THE ROLE OF PLATELETS IN LYMPHOCYTE RECRUITMENT DURING LIVER INFLAMATION

by

Joseph Robinson

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School of Clinical and Experimental Medicine

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ABSTRACT

Platelets have long been known to be capable of mediating lymphocyte recruitment. However, their role in recruitment of lymphocytes to the inflamed liver remains unclear. This was investigated *in vivo* in two murine models of hepatic injury: ischaemia reperfusion (IR) and concanavalin A (ConA) injury. Intravital microscopy studies were conducted in normal or thrombocytopenic mice undergoing sham surgery or injury. Hepatic recruitment of fluorescently labelled donor spleen derived T- and B-cells or donor platelets was determined. Platelet adhesion was observed in healthy and injured livers, but was not increased following injury when the whole region viewed microscopically in the liver was assessed. However, in areas of sustained blood flow, platelet adhesion was significantly increased in IR livers. T-cell adhesion was significantly increased in IR injured livers, with no increases in T- or B-cell adhesion following ConA injury. In thrombocytopenic mice, a significant reduction in T- and B-cell adhesion was observed in both IR and ConA livers. Blocking CD162 (PSGL-1) on T-cells significantly decreased their recruitment in both injury models. Our data demonstrates adhesion of both T- and B-cells within the hepatic microcirculation in both injury models is platelet dependent. Interestingly, lymphocyte adhesion within healthy liver was not platelet dependent. Collectively, our results suggest platelets play a key role in mediating lymphocyte recruitment to injured liver.

Papers

Robinson J, Kavanagh D, Adams D and Kalia N. The role of platelets in recruitment of lymphocytes to the injured liver. (In preparation)

Oral Presentations

14th UK Platelet Group Meeting, 2013, University Birmingham
Robinson J., Adams D.H., Kalia N.
"Lymphocyte recruitment to ischaemia-reperfusion injured liver is platelet dependent."

27th UK Cell Adhesion Society Meeting, 2013, University of Birmingham
Robinson J., Adams D.H., Kalia N.
"Lymphocyte recruitment to the injured liver is platelet dependent."

64th British Microcirculation Society Meeting, 2014, University of Bristol
Robinson J., Adams D.H., Kalia N.
"Lymphocyte recruitment to the injured liver is platelet dependent."

Poster Presentations

62nd British Microcirculation Society Meeting - 2nd Joint meeting with The Microcirculation Society, 2012, University of Oxford
Robinson J, Adams D and Kalia N.
"Lymphocyte adhesion to endothelial counter-ligands can be enhanced by adherent platelets or by pre-incubating lymphocytes with platelets."

37th International Congress of Physiological Societies, 2013, Birmingham
Robinson J, Adams D and Kalia N.
"Intravital investigations of lymphocyte and platelet recruitment to murine liver following ischemia-reperfusion injury"

Awards

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ABBREVIATIONS

Abreviation	Definition
ACD	Acid citrate dextrose
ALT	Alanine aminotransferase
BCR	B-cell receptor
B_{reg}	Regulatory B-cell
BSA	Bovine serum albumin
ConA	Concanavalin A
ECM	Extra cellular matrix
FCS	Foetal calf serum
IL	Interleukin
INF	Interferon
IR	Ischemia reperfusion
MACS	Magnetic activated cell sorting
MAdCAM	Mucosal vascular addressin cell adhesion molecule 1
NK	Natural killer
NKT	Natural killer T-cell
NO	Nitric oxide
PAF	Platelet activating factor
PMP	Platelet micro particle
PBS	Phosphate buffered saline
PRP	Platelet rich plasma
ROS	Reactive oxygen species
TNF	Tumour necrosis factor
NK	Natural killer
T_c	Cytotoxic T-cell
TCR	T-cell receptor
T_h	Helper T-cell
T_{reg}	Regulatory T-cell
VEGF	Vascular endothelial growth factor
VWF	Von willebrand factor

CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1. Liver Disease and Hepatic Inflammation

Liver disease is the United Kingdom's 5th biggest killer and the number of cases is rising (British Liver Trust, 2008). Liver disease covers a range of conditions with varying causes but a common feature of all liver diseases is hepatitis, or inflammation of the liver. Liver inflammation is most commonly associated with viral infection (hepatitis virus A-E) but can also be caused by other infectious agents, toxic substances such as alcohol, autoimmune disorders and liver hypoxia. Many cases of hepatitis can resolve spontaneously including most cases of hepatitis A and E. However, other cases such as autoimmune hepatitis and hepatitis C can become chronic. These can then progress to cirrhosis and chronic liver failure. In certain cases, such as paracetamol overdose, hepatitis A or B infection or excessive alcohol intake, hepatocellular damage can occur rapidly leading to acute or sub-acute liver failure. When liver failure does not resolve itself, treatment is limited to liver transplantation. However, a shortage of donor organs in recent years has led to increased usage of marginal organs (those not are not ideal and include donors who are older, have diabetes, hepatitis etc.) or live donor transplantation (Angelico, 2005, Abbasoglu, 2008). Regardless of the cause, liver inflammation results in the local accumulation of inflammatory effector cells leading to hepatocellular damage. Although neutrophils have long been considered the main effector cell in hepatic inflammation, emerging evidence suggests other blood cells recruited by the liver microcirculation play a role. These include lymphocytes and platelets which will be the focus of the research contained within this thesis.

1.2. Hepatic Microcirculation

The human liver makes up 2.5% of the body's weight and as such is its largest organ. It receives approximately 25% of the cardiac output. The microcirculation of the liver has many functions including supplying the liver parenchyma with oxygen and nutrients, clearing toxins and foreign bodies from the circulation and also accepting the nutrients absorbed by the small intestine for the liver to metabolise. The microcirculation (of any organ) also permits leukocyte entry in to the surrounding interstitium during inflammatory processes. To ensure these various roles can be performed, the liver receives blood from two sources. The hepatic portal vein drains blood from the intestine, spleen, pancreas and gallbladder and supplies almost 80% of the total blood flow to the liver. The hepatic artery, a branch of the aorta, supplies oxygenated blood to the liver. Within the liver, these large vessels become hepatic portal venules and hepatic arterioles which mix blood from both sources in hepatic capillaries called sinusoids. Blood entering the sinusoids is then drained into the central veins (Vollmar and Menger, 2009).

It is within the sinusoids that the supply of nutrients to the hepatocytes and removal of metabolic products takes place. Since smooth muscle is absent in these capillaries, blood flow within them is controlled by contractile sphincters composed of sinusoidal lining cells at both the entry and exit points (McCuskey, 2000). The endothelium lining the hepatic microcirculation is markedly heterogeneous. The endothelial cells lining the sinusoids are

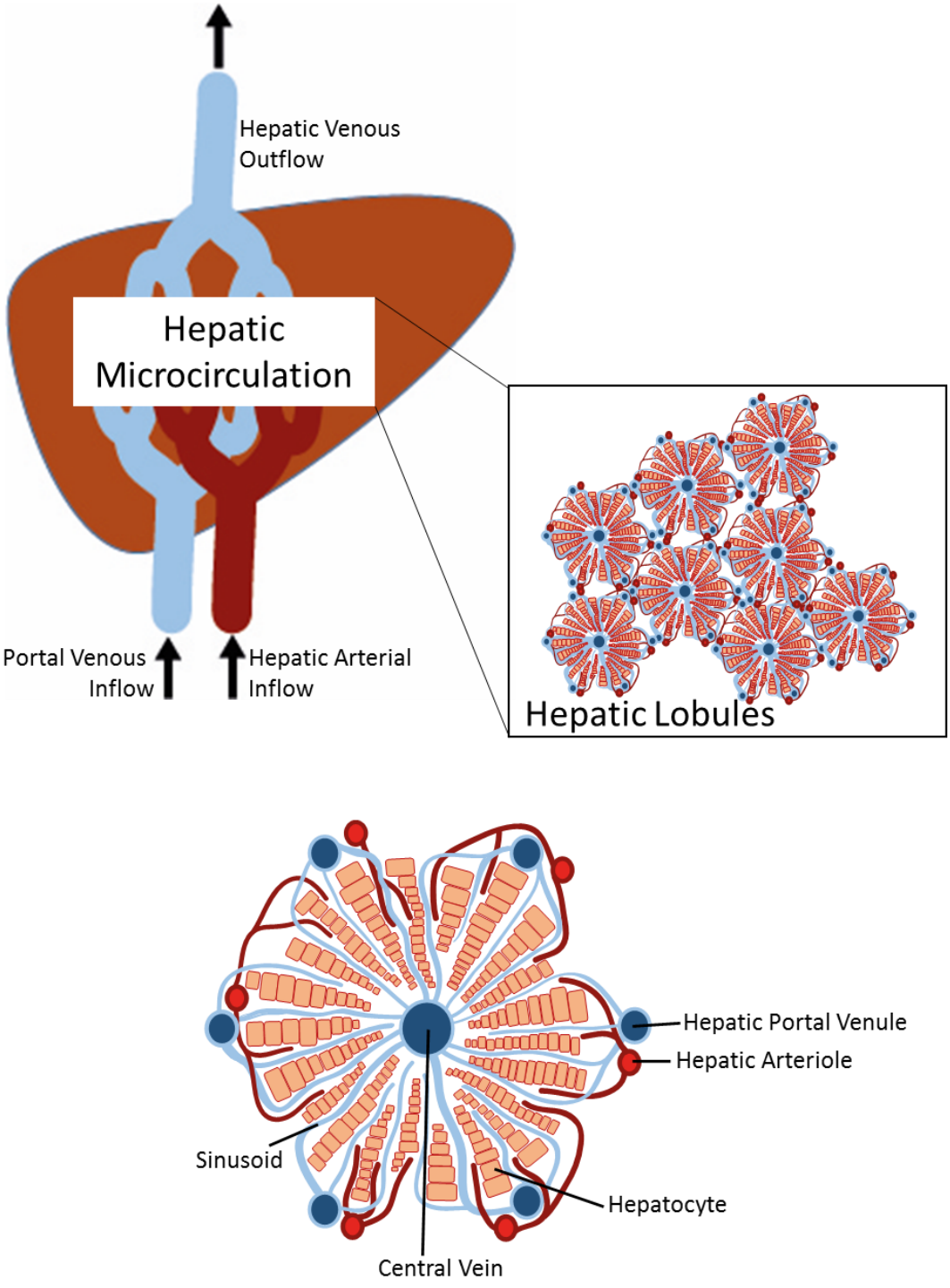


Figure 1:1 The Hepatic Microcirculation. Blood enters the liver via the hepatic artery providing oxygenated blood and the hepatic portal vein carrying blood from the intestines. The microcirculation of the liver can be divided into hexagonal subunits called lobules. Within these lobules blood from the hepatic artery and portal vein mix in capillaries called sinusoids that have a characteristic morphological pattern.

functionally specialised cells that lack tight junctions as well as a basal lamina. This facilitates their role in the clearance of endotoxin/bacteria, regulation of inflammation, leukocyte recruitment and their transmigration into the surrounding parenchyma and host immune responses to pathogens. In contrast the vascular endothelial cells lining the larger portal venules and hepatic arterioles have a continuous basal membrane and tight junctions between cells (Lalor *et al*., 2002a). This is appropriate to their main function of distributing blood evenly throughout the liver parenchyma (Lalor *et al*., 2006).

Between the sinusoidal endothelial cells and hepatocytes lies a blood plasma filled area called the perisinusoidal space. Hepatocyte microvilli extend into this space facilitating the absorption of plasma components. It is also within this space that hepatic stellate cells (HSCs) reside. These cells perform a number of important functions. They are involved in antigen presentation (Viñas et al., 2003), they are the main mediators of liver fibrosis through their release of collagen (Bataller and Brenner, 2001), and they are important mediators of liver regeneration (Balabaud et al., 2004). They also contract following activation during liver injury increasing intrahepatic resistance and portal pressure (Rockey and Weisiger, 1996).

1.3. Leukocytes in Hepatitis

Inflammation is a localised response to pathogens, irritants or cellular damage. The liver is highly prone to inflammation due to its roles in metabolism of toxins and immune surveillance of the blood leaving the intestine. During inflamation leukocytes invade the liver parenchyma after exiting from the sinusoids and the post-sinusoidal venules. Although this is an important

and much needed process, unwanted or an overwhelmingly excessive activation of these inflammatory cells leads to liver damage.

Leukocytes are derived from haematopoietic stem cells in the bone marrow that collectively make up the immune system. They can be divided into five main subsets which can be separated into two distinct groups: granular leukocytes (neutrophils, basophils and eosinophils) and non-granular leukocytes (monocytes and lymphocytes). Evidence suggests that neutrophils, monocytes and lymphocytes are the most important of these cell types in mediating hepatitis. Although eosinophil recruitment is observed in several types of hepatitis, especially drug induced hepatitis, (Brunt *et al*., 1999), their role, if any, is poorly understood.

1.3.1. Neutrophils in Hepatitis

Neutrophils are phagocytic polymorphonuclear cells which account for 50-60% of the total circulating leukocytes in humans (Smith, 1994) and approximately 13% of circulating leukocytes in C57BL/6 mice (Carp *et al*., 1972). They are major players of the rapidly responding innate immune system which is the first line of defense against initial environmental challenges and injury. They are recruited to the site of inflammation within minutes to hours and are generally the first leukocytes to accumulate in the tissue (Kaplanski *et al*., 2003). Neutrophils play a vital role in fighting infections. However during inflammation, neutrophils can inadvertently mediate cellular damage and death through production of ROS and release of other cytotoxic factors (Smith, 1994). They also play a role in the recruitment

of other leukocytes to the site of inflammation through the release of various pro-inflammatory cytokines (Scapini *et al*., 2000).

Neutrophils have been identified as key mediators of tissue damage in a number of animal models of hepatitis including endotoxin induced hepatitis (Jaeschke *et al*., 1991), hepatic ischaemia-reperfusion (IR) injury (Jaeschke *et al*., 1990) and concanavalin A (ConA)-induced hepatitis (Bonder *et al*., 2005). The latter two models will be discussed later in *Sections 1.4 and 1.5.* Hepatic accumulation and activation of neutrophils is also observed in patients with hepatitis (Taïeb *et al*., 2000, Nagore and Scheuer, 1988, Lee, 1989). Neutrophil-mediated liver damage follows their activation by inflammatory chemokines and subsequent accumulation within the hepatic vasculature, specifically within the hepatic sinusoids. From here they are able to transmigrate into the surrounding parenchyma to cause hepatocyte damage. A neutrophil elastase inhibitor, Sivelestat, has been shown to confer benefit in patients following liver resectioning surgery (Tsujii *et al*., 2012). However, despite strong experimental and pre-clinical data, clinical trials for anti-neutrophil adhesion therapy in different organ injuries, including hepatic IR injury, have failed to show a significant benefit (Harlan and Winn, 2002). This suggests other pathophysiological mechanisms, possibly involving different blood cells are involved in hepatitis.

1.3.2. Monocytes and Macrophages in Hepatitis

During acute inflammation, neutrophil recruitment is often followed by monocyte accumulation (Kaplanski *et al*., 2003). Monocytes produce inflammatory cytokines such as TNF-α and IL-6 during hepatic inflammation which may contribute to both liver injury and

recruitment of additional inflammatory cells. Their significant presence has been identified immunohistologically in liver biopsies of patients with hepatitis and when isolated were found to possess a very pro-migratory and activated phenotype (McClain and Cohen, 1989, Longhi *et al*., 2009).

Macrophages are phagocytic mononuclear cells which form through differentiation of monocytes that have transmigrated from the circulation into tissue. Kupffer cells are abundant specialised liver resident macrophages which line the walls of the hepatic sinusoids and make up 10-15% of the total liver cell population (Heydtmann, 2009). They are the largest group of fixed macrophages in the body. Kupffer cells have long been considered as mostly scavenger cells, responsible for removing particulate material from the portal blood entering the liver from the gut. However, increasing experimental evidence suggests these cells may be implicated in the pathology of various inflammatory liver diseases including viral hepatitis, steatohepatitis, alcoholic liver disease and rejection of the liver following transplantation (Kolios *et al*., 2006). Both Kupffer cells and non-liver resident macrophages appear to contribute directly to liver injury and hepatocyte death through release of lysosomal and proteolytic enzymes. They also exacerbate injury through mediating inflammatory cell recruitment during hepatitis. The latter occurs as a result of cytokine release as well as generation of ROS, nitric oxide (NO), interferons and other cytokines (Heydtmann, 2009, Nanji, 2002, Schumann *et al*., 2000, Sass *et al*., 2002). Indeed, Kupffer cells are a significant source of chemoattractants for cytotoxic lymphocytes, particularly CD8+ cells and can induce their activation (Kolios *et al*., 2006).

1.3.3. Lymphocytes in Hepatitis

In addition to neutrophils, monocytes and macrophages, lymphocytes also play a role in hepatitis. Unlike the other leukocytes, lymphocytes originate from the common lymphoid progenitor as opposed to the common myeloid progenitor during haematopoiesis. In adult humans they make up ~31% of circulating leukocytes (Hulstaert *et al*., 1994) whereas in mice they account for ~54% (Domínguez-Gerpe and Rey-Méndez, 2001). Lymphocytes can be split into three main groups: natural killer (NK) cells, a component of the innate immune system, and T-cells and B-cells, which are part of the adaptive immune system (with the exception of natural killer T (NKT) –cells, which bridge the two systems). The healthy liver has a large resident population of lymphocytes representing ~25% of non-hepatocytes. This population is made up of ~63% T-cells, ~6% B-cells and ~31% NK cells (Racanelli and Rehermann, 2006). These lymphocytes reside in the liver parenchyma, portal tracts and sinusoids. The large population of functionally active resident lymphocytes, in addition to the liver resident macrophages, provides vital immune surveillance against foreign antigens, particularly those arriving from the gut. Additional lymphocytes can also be recruited from the peripheral circulation in response to infection or injury. Clearly, this process requires lymphocytes to be able to adhere to and transmigrate across the endothelial cells that line the hepatic microvasculature. Studies also suggest that terminally differentiated lymphocytes can be removed from the body by the liver, where they are cleared by apoptosis (Crispe *et al*., 2000).

Liver infiltration by lymphocytes is a certainly a feature of hepatitis, however the extent and type of lymphocytes involved varies from case to case. In viral hepatitis CD8+ T-cell infiltration is responsible for viral clearance and disease pathogenesis (Chisari, 1997, Thimme *et al*.,

2003), whereas in experimental models of ConA induced liver injury, little CD8+ T-cell infiltration is observed whereas high levels of CD4+ T-cell are observed (Mizuhara *et al*., 1994). Similar results are seen in the post-ischaemic liver where the majority of infiltrating T-cells are also CD4+ cells (Zwacka *et al*., 1997). B-cell infiltration during hepatic inflammation is relatively poorly studied. However, B-cell deficiency does attenuate liver fibrosis in an experimental, carbon tetrachloride (CCL_4) induced, model of liver inflammation (Novobrantseva *et al*., 2005). The role of lymphocytes in the two experimental models of injury that will be used in this thesis will be detailed later in *Sections 1.4* and *1.5*. The major subsets of lymphocytes will be outlined in the following sections and are also summarised in *Figure 1.2.*

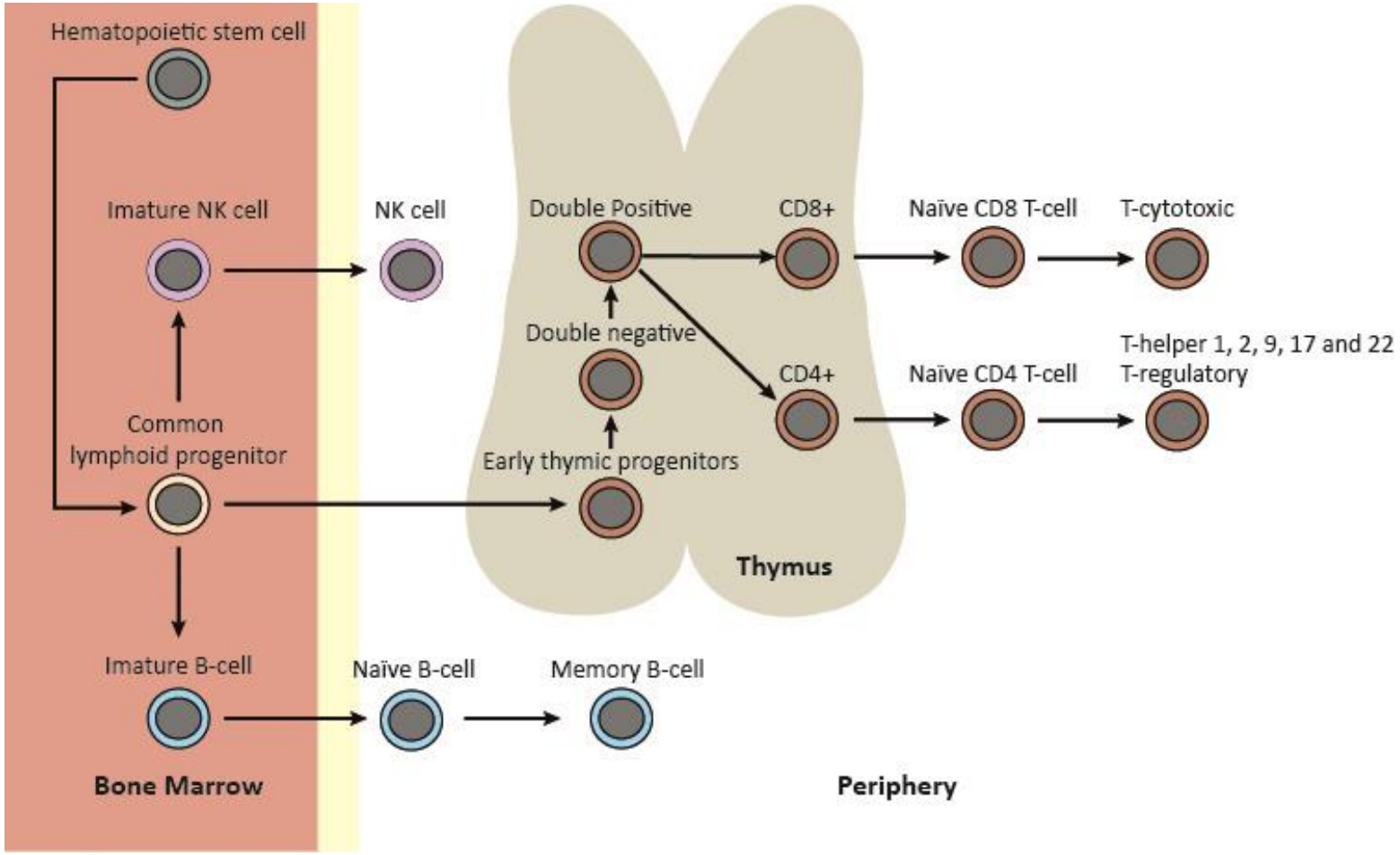


Figure 1:2 Lymphocyte Development (lymphopoiesis). Lymphocytes originate through differentiation of hematopoietic stem cells which reside in the bone marrow. B-cells and most NK cells mature within the bone marrow, but T-cells mature within the thymus. Following maturation lymphocytes enter the circulation and peripheral lymph organs.

1.3.3.1. T-Cell subsets: CD4+ (T_{helper} and T_{reg}), CD8+, NK and NKT-cells

T- and B-cells both originate in the bone marrow with B-cells maturing there and T-cells migrating to the thymus to mature and differentiate. After leaving the bone marrow or thymus the naïve cells circulate between the blood and secondary lymphoid organs. The naïve cells continue to circulate until they encounter their specific antigen at which point they become activated, proliferate and differentiate into effector cells. T-cells (CD3+) can be distinguished from other lymphocytes by their surface expression of the T-cell receptor (TCR) found on all T-cells. The T-cell receptor is a heterodimer made up of α and β or γ and δ chains. The TCR associates non-covalently with CD3 signalling proteins, $CD3_{\gamma}$, $CD3_{\delta}$ and two $CD3_{\varepsilon}$ chains, to make up the TCR complex (Clevers *et al*., 1988). This CD3/TCR protein complex is a defining feature of the T-cell lineage and as such anti-CD3 antibodies can be used effectively as T-cell markers. T-cells can be split into a number of subsets based on their function in the immune system and expression of various cell surface molecules.

The CD4+ T-cells are the most common group of T-cells which express the glycoprotein CD4. These are non-phagocytic cells whose main role in the immune system is to secrete a variety of different cytokines that can then stimulate other immune cells. The CD4+ T-cells can be separated into T-helper (T_h) cells and T-regulatory (T_{reg}) cells. T_h cells function primarily to stimulate other immune cells e.g. stimulate B-cells to produce antibodies against antigens, stimulate cytotoxic cells such as macrophages to destroy pathogens etc. T_h cells can be further separated into subsets based on the profile of cytokines they produce. Two of the main T_h cell subsets are the T_h1 and T_h2 cells. T_h1 cells release interferon-γ (INF-γ), TNFα, interleukin 2 (IL-2) and lymphotoxin through which they can activate macrophages and promote cell

mediated immunity i.e. destruction of infected or damaged cells (Romagnani, 1999). T_h2 cells release IL-4, -5, -6, -10 and -13 which inhibit certain macrophage functions and induce humoral mediated immunity i.e. antibody production from B-cells to remove antigens (Romagnani, 1999). Until recently, it was thought that T_h1 and T_h2 were the only CD4+ T-helper cells. However, this was challenged with the identification of T_h17 which could contribute to inflammatory diseases such as rheumatoid arthritis and were defined by their production of the pro-inflammatory cytokine IL-17 (Furuzawa-Carballeda *et al*., 2007). The sub-set field expanded further with the identification of IL-9 producing T_h cells, termed T_h9 cells, which have been implicated in allergic inflammation and autoimmune diseases (Kaplan, 2013). Follicular helper T-cells are IL-21 producing cells found in the B-cell follicles of lymphoid organs. They are involved in stimulation of B-cell differentiation (King *et al*., 2008). T_h22 cells are a recently characterised group of IL-22 producing CD4+ T cells (Duhen *et al*., 2009), thought to be involved in the pathogenesis of inflammatory skin disorders such as psoriasis, atopic eczema and allergic contact dermatitis.

T_{reg} cells (CD4+, CD25+, FoxP3+) act as suppressors of the immune response of other cells and prevent autoimmunity. They are critical in the maintenance of immune cell homeostasis as they maintain order in the immune system by negatively regulating other immune cells. Therefore, dysfunction of T_{reg} cells can lead to auto-immune disorders (Wing and Sakaguchi, 2010). They can be divided into naturally arising T_{reg} cells, which are generated in the thymus and function to downregulate the overall immune system, and inducible T_{reg} cells, which are generated from naïve CD4+ T-cells following activation and function to prevent immune responses in the gut (Wing and Sakaguchi, 2010). Precise understanding of the role of T_{regs} is

unclear although they are thought to confer immunosuppression through release of soluble factors such as IL-9, IL-10 and transforming growth factor β (TGFβ).

Another group of T-cells are the CD8+ T-cells which express the CD8 glycoprotein on their surface. Following activation naïve CD8+ T-cells differentiate into cytotoxic T-cells (T_c). Unlike T_h cells, the role of CD8+ T_c cells is to kill infected, cancerous or damaged cells, which they do through inducing perforin/granzyme mediated apoptosis of the target cell (Trapani, 1995). Like T_h and T_{reg} cells, T_c cells can also secrete cytokines although in far smaller quantities (Mosmann *et al*., 1997).

In addition to macrophages and CD8+ Tc cells, NK lymphocytes are also large cytotoxic cells containing numerous granules. They are capable of killing virally infected cells and malignant cells. They are also capable of releasing several cytokines including INF-γ, TNF-α, CCL5 and CCL3 (Fauriat et al., 2010). Human NK cells can be identified by surface expression of CD56 and CD16 and NK cells from C57BL/6 mice can be identified by NK 1.1 and NK 1.2 expression. After the lungs, the liver contains the second largest population of NK cells in the body. NK cells account for 25-40% of hepatic lymphocytes in humans and 10-20% in mice (Tian et al., 2013). NK cells play and important role in control of viral hepatitis (Krämer et al., 2012) and can inhibit liver fibrosis (Melhem et al., 2006). Suppression of NK cells by chronic alcohol consumption is also a contributor to the pathogenesis of alcoholic liver disease (Jeong et al., 2008).

Natural Killer T-cell (NKT) are another type of T-cell, so named due to similarities with NK cells in function and surface receptor expression. They are CD1d restricted, express TCRαβ (often,

but not always, with an invariant Vα14-Jα18 TCRα chain in mice or Vα24-Jα18 in humans), and generally express NK1.1 (CD161-human); some NKT cells also express CD4 or CD8 (Emoto et al., 2000, Pellicci et al., 2002, Godfrey et al., 2004). In mice they are most frequent in the liver where they make up 50% of all mature T-cells but only account for 4% of all hepatic T-cells in humans (Godfrey *et al*., 2000). NKT cells can rapidly release high levels of TNF, IL-4 and INF-γ and are implicated in cancer cell killing and the immune responses to various infections (Godfrey *et al*., 2000). They also play a pathogenic role in viral hepatitis (de Lalla et al., 2004, Baron et al., 2002) and produce large amounts of inflammatory cytokines during concanavalin A induced liver injury (Yamanaka et al., 2004). Mucosal-associated invariant T (MAIT) are a group of NKT like T-cells which are MR1 restricted, often express NK1.1 (CD161), and express an invariant Vα19-Jα33 TCR α chain in mice or Vα7.2-Jα33 in humans (Treiner et al., 2003, Treiner et al., 2005, Dusseaux et al., 2011). MAIT cells are enriched within the human liver in which the majority of NK marker expressing T-cells have been shown to be MAIT cells (Tang et al., 2013). They have previously been shown to be present in the liver during hepatitis C infection and are known to produce the pro-inflammatory cytokine IL-17 (Le Bourhis et al., 2013).

1.3.3.2. B-Cells

B-cells (CD19+) can be distinguished from other lymphocytes by their expression of the B-cell receptor (BCR) which is responsible for B-cell antigen recognition. The BCR is made up of a membrane bound antibody and a CD79A/CD79B signal transduction moiety (Pleiman *et al*., 1994). The primary function of B-cells in the immune response is antibody production (Mauri and Bosma, 2012). In addition to this role, B-cells can also secrete cytokines including TNFα,

INFγ, IL-2, -4, -6 and -12 (Lund, 2008). A small sub-group of B-cells which secrete the anti-inflammatory cytokine 1L-10 has been termed regulatory B-cells (B_{reg}). These cells have proven to be important suppressors of inflammation in a number of models including colitis, collagen induced arthritis and autoimmune encephalomyelitis (Mauri and Bosma, 2012). IL-10 producing B_{reg} cells account for only a small proportion of hepatic B-cells in stark contrast to the B-cells of the secondary lymphoid organs (Zhang *et al*., 2013).

1.3.4. Leukocyte Adhesion Cascade

Lymphocytes circulate continuously from blood into tissues and secondary lymphoid organs, and then return back to the blood to patrol the body in search for antigens, thus providing an effective immune surveillance. However, during inflammation, circulating lymphocytes adhere to vascular endothelium in a dynamic process known as the leukocyte adhesion cascade (*Figure 1.3*). There are three main stages to leukocyte adhesion: slow rolling, leukocyte activation and firm adhesion. The neutrophil adhesion cascade has been very well characterised and described. Since this thesis will be focussing on lymphocytes in the liver, the following section will describe the adhesion cascade specifically utilised by lymphocytes for hepatic recruitment. It is apparent that there are many similarities to the adhesive events governing neutrophil recruitment. However, the molecular mechanisms that control the recruitment of lymphocytes via the hepatic circulation, under both physiological and pathological conditions, have yet to be elucidated fully. Lymphocytes can be recruited to the liver by adhesion to hepatic portal vessels and also sinusoidal capillaries. However, both these microvessels express and utilise different adhesion molecules and cytokines for lymphocyte recruitment (Table 1.1).

1.3.4.1. Lymphocyte Capture and Rolling

Leukocyte capture and rolling on endothelium slows the velocity of trafficking leukocytes giving them time to form the more stable interactions required for firm adhesion. Leukocyte rolling is primarily mediated by CD62L (L-selectin), CD62E (E-selectin) and CD62P (P-selectin) and their counterligands such as CD162 (P-selectin glycoprotein ligand-1; (Kansas, 1996). All four of these are important in mediating neutrophil rolling in other inflamed vascular beds (Ley *et al*., 2007). In CD62L deficient mice, defects in lymphocyte rolling on the high endothelial venules of peripheral lymph nodes is observed (Arbonés *et al*., 1994). However, the velocity of blood flow in the hepatic sinusoids is lower than that observed in other capillaries. In rats it is around 0.28mm/s compared to 0.96mm/s in the brain and 1.07mm/s in the renal peritubular capillaries (Ivanov *et al*., 1985, Yamamoto *et al*., 2002). Due to this low rate of blood flow, rolling therefore plays only a minimal pre-requisite role in mediating lymphocyte adhesion within the hepatic microcirculation (Wong *et al*., 1997) and occurs primarily in the hepatic portal venules (Sawaya *et al*., 1999). Indeed, selectins are generally absent from sinusoidal capillaries.

When not inflamed, the endothelial cells of the hepatic portal vessels and sinusoids constitutively express CD54 (intercellular adhesion molecule-1) and vascular adhesion protein-1 (VAP-1). However, during inflammation, a whole host of additional adhesive molecules are expressed including CD62E, CD62P, CD106 (vascular cell adhesion molecule-1) and higher levels of CD54. The local presence of inflammatory chemokines is also increased. CD62P and CD162 appear most relevant in inducing lymphocyte rolling in the liver portal vessels. CD62P is stored in the Weibel-Palade bodies of un-activated endothelial cells and α-

granules of un-activated platelets, and upon their activation is translocated to the plasma membrane (Ley *et al*., 2007). CD62P mediated leukocyte rolling is dependent on the leukocyte expressed high affinity CD62P ligand CD162 (Norman *et al*., 1995).

On sinusoids, where selectins are absent, VAP-1 was identified by Lalor and colleagues as another candidate that could support lymphocyte rolling in the liver in a sialic acid dependent manner, although it is not as effective as selectins (Lalor *et al*., 2002). In addition to selectins a number of other cell surface molecules are capable of supporting lymphocyte rolling. The integrins CD49d/CD29 ($\alpha_4\beta_1$, VLA-4) and CD49d/beta7 ($\alpha_4\beta_7$, LPAM-1) have been shown to support lymphocyte rolling independently of selectins on CD106 and mucosal vascular addressin molecule 1 (MAdCAM-1) respectively (Berlin *et al*., 1995). MAdCAM-1 is an endothelial cell addressin that was initially recognised to be key player in gut inflammation. However it has subsequently been identified in inflammatory liver diseases which occur as a result of bowel disorders.

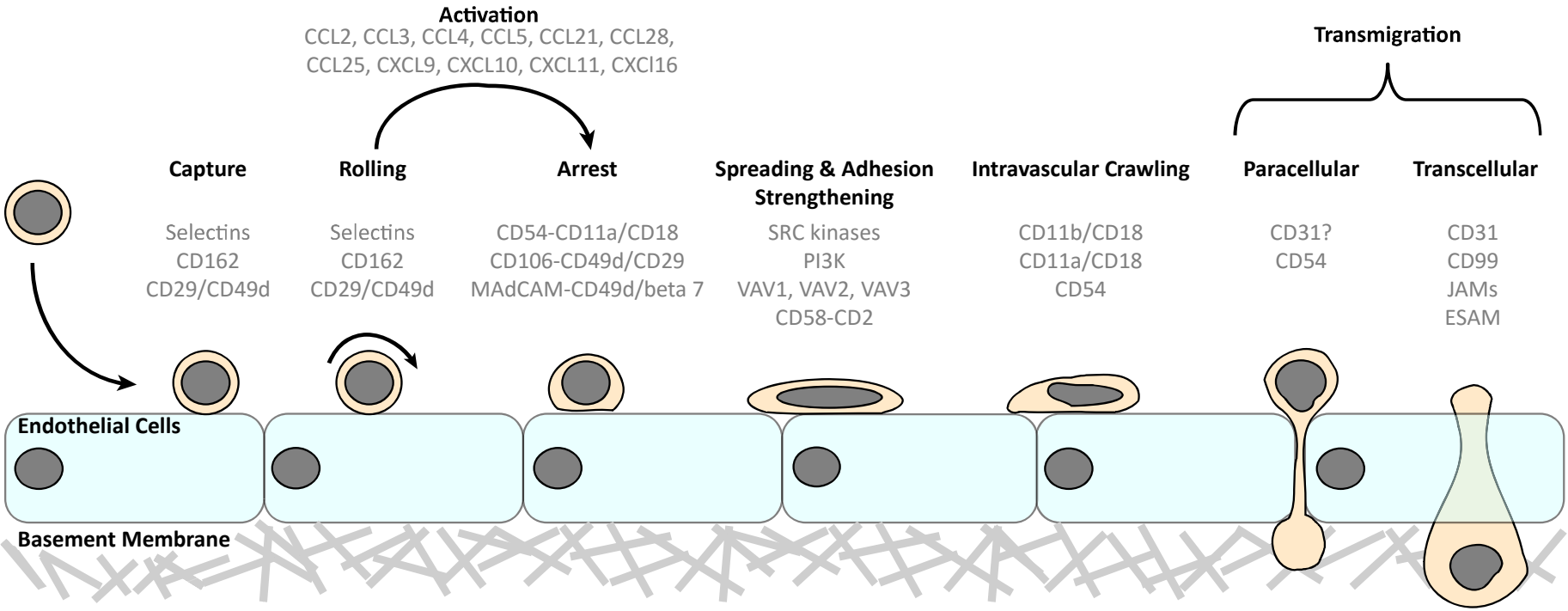


Figure 1:3 The Leukocyte Adhesion Cascade. The stages of leukocyte adhesion are shown in bold, key molecules involved in each stage are listed underneath. MAdCAM, mucosal vascular addressin molecule 1; PI3K, phosphoinositide 3-kinase; JAM, junctional adhesion molecule; ESAM, endothelial cell-selective adhesion molecule. Figure adapted from Ley *et al.* (2007).

HEPATIC SINUSOIDS		*HEPATIC PORTAL VESSELS*	
Normal Liver	***Inflamed Liver***	***Normal Liver***	***Inflamed Liver***
VAP-1	VAP-1	VAP-1	VAP-1
CD106 (VCAM-1; low levels)	CD106	CD106 (low levels)	CD106
CD54 (ICAM-1)	CD54	CD54	CD62E (E-selectin)
CD102 (ICAM-2)	CD102	CD102	CD62P (P-selectin)
Stabilin-2	Stabilin-2	PECAM-1	CD54
CLEVER-1	MAdCAM-1	JAMs	CD102
CD31 (PECAM-1; low levels)	CLEVER-1	CCL3	MAdCAM-1
JAMs (low levels)	CD31	CCL4	CD31
CXCL9	CXCL9, CXCL10	CCL5 (low levels)	JAMs
CXCL10	CXCL11		CCL3
CXCL11	CXCL16		CCL4
CXCL16	CCL25		CCL5
			CCL25
			CCL28
			CCL21

Table 1:1 Molecules involved in the recruitment of lymphocytes to the different hepatic anatomical compartments. Adhesion molecules and chemokines implicated in lymphocyte recruitment via hepatic sinusoids and portal vessels under normal and inflamed conditions (Liaskou, 2010)

The integrins CD11a/CD18 ($\alpha_L\beta_2$, LFA-1) and CD11b/CD18 ($\alpha_M\beta_2$, Mac-1), in addition to CD62E, are required for slow rolling of leukocytes following TNFα induced inflammation (Dunne *et al*., 2002, Kunkel and Ley, 1996) and in inflamed arterioles the CD11a/CD18 and CD11b/CD18 ligand ICAM-1 is required for leukocyte rolling (Sumagin and Sarelius, 2007). In fact at low shear rates CD11/CD18 and not selectins may be the dominant mediator of leukocyte rolling (Gaboury and Kubes, 1994).

1.3.4.2. Lymphocyte Activation

During rolling, lymphocytes are exposed to cytokines, released by the inflamed endothelium and other inflammatory cells, which trigger their activation. Cytokines are small secreted peptides (5-30kDa) which exert there biological effects through interactions with specific G-protein linked transmembrane receptors on target cells. Chemokines are a specific group of cytokines which are able to induce directed chemotaxis and are classified based on their structure into four subfamilies: CXC, CC, CX3C and XC. A large number of cytokines have been implicated in lymphocyte activation including CCL5 (RANTES), TNF-α, INF-γ, CXCL12 (SDF-1), IL-1, IL-2, IL-3, IL-4, IL-6 and IL-10 (Martino *et al*., 1998, Turner *et al*., 1995, Bleul *et al*., 1996, Haas *et al*., 2011).

Some of the key players involved in activating lymphocytes in the liver are CXCL12, CCL5, CCL2 (MCP-1), and CCL3 (MIP-1α) with their expression increasing markedly in the inflamed liver (Lalor *et al*., 2002). Within the liver vasculature the profile of chemokine expression varies greatly between vessel types and inflammatory states (Table 1.1), it is this pattern of expression that regulates selective recruitment of different lymphocyte subsets to the liver

during inflammation. Increased expression of chemokines during inflammation can result in accumulation of lymphocytes expressing corresponding chemokine receptors within the liver. During hepatitis C infection the CXCR3 ligands CXCL9 and CXCL10 are expressed by sinusoidal endothelium and result in the selective recruitment of CXCR3 positive T-cells. Whereas, the CCR5 ligands CCL3 and CCL4 are expressed in portal vessels this results in selective recruitment of CCR5 positive T-cells to the liver (Shields et al., 1999). Indeed, both anti-CCR5 anti-CCL3 antibodies protect against graft versus host disease associated liver injury by preventing selective recruitment of CCR5 positive T-cells (Murai et al., 1999) . High levels of CXCR6 expressing CD4 and CD8 T-cells have been observed within the inflamed human liver. The CXCR6 ligand CXCL16 is upregulated on inflamed bile ducts and hepatocytes and has been suggested to be important for the recruitment of effector T-cells within the liver (Germanov et al., 2008).

Although cytokines are soluble, a significant proportion becomes bound to proteoglycans, such as heparin sulphate on endothelial cell. These proteoglycans constitute the glycocalyx on the apical or lumenal side of the endothelial cells. They present the chemokines, possibly enhancing their biological activity, to the circulating cells thereby creating a localised inflammatory environment (Middleton *et al*., 2002, Wang *et al*., 2005).

Binding of cytokines to their receptors on the leukocyte can change their adhesive properties allowing them to firmly adhere. The overall strength of cellular adhesion is determined by both the strength of the individual interactions and the total number of interactions (Carman and Springer, 2003). The key mechanism by which cytokines can alter cellular adhesion is

through modulation of integrin interactions. Integrins are transmembrane receptors which mediate adhesion of cells to their surroundings. They are made up of two non-covalently linked transmembrane glycoproteins known as α and β subunits. Integrins can switch between low affinity, inactive forms and high affinity, active forms via conformational changes. Treatment with CXCL12, CCL19 and CCL21 transiently increases the affinity of the integrin CD18/CD11a on lymphocytes for ICAM-1 (Constantin *et al*., 2000). CCL2 activates the fibronectin receptors CD49d/CD29 and CD49e/CD29 on T-cells (Carr *et al*., 1996).

Cytokines can also trigger changes in integrin mobility within the cell membrane. Increased integrin mobility can increase the total number of integrin-ligand interaction that can occur (Carman and Springer, 2003). Treatment with CXCL12, CCL19 and CCL21 rapidly (within 10s) increases the lateral mobility of CD18/CD11a within the plasma membrane of lymphocytes (Constantin *et al*., 2000). Clustering of integrins can increase the strength of adhesion by increasing the number of adhesive integrins at the point of cell-cell contact. Clustering of integrins can occur extremely rapidly in response to cytokines, for example immobilised CXCL12 can trigger sub-second clustering of CD49d/CD29 on T-cells (Grabovsky *et al*., 2000).

Stimulation with cytokines can also up-regulate cell surface expression of integrins on leukocytes. Up-regulation of integrin expression following cytokine stimulation is well described in both neutrophils and monocytes (Vaddi and Newton, 1994, Jiang *et al*., 1992, Montecucco *et al*., 2008, Conklyn *et al*., 1996). There is limited evidence of lymphocyte up-regulation of integrin expression in response to cytokine stimulation. Following stimulation with TNF-α expression of CD11b on human lymphocytes is increased (Armstrong *et al*., 2001).

Lymphocytes can also show increased integrin expression during infection by a pathogen (Ueda *et al*., 2010).

1.3.4.3. Lymphocyte Firm Adhesion

Following activation lymphocytes firmly adhere to the endothelium, this process is primarily mediated by integrins and their ligands (Ley *et al*., 2007). Integrins can bind to endothelium expressed members of the immunoglobulin superfamily: a group of proteins which all have an immunoglobulin-like domain (Barclay, 2003). The immunoglobulin superfamily members CD54, CD102 (ICAM-2), CD106 and MAdCAM can all be expressed on endothelium within the liver (Scoazec *et al*., 1994, Steinhoff *et al*., 1993, Grant *et al*., 2001).

CD54 is constitutively expressed on hepatic sinusoidal endothelial cells and at low levels on hepatic portal vascular endothelial cells (Scoazec *et al*., 1994). Its expression on sinusoidal endothelial cells is significantly increased during inflammation (Steinhoff *et al*., 1993, Volpes *et al*., 1990). Lymphocyte adhesion to CD54 is mediated by the integrin CD11a/CD18 (LFA-1) (Dustin and Springer, 1988). In the non-inflamed liver, CD102 is strongly expressed on the endothelium of hepatic portal arteries and weakly expressed on sinusoidal endothelium, a similar expression pattern is observed in the inflamed liver (Steinhoff *et al*., 1993). Lymphocyte binding to CD102 is also mediated by the integrin CD11a/CD18 (Binnerts *et al*., 1994).

In the normal liver, CD106 is expressed at very low levels on vascular endothelium and not at all on sinusoidal endothelium (Steinhoff *et al*., 1993). During inflammation its expression on both hepatic vascular, portal and sinusoidal endothelium is increased (Steinhoff *et al*., 1993, García-Monzón *et al*., 1998). Lymphocyte adhesion to endothelium expressed CD106 is mediated by the lymphocyte expressed integrin CD49d/CD29 (van Dinther-Janssen *et al*., 1991). MAdCAM-1 is generally only expressed on gut endothelium and is responsible for targeting lymphocytes to the gut trough interaction with the lymphocyte expressed integrin CD49d/beta7 (integrin $\alpha_4\beta_7$) (Hamann *et al*., 1994). However, in certain inflamed states MAdCAM-1 is expressed by hepatic endothelium leading to recruitment of mucosal lymphocytes (Grant *et al*., 2001).

Non-integrin mediated interactions are also involved in firm adhesion. CD58 (LFA-3) is constitutively expressed at low levels on hepatic sinusoidal endothelium and, like CD54, during inflammation its expression is up-regulated (Volpes *et al*., 1990, Steinhoff *et al*., 1993). T-cell expressed CD2 (LFA-2) can bind CD58 with a low affinity, these interactions serve to strengthen cellular adhesion (van der Merwe *et al*., 1994, Shao *et al*., 2005).

Expression of cell adhesion molecules on the surface of endothelial cells may not, on its own, be sufficient to support leukocyte adhesion. The endothelial glycocalyx is a layer of glycoproteins, glycosaminoglycans, proteoglycans and soluble plasma proteins which extends ≈500nm into the vascular lumen (Weinbaum *et al*., 2007). As the thickness of glycocalyx far exceeds the dimensions of the endothelial cell adhesion molecules it is likely to act as a barrier to leukocyte adhesion (Mulivor and Lipowsky, 2002). However, glycocalyx shedding can occur

in disease states such as IR injury, inflammation and acute hyperglycaemia (Nieuwdorp *et al*., 2006, Mulivor and Lipowsky, 2004). Components of the endothelial glycocalyx may also be involved in leukocyte adhesion. CD44 is a glycoprotein expressed on both lymphocytes and hepatic endothelium (Couvelard *et al*., 1993, Joensuu *et al*., 1993). It can support firm adhesion through binding either hyaluronic acid or the integrin CD49d/CD29 (Nandi *et al*., 2004, McDonald *et al*., 2008). Compared to other tissues hyaluronic acid is present at very high concentrations in both the non-inflamed and inflamed liver sinusoids (McDonald *et al*., 2008). CD44 interactions with hyaluronic acid have been shown to be import in neutrophil recruitment to the inflamed liver and lymphocyte recruitment to other inflamed tissues (McDonald *et al*., 2008, Milinkovic *et al*., 2004, Stoop *et al*., 2002).

