liver disease

Programmed cell death and ROS have been implicated in normal physiology of the liver as well as during chronic liver disease. Liver injury is often triggered by inflammatory cell products. For example; hepatocellular injury induced by toxins show enhanced ROS production and inflammatory cell cytokines. During liver disease, TNF mediated apoptosis is known to play a significant role during liver disease pathogenesis (Schattenberg, Galle e Schuchmann, 2006). Briefly; in carbon tetra chloride injury models, NF-kB and Jnk signalling pathways were shown to mediate liver injury, TNF induced apoptosis (TNFIA) was shown to induce hepatocyte death (Czaja, Xu e Alt, 1995). Hepatocyte immunity to apoptosis was subsequently observed in TNF receptor and ligand null mice (Morio *et al.*, 2001). In cases of NASH, over expression of a pro oxidising agent cytochrome (Cyt) P450 2E1 was observed in a MAPK signalling dependent mechanism in humans and mouse models. In response to Cyt P450 2E1 overexpression in these cases, induced TNFIA was observed to be dependent on the up-regulation of ROS in the liver tissue. Subsequent decreased TNFIA in antioxidant

treated hepatocytes was observed in NASH models (Liu *et al.*, 2002; Schattenberg *et al.*, 2004). HCC cell lines have shown inherent resistance to TRAIL induced apoptosis and down-regulated expression of TRAIL receptors. Subsequently in HCC cases defective apoptotic signalling has been shown to enhance cellular proliferation (Shin *et al.*, 2002). During viral hepatitis infection apoptosis inducing proteins (such as HBV x) are up-regulated in hepatocytes to protect them from infection (Su e Schneider, 1997), and defective apoptosis has been shown to be one of the causes of HCC development post viral infection (Yoo e Lee, 2004). It has over the years become apparent that apoptosis and necrosis are two different pathways leading to the same outcome; which is cell death. In cases of liver disease, cells which undergo apoptosis in an ATP dependent manner, follow a necrotic pathway when ATP is depleted from metabolic processes. For instance, during liver ischaemia reperfusion injury in hepatocytes, glycine and fructose administration prevents necrotic cell death by enhancing ATP and preventing ATP depletion mediated membrane destruction. This process leads to the activation of apoptosis, mediated by caspase activation. Subsequently apoptosis was shown to be inhibited by depletion of ATP (Kim *et al.*, 2003; Malhi, Gores e Lemasters, 2006).

Defective autophagy in liver cells is a key to disease progression in the liver, for instance during ischaemia reperfusion liver injury, autophagy markers show a specific autophagic response where they are found to be up-regulated or decreased depending on the context and conditions (Rautou *et al.*, 2010). During cases of ALD and non-alcoholic fatty liver disease, several studies have shown autophagy inhibition. Enhanced uptake of ethanol may

result in inhibition of autophagy by decreased autophagic vacuoles demonstrated by rats fed on a high ethanol diet, decreased catabolism of proteins (Pösö e Hirsimäki, 1991), and enhanced protein accumulation (Baraona *et al.*, 1975). In NAFLD mouse models of chronic obesity and insulin resistance defective autophagy has been demonstrated by sustained lipid availability, and down-regulated autophagy indicators in the liver (Singh *et al.*, 2009; Codogno e Meijer, 2010). During hepatitis C infection, autophagy can play an infection enhancing role. Accumulated autophagic vacuoles were observed in HCV infected hepatocytes, however these were found to be functionally redundant as the virus can evade detection by the autophagosome and prevents subsequent autolysosomal formation. The virus actually has been found to use the autophagy process to enhance its own replication by triggering cells to produce required proteins for viral enhancement (Dreux *et al.*, 2009).

Reactive oxygen species accumulation has been shown to contribute to liver disease. For instance during ALD, oxidative stress is induced by alcohol consumption. This leads to enhanced ROS and RNS production and the peroxidisation of DNA, protein, and lipids. Enhanced ROS production has been shown to contribute to necrosis and apoptosis of hepatocytes, and enhanced fibrosis by the activation of HSC (Bataller *et al.*, 2003; Muriel, 2009). ROS contribution to malignancy is well documented. Specifically chronic inflammation paired with oxidative stress is a large contributing factor to the cause of liver cirrhosis which can subsequently lead to HCC by the persistent up-regulation of oxidative stress and inflammation (Marx, 2004; Seitz e Stickel, 2006).

6.1.7 TWEAK mediated cell fate responses

TWEAK activated responses to ROS production and programmed cell death have been investigated extensively since the initial discovery that TWEAK can induce apoptotic activity in activated cells (Chicheportiche *et al.*, 1997). The ability of TWEAK to induce apoptosis alone or in combination with activating cytokines such as IFN-γ is intriguing as TWEAK receptor Fn14 does not possess a death domain, and so TWEAK induction of apoptosis is still a process which is not fully understood. There have been suggestions that TWEAK induces apoptosis indirectly by the activation of other ligands which have receptors possessing the death domain such as TNF-α. In Kym-1 cells, TWEAK activation showed enhanced apoptosis mediated by TNF ligand and receptor activation. Neutralising antibodies to TNF showed decreased subsequent apoptosis (Schneider *et al.*, 1999). It was further shown that TWEAK and Fn14 can induce signalling to induce lysosomal degradation of the cellular IAP-TRAF2 which binds Fn14. This process lead to immortalised cells sensitized to TNF-α mediated cell death (Vince *et al.*, 2008). Recently it was shown that TWEAK activation can activate the intrinsic and extrinsic apoptotic pathways to induce cell death. TWEAK induced apoptosis was found to be mediated by a death signalling complex consisting of TWEAK and Fn14 interactions with RIP-1 (receptor interacting protein – 1) and FADD, promoting TNF activated apoptosis via caspase 8. siRNA depletion of components of the extrinsic (Caspase 8 and FADD) and intrinsic (BID, BAX, BAK) apoptosis pathway showed inhibition of apoptosis after TWEAK activation (Ikner e Ashkenazi, 2011).

Nakayama et al were one of the studies to suggest an alternative receptor to TWEAK as they found that TWEAK could induce caspase dependent apoptosis and cathepsin B mediated necrosis in DR3 negative cell lines and HT-29 cells (Nakayama *et al.*, 2002). They then further characterised death pathways regulated by TWEAK and found Fn14 transfected cells could induce cell death via multiple pathways. They found Fn14 expression on tumour cell surface and confirmed their previous study which showed positively regulated cell death via cathepsin B mediated necrosis, and ROI expression. They showed that TWEAK activated cells induced ROI expression induced lysosomal cathepsin B release, and in response to TWEAK activation, cathepsin B activation was required for subsequent reactive oxygen intermediate intracellular release. They showed that these positive cell death mediators were negatively regulated by caspase activation and TWEAK induced cell death was successfully blocked by Fn14 antibodies, suggesting that Fn14 mediated TWEAK induced cell death activation (Nakayama *et al.*, 2003). It has been shown that TWEAK in combination with TNF-α and IFN-γ activation of renal tubular cells induce Fn14 dependent apoptosis. TWEAK induced apoptosis was shown to be in a caspase 3, 8, and 9 dependent manner which lead to cytochrome C release from the mitochondria in response to truncated Bid expression. They showed that necrosis was induced in response to caspase inactivation. When cells were activated with TNF-α and IFN-γ alone, no changes in cell fate responses in renal tubular cells were observed suggesting a TWEAK specific response (Justo *et al.*, 2006).

TWEAK has subsequently been shown to be a key regulator of multiple cell fate responses in numerous studies. Shimada et al 2012 showed that ROS production in a NOX2 dependent

mechanism was functionally implicated during ALKBH3 (a repair enzyme which promotes repair of DNA damage induced by methylation) induced survival and angiogenic potential in urothelial carcinoma cell lines. This process was dependent upon TWEAK-Fn14-VEGF interactions (Shimada *et al.*, 2012). Alternatively in human dermal microvascular endothelial cells no changes in apoptosis, necrosis or ROS production was observed in response to TWEAK activation (Chen *et al.*, 2013). In colon cancer cell lines, TWEAK activation was found to induce apoptosis in an ROS dependent pathway when these cells were treated with 15d-PGJ2; a peroxisome proliferator activated receptor γ ligand (Dionne *et al.*, 2010).

TWEAK activated autophagy functions have been less characterised. Recently it was shown that TWEAK activation promoted skeletal muscle wasting. Skeletal muscle atrophy was functionally characterised by TWEAK activated pathways. They found that TWEAK activation induced the activation of the ATP dependent ubiquitin-proteasome pathway and induced autophagic responses by enhanced autophagy inducing genes, and caspase activation (characterised by a caspase inhibitor z VAD FMK) mediating skeletal muscle wasting. Inhibition of these pathways showed down-regulated muscle wasting via TWEAK activation. They showed that TWEAK induced skeletal muscle wasting was dependent on TWEAK activated NF-kB signalling pathways (Bhatnagar *et al.*, 2012).

These studies which have characterised TWEAK regulated cell fate have described TWEAK mediated cell fate responses to be cell and context dependent. It was highly important to understand TWEAK mediated cell fate responses in HIEC isolated from diseased liver tissue,

to help further understand the potential of TWEAK mediated inflammatory responses during chronic liver disease via HIEC.

6.1.8 Aims

The aims of this chapter were:

- To determine if TWEAK activated HIEC undergo cell fate responses; specifically autophagy, apoptosis, necrosis and ROS production.
- To determine if these processes were regulated via Erk and NF-kB Rel A signalling pathways.

6.2 MATERIALS AND METHODS

Materials and Methods for this chapter; please refer to sections: 2.1-2.4, 2.7 and 2.12,

Tables: 2.1-2.4.

6.3 RESULTS

To determine and analyse the effects of TWEAK activation on HIEC cell fate. HIEC were activated with TNF-α and TWEAK; apoptosis, necrosis, ROS production and autophagy responses were determined and compared (Figure 6.3.1). These were measured by markers for Annexin-V, 7-AAD, 2', 7' dichlorofluoroscin (DCF) and monodansylcadaverine (MDC), respectively. TNF-α was used as a positive control and unstimulated HIEC were used as a negative control. TWEAK activation enhanced ROS production, necrosis, apoptosis and autophagy in HIEC in comparison to unstimulated HIEC controls. TWEAK activated HIEC significantly enhanced expression of DCF, which emits fluorescence in response to oxidation; unstimulated HIEC showed 31.4 percentage of positive fluorescent cells in comparison to TWEAK activated HIEC which were 45.2 ± SEM (*p=0.02). TNF-α activated HIEC showed a higher expression of DCF at 51.2 percentage positive HIEC, but this was not statistically significant. TWEAK activated HIEC also showed a significantly high percentage of HIEC undergoing necrosis from 46 percent positive unstimulated HIEC to 63.5 percent TWEAK activated HIEC ± SEM (*p=0.04). TNF-α activated HIEC showed down-regulated necrosis at 38 percent HIEC. TWEAK activated HIEC also showed enhanced apoptosis marker Annexin-V expression (46.8 percent unstimulated HIEC to 60.4 percent TWEAK activated HIEC, and 51.1 percent TNF-α activated HIEC ± SEM). TWEAK activated HIEC also showed enhanced autophagic vacuole marker MDC in comparison to unstimulated HIEC (64.3 percent HIEC to 79.9 percent TWEAK activated HIEC ± SEM). TNF-α activated HIEC down-regulated autophagy to 40.8 percent HIEC. TWEAK activated responses to autophagy and apoptosis were not statistically significant. The MFI data showed a similar pattern of expression to TWEAK and TNF-α activation.

We wanted to determine if TWEAK activated cell fate responses were mediated by signalling via NF-kB Rel A and Erk 1/2 pathways. To do this, the four colour reporter assay was repeated with inhibitors for these signalling pathways (Figure 6.3.2). TWEAK activated HIEC all showed enhanced expression of ROS, necrosis, apoptosis, and autophagy markers during percentage positive HIEC determination. In response to TWEAK activation in combination with Erk and NF-kB Rel A inhibition, all four cell fate determinants except the autophagy marker were up-regulated. The data obtained in this assay was not statistically significant, but a trend in observed expression patterns was seen. In TWEAK activated HIEC with Erk signalling inhibited, autophagy remained unchanged to the unstimulated control. When NF-kB signalling was inhibited, autophagy decreased. Erk inhibited HIEC without TWEAK activation still showed up-regulated cell fate markers apart from autophagy which showed down-regulated responses. NF-kB inhibited HIEC showed up-regulated necrosis and apoptosis markers but down-regulated ROS and autophagy markers. As this data was not statistically significant, we can confirm that Erk 1/2 and NF-kB Rel A do not regulate HIEC cell fate responses.

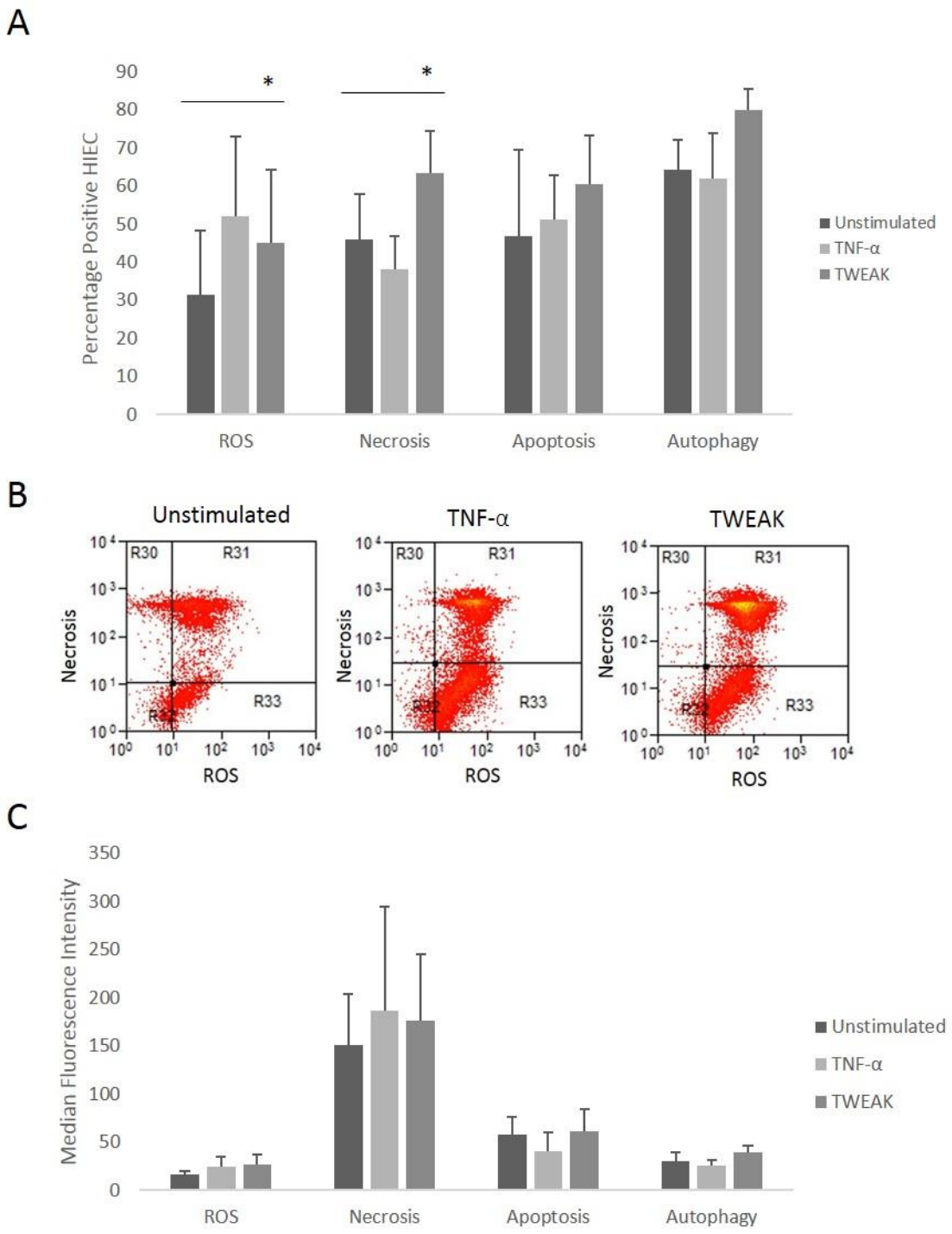


Figure 6.3.1 TWEAK activation promotes necrosis and the production of ROS in HIEC: HIEC were stimulated with TNF-α (10ng/ml) and TWEAK (100ng/ml) for 24 hours prior to FACS analysis. TNF-α was used as a positive control and unstimulated HIEC were used as a negative control. A) Percentage positive population of HIEC expressing markers for ROS (2',7'-dicholofluoroscin), necrosis (7-AAD), apoptosis (Annexin V), and autophagy (monodansylcadaverine), in response to TWEAK and TNF-α activation. HIEC activated with TWEAK significantly enhanced ROS production (*p=0.02) and necrosis (*p=0.04). B) Representative histograms of unstimulated HIEC and HIEC stimulated with TWEAK, showing ROS and necrosis marker increase on HIEC cell surface in response to TWEAK. C) Median fluorescence intensity from HIEC expressing markers for ROS, necrosis, apoptosis and autophagy in response to TWEAK and TNF-α activation. Data is shown as ± SEM n=6. Statistical analysis was carried out using a paired Student's t test.

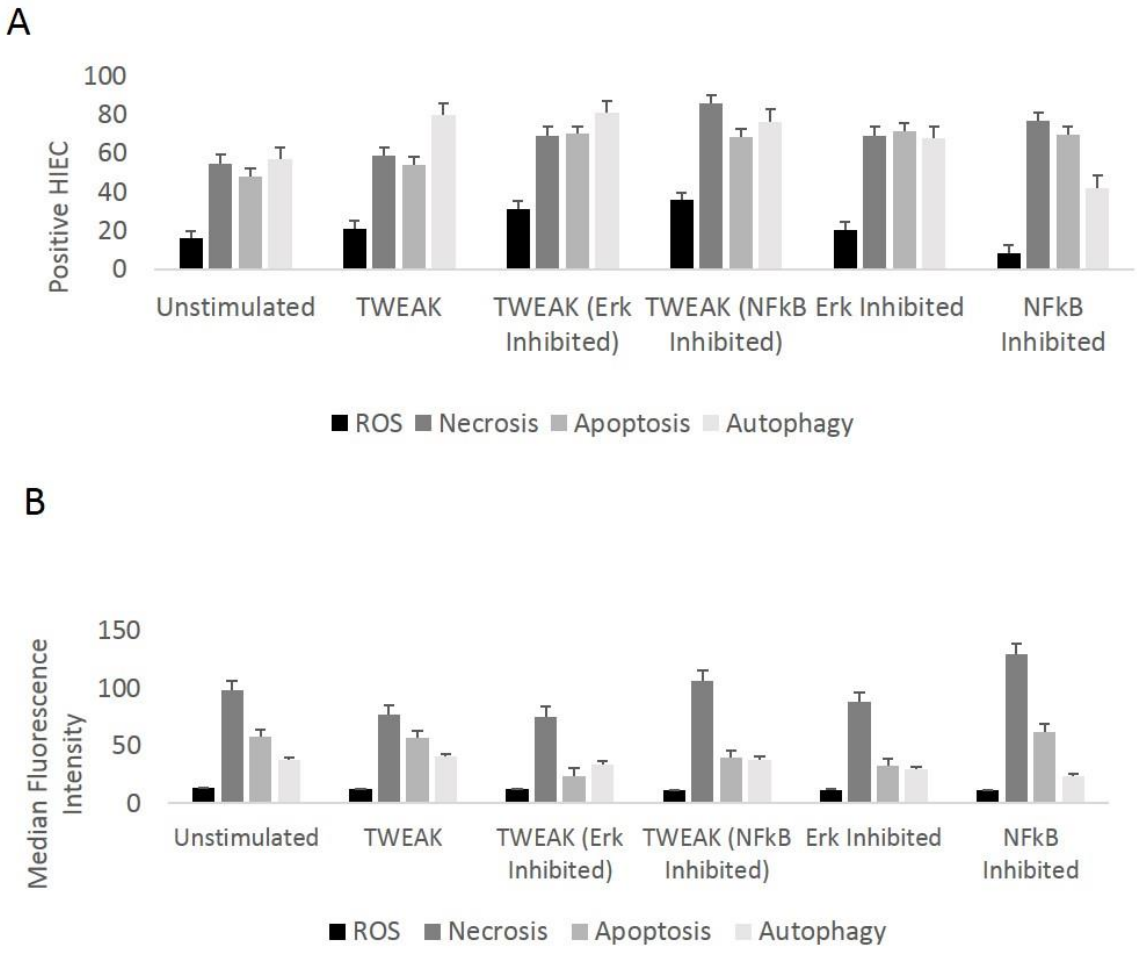


Figure 6.3.2 TWEAK promoted ROS and necrosis are not dependent on Erk and NF-kB Rel A signalling mechanisms: HIEC were stimulated with TWEAK (100ng/ml) ± Erk Inhibitor and ± NF-kB Inhibitor for 24 hours prior to FACS analysis. Unstimulated HIEC were used as a negative control. A) The percentage positive population of HIEC expressing markers for ROS (2',7'-dicholofluoroscin), necrosis (7-AAD), apoptosis (Annexin V), and autophagy (monodansylcadaverine), in response to TWEAK activation. Erk and NF-kB inhibition showed patterns of enhanced apoptosis, ROS and necrosis markers. Erk inhibition had no effect on autophagy and NF-kB inhibition in TWEAK activated cells down-regulated autophagy. B) Median fluorescence Intensity from HIEC expressing markers for ROS, necrosis, apoptosis and autophagy in response to TWEAK activation. Data is shown as ± SEM n=3. This data was not statistically significant.