In addition to endothelium, leukocytes can also adhere to components of the endothelial extracellular matrix (ECM). During liver inflammation endothelial denudation can occur leaving the ECM exposed (Tiegs et al., 1992). Lymphocytes can adhere to the ECM component fibronectin through the integrins CD49d/CD29 and CD49e/CD29 (Szekanecz *et al*., 1992, van de Wiel-van Kemenade *et al*., 1992). This interaction appears to be important in the recruitment of T-cells to areas of inflammation (Rodriguez *et al*., 1992, Hfrshkoviz *et al*., 1994). Activated T-cells are also able to adhere to both type I and type IV collagen through the integrins CD49a/CD29 and CD49b/CD29 (de Fougerolles *et al*., 2000).

1.3.4.4. Lymphocyte Intravascular Crawling and Transmigration

Once a leukocyte has become firmly adherent it can then crawl along the endothelium or transmigrate trough the endothelium and into the tissue. Crawling along the inside of the

blood vessel allows leukocytes to position themselves for transmigration, in monocytes and neutrophils this is mediated by CD11b/CD18 and CD54 (Ley *et al*., 2007). Lymphocyte intravascular crawling appears to be mediated by high affinity CD11a/CD18 (Shulman *et al*., 2009). Transmigration occurs most commonly at the junctions between endothelial cells (paracellular transmigration) but there is also evidence for leukocytes, including lymphocytes, migrating trough a single endothelial cell (transcellular transmigration) (Feng *et al*., 1998, Millan *et al*., 2006). Transmigration is mediated by a number of molecules including integrins, endothelial cell-selective adhesion molecule (ESAM), PECAM-1 (CD31), CD99 and junctional adhesion molecules (JAMs) (Ley *et al*., 2007).

1.4. Platelets in Hepatitis

In addition to leukocyte accumulation in the liver, a few studies have identified hepatic platelet accumulation in a number of models of liver inflammation including lymphocytic choriomeningitis virus infection (Lang *et al*., 2008), hepatic IR injury (Cywes *et al*., 1993), ConA-induced liver injury (Massaguer et al., 2002) and lipopolysaccharide induced liver injury (Eipel *et al*., 2004). Tamura and colleagues reported that platelet-endothelial interactions occurred earlier than leukocyte responses after hepatic IR injury and that, interestingly, adhesion of platelets required the presence of activated Kupffer cells. Indeed, half of the adherent platelets located were attached to Kupffer cells (Tamura *et al*., 2012). Several recent studies by Iannacone and colleagues have reported that the intrahepatic recruitment of CD8+ T-cells was critically dependent on platelets, particularly after acute viral hepatitis (Iannacone *et al*., 2005).

1.4.1. Role of Platelets in Inflammation

Platelets are discoid shaped blood cells between 0.5-3µm in size. They are formed by cytoplasmic fragmentation of precursor megakaryocytes found within the bone marrow. The major role of circulating platelets in the vasculature is to maintain haemostasis or prevent blood loss at sites of vessel injury through the formation of platelet plugs or thrombi. They do this by adhering at sites of vascular injury, becoming activated and subsequently aggregating to form a thrombus. Further to their role in maintaining haemostasis, platelets also play a diverse role in many other physiological and pathological processes. This includes emerging evidence for platelets contributing to angiogenesis, tumour metastasis, lymphangiogenesis, tissue regeneration, wound repair and in maintaining vascular integrity (McNicol and Israels, 2008). However, it is their role as immune cells that can initiate and accelerate many vascular inflammatory conditions that has received a lot of recent attention. Indeed, platelets are linked to the pathogenesis of many inflammatory diseases such as atherosclerosis, malaria infection, transplant rejection, inflammatory bowel disease, IR injury and rheumatoid arthritis. They carry out this inflammatory role through direct interactions with leukocytes and endothelial cells and/or through release of soluble and vesicular inflammatory factors. The next sections describe how platelets can adhere to damaged and intact blood vessels to either form aggregates/thrombi or platelet-endothelial interactions respectively.

1.4.2. Platelet Adhesion, Activation and Aggregation

Platelet adhesion to the subendothelium, exposed after vascular damage, initiates thrombus formation and haemostasis. In order to carry out this process, platelets express high concentrations of integrins and other glycoproteins on their membrane surface. Similarly to the leukocyte adhesion cascade, thrombus formation also involves platelet tethering/rolling, activation, firm adhesion and an additional aggregation step.

1.4.2.1. Platelet Adhesion to Exposed Subendothelial Matrix

In the uninjured vasculature, platelets show no physical interaction with endothelial cells. The intact, non-activated endothelium prevents platelet adhesion and controls platelet reactivity through inhibitory and modulating mechanisms involving NO, cyclooxygenase-2 (COX-2) and prostacyclin (PGI_2). However, disturbances in vascular integrity leading to loss or denudation of endothelium, exposes the underlying extracellular matrix (ECM) proteins. Adhesive rolling interactions of platelets with these proteins is the first step of haemostasis. Platelets can adhere to many components of the ECM including immobilised von Willebrand factor (VWF), collagen, laminin and fibronectin (Ruggeri and Mendolicchio, 2007) using a number of specific platelet glycoproteins and integrins.

Platelet rolling or tethering occurs via the interaction of the platelet glycoprotein CD42b (GPIbα; part of the GPIb-IX-V receptor complex) with immobilised VWF (Girma *et al*., 1986, Huizinga *et al*., 2002). These initial weak interactions allow platelets to remain attached to the vessel wall despite the high shear forces generated within the vascular lumen of arteries

and arterioles. Within the sub-endothelial matrix, VWF is immobilised primarily to collagen type I, III and VI filaments (Rand *et al*., 1997). However, soluble VWF in plasma can also bind to other ECM components including sulfatides (Roberts *et al*., 1986), heparin (Fujimura *et al*., 1987), fibrin (Hada *et al*., 1986) and even to VWF already bound in the sub-endothelial matrix (Savage *et al*., 2002). The binding of CD42b to VWF has a fast off-rate and is therefore insufficient to mediate stable adhesion but rather maintains the platelet in close contact with the surface. Indeed, interactions between CD42b and immobilised VWF typically slow platelet velocity to less than 2% of their free flow velocity allowing slower forming but more stable adhesive bonds to occur (Ruggeri and Mendolicchio, 2007). At shear rates of greater than 500-800s^{-1} for human blood (Savage *et al*., 1998) and 2000-5000s^{-1} for mouse blood (Konstantinides *et al*., 2006) only CD42b-VWF interactions have sufficiently rapid on-rate to initiate platelet adhesion.

In addition to collagen-bound VWF, platelets are also capable of adhering directly to collagen itself (Saelman *et al*., 1994). Indeed, among the various constituents of the subendothelial matrix, collagen is the most abundant vessel wall protein and the most thrombogenic. Collagen not only supports weak platelet adhesion, but also acts as a powerful activator of platelets which leads to their subsequent firm adhesion and aggregation (Nieswandt and Watson, 2003, Watson, 1999, Nievelstein *et al*., 1988). Platelets express a number of collagen receptors including the CD49b/CD29 (integrin $\alpha_2\beta_1$, VLA-2; GPIa/IIa), GPVI, CD36 (GPIV) and p65 (Ruggeri and Mendolicchio, 2007). Of these, CD49b/CD29 and GPVI are the most important, with blockade of either resulting in significantly reduced platelet adhesion and aggregation (Sarratt *et al*., 2005).

ECM fibronectin can also support platelet adhesion as blocking it on exposed arterial sub-endothelium or its depletion markedly reduces platelet adhesion, delays thrombus formation and produces an unstable thrombus (Houdijk and Sixma, 1985, Ni *et al*., 2003). Platelet adhesion to fibronectin is mediated by the platelet receptors CD41/CD61 (integrin $\alpha_{IIb}\beta_3$, GPIIb/IIIa,), CD49e/CD29 (integrin $\alpha_5\beta_1$, VLA-5) and GPIbα (Beumer *et al*., 1995). Circulating fibrinogen, produced by the liver, is cleaved by thrombin to form insoluble fibrin (Blomback *et al*., 1978). Both fibrin and fibrinogen also act as a substrate for platelet adhesion through binding of CD41/CD61 (Hantgan *et al*., 1990, Savage *et al*., 1996). At low shear rates of 600-900s^{-1}, arrest on fibrinogen and not GPIbα-VWF interactions is the primary initiator of platelet adhesion (Savage *et al*., 1996)

1.4.2.1. Platelet Activation

Under normal conditions platelet activation is inhibited by NO and PGI2 and also the endothelial ecto-ADPase CD39 (Furie and Furie, 2008). However, the tethering events described above allow platelet surface signalling receptors to establish contact with exposed collagen, which acts as a potent activator of platelets. Platelet activation is characterised by their shape change from discoid to spiculated spheres, a shape more conducive to platelet aggregation. Activation also results in α and dense granule release of additional secondary activators such adenosine diphosphate (ADP) and thromboxane A_2 (TXA_2). The ultimate aim of these activatory signalling events is to up-regulate platelet integrins that will enable the platelet to establish firm adhesion with the vessel wall followed by thrombus growth.

Platelet GPVI appears to be the main signalling receptor mediating platelet activation through a tyrosine kinase-based signaling pathway that involves the kinase Syk and phospholipase Cγ2 (PLCγ2) (Inoue *et al*., 2003) Indeed GPVI deficient platelets do not aggregate in response to collagen (Kehrel *et al*., 1998). There is also evidence that CD49b/CD29-collagen interactions can also lead to partial platelet activation (Nakamura *et al*., 1999). Binding of collagen by either GPVI or CD49b/CD29 results in the activation of the dominant aggregatory platelet integrin CD41/CD61 (Kehrel *et al*., 1998, Nakamura *et al*., 1999). CD42c (GPIbβ)-VWF interactions can also lead to activation of platelets (Kroll *et al*., 1991). This occurs through activation of phospholipase-C which leads to activation of protein kinase-C and an increase in intracellular calcium which promotes aggregation and platelet secretion (Kroll *et al*., 1991).

Platelets can be activated by other pathways in addition to those mentioned above. Thrombin is a serine protease which acts as a platelet activator through the G-protein-linked protease activated receptors PAR1, PAR3 and PAR4 (Coughlin, 2000). Cleavage of the N-terminal of the PAR receptors by thrombin leads to activation of phospholipase-C_β leading to activation of protein kinase-C and an increase in intracellular calcium (Coughlin, 2000). Prothrombin is cleaved to form thrombin at the site of vascular injury by factor-X which is activated by a factor-VIIa and tissue factor complex (Coughlin, 2000). Both factor-X and factor-VIIa are produced in the liver and are present in the plasma (Olson *et al*., 1966). Tissue factor is constitutively expressed in the vascular adventitia (Drake *et al*., 1989), by arterial smooth muscle following injury (Marmur *et al*., 1993) and by cytokine activated endothelial cells (Zucker *et al*., 1998). Vascular injury therefore allows factor-VIIa and factor-X to come into contact with tissue factor leading to prothrombin cleavage.

Platelet activation by potent agonists such as thrombin or collagen causes the secretion of ADP from platelet dense granules where it is stored. ADP also activates platelets through its interaction with its G protein–coupled receptors $P2Y_{12}$, $P2Y_1$ and $P2X_1$ receptors (Gachet, 2001, Mahaut-Smith *et al*., 2011). This amplifies the platelet response to other agonists (Andre *et al*., 2003). Clinically utilised drugs such as Clopidogrel block activation of $P2Y_{12}$.

1.4.2.2. Thrombus Formation

Following initial platelet adhesion and activation, platelets aggregate in increasing numbers to form a thrombus. Thrombi are composed primarily of platelets, fibrin and trapped erythrocytes and leukocytes. The developing thrombus binds unstimulated platelets from the circulation and a proportion of these become activated - some platelets remain un-activated and may become detached (Dubois *et al*., 2007). Platelet activation leads to an increase in platelet-platelet affinity which stabilises the thrombus (Furie and Furie, 2008). Platelet-platelet interactions are mediated by CD41/CD61 on platelets through a fibrinogen bridge (Goto *et al*., 1998) or at high shear rates by CD41/CD61 and GPIbα through a VWF bridge (De Marco *et al*., 1986, Goto *et al*., 1998). The dominant role of the integrin CD41/CD61 in mediating thrombus growth is evident by the clinically available CD41/CD61 inhibitors such as abciximab, eptifibatide and tirofiban that are used as potent anti-platelet drugs. The rolling, adhesion, activation and subsequent aggregatory events of platelets are summarised in *Figure 1.5*.

1.4.2. Platelet-Endothelial Cell Interactions

In recent years it has also become increasingly evident that endothelial denudation is not an absolute pre-requisite to allow platelet attachment to the arterial wall. Damaged or inflamed endothelium is known to develop properties that render it pro-adhesive for platelets (Massberg *et al*., 1999, Massberg *et al*., 1998, Frenette *et al*., 1998). Platelets not only can roll on ECM components, but similarly to leukocytes, can also roll on intact but activated endothelium. This is dependent primarily on endothelial CD62P interacting with CD162 constitutively expressed on platelets (Frenette *et al*., 2000). Activated platelets are also able to firmly adhere to endothelium using platelet CD41/CD61 binding endothelial adhesion molecules CD51/CD61 ($\alpha_v\beta_3$), CD54 and GPIbα (Bombeli *et al*., 1998). This interaction uses soluble fibrinogen, fibronectin or VWF as bridging molecules. The fact that platelets can adhere to subendothelial and endothelial layers suggests they are crucial to haemostasis as well as to an inflammatory response (Wagner and Burger, 2003).

Platelets can potentially regulate leukocyte recruitment through activation of endothelial cells. Interactions between CD40L on activated platelets and CD40 on endothelial cells up-regulates endothelial expression of the adhesion molecules CD62E, CD54 and CD106 and induces release of the cytokines CXCL8 (IL-8) and CCL2 by the endothelial cells (Henn *et al*., 1998). It has been suggested that this interaction is an important mediator of inflammation in

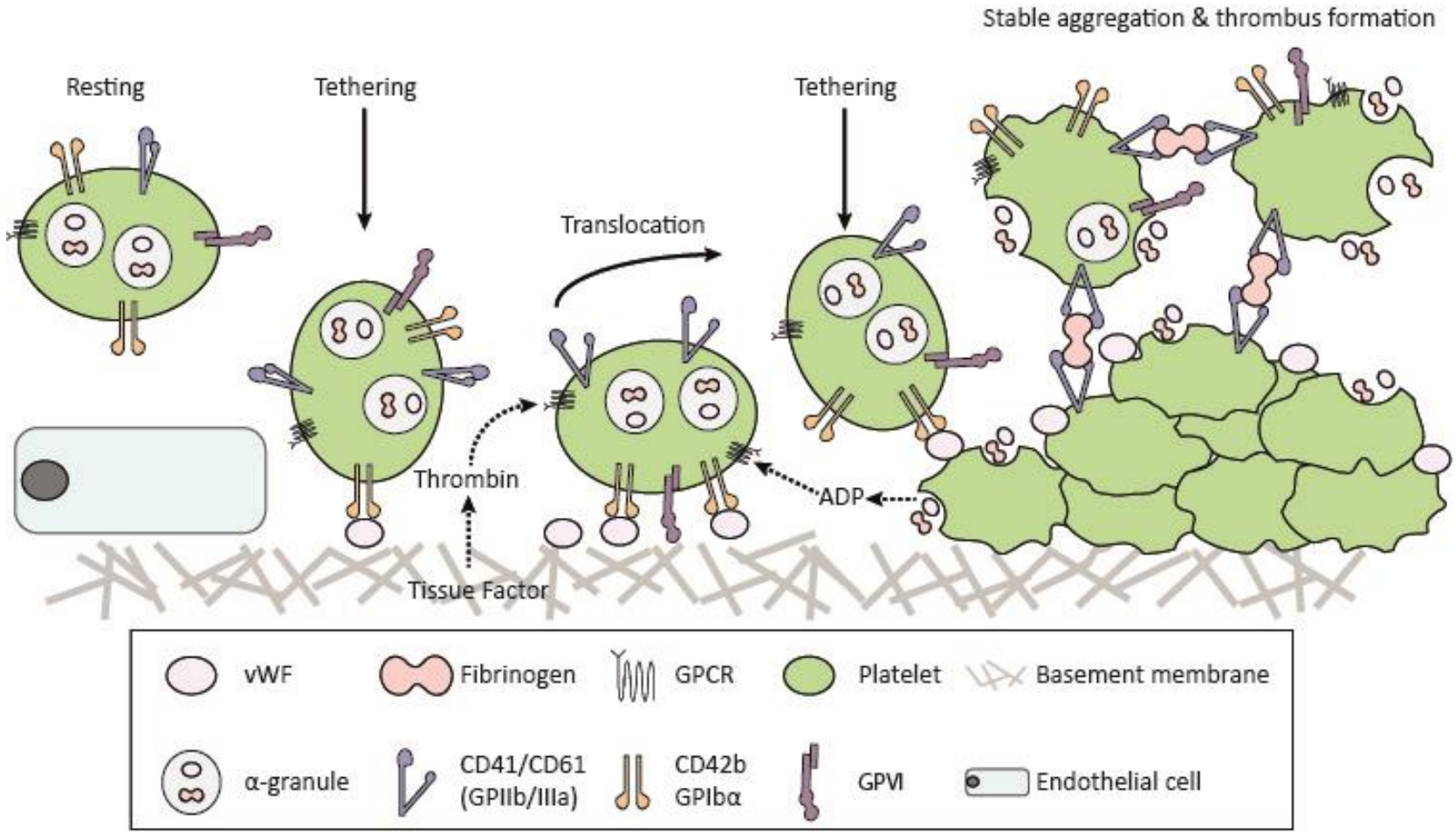


Figure 1:4 Platelet adhesion and aggregation on the ECM components such as collagen. The CD42b-vWF interaction mediates platelet tethering thereby enabling GPVI interaction with collagen. This triggers the shift of platelet integrins to a high-affinity state and release of ADP and TXA2 (not shown). In parallel, tissue factor locally triggers thrombin formation which also contributes to platelet activation. GPCR, g-protein coupled receptor; vWF, von Willebrand factor; GP, glycoprotein. Adapted from Varga-Szabo *et al*. (2008) and Jackson and Schoenwaelder (2003).

disease states such as inflammatory bowel disease, and atherosclerosis (Danese *et al*., 2003, Phipps, 2000).

1.4.3. Platelet-Leukocyte Interactions

Leukocytes and platelets are well known to directly interact with each other. Even in the normal circulation about 3% of lymphocytes are conjugated to platelets. ADP activation of platelets increases conjugation to CD8+ T-cells (from 3% to 7%) and NK cells (from 3% to 12%) but has little effect on conjugation of CD4+ T-cells or B-cells (Li *et al*., 2006). CD8+ T-cell release of interferon-γ (IFNγ) is augmented in response to binding CD40 on its surface to CD40L (CD154) expressed on the platelet in a mouse model of adenoviral infection (Elzey *et al*., 2003). In the same study, Elzey and colleagues also demonstrated that B-cells required B-cell CD40 to interact with platelet CD40L in order to switch from IgM/IgD production to production of IgG and other Ig isotypes. This was demonstrated eloquently by transfusing activated wild type platelets into $CD40L^{-/-}$ mice (Elzey *et al*., 2003). Lymphocyte activation could also increase conjugation to platelets of CD4+ T-cells (from 3% to 12%) and CD8+ T-cells (from 3% to 15%), but had little effect on the percentage of B-cells and NK cells conjugated to platelets (Li *et al*., 2006). Neutrophils also form circulating complexes with platelets. About 15% of circulating neutrophils are conjugated to platelets (Tuttle *et al*., 2003). These neutrophils, derived from healthy individuals, have been shown to exhibit a greater expression of the integrin subunit CD11b, a greater capacity for phagocytosis and ROS release than non-platelet attached neutrophils (Peters *et al*., 1999). These collective findings are indicative that neutrophils and lymphocytes within platelet-leukocyte complexes represent an activated subset of cells, where their function is enhanced by the attachment of platelets (*Figure 1.6*).

In vitro studies have demonstrated that immobilised platelets are capable of supporting the rolling and adhesion of leukocytes. T-cells and NK-cells can both roll on and adhere to immobilised activated platelets under physiological flow in a CD62P dependant manner (Diacovo *et al*., 1996b, Sheikh *et al*., 2004). Firm adhesion of NK cells on activated platelets is mediated by NK cell expressed integrins CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1) binding to CD102 (ICAM-2) expressed on platelets (Sheikh *et al*., 2004, Diacovo *et al*., 1994). Platelets also support T- and B-cell rolling and adhesion on immobilised collagen under arterial flow in a CD62P, CD40L and CD41/CD61 dependent manner (Hu *et al*., 2010). CD62P dependent lymphocyte rolling on platelets also occurs *in vivo.* Platelets immobilised on the high endothelial venules of peripheral lymph nodes have also been shown to mediate lymphocyte capture and rolling (Diacovo *et al*., 1996c). Neutrophils also manifest CD62P dependent rolling on activated platelets as well as CD11a/CD18 and CD11b/CD18 mediated firm adhesion to platelet expressed CD102 and platelet bound fibrinogen (Diacovo *et al*., 1996a, Weber and Springer, 1997).

Occlusion of vessels by platelet aggregates may also result in entrapment of leukocytes behind the thrombus without the need for receptor mediated interactions. Indeed, microvascular plugging by aggregates of platelets is frequently observed during inflammation in the liver following ischaemia (Cywes *et al*., 1993, Nakano *et al*., 2008), in the colitic gut (Miele *et al*., 2009) and during acute renal failure (Clarkson *et al*., 1970).

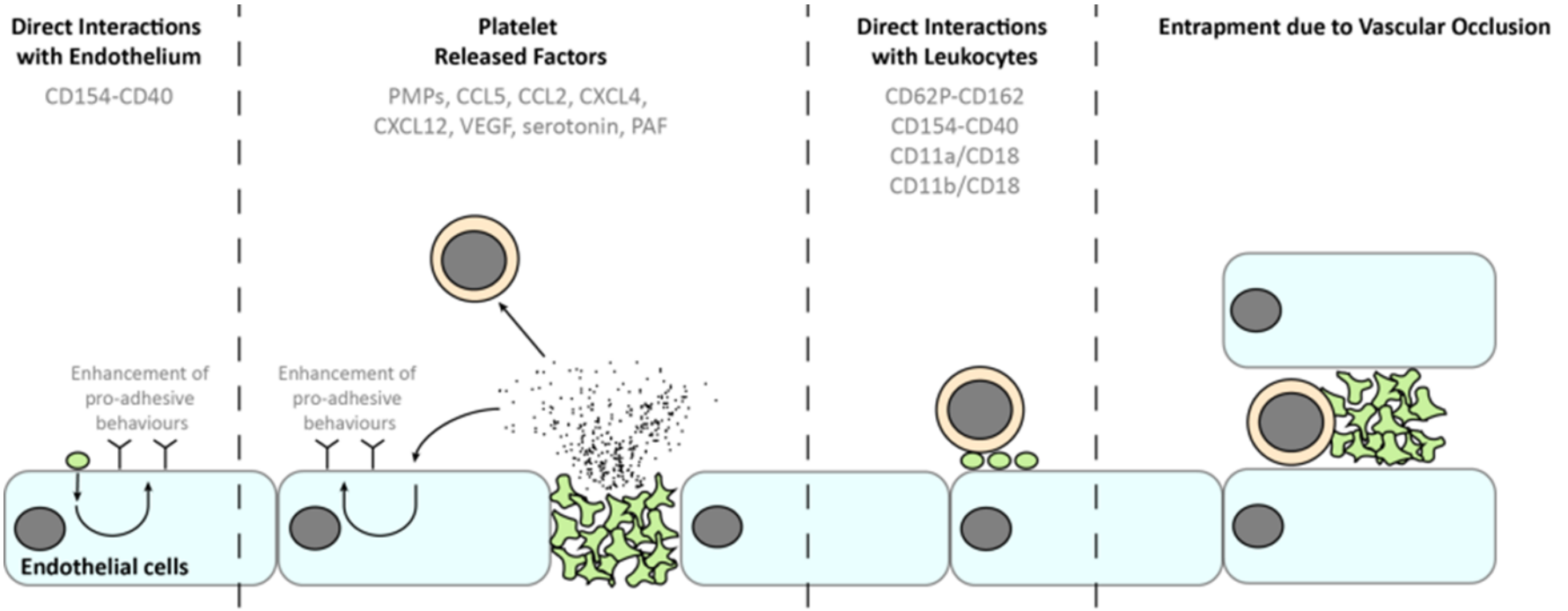


Figure 1:5 Platelet mediated leukocyte recruitment and activation. Platelets (green) are capable of mediating leukocyte (orange) recruitment through direct interactions with endothelial cells and leukocytes and through release of inflammatory factors. The major mediators of these interactions are listed in grey. Occlusion of capillaries due to platelet aggregation may also result in entrapment of lymphocytes. PMP, platelet micro-particle; VEGF, vascular endothelial growth factor; PAF, platelet activating factor.

1.4.4. Platelet Released Factors

In addition to mediating leukocyte recruitment through direct cell contact, platelets also release a range of soluble factors which can act upon both leukocytes and the endothelium. Activated platelets release a number of soluble factors including cytokines and growth factors. The α-granules of platelets contain a number of cytokines capable of regulating lymphocyte function. CXCL12 (SDF-1α), a powerful lymphocyte chemoattractant, is released by activated platelets. Indeed, platelets are a major source of CXCL12 at sites of vascular injury (Bleul *et al*., 1996, Massberg *et al*., 2006). Activated platelets also release CCL5 (RANTES) which acts as a chemoattractant for CD4+ T-cells but not CD8+ T-cells (Kameyoshi *et al*., 1992, Schall *et al*., 1990). Platelets are also the sole source of CXCL4 (PF4), an important regulator of T-cell proliferation, which stimulates proliferation of T_{reg} cells but inhibits proliferation of non-regulatory CD4+ T-cells (Liu *et al*., 2005).

Activated platelets also release several non-cytokine mediators of lymphocyte function such as vascular endothelial growth factor (VEGF) which enhances recruitment of T-cells (Wartiovaara *et al*., 1998, Zhang *et al*., 2010). Platelet serotonin release modulates a range of lymphocyte functions (Holmsen and Weiss, 1979), namely promoting proliferation of T-cells, B-cells and NK cells as well as NK cell cytotoxicity (Iken *et al*., 1995, Aune *et al*., 1994, Hellstrand and Hermodsson, 1993). Serotonin can also elicit anti-inflammatory effects. In a CD8+ dependent model of viral hepatitis, platelet derived serotonin delayed CD8+ T-cell invasion, prolonging virus persistence and aggravating the injury (Lang *et al*., 2008). Platelet activating factor (PAF) is also capable of stimulating lymphocyte migration (McFadden *et al*., 1995, Chignard *et al*., 1980).

Further to their effects on lymphocytes, many of the molecules released following platelet activation can also mediate activation of other leukocytes and endothelial cells. For example platelet released IL-1β is a potent activator of endothelial cells and CXCL4, CXCL7 and CXCL8 can mediate neutrophil recruitment (Zarbock *et al*., 2007). Clearly a significant number of platelet-derived soluble factors exist that can modify the leukocyte and vascular response during inflammation

1.4.5. Platelet Microparticles

In addition to soluble factors, activated and apoptotic platelets can shed membrane vesicles called platelet microparticles (PMPs) from their surface into the circulation. These are membrane fragments of 0.1μm-1μm in diameter (Diamant *et al*., 2004). Although initially considered as cell debris, a central role in haemostasis has been identified for PMPs. Plasma PMP numbers are significantly increased in a number of disease states including rheumatoid arthritis (Knijff-Dutmer *et al*., 2002), atherosclerosis (Nomura *et al*., 2000) and cancer (Kim *et al*., 2003). They express a number of platelet receptors including CD62P, GPIbα, CD41/CD61 and CD31 (PECAM) (Diamant *et al*., 2004).

There is good evidence that PMPs can mediate cell-cell interactions of blood cells through binding to their membrane. This can occur through binding of PMP expressed CD62P to its ligand on that cell (Janowska-Wieczorek *et al*., 2001, Forlow *et al*., 2000). Jy and colleagues presented seminal data that PMPs can 'coat' neutrophils and induce their activation, as measured by increased integrin CD11b (α_M) expression, induce neutrophil aggregation and

increase their phagocytic activity (Jy *et al*., 1995). Neutrophil aggregation is normally mediated by CD62L and CD162 (Guyer *et al*., 1996). However, in the absence of CD62L, neutrophil bound PMPs can support neutrophil aggregation, most likely through PMP expressed CD62P and neutrophil expressed CD162 (Forlow *et al*., 2000). Additionally PMPs on haematopoietic stem cells can increase their adhesion to endothelium and engraftment in lethally irradiated mice (Janowska-Wieczorek *et al*., 2001). Although PMPs can bind to lymphocytes, the binding efficacy is at much lower levels than that seen with neutrophils (Jy *et al*., 1995). However, it is not currently known whether lymphocyte bound PMPs can modulate lymphocyte-endothelial interactions.

PMPs can also modulate leukocyte adhesion through delivery of inflammatory cytokines to the local endothelial environment. Monocyte recruitment can be mediated by CCL5 deposition on endothelium by PMPs in an GPIbα and CD41/CD61 dependent process (Mause *et al*., 2005).

1.5. Platelet-Lymphocyte Interactions in Hepatitis

It is clear from the literature that both lymphocytes and platelets can contribute to the pathology of a number of inflammatory liver conditions. It is also well known that platelets can actually mediate the recruitment of inflammatory leukocytes, with the evidence stronger for neutrophils and emerging for lymphocytes. However, very little is known about the role of platelets in mediating recruitment of lymphocytes in hepatitis. Certainly recruitment of T_c cells in a mouse model of hepatitis B virus infection is platelet dependant and liver injury in

this model is reduced following platelet depletion but not after treatment with the anticoagulant warfarin (Iannacone *et al*., 2005). Recruitment of T_c cells to the liver was also greatly reduced by treatment with the anti-platelet drugs aspirin and clopidigrel in mice infected with a hepatotropic, replication deficient adenovirus (Iannacone *et al*., 2007). Platelets have also been shown to co-localise within the sinusoids following hepatic IR injury (Khandoga et al., 2006a).

However, evidence of the role of platelets in recruiting lymphocytes to non-viral mediated liver inflammation remains limited. Although T-cells are considered prime candidates in hepatitis, no studies have directly investigated whether B-cells are recruited post-injury and whether platelets can influence their presence in the liver. The dynamics of the recruitment of both these cells types, and their spatial-temporal correlation, is also not characterised *in vivo.* In this thesis, the contributory role of platelets in mediating lymphocyte recruitment to the murine liver will be investigated in two distinctly different inflammatory injuries of the liver, namely the hepatic IR injury and the concanavalin A (ConA) injury models.

1.6. Hepatic Ischaemia-Reperfusion (IR) Injury

Hepatic IR injury is a clinically important problem; it is a feature of both liver re-sectioning surgery and cadaveric liver transplantation (Serracino-Inglott *et al*., 2001). It is associated with post-operative morbidity and mortality and is increasingly known to impact on the functioning of many remote organs. Although significant damage does occur during ischaemia it is the reperfusion of the liver with oxygenated blood that results in the majority of the injury

(Eltzschig and Collard, 2004). IR injury refers to tissue damage caused paradoxically when blood flow is returned to the tissue after a period of restricted blood flow (greek. *isch*- restriction, *haema*- blood). Lack of oxygen and nutrients creates a condition in which the restoration of blood flow results in inflammation and oxidative damage through generation of ROS. Reperfusion therefore exacerbates the ischaemic injury. Indeed, in the liver, the level of hepatic damage seen after 3 hours of ischaemia and 1 hour of reperfusion is much greater than after 4 hours of ischaemia alone (Eltzschig and Collard, 2004).

Initially, ischaemia causes metabolic disturbances within cells resulting in dysregulation of energy pathways and ROS release. At the outset, and within 15 minutes, hepatic cells experience a loss of ATP, rise of intracellular sodium and cell oedema (Eltzschig and Collard, 2004, Fondevila *et al*., 2003, Kamiike *et al*., 1982). Ischaemic metabolic failure of the mitochondrial respiratory chain leads to the generation of hydroxyl radicals during reperfusion through an increased rate of hydrogen peroxide (H_2O_2) production. Evidence suggests that hepatocytes and Kupffer cells are also important origins of oxidative stress, although the latter appear to play a minor role as depleting Kupffer cells fails to attenuate IR injury (Fondevila *et al*., 2003).

Hepatic sinusoidal perfusion failure is also common during the post-reperfusion phase, resulting in microcirculatory failure (Jaeschke, 2003). The vascular tone of the hepatic sinusoids is a result of a delicate balance between vasodilatory molecules (such as NO and PGI2) and vasoconstrictory molecules (such as endothelin-1; ET-1). Any imbalance between these factors during IR results in dysregulation of vascular tone and sinusoidal blood flow,

promoting mechanical leukostasis. Indeed, pharmacological restoration of vascular tone following IR has been shown to improve blood flow and attenuates injury (Shiraishi *et al*., 1997).

Injury is also exacerbated by the substantial endothelial recruitment of platelets. Up to one third of circulating platelets were found adherent in the rat isolated liver perfused with platelets after a period of ischaemia (Sindram *et al*., 2000). These platelets form microaggregates and appear activated when examined by electron microscopy (Cywes *et al*., 1993). Platelets can induce apoptosis in sinusoidal endothelial cells during tissue reperfusion which can be prevented by blocking their adhesion (Sindram *et al.*, 2000). As a result of both the oxidatively stressed environment and the vascular mechanical disturbances during IR, hepatic microvessels display marked neutrophil accumulation, at least in part as a consequence of increased capture by endothelial cell adhesion molecules. During the reperfusion phase, neutrophil activation occurs which also results in the generation of further damaging ROS (Martí *et al*., 2004). Leukocyte recruitment is additionally amplified by the release of cytokines from the injured tissue, such as TNF-α and IL-1. During IR injury, plasma levels of TNF-α and IL-1 are raised within 5 minutes of the reperfusion phase (Toledo-Pereyra and Suzuki, 1994). Both TNF-α and IL-1 mediate activation of the pro-inflammatory transcription factor NF-κB which leads to the generation of further cytokines and chemokines, as well as synthesis of adhesion molecules. Hypoxia itself activates a number of additional transcription factors, such as hypoxia-inducible-factor-1α (HIF-1α). Hypoxic induction of HIF-1α drives a robust release of CXCL12, establishing a chemokine gradient for inflammatory cell recruitment (Ceradini *et al*., 2004).

Reperfusion also sees a dramatic increase in inflammatory cell numbers within the liver. Neutrophils are the major mediators of the inflammatory injury that occurs during reperfusion (Jaeschke *et al*., 1990). They accumulate in the liver vasculature in response to a number of inflammatory mediators including TNF-α, CCL3, CXCL2 (MIP-2), CXCL1, CXCL8 (IL-8), activated compliment factors, PAF and cytokine-induced neutrophil chemoattractant amongst others (Jaeschke, 2006). Their adhesion is mediated by endothelial adhesion molecules interacting with integrins on their surface. Under basal conditions, sinusoidal endothelium expresses low levels of CD54 and CD106, with no expression of any of the selectin family members (Lalor and Adams, 1999). Following insult, levels of CD54 and CD106 are raised, and although some vessels express CD62E and CD62P, selectins are largely absent from the sinusoids (Wang *et al.*, 1999, Adams *et al.*, 1996, Grant *et al.*, 2001). Other endothelial adhesion molecules such as MAdCAM-1 can be detected under certain specific inflammatory conditions and could play a role in IR injury (Ando *et al.*, 2007). Additionally, VAP-1 is constitutively expressed in the liver and is one of the few endothelial beds to exhibit constitutive expression (Lalor and Adams, 1999). VAP-1 has been shown to play an important role in lymphocyte recruitment to rat and murine liver allografts (Martelius *et al.*, 2004) and during inflammation in cerebral IR injury (Xu *et al.*, 2006), but it is unclear whether it has any role in hepatic IR injury. Once neutrophils have accumulated within the hepatic vascular they extravasate into the surrounding parenchyma and induce cytotoxic effects (Jaeschke and Smith, 1997). Killing of hepatocytes by activated neutrophils is mediated by both ROS production and neutrophil derived proteases (Jaeschke, 2006).

1.6.1. Lymphocytes and Hepatic IR Injury

Although the major effector cell associated with hepatic IR injury is the neutrophil, recent studies have identified a critical role for lymphocytes, particularly T-cells, in the pathophysiology of IR injury, with their presence detected post-reperfusion in various organs including the brain (Yilmaz *et al*., 2006), lung (de Perrot *et al*., 2003) and heart (Yang *et al*., 2006). Previously thought to be "passive observers" in the inflammatory response, there is now an overwhelming body of literature demonstrating the role of T-cells as direct mediators of IR injury.

1.6.1.1. T-cells and Hepatic IR Injury

Significant involvement of T-cells in hepatic IR injury was first demonstrated in 1997 in a report showing their accumulation in reperfused liver (Zwacka *et al*., 1997). This immunohistological study demonstrated peripheral $CD4^+$, but not $CD8^+$, T-cells were recruited as early as 1 hour post-reperfusion. This rapid recruitment was suggestive of lymphocytes promoting activation of the inflammatory cascade rather than being recruited inadvertently as a consequence of it. Indeed, lymphocyte recruitment preceded the influx of any appreciable numbers of potently inflammatory neutrophils, whose subsequent recruitment was dependent upon IL-17, a cytokine preferentially expressed and secreted by activated $CD4^+$ T cells and Kupffer cell activation (Zwacka *et al*., 1997, Le Moine *et al*., 2000, Kuboki *et al*., 2009). The fast reponse of lymphocytes and their ability to recruit neutrophils was further demonstrated in mice lacking CD4+ T-cells in which hepatic neutrophil accumulation was significantly decreased. Interestingly, however, both neutrophil oxidative burst activity and liver injury was

significantly increased. (Caldwell *et al*., 2005). Furthermore, the use of immunomodulators that sequestered peripheral blood T cells (eg. FTY720) into lymphoid organs, was associated with improved hepatic structure and function and overall survival (Mizuta *et al*., 1999, Anselmo *et al*., 2002, Martin *et al*., 2010). Hepatic IR injury conducted in $CD4^{-/-}$ mice was also associated with improved histological scores, reduced neutrophil infiltration and improvements in sinusoidal perfusion (Khandoga *et al*., 2006a).

Binding of CXCR3 by its ligands CXCL9, 10 and 11 released during IR injury appears to be at least partially responsible for T-cell activation during IR injury (Zhai *et al*., 2006). Antibody blockade of CXCR3 in a rat model of liver transplant significantly reduced both CXCR3+ CD4+ T-cell accumulation within the liver and also liver injury (Zhai *et al*., 2006). In addition to cytokines matrix metalloproteinase-9 is required for T-cell recruitment and transmigration during hepatic IR injury (Khandoga *et al*., 2006b). There is also some evidence in other organs for antigen mediated activation of T-cells during IR injury however, it remains unclear if this occurs during hepatic IR injury (Caldwell *et al*., 2007).

1.6.1.2. B-cells and Hepatic IR Injury

The other major cell of the adaptive immune system is the B-cell. Activated B-cells primarily function in antibody production and antigen presentation. However, due to their ability to secrete the potently anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokines IL-6, CXCL8 and TNFα, the role of a positive and negative regulator of inflammation has been ascribed to this cell (Hamze *et al*., 2013). In IR injuries of the gut and hind-limb, B-cell produced

IgM is responsible for activation of the classical complement pathway resulting in injury (Zhang *et al*., 2004, Austen Jr *et al*., 2004). Burne-Taney and colleagues demonstrated reduced injury scores following renal IR injury in mice unable to develop peripheral mature B-cells (Renner *et al*., 2010, Burne-Taney *et al*., 2003). In the kidney IgM release by B-cells exacerbated injury independently of compliment. However, IL-10 release by B-cells appeared to have protective effects (Renner *et al*., 2010). This dual role of B-cells in renal IR injury may account for findings that B-cell depletion can increase or decrease renal IR injury (Renner *et al*., 2010, Burne-Taney *et al*., 2003). IgM also plays a pathogenic role in myocardial IR injury (Zhang *et al*., 2006). Further demonstrations of a B-cell pathogenic role was shown in IR injured intestine, heart and skeletal muscle (Linfert *et al*., 2009).

Despite frequent histological observations of B-cells at inflammatory sites, there is little known about the recruitment kinetics of circulating B-cells to IR injured liver, even though the liver has a very large resident B cell population (Novobrantseva *et al*., 2005). They have been observed to home to the liver following parasitic hepatic infection (Moore *et al*., 2012) and are known to contribute to liver fibrosis, a late presenting feature of hepatic IR injury (Novobrantseva *et al*., 2005, Cheng *et al*., 2008).

Collectively, these studies are consistent with an emerging pivotal role for lymphocytes in IR injury. However, the question remains as to how they are recruited to the ischaemically injured sinusoidal microcirculation *in vivo*. The importance of Kupffer macrophages has been demonstrated intravitally whereby their depletion using gadolinium chloride resulted in

reduced T-cell recruitment (Hanschen *et al*., 2008). However, lymphocytes have also long been known to roll and adhere under flow conditions to immobilised platelets *in vitro* and to captured platelets in high endothelial venules of peripheral lymph nodes *in vivo* (Sheikh *et al*., 2004, Diacovo *et al*., 1996c). Platelets are also recruited in abundance during hepatic IR injury (Khandoga *et al*., 2003) and more recently have been demonstrated to recruit T-cells following hepatitis B infection (Iannacone *et al*., 2005, Sitia *et al*., 2012). However, the role of platelets in non-viral or non-antigen induced T-or B-cell recruitment to the liver is not clear.

1.7. Concanavalin-A Induced Liver Injury

In order to compare the mechanisms of lymphocyte recruitment in a primarily neutrophil mediated liver injury (hepatic IR injury) to a primarily lymphocyte mediated liver injury, studies within this thesis have also utilised an acute mouse model of ConA induced hepatitis. ConA is a lectin originally isolated from the jack bean (*Canaualia ensi-formis*) which triggers T-cell activation and proliferation *in vitro* (Palacios, 1982). Following intravenous injection with ConA at doses greater than 1.5mg/kg, mice develop a severe T-cell dependant liver injury within 8 hours (Tiegs *et al*., 1992). It has been used as a model for T-cell mediated hepatitis including autoimmune hepatitis, viral hepatitis and fulminant hepatitis (Trautwein *et al*., 1998, Ajuebor *et al*., 2005, Diao *et al*., 2004). ConA induced liver injury is characterised by leukocyte and platelet recruitment followed by endothelial detachment, hepatocyte death and obstruction of the microcirculation (Tiegs et al., 1992, Massaguer et al., 2002).

The injury appears to be mainly mediated by CD4+ T cells as injection of anti-CD4, but not anti-CD8, mab significantly reduces injury (Tiegs *et al*., 1992). Significant numbers of CD4+ T, and much fewer CD8+ T-cells, infiltrate the liver following ConA injection (Mizuhara *et al*., 1994). NKT cells are also required for induction of ConA injury (Kaneko *et al*., 2000, Toyabe *et al*., 1997) through osteopontin release, which triggers neutrophil activation and infiltration into the liver (Diao *et al*., 2004). ConA injury is dependent on the activation of circulating T-cells by ConA (Mizuhara *et al*., 1994). In the liver, ConA binds to mannose receptors on sinusoidal endothelial cells resulting in the liver specific activation of T-cells (Knolle *et al*., 1996). Although, like sinusoidal endothelial cells, Kupffer cells bind ConA *in vitro,* they do not significantly bind ConA *in vivo* (Knolle *et al*., 1996). The activation of T-cells in the liver leads to increases in several cytokines involved in inflammation including: CXCL9 (MIG), CCL20 (MIP3A), CXCL5 (ENA-78), CXCL11 (I-TAC), CXCL10 (IP-10), CCL3 (MIP1A), INF-γ, TNF-α, IL-12, IL-2 and IL-10 (Jaruga *et al*., 2004, Ajuebor *et al*., 2004, Gantner *et al*., 1995, Tagawa *et al*., 1997, Louis *et al*., 1997). In INF-$\gamma^{-/-}$ mice there is a significant reduction in injury caused by ConA injection when compared to wild type mice and hepatocyte apoptosis is also significantly reduced (Tagawa *et al*., 1997).

ConA injury is associated with increased expression of a number of endothelial cell adhesion molecules in the liver including CD54, CD106 and VAP1 (Jaruga *et al*., 2004, Bonder *et al*., 2005). Injury is reduced in CD62L$^{-/-}$ and CD54$^{-/-}$ mice and is greatly reduced in double knockout mice when compared to wild type controls. These reductions in injury correlate to a decrease in number of CD4+ T-cells but not CD8+ T-cells or neutrophils within the liver (Kawasuji *et al*., 2006). Th1 and Th2 adhesion within the liver sinusoids following ConA injection is mediated

by VAP1 and CD49d/CD29 respectively (Bonder *et al*., 2005). ConA injury is also severely impaired in mice lacking P-selectin (Massaguer et al., 2002) which mediates leukocyte rolling within the ConA injured liver (March *et al*., 2005).

1.8. Summary

Given the emerging evidence for the role of the adaptive immune system in the pathology of inflammation in the liver, this study examined intravitally the dynamics of circulating T- and B-cell recruitment. Furthermore, whether this was mechanistically dependent on platelets was also investigated. Platelets have long been known to be capable of mediating lymphocyte adhesion and platelet accumulation within the liver is a common feature of liver inflammation. However, to date little is known about the role of platelets, their microparticles or their releasate as mediators of lymphocyte recruitment in hepatic inflammation. Determining the mechanisms of lymphocyte recruitment to the hepatic microcirculation during inflammation is an important first step in developing strategies to limit their invasion of the liver. In the first study of its kind, the kinetics and mechanisms of lymphocyte recruitment following IR injury and ConA-induced liver injury was compared.

1.9. Aims and Hypotheses

This thesis aimed to test the following hypotheses:

1. Platelets, their microparticles or their releasate can enhance lymphocyte adhesion to endothelial cells or immobilised endothelial counterligands *in vitro.*

2. T- and B-cell recruitment to the hepatic microvasculature is significantly increased following IR and ConA liver injuries.

3. Platelet recruitment to the hepatic microvasculature is significantly increased following injury.

4. T- and B-cell recruitment to the injured hepatic vasculature is platelet dependent.

5. Lymphocytes are recruited to the liver via similar mechanisms in ConA and IR injury.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Materials

All antibodies were sourced from the suppliers listed in *Table 2.1* and used at the stated concentrations. Materials commonly used in this thesis are listed in *Table 2.2*; materials mentioned in the text were obtained from the indicated supplier unless stated otherwise.

2.2. Methods

2.2.1. Animals

Animal experiments were conducted with the prior approval of the United Kingdom Home Office and local ethics committee in accordance with the Animals (scientific procedures) Act of 1986 under project licence 40/2749 (held by Dr. Neena Kalia) for intravital studies and project Licence 30/2721 (held by Prof. Steve Watson) for terminal bleeding. Tissue samples were obtained from colitis mice which were generated under project licence 40/3226 (held by Dr. Bertus Eksteen) and transferred to project licence 40/2749 for intravital experiments (carried out by [redacted] prior to tissue isolation. For all animal experiments 8-12 week old male C57BL/6 mice were obtained from [redacted] ([redacted]) and housed [redacted] in the [redacted] with *ad libitum* access to food and water. The mice were allowed to rest for at least one week prior to experimentation following their arrival at the [redacted].