6.3.1 Summary of Results

- TWEAK stimulated HIEC may induce ROS production and undergo necrosis over prolonged exposure to TWEAK.

6.4 DISCUSSION

Our data showed TWEAK activated HIEC trigger cell fate responses, specifically enhanced ROS production and necrosis in HIEC (Figure 6.3.1). Autophagy and apoptosis responses were also enhanced by TWEAK activation but this data was not statistically significant. The percentage positive data obtained from TWEAK activated HIEC showed that the cell fate responses were all highly responsive to TWEAK in comparison to TNF-α. This data was in confirmation with all previous studies which have suggested that prolonged TWEAK stimulation can lead to cell fate and subsequent pathological consequences.

Data obtained and discussed in this chapter showed enhanced ROS production which is a known mediator of inflammatory responses during pathological environments, and leads to damage to cells by induced cytotoxicity, enhanced production of inflammatory mediators, and enhancing sensitisation to inflammation (Nathan e Cunningham-Bussel, 2013). Our previous data showed that TWEAK may regulate chronic inflammatory liver responses via HIEC by the recruitment of inflammatory and angiogenic mediators, this data showed that these responses may be regulated via ROS production.

TWEAK activated HIEC also showed significantly enhanced necrosis. Necrosis is a response associated to tissue damage and even fatality and is triggered when apoptosis is absent or deregulated. Necrotic cells can subsequently trigger an inflammatory response (Golstein e Kroemer, 2007). TWEAK activated HIEC may be undergoing subsequent necrosis in response to prolonged TWEAK activation, and using the necrotic response to further promote

inflammation. Necrosis results in the activation of ROS production, and ROS production leads to activated necrosis (Portt *et al.*, 2011). Our data showed that the majority of cells analysed were expressing markers for both necrosis and ROS on the same cell (Figure 6.3.1 B), suggesting that these processes may be regulated by one another. Our data showed enhanced apoptosis but it was not statistically significant, therefore our data confirms that in the absence or deregulation of TWEAK activated apoptosis, HIEC may undergo necrosis to promote inflammatory responses. It would appear that TWEAK activated HIEC may be regulating inflammatory responses and as a consequence, the induction of these cell fate responses were observed, and a positive feedback loop of necrosis and ROS production may contribute to the prolonged inflammatory response via TWEAK in the liver leading to pathological consequences. This data is agreement with findings of Nakayama et al 2003 and Shimada et al 2012 who showed that ROS production and necrosis activated by TWEAK may lead to varying functional outcomes depending on the cell types and contexts such as, detrimental tumour progression and endothelial cell survival, and mediated angiogenic functions (Nakayama *et al.*, 2003; Shimada *et al.*, 2012). It is clear from this investigation that TWEAK activation lead cell fate responses may also be a context dependent response.

The complexity of TWEAK mediated HIEC cell fate responses was highlighted when our data also showed that these responses could be influenced by Rel A and Erk (Figure 6.3.2). This data was not statistically significant, however we found that TWEAK activation enhanced all cell fate responses and Erk and NF-kB may suppress the HIEC cell fate responses of apoptosis, ROS production, and necrosis, as enhanced expression of specific markers for

these were observed when Erk and NF-kB signalling was inhibited. This data complimented our angiogenesis data where we showed that Erk and NF-kB may regulate HIEC potential to undergo inflammatory mediated angiogenesis, which would be advantaged by a down-regulated response of cell death pathways and ROS production, indicating possible HIEC protection by these signalling pathways. Autophagy in response to Erk inhibition in TWEAK activated HIEC showed no change, but in NF-kB inhibited cells the autophagy response was inhibited. These findings were further complicated when we observed changes in cell fate mediation in HIEC in response to Erk and NF-kB inhibition alone without TWEAK activation, which showed varying responses to each marker. Furthermore our previous data had shown that Erk and NF-kB also regulated Fn14 cell surface HIEC expression (Chapter 5), which indicates the high level of regulation by Erk and NF-kB in TWEAK and Fn14 HIEC responses. Further experiments and statistically significant data will be necessary before drawing any conclusions from this preliminary data.

An interesting question which has arisen from this study is, if TWEAK activation is activating a necrotic response in HIEC, how were angiogenic responses mediated by TWEAK induction which require cell survival processes such as proliferation and migration. Data from this investigation showed that TWEAK activated HIEC were still viable (Appendix 2) and could contribute to angiogenic responses such as proliferation, migration, and tubule formation specifically under inflammatory conditions. Under the same conditions however, TWEAK can activate necrosis to a significant number of HIEC. One explanation would be that both responses of angiogenesis and necrosis are mediated by or mediate the inflammatory

response via NF-kB. Therefore all the subsequent responses that we observe in response to TWEAK activation may all be a product of a specific inflammatory response in the liver. From our data it would appear that TWEAK activated responses of HIEC may be time course dependent, where over shorter time periods TWEAK may promote angiogenesis to support liver regeneration and repair, however over longer periods of time TWEAK activation leads to detrimental cell fate responses. This finding would be in agreement with the large number of studies which often cite that TWEAK can regulate often conflicting functions, and is dependent upon environment, conditions, and signalling pathways.

To conclude this chapter, we have found that TWEAK activated HIEC may regulate cell fate responses during inflammatory liver disease in particular necrosis and ROS production. It would be of importance to further determine which specific pathways and mechanisms mediate the necrosis response in HIEC, and if ROS and necrosis are dependent on the activation of one another in response to TWEAK activated HIEC. It would also be highly important to determine if these TWEAK mediated cell fate responses were dependent on Fn14, and if they can be further regulated via inflammatory induction by additional cytokines.

CHAPTER 7

Final Summary and Further Work

7.1 SUMMARY

TWEAK and Fn14 are recently identified TNFSF members which have been characterised in several functions. There are limited studies observing the role of TWEAK and Fn14 in the liver, however studies to date have shown that TWEAK and Fn14 may regulate hepatic proliferative and regenerative responses, and mediate hepatic pathogenesis via inflammatory regulation. This has been determined by several *in vitro* and *in vivo* assays, specifically PHx and liver injury models where elevated TWEAK and Fn14 expression has been observed in comparison to normal controls, and TWEAK and Fn14 have been shown to support inflammatory cell and factor mediation and recruitment, and hepatic and progenitor cell proliferation (Jakubowski, Ambrose, Parr, Lincecum, Wang, Zheng, Browning, Michaelson, Baetscher, Baestcher*, et al.*, 2005; Tirnitz-Parker *et al.*, 2010; Kuramitsu *et al.*, 2013; Karaca *et al.*, 2014; Tirnitz-Parker, Olynyk e Ramm, 2014). Furthermore, TWEAK and Fn14 have been shown to critically mediate angiogenesis directly in several *in vitro* and *in vivo* assays, and have also been shown to indirectly mediate angiogenesis by enhancing other angiogenesis regulators (Lynch *et al.*, 1999; Wiley *et al.*, 2001). TWEAK and Fn14 have also been shown to specifically mediate tumourigenesis which requires inflammatory, angiogenic, and proliferative responses (Feng *et al.*, 2000; Ho *et al.*, 2004).

This investigation set out to further understand TWEAK and Fn14 regulation of hepatic inflammatory and regenerative responses. It can be suggested from the literature that under normal conditions Fn14 and TWEAK may regulate normal liver homeostasis, and under chronic insult and injury conditions these processes may be deregulated by sustained

TWEAK and Fn14 expression, leading to pathological remodelling and loss of liver function, which has also been shown in several studies of inflammatory diseases (Desplat-Jégo *et al.*, 2005; Kamata *et al.*, 2006; Michaelson *et al.*, 2012). More specifically we wanted to determine if TWEAK and Fn14 can regulate specific inflammatory and angiogenic functions via HIEC. HIEC function in hepatic pathogen detection and clearance, regulation and exchange of fluids, solutes, and molecules between the sinusoidal lumen and parenchyma, therefore they can regulate hepatic immune and inflammatory responses which is further facilitated by a host of immune receptors on their cell surface (Jenne e Kubes, 2013). Notably they can facilitate hepatic regeneration by remodelling of vascular structures by angiogenesis post liver insult, facilitated by expression of growth and angiogenic factors (Sato *et al.*, 2001). The incidence of chronic liver disease is an increasing problem as patients requiring liver transplants are exceeding the supply of available livers. Therefore, understanding TWEAK and Fn14 regulated HIEC responses will be essential to developing target specific therapeutics in future, aimed at facilitating liver regenerative responses to ease the demand on liver transplants.

Data obtained from this investigation has contributed towards the limited knowledge we currently have of TWEAK and Fn14 mediated functional responses during inflammatory liver conditions. Using IHC techniques we showed TWEAK expression in liver diseased tissue was predominantly found in areas of mono-nuclear cell infiltration in close proximity to portal vessels. Fn14 expression was found in hepatocytes, and high Fn14 expression was observed surrounding portal vessels, neovessels, ductular reactive cells, and areas surrounding

granuloma formation. This data indicated that TWEAK and Fn14 may regulate inflammatory responses, angiogenesis, and the subsequent formation of PALT. We confirmed previous findings of several studies where TWEAK and Fn14 up-regulated expression during pathogenesis has been seen, as our IHC data revealed that normal donor tissue showed milder Fn14 expression. This was further supported by TWEAK and Fn14 mRNA expression detected using quantitative PCR, which was significantly up-regulated in diseased liver tissue in comparison to normal liver tissue. Fn14 expression was highly up-regulated in comparison to TWEAK expression, indicating that TWEAK and Fn14 may play specific roles during inflammatory liver disease progression, perpetuation, and pathogenesis; with Fn14 possibly playing a more local role during normal liver physiology and TWEAK responding to Fn14 via inflammatory cells in settings of acute to chronic inflammation.

Our investigation made novel observations and highlighted the importance of TWEAK and Fn14 regulated HIEC expression and functional responses. Fn14 expression was highly induced by cytokines and growth factors on HIEC. We found Fn14 protein expression up-regulated on HIEC cell surface using flow cytometry and IF techniques in response to IL-1β, TNF-α, and FGF. We observed Fn14 present as a punctate stain in the cytoplasm indicating compartmentalised storage of Fn14 within HIEC, and further found that Fn14 protein may be shuttled from the Golgi to endoplasmic reticulum in the cytoplasm, and to the cell surface. It appeared that Fn14 stored in the Golgi was subsequently released into the cytoplasm upon cytokine stimulation, and may be transported to the cell membrane for protein-protein interactions. TGF-β, TWEAK, and IL-17A cytokine activation on the other hand, significantly

down-regulated Fn14 cell surface protein expression on HIEC, indicating possible contribution to the promotion of Fn14 degradation on HIEC cell surface. This data was in confirmation with a recent study observing Fn14 transport within the Golgi apparatus and lysosomal mediated degradation of Fn14, accelerated in response to TWEAK (Gurunathan *et al.*, 2014). Our data indicated the possibility of TGF-β and IL-17A enhancing TWEAK responses to HIEC, facilitating this process.

No TWEAK protein expression was observed on HIEC, however our IHC data revealed TWEAK presence in inflammatory infiltrates, therefore we wanted to determine TWEAK expression from total PBMC. We observed TWEAK mRNA expression present in total PBMC, which was highly inducible by IFN-γ. Monocytes have been found to highly express TWEAK, and specific monocyte subsets have been found to contribute to chronic inflammation in the liver (Liaskou *et al.*, 2013). We therefore examined TWEAK mRNA expression and responses to inflammatory cytokine IFN-γ which highly induces TWEAK expression. We observed TWEAK expression present in different monocyte subsets which were differentially regulated by IFN-γ activation. In CD14+ and dual positive monocyte subsets IFN-γ activation up-regulated TWEAK mRNA expression. In CD16+ monocytes, IFN-γ activation down-regulated TWEAK mRNA expression.

We further wanted to determine TWEAK and HIEC regulated leukocyte recruitment functions to understand the presence of TWEAK in inflammatory infiltrates. We found that TWEAK activated HIEC marginally but significantly contributed to leukocyte adhesion with a

higher affinity for monocyte recruitment, and leukocyte recruitment via TWEAK activated HIEC was found to be highly dependent on the interactions of TWEAK with other cytokines such as TNF-α, suggesting that TWEAK plays a more supportive role during leukocyte recruitment and adhesion rather than an integral role. This data indicated that specific TWEAK mediated inflammatory responses via leukocyte interactions is a highly regulated process and may contribute to inflammatory liver disease pathogenesis, and further proved that TWEAK and Fn14 functions may be regulated via paracrine mechanisms; where Fn14 present on HIEC may interact with TWEAK present on leukocytes which infiltrate liver tissue from circulation during liver insult.

TWEAK regulated angiogenesis has been characterised in many studies, but has not been characterised extensively in the liver and specifically in HIEC. Our data showed that TWEAK may regulate angiogenesis via HIEC, which complimented our IHC data which revealed Fn14 presence in areas of neovascularisation. We found that TWEAK activation significantly enhanced HIEC tube formation which was mediated by Fn14. We then found that these processes were highly regulated via NF-kB and Erk transcription factors. Our data further suggested that TWEAK activation of HIEC may induce angiogenic cytokine induction responses specific to liver tissue, depending on the tissue histology; with diseased liver tissue showing a higher response to TWEAK mediated angiogenic cytokine expression, in comparison to normal tissue which showed a milder angiogenic cytokine response. We further showed that TWEAK may functionally regulate angiogenesis in inflammatory liver disease isolated HIEC via a specific set of angiogenesis mediators; Angiopoietin-1, CXCL16, IL-

8, MCP-1, and VEGF. In tissue where granulomas were not present the cytokine induction in response to TWEAK was of a wider range and not specific. We further showed using IHC that Fn14 expression was highly up-regulated in diseased liver in portal vessels surrounding granulomas. There were limitations to the angiogenic cytokine array which would influence any conclusions we could draw. We would need a larger number of samples to analyse from normal donor patients and patients with CILD, we would need more statistically significant data and follow up studies of the influences of the significant cytokines regulating HIEC angiogenesis via TWEAK.

We further found that TWEAK and Fn14 functions were regulated by NF-kB Rel A and Erk signalling; from expression of this ligand and receptor on HIEC and angiogenesis inducing functions. Our data found that TWEAK activated HIEC induce pro-inflammatory NF-kB Rel A activation early on, and then subsequently activated Erk as a later response which may contribute to inflammatory angiogenesis regulation. We found that exposure to TWEAK over time induced ROS production and necrosis in HIEC, this may contribute to potential pathogenesis in the liver via TWEAK activated responses. Our signalling data revealed that NF-kB and Erk regulated HIEC responses were complex and as other studies have confirmed, these responses are dependent on the cellular environment and conditions, as often conflicting functions in response to TWEAK activation in HIEC could be observed. Our data has shown that TWEAK and Fn14 are indeed a highly complex, highly regulated, multifunctional system which can potentially be manipulated in the development of future novel therapeutics to aid inflammatory liver disease regulation.

Current data reviewed in this thesis suggests that excessive TWEAK and Fn14 expression leads to detrimental consequences, in our study these consequences suggest inflammatory liver disease progression. Future TWEAK and Fn14 based therapeutics would ideally target enhanced TWEAK and Fn14 expression, and the potential to regulate this. It has been found using *in vivo* mouse models that TWEAK and Fn14 do not interfere with normal growth and development (Maecker *et al.*, 2005), however several studies have confirmed that they significantly contribute to disease maintenance and progression rather than disease initiation. Therefore it is hoped that TWEAK and Fn14 inhibition would have limited adverse effects in patients. TWEAK and Fn14 suppression can be employed by using RNAi technology, or interfering with TWEAK and Fn14 protein-protein interactions preventing ligand and receptor multimerisation, TRAF association with Fn14, and TWEAK-Fn14 interactions, which all lead to signal transduction. Current TWEAK and Fn14 based clinical trials have used TWEAK mAb's to inhibit TWEAK activity. In RA patients the use of BIIB023; a TWEAK blocking mAb in a clinical phase I trial has shown the ability to safely down-regulate soluble TWEAK serum expression, which is usually associated with chronic inflammation and tissue destruction during RA. Up to 20mg/kg dosage had the ability to suppress TWEAK expression for up to 28 days, and also was found to down-regulate other inflammatory inducing proteins including MCP-1, MIP-1β and TIMP-1. This antibody was found to have minimal adverse effects and no infections were reported, suggesting that BIIB023 does not interfere with the adaptive immune response (Wisniacki *et al.*, 2013). A phase II trial is scheduled to observe BIIB023 efficacy in the renal response during LN (Michaelson *et al.*, 2012). Another phase I trial has tested the ability of RG7212 anti TWEAK mAb in Fn14 positive tumours. They found tumour shrinkage accompanied by enhanced host immune responses and no

toxicity (Yin *et al.*, 2013). Anti-TWEAK mAb technology has its own limitations as it may not be able to fully inhibit Fn14 responses which have been occasionally shown to be independent of TWEAK. Therefore continued development and research leading to potential TWEAK and Fn14 based therapies is essential.

This investigation set out to determine the potential functions that TWEAK and Fn14 may have during inflammatory liver disease, and how these functions may be regulated via HIEC interactions with this ligand and receptor. This thesis has successfully started to develop a better understanding of TWEAK and Fn14 regulated responses in liver disease via HIEC, and indicates that they may contribute to inflammatory, angiogenic, and the regulation of potential pathogenic functions. It is essential to continue to further understand TWEAK and Fn14 regulation in the liver, furthermore in individual hepatic cells, their crosstalk and communication, and how this may translate into human studies as an aim for developing novel liver regeneration promoting therapies.