Table 2.1 Antibodies used in this thesis

Antibody	*Conj*	*Clone*	*Host*	*Target*	*Isotype*	*Supplier*	*Conc (mg/ml)*
Anti-CD19	PE	eBio 1D3	Rat	Mouse	IgG2a,κ	eBioscience	0.2
Anti-CD3e	PE	145-2C11	Armenian hamster	Mouse	IgG	eBioscience	0.2
Anti-CD19	NA	MB19-1	Mouse	Mouse	IGAκ	eBioscience	0.5
Anti-CD3e	biotin	145-2C11	Armenian hamster	Mouse	IgG	ebioscience	0.5
Anti-CD41	NA	MwReg30	Rat	Mouse	Rat IgG_1, κ	BD biosciences	0.5
Anti-Rat IgG (H+L)	Alexa Flour 555	Polyclonal	Goat	Rat	IgG	Life technologies	2
Streptavidin	Alexa Flour 647	NA	NA	Biotin	NA	Life technologies	1
Anti-Mouse IgG (H+L)	Alexa Fluor 488	Poly clonal	Rabbit	Mouse	IgG	Life technologies	2
Anti-CD16/32	NA	93	Rat	Mouse	IgG2a, λ	Biolegend	0.5
Anti-CD18	FITC	M18/2	Rat	Mouse	IgG2a, κ	eBioscience	0.5
Anti-CD49d	FITC	R1-2	Rat	Mouse	IgG2b, κ	eBioscience	0.5
Rat IgG2a, κ isotype control	FITC	eBR2a	Rat	Mouse	IgG2a, κ	eBioscience	0.5
Armenian hamster IgG isotype control	PE	eBio299Arm	Armenian hamster	Mouse	IgG	eBioscience	0.2
Rat IgG2a κ isotype control	PE	eBR2a	Rat	Mouse	IgG2a, κ	eBioscience	0.2

Antibody	*Conj*	*Clone*	*Host*	*Target*	*Isotype*	*Supplier*	*Conc (mg/ml)*
Anti CD42b	NA	Xia.B2	Rat	Mouse	IgG2a	emfret	0.5
Platelet depletion antibody (anti CD42b)	NA	Polyclonal/ Cocktail (R300)	Rat	Mouse	IgG	emfret	0.5
Rat IgG1 κ isotype control	NA	eBRG1	Rat	Mouse	IgG1 κ	eBioscience	0.5
Anti CD162	NA	4RA10	Rat	Mouse	IgG1 κ	BD biosciences	0.5
CD8a	PE	53-6.7	Rat	Mouse	IgG2a κ	eBioscience	0.2
CD4	PE	GK1.5	Rat	Mouse	IgG2b κ	eBioscience	0.2
Anti CD62L	FITC	MEL-14	Rat	Mouse	IgG2a κ	eBioscience	0.2
Anti CD44	PE	IM7	Rat	Mouse	IgG2b κ	Biolegend	12.5
Ly-6G	PE	RB6-8C5	Rat	Mouse	IgG2b κ	eBioscience	0.2
CD19	NA	6D5	Rat	Mouse	IgG2a	Life technologies	0.2
Armenian hamster IgG isotype control	biotin	eBio299Arm	Armenian hamster	Mouse	IgG	eBioscience	0.2
Mouse IgA isotype control	NA	NA	Mouse	Mouse	IgA κ	eBioscience	0.2

Table 2.2 Materials frequently used in this thesis

Materials	*Abbreviation*	*Manufacturer*	*Location*
Acetone	-	Fisher Scientific	Leicestershire, UK
Alexa-647 Bovine Serum Albumin	-	Life Technologies	Paisley, UK
Bovine Serum Albumin	BSA	Sigma	Poole, UK
5'6-carboxyfluorescein diacetate succinimidyl ester	CFDA-SE	Life Technologies	Paisley, UK
Cell Tracker Orange (5-(and-6)-(((4-Chloromethyl)Benzoyl)Amino)Tetramethylrhodamine)	CMTMR	Life Technologies	Paisley, UK
Concanavalin A	ConA	Sigma	Poole, UK
Dimethyl Sulphoxide	DMSO	Sigma	Poole, UK
DMEM (4.5g/l glucose)	-	Sigma	Poole, UK
D-valine MEM	-	Stratech	Newmarket, UK
Ethylenediaminetetraacetic acid	EDTA	Sigma	Poole, UK
Fluorescein isothiocyanate isomer I–Celite	FITC on celite	Sigma	Poole, UK
Heat Inactivated Fetal Calf Serum	FCS	Sigma	Poole, UK
Heparin (multiparin)	-	CP Pharmaceuticals	Wrexham, UK
2% Gelatin (Type I from porcine skin)	-	Sigma	Poole, UK
Ketamine Hydrochloride (Vetalar V)	-	Pfizer	Kent, UK
L-Glucose	-	Sigma	Poole, UK
L-Glutamine	-	Sigma	Poole, UK
Non-essential Amino Acids Solution	-	Life Technologies	Paisley, UK
Penicillin/Streptomycin	P/S	Life Technologies	Paisley, UK

Materials	*Abbreviation*	*Manufacturer*	*Location*
Phosphate Buffered Saline	PBS	Sigma	Poole, UK
Prostacyclin	-	Sigma	Poole, UK
Propridium Iodide	PI	eBioscience	Hatfield, UK
Saline	-	MacoPharma	Middlesex, UK
Thrombin	-	Sigma	Poole, UK
Trypan Blue	-	Sigma	Poole, UK
Trypsin EDTA solution	-	Sigma	Poole, UK
MEM Vitamin Solution	-	Life Technologies	Paisley, UK
Xylazine Hydrochloride (Xylacare 2%)	-	Animalcare	York, UK

2.2.2. Cell Isolation and Culture

2.2.2.1. Endothelial Cell Culture

Immortalised colonic endothelial cells (kindly provided by Professor Alexander, USA) were isolated as previously described from the Immortomouse (Ando *et al*., 2005). The cells were expanded at 33^{o}C and 5% CO_2 in endothelial growth media (D-Valine MEM, 10% foetal calf serum (FCS), 1% penicillin streptomycin (P/S), 1% L-glutamine, 1% non-essential amino acids, 1% vitamin mix and 10 units/ml interferon γ). At confluence, cells were dissociated with trypsin-EDTA washed with growth media and split 1 in 3. For static adhesion assays the cells were de-immortalised by transferring them to experiment media (450ml DMEM (4.5g/l glucose), 1% FCS, 1% P/S) splitting them one in six and allowing them to grow to confluence over 2-5 days at 37^{o}C and 5% CO_2. All tissue culture plastics were treated with a 2% gelatine solution before use to aid cell adhesion.

2.2.2.2. Lymphocyte Isolation

For some experiments lymphocytes were isolated from the inguinal lymph nodes of sacrificed C57BL/6 mice on the day of use. The lymph nodes were pushed through a CD-1 mesh size 100 screen (Sigma) into ice cold PBS containing 2% FCS using the plunger from a 5 ml syringe. The screen was then washed thoroughly with 2% FCS in PBS and the resulting cell suspension collected. The suspension was then spun at 400g for 10 minutes and the pellet was responded in 1ml of 2% FCS in PBS. The cell suspension was stored on ice until use.

For other experiments T-cells, B-cells, CD4+ T-cells or CD8a+ T-cells were isolated through negative selection magnetic activated cell sorting (MACS). MACS has been frequently used to isolated lymphocytes for intravital microscopy studies (Worbs *et al*., 2007, Ogino *et al*., 2004, Chtanova *et al*., 2014). Negative selection kits were used to limit the possible effects of isolation on lymphocyte function (Šafařík and Šafaříková, 1999). The B cell isolation kit, Pan T cell isolation kit II, CD4+ T cell Isolation Kit II and CD8a+ T cell Isolation Kit II were used as per the manufacturer's protocol (Miltenyi Biotec, Surrey, UK). Spleens were taken from C57BL/6 mice and pressed through CD-1 mesh size 100 screens into ice cold MACS buffer (PBS, pH7.2 supplemented with 0.5% bovine serum albumin (BSA) and 2mM EDTA). The cell suspension was then passed through a 7.5µm filter to remove cell clumps, counted and centrifuged at 300g for 10 minutes. The cells were resuspended in MACS buffer at $2.5x10^9$cells/ml. 10µl of the required biotinylated antibody cocktail (*Table 2.3*) was added per 40µl of cell suspension and incubated at 4^oC for 10 minutes. After incubation with the antibody cocktail 20µl of anti-biotin MicroBeads and 30µl of MACS buffer per 40µl cell suspension was added and the cells were incubated for a further 10 minutes at 4^oC. 1ml of MACS buffer was added and the cells were centrifuged at 400g for 10 minutes. The cell pellet was resuspended in 500µl of MACS buffer and run through an MS or LS MACS column in a MiniMACS or MidiMACS separator respectively (Miltenyi Biotec). The column was washed 4 times with MACS buffer and the eluted cells collected. The purity of the isolated cells was checked through flow cytometric identification of the cell specific markers CD3ε (T-cells), CD19 (B-cells), CD4 and CD8a (Section 2.2.3).

Table 2.3 MACS antibody cocktails

Kit	*Antibody Cocktail against*
B Cell Isolation Kit	CD43, CD4, Ter-119
Pan T cell Isolation Kit II	CD11b, CD11c, CD19, CD45R, CD49b CD105, MHC class II, Ter-119
CD4+ T Cell Isolation Kit II	CD8a, CD11b, CD11c, CD19, CD45R, CD49b, CD105, MHC Class II, Ter-119
CD8a+ T Cell Isolation Kit II	CD4, CD11b, CD11c, CD19, CD45R, CD49b, CD105, MHC Class II, Ter-119

2.2.2.3. Platelet Isolation

In order to isolate platelets isoflourane anesthetised mice were terminally CO_2-narcosed. A laparotomy was performed and blood was taken from the descending aorta into 100µl of acid citrate dextrose (ACD; 120mM sodium citrate, 110mM glucose, 80mM citric acid). The blood was added to 200µl of Tyrode's Buffer (134mM NaCl, 0.34mM Na_2HPO_4, 2.9mM KCl, 12mM $NaHCO_3$, 20mM HEPES, 1mM $MgCl_2$, 5mM glucose; pH 7.3 at 37^{o}C) and centrifuged at 200g for 5 minutes. The platelet rich plasma (PRP) and the top third of erythrocytes were removed and centrifuged again at 200g for 6 minutes. PRP was retained and a further 200µl of Tyrode's buffer was added to the remaining erythrocytes which were then centrifuged again at 200g for 6 minutes. The PRP from this spin was pooled with that of the previous spin, 10ng/ml of prostacyclin was added to prevent aggregation and the platelets were pelleted through centrifugation at 1000g for 6 minutes. The pellet was resuspended in Tyrode's buffer, platelet counts were obtained using a coulter counter (Beckman Coulter, High Wycombe) and the platelets were diluted to the required concentration with Tyrode's buffer. Platelets were left to rest for at least 30 minutes after the addition of prostacyclin prior to their use in experiments to ensure no prostacyclin remained.

2.2.2.4. Lymphocyte Staining

For adhesion assays and intravital studies lymphocytes were fluorescently labelled with either CMTMR or CFDA-SE, both of which are cytoplasmic dyes which enter the cell through diffusion. Once in the cell they are converted to cell-impermeant reaction products preventing them from leaving. CMTMR and CFDA-SE were dissolved at 10mM in DMSO,

divided into single use aliquots and stored at -20^{o}C. Cells were stained by incubating them in a solution of 10µM CFDA-SE or 5µM CMTMR in PBS for 40 minutes at room temperature in the dark. These staining concentrations have previously been used to label lymphocyte for intravital microscopy experiments (Gunzer *et al*., 2004, Jones *et al*., 2003).

2.2.2.5. Platelet Staining

Platelets were also fluorescently labelled for use in adhesion assays and intravital studies. The initial steps of staining follow the platelet isolation protocol above up to the point of centrifugation at 1000g. After centrifugation the platelets were resuspended in 1ml Tyrode's buffer with 10µM CFDA-SE and incubated in the dark at room temperature for 20 minutes. 10ng/ml of prostacyclin was added, and the platelets were pelleted through centrifugation at 1000g for 6 minutes. The pellet was resuspended in Tyrode's buffer, platelet counts were obtained using a coulter counter, and the platelets were diluted to the required concentration with Tyrode's buffer. Platelets were left to rest for at least 30 minutes after the addition of prostacyclin prior to their use in experiments. This method of staining platelets has previously been shown not to cause platelet activation (Holyer, 2010).

2.2.3. Flow Cytometry

Flow cytometry was used to measure the purity of cell separations and changes in surface receptor expression on cells following treatment. 5x10^{5} cells in 100µl FACS buffer (2% FCS, 0.01% sodium azide in PBS) were incubated for 20min on ice with a 1:50 dilution of the required antibody (*Table 2.1*). If a secondary antibody was required this was added at a dilution of 1:50 and incubated for a further 20min on ice. Cells were then centrifuged at 400g

for 10 minutes and resuspended in 300µl FACS buffer. The samples were then loaded into a FACSCalibur flow cytometer (BD) and analysed with Summit 4.0 (DakoCytomation; Colorado, USA). Flow cytometry plots in this thesis were produced using Flowing Software 2.5.1 (University of Turku, Finland) and Summit 4.0.

2.2.4. Static Adhesion Assays

These assays were conducted to determine the effects of (i) adherent platelets on subsequent lymphocyte adhesion and (ii) incubating lymphocytes with platelets, cytokines and H_2O_2 on their subsequent adhesion. Static adhesion assays were carried out on both colonic endothelial cells and immobilized ligands (CD54, CD106 and MAdCAM-1). Three different types of assay were carried out. Firstly, naïve and thrombin activated (5 minute incubation in 0.2U/ml thrombin) CFSE-labelled platelets ($2x10^7$platelets/ml) in Tyrode's were incubated on the ligands or endothelium for 20 minutes followed by 3 washes with PBS.

In the second assay the ligands or endothelium were incubated with platelets as above, the wells were then washed 3 times with PBS and then incubated for 20 minutes with lymphocytes ($1x10^6$lymphocytes/ml) in PBS followed by a further 3 washes with PBS (*Figure 2.1 A*).

In the final assay the lymphocytes ($1x10^6$lymphocytes/ml) were incubated with a pre-treatment (*Table 2.4*) for 5 or 30 minutes. For this, thrombin activated platelet supernatant was produced by incubation of washed platelets with 0.2U/ml thrombin in Tyrode's buffer for 5 or 30 minutes, the platelets were then spun down (1500g, 15 minutes) and the supernatant isolated. Platelet micro particles (PMPs) were produced by incubation of $3x10^8$ washed

platelets/ml with 2U/ml thrombin for 30 minutes at 37°C. Platelets were then spun down (1500g, 15 minutes) and the resulting supernatant was centrifuged again at 1800g for 30 minute. The supernatant was discarded and the pellet (PMPs) was resuspended in an equivalent volume of Tyrode's. Following pre-treatment the cells were added to endothelium or immobilised ligands at a concentration of $1x10^6$lymphocytes/ml and incubated for a further 20 minutes (*Figure 2.1 B*).

Following each of the assays, cells were fixed overnight in 5% formalin at 4^oC then washed 3 times in PBS prior to imaging with an Olympus IX81 inverted microscope fitted with a 20X objective lens (Olympus, UK). 5 images were taken per well in a pre-defined pattern (*Figure 2.2*) starting at the centre of the well. To eliminate bias, images were analysed using blind counting software developed by Dr Kavanagh (University of Birmingham).

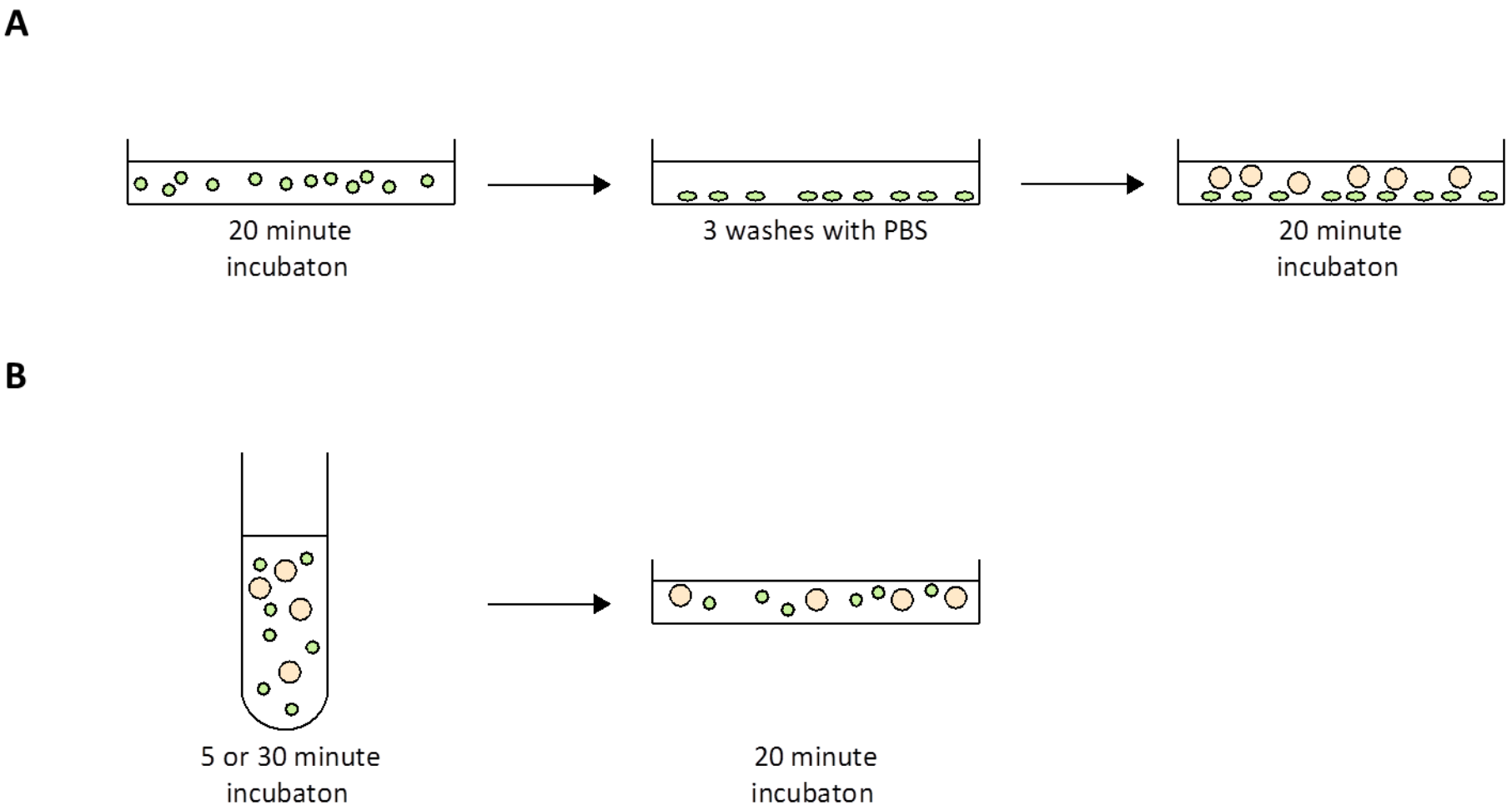


Figure 2:1 Schematic of static adhesion assays. Assays were conducted to determine the effects of (**A**) adherent platelets (green) on subsequent lymphocyte (orange) adhesion and (**B**) incubating lymphocytes with platelets, cytokines and H_2O_2 on their subsequent adhesion.

Table 2.4 Static adhesion assay pre-treatments

Pre-treatment Name	***Description***
Thrombin	0.2U/ml thrombin
Naïve Platelets	$2x10^7$ platelets/ml
Thrombin activated Platelets	$2x10^7$ platelets/ml + 0.2U/ml thrombin
Thrombin activated Platelet Supernatant	The supernatant of $2x10^7$ platelets/ml + 0.2U/ml thrombin
Platelet micro particles (PMPs)	Released following activation of $3x10^8$ platelets/ml with 2U/ml thrombin for 30 minutes at 37°C
CXCL1 (KC)	25 ng/ml CXCL1 (PeproTech; London, UK)
CXCL12 (SDF)	25 ng/ml CXCL12 (PeproTech; London, UK)
CCL5 (RANTES)	25 ng/ml CCL5 (PeproTech; London, UK)
H_2O_2	100μM H_2O_2

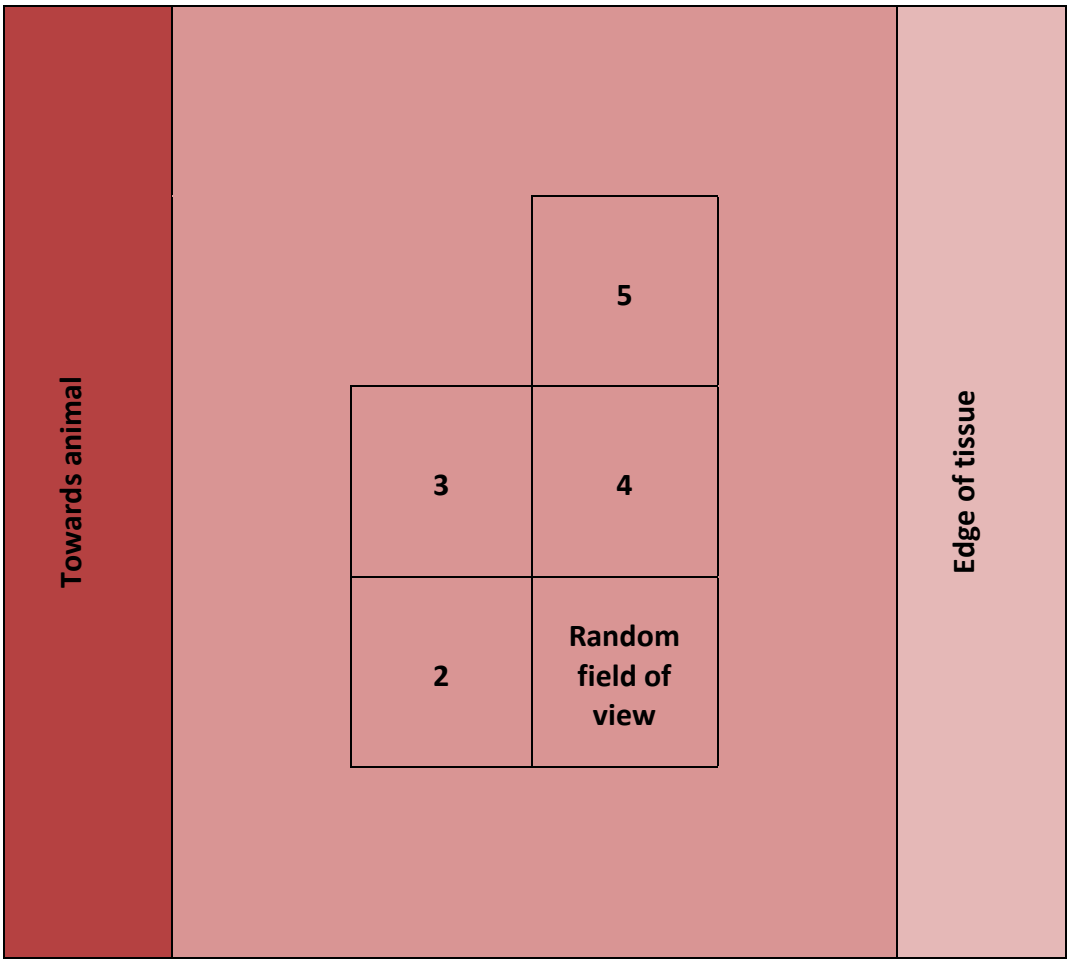


Figure 2:2 Pre-defined imaging pattern. For intravital studies, adhesion assays and immunofluorescent staining 5 fields of view were imaged following this pre-defined pattern.

2.2.4.1. Ligand Static Adhesion Assay

For ligand static adhesion assays, 96 well plates were treated with 50µl of 3% BSA or 10µg/ml recombinant murine CD54, CD106 or MAdCAM-1 for 1 hour at room temperature. The treatments were then removed and the remaining uncoated plastic was blocked by incubation with 50µl of 3% BSA for a further hour. This concentration was determined as the minimum concentration that could block activated platelet (5 minutes, 0.2U/ml thrombin) adhesion to tissue culture plastic. The wells were then washed three times with PBS and the assays were carried out as above adding 100µl of cell suspension to each well.

2.2.4.2. Endothelial Static Adhesion Assay

Immortomouse colonic endothelial monolayers in 24 well plates were washed with PBS and incubated at 37^{o}C for 4 hours in experiment media with or without 10ng/ml TNF-α. The wells were then washed three times with PBS and the assays were carried out as above adding 500µl of cell suspension to each well.

2.2.4.3. Stamper Woodruff Assays

Gut sections were taken from sham, IR injured and colitis mice. IR injury was induced by clamping of the superior mesenteric artery for 45 minutes followed by 2 hours of reperfusion, for uninjured controls sham surgery was carried out. Colitis was induced by replacement of drinking water with water containing 3% dextran sodium sulphate for 5 days (MW 36000-50000, MP Biomedicals, UK), sham controls were given normal drinking water. The terminal Ileum and terminal jejunum were taken from IR injured mice and the descending colon was

taken from colitis mice. Gut sections were then cut lengthways along one side and laid flat, mucosal side, up on a cork board. The tissue samples were then snap frozen in liquid nitrogen and stored at -80^{o}C. Prior to use the top 60µm of the mucosal side of the sections was removed using a cryostat in order to expose the vasculature. Tissue sections were then fixed in acetone for 20 minutes, air dried and stored at -20^{o}C.

On the day of use tissue sections were placed at room temperature for 20 minutes to allow them to thaw. The surfaces of the sections were than washed 3 times with PBS. 100µl of PBS containing 2x10^{6} naïve or activated (0.2U/ml; 5 minutes) CFSE labelled platelets or 100,000 lymphocytes were then placed onto the sections for 20 minutes in the dark in a humidified box. The cell suspension was then removed and the sections were washed 3 times with PBS. The sections were fixed with acetone for 20 minutes and air dried before imaging.

Images were captured with an Olympus IX81 inverted microscope fitted with a 20X objective lens. 5 images were taken per section in a pre-defined pattern (*Figure 2.2*) starting at the centre of the section. To eliminate bias images were analysed using blind counting software developed by Dr Kavanagh (University of Birmingham).

2.2.5. *In vivo* Studies

2.2.5.1. Surgical Preparation

Anaesthesia was induced through intraperitoneal injection of ketamine (100mg/Kg) and xylazine hydrochloride (10mg/Kg) delivered in 0.9% saline solution. Anaesthesia was monitored through regular testing of the pedal reflex and maintenance anaesthesia was

delivered intra-arterially as required. The trachea and right common carotid artery were cannulated using heparinised polyethylene portex tubing (Smiths Medical, Hythe, UK) in order to assist breathing and allow introduction of anaesthetic, cells and antibodies. Skin was removed for the abdomen and a midline laparotomy was performed by cutting along the avascular linea alba. The left and right rectus abdominis muscles were then removed using a cautery exposing the liver. The falciform ligament and round ligament of the liver were cut freeing the liver from its attachment to the diaphragm.

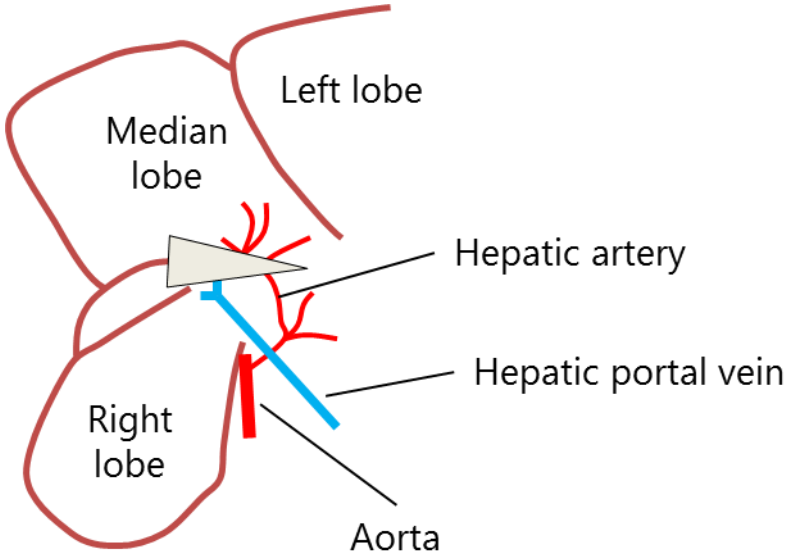
2.2.5.2. Hepatic Ischemia Reperfusion

As total hepatic ischemia for periods of greater than 45 minutes results in 100% mortality (Kavanagh, 2009) injury was induced through partial hepatic ischemia. Ischemia of the left and median lobes of the liver was induced through application of an atraumatic vascular clamp to the hepatic artery and portal vein supplying these lobes for 90 minutes (*Figure 2.3 A*). Ischemia was confirmed by colour change of the left and median liver lobes (*Figure 2.3 B*). Sham animals were prepared in the same way as injured animals except the vascular clamp was not applied. After 90 minutes ischemia the clamp was removed and intravital observations carried out.

2.2.5.3. Concanavalin A induced Liver Injury

ConA was dissolved in sterile saline, divided into single use aliquots and stored at -20^{o}C until use. To induce injury a dose of 20mg/kg was introduced via the carotid cannula (Tiegs *et al*., 1992). For uninjured vehicle control mice an equivalent volume of sterile saline was

A



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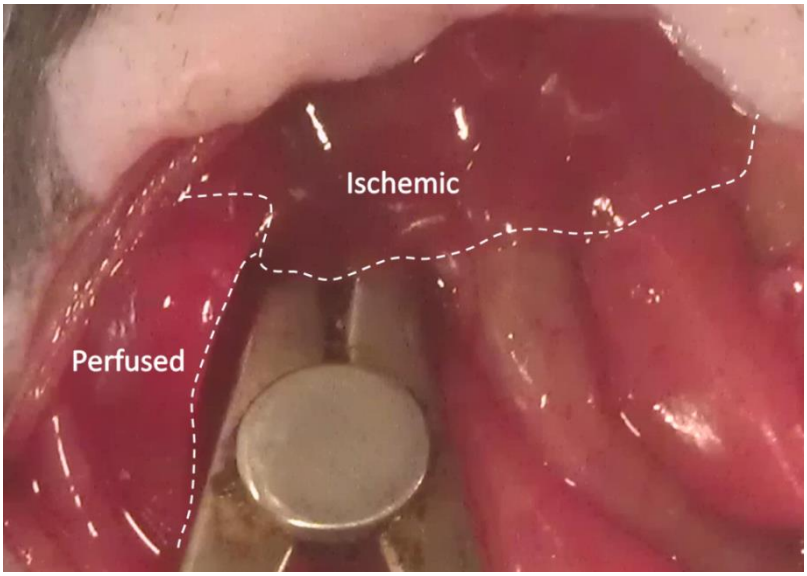


Figure 2:3 Ischemia of the liver was induced through application of a vascular clamp. IR injury was induced through clamping of the blood supply to the left and median lobes but not the right lobe of the liver (**A**; adapted from Cook (1965)). Ischemia was confirmed through a colour change of the tissue (**B**).

introduced into the carotid artery. Injury was then allowed to develop for 2 or 4 hours prior to intravital observation.

2.2.5.4. Induction of Thrombocytopenia

For some experiments platelet depleted mice were used. R300 is a cocktail of monoclonal CD42b antibodies which, when injected into mice, cause profound irreversible thrombocytopenia in an Fc-receptor independent manner (Nieswandt *et al*., 2000). Thrombocytopenia was induced by a dose of 1.5mg/Kg R300 antibody introduced via the carotid cannula. After 1 hour liver injury or sham surgery was carried out. Thrombocytopenia was confirmed using an ABX Pentra 60 automated blood counter (Horiba; Northampton, UK). Platelet depletion with this antibody does not affect the number of other blood cells in circulation (Holyer, 2010).

2.2.5.5. CD162 Blocking

For CD162 blocking experiments lymphocytes were incubated in 200µl of 20µg/ml CD162 blocking antibody (4RA10) or rat IgG1κ for 20 minutes at 4^{o}C (Engelhardt *et al*., 2005). Complete blocking was confirmed using flow cytometry.

2.2.5.6. Intravital Microscopy

15 minutes prior to the end of injury induction the left lobe of the liver was exteriorised onto a petri dish and the mouse was transferred to the stage of an Olympus IX81 inverted microscope. The microvasculature of the liver was then viewed through a 20X objective.

For monitoring of T-cell and B-cell recruitment $0.5x10^6$ fluorescently labelled (section 2.2.2.4) T-cells and B-cells were injected into the carotid artery 2 or 4 hours post ConA injection or at the point of clamp removal for IR injury as previously described (Chimen *et al*., 2015). For some experiments cells were injected directly into the portal vein at 60 minutes post reperfusion. Following cell injection a random field was selected every 10 minutes and imaged for 20 seconds. 5 additional fields of view in a pre-defined pattern were then imaged for 20 seconds each (*Figure 2.2*).

To monitor the effect of PMP pre-treatment on lymphocyte adhesion in the injured liver, $1.8x10^6$ CFDA-SE labelled inguinal node lymphocytes were treated with PMPs for 5 minutes using the same method used for adhesion assays (section 2.2.4). These cells were then injected into the carotid artery prior to 90 minutes partial hepatic ischemia. Following reperfusion one random field was imaged every 10 minutes for 2.5 hours.

For monitoring of platelet recruitment CFDA-SE labelled platelets were injected into the carotid artery either 5 minutes before application of the clamp for IR injury or 2 or 4 hours post ConA injection. One field of view was randomly selected every 10 minutes over 2 hours and imaged for 1 minute. After each minute of imaging 5 additional fields of view in a predefined pattern were captured in order to ensure that the random field was representative.

For labelling of endogenous platelets and lymphocytes 20µl of rat anti mouse CD41 antibody (platelet specific), biotin conjugated Armenian hamster anti mouse CD3e (T-cell specific), and mouse anti mouse CD19 antibody (B-cell specific) were incubated for 5 minutes with 40µl

alexa 488 anti mouse antibody, alexa 555 anti rat antibody, and alexa 647 streptavidin. IgG controls for the primary antibodies were prepared in the same way. In a separate experiment a rat anti mouse CD19 antibody was also used, 20µl of the antibody was incubated with 40µl alexa 488 anti rat for 5 minutes. In another experiment 8µl of PE conjugated rat anti mouse Lye6G (neutrophil specific) was made up to 100µl with PBS. The antibody cocktails were injected 10 minutes prior to induction of ischemia or sham surgery to allow time for the antibody to circulate. Following 90 minutes of ischemia one field of view was imaged for 2 minutes every 10 minutes for 2.5 hours.

2.2.5.7. Analysis of Intravital Experiments

Intravital data was analysed using Slidebook V software (3i; Göttingen, Germany). Free flowing and adherent cells/platelets were counted blind to remove possibility of bias, to do this file names were obfuscated using software developed by Dr Kavanagh (University of Birmingham). Free flowing cells/platelets were defined as cells that were present in the field of view but were not static or were static for less than 20 seconds. Adherent cells/platelets were defined as cells that were static for at least 20 seconds. Cells/platelets at the edge of the field of view that were not entirely within the field of view were not counted. Out of focus cells/platelets (lacking a punctate, ovoid appearance) were not counted. Platelet velocity was calculated using the manual single particle tracking feature of Slidebook software. The velocities of at least the first 5 free flowing platelets to enter the field of view which were visible for more than one time point were measured. To calculate the area of CD41 antibody staining the masking feature of Slidebook software was used (*Figure 2.4*), 'masks' were drawn over points

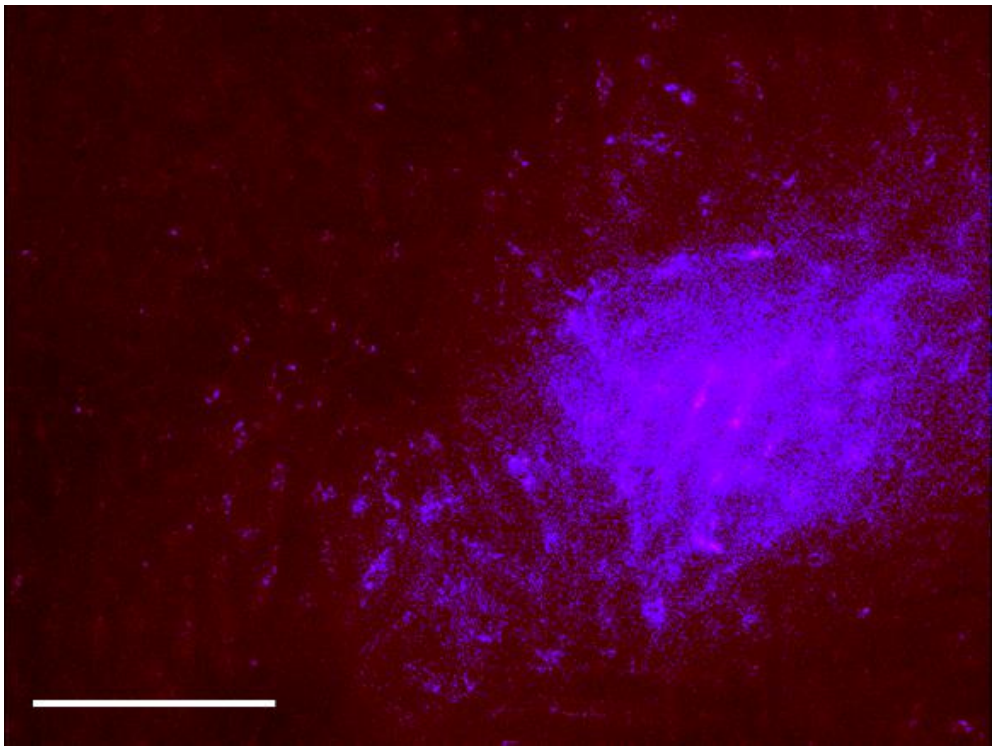


Figure 2:4 Quantification of fluorescence using Slidebook's masking tool. A mask (blue) was drawn over areas of fluorescence (red) with a pixel intensity of greater than 180. Scale bar = 100µm.

in the Cye3 channel with intensities greater than 180. This intensity range was chosen as it excluded background fluorescence.

2.2.5.8. Laser Doppler Measurements

In order to measure perfusion of the liver during injury measurements were taken using a moorVMS-LDF laser Doppler blood flow monitor (Moor instruments; Devon, UK), measurements were taken prior to injury and then every hour post injury. Measurements were taken at five random locations for every time point. During laser Doppler measurements the room was kept dark to reduce interference from outside light, damp gauze was placed under the liver lobe to minimise movement due to respiration.

2.2.5.9. Visualisation of Perfusion

In order to visualise areas of flow within the hepatic vasculature 100µl FITC-BSA or 100µl 2.5mg/ml Alexa-647 BSA were injected intra arterially. It was then possible to visualise the BSA within the liver using intravital microscopy. When introduced systemically the BSA is retained within the vasculature, provided that the integrity of the vessel is not disturbed (Kalia *et al*., 2002). It does not enter areas of the vasculature without flow. To produce FITC-BSA 170mg 10% FITC on celite was conjugated to 1g BSA by mixing the FITC, BSA and 10ml of bicarbonate buffer (0.6g Na_2CO_3, 3.7g $NaHCO_3$, 100ml distilled water, pH 9.0) overnight at 4^{o}C. This was then centrifuged at for 10 minutes at 16000g and conjugated/unconjugated BSA separated by dialysis (12,000MW cut off; Spectrum Laboratories, CA, USA). Prepared FITC-BSA was split into single use aliquots, frozen and stored until use.

2.2.5.10. Immunofluorescent Staining of Tissue Sections

Following injury or sham surgery livers were snap frozen in liquid nitrogen and stored at -80^{o}C. 10µm sections of the livers were taken using a cryostat, mounted on slides and fixed in acetone for 20 minutes. Sections were then incubated with 1:50 dilutions of CD42b, CD41 or IgG2a antibody for 3 hours at 4^{o}C and washed 3 times in PBS. Sections were then incubated with a 1:50 dilution of Alexa 488 rabbit anti rat antibody for 1 hour at 4^{o}C. Sections were then washed 3 times with PBS and dehydrated for 5 minutes in 95% ethanol. Sections were then imaged with a fluorescence microscope fitted with a 20X objective lens (EVOS microscope; AMG; Washington, USA). 5 images were taken per section in a pre-defined pattern (*Figure 2.2*) starting at the centre of the section. To eliminate bias images were analysed using blind counting software developed by Dr Kavanagh (University of Birmingham).

2.2.5.11. Plasma Alanine Aminotransferase Tests

Following intravital experiments blood samples were taken from the mice into 100µl ACD. The blood was then centrifuged at 2000g for 20 minutes at 4^{o}C. The plasma fraction was then removed and stored at -80^{o}C. In order to quantitate the level of injury alanine aminotransferase (ALT) activity tests were carried on the plasma samples by the clinical chemistry department at Birmingham Women's Hospital (UK) using an Olympus Au400 analyser.

2.2.5.12. Blood Counts

At the end of some intravital experiments blood samples were taken from the mice into 100µl ACD. The samples were analysed using an ABX Pentra 60 automated blood counter to obtain erythrocyte, leukocyte and platelet counts.

2.2.6. Statistics

Statistical tests were carried out using GraphPad Prism 5 software (GraphPad; San Diego, USA). Serial data was analysed using two-way ANOVA. Static adhesion assay data and some flow cytometry data was analysed using one-way ANOVA with Dunnet's post-hoc tests. Survival curves were analysed using Mantel-Cox tests with Bonferroni corrections for multiple comparisons. All remaining data was compared using unpaired student's t-tests. p-values of <0.05 (*), <0.005 (**) and <0.001 (***) were considered significant.

CHAPTER 3

PLATELET MODULATION OF LYMPHOCYTE ADHESION *IN VITRO*

3. PLATELET MODULATION OF LYMPHOCYTE ADHESION *IN VITRO*

3.1. Introduction and Hypotheses

It is well established that immobilised activated platelets can support rolling and adhesion of lymphocytes under physiological flow in a CD62P, CD11a/CD18 and CD11b/CD18 dependant manner (Diacovo *et al*., 1996b, Sheikh *et al*., 2004). Lymphocytes can also adhere to activated endothelium, a process which is mediated by endothelial cell expressed adhesion molecules such as CD54, CD106 and MAdCAM-1 (van Dinther-Janssen *et al*., 1991, Dustin and Springer, 1988, Grant *et al*., 2001). However, little is known about whether platelets can modify directly or indirectly lymphocyte adhesion to cell adhesion molecules or endothelial cells. There are a number of ways in which platelets could potentially modulate lymphocyte adhesion. Platelets may act as a bridge between the endothelial surface and the lymphocyte. Indeed, this has previously been shown to mediate the capture of lymphocytes from flow on the high endothelial venules of peripheral lymph nodes (Diacovo *et al*., 1996c). Platelets also release a number of factors including cytokines and PMPs which may modulate lymphocyte adhesion (Zarbock *et al*., 2007, Diamant *et al*., 2004). Platelet adhesion on endothelium can also result in endothelial activation via the CD40/CD154 pathway (Henn *et al*., 1998). The experiments in this chapter aim to assess the effect of platelets and platelet released factors on lymphocyte adhesion to immobilised cell adhesion molecules and endothelium *in vitro.*

For the work included in this chapter we hypothesised that:

1. Adhesion of lymphocytes to endothelium and immobilised cell adhesion molecules is increased following their incubation with activated platelets or their releasate.
2. Adhesion of lymphocytes on immobilised cell adhesion molecules is increased following their incubation with PMPs.
3. Expression of integrins on lymphocytes is increased following their incubation with activated platelets or their releasate.

3.2. Methods

The methods used in this chapter are discussed in detail in Chapter 2 and were used to investigate *in vitro* whether lymphocyte adhesion to immobilised endothelial counter-ligands could be modulated by (i) pre-incubation with platelets (ii) previously adherent platelets (iii) pre-incubation with PMPs or factors released from activated platelets. Briefly, murine lymphocytes were isolated from the inguinal lymph nodes and platelets from the whole blood of C57BL/6 mice. Static adhesion assays were carried out on endothelium, immobilised cell adhesion molecules and intestinal tissue sections to determine the effects of adherent platelets on subsequent lymphocyte adhesion and incubating lymphocytes with platelets or platelet releasate on their subsequent adhesion. Lymphocyte expression of CD49d and CD18 following incubation with platelets or platelet releasate was determined by flow cytometry.

To investigate whether PMPs affect lymphocyte adhesion, lymphocytes were incubated with PMPs generated through thrombin stimulation of washed platelets and their adhesion to

endothelial counter ligands was quantitated. Flow cytometry was used to determine whether PMPs form conjugates with lymphocytes using antibodies against the platelet specific marker CD41.

3.3. Results

3.3.1. 3% BSA is Sufficient to Block Platelet Adhesion to Tissue Culture Plastic

In static adhesion assays using protein coated tissue culture plastic, BSA can be used to block uncoated plastic. Previously, a concentration of 1% BSA has been used in this lab to block tissue culture plastic for static adhesion assays. Large numbers of adherent platelets were observed on untreated tissue culture plastic (*Figure 3.1* A) as well as on tissue culture plastic blocked with 1% BSA, indicating incomplete blocking of the plastic (*Figure 3.1 B*). 3% BSA was found to almost completely block activated platelet adhesion to tissue culture plastic (*Figure 3.1 C*). 3% BSA was therefore used for blocking in all future static adhesion assays on immobilised protein.

3.3.2. Activation with Thrombin Significantly Increases Platelet Adhesion on Immobilised Cell Adhesion Molecules

To determine whether platelet activation increases platelet adhesion to cell adhesion molecules naïve and activated platelets were incubated under static condition on immobilised CD54, CD106 and MAdCAM-1 for 20 minutes and their adhesion was quantitated. Platelet activation significantly increased platelet adhesion to both CD54 *(Figure 3.2 A-C*; $p<0.01$) and MAdCAM-1 (*Figure 3.2 G-I*; $p<0.01$). Adhesion of activated platelet to CD106 was also greater

than that of naïve platelets although this difference did not reach significance (*Figure 3.2 D-F*; p>0.05). Activated platelet adhesion was highest on CD54 (adherent cells / field of view: 88±25), 4 times greater than the number of adherent platelets observed on MAdCAM-1 (adherent cells / field of view: 22±6), few adherent platelets were observed on CD106 (adherent cells / field of view: 2±1).

3.3.3. T-cells Make Up the Majority of Cells in Lymph Node Cell Isolates

For the following experiments in which lymphocyte adhesion was investigated, lymphocytes were isolated via mechanical digestion of inguinal lymph nodes taken from C57BL/6 mice. Flow cytometry showed these cell isolates to be made up of 76±1% T-cells (*Figure 3.3 A*; CD3ε+ cells) and 26±3% B-cells (*Figure 3.3 B*; CD19+ cells). NK cells were undetectable in this population of murine inguinal lymph node cells (*Figure 3.3 C*; NK1.1+ cells).

3.3.4. Adherent Activated Platelets Significantly Enhance Lymphocyte Adhesion to Immobilised Cell Adhesion Molecules

In order to investigate whether adherent platelets can affect lymphocyte adhesion to cell adhesion molecules naïve or activated platelets where added to the immobilised ligand and non-adherent platelets washed away. Lymphocytes were then added and their adhesion after 20 minutes quantitated. There was significantly greater adhesion of lymphocytes to CD54 (*Figure 3.4 A*; p<0.05) and MAdCAM-1 (*Figure 3.4 E*; p<0.05) when activated platelets had previously been incubated on the ligands compared to lymphocytes on their own. Naïve platelets had no significant effect on adhesion to CD54 (*Figure 3.4 A*; p>0.05) or MAdCAM-1 (*Figure 3.4 E*; p>0.05). On CD106 no adhesion of lymphocytes was observed in the absence of

platelets; however, some adhesion did occur when the immobilised ligand had previously been incubated with naïve and activated platelets (*Figure 3.4 C*). The vast majority of adherent lymphocytes were not bound to platelets (*Figure 3.4 D & F)*.

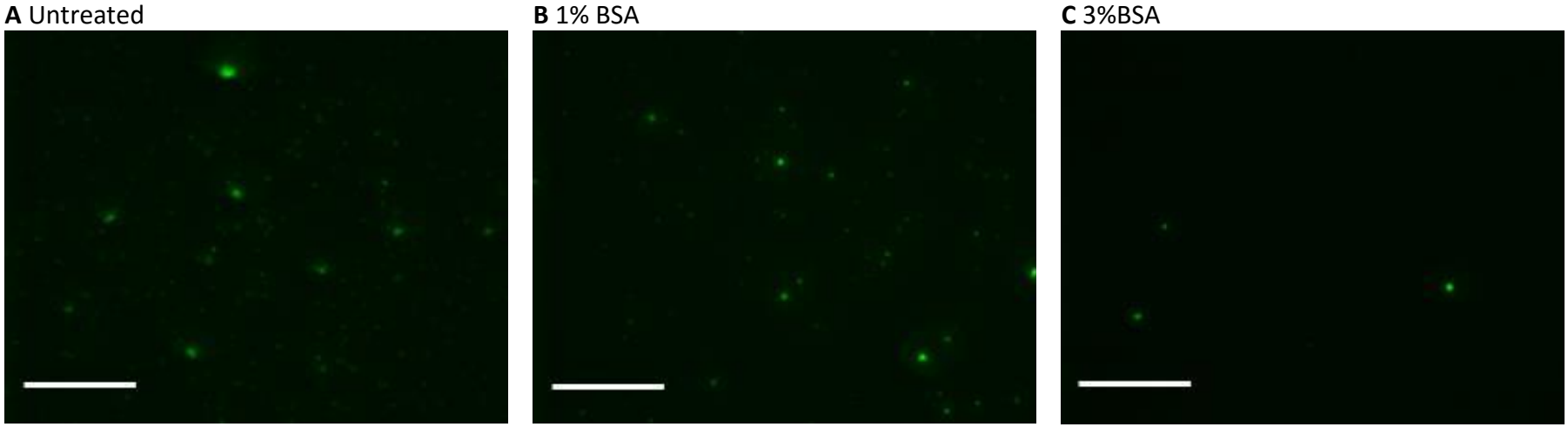


Figure 3:1 3% BSA is sufficient to block platelet adhesion to tissue culture plastic. CFSE labelled activated platelets (0.2U/ml thrombin; 5 minutes) were incubated on untreated tissue culture plastic (**A**) and tissue culture plastic blocked with 1% (**B**) and 3% BSA (**C**). Blocking the plastic with 3% BSA greatly reduced the number of adherent platelets observed. n=1, scale bars=100µm.

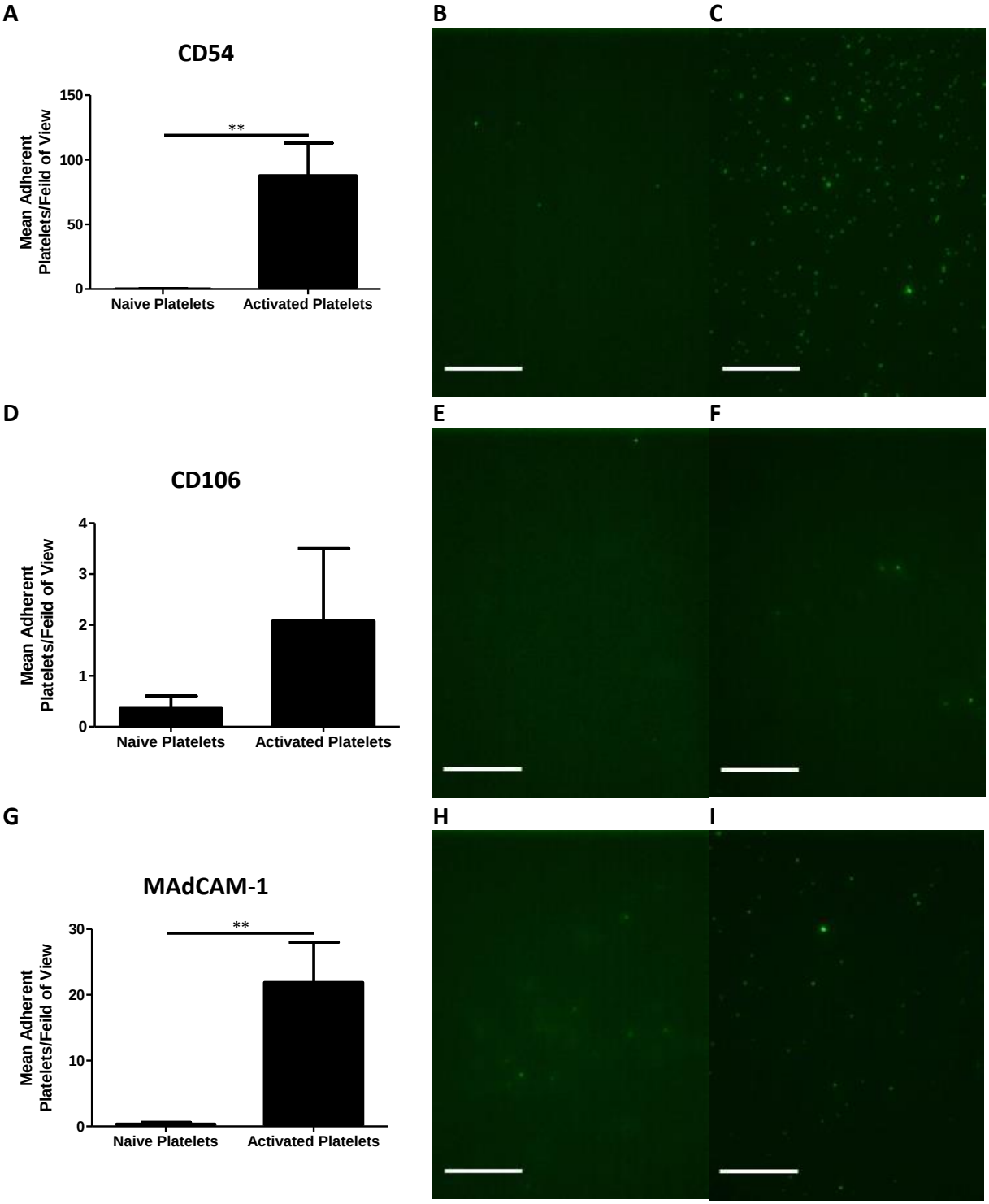


Figure 3:2 Thrombin significantly increases platelet adhesion to cell adhesion molecules. Adhesion of naïve and activated (0.2U/ml thrombin; 5 minutes) platelets after 20 minutes incubation on immobilised cell adhesion molecules was quantitated. Activation significantly increased platelet adhesion on CD54 (**A**) and MAdCAM-1 (**G**) but not CD106 (**D**). Images show CFDA-SE stained naïve/activated platelets on CD54 (**B/C**) CD106 (**E/F**) and MAdCAM (**H/I**). Data presented as mean ±SEM, n=5, **p<0.01 (t-tests), scale bars=100µm.

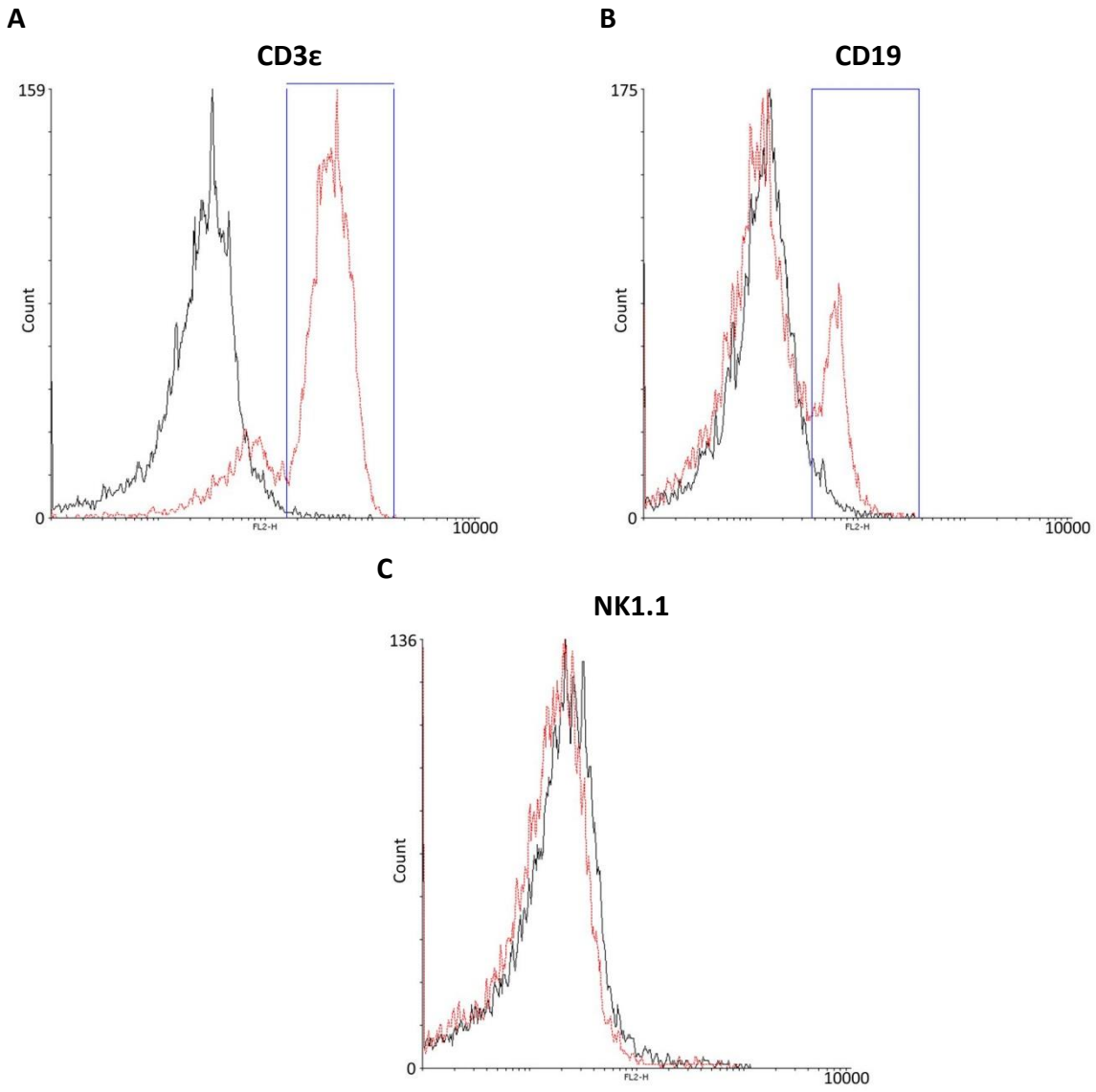


Figure 3:3 T-cells make up the majority of cells in lymph node cell isolates. Flow cytometry was carried out on lymph node cell isolates to analyse their composition. Representative plots are shown. Isolates were found to contain 76±1% T-cells (**A**; CD3ε+ cells; n=4) and 26±3% B-cells (**B**; CD19+ cells; n=4); NK cells were undetectable (**C**; NK1.1+ cells; n=4). Black lines: isotype controls, red lines: antibody against relevant cell marker, blue gates: cell marker positive cells,.

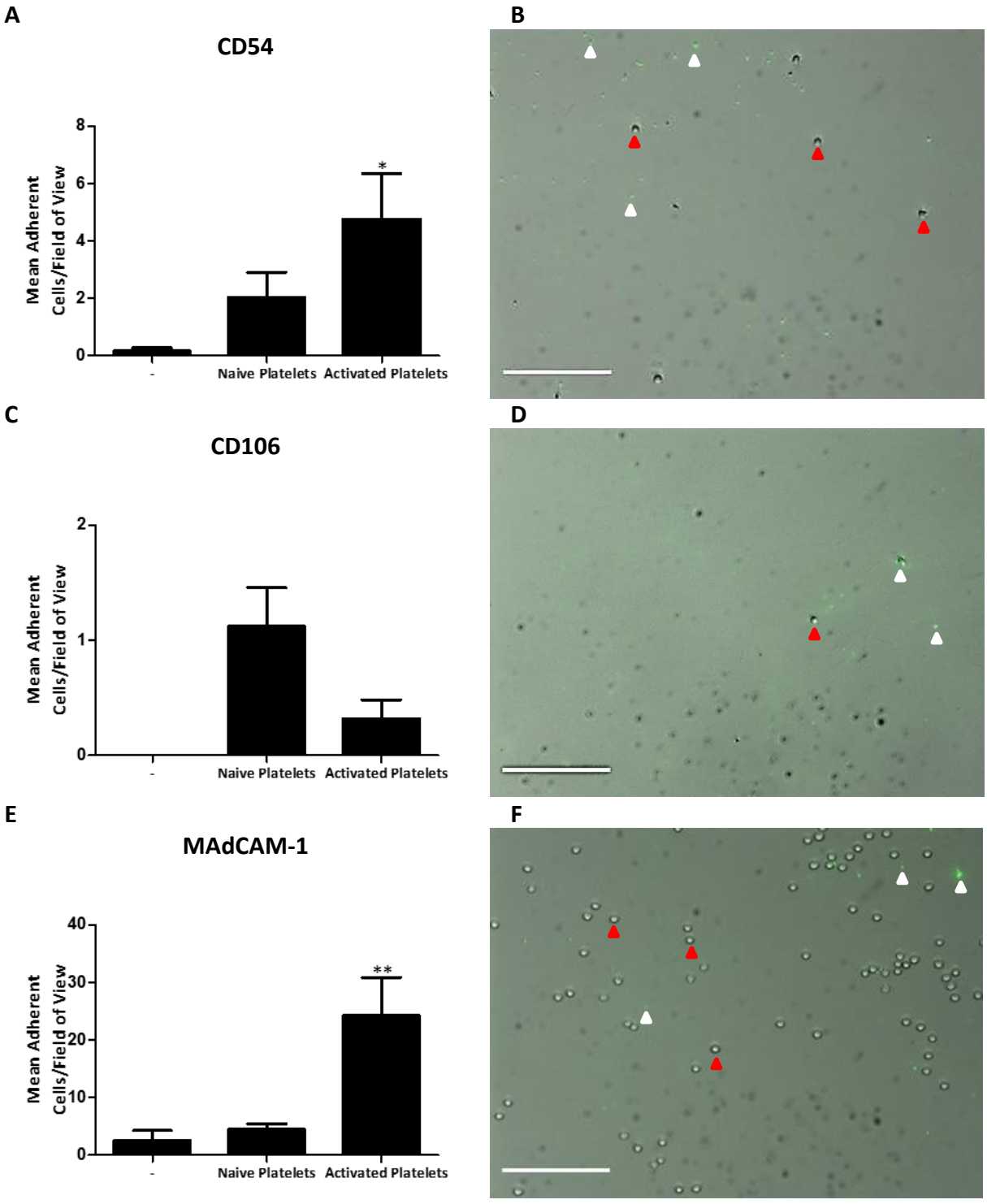


Figure 3:4 Adherent activated platelets significantly increase lymphocyte adhesion to immobilised cell adhesion molecules. The presence of activated (0.2U/ml thrombin; 5 min) adherent platelets significantly increased lymphocyte adhesion on immobilised CD54 (**A**) and MAdCAM-1 (**E**) but not CD106 (**C**). Representative images are shown of lymphocyte (red arrowheads / colourless cells) adhesion in the presence of activated adherent platelets (white arrowheads / green cells) on CD54 (**B**), CD106 (**D**) and MAdCAM-1 (**F**). Data presented as mean ±SEM, n=5, *p<0.05 **p<0.01 (one-way ANOVA with Dunnett's post-tests), scale bars=100µm.

3.3.5. Incubation with Platelets or Activated Platelet Supernatant Significantly Enhances Lymphocyte Adhesion to Immobilised Cell Adhesion Molecules

Lymphocytes were pre-incubated with thrombin (control) naïve platelets, thrombin activated platelets or thrombin activated platelet releasate for 5 or 30 minutes to determine whether this modified their subsequent adhesion. Although increases in lymphocyte adhesion to CD54 were observed after 5 and 30 minutes incubation with activated platelets and platelet releasate, this did not reach statistical significance between treatments (*Figure 3.5 A;* $p>0.05$). Incubation with the releasate of thrombin activated platelets for 5 and 30 minutes increased lymphocyte adhesion to CD106 but reached statistical significance at 5 minutes only when compared to incubation with thrombin alone (*Figure 3.5 B;* $p<0.01$). Similarly, incubation with the releasate of thrombin activated platelets increased lymphocyte adhesion to MAdCAM-1, which was significantly different at 5 minutes (*Figure 3.5 C;* $p<0.001$) and 30 minutes (*Figure 3.5 C;* $p<0.05$). Incubation for 5 minutes with naïve platelets also significantly increased lymphocyte adhesion to MAdCAM-1 when compared to untreated cells (*Figure 3.5 C;* $p<0.05$).

3.3.6. Incubation with PMPs Significantly Increased Lymphocyte Adhesion to Immobilised Cell Adhesion Molecules

As activated platelet releasate was shown to significantly increase lymphocyte adhesion to cell adhesion molecules the effect of purified PMPs, vesicles released from activated platelets, on lymphocyte adhesion was investigated. Incubation with PMPs for 5 minutes significantly increased lymphocyte adhesion to CD54 (*Figure 3.6 A;* $p<0.05$) and MAdCAM-1 (*Figure 3.6 C;* $p<0.05$) and to CD106 following 30 minutes incubation (*Figure 3.6 B;* $p<0.05$). In order to

determine if PMPs were physically interacting with the membrane of the lymphocytes flow cytometry was used to look for the presence of the platelet specific marker CD41. Flow cytometry following incubation with PMPs did not detect CD41 on the surface of lymphocytes (*Figure 3.6 D;* p>0.05). To determine if this was due to the low concentration of PMPs lymphocytes were incubated with 10 times the concentration of PMPs; this also did not result in surface CD41 expression (*Figure 3.6 E;* p>0.05).

3.3.7. Neither Endothelial Activation nor Platelet Activation Significantly Alters Platelet Adhesion to Endothelium

As previously adherent platelets or incubation with platelets or platelet releasate enhanced lymphocyte adhesion to immobilised cell adhesion molecules found on endothelial cells, further experiments were carried out to determine if platelets could also enhance lymphocyte adhesion on endothelial monolayers as well. Firstly, platelet adhesion to untreated and TNFα stimulated murine colonic endothelial cells using static adhesion assays was investigated. Considerable adhesion of naïve platelets was observed on untreated endothelial cells. Although increased adhesion of thrombin activated platelets was observed on untreated endothelial cells, this did not reach statistical significance (*Figure 3.7*; p>0.05). TNF-α stimulation of the endothelium also had no effect on the adhesion of naïve or activated platelets (*Figure 3.7*; p>0.05).

3.3.8. The Presence of Adherent Platelets does not Significantly Affect Lymphocyte Adhesion to Endothelium

As adherent platelets significantly enhanced lymphocyte adhesion to cell adhesion molecules their effect on lymphocyte adhesion to endothelium was also investigated. The presence of adherent platelets on the endothelium had no significant effect on lymphocyte adhesion to endothelium (*Figure 3.8*; $p>0.05$). Stimulation of the endothelium with TNF-α also had no significant effect on lymphocyte adhesion to endothelium (*Figure 3.8*; $p>0.05$).

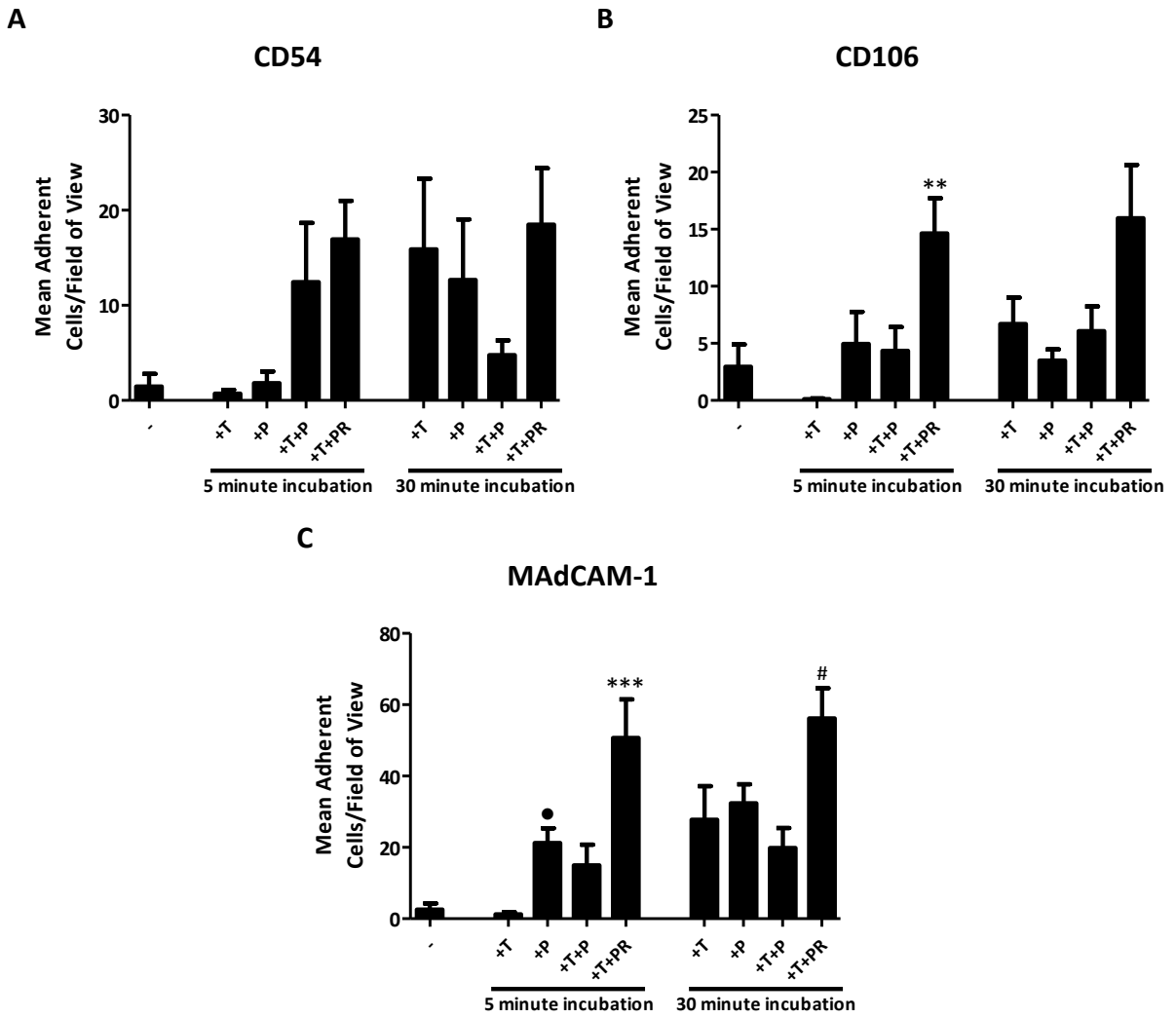


Figure 3:5 Incubation with platelets or activated platelet supernatant significantly enhances lymphocyte adhesion on immobilised cell adhesion molecule. The adhesion on CD54 (**A**), CD106 (**B**) and MAdCAM-1 (**C**) of untreated lymphocytes (-) and lymphocytes incubated for 5 or 30 minutes with thrombin (+T), platelets (P), platelets and thrombin (+T+P) or thrombin activated platelet releasate (+T+PR) was quantitated. Data presented as mean ±SEM, n≥4, **p<0.05 compared to 5 minute +T, ***p<0.001 compared to 5 minute +T, •p<0.05 compared to -, #p<0.05 compared to 30 minute +T (one-way ANOVA with Bonferroni post-tests).

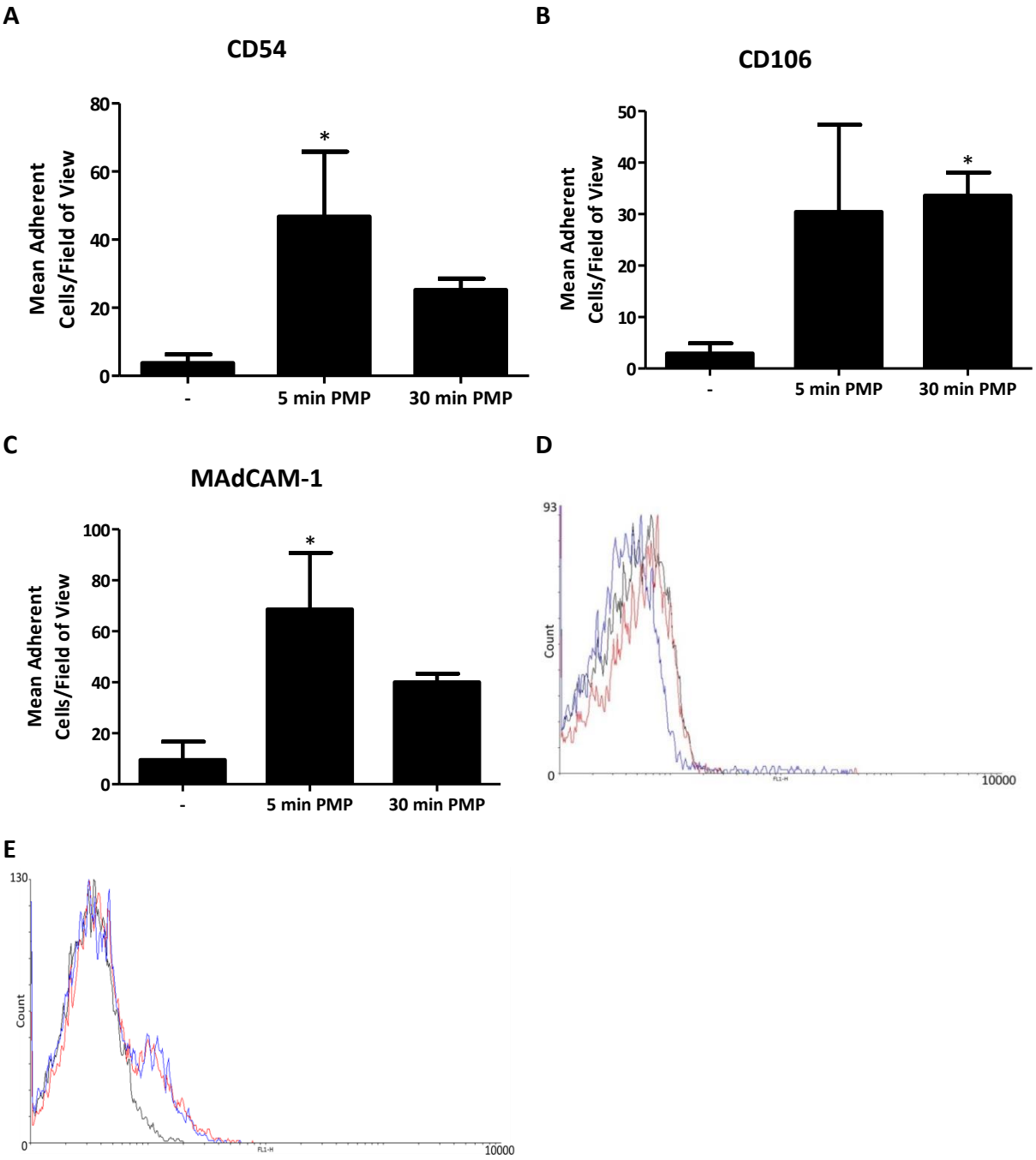


Figure 3:6 Lymphocyte adhesion on immobilised cell adhesion molecules was significantly increased following incubation with PMPs. The adhesion of untreated lymphocytes (-) and lymphocytes incubated with PMPs for 5 and 30 minutes on immobilised CD54 (**A**), CD106 (**B**) and MAdCAM-1 (**C**) was quantitated. Panels D and E show representative flow cytometry plots, black line: isotype control, red lines: untreated lymphocytes with CD41 antibody, blue line: PMP treated lymphocytes (**D**) or lymphocytes treated with a 10X concentration of PMPs (**E**) with CD41 antibody. Data presented as mean ±SEM, n≥3, *p<0.05 (one-way ANOVA with Dunnett's post-tests (A, B & C) and t-tests (D&E)).

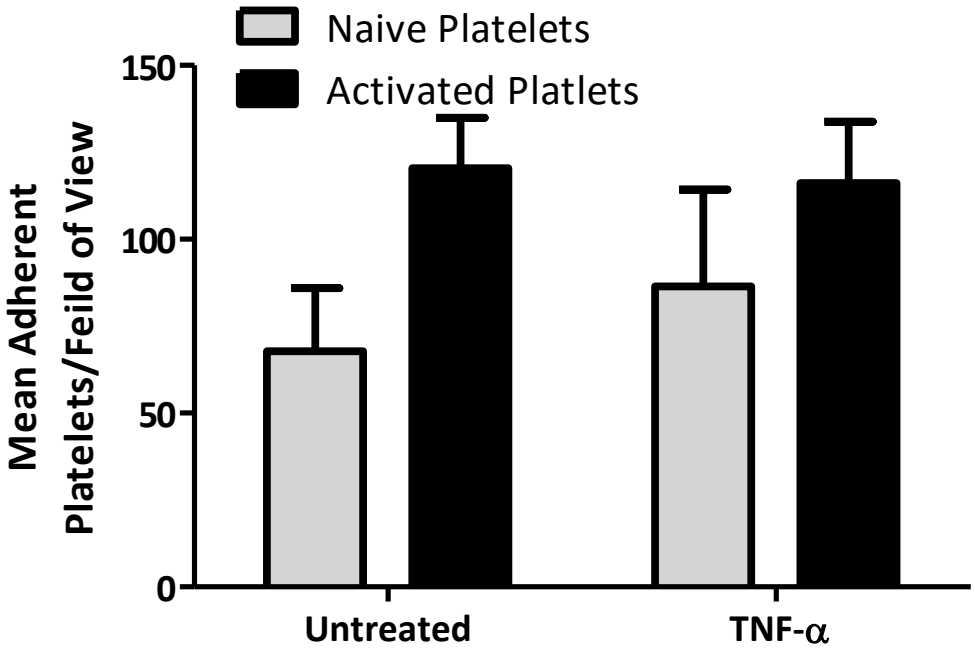


Figure 3:7 Neither endothelial activation nor platelet activation significantly alters platelet adhesion on endothelium. Activation of platelets (0.2U/ml; 5 minutes) and/or endothelium (10ng/ml; 4 hours) had no significant effect on platelet adhesion to endothelium after 20 minutes incubation. Data presented as mean ±SEM, n≥5 (two-way ANOVA).

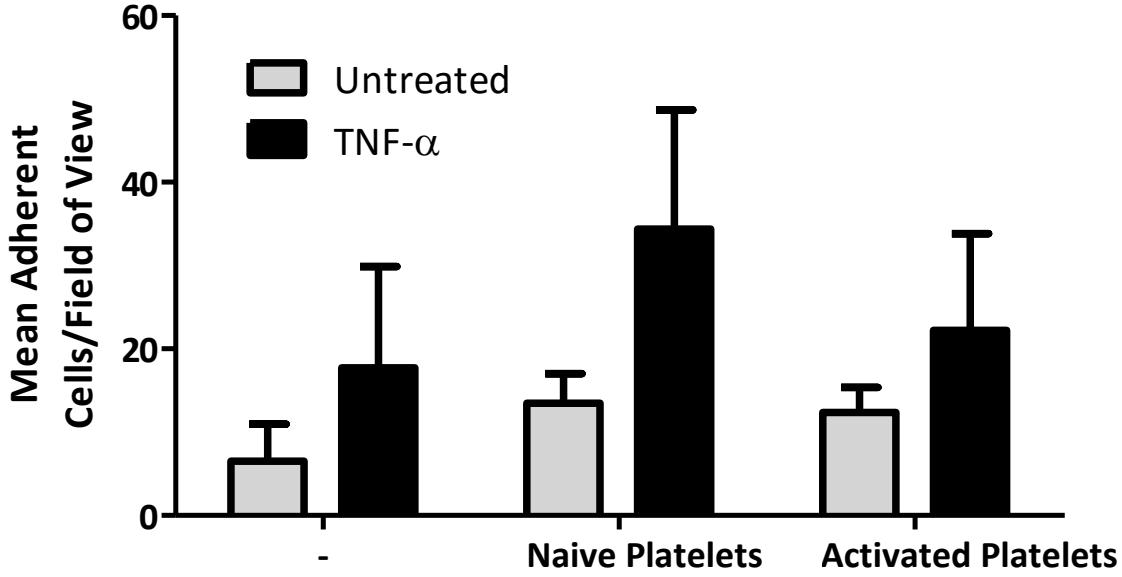


Figure 3:8 The presence of adherent platelets does not significantly affect lymphocyte adhesion on endothelium. The presence of adherent naïve or activated (0.2U/ml thrombin; 5 min) platelets had no significant effect on lymphocyte adhesion to endothelium after 20 minutes. Data presented as mean ±SEM, n≥3 (two-way ANOVA).

3.3.9. Incubation with Platelets has no Significant Effect on Lymphocyte Adhesion to Endothelium

As incubation with platelets increased lymphocyte adhesion to immobilised cell adhesion molecules their effect on lymphocyte adhesion on endothelium was investigated. No significant difference in lymphocyte adhesion was observed following incubation with platelets for either 5 or 30 minutes (*Figure 3.9*; $p>0.05$). TNF-α stimulation of the endothelium had no significant effect on lymphocyte adhesion, although there was a trend for increased lymphocyte adhesion when endothelium had been activated (*Figure 3.9*; $p>0.05$).

3.3.10. Thrombin Activation and Tissue Injury Increase Platelet Adhesion to Gut Tissue

Interestingly, the static adhesion assay data presented so far demonstrated that platelets could modulate lymphocyte adhesion to immobilised endothelial cell adhesion molecules but this effect was not reproduced on endothelial cells themselves. Further assays were carried out to determine whether platelets modulated lymphocyte adhesion on frozen sections of gastrointestinal tissue (Stamper Woodruff assays). Firstly, platelet adhesion to sham and IR injured jejunum and ileum was assessed as well as platelet adhesion to sham and colitis injured colon tissue. Generally, there was a trend for naïve and activated platelets to adhere more so on injured tissue sections than healthy sham sections but this did not always attain statistical significance. Certainly adhesion of naïve platelets was significantly greater on IR ileum than sham ileum sections (*Figure 3.10 D*; $p<0.05$). The most adhesion of platelets was observed on ileum tissue rather than jejunum or colon. Also, thrombin activation significantly

increased adhesion of platelets on sham jejunum (*Figure 3.10 A*; $p<0.01$) and colitis injured colon (*Figure 3.10 G*; $p<0.01$) sections.

3.3.11. Incubation with Platelets Increases Lymphocyte Adhesion to Ileum

Interestingly, untreated lymphocytes did not adhere to any of the intestinal sham or injured tissue sections. However, a very small number of lymphocytes did adhere to ileal sections after they were incubated with platelets or thrombin activated platelets, but this failed to reach statistical significance between sham and IR injured ileum (*Figure 3.11*; $p>0.05$). Adhesion to other tissue sections was not modified by the presence of naïve or activated platelets.

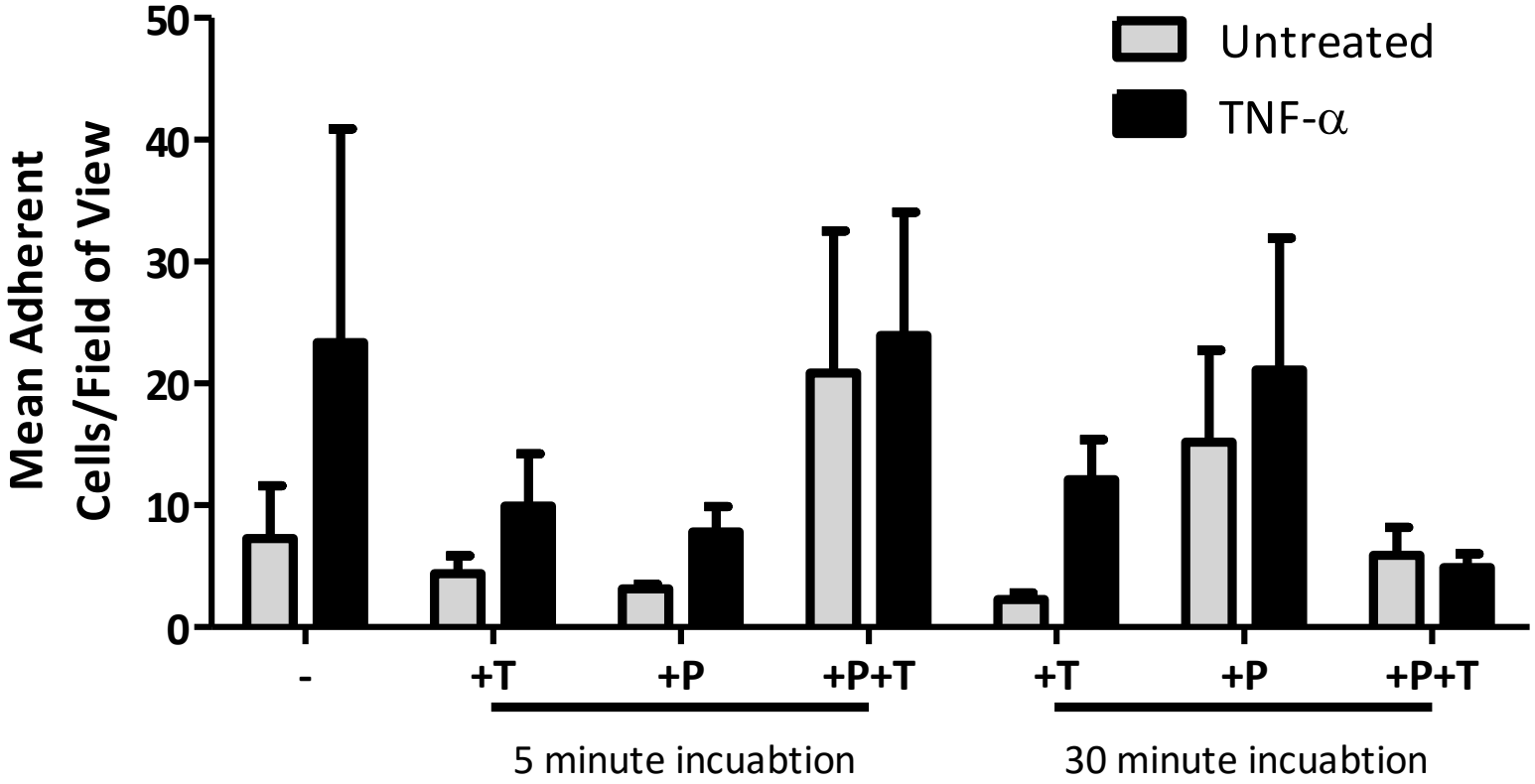


Figure 3:9 Incubation with platelets has no significant effect on lymphocyte adhesion on endothelium. The adhesion on untreated and TNF-α stimulated (10ng/ml; 4 hours) colonic endothelium of untreated lymphocytes (-) and lymphocytes incubated for 5 or 30 minutes with thrombin (+T), platelets (P) or platelets and thrombin (+T+P) was quantitated. Data presented as mean ±SEM, n≥3 (two-way ANOVA).

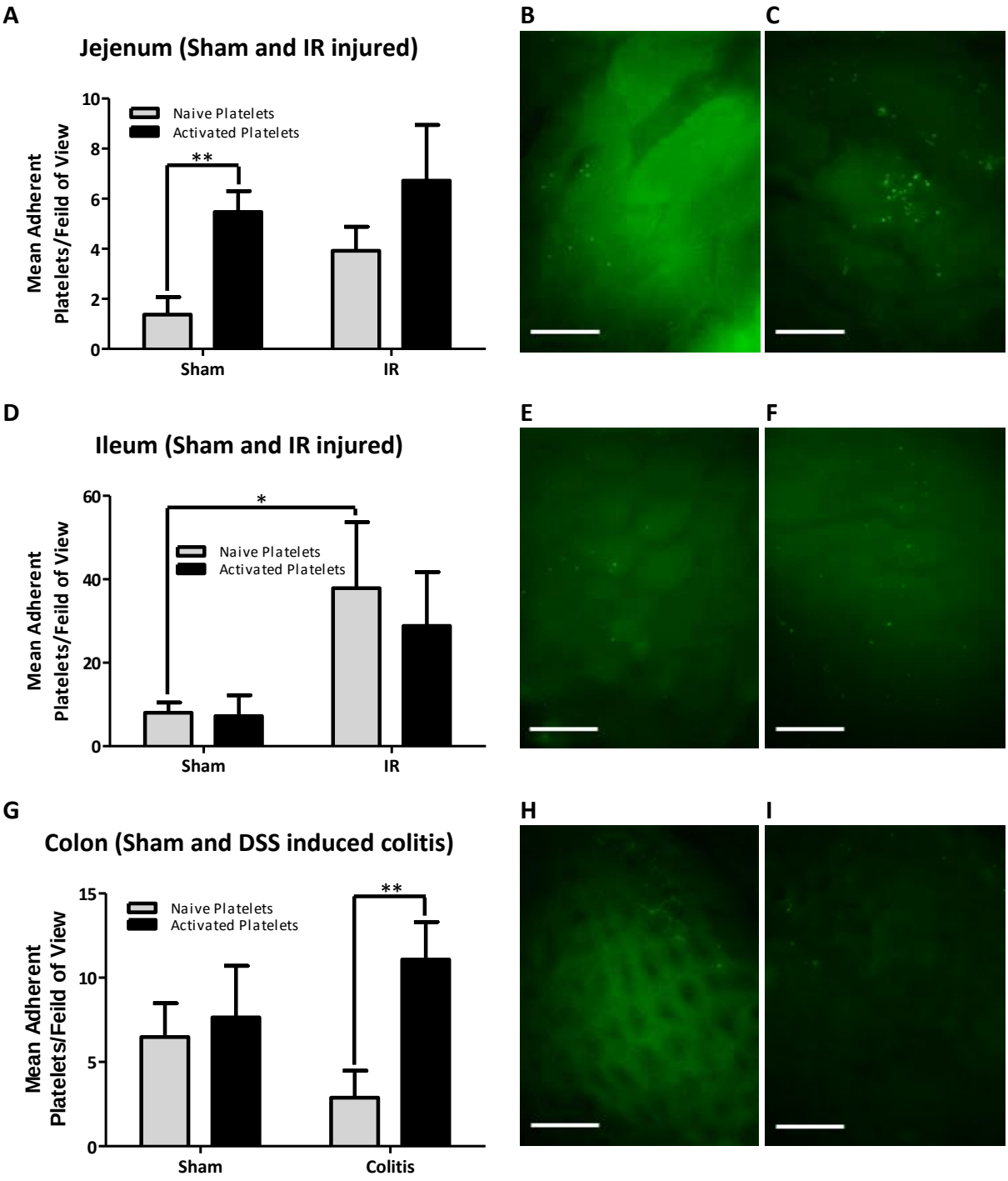


Figure 3:10 Thrombin activation and tissue injury increase platelet adhesion to gut tissue. Sham and Ischemia reperfusion (IR) injured ileum (**A**) and jejunum (**D**) sections and sham and colitis colon (**G**) sections were incubated with naïve and activated CFSE labelled platelets and their adhesion quantitated. Images show naïve (**B**) and activated (**C**) platelets (geen) on sham jejunum; naïve platelets on sham ileum (**E**) and IR Ileum (**F**) and naïve platelets (**H**) and activated platelets (**I**) on colitis colon. Data presented as mean ±SEM, n≥4, *p<0.05 **p<0.01 (one-way ANOVA with Bonferroni post-tests).

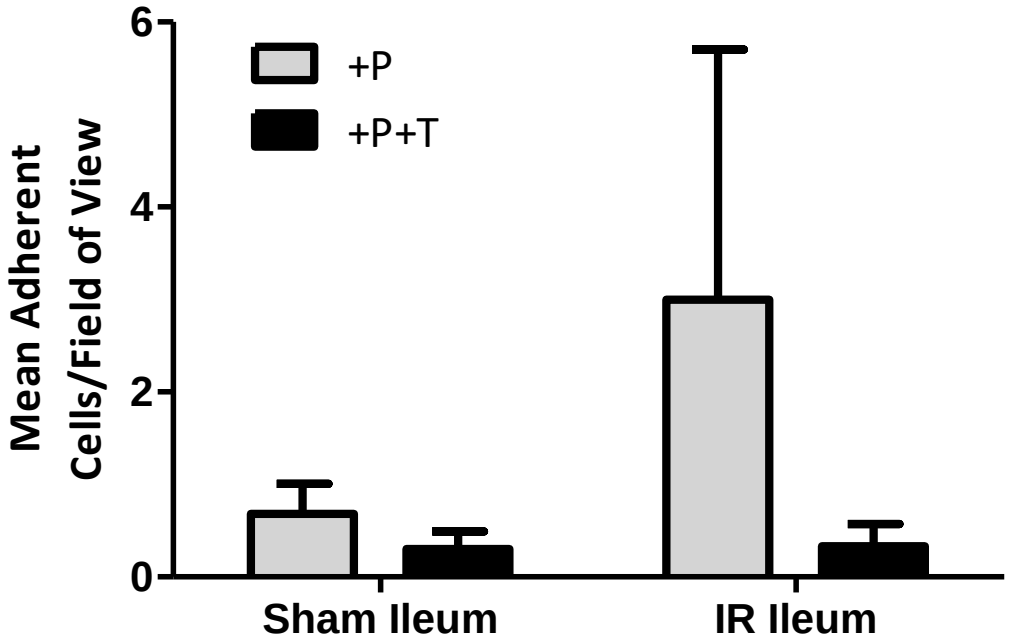


Figure 3:11 Lymphocytes adhere to ileal tissue sections following incubation with platelets. Lymphocytes were incubated with platelets (+P) or platelets and thrombin (+P+T) for 5 minutes. Adhesion of the lymphocytes to Sham and Ischemia reperfusion (IR) injured ileum sections was then quantitated. Data presented as mean ±SEM, n≥3, (one-way ANOVA with Bonferroni post-tests).

3.3.12. Incubation with Platelets had no Significant Effect on CD18 or CD49d Expression on Lymphocytes

In order to determine how platelets increase lymphocyte adhesion, their effect on lymphocyte integrin expression was investigated using flow cytometry. Both CD18 (*Figure 3.12*) and CD49d (*Figure 3.13*) were expressed on both T-cells and B-cells. However, incubation with thrombin, platelets or thrombin and platelets for 5 or 30 minutes had no significant effect on either CD18 (*Figure 3.12*; $p>0.05$) or CD49d (*Figure 3.13*; $p>0.05$) expression on T-cells or B-cells.

The results outlined above are also summarised in **Table 3.1.**

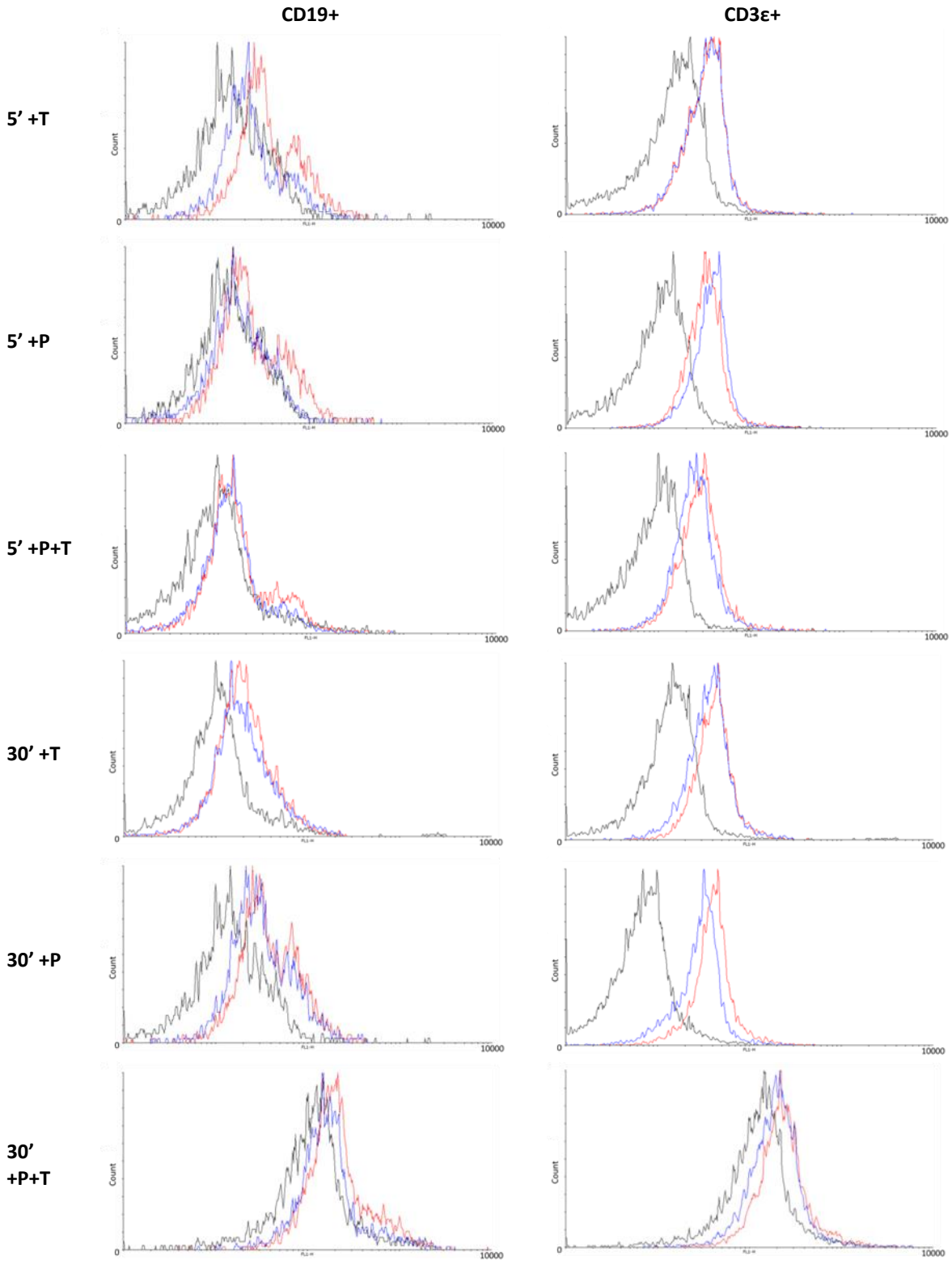


Figure 3:12 Incubation with platelets or thrombin had no effect on lymphocyte CD18 expression. Lymphocytes were incubated with platelets (+P) thrombin (+T) or platelets and thrombin (+P+T) for 5 or 30 minutes. CD18 expression on T-cells (CD3ε+) and B-cells (CD19+) was then quantitated using flow cytometry. Panels show representative flow cytometry plots, black line: isotype control, red lines: untreated lymphocytes with CD18 antibody, blue line: treated lymphocytes with CD18 antibody. n≥3, (t-tests).