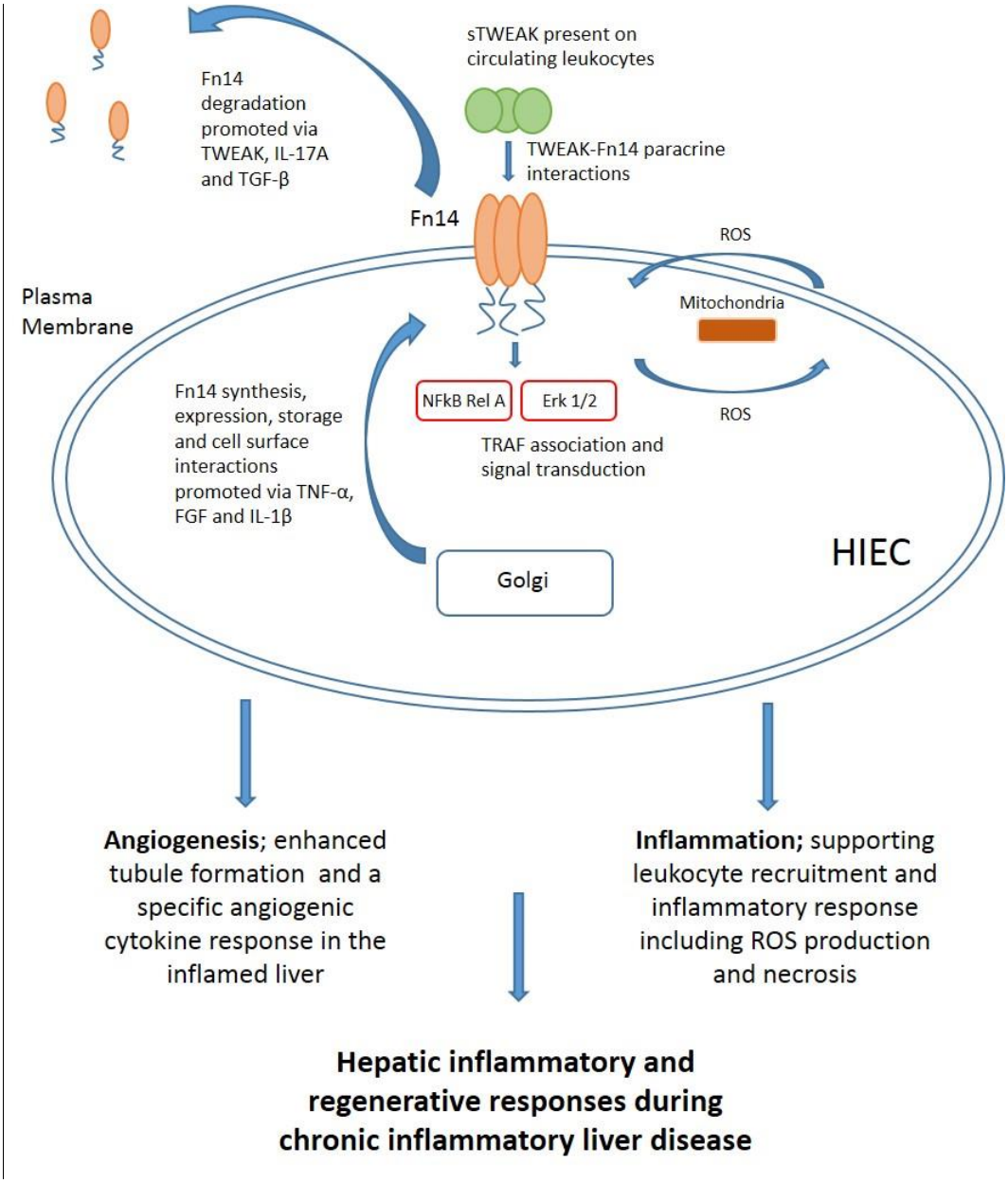


Figure 7.1 TWEAK and Fn14 regulated HIEC functional responses during inflammation: Fn14 was expressed on HIEC cell surface and interacts with TWEAK expressed on circulating leukocytes via paracrine interactions. Fn14 expression on HIEC was highly regulated by TNF-α, IL-1β and FGF, promoting Fn14 synthesis and Golgi transport to the cell surface for protein-protein interactions. Fn14 protein in HIEC may subsequently be degraded by TWEAK activation possibly promoted by IL-17A and TGF-β activation. TWEAK and Fn14 function via Erk 1/2 and NF-kB Rel A signalling. TWEAK activated HIEC promote angiogenic responses and leukocyte adhesion during inflammation. Sustained TWEAK activation promotes HIEC ROS production and necrosis.

7.2 FURTHER WORK

In future there is a lot to still learn about the functional regulation of inflammatory liver disease by TWEAK and Fn14 via HIEC. Data in this thesis has suggested that TWEAK and Fn14 may regulate hepatic inflammatory and regenerative responses via HIEC, which may subsequently contribute to portal associated lymphoid tissue formation. Following this there still remain important questions, the answers of which will support our current data and enhance our understanding.

- It would be of importance to further characterise normal liver and liver disease specific TWEAK and Fn14 expression using IHC and mRNA analysis, to draw significant conclusions of any differences found between tissue types.
- We showed different responses of TWEAK mRNA expression in monocyte subsets to IFN-γ, more experiments will be required to further characterise these differences and to obtain statistically significant results. It will then be important to determine the potential effects of TWEAK on trans-endothelial migration of monocyte subsets to HIEC, using transwell assays to determine the phenotypic traits of the receptive monocytes when they are regulated by TWEAK. We determined that TWEAK activated HIEC may facilitate leukocyte recruitment, it will be important to further characterise the TWEAK inflammatory response by further experiments and using cytokines which have shown Fn14 expression regulation. It will also be essential to further characterise monocyte subset responses to TWEAK activated HIEC using flow based adhesion assays. This will help to further understand and determine

mechanistic details and regulation of HIEC recruitment of leukocytes. It will also be important to further characterise any differences of TWEAK activated HIEC under flow between normal and diseased liver isolated HIEC.

- We characterised the Erk and p38 MAPK signalling pathways by TWEAK activated HIEC, it will also be important to determine if TWEAK activated HIEC signal through the Jnk Pathway. Our data showed that Erk and NF-kB potentially regulate Fn14 expression via cytokine and growth factor activation, however further experiments will be required to determine this. TWEAK and Fn14 responses are highly regulated by TRAF association, therefore profiling TWEAK and Fn14 HIEC response activated TRAFs will be ideal.
- We determined that TWEAK activated HIEC induce ROS production and are susceptible to necrosis, it will be important to determine if these cell fate responses of TWEAK activation are mediated by Fn14, and to further characterise if TWEAK responses are further regulated by inflammatory cytokines which have been previously implicated in TWEAK and Fn14 functional responses. It will then be essential to characterise signalling during TWEAK activated necrosis and ROS production by the use of cathepsin inhibitors and calcium probes.
- We determined that TWEAK activated HIEC may contribute to angiogenesis, it will be important to further characterise if TWEAK activated angiogenesis via HIEC is mediated by VEGF and FGF dependent pathways. It is highly important to further characterise the angiogenic cytokine profiles, more assays will be required and inhibitors to the specific angiogenic proteins which showed a significant result will be ideal for proof of concept experiments. It will also be beneficial to determine if

TWEAK and Fn14 knock-out mice liver tissue after inflammatory liver injury, also show down-regulated angiogenesis protein responses using IHC, to further confirm the regulated inflammatory angiogenesis responses via TWEAK. It will then be highly important to carry out *in vivo* angiogenesis experiments using TWEAK, and determine HIEC responses, to further confirm findings from our *in vitro* pro-angiogenesis data.

- Data suggested TWEAK and Fn14 regulate PALT formation in the liver. Proof of concept experiments will be essential to determine TWEAK and Fn14 involvement in PALT formation in inflammatory liver disease, using TWEAK and Fn14 knock out mouse models with induced liver injury by *propioni bacterium acnes*.
- It will be beneficial to determine TWEAK and Fn14 expression patterns in TWEAK and Fn14 knock out mouse liver tissue with induced liver injury models, to further characterise TWEAK and Fn14 specific inflammatory disease regulation.
- Translational studies will be essential to develop future therapeutics targeting the TWEAK and Fn14 pathway during CLD. A start will be to determine soluble TWEAK levels in patient serum with chronic inflammatory liver disease, to analyse if TWEAK can be a potential marker of CLD.

Appendix 1

Angiogenesis Protein	Function
Activin A	Activin A is a multifunctional protein involved in processes such as follicle stimulating hormone biosynthesis and secretion, regulation of the menstrual cycle, cell fate, homeostasis, and wound repair (Chen *et al.*, 2006).
ADAMTS-1	ADAMTS-1 interacts with VEGF and may have functions during normal growth, function and development of organs (Vázquez *et al.*, 1999).
Angiogenin	Angiogenin is a stimulator of new vessel formation (Gao e Xu, 2008).
Angiopoietin-1	Angiopoetins are proteins involved in angiogenesis specifically during blood vessel maturation and stability (Thurston, 2003; Barton, Tzvetkova e Nikolov, 2005).
Angiopoietin-2	
Angiostatin/Plasminogen	Angiostatin is an endogenous inhibitor of angiogenesis (Cao *et al.*, 1996).
Amphiregulin	Amphiregulin may promote the growth of epithelial cells and may also act as a tumour suppressor gene (Zaiss *et al.*, 2013).
Artemin	Artemin is part of the GDNF family of ligands and has been shown to promote angiogenesis (Banerjee *et al.*, 2012).
C5a	Complement c5a has been shown to regulate inflammation and angiogenesis during pathogenesis (Conroy *et al.*, 2009).
Coagulation Factor III	CFIII can regulate angiogenesis directly by clotting independent mechanisms, and indirectly via clotting dependent mechanisms, or by regulating growth regulatory molecules (Bluff *et al.*, 2008).
CXCL16	CXCL16 is a chemokine produced by dendritic cells and has been found to be an angiogenesis factor in HUVEC (Zhuge *et al.*, 2005).
DPPIV	Dipeptidyle peptidase 4 has been found to regulate immune regulation, apoptosis and signal transduction. Recently it has been associated with the development of cancers and tumours (Kitlinska *et al.*, 2003).
EGF	Epidermal growth factor regulates cell survival, proliferation, and differentiation (Herbst, 2004).
EG-VEGF	Endocrine gland derived VEGF has been shown to regulate endocrine specific angiogenesis (Brouillet *et al.*, 2010).
Endoglin	Endoglin in part of the TGF beta 1 receptor complex and has been found to be involved in cardiac development and vascular remodelling (ten Dijke, Goumans e Pardali, 2008).
Endostatin/ Collagen XVIII	Endostatin is an endothelial cell proliferation, angiogenesis, and tumourigenesis inhibitor (Folkman, 2006).
Endothelin-1	Endothelin is primarily produced by endothelial cells and is involved in vascular homeostasis and has been found to stimulate angiogenesis (Boron e Boulpaep, 2009).
FGF acidic	Fibroblast growth factor; involved in wound healing, embryonic development and angiogenesis, they also regulate cell survival, proliferation, differentiation, and *in vivo* development (Dorey e Amaya, 2010).
FGF basic	
FGF-4	
FGF-7	

GDNF	Glial cell line derived neutrophic factor; is a neutrotrophic factor which has been shown to regulate cell survival, differentiation, migration and neurite outgrowth (Shang *et al.*, 2011).
GM-CSF	Granulocyte macrophage colony stimulating factor is secreted by immune cells, endothelial cells and fibroblasts. It is a white blood cell growth factor .
GroA	GroA is a chemoattractant regulating leukocyte trafficking and an angiogenesis regulator (Paleolog, 1996).
HB-EGF	Heparin binding like EGF growth factor; has various functions which are dependent on its interactions with other proteins. One of its critical functions have been in promoting wound healing and angiogenesis (Mehta e Besner, 2007).
HGF	Hepatocyte growth factor functions to regulate cell growth, motility and morphogenesis and subsequently has a significant role during angiogenesis, tumourigenesis, and tissue regeneration (Rosen *et al.*, 1997)(Rosen *et al.*, 1997)(Rosen *et al.*, 1997).
IGFBP-1	There are suggestions of IGFBP-1 (insulin like growth factor binding protein) having anti angiogenic properties (Shay *et al.*, 2011).
IGFBP-2	IGFBP-2 showed enhanced VEGF gene promoter activity and subsequent angiogenesis promotion (Azar *et al.*, 2011).
IGFPB-3	IGFBP-3 showed enhanced angiogenesis through IGF-1 signaling and SphK1 activation (Granata *et al.*, 2007).
IL-1β	IL-1β has been shown to regulate tumour invasiveness and angiogenesis (Voronov, Carmi e Apte, 2007).
IL-8	IL-8 has been shown to promote angiogenesis, tumour growth and metastasis (Heidemann *et al.*, 2003).
IP-10	Plasma interferon gamma inducible protein 10 has been shown to inhibit VEGF functions (Bodnar, Yates e Wells, 2006)
LAP(TGF-B1)	Latency associated peptide (TGF b1); stimulate cell growth, differentiation, proliferation and apoptosis (Pepper, 1997).
Leptin	Leptin has been shown to regulate endothelial cell proliferation and increase the expression of MMP's and subsequently regulate angiogenesis (Anagnostoulis *et al.*, 2008)(Anagnostoulis *et al.*, 2008)(Anagnostoulis *et al.*, 2008)(Anagnostoulis *et al.*, 2008)(Anagnostoulis *et al.*, 2008)(Anagnostoulis *et al.*, 2008)(Anagnostoulis *et al.*, 2008)(Anagnostoulis *et al.*, 2008)(Anagnostoulis *et al.*, 2008)(Anagnostoulis *et al.*, 2008).
MCP-1 (CCL2)	MCP-1 has been shown to regulate the angiogenic potential of TGF-b by the recruitment of vascular smooth muscle cells and mesenchymal cells towards endothelial cells (Niu *et al.*, 2008).
MIF	Macrophage migratory inhibitory factor has important inflammatory, immune and cell growth responses. Specifically anti-tumour and anti-angiogenesis functions (Nishihira, 2000).
MIP-1a	Macrophage inflammatory protein 1 alpha; has been shown to decrease angiogenesis and subsequent wound healing by a reduction of macrophages at the wound site (Yoshida *et al.*, 2003).
MMP-8	Matrix metalloproteinase family function during the breakdown of the extracellular matrix (Rundhaug, 2005).
MMP-9	
NRG1-β	Neuregulin 1 beta is known to stimulate the up-regulation of VEGF A and therefore can promote in vivo and in vitro angiogenesis (Hedhli *et al.*, 2012).
Pentraxin 3 (PTX3)	PTX 3 is released in response to inflammatory signals and has been known to inhibit angiogenesis (Leali *et al.*, 2011).
PD-ECGF	Platelet derived endothelial cell growth factor is known to stimulate angiogenesis and promote the growth of endothelial cells (Fujimoto *et al.*, 1999).

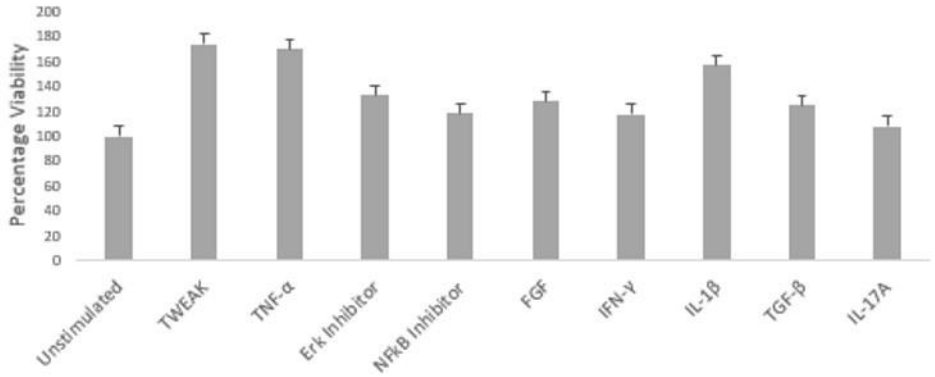
PDGF-AA	Platelet derived growth factor. Regulate cell growth and division and have significant roles during angiogenesis (Sato *et al.*, 1993).
PDGF-AB/BB	
Persephin	Persephin is part of the GDNF family of ligands which have been found to regulate a number of processes such as cell survival, growth, differentiation and migration (Milbrandt *et al.*, 1998).
Platelet factor 4	PF4 is released from activated platelets and promotes blood coagulation and may have roles during wound repair and inflammation. It has also been found to inhibit cell proliferation, migration and angiogenesis (Bikfalvi, 2004).
PlGF	Placental growth factor is an inflammation and angiogenesis regulator (Luttun, Tjwa e Carmeliet, 2002)
Prolactin	Prolactin has been found to stimulate and inhibit angiogenesis in different settings (Corbacho, Martínez De La Escalera e Clapp, 2002).
Rantes	Rantes has shown pro-angiogenic functions specifically endothelial cell migration, spreading and neovessel formation (Suffee *et al.*, 2012).
sICAM-1	Soluble Intracellular adhesion molecule 1 can regulate in vitro and in vivo angiogenesis (Deng *et al.*, 2007).
Serpin B5	Also known as Maspin which functions as an angiogenesis inhibitor and a tumour suppressor (Richardson *et al.*, 2007).
Serpin E1	Plasminogen activator inhibitor-1; mainly produced by endothelium and inhibits serine proteases and the activity or matrix metalloproteinases.
Serpin-F1	Also known as pigment epithelium derived factor which may function as an anti tumourigenic, anti angiogenic and neutrophic protein (Richardson *et al.*, 2007).
TIMP-1	Metalloproteinase inhibitor; functions to promote cell proliferation, may be involved in platelet aggregation and recruitment in endometrial tissue remodelling, and may have anti apoptotic function (Reed *et al.*, 2003) .
TIMP-4	
Thrombospondin-1	Thrombospondins may inhibit angiogenesis and tumourigenesis. These may regulate cell adhesion, migration and growth (Lawler, 2000).
Thrombospondin-2	
uPA	Urokinase-type plasminogen activator may function during thrombolysis and extracellular matrix degradation (Ribatti *et al.*, 1999).
Vasohibin	Vasohibin is an inhibitor of angiogenesis (Shimizu *et al.*, 2005).
VEGF	Vascular endothelial growth factor; a signalling protein expressed by cells during vasculogenesis and angiogenesis. VEGF-C functions during lymphangiogenesis (Ferrara, 2002).
VEGF-C	

Appendix 2

Activated HIEC do not alter HIEC viability

To determine if any of the stimulations used in all of the experiments in this study altered HIEC viability, an MTT assay was performed on HIEC. Stimulations with TWEAK, TNF-α, IFN-γ, FGF, IL-1β, TGF-β, IL-17A, Erk Inhibitor and NF-kB Inhibitor were compared to an unstimulated HIEC control. No negative change in HIEC viability was observed when the cells were stimulated in comparison to un-stimulated HIEC (n=3).

MTT Percentage Viability Mean									
Unstimulated	TWEAK	TNF-α	Erk Inhibitor	NFkB Inhibitor	FGF	IFN-γ	IL-1β	TGF-β	IL-17A
100	173.6	169.9	132.9	118.3	127.6	117.1	156.5	124.4	107.7



HIEC are viable after stimulation with TWEAK and TNF-α: An MTT assay was used to determine if the HIEC used in the angiogenesis assay were viable after stimulation with TNF-a (10ng/ml) and TWEAK (100ng/ml) for 8 hours. TNF-α and TWEAK stimulated HIEC showed at least 100% viablilty after 8 hours, mean ± SE.

LIST OF REFERENCES

Adachi, M. et al. Enhanced and accelerated lymphoproliferation in Fas-null mice. **Proc Natl Acad Sci U S A,** v. 93, n. 5, p. 2131-6, Mar 1996. ISSN 0027-8424. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8700897 >.

Adams, D. H.; Afford, S. C. The role of cholangiocytes in the development of chronic inflammatory liver disease. **Front Biosci,** v. 7, p. e276-85, Jul 2002. ISSN 1093-9946. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12086915 >.

Afford, S. C. et al. CD40 activation induces apoptosis in cultured human hepatocytes via induction of cell surface fas ligand expression and amplifies fas-mediated hepatocyte death during allograft rejection. **J Exp Med,** v. 189, n. 2, p. 441-6, Jan 1999. ISSN 0022-1007. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9892626 >.

Aggarwal, B. B. Signalling pathways of the TNF superfamily: a double-edged sword. **Nat Rev Immunol,** v. 3, n. 9, p. 745-56, Sep 2003. ISSN 1474-1733. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12949498 >.

Aggarwal, B. B.; Gupta, S. C.; Kim, J. H. Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. **Blood,** v. 119, n. 3, p. 651-65, Jan 2012. ISSN 1528-0020. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22053109 >.

Akdis, M. et al. Interleukins, from 1 to 37, and interferon-γ: receptors, functions, and roles in diseases. **J Allergy Clin Immunol,** v. 127, n. 3, p. 701-21.e1-70, Mar 2011. ISSN 1097-6825. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21377040 >.

Aleffi, S. et al. Upregulation of proinflammatory and proangiogenic cytokines by leptin in human hepatic stellate cells. **Hepatology,** v. 42, n. 6, p. 1339-48, Dec 2005. ISSN 0270-9139. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16317688 >.

Algeciras-Schimnich, A. et al. Molecular ordering of the initial signaling events of CD95. **Mol Cell Biol,** v. 22, n. 1, p. 207-20, Jan 2002. ISSN 0270-7306. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11739735 >.

Anagnostoulis, S. et al. Human leptin induces angiogenesis in vivo. **Cytokine,** v. 42, n. 3, p. 353-7, Jun 2008. ISSN 1096-0023. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18448353 >.

Ando, T. et al. TWEAK/Fn14 interaction regulates RANTES production, BMP-2-induced differentiation, and RANKL expression in mouse osteoblastic MC3T3-E1 cells. **Arthritis Res Ther,** v. 8, n. 5, p. R146, 2006. ISSN 1478-6362. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16945157 >.

Aronin, A. et al. Fn14•TRAIL effectively inhibits hepatocellular carcinoma growth. **PLoS One,** v. 8, n. 10, p. e77050, 2013. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24130833 >.

Asrani, K. et al. The HER2- and heregulin β1 (HRG)-inducible TNFR superfamily member Fn14 promotes HRG-driven breast cancer cell migration, invasion, and MMP9 expression. **Mol Cancer Res,** v. 11, n. 4, p. 393-404, Apr 2013. ISSN 1557-3125. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23378579 >.

Azar, W. J. et al. IGFBP-2 enhances VEGF gene promoter activity and consequent promotion of angiogenesis by neuroblastoma cells. **Endocrinology,** v. 152, n. 9, p. 3332-42, Sep 2011. ISSN 1945-7170. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21750048 >.

Bancroft, J. D.; Gamble, M. **Theory and practice of histological techniques**. 6th ed. [Edinburgh]: Churchill Livingstone, 2008. ISBN 9780443102790 (hbk.) : ¹110.00

0443102791 (hbk.) : ¹110.00.

Banerjee, A. et al. ARTEMIN promotes de novo angiogenesis in ER negative mammary carcinoma through activation of TWIST1-VEGF-A signalling. **PLoS One,** v. 7, n. 11, p. e50098, 2012. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23185544 >.

Baraona, E. et al. Alcoholic hepatomegaly: accumulation of protein in the liver. **Science,** v. 190, n. 4216, p. 794-5, Nov 1975. ISSN 0036-8075. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1198096 >.

Bartley, P. B. et al. A contributory role for activated hepatic stellate cells in the dynamics of Schistosoma japonicum egg-induced fibrosis. **Int J Parasitol,** v. 36, n. 9, p. 993-1001, Aug 2006. ISSN 0020-7519. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16806222 >.

Barton, W. A.; Tzvetkova, D.; Nikolov, D. B. Structure of the angiopoietin-2 receptor binding domain and identification of surfaces involved in Tie2 recognition. **Structure,** v. 13, n. 5, p. 825-32, May 2005. ISSN 0969-2126. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15893672 >.

Basuroy, S. et al. Nox4 NADPH oxidase-derived reactive oxygen species, via endogenous carbon monoxide, promote survival of brain endothelial cells during TNF-α-induced apoptosis. **Am J Physiol Cell Physiol,** v. 300, n. 2, p. C256-65, Feb 2011. ISSN 1522-1563. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21123734 >.

Bataller, R. et al. NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. **J Clin Invest,** v. 112, n. 9, p. 1383-94, Nov 2003. ISSN 0021-9738. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14597764 >.

Beaudry, P. et al. Endothelial progenitor cells contribute to accelerated liver regeneration. **J Pediatr Surg,** v. 42, n. 7, p. 1190-8, Jul 2007. ISSN 1531-5037. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17618879 >.

Beg, A. A. et al. Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice. **Genes Dev,** v. 9, n. 22, p. 2736-46, Nov 1995. ISSN 0890-9369. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/7590249 >.

Berra, E.; Pagès, G.; Pouysségur, J. MAP kinases and hypoxia in the control of VEGF expression. **Cancer Metastasis Rev,** v. 19, n. 1-2, p. 139-45, 2000. ISSN 0167-7659. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11191053 >.

Bhatnagar, S. et al. TWEAK causes myotube atrophy through coordinated activation of ubiquitin-proteasome system, autophagy, and caspases. **J Cell Physiol,** v. 227, n. 3, p. 1042-51, Mar 2012. ISSN 1097-4652. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21567392 >.

Biancone, L. et al. Activation of CD40 favors the growth and vascularization of Kaposi's sarcoma. **J Immunol,** v. 163, n. 11, p. 6201-8, Dec 1999. ISSN 0022-1767. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10570312 >.

Biancone, L. et al. Development of inflammatory angiogenesis by local stimulation of Fas in vivo. **J Exp Med,** v. 186, n. 1, p. 147-52, Jul 1997. ISSN 0022-1007. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9207009 >.

Bikfalvi, A. Platelet factor 4: an inhibitor of angiogenesis. **Semin Thromb Hemost,** v. 30, n. 3, p. 379-85, Jun 2004. ISSN 0094-6176. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15282661 >.

Bilzer, M.; Roggel, F.; Gerbes, A. L. Role of Kupffer cells in host defense and liver disease. **Liver Int,** v. 26, n. 10, p. 1175-86, Dec 2006. ISSN 1478-3223. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17105582 >.

Bird, T. G. et al. Bone marrow injection stimulates hepatic ductular reactions in the absence of injury via macrophage-mediated TWEAK signaling. **Proc Natl Acad Sci U S A,** v. 110, n. 16, p. 6542-7, Apr 2013. ISSN 1091-6490. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23576749 >.

Björnsson, E. S.; Kilander, A. F.; Olsson, R. G. Bile duct bacterial isolates in primary sclerosing cholangitis and certain other forms of cholestasis--a study of bile cultures from ERCP. **Hepatogastroenterology,** v. 47, n. 36, p. 1504-8, 2000 Nov-Dec 2000. ISSN 0172-6390. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11148988 >.

Blanco-Colio, L. M. TWEAK/Fn14 Axis: A Promising Target for the Treatment of Cardiovascular Diseases. **Front Immunol,** v. 5, p. 3, 2014. ISSN 1664-3224. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24478772 >.

Block, G. D. et al. Population expansion, clonal growth, and specific differentiation patterns in primary cultures of hepatocytes induced by HGF/SF, EGF and TGF alpha in a chemically defined (HGM) medium. **J Cell Biol,** v. 132, n. 6, p. 1133-49, Mar 1996. ISSN 0021-9525. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8601590 >.

Bluff, J. E. et al. Tissue factor, angiogenesis and tumour progression. **Breast Cancer Res,** v. 10, n. 2, p. 204, 2008. ISSN 1465-542X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18373885 >.

Bobé, P. et al. Fas-mediated liver damage in MRL hemopoietic chimeras undergoing lpr-mediated graft-versus-host disease. **J Immunol,** v. 159, n. 9, p. 4197-204, Nov 1997. ISSN 0022-1767. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9379013 >.

Bodnar, R. J.; Yates, C. C.; Wells, A. IP-10 blocks vascular endothelial growth factor-induced endothelial cell motility and tube formation via inhibition of calpain. **Circ Res,** v. 98, n. 5, p. 617-25, Mar 2006. ISSN 1524-4571. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16484616 >.

Bonizzi, G.; Karin, M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. **Trends Immunol,** v. 25, n. 6, p. 280-8, Jun 2004. ISSN 1471-4906. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15145317 >.

Boron, W. F.; Boulpaep, E. L. **Medical physiology : a cellular and molecular approach**. 2nd ed., International ed. Philadelphia, Pa. ; London: Saunders, 2009. ISBN 9781416031154 (hbk.) : ¹49.99

1416031154 (hbk.) : ¹49.99.

Bradley, J. R. TNF-mediated inflammatory disease. **J Pathol,** v. 214, n. 2, p. 149-60, Jan 2008. ISSN 0022-3417. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18161752 >.

Bremer, E. Targeting of the tumor necrosis factor receptor superfamily for cancer immunotherapy. **ISRN Oncol,** v. 2013, p. 371854, 2013. ISSN 2090-5661. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23840967 >.

Brooks, P. C. Role of integrins in angiogenesis. **Eur J Cancer,** v. 32A, n. 14, p. 2423-9, Dec 1996. ISSN 0959-8049 (Print)

0959-8049 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9059330 >.

Brouillet, S. et al. Molecular characterization of EG-VEGF-mediated angiogenesis: differential effects on microvascular and macrovascular endothelial cells. **Mol Biol Cell,** v. 21, n. 16, p. 2832-43, Aug 2010. ISSN 1939-4586. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20587779 >.

Brown, S. A. et al. TWEAK-independent Fn14 self-association and NF-κB activation is mediated by the C-terminal region of the Fn14 cytoplasmic domain. **PLoS One,** v. 8, n. 6, p. e65248, 2013. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23750247 >.

Brown, S. A. et al. TWEAK binding to the Fn14 cysteine-rich domain depends on charged residues located in both the A1 and D2 modules. **Biochem J,** v. 397, n. 2, p. 297-304, Jul 2006. ISSN 1470-8728. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16526941 >.

Brown, S. A. et al. The Fn14 cytoplasmic tail binds tumour-necrosis-factor-receptor-associated factors 1, 2, 3 and 5 and mediates nuclear factor-kappaB activation. **Biochem J,** v. 371, n. Pt 2, p. 395-403, Apr 2003. ISSN 0264-6021. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12529173 >.

Bruix, J. et al. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. **J Hepatol,** v. 35, n. 3, p. 421-30, Sep 2001. ISSN 0168-8278. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11592607 >.

Burkly, L. C.; Dohi, T. The TWEAK/Fn14 pathway in tissue remodeling: for better or for worse. **Adv Exp Med Biol,** v. 691, p. 305-22, 2011. ISSN 0065-2598. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21153335 >.

Burkly, L. C. et al. TWEAKing tissue remodeling by a multifunctional cytokine: role of TWEAK/Fn14 pathway in health and disease. **Cytokine,** v. 40, n. 1, p. 1-16, Oct 2007. ISSN 1096-0023. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17981048 >.

Burkly, L. C.; Michaelson, J. S.; Zheng, T. S. TWEAK/Fn14 pathway: an immunological switch for shaping tissue responses. **Immunol Rev,** v. 244, n. 1, p. 99-114, Nov 2011. ISSN 1600-065X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22017434 >.

Byun, J. S.; Jeong, W. I. Involvement of hepatic innate immunity in alcoholic liver disease. **Immune Netw,** v. 10, n. 6, p. 181-7, Dec 2010. ISSN 2092-6685. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21286378 >.

Cao, Y. et al. Kringle domains of human angiostatin. Characterization of the anti-proliferative activity on endothelial cells. **J Biol Chem,** v. 271, n. 46, p. 29461-7, Nov 1996. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8910613 >.

Carmeliet, P. Basic Concepts of (Myocardial) Angiogenesis: Role of Vascular Endothelial Growth Factor and Angiopoietin. **Curr Interv Cardiol Rep,** v. 1, n. 4, p. 322-335, Dec 1999. ISSN 1523-3839. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11096639 >.

Chacón, M. R. et al. Expression of TWEAK and its receptor Fn14 in human subcutaneous adipose tissue. Relationship with other inflammatory cytokines in obesity. **Cytokine,** v. 33, n. 3, p. 129-37, Feb 2006. ISSN 1043-4666. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16503147 >.

Chaplin, D. D. Overview of the immune response. **J Allergy Clin Immunol,** v. 125, n. 2 Suppl 2, p. S3-23, Feb 2010. ISSN 1097-6825. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20176265 >.

Chapman, M. S. et al. TWEAK signals through JAK-STAT to induce tumor cell apoptosis. **Cytokine,** v. 61, n. 1, p. 210-7, Jan 2013. ISSN 1096-0023. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23107828 >.

Chen, C. et al. Soluble CD40 ligand induces endothelial dysfunction in human and porcine coronary artery endothelial cells. **Blood,** v. 112, n. 8, p. 3205-16, Oct 2008. ISSN 1528-0020. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18658029 >.

Chen, T. et al. TWEAK enhances E-selectin and ICAM-1 expression, and may contribute to the development of cutaneous vasculitis. **PLoS One,** v. 8, n. 2, p. e56830, 2013. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23457623 >.

Chen, Y. G. et al. Activin signaling and its role in regulation of cell proliferation, apoptosis, and carcinogenesis. **Exp Biol Med (Maywood),** v. 231, n. 5, p. 534-44, May 2006. ISSN 1535-3702. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16636301 >.

Chicheportiche, Y. et al. TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. **J Biol Chem,** v. 272, n. 51, p. 32401-10, Dec 1997. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9405449 >.

Chicheportiche, Y. et al. Proinflammatory activity of TWEAK on human dermal fibroblasts and synoviocytes: blocking and enhancing effects of anti-TWEAK monoclonal antibodies. **Arthritis Res,** v. 4, n. 2, p. 126-33, 2002. ISSN 1465-9905. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11879548 >.

Chicheportiche, Y. et al. Down-regulated expression of TWEAK mRNA in acute and chronic inflammatory pathologies. **Biochem Biophys Res Commun,** v. 279, n. 1, p. 162-5, Dec 2000. ISSN 0006-291X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11112433 >.

Claesson-Welsh, L. Signal transduction by vascular endothelial growth factor receptors. **Biochem Soc Trans,** v. 31, n. Pt 1, p. 20-4, Feb 2003. ISSN 0300-5127. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12546646 >.

Claesson-Welsh, L. et al. Angiostatin induces endothelial cell apoptosis and activation of focal adhesion kinase independently of the integrin-binding motif RGD. **Proc Natl Acad Sci U S A,** v. 95, n. 10, p. 5579-83, May 1998. ISSN 0027-8424. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9576925 >.

Codogno, P.; Meijer, A. J. Autophagy: a potential link between obesity and insulin resistance. **Cell Metab,** v. 11, n. 6, p. 449-51, Jun 2010. ISSN 1932-7420. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20519116 >.

Conroy, A. et al. C5a enhances dysregulated inflammatory and angiogenic responses to malaria in vitro: potential implications for placental malaria. **PLoS One,** v. 4, n. 3, p. e4953, 2009. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19308263 >.

Conti, P.; DiGioacchino, M. MCP-1 and RANTES are mediators of acute and chronic inflammation. **Allergy Asthma Proc,** v. 22, n. 3, p. 133-7, May-Jun 2001. ISSN 1088-5412 (Print)

1088-5412 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11424873 >.

Corbacho, A. M.; Martínez De La Escalera, G.; Clapp, C. Roles of prolactin and related members of the prolactin/growth hormone/placental lactogen family in angiogenesis. **J Endocrinol,** v. 173, n. 2, p. 219-38, May 2002. ISSN 0022-0795. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12010630 >.

Coulon, S. et al. Angiogenesis in chronic liver disease and its complications. **Liver Int,** v. 31, n. 2, p. 146-62, Feb 2011. ISSN 1478-3231. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21073649 >.

Courtneidge, S. A. et al. Activation of Src family kinases by colony stimulating factor-1, and their association with its receptor. **EMBO J,** v. 12, n. 3, p. 943-50, Mar 1993. ISSN 0261-4189. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/7681396 >.

Culp, P. A. et al. Antibodies to TWEAK receptor inhibit human tumor growth through dual mechanisms. **Clin Cancer Res,** v. 16, n. 2, p. 497-508, Jan 2010. ISSN 1078-0432. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20068083 >.

Czaja, M. J. et al. Monocyte chemoattractant protein 1 (MCP-1) expression occurs in toxic rat liver injury and human liver disease. **J Leukoc Biol,** v. 55, n. 1, p. 120-6, Jan 1994. ISSN 0741-5400 (Print)

0741-5400 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8283136 >.

Czaja, M. J.; Xu, J.; Alt, E. Prevention of carbon tetrachloride-induced rat liver injury by soluble tumor necrosis factor receptor. **Gastroenterology,** v. 108, n. 6, p. 1849-54, Jun 1995. ISSN 0016-5085. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/7768392 >.

Dai, L. et al. TWEAK promotes ovarian cancer cell metastasis via NF-kappaB pathway activation and VEGF expression. **Cancer Lett,** v. 283, n. 2, p. 159-67, Oct 2009. ISSN 1872-7980. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19398263 >.

Danese, S. et al. Critical role of the CD40 CD40-ligand pathway in regulating mucosal inflammation-driven angiogenesis in inflammatory bowel disease. **Gut,** v. 56, n. 9, p. 1248-56, Sep 2007. ISSN 0017-5749 (Print)

0017-5749 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17317789 >.

Davila, J. A. et al. Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study. **Gut,** v. 54, n. 4, p. 533-9, Apr 2005. ISSN 0017-5749. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15753540 >.

Dawson, D. W. et al. CD36 mediates the In vitro inhibitory effects of thrombospondin-1 on endothelial cells. **J Cell Biol,** v. 138, n. 3, p. 707-17, Aug 1997. ISSN 0021-9525. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9245797 >.

De Duve, C.; Wattiaux, R. Functions of lysosomes. **Annu Rev Physiol,** v. 28, p. 435-92, 1966. ISSN 0066-4278. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/5322983 >.

De Ketelaere, A. et al. Involvement of GSK-3beta in TWEAK-mediated NF-kappaB activation. **FEBS Lett,** v. 566, n. 1-3, p. 60-4, May 2004. ISSN 0014-5793. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15147869 >.

De Spiegelaere, W. et al. Detection of hypoxia inducible factors and angiogenic growth factors during foetal endochondral and intramembranous ossification. **Anat Histol Embryol,** v. 39, n. 4, p. 376-84, Aug 2010. ISSN 1439-0264. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20545637 >.

DeLeve, L. D. Liver sinusoidal endothelial cells and liver regeneration. **J Clin Invest,** v. 123, n. 5, p. 1861-6, May 2013. ISSN 1558-8238. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23635783 >.

DeLeve, L. D. et al. Rat liver endothelial cells isolated by anti-CD31 immunomagnetic separation lack fenestrae and sieve plates. **Am J Physiol Gastrointest Liver Physiol,** v. 291, n. 6, p. G1187-9, Dec 2006. ISSN 0193-1857. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16782698 >.

Deng, C. et al. Angiogenic effect of intercellular adhesion molecule-1. **J Huazhong Univ Sci Technolog Med Sci,** v. 27, n. 1, p. 9-12, Feb 2007. ISSN 1672-0733. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17393097 >.

Desplat-Jégo, S. et al. Anti-TWEAK monoclonal antibodies reduce immune cell infiltration in the central nervous system and severity of experimental autoimmune encephalomyelitis. **Clin Immunol,** v. 117, n. 1, p. 15-23, Oct 2005. ISSN 1521-6616. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16027043 >.

Desplat-Jégo, S. et al. TWEAK is expressed at the cell surface of monocytes during multiple sclerosis. **J Leukoc Biol,** v. 85, n. 1, p. 132-5, Jan 2009. ISSN 0741-5400. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18945822 >.

Desplat-Jégo, S. et al. TWEAK is expressed by glial cells, induces astrocyte proliferation and increases EAE severity. **J Neuroimmunol,** v. 133, n. 1-2, p. 116-23, Dec 2002. ISSN 0165-5728. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12446014 >.

Dhanabal, M. et al. Cloning, expression, and in vitro activity of human endostatin. **Biochem Biophys Res Commun,** v. 258, n. 2, p. 345-52, May 1999. ISSN 0006-291X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10329390 >.

Dharmapatni, A. A. et al. TWEAK and Fn14 expression in the pathogenesis of joint inflammation and bone erosion in rheumatoid arthritis. **Arthritis Res Ther,** v. 13, n. 2, p. R51, 2011. ISSN 1478-6362. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21435232 >.

Dionne, S. et al. PPARgamma ligand 15-deoxy-delta 12,14-prostaglandin J2 sensitizes human colon carcinoma cells to TWEAK-induced apoptosis. **Anticancer Res,** v. 30, n. 1, p. 157-66, Jan 2010. ISSN 1791-7530. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20150631 >.

Dogra, C. et al. Tumor necrosis factor-like weak inducer of apoptosis inhibits skeletal myogenesis through sustained activation of nuclear factor-kappaB and degradation of MyoD protein. **J Biol Chem,** v. 281, n. 15, p. 10327-36, Apr 2006. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16461349 >.

Dogra, C. et al. Fibroblast growth factor inducible 14 (Fn14) is required for the expression of myogenic regulatory factors and differentiation of myoblasts into myotubes. Evidence for TWEAK-independent functions of Fn14 during myogenesis. **J Biol Chem,** v. 282, n. 20, p. 15000-10, May 2007. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17383968 >.

Doherty, D. G.; O'Farrelly, C. Innate and adaptive lymphoid cells in the human liver. **Immunol Rev,** v. 174, p. 5-20, Apr 2000. ISSN 0105-2896. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10807503 >.

Dohi, T. et al. TWEAK/Fn14 pathway: a nonredundant role in intestinal damage in mice through a TWEAK/intestinal epithelial cell axis. **Gastroenterology,** v. 136, n. 3, p. 912-23, Mar 2009. ISSN 1528-0012. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19109961 >.

Donohue, P. J. et al. TWEAK is an endothelial cell growth and chemotactic factor that also potentiates FGF-2 and VEGF-A mitogenic activity. **Arterioscler Thromb Vasc Biol,** v. 23, n. 4, p. 594-600, Apr 2003. ISSN 1524-4636. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12615668 >.

Dorey, K.; Amaya, E. FGF signalling: diverse roles during early vertebrate embryogenesis. **Development,** v. 137, n. 22, p. 3731-42, Nov 2010. ISSN 1477-9129. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20978071 >.

Dowman, J. K.; Tomlinson, J. W.; Newsome, P. N. Systematic review: the diagnosis and staging of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. **Aliment Pharmacol Ther,** v. 33, n. 5, p. 525-40, Mar 2011. ISSN 1365-2036. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21198708 >.