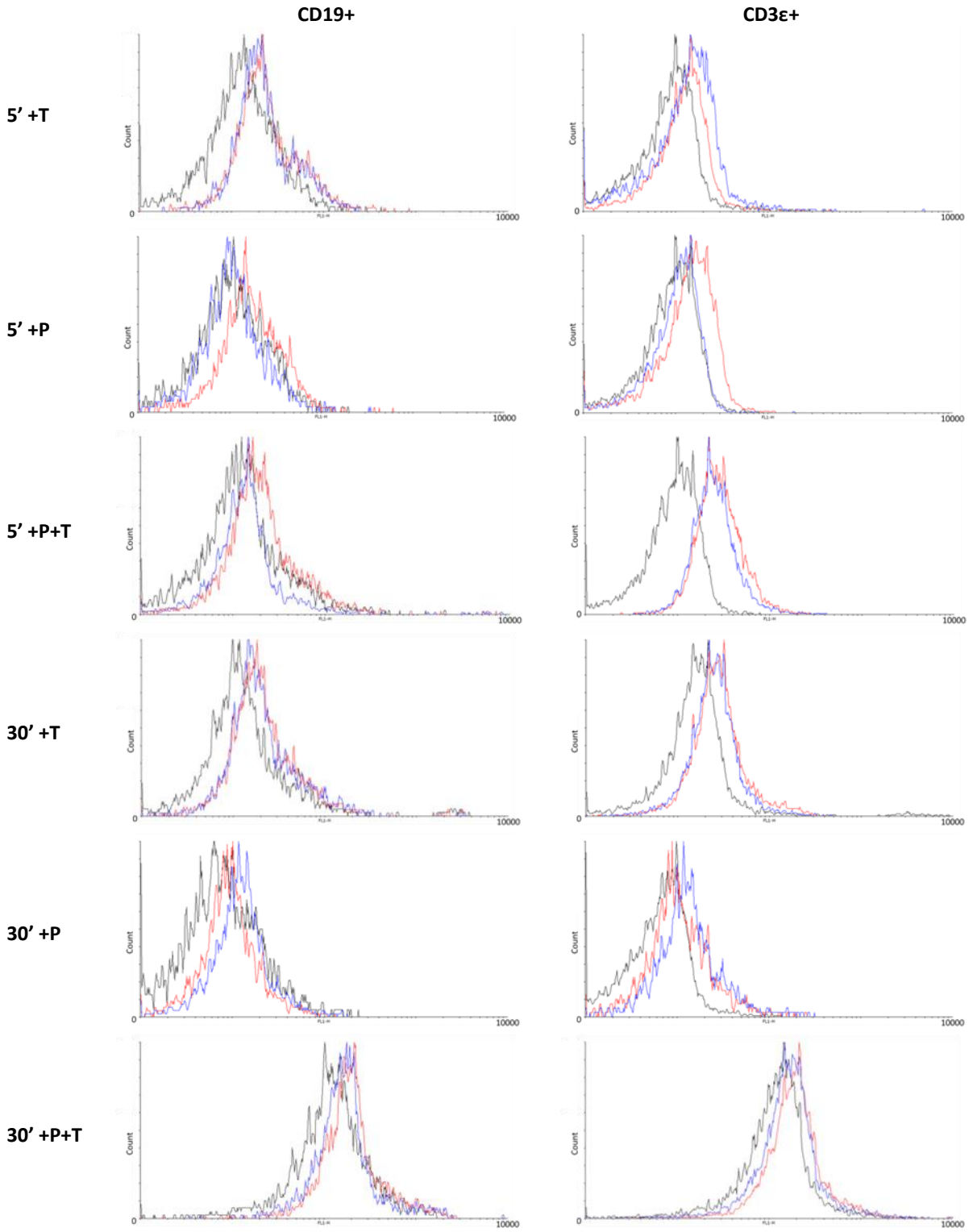


Figure 3:13 Incubation with platelets or thrombin had no effect on lymphocyte CD49d expression. Lymphocytes were incubated with platelets (+P) thrombin (+T) or platelets and thrombin (+P+T) for 5 or 30 minutes. CD49d expression on T-cells (CD3ε+) and B-cells (CD19+) was then quantitated using flow cytometry. Panels show representative flow cytometry plots, black line: isotype control, red lines: untreated lymphocytes with CD49d antibody, blue line: treated lymphocytes with CD49d antibody. n≥3, (t-tests).

	ICAM-1	VCAM-1	MAdCAM-1	Stimulated Endothelium	Intestinal Sections
Naïve Platelets	No adhesion	No adhesion	No adhesion	~ 60-80 platelets	Generally, there was a trend for naïve / activated platelets to adhere to injured tissue, with most adhesion observed on ileum (~10-40 platelets).
Activated Platelets	**↑~ 100 platelets p<0.01**	↑ 1-2 platelets NS	**↑~ 20 platelets p<0.01**	**↑~ 100 platelets NS**	
Lymphocytes	No adhesion	No adhesion	No adhesion	~ 20 lymphocytes	No adhesion
Lymphocytes when naïve platelets already adherent	**↑ ~1-2 lymphocytes NS**	No adhesion	No adhesion	**↑ ~ 30 lymphocytes NS**	NOT INVESTIGATED
Lymphocytes when activated platelets already adherent	**↑~ 4 lymphocytes p<0.05**	No adhesion	**↑ ~ 20 lymphocytes p<0.01**	No increase	NOT INVESTIGATED
Lymphocytes incubated with thrombin	No adhesion (but thrombin can increase lymphocyte adhesion with long incubations).	No adhesion (but thrombin can increase lymphocyte adhesion with long incubations).	No adhesion (but thrombin can increase lymphocyte adhesion with long incubations).	No increase	~ 1-2 lymphocytes adhere to injured ileum when incubated with activated platelets
Lymphocytes incubated with naïve platelets	No adhesion	No adhesion	**↑ ~ 20 lymphocytes p<0.05**	No increase	
Lymphocytes incubated with activated platelets	**↑~ 10 lymphocytes NS** (5 mins)	No adhesion	**↑ ~ 15 lymphocytes NS** (5 mins)	No increase	
Lymphocytes incubated with activated platelet releasate	**↑~ 15 lymphocytes NS** (5 mins)	**↑~ 15 lymphocytes p<0.01** (5 mins)	**↑ ~ 50 lymphocytes p<0.001** (5 & 30 mins)	NOT INVESTIGATED	
Lymphocytes incubated with PMPs generated after activation of platelets	**↑ ~ 40 lymphocytes p<0.05** (5 mins)	**↑ ~ 30 lymphocytes p<0.05** (30 mins)	**↑ ~ 60 lymphocytes p<0.05** (5 mins)	NOT INVESTIGATED	NOT INVESTIGATED

Table 3.1 Summary of results from all *in vitro* studies highlighting platelet, lymphocyte and platelet modified lymphocyte adhesion to endothelial counterligands, endothelium and intestinal tissue sections. Generally, it seems as if platelets are better able to modify lymphocyte adhesion to ICAM-1 and MAdCAM-1 rather than endothelium and tissue sections. NS = Not significant. Endothelium was stimulated with TNFα.

3.4. Discussion

Recent evidence suggests lymphocytes may play a role in inflammatory disorders. The mechanisms by which they are recruited are not clear. Platelets and their micro particles (PMPs) are known to mediate recruitment of inflammatory leukocytes, particularly neutrophils. However, their ability to influence lymphocyte adhesion is unclear. The data presented in this chapter provides clear evidence for platelet modulation of lymphocyte adhesion *in vitro*. The results demonstrated that previously adherent activated platelets increased lymphocyte adhesion to endothelial counter-ligands, namely CD54 and MAdCAM-1. These results were also repeated when lymphocytes were pre-incubated with platelet releasate and PMPs. Modified adhesion did not appear to be related to platelets inducing an increased expression of adhesion molecules on lymphocytes. Interestingly, lymphocyte adhesion to endothelial monolayers could not be modified by any of the platelet treatments tested. These results suggest that peripherally circulating activated platelets may be able to modify lymphocyte adhesion *in vivo* when endothelial cell adhesion molecules are expressed at high density. Clearly this will have pathophysiological consequences.

The experiments presented in this chapter firstly tested platelet adhesion to endothelial counterligands. As expected adhesion of naïve platelets to CD54, CD106 and MAdCAM-1 was low. Following activation with thrombin, platelet adhesion to ICAM-1 was greatly increased. This is likely to be mediated by platelet adhesion to ICAM-1 bound fibrinogen (Bombeli *et al*., 1998). Fibrinogen would be present in this assay as it is found within α-granules which are released by platelets following activation. Adhesion to MAdCAM-1 was also greatly increased following platelet activation. This was unexpected as platelets do not express the receptors

usually associated with adhesion to MAdCAM-1, namely CD62L, CD49d/beta7, and CD49d/CD29. This may be non-specific binding or mediated by an as yet unknown receptor. Unsurprisingly platelet adhesion to VCAM-1 was very low as platelets are not known to express the VCAM-1 binding integrins CD49d/beta7 and CD49d/CD29.

More importantly, we demonstrated that both activated previously adherent platelets and incubation with platelets or platelet releasate significantly enhance lymphocyte adhesion to immobilised cell adhesion molecules. Interestingly incubation with activated platelet releasate appeared to result in a greater increase in platelet adhesion than incubation with activated platelets for the equivalent length of time. Although the presence of activated platelets on ligand increased subsequent lymphocyte adhesion to CD54 and MAdCAM-1, incubation with platelet releasate increased adhesion to VCAM-1 as well. This suggests that it is factors released from activated platelets that result in the increase in lymphocyte adhesion observed in these static adhesion assays. This is also supported by the observation that in these experiments few direct lymphocyte-platelet conjugates were observed. This is in agreement with previous findings that only about 3% of lymphocytes (be they T-cells, B-cells and NK-cells) are conjugated to platelets in the peripheral circulation, with only a slight rise in this percentage following platelet activation (Li *et al*., 2006). Indeed, it has been demonstrated that lymphocytes have a considerably lower potential to form heterotypic aggregates, with other cells such as platelets, than monocytes and granulocytes (Li *et al*., 1999). This is not to say that platelets cannot modify lymphocyte adhesion through direct interactions with platelets. Certainly, under flow conditions, platelet-lymphocyte conjugates have been shown to influence lymphocyte adhesion (Diacovo *et al*., 1996b, Sheikh *et al*., 2004,

Hu *et al*., 2010). It is possible that both mechanisms are important in the recruitment of lymphocytes during inflammation.

It is likely that one of the factors in activated platelet releasate that affect lymphocyte adhesion are PMPs. Indeed, Jy *et al*. (1995) presented seminal data that PMPs can 'coat' neutrophils and induce their activation, as measured by increased integrin CD11b (α_M) expression and phagocytic activity. However the effect of PMPs on lymphocyte adhesion has not been previously described. In the current study, purified PMPs increased lymphocyte adhesion to CD54, CD106 and MAdCAM-1. PMPs are known to bind to lymphocytes albeit at much lower levels than to neutrophils (Jy *et al*., 1995). To confirm lymphocyte coating with PMPs, flow cytometry was used to detect the platelet specific marker on the lymphocytes. This is a glycoprotein integrin subunit found specifically on platelets (and megakaryocytes). It forms half of the CD41/CD61 complex that interacts with fibrinogen and thus plays an important role in platelet aggregation and adhesion to endothelium. However, in our experiments, CD41 was not found on lymphocytes treated with PMPs generated by treating platelets with 2U/ml thrombin for 30 minutes. This may indicate that PMPs were not coating the surface of the lymphocytes or alternatively, the number of CD41 molecules present on lymphocytes were too low to detect. This may be a result of low numbers of PMPs being present. Future studies could focus on using higher doses of thrombin or alternative agonists to see if this leads to the detection of PMPs on lymphocytes. Indeed, recent studies from the Kalia lab have shown that calcium ionophore generated more than double the number of PMPs than thrombin as determined by flow cytometry and nanoparticle tracking.

Furthermore, increased efficacy of coating of mesenchymal stem cells was observed when higher number of PMPs were generated (unpublished results).

As the releasate of platelets stimulated with 0.2U/ml thrombin for 5 minutes is unlikely to contain high concentrations of PMPs (Matzdorff *et al*., 1998) it is expected that other factors, such as platelet released cytokines, also contribute to the observed effects of this releasate on lymphocyte adhesion. Activated platelets are known to release a number of well-known regulators of lymphocyte recruitment and migration including the chemokines CCL5 (RANTES) and CXCL12 (SDF-1α) and also VEGF, and PAF (Bleul *et al*., 1996, Massberg *et al*., 2006, Szabo *et al*., 1997, Kameyoshi *et al*., 1992, Webb *et al*., 1998, Zhang *et al*., 2010, McFadden *et al*., 1995, Chignard *et al*., 1980). These factors appear to primarily influence lymphocyte adhesion through mediating increases in surface integrin expression. Certainly RANTES has been shown to induce *de novo* expression of adhesion molecules such as CD11b and CD62L or upregulate expression of integrins such as CD49d on human lymphocytes (Szabo *et al*., 1997). In contrast, in the current study flow cytometry did not detect changes in the expression of CD49d or CD18 – these differences could simply be due to murine rather than human lymphocytes being tested. It is also possible for increased lymphocyte adhesion to be mediated independently of changes in integrin expression. Indeed SDF-1α induces clustering of integrins such as VLA-4 on the surface of human lymphocytes has been shown to result in enhanced avidity for endothelial CD106 (Grabovsky *et al*., 2000).

Interestingly, the number of lymphocytes observed to adhere on CD106, either following pre-incubation with platelets or subsequent to platelet adhesion, was considerably lower than

that observed on CD54 or MAdCAM-1. This was an unexpected result as the cells used in this study did express CD49d, half of the CD106 binding integrins CD49d/beta7 and CD49d/CD29 , and lymphocytes have previously been shown to adhere to CD106 (Chan *et al*., 2000). This result may be due to a low density of CD106 coating on the tissue culture plastic as lymphocyte adhesion on immobilised CD106 is heavily dependent on this density. In a study by Chan *et al*. (2000) lymphocyte adhesion was found only to occur at CD106 densities of greater than 100 molecules/μm^2. Unfortunately, due to the method of coating used in our work it is not possible to know the density at which the plates were coated with CD106. Lalor *et al*. (1997) found that coating plastic with 10µg/ml CD106, the same as used in our experiments, supported lymphocyte rolling but not firm adhesion under flow; however, a concentration of 100µg/ml was found to be sufficient for lymphocyte arrest (Chan *et al*., 2000).

In contrast to lymphocyte adhesion on cell adhesion molecules, platelets had no significant effect on lymphocyte adhesion to colonic endothelium. It may be the case that in the absence of platelets, maximal lymphocyte adhesion was already achieved and therefore adhesion could not be further increased by addition of platelets. Carrying out further experiments under flow instead of static conditions may reduce lymphocyte adhesion sufficiently for platelets to have a modulatory effect on their adhesion. Interestingly, TNF-α stimulation of the endothelium had no effect on lymphocyte adhesion or platelet adhesion. These results are similar to those seen previously in the lab with haematopoietic and mesenchymal stem cells (Yemm, 2013). One possible reason for this is that 4 hours stimulation was not a sufficient period of time for up-regulation of adhesion molecule expression to occur. MAdCAM-1 expression on these cells only increases following 24 hours of TNF-α stimulation (Ando *et al*.,

2005). However, 4 hours stimulation is sufficient to increase CD54 expression and neutrophil adhesion on colonic endothelium (Goebel, 2006). Another possibility is that the endothelium was already in an activated state due to the INF-γ in its culture media (Thornhill and Haskard, 1990).

Both naïve and activated platelets were found to adhere to the endothelial monolayers. It is well known that activated platelets can adhere to endothelium and that this adhesion is mediated by platelet CD41/CD61 through its binding of fibrinogen, fibronectin and VWF which can then become bound by the endothelial adhesion molecules CD51/CD61, CD54 and CD42b (Bombeli *et al*., 1998). Platelet activation with thrombin and endothelial stimulation with TNF-α had no effect on the adhesion of platelets on endothelium. Naïve platelets do not adhere to endothelium under normal physiological conditions so this adhesion is likely an artefact of the static nature of this assay.

Finally, this chapter assessed platelet modulation of lymphocytes to frozen sections of injured gut. Adhesion of platelets to tissue sections was significantly increased by both platelet activation and IR injury of the tissue. This is consistent with previous work carried out in the Kalia lab which showed platelet accumulation does occur in the small intestine following IR injury *in vivo* (Holyer, 2010). This is likely due to endothelial denudation caused by the IR injury, which subsequently exposes extracellular matrix components such as collagen that platelets can adhere to (Grootjans *et al*., 2010, Ruggeri and Mendolicchio, 2007). Interestingly untreated lymphocytes did not adhere to injured or uninjured tissue sections despite adhering to cell adhesion molecules and cultured endothelial cells. However, following incubation with

naïve and activated platelets some adhesion was observed on ileum sections, supporting our hypothesis that incubation with platelets can increase lymphocyte adhesion.

As direct adhesion of lymphocytes to platelets did not appear to be required for the increased lymphocyte adhesion observed on cell adhesion molecules, it was hypothesised that platelets were modulating lymphocyte integrin expression. Both T-cells and B-cells were found to express CD18, which is a subunit of the CD54 binding integrins C11a/CD18 and CD11b/CD18, and CD49d, which is a subunit of the MAdCAM-1 and CD106 binding integrins CD49d/beta7 and CD49d/CD29. Although platelets were capable of modulating lymphocyte adhesion in static adhesion assays on CD54 and MAdCAM-1, treatment with naïve and thrombin activated platelets had no significant effect on CD18 or CD49d expression. Although the increases in lymphocyte adhesion observed do not appear to be due to platelet modulation of lymphocyte integrin expression, they may be due to modulation of lymphocyte integrin function through integrin activation or integrin mobility changes. For example the platelet released cytokines CXCL12 and CCL5 can induce subsecond clustering of CD49d/CD29 on lymphocytes leading to increased rolling and tethering on endothelium (Grabovsky *et al*., 2000).

The results presented in this chapter clearly show that platelets are able to modulate the adhesion of lymphocytes *in vitro*. Previous work on the role of platelets in lymphocyte adhesion has shown that a direct interaction between platelets and lymphocytes is a key mediator of lymphocyte adhesion. The evidence presented in this chapter indicates that factors released by activated platelets are also important mediators of lymphocyte adhesion. Platelet released PMPs appear to be a strong candidate for the factor which modulates

lymphocyte adhesion although other factors such as platelet released cytokines are also likely to play a role. The cells used in the adhesion assays were a mixed population of lymphocytes taken from the inguinal lymph nodes. It is therefore not possible to define which subsets of lymphocytes were actually adhering in these experiments. However, as T-cells made up the majority of the isolated cells and appeared to have greater levels of CD18 and CD49d expression than B-cells it is probable that the majority of adherent cells were T-cells. The following chapters will address whether platelet modulation of T- and B-cells adhesion occurs *in vivo* in models of liver inflammation.

CHAPTER 4

LYMPHOCYTE RECRUITMENT TO THE LIVER DURING ISCHEMIA REPERFUSION INJURY IS MEDIATED BY PLATELETS

4. LYMPHOCYTE RECRUITMENT TO THE LIVER DURING ISCHEMIA REPERFUSION INJURY IS MEDIATED BY PLATELETS

4.1. Introduction and Hypotheses

Hepatic IR injury is a clinically relevant problem that occurs following a transient cessation of blood flow to the liver. This results in accumulation of inflammatory cells and platelets within the hepatic microvasculature. The majority of the injury is thought to be caused by a neutrophil mediated inflammatory response following reperfusion (Jaeschke *et al*., 1990, Eltzschig and Collard, 2004). However, there is a growing body of evidence that lymphocytes, particularly T-cells, are also important mediators of hepatic IR injury. Their presence has also been detected post-reperfusion in various other organs including the brain, lungs, intestine and heart (Linfert *et al*., 2009). Previously thought to be "passive observers" in the inflammatory response, there is now an overwhelming body of literature demonstrating the role of T-cells as direct mediators of IR injury (Linfert *et al*., 2009). Indeed, depletion of circulating T-cells significantly reduces hepatic injury following 60 and 150 minutes ischaemia (Anselmo *et al*., 2002). Furthermore, in mice lacking CD4+ T-cells, hepatic neutrophil accumulation is significantly reduced (Caldwell *et al*., 2005).

The other major cell of the adaptive immune system is the B-cell. Activated B-cells primarily function in antibody production and antigen presentation. However, due to their ability to secrete the potently anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokines IL-6, IL-8 and TNFα, the role of a positive and negative regulator of inflammation has been ascribed to this cell (Hamze *et al*., 2013). Despite frequent histological observations of B-cells at inflammatory sites, there is little known about the recruitment kinetics of circulating B-cells

to IR injured liver, even though the liver has a very large resident B-cell population (Novobrantseva *et al*., 2005). Burne-Taney and colleagues demonstrated reduced injury scores following renal IR injury in mice unable to develop peripheral mature B-cells (Burne-Taney *et al*., 2003). Further demonstrations of their pathogenic role was shown in IR injured intestine, heart and skeletal muscle (Linfert *et al*., 2009). However, no studies have currently assessed the spatial and temporal kinetics of peripheral B-cells in acute hepatic IR injury, although they have been observed to home to the liver following parasitic hepatic infection (Moore *et al*., 2012) and are known to contribute to liver fibrosis, a late presenting feature of hepatic IR injury (Novobrantseva *et al*., 2005).

Collectively, these studies are consistent with an emerging pivotal role for lymphocytes in IR injury. However, the question remains as to how they are recruited to ischaemically injured sinusoidal microcirculation *in vivo.* Platelets are also recruited in abundance during hepatic IR injury and more recently have been demonstrated to recruit T-cells to the liver during hepatitis B infection (Iannacone *et al*., 2005, Sitia *et al*., 2012). However, the role of platelets in non-viral or non-antigen induced T- or B- cell recruitment to the liver has not been investigated in detail.

The data presented in the previous chapter suggests that platelets, in particular activated platelets, can increase the adhesion of lymphocytes *in vitro* under static conditions. In hepatic IR injury significant numbers of activated platelets are found within the liver (Cywes *et al*., 1993) which may play a role in lymphocyte recruitment. Given the emerging evidence for the role of the adaptive immune system in hepatic IR injury, this chapter examined intravitally the dynamics of circulating T- and B-cell recruitment *in vivo* and investigated whether this was

mechanistically dependent on platelets. The experiments in this chapter aimed firstly to develop a method for monitoring the trafficking of lymphocytes and platelets *in vivo* within the murine hepatic microcirculation. Data are presented in this chapter comparing lymphocyte recruitment in the IR injured and uninjured hepatic microvasculature. Further to this, data comparing lymphocyte recruitment in the hepatic microcirculation in both normal and platelet depleted mice are presented.

For the work included in this chapter we hypothesised that:

1. Lymphocyte recruitment to the liver is increased following IR injury.
2. Lymphocyte recruitment in IR injured liver is reduced following platelet depletion.
3. Lymphocyte recruitment to the IR injured liver is dependent on lymphocyte expressed CD162 (PSGL-1).
4. Treatment with PMPs can increase lymphocyte recruitment to the IR injured liver.

4.2. Methods

The methods used in this chapter are described in detail in Chapter 2. Briefly, lymphocyte and platelet recruitment to the injured liver was monitored in the anaesthetised mouse using fluorescent intravital microscopy. Injury was induced by clamping off the blood supply to the left and median lobes of the liver for 90 minutes. The clamp was then removed to allow reperfusion. Platelet and lymphocyte trafficking were tracked via two methods: (i) endogenous platelets, T-cells and B-cells were labelled by injection of fluorescent antibodies against CD41, CD3ε and CD19 respectively in mice undergoing intravital experimentation (ii)

fluorescently labelled donor cells were injected into the systemic circulation of recipient mice undergoing intravital experimentation. For the latter, T-cells and B-cells were isolated from the spleen of donor mice using MACS and platelets were isolated from the whole blood. For some experiments, purified CD4+ and CD8+ T-cells were used, which were again isolated from the spleen of donor mice and purified using MACS.

To investigate if platelets play a role in lymphocyte recruitment, in some experiments systemic platelets were depleted prior to injury by injection of a mix of platelet-depleting CD42b antibodies. Platelets may mediate the recruitment of lymphocytes by direct interactions between CD62P on the platelet and CD162 on the lymphocyte. Therefore, in other experiments CD162 on the surface of lymphocytes was blocked via incubation with a blocking antibody prior to their infusion. To investigate the role of pre-treatment with PMPs on lymphocyte recruitment to the IR injured liver, lymphocytes isolated from the inguinal lymph nodes of donor mice were incubated with PMPs generated by thrombin stimulation of washed platelets.

Blood flow within the whole liver was measured using laser Doppler and areas of blood flow were visualised intravitally following injection of FITC-BSA. The velocity of free flowing fluorescently labelled donor platelets was also used as a quantitative measure of overall blood flow. At the end of the experiments, blood samples were taken for blood counts and analysis of serum alanine transaminase (ALT) activity. ALT is an enzyme produced by the liver and released into the bloodstream following hepatocellular injury. Liver sections obtained from

mice at the end of experiments were stained with fluorescent CD41 and CD42b antibodies to further analyse the number of platelets present.

Static adhesion assays were carried out to determine the effects of inflammatory mediators on lymphocyte adhesion. CFDA-SE labelled T-cells or B-cells isolated from the spleens of mice were incubated for 5 minutes with 100μM H_2O_2 or 25ng/ml CCL5 (RANTES), CXCL12 (SDF-1α) or CXCL1 and their adhesion to CD54, CD106 or MAdCAM was quantitated.

4.3. Results

4.3.1. IR Results in Macroscopically Visible Injury and an Increase in Serum ALT Activity

The liver is visibly injured 2 hours after reperfusion (*Figure 4.1*). Blood counts did not significantly change following IR injury compared to sham controls (*Figure 4.2 A*; $p>0.05$).

Platelet depletion using a depleting antibody had no significant effect on the numbers of circulating leukocytes or erythrocytes in either sham or IR mice (*Figure 4.2 A*; $p>0.05$). However, as was desired, numbers of circulating platelets were reduced to below detectable levels ($<1x10^3/mm^3$). Circulating lymphocyte numbers did show a trend to go down in injured mice, but this did not prove to be statistically significant. Following IR injury, survival of mice was not significantly different from sham treated animals up to two hours post-reperfusion (*Figure 4.2 B*; $p>0.05$). There was also no significant difference between the survival of sham treated thrombocytopenic mice and IR injured thrombocytopenic mice (*Figure 4.2 B*; $p>0.05$). Depletion of platelets did appear to reduce survival of mice compared to non-thrombocytopenic mice; however, these differences were not significant (*Figure 4.2 B*;

$p>0.05$). Serum ALT activity, a biochemical marker of liver injury, was significantly increased in both normal (*Figure 4.2 C*; $p<0.01$) and thrombocytopenic mice (*Figure 4.2 C*; $p<0.05$) following IR injury when compared to that of uninjured controls. Platelet depletion did not have a significant effect on serum ALT activity following injury ($p>0.05$). However, following injury serum ALT activity did appear to be higher in thrombocytopenic mice than normal mice but this was not significant.

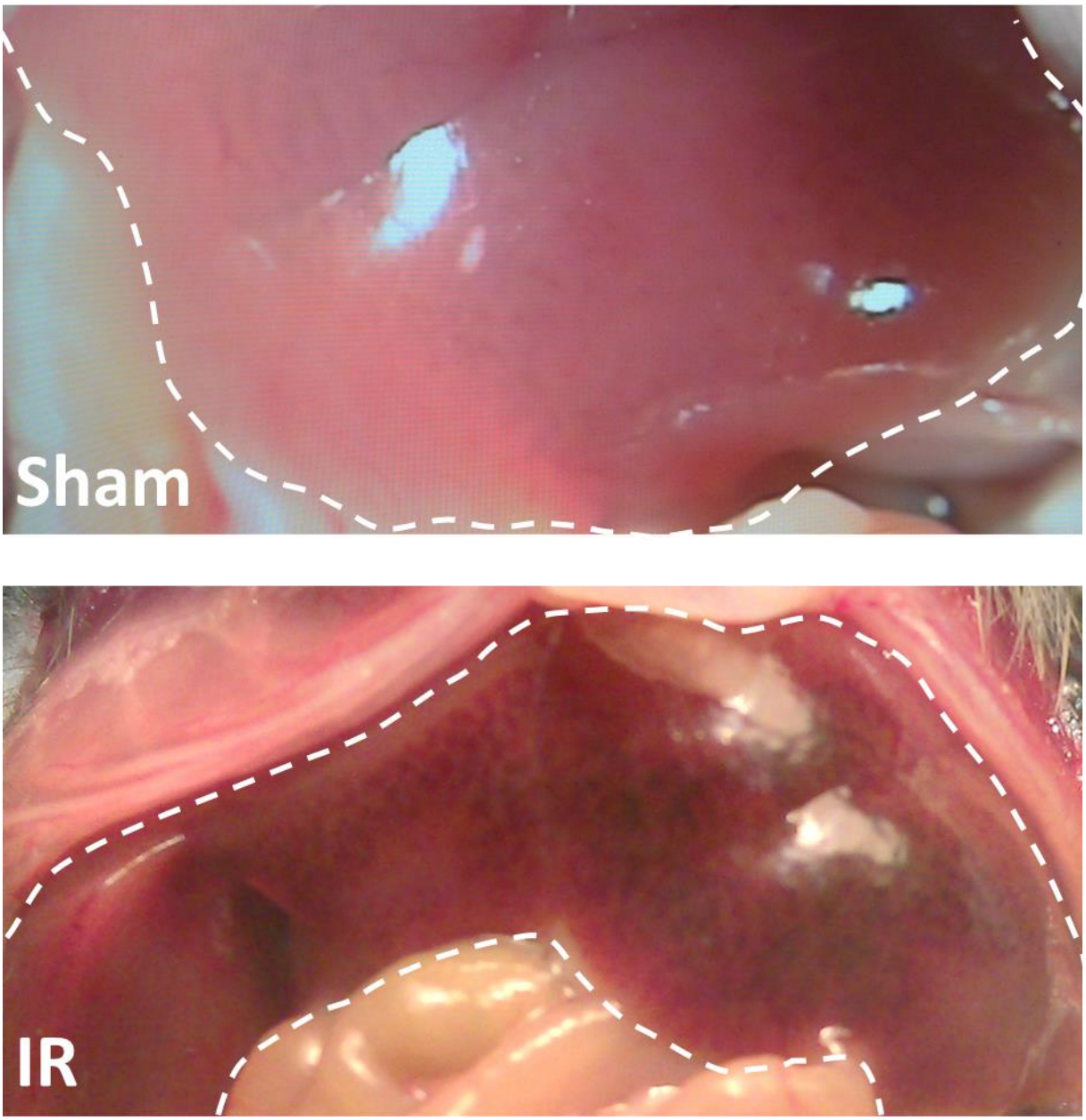


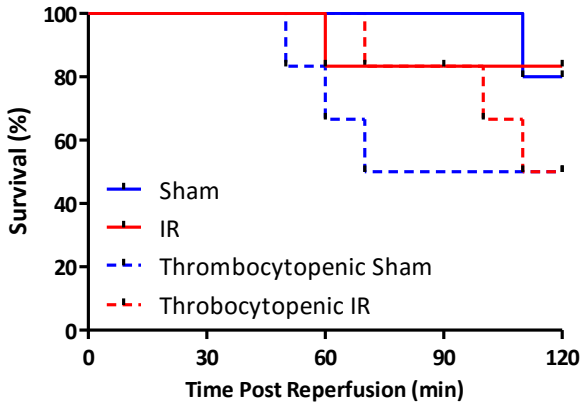
Figure 4:1 2 hours after ischemia the liver is visibly injured. IR injury was induced by clamping off the blood supply to the left and median lobes of the liver for 90 minutes. 2 hours after rep the IR injured liver is darker and less uniform in colouration than the uninjured liver.

A

	RBC (10^6/mm^3)	*PLT (10^3/mm^3)*	*WBC (10^3/mm^3)*	*HCT (%)*	*MPV (µm^3)*	*LYM%*	*MON%*	*NEU%*	*EOS%*	*BAS%*
Sham	9.5±0.5	768±4	3.4±0.9	36.0±1.2	5.7±0.2	83.5±6.0	3.2±0.5	8.5±1.2	0.1±0.0	5.7±5.1
IR	8.9±0.2	782±64	4.5±0.8	33.2±1.5	5.6±0.3	75.9±7.2	4.0±1.2	19.0±8.0	0.3±0.1	0.7±0.2
Sham Thrombocytopenic	9.5±0.7	ND	5.4±1.6	36.0±2.8	ND	83.7±1.7	10.2±1.5	8.3±2.5	0.5±0.2	0.4±0.1
IR Thrombocytopenic	9.3±0.7	ND	3.8±0.3	34.9±2.3	ND	75.8±4.4	13.6±5.4	9.3±2.2	0.4±0.3	1.0±0.1

RBC=red blood cells, **PLT**=platelets, **WBC**=white blood cells, **HCT**=haematocrit, **MPV**=mean platelet volume, **LYM**=lymphocytes, **MON**=monocytes, **NEU**=neutrophils, **EOS**=eosinophils, **BAS**=basophils, **ND**=not detected.

B



C

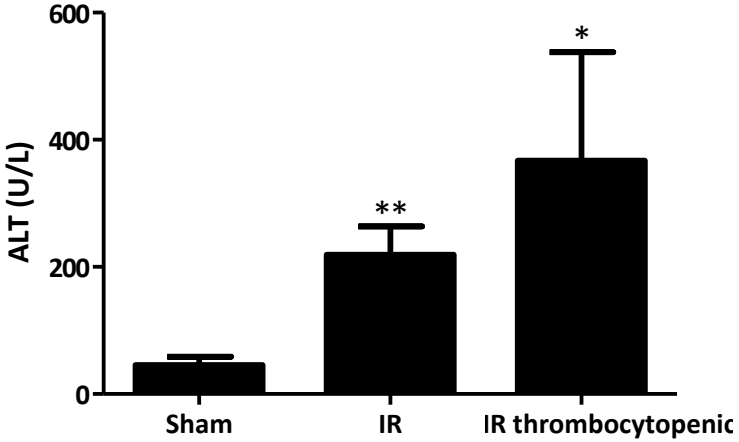


Figure 4:2 IR injury causes a significant increase in serum ALT activiy. (**A**) Blood counts of mice 2hours after 90 minutes ischemia or sham surgery for both untreated and platelet depleted mice. (**B**) Survival of mice following sham surgery or IR injury for both untreated and platelet depleted mice. (**C**) Alanine transferase activity in serum samples taken from mice 2 hours after sham surgery or IR injury in both untreated and platelet depleted mice. Data presented a mean ±SEM, n≥3, *p<0.05 **p<0.01 compared to sham (one-way ANOVA with Bonferroni post-tests (A&C) and Mantel Cox tests with Bonferroni correction (B))

4.3.2. Following Ischemia Blood Flow within the Liver Vasculature is Severely Disrupted

Immediately following reperfusion, laser Doppler measurements show no significant difference in perfusion of the IR injured liver compared to the sham liver (*Figure 4.3 A*; $p>0.05$). However, perfusion in the IR injured liver is significantly reduced compared to the sham at 1 hour (*Figure 4.3 A*; $p<0.05$) and 2 hours (*Figure 4.3 A*; $p<0.01$) post reperfusion. The velocity of free flowing platelets can be used as a measure of the rate of blood flow (Holyer, 2010). The velocity of fluorescently labelled platelets within the hepatic microcirculation was significantly greater in IR injured animals than sham animals at the point of reperfusion and 30 minutes post reperfusion (*Figure 4.3 B;* $p<0.05$). This difference decreased over time, and by 120 minutes post reperfusion platelet velocities were almost equal between sham and IR injured animals.

To parallel the above quantitative data, areas of non-perfused hepatic tissue were not observed in the sham animals intravitally when FITC-BSA was used to identify areas of flow within the microvasculature (*Figure 4.3 C & E*). However, injection of FITC-BSA revealed large areas of non-perfused tissue, which appeared as 'black' vessels, both immediately following reperfusion (*Figure 4.3 D*) and 2 hours after reperfusion (*Figure 4.3 F*).

Platelet depletion did not significantly affect hepatic tissue perfusion following ischaemia as measured by laser Doppler (*Figure 4.3 H*; $p>0.05$). Areas of non-perfused tissue are also visible in platelet depleted mice immediately after ischemia (*Figure 4.3 G*).

4.3.3. Incubation with PMPs has no Significant Effect on Lymphocyte Recruitment to the IR Injured Liver

As incubation with PMPs was shown to increase lymphocyte adhesion to cell adhesion molecules in chapter 3, the effect of incubation with PMPs on lymphocyte recruitment to the IR injured liver was investigated. Incubation with PMPs was found to have no significant effect on lymphocyte adhesion within the post ischemic hepatic vasculature (*Figure 4.4 A;* $p>0.05$). Incubation with PMPs also had no significant effect on the number of free flowing lymphocytes in hepatic vasculature (*Figure 4.4 B;* $p>0.05$).

4.3.4. Increased Endogenous T-cell Recruitment to the Liver Following Ischemia

Previous studies have tracked lymphocytes *in vivo* by labelling donor cells and imaging their trafficking in recipient experimental mice. However, not only does this require more mice to be used, it may also underestimate cellular events as it does not image the endogenous cells. To avoid these issues, and also to reduce the number of mice required for experiments even further, we aimed to track the recruitment of *endogenous* T-cells, B-cells and platelets within the *same mouse*. Antibody labelling was used to allow the imaging of these three endogenous populations of cells. Mice were injected with antibodies against CD3ε (T-cells), CD19 (B-cells) or CD41 (platelets) which had been conjugated to fluorescent secondary antibodies or streptavidin. The results showed a significant increase in endogenous T-cell recruitment (*Figure 4.5 A;* $p<0.001$) but not endogenous B-cells recruitment (*Figure 4.5 B;* $p>0.05$). Interestingly, the number of B-cells observed within the hepatic microcirculation was far higher than the number of T-cells. Approximately four endogenous T-cells were observed per

field of view in IR injured mice whereas 70-80 B-cells were observed in sham and IR injured mice. Free flowing cells were not observed in either the injured or the sham animals. No significant difference in platelet adhesion was observed between sham and IR injured livers (*Figure 4.5 E;* $p>0.05$). This was quantitated as the area of liver labelled by CD41 antibody as it was not possible to identify individual platelets.

4.3.5. Increased Endogenous T-cell Recruitment to the Liver Following Platelet Depletion

In order to determine whether platelets were involved in mediating the increased endogenous lymphocyte recruitment to the IR injured liver, mice were depleted of systemic platelets by injection of a platelet depleting CD42b antibody. Platelet depletion was confirmed using an automated blood counter (see data presented in *Figure 4.2*). Platelet depletion had no effect on leukocyte or erythrocyte numbers. Interestingly, following platelet depletion, a significant increase in endogenous T-cell recruitment to the injured liver was observed (*Figure 4.6 A;* $p<0.001$). No significant difference in endogenous B-cell recruitment to the injured liver following platelet depletion was noted (*Figure 4.6 B;* $p>0.05$).

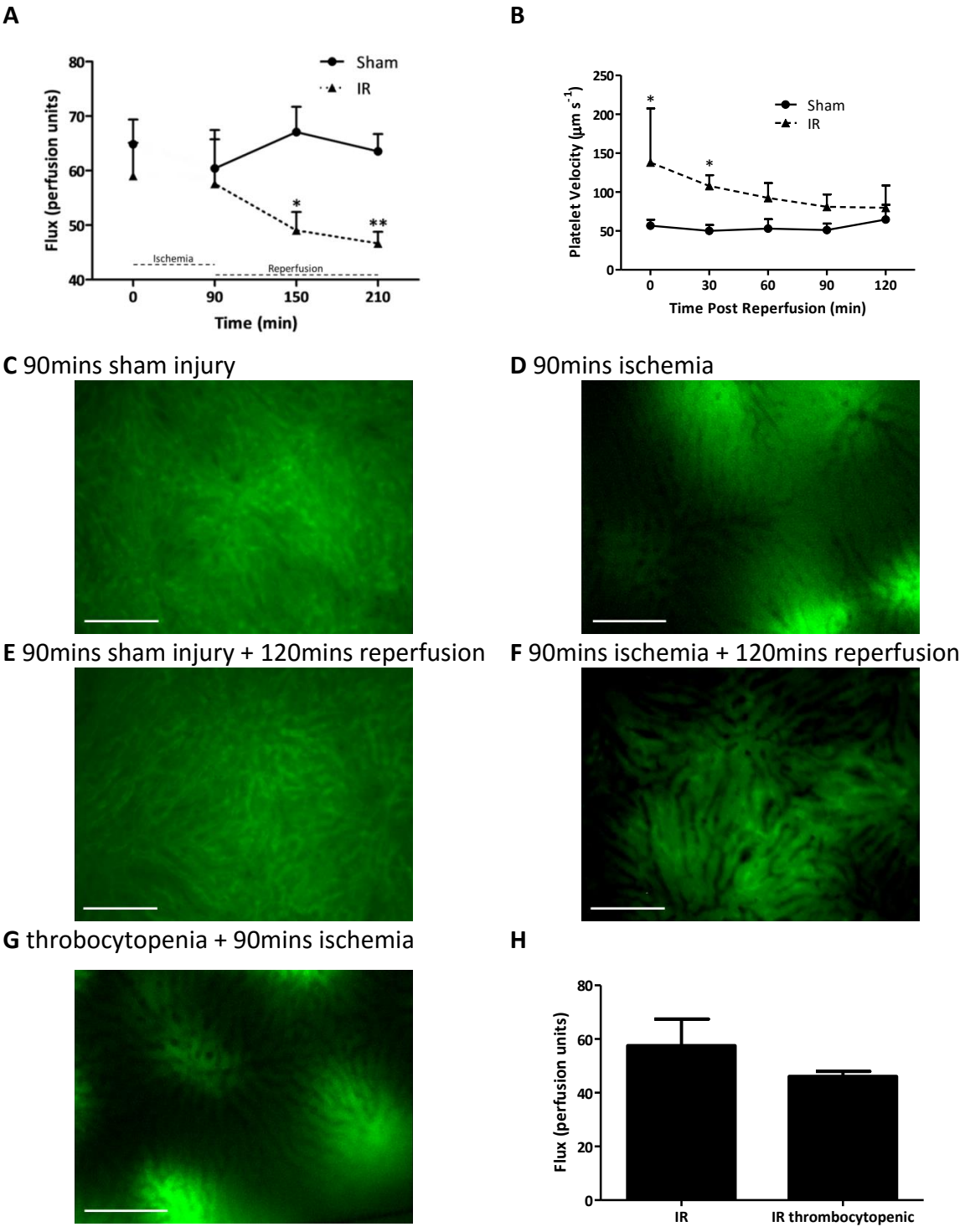
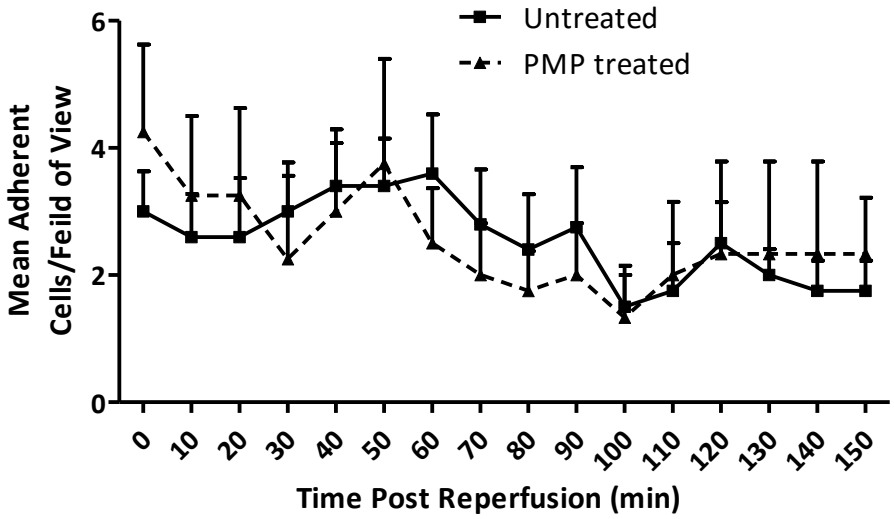


Figure 4:3 Following ischemia blood flow within the liver vasculature is severely disrupted. Laser Doppler for IR injured liver compared to the sham control after 150 and 210 minutes (**A**). Velocities of free flowing platelets in the IR and sham livers were determined by intravital microscopy (**B**). Images show FITC-BSA (green) within the hepatic vasculature following 90 minutes sham treatment (**C**); 90 minutes ischemia (**D**); 120 minutes after 90 minutes sham treatment (**E**); after 90 minutes ischemia and 120 minutes reperfusion (**F**) and after platelet depletion and 90 minutes ischemia (**G**). Perfusion of the IR liver in normal and platelet depleted mice measured by laser Doppler (**H**). Data presented a mean ±SEM, n≥3, *p<0.05 **p<0.01 (one-way ANOVA with Bonferroni post-tests (A&B) and t-test (**H**)) scale bars=100µm.

A



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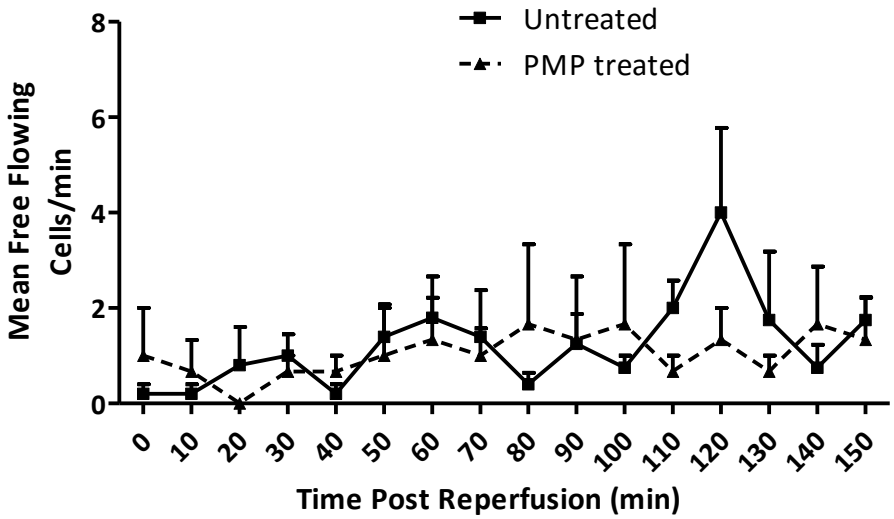


Figure 4:4 Incubation with PMPs has no significant effect on lymphocyte recruitment to the IR injured liver. 1.8 million CFSE stained lymphocytes were incubated for 5 minutes with PMPs or PBS (untreated) and their adhesion (**A**) and the number of free flowing cells (**B**) within the IR injured liver was quantitated using intravital microscopy. Data presented as mean ± SEM, n≥3 (two-way ANOVA).