Dreux, M. et al. The autophagy machinery is required to initiate hepatitis C virus replication. **Proc Natl Acad Sci U S A,** v. 106, n. 33, p. 14046-51, Aug 2009. ISSN 1091-6490. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19666601 >.

Ebihara, N. et al. Proinflammatory effect of TWEAK/Fn14 interaction in human retinal pigment epithelial cells. **Curr Eye Res,** v. 34, n. 10, p. 836-44, Oct 2009. ISSN 1460-2202. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19895311 >.

Echeverry, R. et al. The cytokine tumor necrosis factor-like weak inducer of apoptosis and its receptor fibroblast growth factor-inducible 14 have a neuroprotective effect in the central nervous system. **J Neuroinflammation,** v. 9, p. 45, 2012. ISSN 1742-2094. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22394384 >.

Elgueta, R. et al. Molecular mechanism and function of CD40/CD40L engagement in the immune system. **Immunol Rev,** v. 229, n. 1, p. 152-72, May 2009. ISSN 1600-065X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19426221 >.

Eliceiri, B. P. et al. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. **Mol Cell,** v. 4, n. 6, p. 915-24, Dec 1999. ISSN 1097-2765. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10635317 >.

Elmore, S. Apoptosis: a review of programmed cell death. **Toxicol Pathol,** v. 35, n. 4, p. 495-516, Jun 2007. ISSN 0192-6233. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17562483 >.

,

Exley, M. A.; Koziel, M. J. To be or not to be NKT: natural killer T cells in the liver. **Hepatology,** v. 40, n. 5, p. 1033-40, Nov 2004. ISSN 0270-9139. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15486982 >.

Faint, J. M. et al. Memory T cells constitute a subset of the human CD8+CD45RA+ pool with distinct phenotypic and migratory characteristics. **J Immunol,** v. 167, n. 1, p. 212-20, Jul 2001. ISSN 0022-1767. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11418651 >.

Fajardo, L. F. et al. Dual role of tumor necrosis factor-alpha in angiogenesis. **Am J Pathol,** v. 140, n. 3, p. 539-44, Mar 1992. ISSN 0002-9440 (Print)

0002-9440 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1372154 >.

Fang, C. et al. An important role of matrix metalloproteinase-8 in angiogenesis in vitro and in vivo. **Cardiovasc Res,** v. 99, n. 1, p. 146-55, Jul 1 2013. ISSN 1755-3245 (Electronic)

0008-6363 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23512982 >.

Fausto, N.; Campbell, J. S. The role of hepatocytes and oval cells in liver regeneration and repopulation. **Mech Dev,** v. 120, n. 1, p. 117-30, Jan 2003. ISSN 0925-4773. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12490302 >.

Fava, R. A. et al. Vascular permeability factor/endothelial growth factor (VPF/VEGF): accumulation and expression in human synovial fluids and rheumatoid synovial tissue. **J Exp Med,** v. 180, n. 1, p. 341-6, Jul 1994. ISSN 0022-1007. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8006592 >.

Felli, N. et al. Multiple members of the TNF superfamily contribute to IFN-gamma-mediated inhibition of erythropoiesis. **J Immunol,** v. 175, n. 3, p. 1464-72, Aug 2005. ISSN 0022-1767. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16034083 >.

Feng, S. L. et al. The Fn14 immediate-early response gene is induced during liver regeneration and highly expressed in both human and murine hepatocellular carcinomas. **Am J Pathol,** v. 156, n. 4, p. 1253-61, Apr 2000. ISSN 0002-9440. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10751351 >.

Fernandez, M. et al. Anti-VEGF receptor-2 monoclonal antibody prevents portal-systemic collateral vessel formation in portal hypertensive mice. **Gastroenterology,** v. 126, n. 3, p. 886-94, Mar 2004. ISSN 0016-5085. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14988842 >.

Fernández, M. et al. Angiogenesis in liver disease. **J Hepatol,** v. 50, n. 3, p. 604-20, Mar 2009. ISSN 1600-0641. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19157625 >.

Ferrara, N. Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications. **Semin Oncol,** v. 29, n. 6 Suppl 16, p. 10-4, Dec 2002. ISSN 0093-7754. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12516033 >.

Festjens, N.; Vanden Berghe, T.; Vandenabeele, P. Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response. **Biochim Biophys Acta,** v. 1757, n. 9-10, p. 1371-87, 2006 Sep-Oct 2006. ISSN 0006-3002. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16950166 >.

Flory, C. M.; Jones, M. L.; Warren, J. S. Pulmonary granuloma formation in the rat is partially dependent on monocyte chemoattractant protein 1. **Lab Invest,** v. 69, n. 4, p. 396-404, Oct 1993. ISSN 0023-6837 (Print)

0023-6837 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8231108 >.

Folkman, J. Antiangiogenesis in cancer therapy--endostatin and its mechanisms of action. **Exp Cell Res,** v. 312, n. 5, p. 594-607, Mar 2006. ISSN 0014-4827. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16376330 >.

Fortin Ensign, S. P. et al. The Src homology 3 domain-containing guanine nucleotide exchange factor is overexpressed in high-grade gliomas and promotes tumor necrosis factor-like weak inducer of apoptosis-fibroblast growth factor-inducible 14-induced cell migration and invasion via tumor necrosis factor receptor-associated factor 2. **J Biol Chem,** v. 288, n. 30, p. 21887-97, Jul 2013. ISSN 1083-351X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23775076 >.

Fortin, S. P. et al. Tumor necrosis factor-like weak inducer of apoptosis stimulation of glioma cell survival is dependent on Akt2 function. **Mol Cancer Res,** v. 7, n. 11, p. 1871-81, Nov 2009. ISSN 1557-3125. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19861406 >.

Fortin, S. P. et al. Cdc42 and the guanine nucleotide exchange factors Ect2 and trio mediate Fn14-induced migration and invasion of glioblastoma cells. **Mol Cancer Res,** v. 10, n. 7, p. 958-68, Jul 2012. ISSN 1557-3125. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22571869 >.

Fotin-Mleczek, M. et al. Tumor necrosis factor receptor-associated factor (TRAF) 1 regulates CD40-induced TRAF2-mediated NF-kappaB activation. **J Biol Chem,** v. 279, n. 1, p. 677-85, Jan 2004. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14557256 >.

Fujimoto, J. et al. Expression of platelet-derived endothelial cell growth factor (PD-ECGF) related to angiogenesis in ovarian endometriosis. **J Clin Endocrinol Metab,** v. 84, n. 1, p. 359-62, Jan 1999. ISSN 0021-972X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9920107 >.

Gale, R. P.; Sparkes, R. S.; Golde, D. W. Bone marrow origin of hepatic macrophages (Kupffer cells) in humans. **Science,** v. 201, n. 4359, p. 937-8, Sep 1978. ISSN 0036-8075. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/356266 >.

Gao, H. X. et al. TNF-like weak inducer of apoptosis (TWEAK) induces inflammatory and proliferative effects in human kidney cells. **Cytokine,** v. 46, n. 1, p. 24-35, Apr 2009. ISSN 1096-0023. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19233685 >.

Gao, X.; Xu, Z. Mechanisms of action of angiogenin. **Acta Biochim Biophys Sin (Shanghai),** v. 40, n. 7, p. 619-24, Jul 2008. ISSN 1745-7270. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18604453 >.

Garcia, G. E. et al. Inhibition of CXCL16 attenuates inflammatory and progressive phases of anti-glomerular basement membrane antibody-associated glomerulonephritis. **Am J Pathol,** v. 170, n. 5, p. 1485-96, May 2007. ISSN 0002-9440 (Print)

0002-9440 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17456756 >.

Garg, A. K.; Aggarwal, B. B. Reactive oxygen intermediates in TNF signaling. **Mol Immunol,** v. 39, n. 9, p. 509-17, Dec 2002. ISSN 0161-5890. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12431383 >.

Geissmann, F. et al. Development of monocytes, macrophages, and dendritic cells. **Science,** v. 327, n. 5966, p. 656-61, Feb 2010. ISSN 1095-9203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20133564 >.

Glick, D.; Barth, S.; Macleod, K. F. Autophagy: cellular and molecular mechanisms. **J Pathol,** v. 221, n. 1, p. 3-12, May 2010. ISSN 1096-9896. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20225336 >.

Golstein, P.; Kroemer, G. Cell death by necrosis: towards a molecular definition. **Trends Biochem Sci,** v. 32, n. 1, p. 37-43, Jan 2007. ISSN 0968-0004. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17141506 >.

Gomaa, A. I. et al. Hepatocellular carcinoma: epidemiology, risk factors and pathogenesis. **World J Gastroenterol,** v. 14, n. 27, p. 4300-8, Jul 2008. ISSN 1007-9327. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18666317 >.

Granata, R. et al. Insulin-like growth factor binding protein-3 induces angiogenesis through IGF-I- and SphK1-dependent mechanisms. **J Thromb Haemost,** v. 5, n. 4, p. 835-45, Apr 2007. ISSN 1538-7933. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17388800 >.

Granger, N. **Inflammation and the Microcirculation**. Morgan and Claypool Life Sciences, 2010.

Grant, A. J. et al. Hepatic expression of secondary lymphoid chemokine (CCL21) promotes the development of portal-associated lymphoid tissue in chronic inflammatory liver disease. **Am J Pathol,** v. 160, n. 4, p. 1445-55, Apr 2002. ISSN 0002-9440. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11943728 >.

Gu, L. et al. Functional expression of TWEAK and the receptor Fn14 in human malignant ovarian tumors: possible implication for ovarian tumor intervention. **PLoS One,** v. 8, n. 3, p. e57436, 2013. ISSN 1932-6203 (Electronic)

1932-6203 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23469193 >.

Guicciardi, M. E.; Gores, G. J. Apoptosis: a mechanism of acute and chronic liver injury. **Gut,** v. 54, n. 7, p. 1024-33, Jul 2005. ISSN 0017-5749. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15951554 >.

Gurunathan, S. et al. Regulation of Fibroblast Growth Factor-inducible 14 (Fn14) Expression Levels via Ligand-independent Lysosomal Degradation. **J Biol Chem,** v. 289, n. 19, p. 12976-88, May 2014. ISSN 1083-351X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24652288 >.

Hammam, O. et al. The role of fas/fas ligand system in the pathogenesis of liver cirrhosis and hepatocellular carcinoma. **Hepat Mon,** v. 12, n. 11, p. e6132, Nov 2012. ISSN 1735-143X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23300494 >.

Han, E. S.; Mekasha, S.; Ingalls, R. R. Fibroblast growth factor-inducible 14 (Fn14) is expressed in the lower genital tract and may play a role in amplifying inflammation during infection. **J Reprod Immunol,** v. 84, n. 1, p. 16-23, Jan 2010. ISSN 1872-7603. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19963275 >.

Han, S. et al. TNF-related weak inducer of apoptosis receptor, a TNF receptor superfamily member, activates NF-kappa B through TNF receptor-associated factors. **Biochem Biophys Res Commun,** v. 305, n. 4, p. 789-96, Jun 2003. ISSN 0006-291X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12767899 >.

Hao, C. et al. TRAIL inhibits tumor growth but is nontoxic to human hepatocytes in chimeric mice. **Cancer Res,** v. 64, n. 23, p. 8502-6, Dec 2004. ISSN 0008-5472. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15574753 >.

Harada, N. et al. Pro-inflammatory effect of TWEAK/Fn14 interaction on human umbilical vein endothelial cells. **Biochem Biophys Res Commun,** v. 299, n. 3, p. 488-93, Dec 2002. ISSN 0006-291X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12445828 >.

Hayden, M. S.; Ghosh, S. Signaling to NF-kappaB. **Genes Dev,** v. 18, n. 18, p. 2195-224, Sep 2004. ISSN 0890-9369. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15371334 >.

Hedhli, N. et al. Endothelial-derived neuregulin is an important mediator of ischaemia-induced angiogenesis and arteriogenesis. **Cardiovasc Res,** v. 93, n. 3, p. 516-24, Mar 2012. ISSN 1755-3245. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22200588 >.

Heidemann, J. et al. Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. **J Biol Chem,** v. 278, n. 10, p. 8508-15, Mar 2003. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12496258 >.

Helmy, K. Y. et al. CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. **Cell,** v. 124, n. 5, p. 915-27, Mar 2006. ISSN 0092-8674. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16530040 >.

Herbst, R. S. Review of epidermal growth factor receptor biology. **Int J Radiat Oncol Biol Phys,** v. 59, n. 2 Suppl, p. 21-6, 2004. ISSN 0360-3016. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15142631 >.

Heymann, F.; Trautwein, C.; Tacke, F. Monocytes and macrophages as cellular targets in liver fibrosis. **Inflamm Allergy Drug Targets,** v. 8, n. 4, p. 307-18, Sep 2009. ISSN 2212-4055. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19534673 >.

Higuchi, M.; Aggarwal, B. B. TNF induces internalization of the p60 receptor and shedding of the p80 receptor. **J Immunol,** v. 152, n. 7, p. 3550-8, Apr 1994. ISSN 0022-1767. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8144934 >.

Hjelmström, P. Lymphoid neogenesis: de novo formation of lymphoid tissue in chronic inflammation through expression of homing chemokines. **J Leukoc Biol,** v. 69, n. 3, p. 331-9, Mar 2001. ISSN 0741-5400. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11261778 >.

Ho, D. H. et al. Soluble tumor necrosis factor-like weak inducer of apoptosis overexpression in HEK293 cells promotes tumor growth and angiogenesis in athymic nude mice. **Cancer Res,** v. 64, n. 24, p. 8968-72, Dec 2004. ISSN 0008-5472. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15604260 >.

Hofnagel, O. et al. Expression of the novel scavenger receptor SR-PSOX in cultured aortic smooth muscle cells and umbilical endothelial cells. **Arterioscler Thromb Vasc Biol,** v. 22, n. 4, p. 710-1, Apr 1 2002. ISSN 1524-4636 (Electronic)

1079-5642 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11950715 >.

Hoofring, A.; Boitnott, J.; Torbenson, M. Three-dimensional reconstruction of hepatic bridging fibrosis in chronic hepatitis C viral infection. **J Hepatol,** v. 39, n. 5, p. 738-41, Nov 2003. ISSN 0168-8278. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14568255 >.

Horie, Y. et al. Role of Kupffer cells in gut ischemia/reperfusion-induced hepatic microvascular dysfunction in mice. **Hepatology,** v. 26, n. 6, p. 1499-505, Dec 1997. ISSN 0270-9139. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9397990 >.

Hosokawa, Y. et al. Proinflammatory effects of tumour necrosis factor-like weak inducer of apoptosis (TWEAK) on human gingival fibroblasts. **Clin Exp Immunol,** v. 146, n. 3, p. 540-9, Dec 2006. ISSN 0009-9104. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17100776 >.

Huang, M. et al. Overexpression of Fn14 promotes androgen-independent prostate cancer progression through MMP-9 and correlates with poor treatment outcome. **Carcinogenesis,** v. 32, n. 11, p. 1589-96, Nov 2011. ISSN 1460-2180. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21828059 >.

Huh, H. et al. The Effects of TWEAK, Fn14, and TGF-beta1 on Degeneration of Human Intervertebral Disc. **J Korean Neurosurg Soc,** v. 47, n. 1, p. 30-5, Jan 2010. ISSN 1598-7876. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20157375 >.

Ikner, A.; Ashkenazi, A. TWEAK induces apoptosis through a death-signaling complex comprising receptor-interacting protein 1 (RIP1), Fas-associated death domain (FADD), and caspase-8. **J Biol Chem,** v. 286, n. 24, p. 21546-54, Jun 2011. ISSN 1083-351X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21525013 >.

Imamura, M. et al. Suppression of macrophage infiltration inhibits activation of hepatic stellate cells and liver fibrogenesis in rats. **Gastroenterology,** v. 128, n. 1, p. 138-46, Jan 2005. ISSN 0016-5085. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15633130 >.

Isozaki, T. et al. Evidence that CXCL16 is a potent mediator of angiogenesis and is involved in endothelial progenitor cell chemotaxis : studies in mice with K/BxN serum-induced arthritis. **Arthritis Rheum,** v. 65, n. 7, p. 1736-46, Jul 2013. ISSN 1529-0131 (Electronic)

0004-3591 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23633118 >.

Izquierdo, M. C. et al. TWEAK (tumor necrosis factor-like weak inducer of apoptosis) activates CXCL16 expression during renal tubulointerstitial inflammation. **Kidney Int,** v. 81, n. 11, p. 1098-107, Jun 2012. ISSN 1523-1755 (Electronic)

0085-2538 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22278019 >.

Jackson, J. R. et al. Modulation of angiogenesis in a model of chronic inflammation. **Inflamm Res,** v. 46 Suppl 2, p. S129-30, Aug 1997. ISSN 1023-3830. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9297545 >.

Jaeschke, H.; Smith, C. W. Cell adhesion and migration. III. Leukocyte adhesion and transmigration in the liver vasculature. **Am J Physiol,** v. 273, n. 6 Pt 1, p. G1169-73, Dec 1997. ISSN 0002-9513. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9435541 >.

Jakubowski, A. et al. TWEAK induces liver progenitor cell proliferation. **J Clin Invest,** v. 115, n. 9, p. 2330-40, Sep 2005. ISSN 0021-9738. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16110324 >.

Jakubowski, A. et al. TWEAK induces liver progenitor cell proliferation. **J Clin Invest,** v. 115, n. 9, p. 2330-40, Sep 2005. ISSN 0021-9738 (Print)

0021-9738 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16110324 >.

Jakubowski, A. et al. Dual role for TWEAK in angiogenic regulation. **J Cell Sci,** v. 115, n. Pt 2, p. 267-74, Jan 2002. ISSN 0021-9533. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11839778 >.

Jenne, C. N.; Kubes, P. Immune surveillance by the liver. **Nat Immunol,** v. 14, n. 10, p. 996-1006, Oct 2013. ISSN 1529-2916. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24048121 >.

Jeong, W. I. et al. Paracrine activation of hepatic CB1 receptors by stellate cell-derived endocannabinoids mediates alcoholic fatty liver. **Cell Metab,** v. 7, n. 3, p. 227-35, Mar 2008. ISSN 1550-4131. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18316028 >.

Jeong, W. I.; Park, O.; Gao, B. Abrogation of the antifibrotic effects of natural killer cells/interferon-gamma contributes to alcohol acceleration of liver fibrosis. **Gastroenterology,** v. 134, n. 1, p. 248-58, Jan 2008. ISSN 1528-0012. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18166357 >.

Jin, L. et al. Induction of RANTES by TWEAK/Fn14 interaction in human keratinocytes. **J Invest Dermatol,** v. 122, n. 5, p. 1175-9, May 2004. ISSN 0022-202X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15140220 >.

Justo, P. et al. Cytokine cooperation in renal tubular cell injury: the role of TWEAK. **Kidney Int,** v. 70, n. 10, p. 1750-8, Nov 2006. ISSN 0085-2538. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17003819 >.

Juza, R. M.; Pauli, E. M. Clinical and surgical anatomy of the liver: A review for clinicians. **Clin Anat**, Jan 2014. ISSN 1098-2353. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24453062 >.

Kahraman, A. et al. TRAIL mediates liver injury by the innate immune system in the bile duct-ligated mouse. **Hepatology,** v. 47, n. 4, p. 1317-30, Apr 2008. ISSN 1527-3350. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18220275 >.

Kamata, K. et al. Involvement of TNF-like weak inducer of apoptosis in the pathogenesis of collagen-induced arthritis. **J Immunol,** v. 177, n. 9, p. 6433-9, Nov 2006. ISSN 0022-1767. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17056575 >.

Kamijo, S. et al. Involvement of TWEAK/Fn14 interaction in the synovial inflammation of RA. **Rheumatology (Oxford),** v. 47, n. 4, p. 442-50, Apr 2008. ISSN 1462-0332. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18310134 >.

Kanda, H. et al. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. **J Clin Invest,** v. 116, n. 6, p. 1494-505, Jun 2006. ISSN 0021-9738 (Print)

0021-9738 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16691291 >.

Kang, L. I.; Mars, W. M.; Michalopoulos, G. K. Signals and cells involved in regulating liver regeneration. **Cells,** v. 1, n. 4, p. 1261-92, 2012. ISSN 2073-4409. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24710554 >.

Kang, Y. B. et al. Layered long-term co-culture of hepatocytes and endothelial cells on a transwell membrane: toward engineering the liver sinusoid. **Biofabrication,** v. 5, n. 4, p. 045008, Dec 2013. ISSN 1758-5090. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24280542 >.

Kaplan, H. J. et al. Fas ligand (CD95 ligand) controls angiogenesis beneath the retina. **Nat Med,** v. 5, n. 3, p. 292-7, Mar 1999. ISSN 1078-8956 (Print)

1078-8956 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10086384 >.

Kaptein, A. et al. Studies on the interaction between TWEAK and the death receptor WSL-1/TRAMP (DR3). **FEBS Lett,** v. 485, n. 2-3, p. 135-41, Nov 2000. ISSN 0014-5793. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11094155 >.

Karaca, G. et al. TWEAK/Fn14 signaling is required for liver regeneration after partial hepatectomy in mice. **PLoS One,** v. 9, n. 1, p. e83987, 2014. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24416188 >.

Karkkainen, M. J.; Petrova, T. V. Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. **Oncogene,** v. 19, n. 49, p. 5598-605, Nov 2000. ISSN 0950-9232. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11114740 >.

Karlmark, K. R. et al. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. **Hepatology,** v. 50, n. 1, p. 261-74, Jul 2009. ISSN 1527-3350. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19554540 >.

Kaur, S. et al. Increased number and function of endothelial progenitor cells stimulate angiogenesis by resident liver sinusoidal endothelial cells (SECs) in cirrhosis through paracrine factors. **J Hepatol,** v. 57, n. 6, p. 1193-8, Dec 2012. ISSN 1600-0641. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22824816 >.

Kawabe, T. et al. CD40/CD40 ligand interactions in immune responses and pulmonary immunity. **Nagoya J Med Sci,** v. 73, n. 3-4, p. 69-78, Aug 2011. ISSN 0027-7622. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21928689 >.

Kawakita, T. et al. Functional expression of TWEAK in human hepatocellular carcinoma: possible implication in cell proliferation and tumor angiogenesis. **Biochem Biophys Res Commun,** v. 318, n. 3, p. 726-33, Jun 2004. ISSN 0006-291X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15144899 >.

Kawakita, T. et al. Functional expression of TWEAK in human colonic adenocarcinoma cells. **Int J Oncol,** v. 26, n. 1, p. 87-93, Jan 2005. ISSN 1019-6439. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15586228 >.

Kim, I. et al. Molecular cloning, expression, and characterization of angiopoietin-related protein. angiopoietin-related protein induces endothelial cell sprouting. **J Biol Chem,** v. 274, n. 37, p. 26523-8, Sep 1999. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10473614 >.

Kim, J. S. et al. Role of the mitochondrial permeability transition in apoptotic and necrotic death after ischemia/reperfusion injury to hepatocytes. **Curr Mol Med,** v. 3, n. 6, p. 527-35, Sep 2003. ISSN 1566-5240. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14527084 >.

Kim, S. H. et al. TWEAK can induce pro-inflammatory cytokines and matrix metalloproteinase-9 in macrophages. **Circ J,** v. 68, n. 4, p. 396-9, Apr 2004. ISSN 1346-9843. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15056843 >.

Kim, W. U. et al. Soluble Fas ligand inhibits angiogenesis in rheumatoid arthritis. **Arthritis Res Ther,** v. 9, n. 2, p. R42, 2007. ISSN 1478-6362 (Electronic)

1478-6354 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17459170 >.

Kimura, K. et al. Pathogenic role of B cells in anti-CD40-induced necroinflammatory liver disease. **Am J Pathol,** v. 168, n. 3, p. 786-95, Mar 2006. ISSN 0002-9440. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16507894 >.

Kirillova, I.; Chaisson, M.; Fausto, N. Tumor necrosis factor induces DNA replication in hepatic cells through nuclear factor kappaB activation. **Cell Growth Differ,** v. 10, n. 12, p. 819-28, Dec 1999. ISSN 1044-9523. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10616907 >.

Kitlinska, J. et al. Dual role of dipeptidyl peptidase IV (DPP IV) in angiogenesis and vascular remodeling. **Adv Exp Med Biol,** v. 524, p. 215-22, 2003. ISSN 0065-2598. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12675242 >.

Klein, S.; Roghani, M.; Rifkin, D. B. Fibroblast growth factors as angiogenesis factors: new insights into their mechanism of action. **EXS,** v. 79, p. 159-92, 1997. ISSN 1023-294X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9002232 >.

Klover, P. J.; Mooney, R. A. Hepatocytes: critical for glucose homeostasis. **Int J Biochem Cell Biol,** v. 36, n. 5, p. 753-8, May 2004. ISSN 1357-2725. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15061128 >.

Koehler, B. C. et al. TRAIL-induced apoptosis of hepatocellular carcinoma cells is augmented by targeted therapies. **World J Gastroenterol,** v. 15, n. 47, p. 5924-35, Dec 2009. ISSN 2219-2840. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20014456 >.

Kubiczkova, L. et al. TGF-β - an excellent servant but a bad master. **J Transl Med,** v. 10, p. 183, 2012. ISSN 1479-5876. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22943793 >.

Kudo, S. et al. A novel migration pathway for rat dendritic cells from the blood: hepatic sinusoids-lymph translocation. **J Exp Med,** v. 185, n. 4, p. 777-84, Feb 1997. ISSN 0022-1007. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9034155 >.

Kumar, M. et al. TNF-like weak inducer of apoptosis (TWEAK) activates proinflammatory signaling pathways and gene expression through the activation of TGF-beta-activated kinase 1. **J Immunol,** v. 182, n. 4, p. 2439-48, Feb 2009. ISSN 1550-6606. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19201899 >.

Kuramitsu, K. et al. Failure of fibrotic liver regeneration in mice is linked to a severe fibrogenic response driven by hepatic progenitor cell activation. **Am J Pathol,** v. 183, n. 1, p. 182-94, Jul 2013. ISSN 1525-2191. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23680654 >.

Kwon, O. H. et al. Elevated fibroblast growth factor-inducible 14 expression promotes gastric cancer growth via nuclear factor-κB and is associated with poor patient outcome. **Cancer Lett,** v. 314, n. 1, p. 73-81, Jan 2012. ISSN 1872-7980. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21993017 >.

Kyriakakis, E. et al. IL-8-mediated angiogenic responses of endothelial cells to lipid antigen activation of iNKT cells depend on EGFR transactivation. **J Leukoc Biol,** v. 90, n. 5, p. 929-39, Nov 2011. ISSN 1938-3673. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21807744 >.

Lalor, P. F. et al. Human hepatic sinusoidal endothelial cells can be distinguished by expression of phenotypic markers related to their specialised functions in vivo. **World J Gastroenterol,** v. 12, n. 34, p. 5429-39, Sep 2006. ISSN 1007-9327. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17006978 >.

Lalor, P. F. et al. Recruitment of lymphocytes to the human liver. **Immunol Cell Biol,** v. 80, n. 1, p. 52-64, Feb 2002. ISSN 0818-9641. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11869363 >.

Lammens, A. et al. Crystal structure of human TWEAK in complex with the Fab fragment of a neutralizing antibody reveals insights into receptor binding. **PLoS One,** v. 8, n. 5, p. e62697, 2013. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23667509 >.

Lampugnani, M. G. et al. A novel endothelial-specific membrane protein is a marker of cell-cell contacts. **J Cell Biol,** v. 118, n. 6, p. 1511-22, Sep 1992. ISSN 0021-9525. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1522121 >.

Lawler, J. The functions of thrombospondin-1 and-2. **Curr Opin Cell Biol,** v. 12, n. 5, p. 634-40, Oct 2000. ISSN 0955-0674. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10978901 >.

Lawson, N. D. et al. phospholipase C gamma-1 is required downstream of vascular endothelial growth factor during arterial development. **Genes Dev,** v. 17, n. 11, p. 1346-51, Jun 2003. ISSN 0890-9369. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12782653 >.

Le Couteur, D. G. et al. Hepatic artery flow and propranolol metabolism in perfused cirrhotic rat liver. **J Pharmacol Exp Ther,** v. 289, n. 3, p. 1553-8, Jun 1999. ISSN 0022-3565. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10336552 >.

Leali, D. et al. Long pentraxin-3 inhibits FGF8b-dependent angiogenesis and growth of steroid hormone-regulated tumors. **Mol Cancer Ther,** v. 10, n. 9, p. 1600-10, Sep 2011. ISSN 1538-8514. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21764903 >.

LeCouter, J. et al. Angiogenesis-independent endothelial protection of liver: role of VEGFR-1. **Science,** v. 299, n. 5608, p. 890-3, Feb 7 2003. ISSN 1095-9203 (Electronic)

0036-8075 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12574630 >.

Lee, J. S. et al. Sinusoidal remodeling and angiogenesis: a new function for the liver-specific pericyte? **Hepatology,** v. 45, n. 3, p. 817-25, Mar 2007. ISSN 0270-9139. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17326208 >.

Lehmann, G. L. et al. LPS induces the TNF-alpha-mediated downregulation of rat liver aquaporin-8: role in sepsis-associated cholestasis. **Am J Physiol Gastrointest Liver Physiol,** v. 294, n. 2, p. G567-75, Feb 2008. ISSN 0193-1857. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18174273 >.

Leibovich, S. J. et al. Macrophage-induced angiogenesis is mediated by tumour necrosis factor-alpha. **Nature,** v. 329, n. 6140, p. 630-2, Oct 15-21 1987. ISSN 0028-0836 (Print)

0028-0836 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/2443857 >.

Levi-Schaffer, F.; Pe'er, J. Mast cells and angiogenesis. **Clin Exp Allergy,** v. 31, n. 4, p. 521-4, Apr 2001. ISSN 0954-7894. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11359417 >.

Ley, K. et al. Getting to the site of inflammation: the leukocyte adhesion cascade updated. **Nat Rev Immunol,** v. 7, n. 9, p. 678-89, Sep 2007. ISSN 1474-1741. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17717539 >.

Li, A. et al. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. **J Immunol,** v. 170, n. 6, p. 3369-76, Mar 2003. ISSN 0022-1767. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12626597 >.

Li, H. et al. Tumor necrosis factor-related weak inducer of apoptosis augments matrix metalloproteinase 9 (MMP-9) production in skeletal muscle through the activation of nuclear factor-kappaB-inducing kinase and p38 mitogen-activated protein kinase: a potential role of MMP-9 in

myopathy. **J Biol Chem,** v. 284, n. 7, p. 4439-50, Feb 2009. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19074147 >.

Liang, J. et al. [Relationship between liver damage and serum levels of IL-18, TNF-alpha and NO in patients with acute pancreatitis]. **Nan Fang Yi Ke Da Xue Xue Bao,** v. 30, n. 8, p. 1912-4, Aug 2010. ISSN 1673-4254. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20813700 >.

Liaskou, E. et al. Monocyte subsets in human liver disease show distinct phenotypic and functional characteristics. **Hepatology,** v. 57, n. 1, p. 385-98, Jan 2013. ISSN 1527-3350. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22911542 >.

Liu, C. J.; Kao, J. H. Hepatitis B virus-related hepatocellular carcinoma: epidemiology and pathogenic role of viral factors. **J Chin Med Assoc,** v. 70, n. 4, p. 141-5, Apr 2007. ISSN 1726-4901. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17475593 >.

Liu, H. et al. Increased cytochrome P-450 2E1 expression sensitizes hepatocytes to c-Jun-mediated cell death from TNF-alpha. **Am J Physiol Gastrointest Liver Physiol,** v. 282, n. 2, p. G257-66, Feb 2002. ISSN 0193-1857. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11804847 >.

Liu, M. L. et al. Collagenase pretreatment and the mitogenic effects of hepatocyte growth factor and transforming growth factor-alpha in adult rat liver. **Hepatology,** v. 19, n. 6, p. 1521-7, Jun 1994. ISSN 0270-9139. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8188184 >.

Liu, Z. et al. Interleukin-6, hepatocyte growth factor, and their receptors in biliary epithelial cells during a type I ductular reaction in mice: interactions between the periductal inflammatory and stromal cells and the biliary epithelium. **Hepatology,** v. 28, n. 5, p. 1260-8, Nov 1998. ISSN 0270-9139. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9794910 >.

Llauradó, G. et al. Serum levels of TWEAK and scavenger receptor CD163 in type 1 diabetes mellitus: relationship with cardiovascular risk factors. a case-control study. **PLoS One,** v. 7, n. 8, p. e43919, 2012. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22937125 >.

Lodish, H. F. **Molecular cell biology**. 6th ed. / Harvey Lodish ... [et al.]. Basingstoke: W. H. Freeman, 2008. ISBN 9780716776017 : No price

0716776014 : No price.

Ludwig, A.; Weber, C. Transmembrane chemokines: versatile 'special agents' in vascular inflammation. **Thromb Haemost,** v. 97, n. 5, p. 694-703, May 2007. ISSN 0340-6245 (Print)

0340-6245 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17479179 >.

Luttun, A.; Tjwa, M.; Carmeliet, P. Placental growth factor (PlGF) and its receptor Flt-1 (VEGFR-1): novel therapeutic targets for angiogenic disorders. **Ann N Y Acad Sci,** v. 979, p. 80-93, Dec 2002. ISSN 0077-8923. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12543719 >.

Lynch, C. N. et al. TWEAK induces angiogenesis and proliferation of endothelial cells. **J Biol Chem,** v. 274, n. 13, p. 8455-9, Mar 26 1999. ISSN 0021-9258 (Print)

0021-9258 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10085077 >.

Ma, J. et al. MCP-1 mediates TGF-beta-induced angiogenesis by stimulating vascular smooth muscle cell migration. **Blood,** v. 109, n. 3, p. 987-94, Feb 1 2007. ISSN 0006-4971 (Print)

0006-4971 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17032917 >.

MacEwan, D. J. TNF ligands and receptors--a matter of life and death. **Br J Pharmacol,** v. 135, n. 4, p. 855-75, Feb 2002. ISSN 0007-1188. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11861313 >.

Mach, F. et al. T lymphocytes induce endothelial cell matrix metalloproteinase expression by a CD40L-dependent mechanism: implications for tubule formation. **Am J Pathol,** v. 154, n. 1, p. 229-38, Jan 1999. ISSN 0002-9440. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9916937 >.

Maecker, H. et al. TWEAK attenuates the transition from innate to adaptive immunity. **Cell,** v. 123, n. 5, p. 931-44, Dec 2005. ISSN 0092-8674. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16325585 >.

Maffucci, T. et al. A phosphoinositide 3-kinase/phospholipase Cgamma1 pathway regulates fibroblast growth factor-induced capillary tube formation. **PLoS One,** v. 4, n. 12, p. e8285, 2009. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20011604 >.

Mak, K. M.; Lieber, C. S. Alterations in endothelial fenestrations in liver sinusoids of baboons fed alcohol: a scanning electron microscopic study. **Hepatology,** v. 4, n. 3, p. 386-91, May-Jun 1984. ISSN 0270-9139 (Print)

0270-9139 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/6539290 >.

Malarkey, D. E. et al. New insights into functional aspects of liver morphology. **Toxicol Pathol,** v. 33, n. 1, p. 27-34, 2005. ISSN 0192-6233. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15805053 >.

Malhi, H. et al. Free fatty acids sensitise hepatocytes to TRAIL mediated cytotoxicity. **Gut,** v. 56, n. 8, p. 1124-31, Aug 2007. ISSN 0017-5749. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17470478 >.

Malhi, H.; Gores, G. J.; Lemasters, J. J. Apoptosis and necrosis in the liver: a tale of two deaths? **Hepatology,** v. 43, n. 2 Suppl 1, p. S31-44, Feb 2006. ISSN 0270-9139. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16447272 >.

Mandrekar, P.; Szabo, G. Signalling pathways in alcohol-induced liver inflammation. **J Hepatol,** v. 50, n. 6, p. 1258-66, Jun 2009. ISSN 1600-0641. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19398236 >.

Marsters, S. A. et al. Identification of a ligand for the death-domain-containing receptor Apo3. **Curr Biol,** v. 8, n. 9, p. 525-8, Apr 1998. ISSN 0960-9822. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9560343 >.

Martin, A.; Komada, M. R.; Sane, D. C. Abnormal angiogenesis in diabetes mellitus. **Med Res Rev,** v. 23, n. 2, p. 117-45, Mar 2003. ISSN 0198-6325 (Print)

0198-6325 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12500286 >.

Martini, F.; Ober, W. C.; Welch, K. **Fundamentals of anatomy & physiology**. 7th ed., international ed. / Frederic H. Martini ; with William C. Ober, art coordinator and illustrator, Claire W. Garrison, illustrator ; Kathleen Welch, clinical consultant ; Ralph T. Hutchings, biomedical photographer. San Francisco ; London: Pearson/Benjamin Cummings, 2006. ISBN 0321315227 (pbk.) : No price 0321311981.

Marx, J. Cancer research. Inflammation and cancer: the link grows stronger. **Science,** v. 306, n. 5698, p. 966-8, Nov 2004. ISSN 1095-9203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15528423 >.

Matloubian, M. et al. A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. **Nat Immunol,** v. 1, n. 4, p. 298-304, Oct 2000. ISSN 1529-2908 (Print)

1529-2908 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11017100 >.

McClain, C. J. et al. Tumor necrosis factor and alcoholic liver disease. **Alcohol Clin Exp Res,** v. 22, n. 5 Suppl, p. 248S-252S, Aug 1998. ISSN 0145-6008. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9727645 >.

McEver, R. P.; Cummings, R. D. Role of PSGL-1 binding to selectins in leukocyte recruitment. **J Clin Invest,** v. 100, n. 11 Suppl, p. S97-103, Dec 1997. ISSN 0021-9738. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9413410 >.

McInnes, I. B.; Schett, G. Cytokines in the pathogenesis of rheumatoid arthritis. **Nat Rev Immunol,** v. 7, n. 6, p. 429-42, Jun 2007. ISSN 1474-1733. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17525752 >.

McNab, G. et al. Vascular adhesion protein 1 mediates binding of T cells to human hepatic endothelium. **Gastroenterology,** v. 110, n. 2, p. 522-8, Feb 1996. ISSN 0016-5085. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8566600 >.

Medzhitov, R. Origin and physiological roles of inflammation. **Nature,** v. 454, n. 7203, p. 428-35, Jul 2008. ISSN 1476-4687. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18650913 >.

Mehta, V. B.; Besner, G. E. HB-EGF promotes angiogenesis in endothelial cells via PI3-kinase and MAPK signaling pathways. **Growth Factors,** v. 25, n. 4, p. 253-63, Aug 2007. ISSN 0897-7194. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18092233 >.

Meighan-Mantha, R. L. et al. The mitogen-inducible Fn14 gene encodes a type I transmembrane protein that modulates fibroblast adhesion and migration. **J Biol Chem,** v. 274, n. 46, p. 33166-76, Nov 1999. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10551889 >.

Michaelson, J. S. et al. Development of an Fn14 agonistic antibody as an anti-tumor agent. **MAbs,** v. 3, n. 4, p. 362-75, 2011 Jul-Aug 2011. ISSN 1942-0870. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21697654 >.

Michaelson, J. S. et al. Role of TWEAK in lupus nephritis: a bench-to-bedside review. **J Autoimmun,** v. 39, n. 3, p. 130-42, Sep 2012. ISSN 1095-9157. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22727560 >.

Michalopoulos, G. K. Liver regeneration. **J Cell Physiol,** v. 213, n. 2, p. 286-300, Nov 2007. ISSN 0021-9541. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17559071 >.

Michielsen, P. P.; Francque, S. M.; van Dongen, J. L. Viral hepatitis and hepatocellular carcinoma. **World J Surg Oncol,** v. 3, p. 27, May 2005. ISSN 1477-7819. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15907199 >.

Milbrandt, J. et al. Persephin, a novel neurotrophic factor related to GDNF and neurturin. **Neuron,** v. 20, n. 2, p. 245-53, Feb 1998. ISSN 0896-6273. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9491986 >.

Mizushima, N. Autophagy: process and function. **Genes Dev,** v. 21, n. 22, p. 2861-73, Nov 2007. ISSN 0890-9369. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18006683 >.

Moreno, J. A. et al. Peripheral artery disease is associated with a high CD163/TWEAK plasma ratio. **Arterioscler Thromb Vasc Biol,** v. 30, n. 6, p. 1253-62, Jun 2010. ISSN 1524-4636. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20299688 >.

Moreno, J. A. et al. The CD163-expressing macrophages recognize and internalize TWEAK: potential consequences in atherosclerosis. **Atherosclerosis,** v. 207, n. 1, p. 103-10, Nov 2009. ISSN 1879-1484. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19473660 >.

Morio, L. A. et al. Distinct roles of tumor necrosis factor-alpha and nitric oxide in acute liver injury induced by carbon tetrachloride in mice. **Toxicol Appl Pharmacol,** v. 172, n. 1, p. 44-51, Apr 2001. ISSN 0041-008X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11264022 >.