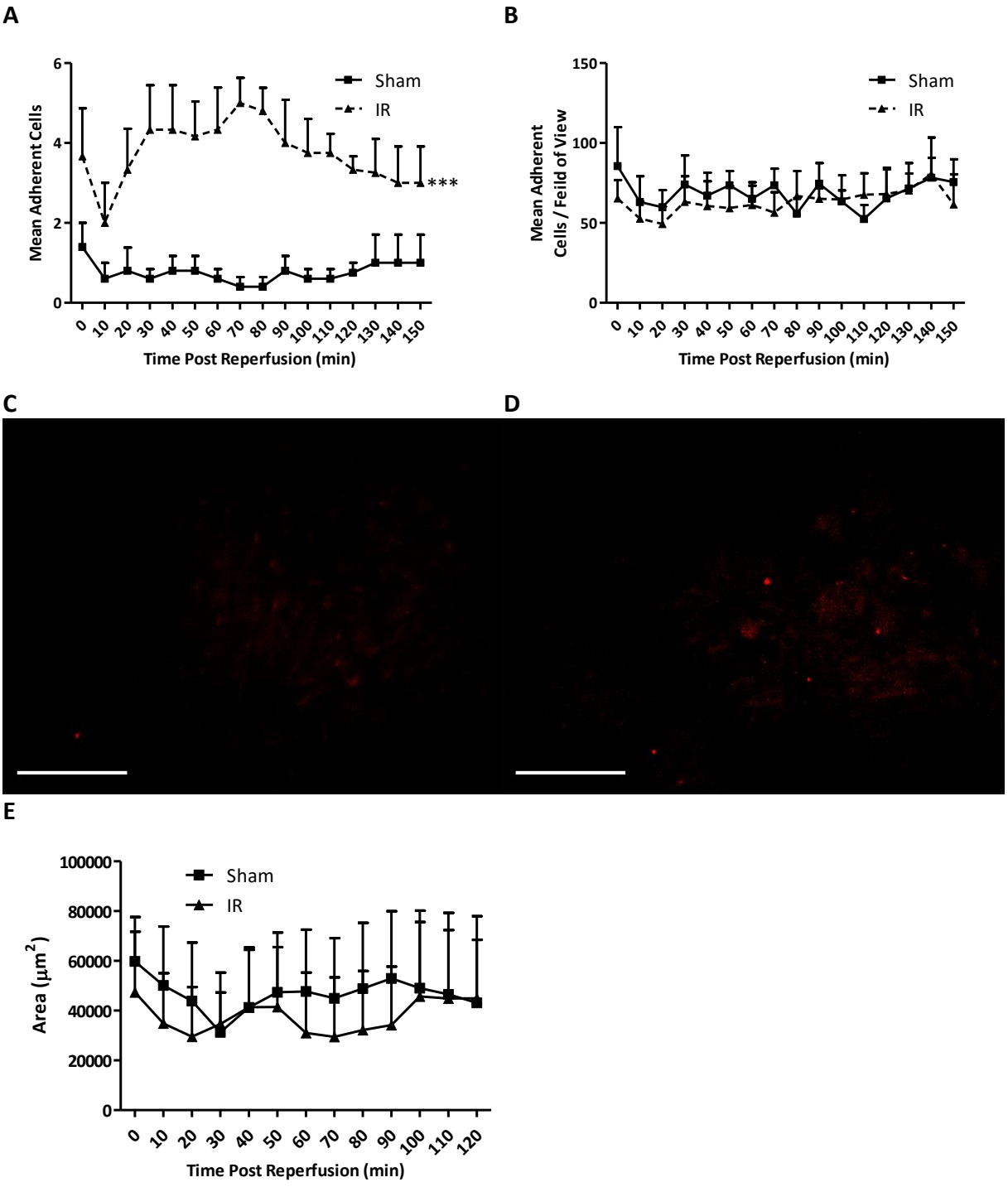


Figure 4:5 Antibody Labelling Shows an Increase in Endogenous T-cell Recruitment to the Liver Following Ischemia. Mice were injected with antibodies against CD3ε, CD19 and CD41 which had been conjugated to fluorescent secondary antibodies or streptavidin. Following 90 minutes ischemia or sham surgery the number or area of fluorescent objects within the liver was quantitated using intravital microscopy. The number of adherent T-cells indicated by CD3ε antibody labelling in the sham and IR liver (**A**). The number of B-cells indicated by CD19 antibody labelling in the sham and IR liver (**B**). Images show CD3ε antibody labelling in the sham (**C**) and IR injured (**D**) liver at 70 minutes post reperfusion. The area of the liver labelled by CD41 antibody in the sham and the IR injured liver (E). Data presented as mean ± SEM, n≥4, ***p<0.001 (two-way ANOVA), scale bars=100µm.

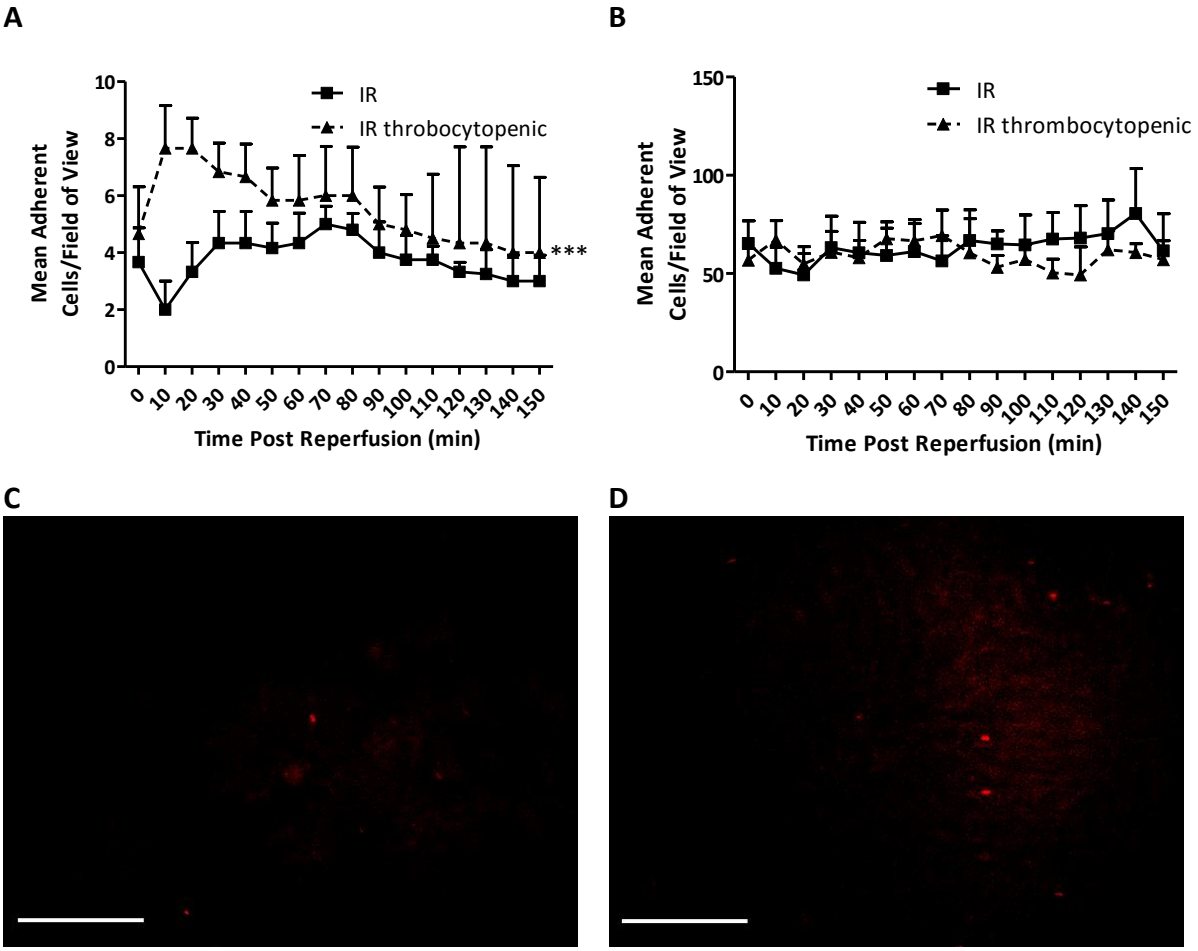


Figure 4:6 Antibody labelling shows an increase in endogenous T-cell recruitment to the IR injured liver following platelet depletion. Prior to the start of the experiment mice were injected with platelet depleting antibodies. Mice were then injected with antibodies against CD3ε and CD19 which had been conjugated to fluorescent secondary antibodies or streptavidin. Following 90 minutes ischemia or sham surgery the number of fluorescent objects within the liver was quantitated using intravital microscopy. The number of adherent T-cells indicated by CD3ε antibody labelling in the livers of normal and platelet depleted mice (**A**). The number of B-cells indicated by CD19 antibody labelling in the IR livers of normal and platelet depleted mice (**B**). Images show CD3ε antibody labelling in the non-platelet depleted (**C**) and thrombocytopenic (**D**) IR injured liver at 10 minutes post reperfusion. Data presented as mean ± SEM, n≥4, ***p<0.001 (two-way ANOVA), scale bars=100μm.

4.3.6. Antibody Labelling Does Not Appear to be the Ideal Method for Studying Lymphocyte and Platelet Trafficking within the Liver

Blood smears taken from animals at the end of these experiments appeared to show that the antibodies used to label T- and B-cells *in vivo* were labelling these specific blood cells (*Figure 4.7 A & B*). However, injection of isotype control antibodies for the CD41, CD3 ε and CD19 antibodies revealed large amounts of non-specific antibody binding within the liver (*Figure 4.7 C, D & E*). Similar binding patterns were also seen with antibodies against other receptors such as the neutrophil specific marker GR1 (*Figure 4.7 F*). One CD19 antibody did however appear to have less non-specific binding and free flowing cells could be observed with this antibody (*Figure 4.7 G*).

As non-specific binding of antibodies appeared to be a problem within the mouse liver, cells isolated from donor mice were fluorescently labelled and used to monitor cell trafficking in all subsequent experiments. T-cells and B-cells isolated from the spleen of donor mice using MACS were routinely >96% pure (*Figure 4.8 A & B*). In static adhesion assays on colonic endothelial cells, incubation with activated platelets significantly increased adhesion of both T-cells (*Figure 4.8 C*; $p<0.05$) and B-cells (*Figure 4.8 D*; $p<0.05$) isolated using this technique. Incubation with activated platelets or activated platelet releasate had no significant effect on expression of CD18, CD49d, CD62L or CD44 on the surface of these cells (*Figure 4.8 E & F*; $p>0.05$).

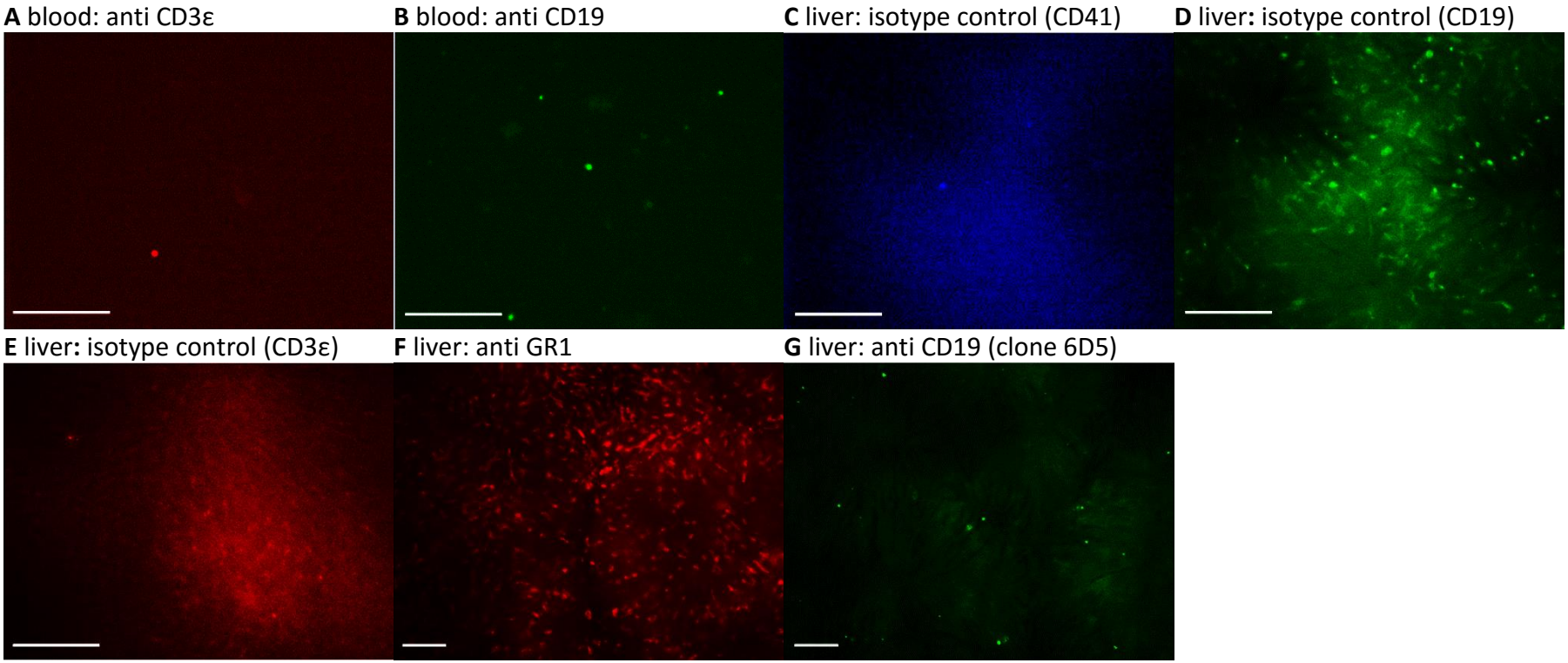


Figure 4:7 Antibodies bind non-specifically within the liver vasculature. Images show Blood taken from mice injected with: (**A**) anti CD3ε antibody and Streptavidin Alexa 647 and (**B**) anti CD19 antibody and anti-mouse alexa488. sham livers with: (**C**) rat IgG and anti-rat IgG-Alexa 555 (**D**) armenian Hamster IgG-Biotin and Streptavidin Alexa 647 (**E**) mouse IgA and anti-mouse Alexa-488 (**F**) PE anti GR1 (**G)** rat anti-mouse CD19 and anti-rat Alexa-488. Scale bars=100µm

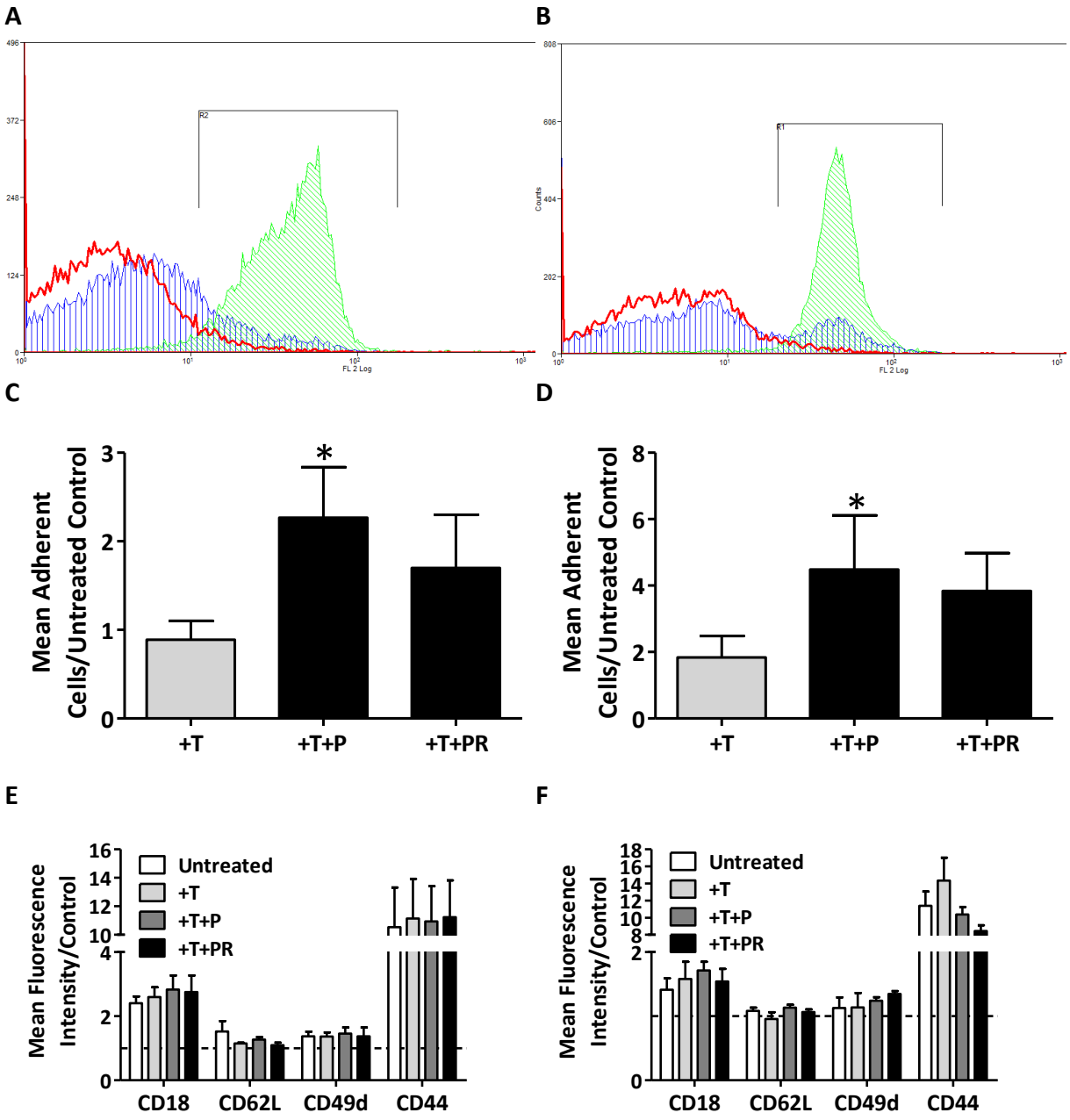


Figure 4:8 Adhesion of purified T-cells and B-cells can be increased by incubation with activated platelets. T-cells and B-cells were purified from the spleens of C57BL/6 mice using MACS. The purity of T-cell (**A**; CD3ε+) and B-cell (**B**; CD19+) separations was determined by flow cytometry and found to be routinely >96%. Plots representative of 3 experiments; red line: isotype control, green line: sorted cells and antibody against relevant marker, blue line: excluded cells and antibody against relevant marker. T-cells (**C**) and B-cells (**D**) were incubated for 5 mins with thrombin (+T), thrombin activated platelets (+T+P) or thrombin activated platelet releasate (+T+PR) and their adhesion on TNF-α stimulated endothelium was quantitated.. The effect of these treatments on expression of CD18, CD62L, CD49d or CD44 on the surface of T-cells (**E**) or B-cells (**F**) was determined by flow cytometry. Data presented a mean ± SEM, n≥3, *p<0.05 (one-way ANOVA with Dunnett's post-tests)

4.3.7. Adhesion of Donor Platelets within the Hepatic Microcirculation is Increased in Areas of Flow Following Hepatic IR Injury

In order to track platelet recruitment to the IR injured liver with a more accurate method than labelling endogenous platelets, CFSE labelled washed donor platelets were injected 5 minutes prior to ischaemia. Overall there was no significant difference in the number of adherent platelets in the sham and IR injured livers (*Figure 4.9 A*; $p>0.05$). Dual labelling of albumin (to identify blood flow) with Alexa 647 and donor platelets with CFSE, demonstrated that the majority of adherent platelets in the IR injured liver were found in areas of blood flow (*Figure 4.9 F*). When platelets in only areas of flow were quantitated, platelet adhesion was found to be significantly higher in IR injured animals than sham animals (*Figure 4.9 C;* $p<0.05$). Interestingly, platelet adhesion was high in sham livers too. The number of free flowing platelets was found to be significantly lower in the injured liver than the sham liver (*Figure 4.9 B;* $p<0.001$). However in areas of flow there was no significant difference between the numbers of free flowing platelets (*Figure 4.9 D*; $p>0.05$). No difference was observed between IR injured and sham treated animals in the number of platelets identified *ex vivo* within the kidney or the mucosa of the jejunum (*Figure 4.9 G;* $p>0.05$). The number of platelets within the spleen was too great to analyse (*Figure 4.9 H*).

In order to confirm these intravital findings, tissue sections were taken from the liver of mice 2 hours after ischaemia or sham surgery. Staining of sections with antibodies against the platelet markers CD41 and CD42b showed no significant difference in the number of platelets present between the sham liver and IR injured liver (*Figure 4.10 A & D*; $p>0.05$) corroborating the intravital data. Again platelet presence was high even in the sham livers.

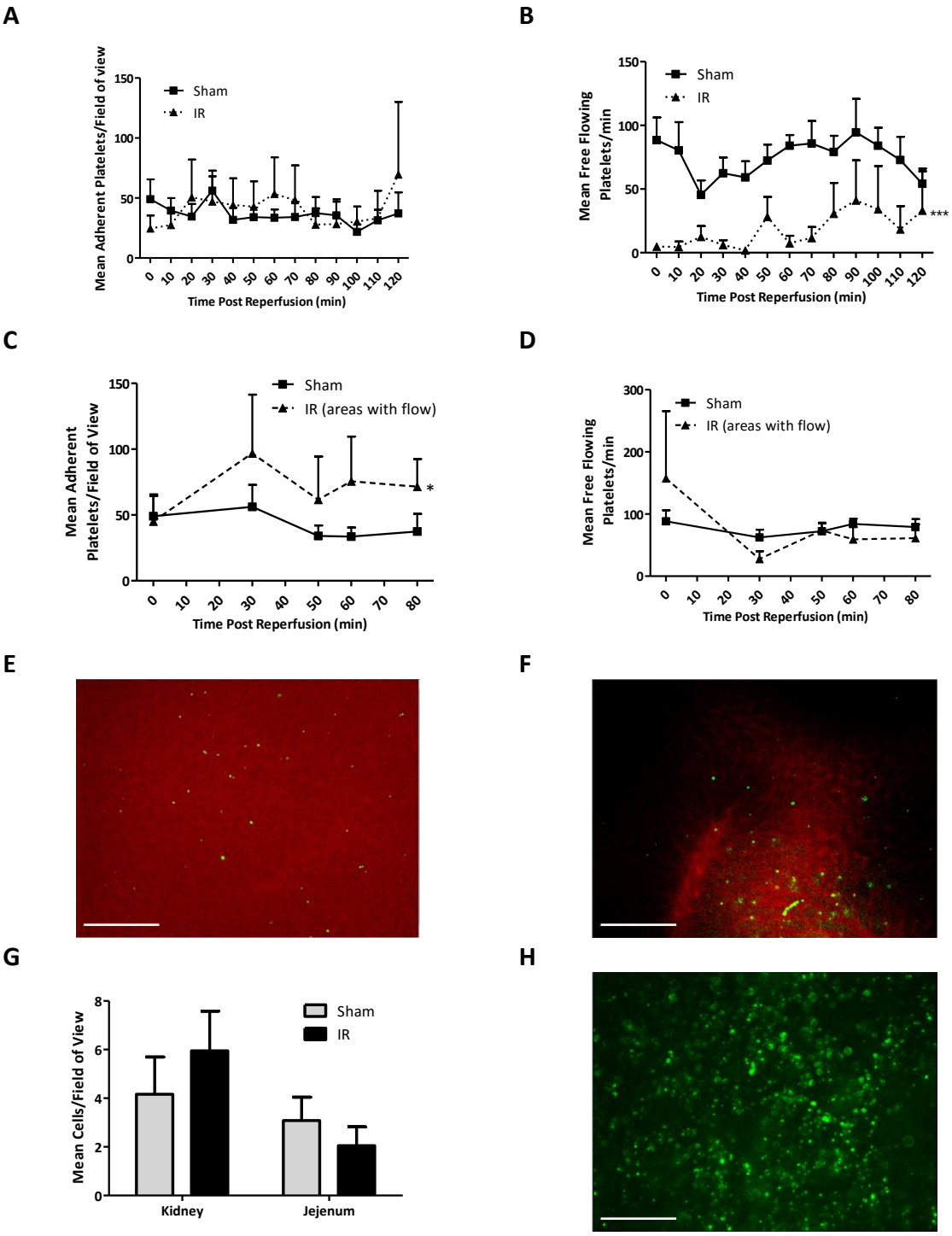


Figure 4:9 Adhesion of donor platelets within the hepatic microcirculation is increased in areas of flow following hepatic IR injury. Trafficking of platelets within the hepatic microcirculation following partial hepatic ischemia was monitored using intravital microscopy. The overall numbers of adherent (**A**) and free flowing (**B**) platelets within the sham and IR liver was quantitated. Numbers of adherent (**C**) and free flowing (**D**) platelets in areas of flow only were also quantitated. Images show platelets (green) and Alexa-647 BSA (red) in the sham (**E**) and IR (**F**) liver at 2 hours post reperfusion. The number of platelets within the kidney and jejunum at 2 hours post reperfusion was quantitated *ex vivo* (**G**), the number of platelets with in the spleen was too great to analyse (**H**). Data presented as mean ±SEM, n≥3, *p<0.05 **p<0.01 (two-way ANOVA), scale bars=100µm.

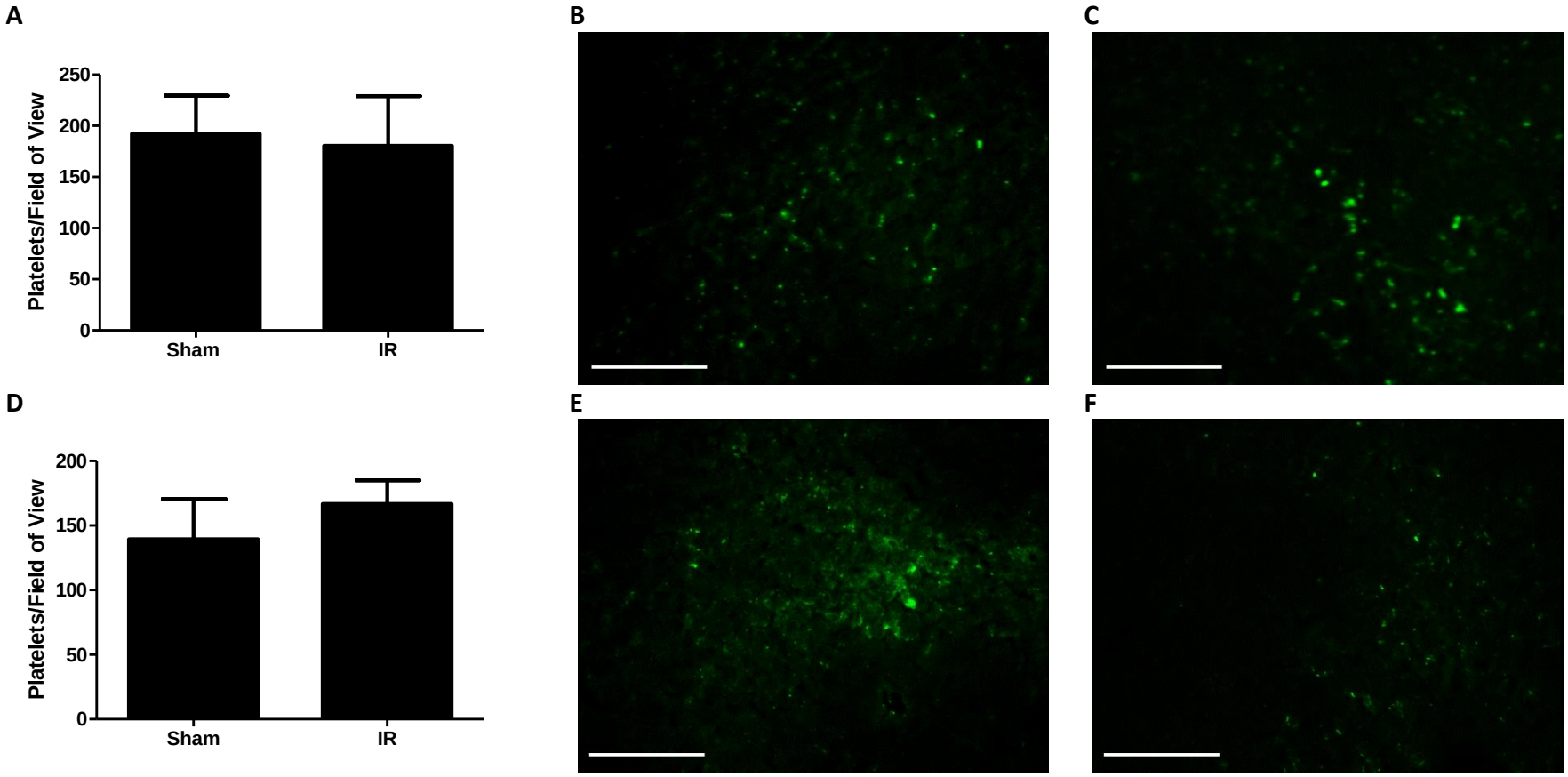


Figure 4:10 Immuno-fluorescent staining shows no difference in platelet accumulation between the sham and IR injured liver. Tissue sections were obtained from the livers of mice at 2 hours post ischemia or sham surgery. Platelet number within the sham and IR livers was quantitated by staining with CD41 (**A**). Platelet number within the sham and IR livers was also quantitated by staining with staining with CD42b(**D**). Images show CD41 (green) staining on a sham liver section (**B**) and IR injured section (**C**) and CD42b staining (green) on a sham liver section (**E**) and IR injured section (**F**). Data presented as mean ±SEM, n=3, (t-tests), scale bars=100µm.

4.3.8. Adhesion of Donor T-cells within the Hepatic Microcirculation is Increased Following IR Injury

In order to track lymphocyte recruitment to the IR injured liver, CMTMR labelled donor spleen T-cells and CFDA-SE labelled B-cells were injected at the point of reperfusion. T-cell adhesion was significantly higher in the IR injured liver than the sham liver (*Figure 4.11 A*; $p<0.001$). There was no significant difference in the number of free flowing T-cells between the post ischemic and sham livers (*Figure 4.11 B*; $p>0.05$); after injection the number of free flowing cells rapidly decreased. *Ex vivo* analysis indicated large numbers of T-cells were becoming adherent in the spleen, although the number of cells present was not significantly affected by liver injury (*Figure 4.11 E*; $p>0.05$).

Unlike T-cells, there was no significant difference in B-cell adhesion between the IR and sham liver (*Figure 4.12 A*; $p>0.05$). Interestingly, a much higher number of B-cells (~10 cells/field of view) were identified in sham liver than T-cells (~2 cells/field of view). There was also no significant difference in the number of free flowing B-cells between the IR injured and sham liver (*Figure 4.12 B*; $p>0.05$). Similarly to T-cells the number of free flowing B-cells rapidly decreased following injection. A large number of B-cells were found in the spleen *ex vivo* and the number of cells present was not significantly affected by liver IR injury (*Figure 4.12 E*; $p>0.05$).

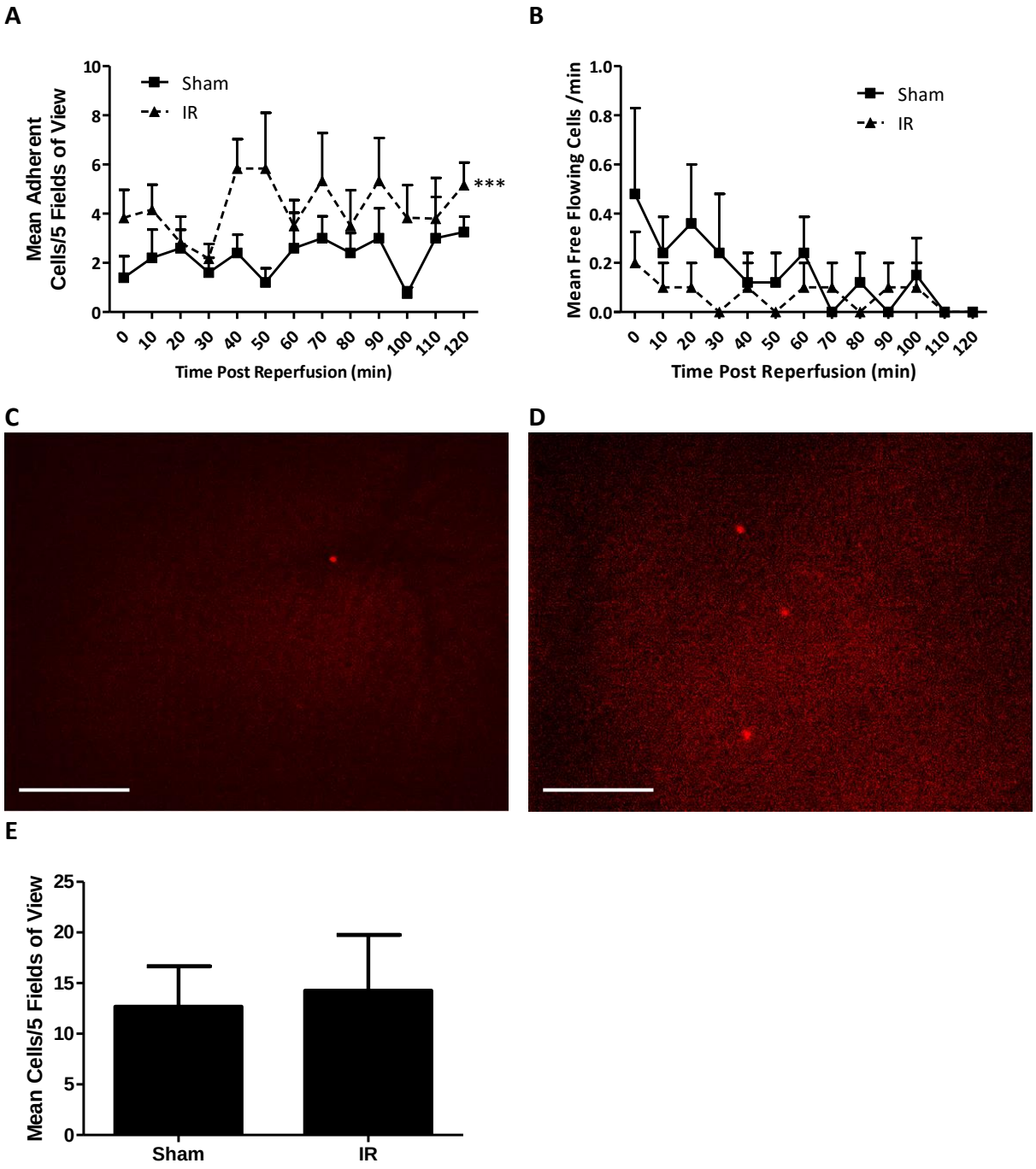


Figure 4:11 Adhesion of donor T-cells in the hepatic microcirculation is significantly increased following IR injury. T-cells isolated from the spleens of donor mice were labelled with CMTMR and injected into the carotid artery of mice following sham surgery or 90 minutes hepatic ischemia. The trafficking of these cells within the liver was monitored using intravital microscopy. Adherent (**A**) and free flowing (**B**) T-cells were quantitated within the IR injured liver and the sham liver. Images show T-cells (red) in the sham (**C**) and IR injured liver (**D**) at 40 minutes post reperfusion. *Ex vivo* analysis of the number of T-cells in the spleen in sham and IR injured animals (**E**). Data presented as mean ±SEM, n≥4, ***p<0.001 (two-way ANOVA (A&B) and t-test (E)), scale bars=100µm

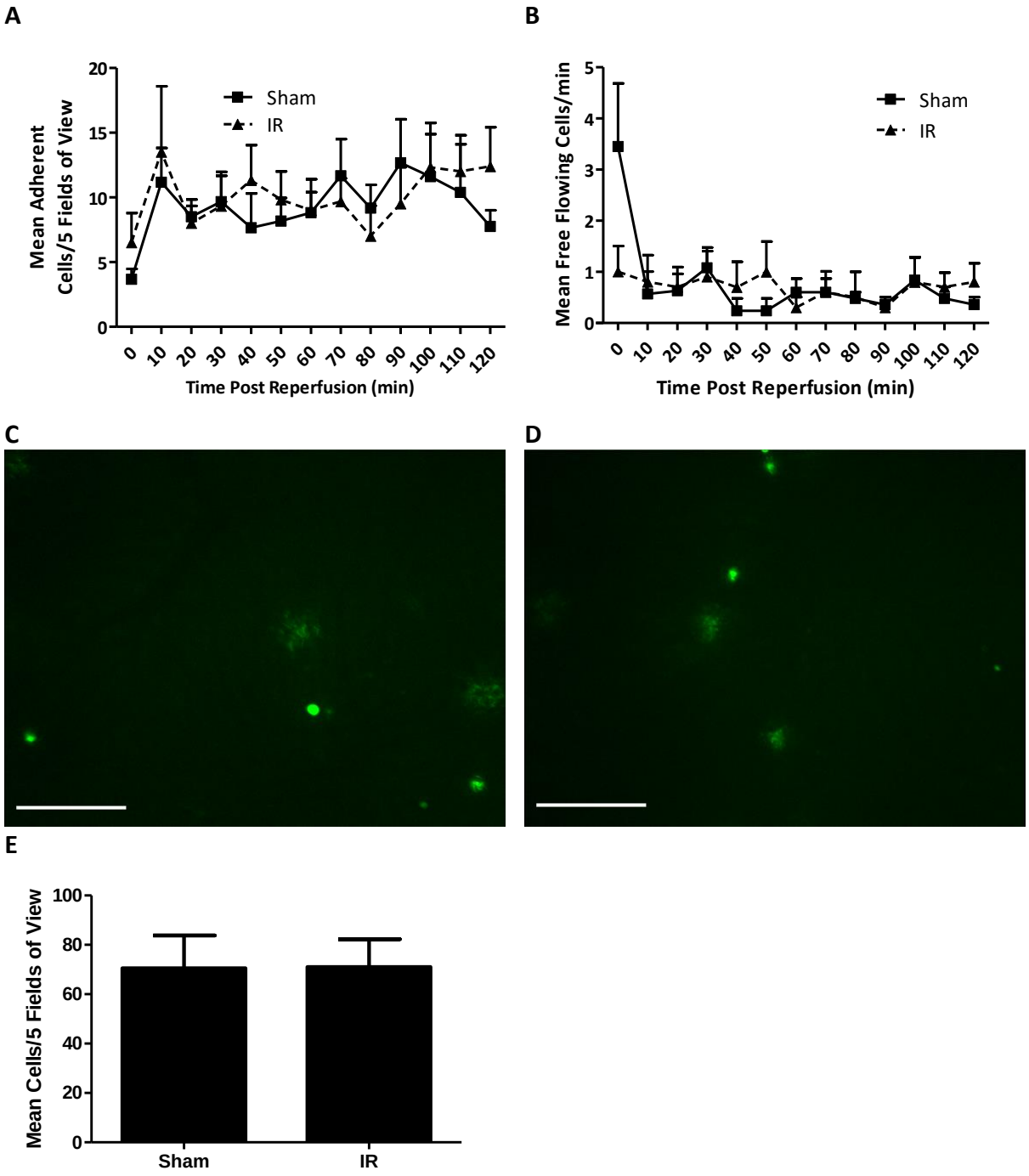


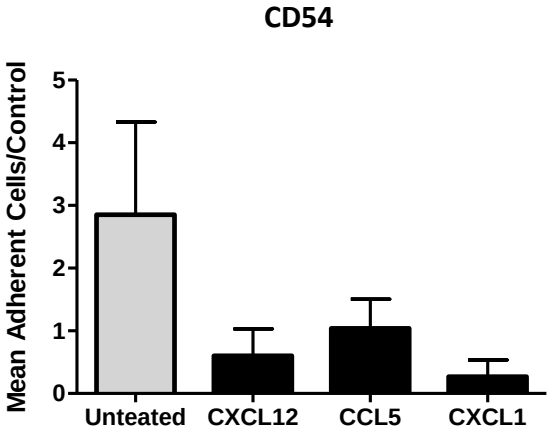
Figure 4:12 Adhesion of donor B-cells in the hepatic microcirculation is not significantly different following IR injury. B-cells isolated from the spleens of donor mice were labelled with CFSE and injected into the carotid artery of mice following sham surgery or 90 minutes hepatic ischemia. The trafficking of these cells within the liver was monitored using intravital microscopy. Adherent (**A**) and free flowing (**B**) B-cells were quantitated within the IR injured liver and the sham liver. Images show B-cells (green) in the sham (**C**) and IR injured liver (**D**) at 40 minutes post reperfusion. *Ex vivo* analysis of the number of B-cells in the spleen of sham and IR injured animals (**E**). Data presented as mean ±SEM, n≥4, *p<0.05 (two-way ANOVA (A&B) and t-test (E)), scale bars=100µm.

4.3.9. Treatment with Inflammatory Cytokines or H_2O_2 had no Significant Effect on Lymphocyte Adhesion to CD54, CD106 or MAdCAM

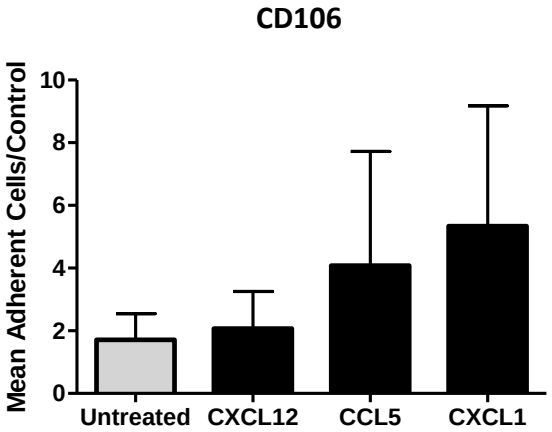
With the aim of determining whether inflammatory cytokines released during IR injury were responsible for the rise in T-cell adhesion seen in the IR injured liver, T-cells were treated with CXCL1, CXCL12 (SDF-1α) and CCL5 (RANTES) and their adhesion on CD54, CD106 and MAdCAM-1 was quantitated. Although none of the pre-treatments elicited a significant effect on T-cell adhesion to the cell adhesion molecules, both CCL5 and CXCL1 resulted in a non-significant increase in adhesion to CD106 and MAdCAM-1 (*Figure 4.13* B & C; $p>0.05$). All of the pre-treatments resulted in a non-significant reduction in adhesion to CD54 (*Figure 4.13* A; $p>0.05$).

In addition to inflammatory cytokines, H_2O_2 is also released during inflammation. H_2O_2 is released by inflammatory cells, including neutrophils, which are able to generate local H_2O_2 concentrations of 200-500μM (Johnston, 1996). Treatment of T-cells or B-cells for 5 minutes with physiological concentrations of H_2O_2 (100μM) did not significantly affect their adhesion to either CD54 or CD106 (*Figure 4.14*; $p>0.05$).

A



B



C

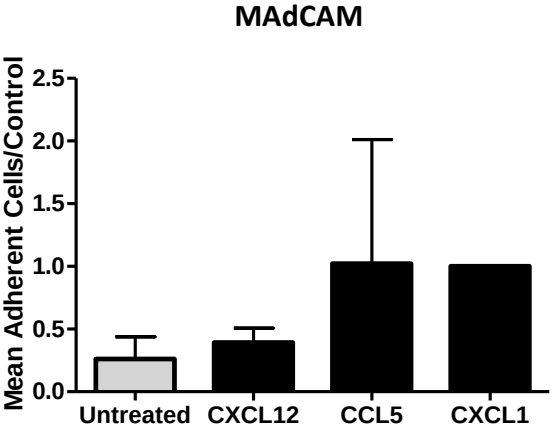
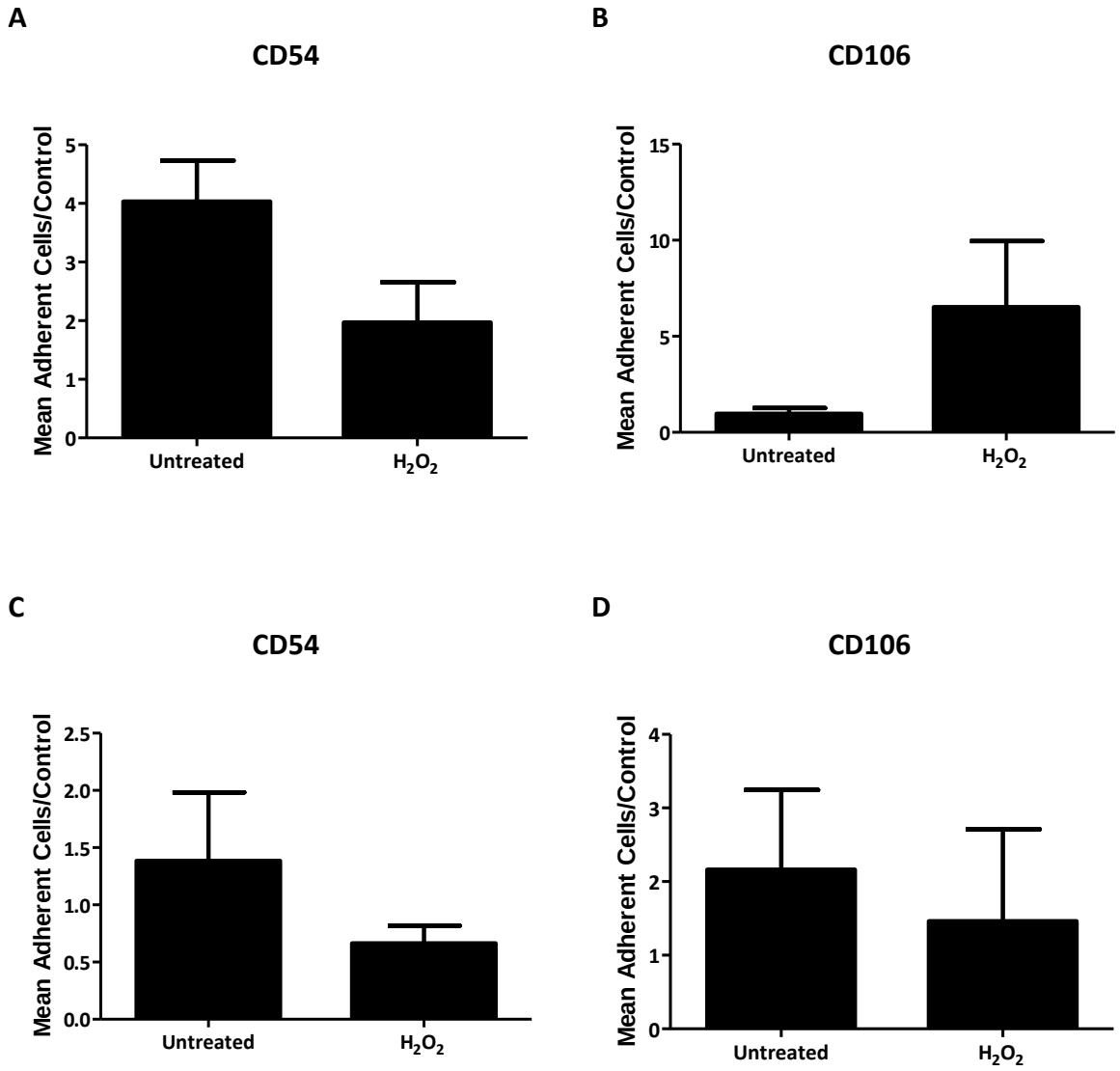


Figure 4:13 Treatment with inflammatory cytokines had no significant effect on T-cell adhesion to CD54, CD106 or MAdCAM-1. CMTMR labelled T-cells were incubated for 5 minute with 25ng/ml CXCL1, CXCL12 or CCL5 before being incubated on immobilised CD54 (**A**), CD106 (**B**) or MAdCAM-1 (**C**) for 20 minutes and their adhesion quantitated. Data presented as mean ±SEM, n≥3 (one-way ANOVA with Dunnet's post-tests).



c

Figure 4:14 Treatment with H_2O_2 had no significant effect on T-cell or B-cell adhesion to CD54 or CD106. CFDA-SE labelled T-cells (**A**&**B**) and B-cells (**C**&**D**) were incubated for 5 minute with 100µM H_2O_2 before being incubated on immobilised CD54 or CD106 for 20 minutes and their adhesion quantitated. Data presented as mean ±SEM, n≥3 (paired t-tests).