Muriel, P. Role of free radicals in liver diseases. **Hepatol Int,** v. 3, n. 4, p. 526-36, Dec 2009. ISSN 1936-0541. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19941170 >.

Muñoz-García, B. et al. Fn14 is upregulated in cytokine-stimulated vascular smooth muscle cells and is expressed in human carotid atherosclerotic plaques: modulation by atorvastatin. **Stroke,** v. 37, n. 8, p. 2044-53, Aug 2006. ISSN 1524-4628. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16809572 >.

Muñoz-García, B. et al. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) enhances vascular and renal damage induced by hyperlipidemic diet in ApoE-knockout mice. **Arterioscler Thromb Vasc Biol,** v. 29, n. 12, p. 2061-8, Dec 2009. ISSN 1524-4636. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19778942 >.

Nakayama, M. et al. Multiple pathways of TWEAK-induced cell death. **J Immunol,** v. 168, n. 2, p. 734-43, Jan 2002. ISSN 0022-1767. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11777967 >.

Nakayama, M. et al. Fibroblast growth factor-inducible 14 mediates multiple pathways of TWEAK-induced cell death. **J Immunol,** v. 170, n. 1, p. 341-8, Jan 2003. ISSN 0022-1767. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12496418 >.

Nakayama, M. et al. Involvement of TWEAK in interferon gamma-stimulated monocyte cytotoxicity. **J Exp Med,** v. 192, n. 9, p. 1373-80, Nov 2000. ISSN 0022-1007. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11067885 >.

Nathan, C.; Cunningham-Bussel, A. Beyond oxidative stress: an immunologist's guide to reactive oxygen species. **Nat Rev Immunol,** v. 13, n. 5, p. 349-61, May 2013. ISSN 1474-1741. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23618831 >.

Neyt, K. et al. Tertiary lymphoid organs in infection and autoimmunity. **Trends Immunol,** v. 33, n. 6, p. 297-305, Jun 2012. ISSN 1471-4981. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22622061 >.

Nishihira, J. Macrophage migration inhibitory factor (MIF): its essential role in the immune system and cell growth. **J Interferon Cytokine Res,** v. 20, n. 9, p. 751-62, Sep 2000. ISSN 1079-9907. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11032394 >.

Nishioji, K. et al. Experimental liver injury induced by Propionibacterium acnes and lipopolysaccharide in macrophage colony stimulating factor-deficient osteopetrotic (op/op) mice. **Dig Dis Sci,** v. 44, n. 10, p. 1975-84, Oct 1999. ISSN 0163-2116. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10548345 >.

Nishioka, K. Hepatitis C virus infection in Japan. **Gastroenterol Jpn,** v. 26 Suppl 3, p. 152-5, Jul 1991. ISSN 0435-1339. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1909256 >.

Niu, J. et al. Monocyte chemotactic protein (MCP)-1 promotes angiogenesis via a novel transcription factor, MCP-1-induced protein (MCPIP). **J Biol Chem,** v. 283, n. 21, p. 14542-51, May 2008. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18364357 >.

Novo, E. et al. Proangiogenic cytokines as hypoxia-dependent factors stimulating migration of human hepatic stellate cells. **Am J Pathol,** v. 170, n. 6, p. 1942-53, Jun 2007. ISSN 0002-9440. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17525262 >.

Németh, Z. et al. Claudin-1, -2, -3, -4, -7, -8, and -10 protein expression in biliary tract cancers. **J Histochem Cytochem,** v. 57, n. 2, p. 113-21, Feb 2009. ISSN 0022-1554. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18854598 >.

O'Hara, S. P. et al. The dynamic biliary epithelia: molecules, pathways, and disease. **J Hepatol,** v. 58, n. 3, p. 575-82, Mar 2013. ISSN 1600-0641. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23085249 >.

Oda, M.; Yokomori, H.; Han, J. Y. Regulatory mechanisms of hepatic microcirculation. **Clin Hemorheol Microcirc,** v. 29, n. 3-4, p. 167-82, 2003. ISSN 1386-0291 (Print)

1386-0291 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14724338 >.

Old, L. J. Tumor necrosis factor (TNF). **Science,** v. 230, n. 4726, p. 630-2, Nov 8 1985. ISSN 0036-8075 (Print)

0036-8075 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/2413547 >.

Olsson, A. K. et al. VEGF receptor signalling - in control of vascular function. **Nat Rev Mol Cell Biol,** v. 7, n. 5, p. 359-71, May 2006. ISSN 1471-0072. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16633338 >.

Otrock, Z. K. et al. Understanding the biology of angiogenesis: review of the most important molecular mechanisms. **Blood Cells Mol Dis,** v. 39, n. 2, p. 212-20, Sep-Oct 2007. ISSN 1079-9796 (Print)

1079-9796 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17553709 >.

Paleolog, E. M. Angiogenesis: a critical process in the pathogenesis of RA--a role for VEGF? **Br J Rheumatol,** v. 35, n. 10, p. 917-9, Oct 1996. ISSN 0263-7103. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8883427 >.

Paleolog, E. M. Angiogenesis in rheumatoid arthritis. **Arthritis Res,** v. 4 Suppl 3, p. S81-90, 2002. ISSN 1465-9905. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12110126 >.

Park, J. S. et al. TWEAK promotes the production of Interleukin-17 in rheumatoid arthritis. **Cytokine,** v. 60, n. 1, p. 143-9, Oct 2012. ISSN 1096-0023. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22819243 >.

Parkin, J.; Cohen, B. An overview of the immune system. **Lancet,** v. 357, n. 9270, p. 1777-89, Jun 2001. ISSN 0140-6736. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11403834 >.

Pearson, G. et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. **Endocr Rev,** v. 22, n. 2, p. 153-83, Apr 2001. ISSN 0163-769X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11294822 >.

Pepper, M. S. Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. **Cytokine Growth Factor Rev,** v. 8, n. 1, p. 21-43, Mar 1997. ISSN 1359-6101. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9174661 >.

Pincus, T.; Callahan, L. F. What is the natural history of rheumatoid arthritis? **Rheum Dis Clin North Am,** v. 19, n. 1, p. 123-51, Feb 1993. ISSN 0889-857X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8356248 >.

Pinzani, M.; Marra, F. Cytokine receptors and signaling in hepatic stellate cells. **Semin Liver Dis,** v. 21, n. 3, p. 397-416, Aug 2001. ISSN 0272-8087. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11586468 >.

Portt, L. et al. Anti-apoptosis and cell survival: a review. **Biochim Biophys Acta,** v. 1813, n. 1, p. 238-59, Jan 2011. ISSN 0006-3002. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20969895 >.

Priester, S.; Wise, C.; Glaser, S. S. Involvement of cholangiocyte proliferation in biliary fibrosis. **World J Gastrointest Pathophysiol,** v. 1, n. 2, p. 30-7, Jun 2010. ISSN 2150-5330. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21607140 >.

Pulford, K. et al. A monocyte/macrophage antigen recognized by the four antibodies GHI/61, Ber-MAC3, Ki-M8 and SM4. **Immunology,** v. 75, n. 4, p. 588-95, Apr 1992. ISSN 0019-2805. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1592433 >.

Purohit, V.; Gao, B.; Song, B. J. Molecular mechanisms of alcoholic fatty liver. **Alcohol Clin Exp Res,** v. 33, n. 2, p. 191-205, Feb 2009. ISSN 1530-0277. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19032584 >.

Pösö, A. R.; Hirsimäki, P. Inhibition of proteolysis in the liver by chronic ethanol feeding. **Biochem J,** v. 273(Pt 1), p. 149-52, Jan 1991. ISSN 0264-6021. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1989576 >.

Racanelli, V.; Rehermann, B. The liver as an immunological organ. **Hepatology,** v. 43, n. 2 Suppl 1, p. S54-62, Feb 2006. ISSN 0270-9139. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16447271 >.

Ramm, G. A. Chemokine (C-C motif) receptors in fibrogenesis and hepatic regeneration following acute and chronic liver disease. **Hepatology,** v. 50, n. 5, p. 1664-8, Nov 2009. ISSN 1527-3350. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19877298 >.

Rautou, P. E. et al. Autophagy in liver diseases. **J Hepatol,** v. 53, n. 6, p. 1123-34, Dec 2010. ISSN 1600-0641. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20810185 >.

Rayego-Mateos, S. et al. TWEAK transactivation of the epidermal growth factor receptor mediates renal inflammation. **J Pathol,** v. 231, n. 4, p. 480-94, Dec 2013. ISSN 1096-9896. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24037740 >.

Razmara, M. et al. Fn14-TRAIL, a chimeric intercellular signal exchanger, attenuates experimental autoimmune encephalomyelitis. **Am J Pathol,** v. 174, n. 2, p. 460-74, Feb 2009. ISSN 1525-2191. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19147815 >.

Reddy, K. B.; Nabha, S. M.; Atanaskova, N. Role of MAP kinase in tumor progression and invasion. **Cancer Metastasis Rev,** v. 22, n. 4, p. 395-403, Dec 2003. ISSN 0167-7659. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12884914 >.

Reed, M. J. et al. Inhibition of TIMP1 enhances angiogenesis in vivo and cell migration in vitro. **Microvasc Res,** v. 65, n. 1, p. 9-17, Jan 2003. ISSN 0026-2862. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12535866 >.

Reinders, M. E. et al. Proangiogenic function of CD40 ligand-CD40 interactions. **J Immunol,** v. 171, n. 3, p. 1534-41, Aug 1 2003. ISSN 0022-1767 (Print)

0022-1767 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12874247 >.

Ribatti, D. et al. In vivo angiogenic activity of urokinase: role of endogenous fibroblast growth factor-2. **J Cell Sci,** v. 112 (Pt 23), p. 4213-21, Dec 1999. ISSN 0021-9533. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10564640 >.

Richardson, M. et al. Viral serpin, Serp-1, inhibits endogenous angiogenesis in the chicken chorioallantoic membrane model. **Cardiovasc Pathol,** v. 16, n. 4, p. 191-202, 2007 Jul-Aug 2007. ISSN 1054-8807. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17637427 >.

Risau, W. et al. Platelet-derived growth factor is angiogenic in vivo. **Growth Factors,** v. 7, n. 4, p. 261-6, 1992. ISSN 0897-7194. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1284870 >.

Rizzo, M. T. Focal adhesion kinase and angiogenesis. Where do we go from here? **Cardiovasc Res,** v. 64, n. 3, p. 377-8, Dec 2004. ISSN 0008-6363. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15537487 >.

Roberts, P. J.; Der, C. J. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. **Oncogene,** v. 26, n. 22, p. 3291-310, May 2007. ISSN 0950-9232. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17496923 >.

Roebuck, M. M. et al. Matrix metalloproteinase expression is related to angiogenesis and histologic grade in spindle cell soft tissue neoplasms of the extremities. **Am J Clin Pathol,** v. 123, n. 3, p. 405-14, Mar 2005. ISSN 0002-9173 (Print)

0002-9173 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15716237 >.

Roos, C. et al. Soluble and transmembrane TNF-like weak inducer of apoptosis differentially activate the classical and noncanonical NF-kappa B pathway. **J Immunol,** v. 185, n. 3, p. 1593-605, Aug 2010. ISSN 1550-6606. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20610643 >.

Rosen, E. M. et al. HGF/SF in angiogenesis. **Ciba Found Symp,** v. 212, p. 215-26; discussion 227-9, 1997. ISSN 0300-5208. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9524773 >.

Roskams, T. A. et al. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. **Hepatology,** v. 39, n. 6, p. 1739-45, Jun 2004. ISSN 0270-9139. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15185318 >.

Ruegg, C. et al. Evidence for the involvement of endothelial cell integrin alphaVbeta3 in the disruption of the tumor vasculature induced by TNF and IFN-gamma. **Nat Med,** v. 4, n. 4, p. 408-14, Apr 1998. ISSN 1078-8956 (Print)

1078-8956 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9546785 >.

Rundhaug, J. E. Matrix metalloproteinases, angiogenesis, and cancer: commentary re: A. C. Lockhart et al., Reduction of wound angiogenesis in patients treated with BMS-275291, a broad spectrum matrix metalloproteinase inhibitor. Clin. Cancer Res., 9: 00-00, 2003. **Clin Cancer Res,** v. 9, n. 2, p. 551-4, Feb 2003. ISSN 1078-0432 (Print)

1078-0432 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12576417 >.

Rundhaug, J. E. Matrix metalloproteinases and angiogenesis. **J Cell Mol Med,** v. 9, n. 2, p. 267-85, 2005 Apr-Jun 2005. ISSN 1582-1838. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15963249 >.

Russo, S. et al. Platelet-activating factor mediates CD40-dependent angiogenesis and endothelial-smooth muscle cell interaction. **J Immunol,** v. 171, n. 10, p. 5489-97, Nov 15 2003. ISSN 0022-1767 (Print)

0022-1767 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14607955 >.

Sabour Alaoui, S. et al. TWEAK affects keratinocyte G2/M growth arrest and induces apoptosis through the translocation of the AIF protein to the nucleus. **PLoS One,** v. 7, n. 3, p. e33609, 2012. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22438963 >.

Sainson, R. C. et al. TNF primes endothelial cells for angiogenic sprouting by inducing a tip cell phenotype. **Blood,** v. 111, n. 10, p. 4997-5007, May 15 2008. ISSN 1528-0020 (Electronic)

0006-4971 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18337563 >.

Saitoh, T. et al. TWEAK induces NF-kappaB2 p100 processing and long lasting NF-kappaB activation. **J Biol Chem,** v. 278, n. 38, p. 36005-12, Sep 2003. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12840022 >.

Sakamoto, T. et al. Mitosis and apoptosis in the liver of interleukin-6-deficient mice after partial hepatectomy. **Hepatology,** v. 29, n. 2, p. 403-11, Feb 1999. ISSN 0270-9139. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9918916 >.

Salcedo, R. et al. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. **Blood,** v. 96, n. 1, p. 34-40, Jul 1 2000. ISSN 0006-4971 (Print)

0006-4971 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10891427 >.

Salzmann, S. et al. TWEAK inhibits TRAF2-mediated CD40 signaling by destabilization of CD40 signaling complexes. **J Immunol,** v. 191, n. 5, p. 2308-18, Sep 2013. ISSN 1550-6606. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23918987 >.

Salzmann, S. et al. Fibroblast growth factor inducible (Fn14)-specific antibodies concomitantly display signaling pathway-specific agonistic and antagonistic activity. **J Biol Chem,** v. 288, n. 19, p. 13455-66, May 2013. ISSN 1083-351X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23532848 >.

Sanz, A. B. et al. TWEAK Promotes Peritoneal Inflammation. **PLoS One,** v. 9, n. 3, p. e90399, 2014. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24599047 >.

Sanz, A. B. et al. TWEAK and the progression of renal disease: clinical translation. **Nephrol Dial Transplant,** v. 29 Suppl 1, p. i54-i62, Feb 2014. ISSN 1460-2385. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24493870 >.

Sanz, A. B. et al. Tweak induces proliferation in renal tubular epithelium: a role in uninephrectomy induced renal hyperplasia. **J Cell Mol Med,** v. 13, n. 9B, p. 3329-42, Sep 2009. ISSN 1582-4934. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19426154 >.

Sanz, A. B. et al. TWEAK activates the non-canonical NFkappaB pathway in murine renal tubular cells: modulation of CCL21. **PLoS One,** v. 5, n. 1, p. e8955, 2010. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20126461 >.

Sasso, F. C. et al. Increased vascular endothelial growth factor expression but impaired vascular endothelial growth factor receptor signaling in the myocardium of type 2 diabetic patients with chronic coronary heart disease. **J Am Coll Cardiol,** v. 46, n. 5, p. 827-34, Sep 2005. ISSN 0735-1097. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16139132 >.

Sato, N. et al. Platelet-derived growth factor indirectly stimulates angiogenesis in vitro. **Am J Pathol,** v. 142, n. 4, p. 1119-30, Apr 1993. ISSN 0002-9440. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/7682762 >.

Sato, T. et al. Sinusoidal endothelial cell proliferation and expression of angiopoietin/Tie family in regenerating rat liver. **J Hepatol,** v. 34, n. 5, p. 690-8, May 2001. ISSN 0168-8278. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11434615 >.

Schapira, K. et al. Fn14-Fc fusion protein regulates atherosclerosis in ApoE-/- mice and inhibits macrophage lipid uptake in vitro. **Arterioscler Thromb Vasc Biol,** v. 29, n. 12, p. 2021-7, Dec 2009. ISSN 1524-4636. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19762780 >.

Schattenberg, J. M.; Galle, P. R.; Schuchmann, M. Apoptosis in liver disease. **Liver Int,** v. 26, n. 8, p. 904-11, Oct 2006. ISSN 1478-3223. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16953829 >.

Schattenberg, J. M. et al. CYP2E1 overexpression alters hepatocyte death from menadione and fatty acids by activation of ERK1/2 signaling. **Hepatology,** v. 39, n. 2, p. 444-55, Feb 2004. ISSN 0270-9139. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14767997 >.

Schmilovitz-Weiss, H. et al. Role of circulating soluble CD40 as an apoptotic marker in liver disease. **Apoptosis,** v. 9, n. 2, p. 205-10, Mar 2004. ISSN 1360-8185. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15004517 >.

Schneider, P. et al. TWEAK can induce cell death via endogenous TNF and TNF receptor 1. **Eur J Immunol,** v. 29, n. 6, p. 1785-92, Jun 1999. ISSN 0014-2980. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10382740 >.

Schoenborn, J. R.; Wilson, C. B. Regulation of interferon-gamma during innate and adaptive immune responses. **Adv Immunol,** v. 96, p. 41-101, 2007. ISSN 0065-2776. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17981204 >.

Schroder, K. et al. Interferon-gamma: an overview of signals, mechanisms and functions. **J Leukoc Biol,** v. 75, n. 2, p. 163-89, Feb 2004. ISSN 0741-5400. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14525967 >.

Schwartz, N. et al. Urinary TWEAK as a biomarker of lupus nephritis: a multicenter cohort study. **Arthritis Res Ther,** v. 11, n. 5, p. R143, 2009. ISSN 1478-6362. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19785730 >.

Seitz, H. K.; Stickel, F. Risk factors and mechanisms of hepatocarcinogenesis with special emphasis on alcohol and oxidative stress. **Biol Chem,** v. 387, n. 4, p. 349-60, Apr 2006. ISSN 1431-6730. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16606331 >.

Selmi, C. et al. Immune-mediated bile duct injury: The case of primary biliary cirrhosis. **World J Gastrointest Pathophysiol,** v. 1, n. 4, p. 118-28, Oct 2010. ISSN 2150-5330. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21607152 >.

Selmi, C. et al. Primary biliary cirrhosis. **Lancet,** v. 377, n. 9777, p. 1600-9, May 2011. ISSN 1474-547X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21529926 >.

Semov, A. et al. Alterations in TNF- and IL-related gene expression in space-flown WI38 human fibroblasts. **FASEB J,** v. 16, n. 8, p. 899-901, Jun 2002. ISSN 1530-6860. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12039873 >.

Serafini, B. et al. Expression of TWEAK and its receptor Fn14 in the multiple sclerosis brain: implications for inflammatory tissue injury. **J Neuropathol Exp Neurol,** v. 67, n. 12, p. 1137-48, Dec 2008. ISSN 0022-3069. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19018248 >.

Serini, G.; Valdembri, D.; Bussolino, F. Integrins and angiogenesis: a sticky business. **Exp Cell Res,** v. 312, n. 5, p. 651-8, Mar 2006. ISSN 0014-4827. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16325811 >.

Shamri, R. et al. Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. **Nat Immunol,** v. 6, n. 5, p. 497-506, May 2005. ISSN 1529-2908. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15834409 >.

Shang, J. et al. Strong neurogenesis, angiogenesis, synaptogenesis, and antifibrosis of hepatocyte growth factor in rats brain after transient middle cerebral artery occlusion. **J Neurosci Res,** v. 89, n. 1, p. 86-95, Jan 2011. ISSN 1097-4547. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20963849 >.

Shashkin, P. et al. Expression of CXCL16 in human T cells. **Arterioscler Thromb Vasc Biol,** v. 23, n. 1, p. 148-9, Jan 1 2003. ISSN 1524-4636 (Electronic)

1079-5642 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12524239 >.

Shay, E. et al. Inhibition of angiogenesis by HC·HA, a complex of hyaluronan and the heavy chain of inter-α-inhibitor, purified from human amniotic membrane. **Invest Ophthalmol Vis Sci,** v. 52, n. 5, p. 2669-78, Apr 2011. ISSN 1552-5783. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21228375 >.

Sheth, K.; Bankey, P. The liver as an immune organ. **Curr Opin Crit Care,** v. 7, n. 2, p. 99-104, Apr 2001. ISSN 1070-5295 (Print)

1070-5295 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11373518 >.

Shimada, K. et al. ALKBH3 contributes to survival and angiogenesis of human urothelial carcinoma cells through NADPH oxidase and tweak/Fn14/VEGF signals. **Clin Cancer Res,** v. 18, n. 19, p. 5247-55, Oct 2012. ISSN 1078-0432. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22850567 >.

Shimizu, K. et al. Gene regulation of a novel angiogenesis inhibitor, vasohibin, in endothelial cells. **Biochem Biophys Res Commun,** v. 327, n. 3, p. 700-6, Feb 2005. ISSN 0006-291X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15649403 >.

Shin, E. C. et al. Human hepatocellular carcinoma cells resist to TRAIL-induced apoptosis, and the resistance is abolished by cisplatin. **Exp Mol Med,** v. 34, n. 2, p. 114-22, May 2002. ISSN 1226-3613. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12085986 >.

Shiojima, I.; Walsh, K. Role of Akt signaling in vascular homeostasis and angiogenesis. **Circ Res,** v. 90, n. 12, p. 1243-50, Jun 2002. ISSN 1524-4571. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12089061 >.

Shiraki, K. et al. CD40 expression in HCV-associated chronic liver diseases. **Int J Mol Med,** v. 18, n. 4, p. 559-63, Oct 2006. ISSN 1107-3756. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16964404 >.

Si-Tayeb, K.; Lemaigre, F. P.; Duncan, S. A. Organogenesis and development of the liver. **Dev Cell,** v. 18, n. 2, p. 175-89, Feb 16 2010. ISSN 1878-1551 (Electronic)

1534-5807 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20159590 >.

Silveira, M. G.; Lindor, K. D. Primary sclerosing cholangitis. **Can J Gastroenterol,** v. 22, n. 8, p. 689-98, Aug 2008. ISSN 0835-7900. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18701947 >.

Singh, R. et al. Autophagy regulates lipid metabolism. **Nature,** v. 458, n. 7242, p. 1131-5, Apr 2009. ISSN 1476-4687. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19339967 >.

Smedsrød, B. Clearance function of scavenger endothelial cells. **Comp Hepatol,** v. 3 Suppl 1, p. S22, Jan 2004. ISSN 1476-5926. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14960174 >.

Steffan, A. M. et al. Mouse hepatitis virus type 3 infection provokes a decrease in the number of sinusoidal endothelial cell fenestrae both in vivo and in vitro. **Hepatology,** v. 22, n. 2, p. 395-401, Aug 1995. ISSN 0270-9139 (Print)

0270-9139 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/7635406 >.

Stephan, D. et al. TWEAK/Fn14 pathway modulates properties of a human microvascular endothelial cell model of blood brain barrier. **J Neuroinflammation,** v. 10, p. 9, 2013. ISSN 1742-2094. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23320797 >.

Strasser, A.; Jost, P. J.; Nagata, S. The many roles of FAS receptor signaling in the immune system. **Immunity,** v. 30, n. 2, p. 180-92, Feb 2009. ISSN 1097-4180. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19239902 >.

Su, F.; Schneider, R. J. Hepatitis B virus HBx protein sensitizes cells to apoptotic killing by tumor necrosis factor alpha. **Proc Natl Acad Sci U S A,** v. 94, n. 16, p. 8744-9, Aug 1997. ISSN 0027-8424. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9238048 >.

Suffee, N. et al. RANTES/CCL5-induced pro-angiogenic effects depend on CCR1, CCR5 and glycosaminoglycans. **Angiogenesis,** v. 15, n. 4, p. 727-44, Dec 2012. ISSN 1573-7209. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22752444 >.

Sun, B.; Karin, M. NF-kappaB signaling, liver disease and hepatoprotective agents. **Oncogene,** v. 27, n. 48, p. 6228-44, Oct 2008. ISSN 1476-5594. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18931690 >.

Tahergorabi, Z.; Khazaei, M. Imbalance of angiogenesis in diabetic complications: the mechanisms. **Int J Prev Med,** v. 3, n. 12, p. 827-38, Dec 2012. ISSN 2008-7802. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23272281 >.

Takahashi, T. et al. A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. **EMBO J,** v. 20, n. 11, p. 2768-78, Jun 2001. ISSN 0261-4189. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11387210 >.

Tarrats, N. et al. Critical role of tumor necrosis factor receptor 1, but not 2, in hepatic stellate cell proliferation, extracellular matrix remodeling, and liver fibrogenesis. **Hepatology,** v. 54, n. 1, p. 319-27, Jul 2011. ISSN 1527-3350. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21523796 >.

Tate, C. M. et al. LY2228820 dimesylate, a selective inhibitor of p38 mitogen-activated protein kinase, reduces angiogenic endothelial cord formation in vitro and in vivo. **J Biol Chem,** v. 288, n. 9, p. 6743-53, Mar 2013. ISSN 1083-351X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23335506 >.

Taura, K. et al. Hepatic stellate cells secrete angiopoietin 1 that induces angiogenesis in liver fibrosis. **Gastroenterology,** v. 135, n. 5, p. 1729-38, Nov 2008. ISSN 1528-0012 (Electronic)

0016-5085 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18823985 >.

Tavora, B. et al. Endothelial FAK is required for tumour angiogenesis. **EMBO Mol Med,** v. 2, n. 12, p. 516-28, Dec 2010. ISSN 1757-4684. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21154724 >.

ten Dijke, P.; Goumans, M. J.; Pardali, E. Endoglin in angiogenesis and vascular diseases. **Angiogenesis,** v. 11, n. 1, p. 79-89, 2008. ISSN 0969-6970. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18283546 >.

Theodosiou, A.; Ashworth, A. MAP kinase phosphatases. **Genome Biol,** v. 3, n. 7, p. REVIEWS3009, Jun 2002. ISSN 1465-6914. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12184814 >.

Thurston, G. Role of Angiopoietins and Tie receptor tyrosine kinases in angiogenesis and lymphangiogenesis. **Cell Tissue Res,** v. 314, n. 1, p. 61-8, Oct 2003. ISSN 0302-766X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12915980 >.

Tirnitz-Parker, J. E.; Olynyk, J. K.; Ramm, G. A. Role of TWEAK in coregulating liver progenitor cell and fibrogenic responses. **Hepatology,** v. 59, n. 3, p. 1198-201, Mar 2014. ISSN 1527-3350. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24038142 >.

Tirnitz-Parker, J. E. et al. Tumor necrosis factor-like weak inducer of apoptosis is a mitogen for liver progenitor cells. **Hepatology,** v. 52, n. 1, p. 291-302, Jul 2010. ISSN 1527-3350. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20578156 >.

Tran, N. L. et al. The human Fn14 receptor gene is up-regulated in migrating glioma cells in vitro and overexpressed in advanced glial tumors. **Am J Pathol,** v. 162, n. 4, p. 1313-21, Apr 2003. ISSN 0002-9440. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12651623 >.

Tran, N. L. et al. Increased fibroblast growth factor-inducible 14 expression levels promote glioma cell invasion via Rac1 and nuclear factor-kappaB and correlate with poor patient outcome. **Cancer Res,** v. 66, n. 19, p. 9535-42, Oct 2006. ISSN 1538-7445. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17018610 >.

Tran, N. L. et al. The tumor necrosis factor-like weak inducer of apoptosis (TWEAK)-fibroblast growth factor-inducible 14 (Fn14) signaling system regulates glioma cell survival via NFkappaB pathway activation and BCL-XL/BCL-W expression. **J Biol Chem,** v. 280, n. 5, p. 3483-92, Feb 2005. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15611130 >.

Vayrynen, J. P. et al. Serum MMP-8 levels increase in colorectal cancer and correlate with disease course and inflammatory properties of primary tumors. **Int J Cancer,** v. 131, n. 4, p. E463-74, Aug 15 2012. ISSN 1097-0215 (Electronic)

0020-7136 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21918979 >.

Viebahn, C. S. et al. Invading macrophages play a major role in the liver progenitor cell response to chronic liver injury. **J Hepatol,** v. 53, n. 3, p. 500-7, Sep 2010. ISSN 1600-0641. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20561705 >.

Viennois, E.; Chen, F.; Merlin, D. NF-κB pathway in colitis-associated cancers. **Transl Gastrointest Cancer,** v. 2, n. 1, p. 21-29, Jan 2013. ISSN 2224-476X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23626930 >.

Vince, J. E. et al. TWEAK-FN14 signaling induces lysosomal degradation of a cIAP1-TRAF2 complex to sensitize tumor cells to TNFalpha. **J Cell Biol,** v. 182, n. 1, p. 171-84, Jul 2008. ISSN 1540-8140. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18606850 >.

Vincent, C. et al. Pro-inflammatory cytokines TNF-related weak inducer of apoptosis (TWEAK) and TNFalpha induce the mitogen-activated protein kinase (MAPK)-dependent expression of sclerostin in human osteoblasts. **J Bone Miner Res,** v. 24, n. 8, p. 1434-49, Aug 2009. ISSN 1523-4681. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19292615 >.

Voronov, E.; Carmi, Y.; Apte, R. N. Role of IL-1-mediated inflammation in tumor angiogenesis. **Adv Exp Med Biol,** v. 601, p. 265-70, 2007. ISSN 0065-2598. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17713014 >.

Vázquez, F. et al. METH-1, a human ortholog of ADAMTS-1, and METH-2 are members of a new family of proteins with angio-inhibitory activity. **J Biol Chem,** v. 274, n. 33, p. 23349-57, Aug 1999. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10438512 >.

Vázquez-Carballo, A. et al. TWEAK prevents TNF-α-induced insulin resistance through PP2A activation in human adipocytes. **Am J Physiol Endocrinol Metab,** v. 305, n. 1, p. E101-12, Jul 2013. ISSN 1522-1555. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23651848 >.

Wack, K. E. et al. Sinusoidal ultrastructure evaluated during the revascularization of regenerating rat liver. **Hepatology,** v. 33, n. 2, p. 363-78, Feb 2001. ISSN 0270-9139. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11172338 >.

Wahl, K. et al. Increased apoptosis induction in hepatocellular carcinoma by a novel tumor-targeted TRAIL fusion protein combined with bortezomib. **Hepatology,** v. 57, n. 2, p. 625-36, Feb 2013. ISSN 1527-3350. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22991197 >.

Wang, L. et al. Liver sinusoidal endothelial cell progenitor cells promote liver regeneration in rats. **J Clin Invest,** v. 122, n. 4, p. 1567-73, Apr 2012. ISSN 1558-8238. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22406533 >.

Wanless, I. R.; Nakashima, E.; Sherman, M. Regression of human cirrhosis. Morphologic features and the genesis of incomplete septal cirrhosis. **Arch Pathol Lab Med,** v. 124, n. 11, p. 1599-607, Nov 2000. ISSN 0003-9985. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11079009 >.

Ware, C. F. The TNF superfamily. **Cytokine Growth Factor Rev,** v. 14, n. 3-4, p. 181-4, 2003 Jun-Aug 2003. ISSN 1359-6101. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12787557 >.

Warren, A. et al. T lymphocytes interact with hepatocytes through fenestrations in murine liver sinusoidal endothelial cells. **Hepatology,** v. 44, n. 5, p. 1182-90, Nov 2006. ISSN 0270-9139. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17058232 >.

Webber, E. M. et al. Tumor necrosis factor primes hepatocytes for DNA replication in the rat. **Hepatology,** v. 28, n. 5, p. 1226-34, Nov 1998. ISSN 0270-9139. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9794905 >.

Whitsett, T. G. et al. Elevated expression of Fn14 in non-small cell lung cancer correlates with activated EGFR and promotes tumor cell migration and invasion. **Am J Pathol,** v. 181, n. 1, p. 111-20, Jul 2012. ISSN 1525-2191. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22634180 >.

Wiley, S. R. et al. A novel TNF receptor family member binds TWEAK and is implicated in angiogenesis. **Immunity,** v. 15, n. 5, p. 837-46, Nov 2001. ISSN 1074-7613 (Print)

1074-7613 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11728344 >.

Wiley, S. R.; Winkles, J. A. TWEAK, a member of the TNF superfamily, is a multifunctional cytokine that binds the TweakR/Fn14 receptor. **Cytokine Growth Factor Rev,** v. 14, n. 3-4, p. 241-9, 2003 Jun-Aug 2003. ISSN 1359-6101. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12787562 >.

Willis, A. L. et al. The fibroblast growth factor-inducible 14 receptor is highly expressed in HER2-positive breast tumors and regulates breast cancer cell invasive capacity. **Mol Cancer Res,** v. 6, n. 5, p. 725-34, May 2008. ISSN 1541-7786. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18505918 >.

Winau, F. et al. Starring stellate cells in liver immunology. **Curr Opin Immunol,** v. 20, n. 1, p. 68-74, Feb 2008. ISSN 0952-7915. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18068343 >.

Winkles, J. A. The TWEAK-Fn14 cytokine-receptor axis: discovery, biology and therapeutic targeting. **Nat Rev Drug Discov,** v. 7, n. 5, p. 411-25, May 2008. ISSN 1474-1784. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18404150 >.

Winkles, J. A.; Tran, N. L.; Berens, M. E. TWEAK and Fn14: new molecular targets for cancer therapy? **Cancer Lett,** v. 235, n. 1, p. 11-7, Apr 2006. ISSN 0304-3835. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15885893 >.

Winkles, J. A. et al. Role of TWEAK and Fn14 in tumor biology. **Front Biosci,** v. 12, p. 2761-71, 2007. ISSN 1093-9946. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17127278 >.

Wisniacki, N. et al. Safety, tolerability, pharmacokinetics, and pharmacodynamics of anti-TWEAK monoclonal antibody in patients with rheumatoid arthritis. **Clin Ther,** v. 35, n. 8, p. 1137-49, Aug 2013. ISSN 1879-114X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23928094 >.

Wisse, E. et al. Structure and function of sinusoidal lining cells in the liver. **Toxicol Pathol,** v. 24, n. 1, p. 100-11, 1996 Jan-Feb 1996. ISSN 0192-6233. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8839287 >.

Xia, L. et al. TRAF2 and cIAP2 involve in TWEAK-induced MMP-9 production in fibroblast-like synoviocytes. **Rheumatol Int,** v. 32, n. 1, p. 281-2, Jan 2012. ISSN 1437-160X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21229359 >.

Xia, L. P. et al. [The expression of TWEAKR/Fn14 in Rheumatoid arthritis fibroblast-like synoviocytes]. **Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi,** v. 26, n. 6, p. 575-7, Jun 2010. ISSN 1007-8738. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20487651 >.

Xia, L. P. et al. [Effects of TWEAK on the synthesis of MMP-3 in fibroblast-like synoviocytes of rheumatoid arthritis]. **Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi,** v. 25, n. 1, p. 46-8, Jan 2009. ISSN 1007-8738. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19126387 >.

Xia, Y. et al. Inhibition of the TWEAK/Fn14 pathway attenuates renal disease in nephrotoxic serum nephritis. **Clin Immunol,** v. 145, n. 2, p. 108-21, Nov 2012. ISSN 1521-7035. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22982296 >.

Xu, B. et al. Capillarization of hepatic sinusoid by liver endothelial cell-reactive autoantibodies in patients with cirrhosis and chronic hepatitis. **Am J Pathol,** v. 163, n. 4, p. 1275-89, Oct 2003. ISSN 0002-9440. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14507637 >.

Xu, H. et al. TWEAK/Fn14 interaction stimulates human bronchial epithelial cells to produce IL-8 and GM-CSF. **Biochem Biophys Res Commun,** v. 318, n. 2, p. 422-7, May 2004. ISSN 0006-291X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15120617 >.

Xu, L. G.; Shu, H. B. TNFR-associated factor-3 is associated with BAFF-R and negatively regulates BAFF-R-mediated NF-kappa B activation and IL-10 production. **J Immunol,** v. 169, n. 12, p. 6883-9, Dec 2002. ISSN 0022-1767. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12471121 >.

Yepes, M. TWEAK and Fn14 in the Neurovascular Unit. **Front Immunol,** v. 4, p. 367, 2013. ISSN 1664-3224. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24273541 >.

Yin, X. et al. RG7212 anti-TWEAK mAb inhibits tumor growth through inhibition of tumor cell proliferation and survival signaling and by enhancing the host antitumor immune response. **Clin Cancer Res,** v. 19, n. 20, p. 5686-98, Oct 2013. ISSN 1078-0432. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23974006 >.

Yin, X. M.; Ding, W. X.; Gao, W. Autophagy in the liver. **Hepatology,** v. 47, n. 5, p. 1773-85, May 2008. ISSN 1527-3350. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18393362 >.

Yoo, Y. G.; Lee, M. O. Hepatitis B virus X protein induces expression of Fas ligand gene through enhancing transcriptional activity of early growth response factor. **J Biol Chem,** v. 279, n. 35, p. 36242-9, Aug 2004. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15173177 >.

Yoong, K. F. et al. Vascular adhesion protein-1 and ICAM-1 support the adhesion of tumor-infiltrating lymphocytes to tumor endothelium in human hepatocellular carcinoma. **J Immunol,** v. 160, n. 8, p. 3978-88, Apr 1998. ISSN 0022-1767. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9558106 >.

Yoriki, R. et al. Therapeutic potential of the TWEAK/Fn14 pathway in intractable gastrointestinal cancer. **Exp Ther Med,** v. 2, n. 1, p. 103-108, 1 2011. ISSN 1792-0981. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22977477 >.

Yoshida, S. et al. Role of MCP-1 and MIP-1alpha in retinal neovascularization during postischemic inflammation in a mouse model of retinal neovascularization. **J Leukoc Biol,** v. 73, n. 1, p. 137-44, Jan 2003. ISSN 0741-5400. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12525571 >.

Yuan, K.; Jin, Y. T.; Lin, M. T. Expression of Tie-2, angiopoietin-1, angiopoietin-2, ephrinB2 and EphB4 in pyogenic granuloma of human gingiva implicates their roles in inflammatory angiogenesis. **J Periodontal Res,** v. 35, n. 3, p. 165-71, Jun 2000. ISSN 0022-3484 (Print)

0022-3484 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10929871 >.

Yumet, G. et al. Tumor necrosis factor mediates hepatic growth hormone resistance during sepsis. **Am J Physiol Endocrinol Metab,** v. 283, n. 3, p. E472-81, Sep 2002. ISSN 0193-1849. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12169440 >.

Zaiss, D. M. et al. Amphiregulin enhances regulatory T cell-suppressive function via the epidermal growth factor receptor. **Immunity,** v. 38, n. 2, p. 275-84, Feb 2013. ISSN 1097-4180. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23333074 >.

Zamara, E. et al. Prevention of severe toxic liver injury and oxidative stress in MCP-1-deficient mice. **J Hepatol,** v. 46, n. 2, p. 230-8, Feb 2007. ISSN 0168-8278 (Print)

0168-8278 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17125873 >.

Zhao, Z. et al. TWEAK/Fn14 interactions are instrumental in the pathogenesis of nephritis in the chronic graft-versus-host model of systemic lupus erythematosus. **J Immunol,** v. 179, n. 11, p. 7949-58, Dec 2007. ISSN 0022-1767. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18025243 >.

Zheng, T. S.; Burkly, L. C. No end in site: TWEAK/Fn14 activation and autoimmunity associated- end-organ pathologies. **J Leukoc Biol,** v. 84, n. 2, p. 338-47, Aug 2008. ISSN 0741-5400. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18483204 >.

Zhi-Chun, L. et al. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) mediates p38 mitogen-activated protein kinase activation and signal transduction in peripheral blood mononuclear cells from patients with lupus nephritis. **Inflammation,** v. 35, n. 3, p. 935-43, Jun 2012. ISSN 1573-2576. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22009442 >.

Zhu, W. H.; MacIntyre, A.; Nicosia, R. F. Regulation of angiogenesis by vascular endothelial growth factor and angiopoietin-1 in the rat aorta model: distinct temporal patterns of intracellular signaling correlate with induction of angiogenic sprouting. **Am J Pathol,** v. 161, n. 3, p. 823-30, Sep 2002. ISSN 0002-9440. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12213710 >.

Zhuge, X. et al. CXCL16 is a novel angiogenic factor for human umbilical vein endothelial cells. **Biochem Biophys Res Commun,** v. 331, n. 4, p. 1295-300, Jun 17 2005. ISSN 0006-291X (Print)

0006-291X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15883016 >.