4.3.10. Injecting Lymphocytes into the Portal Vein at 60 Minutes Post Reperfusion Results in Significantly Fewer Adherent Lymphocytes in the IR Injured Liver Compared to the Sham Liver

For the above intravital studies, donor lymphocytes were introduced systemically following infusion via the carotid artery. However, this resulted in a much lower number of lymphocytes being observed in the liver than expected and also lower than that observed in the spleen. Similar intravital studies were again conducted but this time with cells injected into the hepatic portal vein. This was performed in an attempt to increase the proportion of cells having an opportunity to become adherent in the liver. The cells were also injected at 60 minutes post reperfusion instead of immediately following reperfusion. The number of adherent T-cells in the hepatic microcirculation of sham mice was higher than that previously observed when injected via the carotid artery but the number of B-cells was lower. However, this route of administration resulted in significantly fewer adherent T-cells (*Figure 4.15 A*; $p<0.001$) and B-cells (*Figure 4.16 A*; $p<0.05$) present in the IR injured liver compared to the sham. There was no significant difference in the number of free flow T-cells (*Figure 4.15 B*; $p<0.05$) or B-cells (*Figure 4.16 B*; $p<0.05$) between the IR injured liver and sham liver. *Ex vivo* analysis showed no significant difference in the number of T-cells in a cross-section of the liver (*Figure 4.15 F*; $p>0.05$) or the spleen (*Figure 4.15 E*; $p<0.05$) between sham and IR-injured animals. *Ex vivo* analysis also showed no significant difference in the number of B-cells in the spleen (*Figure 4.16 E*; $p>0.05$) or a cross section of the liver (*Figure 4.16 E*; $p<0.05$) between sham and IR injured animals. Despite the slightly increased adhesion of T-cells within the hepatic microcirculation it was decided that, due to the complicated nature of portal injection, introduction of cells through a carotid cannula would be used for all further experiments.

4.3.11. CD8+ and CD4+ T-cells Adhere in Equal Numbers in the Microvasculature of the IR Injured Liver

As IR injury resulted in a significant increase in T-cell adhesion within the liver (section 4.3.8) further experiments were carried out to determine if there was a difference in adhesion between CD4+ and CD8+ subsets of T-cells. Flow cytometry showed that the T-cells used in previous experiments were 50±5% CD4+ (*Figure 4.17 A*) and 42±1% CD8a+ (*Figure 4.17 B*). MACS was used to isolate purified CD4+ and CD8a+ T-cells from the spleens of donor mice. CD4+ T-cell isolates were found to be >92% pure (*Figure 4.17 C)* and CD8a+ T-cells were found to be >93% pure (*Figure 4.17 D*). Equal numbers of Purified CD4+ and CD8a+ T-cells were introduced into the same mouse following IR injury. The two subsets were differentiated by staining either CFDA-SE or CMTMR; the stain used for each subset was alternated between experiments to control for differences in florescence. There was no significant difference in adhesion in the liver observed between the two subsets (*Figure 4.18 A*; $p>0.05$). There was also no significant difference in the number of free flowing cells between the subsets (*Figure 4.18 B;* $p>0.05$). At 120 minutes post-reperfusion *ex vivo* analysis showed no significant difference between the number of CD8a+ and CD4+ T-cells in the spleen (*Figure 4.18 E;* $p>0.05$) or in a cross section of the liver (*Figure 4.18 F;* $p>0.05$).

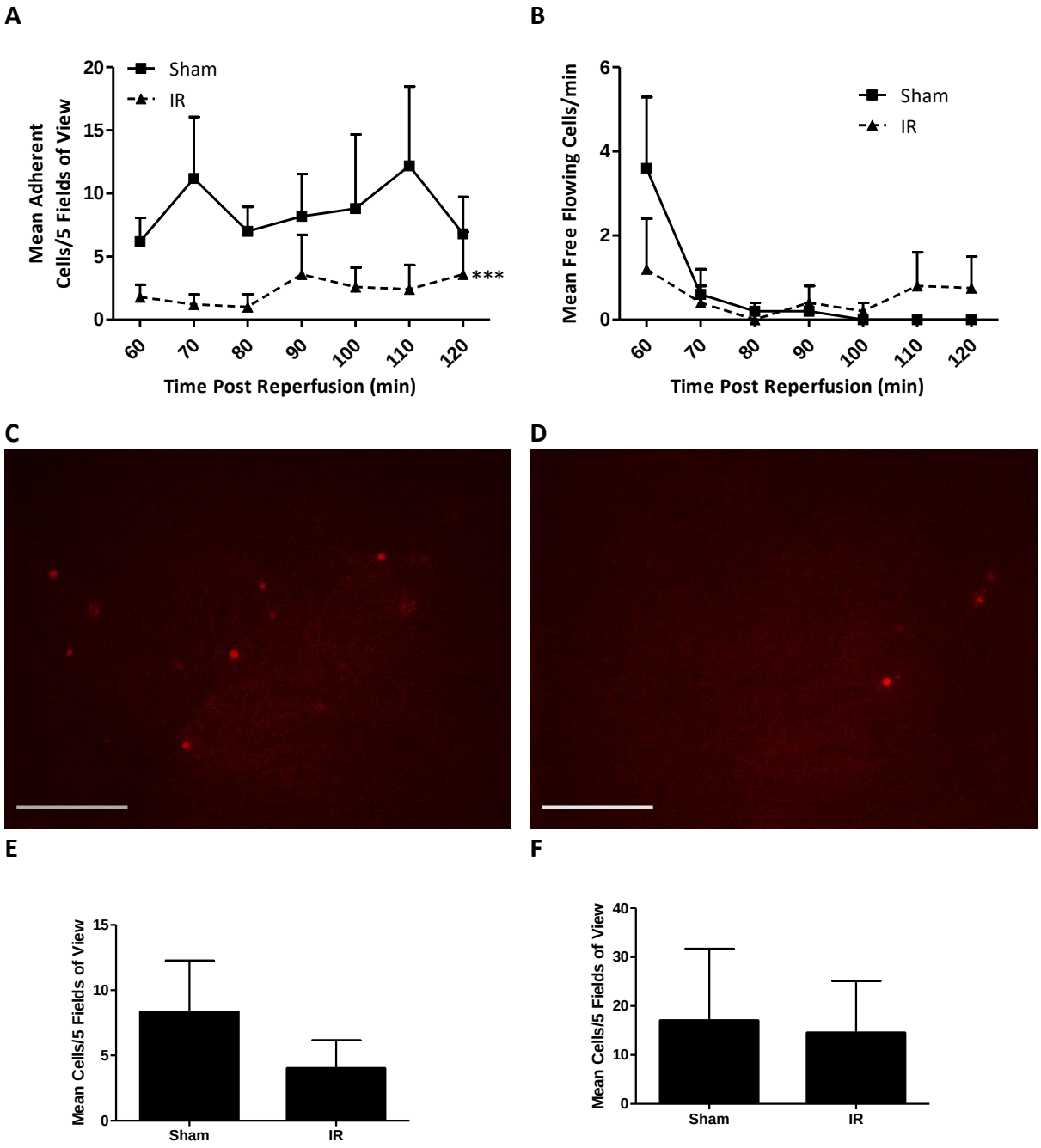


Figure 4:15 Injecting T-cells into the portal vein at 60 minutes post reperfusion results in significantly fewer adherent lymphocytes in the IR injured liver compared to the sham liver. T-cells isolated from the spleens of donor mice were labelled with CMTMR and injected into the portal vein of mice following sham surgery or 90 minutes hepatic ischemia and 60 minutes reperfusion. The trafficking of these cells within the liver was monitored using intravital microscopy. T-cell adhesion (**A**) and free flowing T-cells (**B**) within the liver were quantitated. Images show T-cells (red) in the sham (**C**) and IR injured liver (**D**) at 70 minutes post reperfusion. *Ex vivo* analysis of T-cells in a cross section of the liver (**E**) and the spleen (**F**) between in sham and IR injured animals. Data presented as mean ±SEM, n≥4, ***p<0.001 (two-way ANOVA (A&B) and t-test (E&F)), scale bars=100µm.

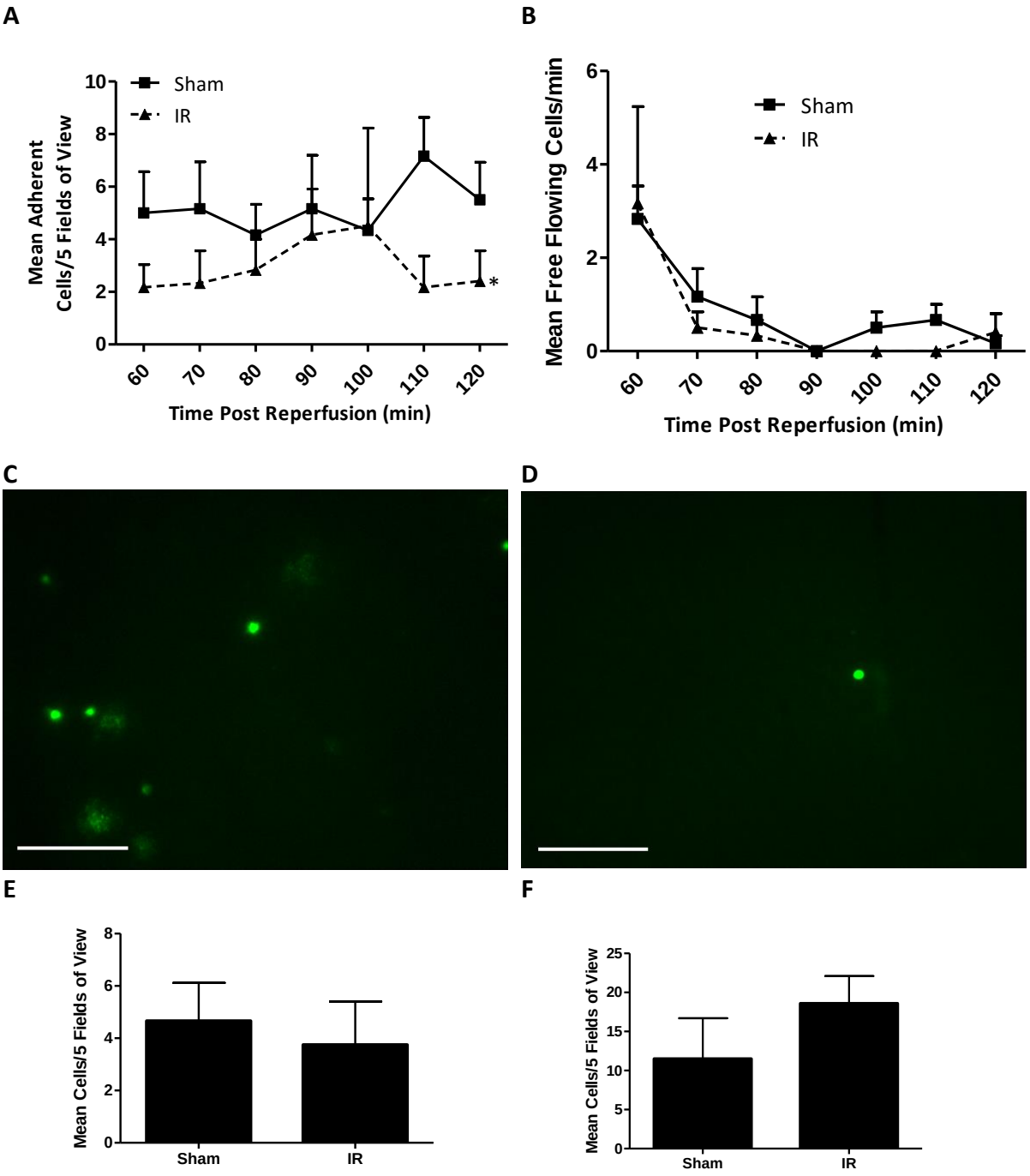


Figure 4:16 Injecting B-cells into the portal vein at 60 minutes post reperfusion results in significantly fewer adherent lymphocytes in the IR injured liver compared to the sham liver. B-cells isolated from the spleens of donor mice were labelled with CFSE and injected into the portal vein of mice following sham surgery or 90 minutes hepatic ischemia and 60 minutes reperfusion. The trafficking of these cells within the liver was monitored using intravital microscopy. B-cell adhesion (**A**) and the number of free flowing B-cells (**B**) in the sham and IR injured livers were quantitated. Images show B-cells (green) in the sham (**C**) and IR injured liver (**D**) at 110 minutes post reperfusion. *Ex vivo* analysis of the number of B-cells in a cross section of the liver (**E**) and the spleen (**F**) in sham and IR injured animals. Data presented as mean ±SEM, n≥4, *p<0.05 (two-way ANOVA (A&B) and t-test (E&F)), scale bars=100µm

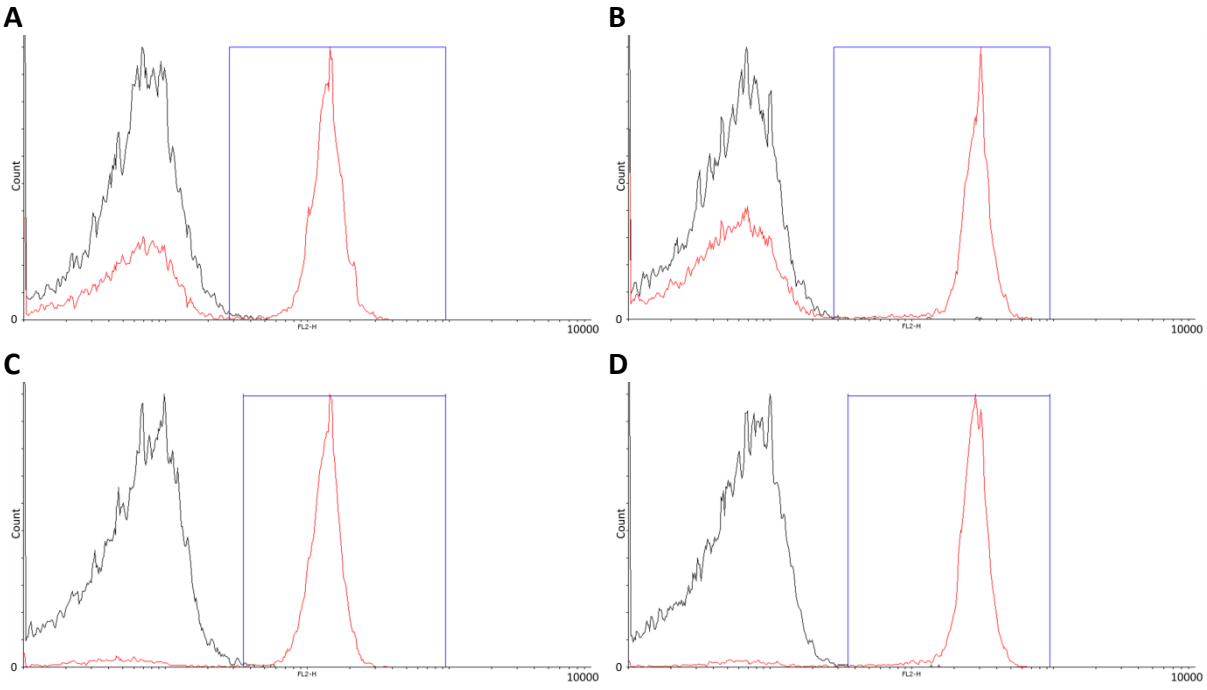


Figure 4:17 CD4 and CD8a expression on T-cells isolated from the mouse spleen using MACS. Flow cytometry was used to determine the percentage of cells expressing CD4 and CD8a from cells isolated from the spleen with several different MACS antibody cocktails. Cells isolated using the pan T-cell isolation kit II were found to be 50±5% CD4+ (**A**) and 42±1% CD8a+ (**B**). Cells isolated with the CD4+ T-cell isolation kit II were found to be >92% CD4+ (**C**) and cells isolated with the CD8a+ T-cell isolation kit II were found to be >93% CD8a+ (**D**). Plots are representative of n=3.

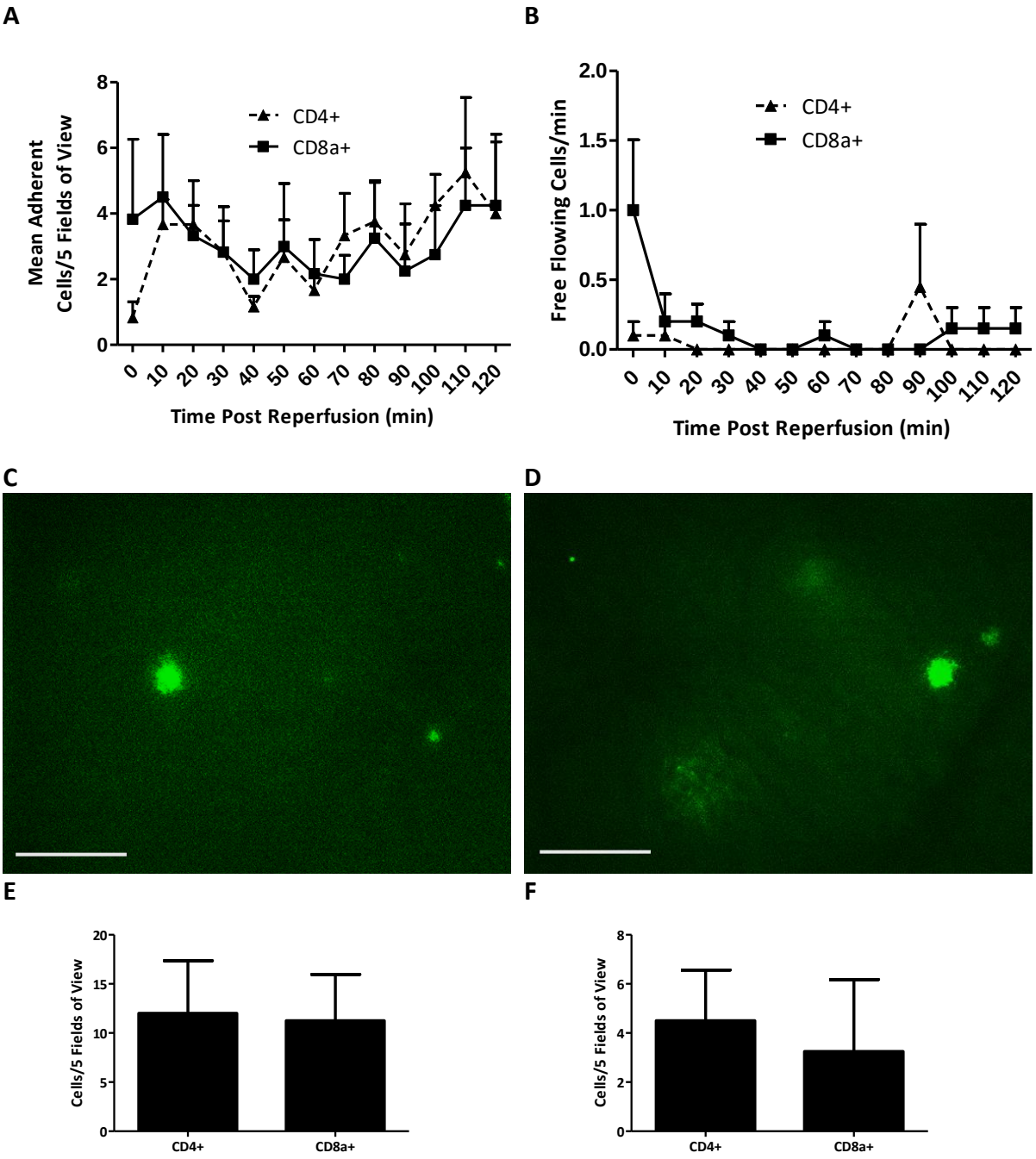


Figure 4:18 CD8+ and CD4+ T-cells are recruited in equal numbers to the IR injured liver. CD4+ and CD8a+ T-cells isolated from the spleens of donor mice were labelled with CFSE or CMTMR and injected into the carotid artery of mice following 90 minutes hepatic ischemia. The trafficking of these cells within the liver was monitored using intravital microscopy. The numbers of adherent CD4+ and CD8a+ T-cells (**A**) and free flowing CD4+ and CD8a+ T-cells (**B**) within the IR injured liver were counted using intravital microscopy. Images show CD4+ (**C**) and CD8a+ (**D**) CFSE labelled T-cells (green) in the IR injured liver at 120 minutes post reperfusion. *Ex vivo* analysis of the number of CD4+ and CD8a+ T-cells in the spleen (**E**) and a cross section of the liver (**F**). Data presented as mean ±SEM, n≥4, (two-way ANOVA (A&B) and t-test (E&F)), scale bars=100µm.

4.3.12. Depletion of Platelets Significantly Reduces Lymphocyte Recruitment to the IR Injured Liver

In order to determine whether platelets play a role in lymphocyte recruitment following hepatic ischemia platelets were depleted using antibodies prior to injury. Fluorescently labelled T-cells and B-cells were then introduced into the circulation and their adhesion within the liver was quantitated. Depletion of platelets was found to significantly reduce both T-cell (*Figure 4.19 A*; $p<0.001$) and B-cell (*Figure 4.20 A*; $p<0.001$) adhesion in the IR injured liver. Depletion of platelets had no significant effect on the number of free flowing T-cells (*Figure 4.19 B*; $p>0.05$) or B-cells (*Figure 4.20 B*; $p>0.05$) within the hepatic vasculature.

4.3.13. Depletion of Platelets has no Significant Effect on T-cell Adhesion within the Sham Liver

In the uninjured liver platelet depletion had no significant effect on adhesion of T-cells (*Figure 4.21 A*; $p>0.05$). Platelet depletion did result in a small significant decrease in B-cell adhesion within the sham liver (*Figure 4.22 A*; $p<0.01$). Depletion of platelets had no significant effect on the number of free flowing T-cells (*Figure 4.21 B*; $p>0.05$) or B-cells (*Figure 4.22 B*; $p>0.05$) within the uninjured hepatic vasculature.

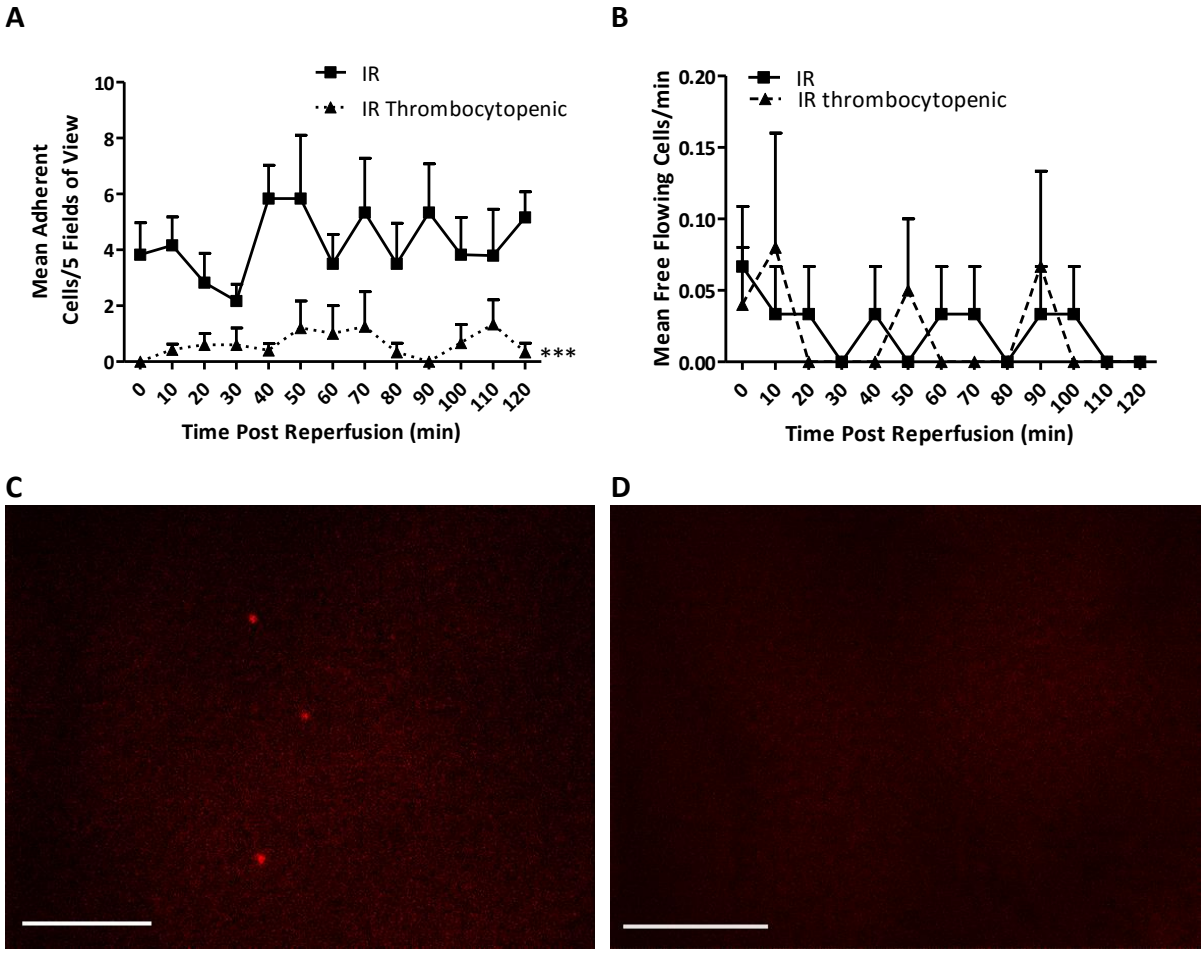


Figure 4:19 T-cell adhesion within the IR liver is significantly reduced following platelet depletion. 0.5 million CMTMR labelled T-cells were introduced via the carotid artery of non-platelet depleted and platelet depleted mice after 90 minutes hepatic ischemia. The trafficking of these cells within the liver was monitored using intravital microscopy. Adherent (**A**) and free flowing (**B**) T-cells within the thrombocytopenic and non-thrombocytopenic IR livers were quantitated. Images show T-cells (red) in the IR injured liver in non-platelet depleted (**C**) and thrombocytopenic mice (**D**) at 40 minutes post reperfusion. Data presented as mean ±SEM, n≥4, ***p<0.001 (two-way ANOVA), scale bars=100µm.

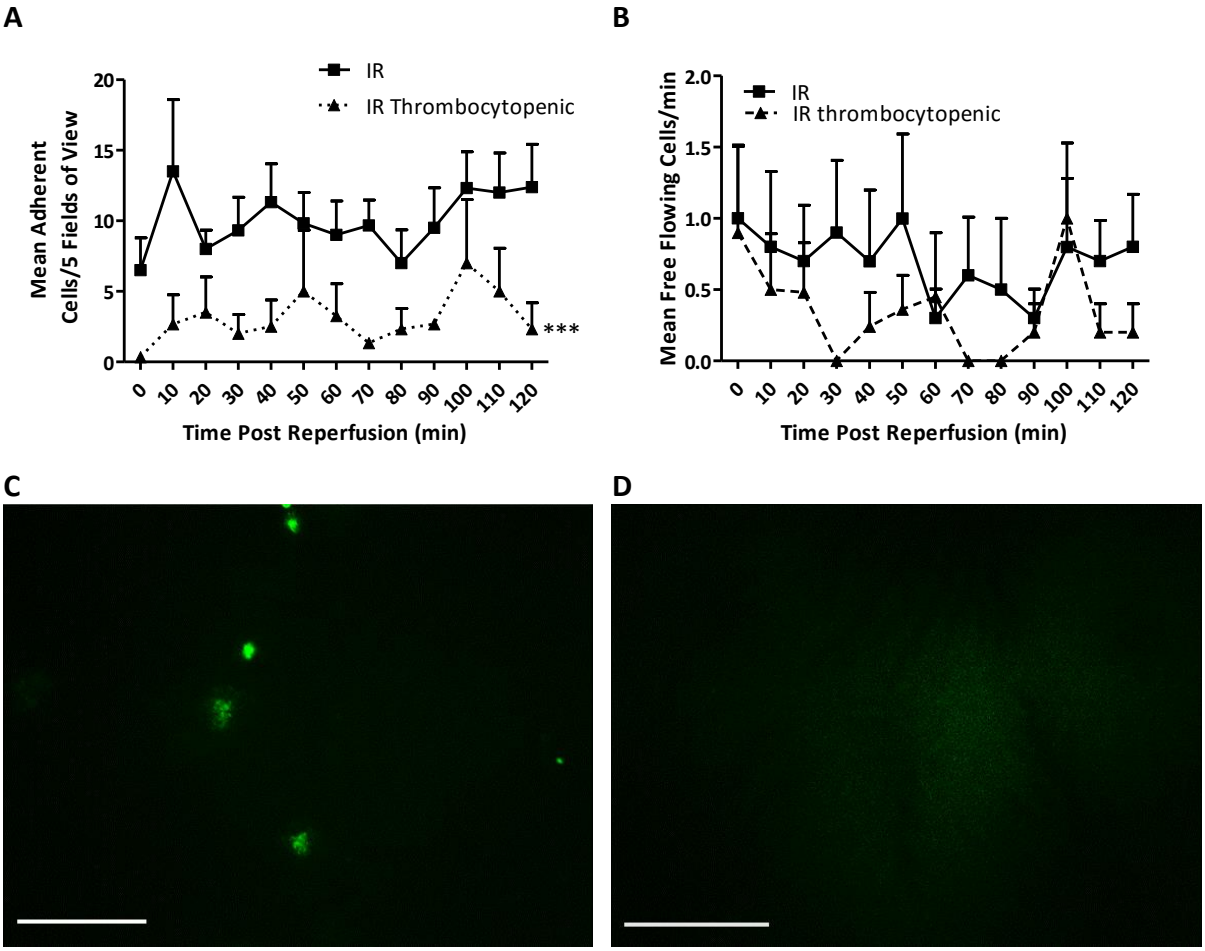


Figure 4:20 B-cell adhesion within the IR liver is significantly reduced following platelet depletion. 0.5 million CFDA-SE labelled B-cells were introduced via the carotid artery of non-platelet depleted and platelet depleted mice after 90 minutes hepatic ischemia. The trafficking of these cells within the liver was monitored using intravital microscopy. Adherent (**A**) and free flowing (**B**) B-cells within the thrombocytopenic and non-thrombocytopenic IR livers were quantitated. Images show B-cells (green) in the IR injured liver in non-platelet depleted (**C**) and thrombocytopenic mice (**D**) at 40 minutes post reperfusion. Data presented as mean ±SEM, n≥4, ***p<0.001 (two-way ANOVA), scale bars=100µm.

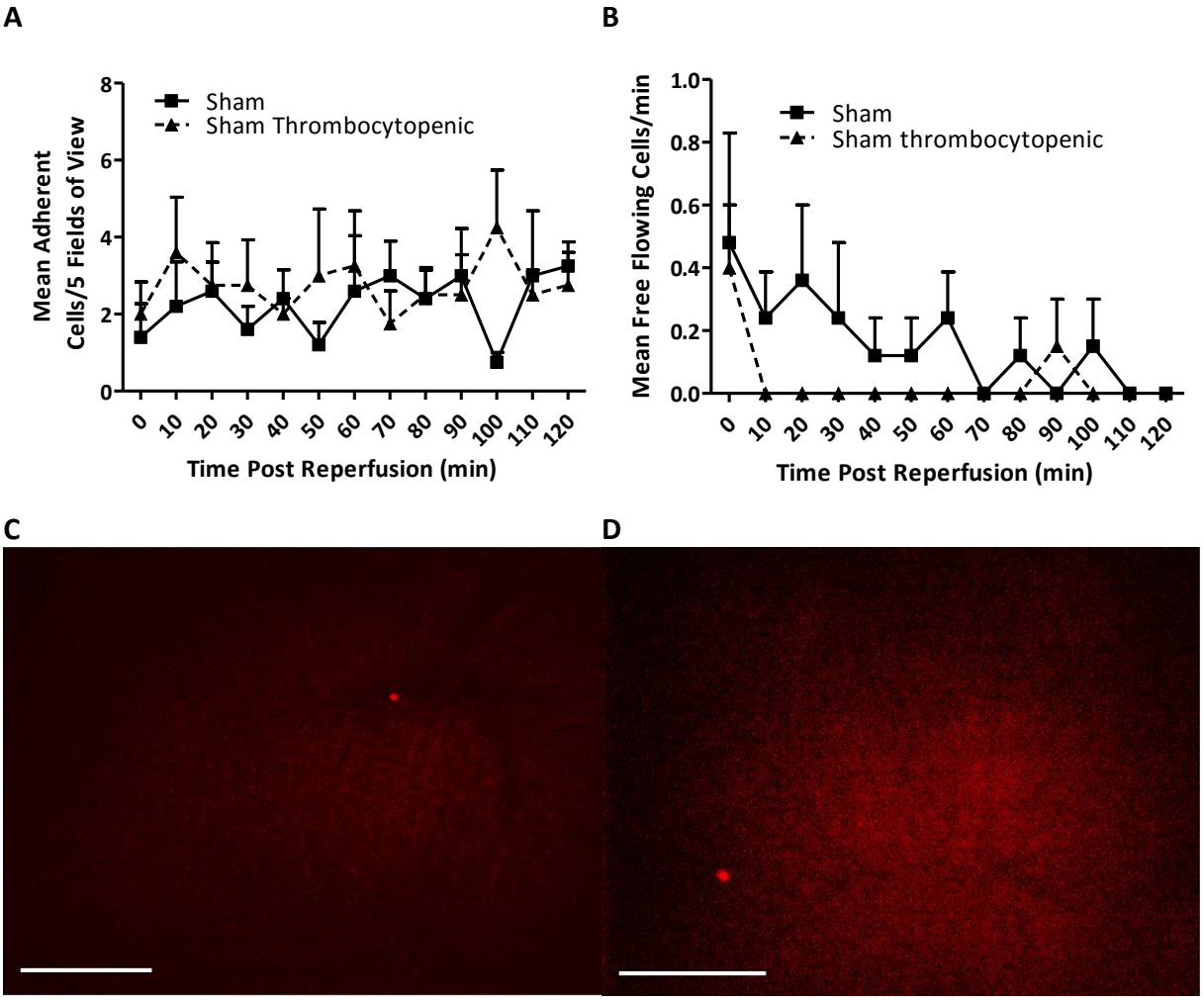


Figure 4:21 Platelet depletion has no significant effect on T-cell adhesion within the sham liver. 0.5 million CMTMR labelled T-cells were introduced via the carotid artery of non-platelet depleted and platelet depleted mice 90 minutes after sham surgery. The trafficking of these cells within the liver was monitored using intravital microscopy. Adherent (**A**) and free flowing (**B**) T-cells within the thrombocytopenic and non-thrombocytopenic sham livers were quantitated. Images show T-cells (red) in the IR injured liver in non-platelet depleted (**C**) and thrombocytopenic mice (**D**) at 40 minutes post reperfusion. Data presented as mean ±SEM, n≥4, (two-way ANOVA), scale bars=100µm.

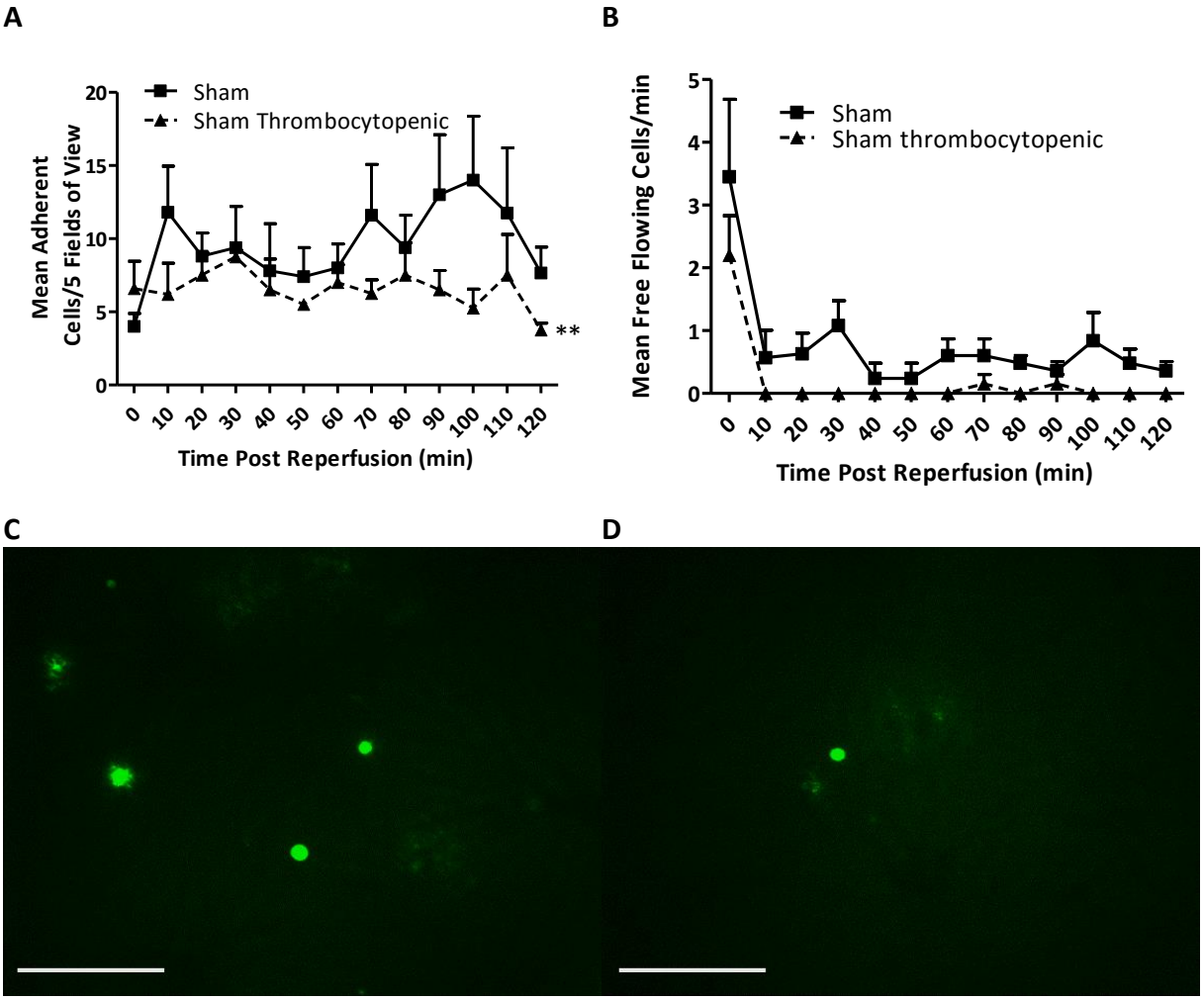


Figure 4:22 Platelet depletion significantly reduces B-cell adhesion within the sham liver. 0.5 million CFDA-SE labelled B-cells were introduced via the carotid artery of non-platelet depleted and platelet depleted mice 90 minutes after sham surgery. The trafficking of these cells within the liver was monitored using intravital microscopy. Adherent (**A**) and free flowing (**B**) B-cells within the thrombocytopenic and non-thrombocytopenic sham livers were quantitated. Images show B-cells (green) in the IR injured liver in non-platelet depleted (**C**) and thrombocytopenic mice (**D**) at 100 minutes post reperfusion. Data presented as mean ±SEM, n≥4, **p<0.01 (two-way ANOVA), scale bars=100µm.

4.3.14. Blocking CD162 (PSGL-1) on T-cells Significantly Reduces their Recruitment to the IR Injured Liver

As depletion of platelets was shown to lead to a significant decrease in lymphocyte adhesion and lymphocytes have previously been shown to adhere to platelet expressed CD62P (Diacovo *et al*., 1996b) experiments were carried out to determine if blocking the CD62P ligand CD162 also lead to a decrease in lymphocyte adhesion. Flow cytometry was used to determine a suitable concentration for blocking all receptors on the surface of T-cells. Increasing the antibody concentrations above 10µg/ml did not result in any increase in antibody binding to T-cell indicating that all C162 receptors were blocked (*Figure 4.23 A*). The B-cells used in our experiments were found not to express CD162 (*Figure 4.23 B*). A concentration of 10µg was used to block T-cells for further experiments. Adhesion within the IR injured liver of T-cell incubated with the CD162 blocking antibody was significantly lower than for T-cells incubated with an IgG control (*Figure 4.23 C* $p<0.01$). However, this decrease in adhesion was far smaller than that observed following platelet depletion. No significant difference was observed in the numbers of free flowing T-cells between those incubated with the blocking antibody and those incubated with the IgG control (*Figure 4.23 D*; $p>0.05$). No significant difference was observed *ex vivo* in the number of T-cells present in the spleen (*Figure 4.23 F*; $p>0.05$) and cross sections of the liver (*Figure 4.23 G*; $p>0.05$) between those incubated with blocking antibody and those incubated with control IgG.

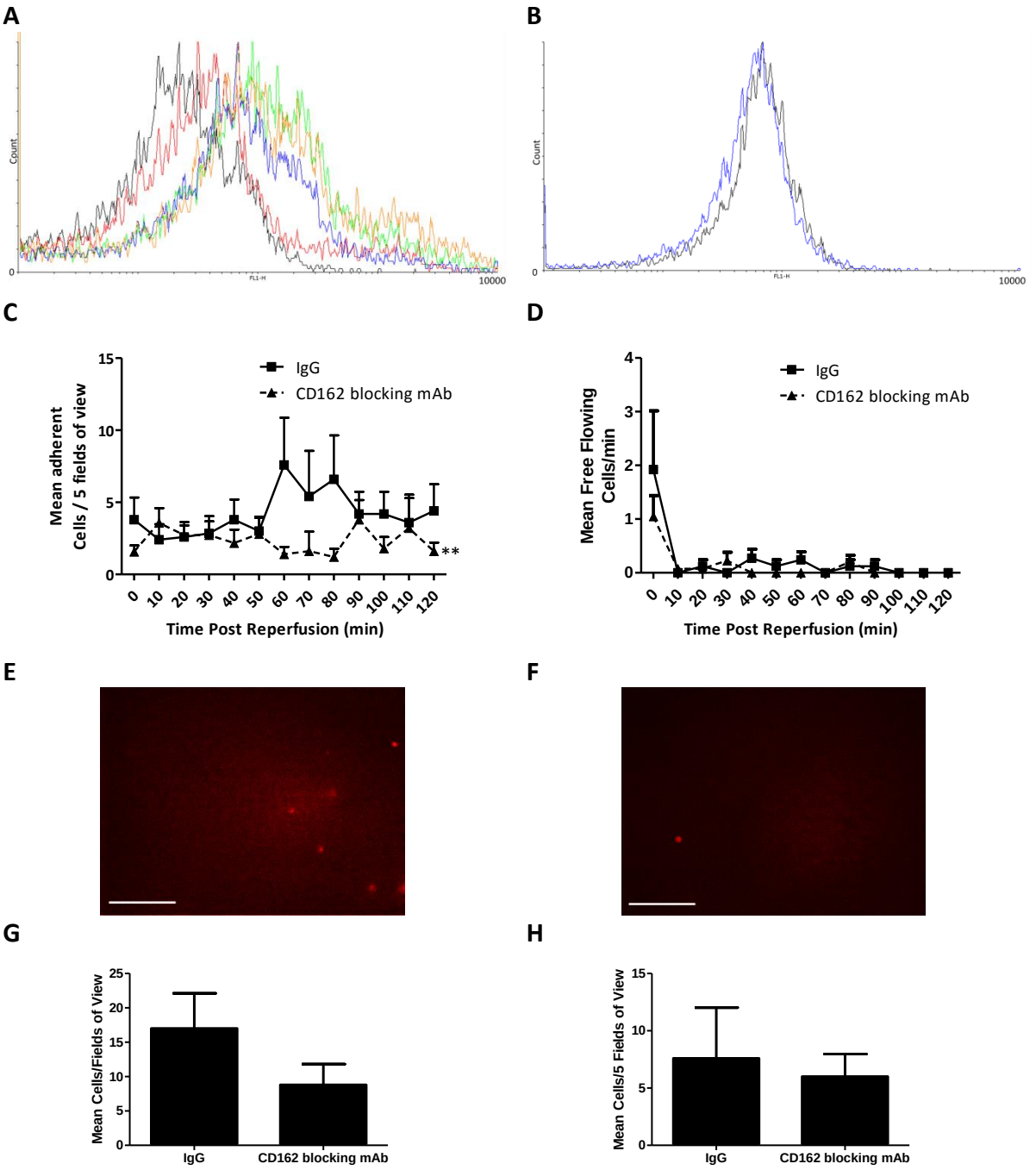


Figure 4:23 Blocking CD162 on T-cells significantly reduces their adhesion within the IR injured liver. (**A**) Representative flow cytometry plots for T-cells incubated with an IgG control (black) and CD162 antibody at concentrations of 5µg/ml (red), 10µg/ml (green), 20µg/ml (blue) and 30µg/ml (orange). (**B**) Representative flow cytometry plots for B-cells incubated with IgG control (black) or CD162 antibody (blue). The effect of incubation with CD162 blocking antibody on T-cells adhesion within the liver compared to the IgG control was determined by intravital microscopy (**C**). The number of free flowing cells following incubation with blocking antibody and control IgG was also quantitated (**D**). Images show T-cells incubated with an IgG control (**E**) and CD162 blocking antibody (**F**) in the liver at 60 minute post reperfusion. *Ex vivo* analysis of T-cells in the spleen (**G**) or a cross section of the liver (H) at 120 minutes post reperfusion following incubation with CD162 blocking antibody or control IgG. Data presented as mean ±SEM, n≥4, **p<0.01 (two-way ANOVA (C&D) and t-test (G&H)), scale bars=100µm.

	Blood Flow as determined by laser Doppler and platelet velocities	***PMP effect on Lymphocyte adhesion***	***Endogenous platelet adhesion***	***Endogenous T-cell adhesion***	***Endogenous B-cell adhesion***	***Donor platelet adhesion***	***Donor T-cell adhesion***	***Donor B-cell adhesion***
Sham livers			Generally seems to be quite high		Generally seems to be higher than endogenous T-cells	Generally seems to be quite high		Generally seems to be higher than donor T-cells
IR injured livers	Laser Doppler suggests overall blood flow within liver is decreased as a result of injury. Platelet velocities suggest the rate of blood flow increases for a short period immediately after reperfusion but decreases with time	No effect on lymphocyte adhesion	No increase compared to sham – not the most sensitive measure of individual platelet adhesion	**↑ (p<0.001) compared to sham**	No increase compared to sham – but numbers much higher than endogenous T-cells	**↑ (p<0.001) compared to sham in areas of blood flow**	**↑ (p<0.001) compared to sham**	No increase compared to sham – but numbers much higher than donor T-cells
Sham Livers in Thrombocytopenic mice							No effect	**↓ (p<0.001) compared to sham mice**
IR injured Livers in Thrombocytopenic mice	No effect on flow which remains poor			**↑ (p<0.001) compared to IR injured mice**	No difference compared to IR injured mice		**↓ (p<0.001) compared to IR injured mice**	**↓ (p<0.001) compared to IR injured mice**

Table 4.1 Summary of results from all *in vivo* studies highlighting platelet, lymphocyte and platelet modified lymphocyte adhesion to sham or IR injured livers *in vivo*. Generally, the presence of platelets within the IR injured increased but, interestingly, was high even in healthy / sham livers. They also played a significant role in the recruitment of lymphocytes, specifically T-cells, to the IR injured liver. Interestingly, platelets could modify basal B-cell presence in healthy and injured livers.

4.4. Discussion

Although there is a large body of evidence to suggest that platelets can interact with various subsets of lymphocytes *in vitro*, there is little evidence to support their cross-talk *in vivo*. Indeed, the data presented in the previous chapter provided clear evidence for platelet modulation of lymphocyte adhesion *in vitro*. The results demonstrated that previously adherent, activated platelets increased lymphocyte adhesion to endothelial counter-ligands. It is therefore possible that platelets may modulate the adhesion of lymphocytes following injury *in vivo*. Hepatic IR injury is an important clinical problem which has been shown to be mediated, in part, by an inflammatory lymphocyte response. The role that platelets may play in mediating lymphocyte recruitment *in vivo* was therefore tested using intravital microscopy in a mouse model of acute hepatic IR injury. The work presented in this chapter provides, for the first time, direct evidence of a role for platelets in the recruitment of lymphocytes to the liver following hepatic IR injury *in vivo*.

Blood flow within the hepatic microcirculation was severely disrupted following ischaemia and subsequent reperfusion. This is a commonly described phenomenon known as 'no-reflow', caused by vasoconstriction and/or cellular aggregation (Cywes *et al*., 1993, Uhlmann *et al*., 2000). The latter compounds the vascular constriction, with the offending cells being congested red blood cells, adherent neutrophils and/or platelet microthrombi. Platelet depletion was found to have no beneficial effect on blood flow within the liver immediately following ischaemia. This seems to correspond with earlier findings that 'no-reflow' within the liver is mainly caused by vascular constriction rather than cellular aggregation (Uhlmann *et al*., 2000). Injection of FITC-BSA, a means of identifying areas of perfusion, revealed areas

of no flow immediately after reperfusion in the IR injured liver. However, laser Doppler measurements showed no overall difference in perfusion between the IR injured and sham livers at the same time point. This discrepancy may be explained by the fact that an increased velocity of blood occurred within the perfused areas as indicated by the increased velocity of free flowing platelets in these areas. Platelet velocity decreased as reperfusion continued, corresponding to the parallel decrease in laser Doppler measurements.

The results in Chapter 3 indicated that PMPs could dramatically increase the adhesion of lymphocytes to endothelial counterligands *in vitro*. However, incubating lymphocytes with PMPs prior to systemic injection did not increase their adhesion to the IR injured liver *in vivo*. PMPs have been identified in the peripheral circulation of patients following various diseases including diabetes, hypertension, arteriosclerosis and sepsis (VanWijk *et al*., 2003). Indeed, a recent study demonstrated that PMPs could be observed in the blood within 30 minutes of reperfusion in mice subjected to hepatic IR injury using a protocol similar to our own model (i.e. 90 mins ischaemia) (Freeman *et al*., 2014). It is possible that some of the adhesion of lymphocytes that was observed following hepatic IR injury was mediated by endogenous circulating PMPs. Therefore, pre-treatment with PMPs may not further increase lymphocyte adhesion – this may explain the results obtained. Another possibility is that incubating lymphocytes with PMPs is only capable of increasing lymphocyte adhesion under static conditions but not under flow conditions where shear stresses are present. Clearly more studies need to be done to investigate whether improving the efficacy of PMP-lymphocyte interactions affects lymphocyte adhesion before completely ruling out a role for PMPs.

Although intravital experiments primarily utilise specific labelled donor cells and monitor their trafficking *in vivo*, this chapter aimed to develop a methodology to visualise the endothelial interactions of the endogenous blood cells of interest. Therefore, fluorescently tagged antibodies to CD3ε and CD19 were injected to visualise endogenous T-cells and B-cells respectively. However, initial experiments using these antibodies demonstrated high levels of non-specific binding within the liver. This non-specific binding was also present when using antibodies previously used successfully by the Kalia group to measure blood cell recruitment to other tissues including the mouse gut and kidney (Holyer, 2010, White, 2014). This implies that non-specific labelling is a liver specific problem. The liver is also known to be one of the main organs involved in simple antibody clearance from the circulation. Indeed Boyle and colleagues demonstrated that following intravenous administration of radiolabelled antibody into rats, 15% of the injected dose was contained within the liver after 2 hours and remained there after 72 hours (Boyle *et al*., 1992). Initially most of this antibody was attached to sinusoidal cells but was taken up by parenchymal cells with time. Interestingly, the rapid hepatic clearance of antibody has been suggested to be a problem requiring consideration in experimental studies of immunotherapy against tumours as it limits the availability of antibody that can bind to the tumour (Sharkey *et al*., 1991). Circulating immune complexes (CICs), comprised of antigen and antibody, are also cleared from the liver. These are produced when an aggressive immune response towards antigen occurs. Human CICs are delivered to the liver sinusoids following their attachment to red blood cells (RBCs). Once within the liver, they detach from the RBCs and are cleared from the circulation by hepatic phagocytes/Kupffer cells. This antibody 'trapping' or clearance role of the liver may explain the high levels of non-specific binding observed within this organ. However, no study has previously provided

intravital images showing such a high non-specific antibody load following infusion within the mouse liver. Given our findings, it was decided that injection of exogenously pre-labelled donor cells was a more appropriate method for tracking cell recruitment within the liver.

The route of delivery used for injection of donor cells greatly affected the results obtained. Injection of cells into the portal vein, as opposed to the carotid artery, delivered them directly to the liver circulation without them having to pass through other tissues first. It was anticipated that this route of delivery would provide more cells to the IR injured liver for adhesion. However, interestingly when cells were injected into the portal vein, fewer adherent T-cells (and B-cells) were observed in the IR injured liver compared to the sham liver. This is in contrast to results obtained from injection of cells into the carotid which showed a small increase in T-cell adhesion in the IR injured liver compared to the sham liver. The manual injection of cells is a relatively 'forceful' mode of delivering cells into the systemic circulation - at the time of entry their velocity was likely higher than that of endogenous circulating cells. However, when cells are introduced via the carotid artery they have an opportunity to adjust their velocity to that of the circulation before they arrive at the liver. Portal injection of cells resulted in large numbers of fast trafficking cells which could be observed intravitally in the liver sinusoids. This may have resulted in a shorter period of time that they were exposed to local (and circulating) inflammatory mediators and could hence explain the reduced adhesion of T-cells in IR injured mice compared to sham mice. In addition to this cell infusion in these experiments was carried out at 60 minutes post reperfusion whereas in experiments using carotid injection, cells were infused immediately after reperfusion. It was hoped that by allowing the injury to develop for longer cellular adhesion would be increased. The later time

point of injection may explain the differing results between the two infusion sites as lower perfusion is observed in the liver 60 minutes after reperfusion compared to immediately after reperfusion. Due to these results we opted to use carotid injection for all experiments.

Following carotid introduction of washed platelets, surprisingly high numbers of adherent platelets were observed within the healthy, sham liver. This was not expected as the Kalia lab (and others) have not previously demonstrated platelets to adhere in such high numbers in sham kidney, small intestine, colon or cremaster muscle vascular beds. It is well known that the reticuloendothelial system, comprising macrophages of the liver and spleen, is the main route for phagocytic removal of damaged or unwanted circulating cells. The liver Kupffer cells are particularly involved in clearing platelets and the presence of Kupffer cells within the sinusoidal blood vessels places them in a prime position to capture circulating platelets. Bearing this in mind, it could be possible that the adherent platelets seen in the sham liver are an artefact of or damaged by the donor platelet washing process. Another possibility is that sham surgery caused damage to the liver leading to platelet activation and adhesion. A similar result was seen in a study by Hoffmeister *et al.* (2003) following injection of washed donor platelets. A significant proportion of the platelets were rapidly cleared from the circulation with the majority of them being found in the healthy mouse liver and the spleen. Interestingly, this study showed that a greater proportion of platelets were found in the liver if platelets were refrigerated prior to injection rather than those stored at room temperature. This study further demonstrated that clearance of platelets by the liver occurred after their ingestion by Kupffer cells, the major phagocytic scavenger cells of the liver. Intravitally they showed platelets colocalised with hepatic sinusoidal Kupffer cells and this colocalisation was greater

with chilled platelets. A later study by the same group showed that following long term refrigeration (>48 hours) platelets are also cleared by hepatocytes (Rumjantseva *et al*., 2009). In contrast, other intravital studies using washed donor platelets have not shown such high numbers of adherent platelets in healthy, sham hepatic sinusoids. Approximately 3-5 Rhodamine-G labelled donor platelets adherent / field of view in sham animals have been noted previously, which is significantly lower than that observed in the current study where approximately 50 adherent platelets / field of view were noted (Pak *et al*., 2010, Ogawa *et al*., 2013, Nakano *et al*., 2008) However, these studies were conducted in rats and the platelets were labelled with Rhodamine 6G, which may explain the discrepancies. In a more recent study conducted in mice, Wong *et al*. (2013) presented intravital images that showed high numbers of endogenous platelets present within the liver sinusoids under basal conditions. Therefore, this phenomenon may be specific to mice.

It was expected that platelet adhesion would increase following hepatic IR injury but this initially did not appear to be the case. This was probably due to the reduced blood flow detected overall in the injured liver as determined by laser Doppler. Also, when platelet adhesion was quantitated in a field of view, in IR livers this area comprised regions of both blood flow and complete stasis. This data was being compared to sham mice in which the whole field of view had blood flow. Therefore, to make comparisons more meaningful, additional analysis was conducted in which platelet adhesion was counted but only in areas of flow. This then demonstrated a significant increase in platelet adhesion between IR and control mice, with almost 100 platelets adherent in a field of view in injured mice at 30 minutes post-reperfusion compared to 50 in sham mice. When free flowing platelets were

analysed in this way, similar numbers were observed between the injured and uninjured liver. In other intravital studies, adhesion of washed platelets was also shown to increase following ischemia. Increases in these studies were more remarkable than the current study which can be explained by the fact that previously published work used a 20 minute ischaemic period which would have resulted in less severely disrupted flow than our 90 minute ischaemic phase (Pak *et al*., 2010, Nakano *et al*., 2008, Ogawa *et al*., 2013). Although we demonstrated an increase in adhesion of donor platelets, which comprised approximately 5% of the circulating platelet population, most likely the actual platelet recruitment was much higher due to adhesion of endogenous platelets too. The role of this significant platelet presence within the injured liver in mediating lymphocyte adhesion was assessed.

Adhesion of both T-cells and B-cells was observed within the sham liver, with presence of the latter being greater. Naïve, memory and activated T-cells are known to continuously migrate from the lymph nodes via the blood and through the liver under normal conditions (Luettig *et al*., 1999). It has also been clearly demonstrated that activated lymphocytes can be cleared by the liver, particularly activated CD8+ T-cells which are selectively retained within normal liver. This event is primarily mediated by CD54 expressed on sinusoidal endothelium and Kupffer cells (Mehal *et al*., 1999). This normal migration to and clearance of lymphocytes by the liver may explain the adhesion of T-cells and B-cells within the sham liver in our experiments. Sham surgery is also likely to result in some minor activatory damage to the liver which may lead to adhesion of lymphocytes. It is interesting that sham liver show high platelet and lymphocyte (particularly B-cell) adhesion. This may also be explained by the unique slow blood velocity found within sinusoidal capillaries which can intermittently stop completely due

to occlusion of the sinusoidal lumen by Kupffer cells. The tortuous structure of the sinusoids may also allow continuous contact between circulating cells and sinusoidal endothelium and thus increase the chances of adhesion. The distribution of adhesion molecules in the sinusoids is also unique as unlike other non-inflamed vessels, liver sinusoids constitutively express abundant ICAM-1 without the need for induction by inflammatory cytokines (Lalor *et al*., 2002b, Panes *et al*., 1995). They also express vascular adhesion protein-1 (VAP-1) which mediates selective adhesion of $CD8^+$ T-cells (Lalor *et al*., 2002b). All of the above increase the likelihood of firm interactions between circulating cells and sinusoidal endothelium even under non-inflamed conditions in the liver.

Following induction of IR injury, a small but nevertheless sustained significant increase in T-cell adhesion was observed. This has previously been reported although all initial experimental studies were immunohistochemical in nature (Zwacka *et al*., 1997). Although the increase in T-cell adhesion in the current study was small, it is worth noting that it was quantitated in a smaller area of the liver as perfused regions were reduced compared to sham. Recruitment was also a rapid response occurring within 1 hour of reperfusion. Indeed, others have shown histologically that lymphocyte infiltration occurs long before any appreciable neutrophil accumulation (Caldwell *et al*., 2005). *In vitro* studies suggested that this increase may not have been mediated by well-known inflammatory mediators including CXCL1, CXCL12 (SDF-1α), CCL5 (RANTES) or H_2O_2 as pre-treatment with them did not increase T-cell adhesion to any of the endothelial cell adhesion molecules tested under static conditions. Interestingly, of these inflammatory factors both CCL5 and CXCL12 are known to be released by platelets (Massberg *et al*., 2006, Kameyoshi *et al*., 1992). No difference in adhesion between CD4+ and CD8a+ T-

cells following hepatic IR injury was identified. This is contrary to the work of Zwacka *et al*. (1997) which showed significant liver infiltration by CD4+ T-cells but very little infiltration by CD8+ T-cells following hepatic IR injury. This discrepancy may be explained by the injection of heparin to prevent clotting in the experiments carried out by Zwacka *et al*. (1997). Also our work used a different mouse strain from the BALB/c mice used in this study. Furthermore, in our experiments CD4+ and CD8a+ cells were injected in equal numbers whereas the circulating T-cells quantitated in the study was Zwacka and colleagues were predominantly CD4+ (Zwacka *et al*., 1997, Chen and Harrison, 2002).

It was not previously know whether B-cells are recruited following hepatic IR injury. However, B-cells are recruited to other tissues such as the kidney and intestine following IR injury (Linfert *et al*., 2009). The results presented in this chapter show that B-cells are not recruited to the IR injured liver in numbers greater than the un-injured liver. However, it is plausible that B-cell recruitment may occur at a later time point as in the kidney B-cell accumulation does not peak until three days post injury (Jang *et al*., 2010). B-cell adhesion may also have been higher if the area of endothelium available for adhesion was higher – this was not the case as blood flow was severely disrupted in the IR livers as evidenced by areas of complete blood flow stasis.

Following platelet depletion remarkable decreases in both T-cell and B-cell adhesion was observed in the IR injured liver. Indeed, T-cell adhesion was lowered to values below that observed basally in sham mice. Iannacone *et al*. (2005) described a similar drop in T-cell recruitment following platelet depletion in a mouse model of hepatitis B infection. Khandoga

et al. (2006a) found that platelets and CD4+ T-cells localise in the post-ischaemic liver. However, this is the first time a role for platelets in recruiting lymphocytes to the IR injured liver has been reported. It is also the first time that a role for platelets in the recruitment of B-cells to liver inflammation has been reported.

Clearly, the decreased lymphocyte presence may have been due to decreased liver injury in the absence of platelets. However, it was surprising that platelet depletion did not decrease injury to the liver as measured chemically by serum ALT activity. Similarly Nocito *et al*. (2007) found that neither platelet depletion nor functional inhibition of platelets with Clopidigrel affected serum aspartate transaminase activity (a marker of liver damage) for 48 hours after 60 minutes partial liver ischaemia. Indeed it appears that in our model, platelet depletion may even increase liver injury as evidenced by a non-significant increase in ALT levels in platelet depleted injured mice compared to injured mice with platelets. This may have been through increased haemorrhage within the liver in the absence of platelets, allowing for greater infiltration of the liver by other populations of inflammatory cells. It may also be due to a reduction in the recruitment of subsets of lymphocytes which may play anti-inflammatory or protective roles during IR injury. For example, Caldwell and colleagues demonstrated histologically that $CD4^{-/-}$ mice, lacking $CD4^{+}$ T lymphocytes and a deficiency in helper T-cells, exhibited increased liver injury (raised ALT levels; widespread hemorrhagic necrosis, parenchymal cell destruction) despite reduced neutrophil recruitment (Caldwell *et al*., 2005). They showed that this was possibly due to the fact that neutrophils from $CD4^{-/-}$ mice had a higher oxidative burst activity.

To assess whether adherent platelets recruited lymphocytes through physically interacting with them and thus bridging them to the endothelium, T-cells were pre-treated with an anti-CD162 blocking antibody prior to injection. This showed that T-cell recruitment appeared to be partially governed by interactions between CD62P the counter-receptor for lymphocyte CD162. As platelets within the IR injured liver express P-selectin (Esch *et al*., 2010), and direct platelet-T-cell interactions are mediated by CD62P (Diacovo *et al*., 1996b, Sheikh *et al*., 2004), it is plausible that physical interactions between the two cells are part of the mechanism by which platelets recruit T-cells to the IR injured liver. Although vascular endothelial cells express CD62P, sinusoidal endothelial cells do not, therefore endothelial cell expressed CD62P is unlikely to play a role in lymphocyte recruitment in our model (Lalor *et al*., 2002b). A role for CD62P has previously been shown in neutrophil infiltration of the IR injured liver and blocking of CD62P results in a reduction in liver damage (Dulkanchainun *et al*., 1998, Garcia-Criado *et al*., 1995). The reduction in adhesion was not as large as that observed following platelet depletion and the B-cells used in our experiments did not express CD162 at all which suggests other mechanisms such as platelet released cytokines may be involved in lymphocyte recruitment. Nocito *et al*. (2007) found that platelet depletion, but not the anti-platelet drug Clopidigrel, reduced the infiltration of neutrophils in the IR injured liver. As Clopidigrel did not affect the expression of pro-inflammatory mediators they concluded from these results that platelet released cytokines were required for neutrophil recruitment.

Interestingly platelet depletion did not affect T-cell adhesion within the un-injured liver but did decrease B-cell adhesion – albeit this was a far smaller reduction than that seen with T-cells in the injured liver. These results imply that the adhesion of lymphocytes within injured

and un-injured liver relies on different mechanisms, being either partially platelet dependent or completely platelet independent. It remains unclear why basal B-cell presence within the liver is somewhat platelet dependent, but in light of our data highlighting a significant platelet presence in sham livers, it may not be so surprising.

The data presented in this chapter have shown for the first time a key role for platelets as mediators of lymphocyte recruitment following hepatic IR injury. In order to determine if the importance of platelets in lymphocyte recruitment is conserved across other injury models, the next chapter will investigate the role of platelets in lymphocyte recruitment following ConA induced liver injury. This injury model is well accepted to be lymphocyte dependent and it is anticipated that a greater lymphocyte recruitment will be induced than that seen following IR injury. This will hopefully allow us to determine whether the ability of platelets to modify lymphocyte recruitment varies depending on the severity of lymphocyte involvement.

CHAPTER 5

LYMPHOCYTE RECRUITMENT TO THE LIVER DURING CONCANAVALIN A INDUCED INJURY CAN BE MODULATED BY PLATELETS

5. LYMPHOCYTE RECRUITMENT TO THE LIVER DURING CONCANAVALIN A INDUCED INJURY CAN BE MODULATED BY PLATELETS

5.1. Introduction and Hypotheses

Concanavalin A (ConA) is a plant lectin from jack bean (*Canaualia ensiformis*), known to activate T lymphocytes *in vitro.* ConA induces liver specific injury in mice and is widely used as a reliable experimental model of acute T-cell mediated diseases on the hepatitis spectrum, including auto-immune hepatitis, viral hepatitis and fulminant hepatitis (Trautwein *et al*., 1998, Ajuebor *et al*., 2005, Diao *et al*., 2004). The cells involved in mediating the injury are primarily $CD4^+$ T-cells that migrate specifically to the liver within hours of exposure, but NK T-cells have also been implicated in the injury (Kaneko *et al*., 2000, Toyabe *et al*., 1997) as have neutrophils (Bonder *et al*., 2004) and Kupffer cells (Schumann *et al*., 2000). These cells have been shown to play roles in a number of human liver diseases, including autoimmune, viral, alcoholic, and IR injury. After recruitment, T-cells release high levels of pro-inflammatory cytokines that induce the development of the hepatic lesions. Indeed, *in vitro* it has been reported that lymphocytes are capable of exerting direct cytotoxicity against ConA–treated hepatocytes.

Tiegs and colleagues were the first to describe this experimental model of liver injury (Tiegs *et al*., 1992). In their seminal study, ConA resulted in T-cell stimulation and particularly IL-2 release *in vivo*. The resultant liver injury could be prevented by immunosuppressive drugs, such as cyclosporin A (FK506). The lymphocyte pathology of this injury was further confirmed by the fact that immunodeficient mice (SCID or athymic nude mice) failed to develop hepatitis

on ConA challenge, with $CD4^+$ T-cells identified as the major effector cell in this model. Many studies thereafter have demonstrated that ConA causes systemic release of cytokines such as TNFαα, GM-CSF, IL-2, and IFN*y.*

Hokari and colleagues demonstrated that activation of T-cells with ConA for 48 hrs prior to their injection led to their highly significant retention within the healthy rat liver (Hokari *et al*., 1999). They further demonstrated that interactions between CD11a/CD18 and CD54 accounted for the activated lymphocyte accumulation in hepatic sinusoids. Since reduced accumulation within the intestinal Peyer's patches and the spleen was observed, the authors concluded that ConA activation diminished lymphocyte entry into lymphoid tissue but enhanced their transit through non-lymphoid sites such as the liver. This makes this a useful experimental model to examine the role that platelets may play in mediating lymphocyte recruitment to the liver.

The work presented in the previous chapters indicates that platelets are important mediators of lymphocyte recruitment both *in vitro* and in a murine hepatic IR injury model. Accumulation of platelets within the hepatic microvasculature has been previously observed during ConA induced liver injury (Massaguer et al., 2002). It is therefore possible that these platelets play a role in the recruitment of lymphocytes to the liver during ConA injury. In this current chapter, data is presented comparing platelet and lymphocyte adhesion in the hepatic microvasculature following both ConA and vehicle control treatment. Further to this, data comparing lymphocyte recruitment in the hepatic microcirculation in both normal and platelet depleted mice is also presented.

For the work included in this chapter, it was hypothesised that:

- Lymphocyte recruitment to the liver is increased following ConA injury.
- CD4+ but not CD8+ T-cells are recruited to the liver following ConA injury.
- Lymphocyte recruitment is significantly reduced following platelet depletion.
- T-cell recruitment to the liver following ConA injury is dependent on lymphocyte expressed CD162.

5.2. Methods

The methods used in this chapter are described in detail in chapter 2. Briefly, injury was induced by introduction of ConA via a carotid artery cannula at a dose of 20mg/kg; injury was then allowed to develop for 2-4 hours prior to intravital observations. T-cells and B-cells were isolated from the spleen of donor mice using MACS and platelets were isolated from the whole blood of donor mice through centrifugation. These cells were then labelled using fluorescent dyes and their trafficking within the liver was monitored using intravital microscopy. Some experiments used purified CD4+ and CD8+ T-cells which were isolated from the spleens of donor mice using MACS. To investigate if platelets play a role in lymphocyte recruitment platelets were depleted prior to injury by injection of a platelet depleting CD42b antibodies. In some experiment CD162 on the surface of T-cells was blocked by incubation with a blocking antibody for 20 minutes.

Blood flow within the liver was measured using laser Doppler. FITC-BSA was injected to visualise areas with flow and the velocities of free flowing platelets were used as a measure

of rate of flow. Blood samples were taken at the end of experiments in order to conduct blood counts. Serum samples were isolated at the end of experiments in order to measure the level of injury through analysis of serum ALT activity.

5.3. Results

5.3.1. Markers of Liver Injury are Increased Following ConA Administration

Within 2 hours of ConA injection the liver was visibly inflamed and sometimes appeared darkened with a spotty haemorrhagic appearance due to vascular congestion (*Figure 5.1 A*). The spleen also became strikingly enlarged and darker in colour (*Figure 5.1 B*). 4 hours after the injection of ConA there were significant reductions in the numbers of circulating erythrocytes ($p<0.001$) and platelets ($p<0.001$) and in haematocrit ($p<0.001$) compared to the vehicle controls (*Figure 5.2 A*). Treatment with ConA also resulted in a decrease in circulating leukocytes although this difference did not reach significance (*Figure 5.2 A*; $p>0.05$). Treatment with ConA significantly increased the proportion of circulating leukocytes made up by basophils ($p<0.05$) but did not significantly alter the proportions of other leukocyte subsets (*Figure 5.2 A*; $p>0.05$). A significant increase in mean platelet volume was also observed (*Figure 5.2 A*; $p<0.01$).

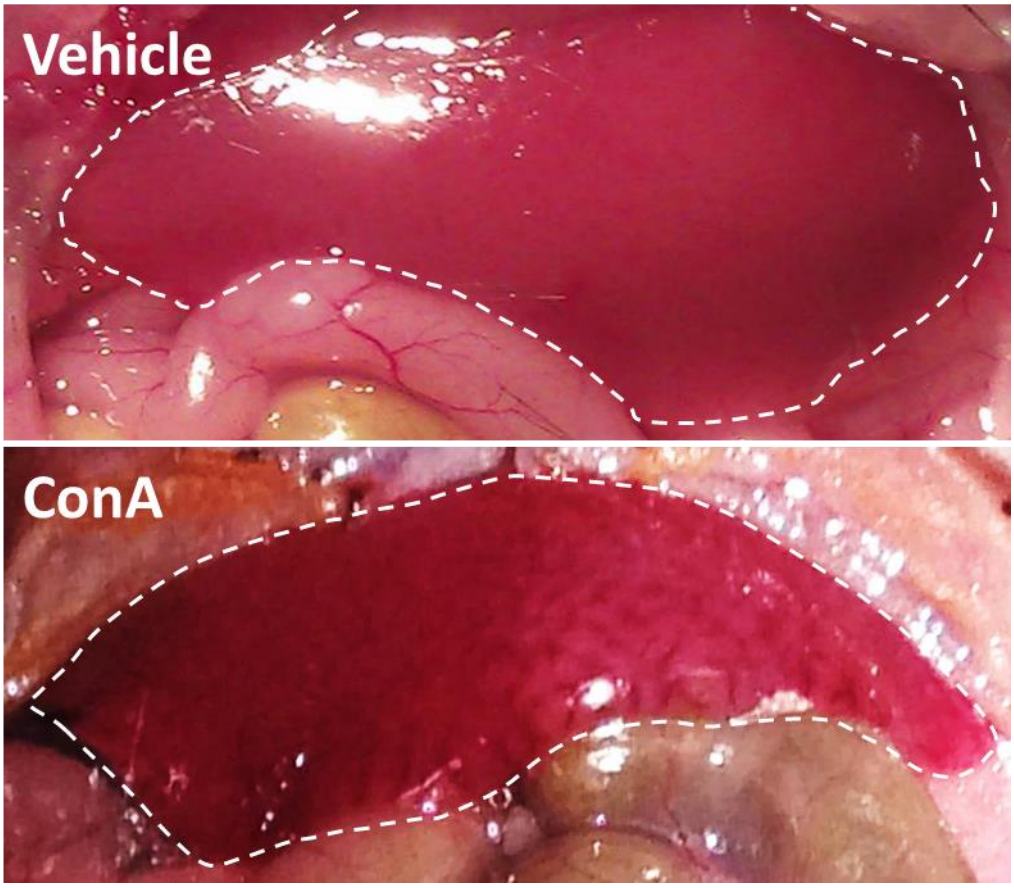
Similar results were observed in platelet depleted mice treated with ConA. Haematocrit and the number of circulating erythrocytes were both significantly reduced compared to the vehicle control (*Figure 5.2 A*; $p<0.05$). There were also significant increases in the proportions of circulating leukocytes made up by monocytes and basophils (*Figure 5.2 A*; $p<0.05$). Blood

counts for platelet depleted ConA mice did not significantly differ from blood counts of non-platelet depleted ConA mice (*Figure 5.2 A*; $p>0.05$).

Survival of ConA injured mice was significantly lower than that of mice treated with a vehicle control (*Figure 5.2 B*; $p<0.05$). For example, 6 hours after ConA administration only 14% of mice survived. Survival of thrombocytopenic ConA treated mice was also significantly reduced compared to those treated with a vehicle control (*Figure 5.2 B*; $p<0.05$). Although platelet depletion did decrease the survival of ConA injured mice this decrease did not reach significance (*Figure 5.2 B*; $p>0.05$).

4 hours after ConA treatment serum ALT activity was significantly increased in both normal (*Figure 5.2 C*; $p<0.05$) and platelet depleted mice (*Figure 5.2 C*; $p<0.05$). Platelet depletion did result in increased serum ALT activity 4 hours after ConA administration; however, this increase was not significant (*Figure 5.2 C*; $p>0.05$).

A



B

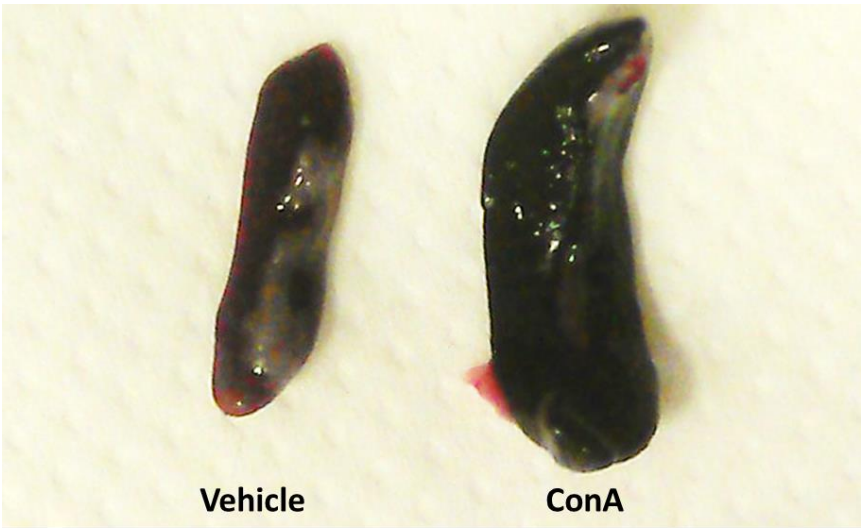


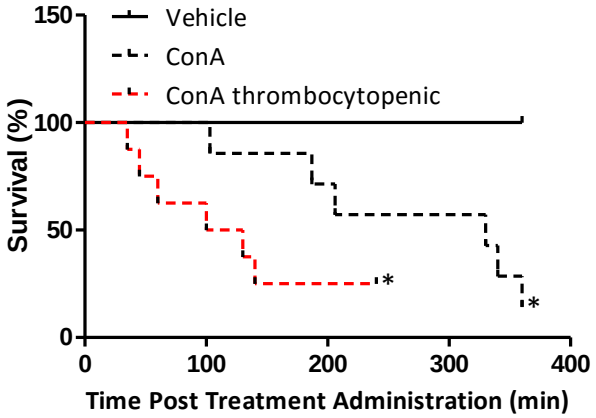
Figure 5:1 Administration of ConA results in liver inflammation and splenomegaly. 2 hours after administration of a 20mg/kg dose of ConA the liver is visibly inflamed compared that of an animal administered with a vehicle control (**A**, liver outlined in white). The spleen of an animal 2 hours after ConA administration is greater in size and darker in colour than that of a control animal (**B**).

A

	RBC (10^6/mm^3)	*PLT (10^3/mm^3)*	*WBC (10^3/mm^3)*	*HCT (%)*	*MPV (µm^3)*	*LYM%*	*MON%*	*NEU%*	*EOS%*	*BAS%*
Vehicle	8.7±0.1	793±29	2.8±0.4	35.5±0.1	5.65±0.1	86.7±0.3	2.7±0.7	10.0±2.7	0.2±0.1	0.3±0.1
ConA	6.6±0.1 ***	144±22 ***	1.8±0.3	25.2±0.7 ***	6.4±0.2 **	69.3±7.8	3.7±1.0	22.9±6.9	0.3±0.0	3.8±1.5 *
Thrombocytopenic ConA	4.7±1.4 *	ND	1.8±0.4	17.8±5.3 *	ND	75.2±1.2	11.4±3.0 *	12.0±1.2	0.4±0.16	1.1±0.2 *

RBC=red blood cells, **PLT**=platelets, **WBC**=white blood cells, **HCT**=haematocrit, **MPV**=mean platelet volume, **LYM**=lymphocytes, **MON**=monocytes, **NEU**=neutrophils, **EOS**=eosinophils, **BAS**=basophils, **ND**=not detected.

B



C

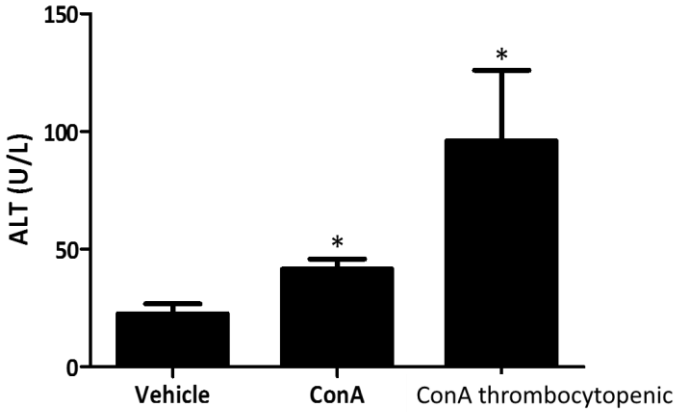


Figure 5:2 ConA treatment significantly increased serum ALT activity and reduced survival. Blood counts of mice 4 hours after vehicle or ConA treatment for both non-platelet depleted and platelet depleted mice (**A**). Survival of mice following vehicle treatment or ConA treatment for both non-platelet depleted and platelet depleted mice (**B**). Alanine transferase activity in serum samples 4 hours after vehicle or ConA treatment for both non-platelet depleted and platelet depleted mice (**C**). Data presented a mean ±SEM, n≥3, *p<0.05 **p<0.01 ***p<0.001 compared to vehicle (one-way ANOVA with Bonferroni post-tests (A&C) and Mantel Cox tests with Bonferroni correction (B))

5.3.2. Hepatic Blood Flow is Disrupted in ConA Induced Liver Injury

Injection of FITC-BSA at 4 hours post-ConA treatment indicated that some areas of the liver were not perfused with blood (*Figure 5.3 B*). However, perfusion of the liver, as measured by laser Doppler, did not significantly differ between the ConA injured liver and uninjured controls at 4 hours following treatment administration (*Figure 5.3 C*; $p>0.05$). Using the velocity of platelets as a measure of flow rate, it was observed that the rate of flow within the ConA injured liver was significantly greater than in control livers (*Figure 5.3 D*; $p<0.001$). Platelet depletion had no significant effect on blood flow within the ConA injured liver at 2 hours post ConA injection (*Figure 5.3 E*; $p>0.05$). Cellular adhesion within the ConA injured liver was only observed within areas of perfusion (*Figure 5.3 F*)

5.3.3. Platelets Injected at 2 Hours Post Treatment Adhere in Significantly Lower Numbers within the Livers of Mice Injected with ConA Compared to Vehicle Control

In order to track platelet recruitment to the ConA injured liver CFDA-SE labelled donor platelets were injected 2 hours after ConA or vehicle control administration. Platelet adhesion was found to be significantly reduced following ConA treatment compared to the control (*Figure 5.4 A*; $p<0.001$). However, towards the end of the experiment the numbers of adherent platelets in ConA injured and control livers were almost equal (*Figure 5.4 A*). In *ex vivo* cross sections of the liver there were fewer platelets following ConA than vehicle control treatment although this difference was not significant (*Figure 5.4 B*; $p>0.05$). Due to their high speed it was not possible to accurately assess the number of free flowing platelets in the ConA injured liver.

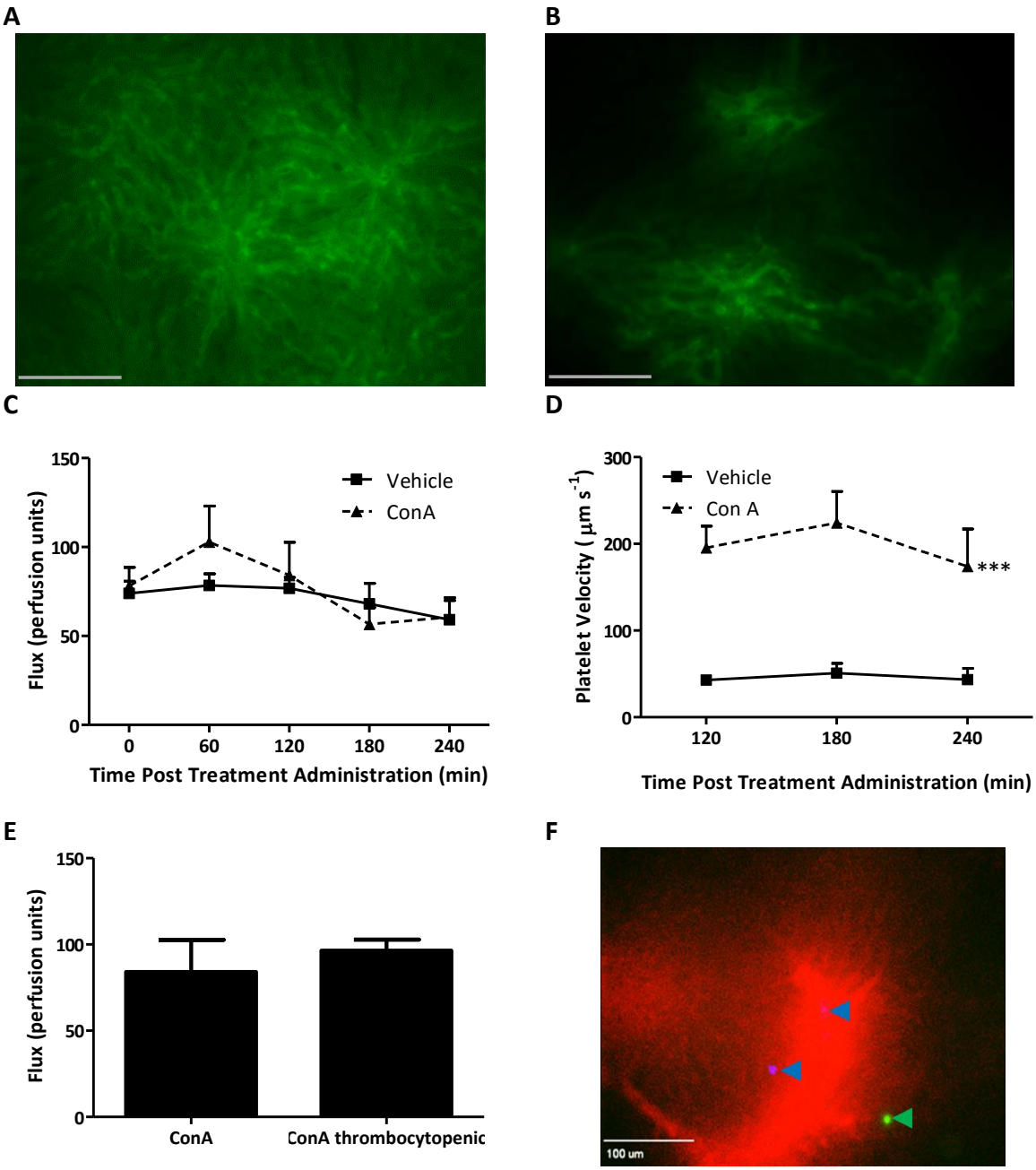


Figure 5:3 Following ConA administration blood flow within the liver is disrupted. 4 hours after administration of a 20mg/kg dose of ConA or a vehicle control FITC-BSA was injected into the circulation and the liver was visualised using intravital microscopy. In the control liver FITC-BSA entered all areas of the tissue (**A**), following ConA treatment FITC-BSA did not enter large areas of the tissue (**B**; dark areas). Laser Doppler was used to measure perfusion of the liver in animals given ConA or a vehicle control (**C**). Velocities of CFDA-SE labelled platelets in livers of mice treated with ConA or vehicle control were determined by intravital microscopy (**D**). Laser Doppler measurements for the ConA injured liver at 120 minutes post administration in throbocytopenic and non-thrombocytopenic mice. T-cells (green) and B-cells (blue) were only observed to adhere in areas of flow – highlighted by Alexa-674 BSA (red) – following ConA administration (**F**). Data presented a mean ±SEM, n≥3, ***p<0.001 (two-way ANOVA) scale bars=100µm.

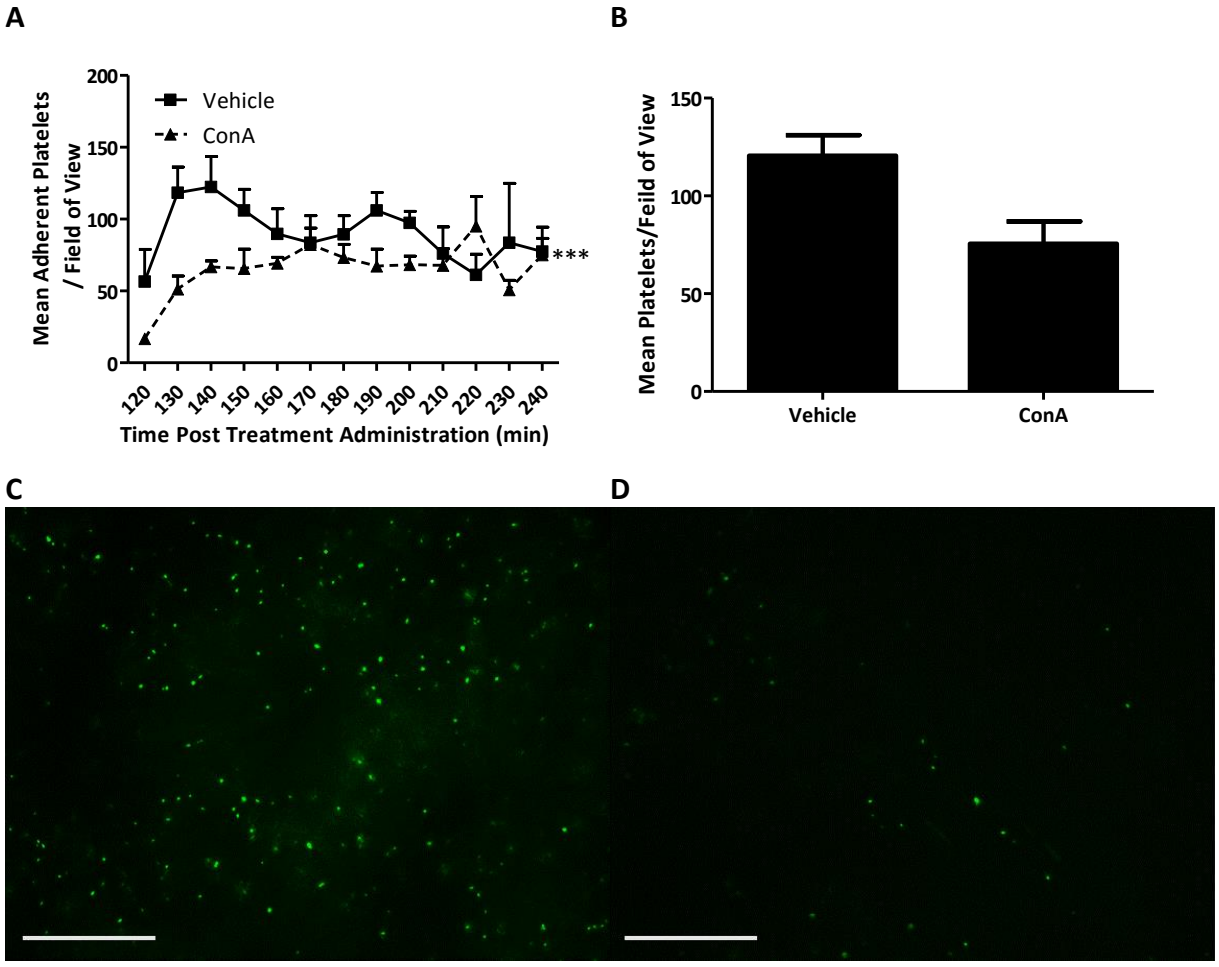


Figure 5:4 Platelets injected at 2 hours post treatment adhere in significantly lower numbers within the livers of mice injected with ConA compared to vehicle control. $1x10^8$ CFDA-SE stained platelets were injected into the carotid artery of mice 2 hours after administration of ConA or a vehicle control. Using intravital microscopy the number of adherent platelets within the hepatic microcirculation was monitored in the ConA injured liver and vehicle control treated liver (**A**). *Ex vivo* analysis of the number of platelets in a cross section of the liver 4 hours after treatment administration (**B**). Images show platelets (green) in the control liver (**C**) and the ConA injured liver (**D**) at 130 minutes post treatment administration. Data presented a mean ±SEM, n≥3, ***p<0.001 (two-way ANOVA (A) and t-test (B)) scale bars=100µm.

5.3.4. There is no Significant Difference in Adhesion of T-cells Injected 2 Hours Post Treatment between the Control and ConA Injured Liver

In order to determine whether ConA injury leads to lymphocyte recruitment the trafficking of CMTMR stained T-cells and CFDA-SE stained B-cells within ConA injured and control livers was tracked using intravital microscopy. When T-cells were injected at 2 hours post-treatment administration there was no significant difference in the adhesion between ConA injured livers and control livers (*Figure 5.5 A*; $p>0.05$). ConA injury also had no significant effect on the number of free flowing cells within the liver compared to control (*Figure 5.5 B*; $p>0.05$). The number of free flowing T-cells rapidly decreased within the first 10 minutes after cell injection. There was no significant difference between ConA injured and control livers in the numbers of T-cells in *ex vivo* liver cross sections taken at 4 hours post treatment administration (*Figure 5.5 E*; $p>0.05$).

Similar results were also obtained with B-cells. There was no significant difference found in the numbers of adherent B-cells between ConA injured and control livers (*Figure 5.6 A*; $p>0.05$). No significant difference was observed in the number of free flowing B-cells between the ConA injured and control livers (*Figure 5.5 B*; $p>0.05$). Similarly to T-cells, the number of free flowing B-cells rapidly decreased within the first 10 minutes following cell injection. There was no significant difference in the numbers of B-cells observed in *ex* vivo liver cross sections between the ConA injured and control livers (*Figure 5.6 E*; $p>0.05$), however the number of B-cells was lower in the ConA injured livers.

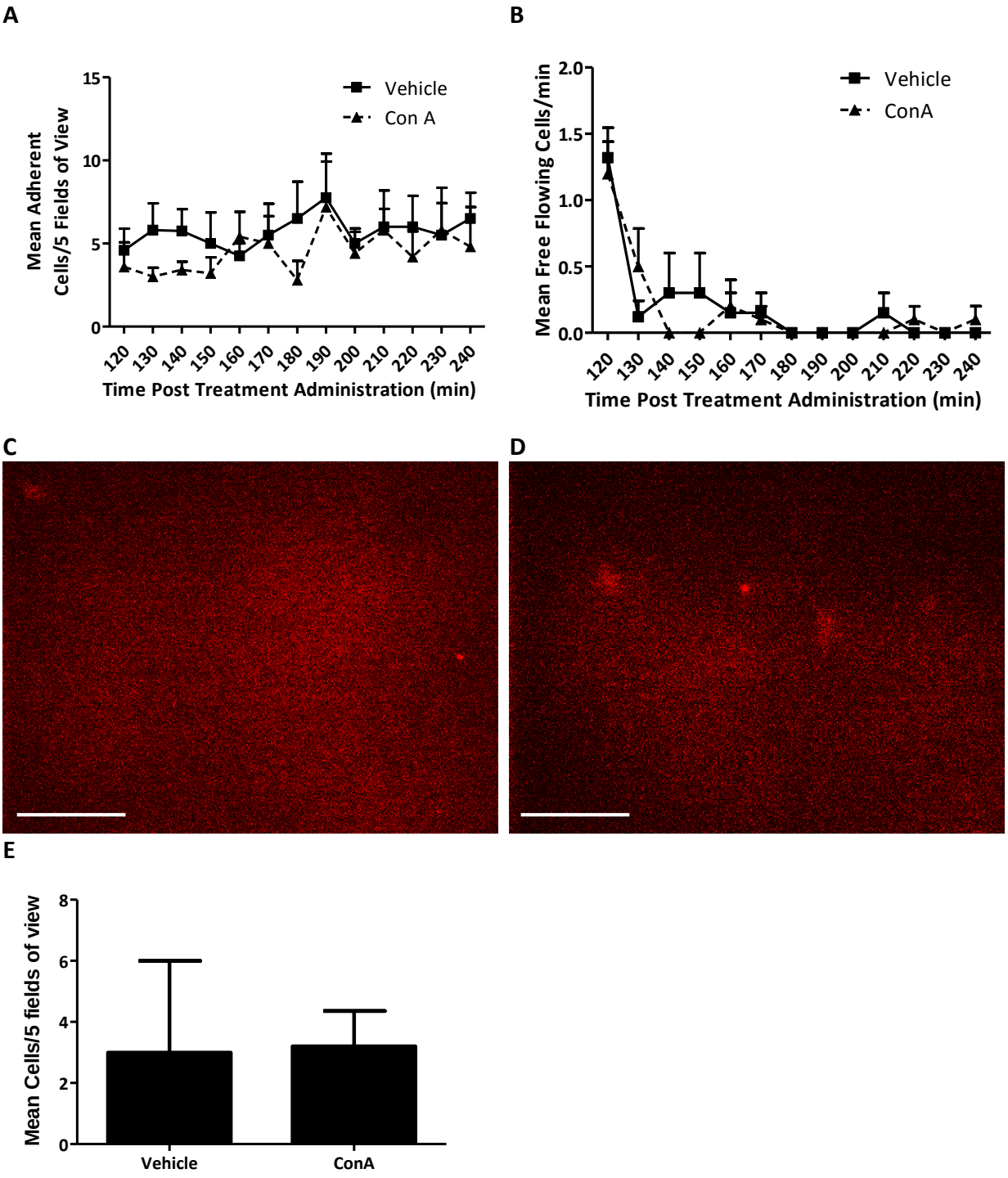


Figure 5:5 There is no significant difference in adhesion of T-cells injected 2 hours post treatment between the control and ConA injured liver. $0.5x10^6$ CMTMR stained T-cells were injected into the carotid artery of mice 2 hours after administration of ConA or a vehicle control. Intravital microscopy was used to monitor their trafficking within the liver. Numbers of adherent cells (**A**) and free flowing cells (**B**) in control and ConA injured livers. Images show T-cells (red) in the control liver (**C**) and the ConA injured liver (**D**) at 240 minutes post treatment administration. Numbers of T-cells in cross sections of the liver taken *ex vivo* 4 hours after treatment administration (**E**). Data presented a mean ±SEM, n≥3, (two-way ANOVA (A&B) and t-test (E)) scale bars=100µm.

A B

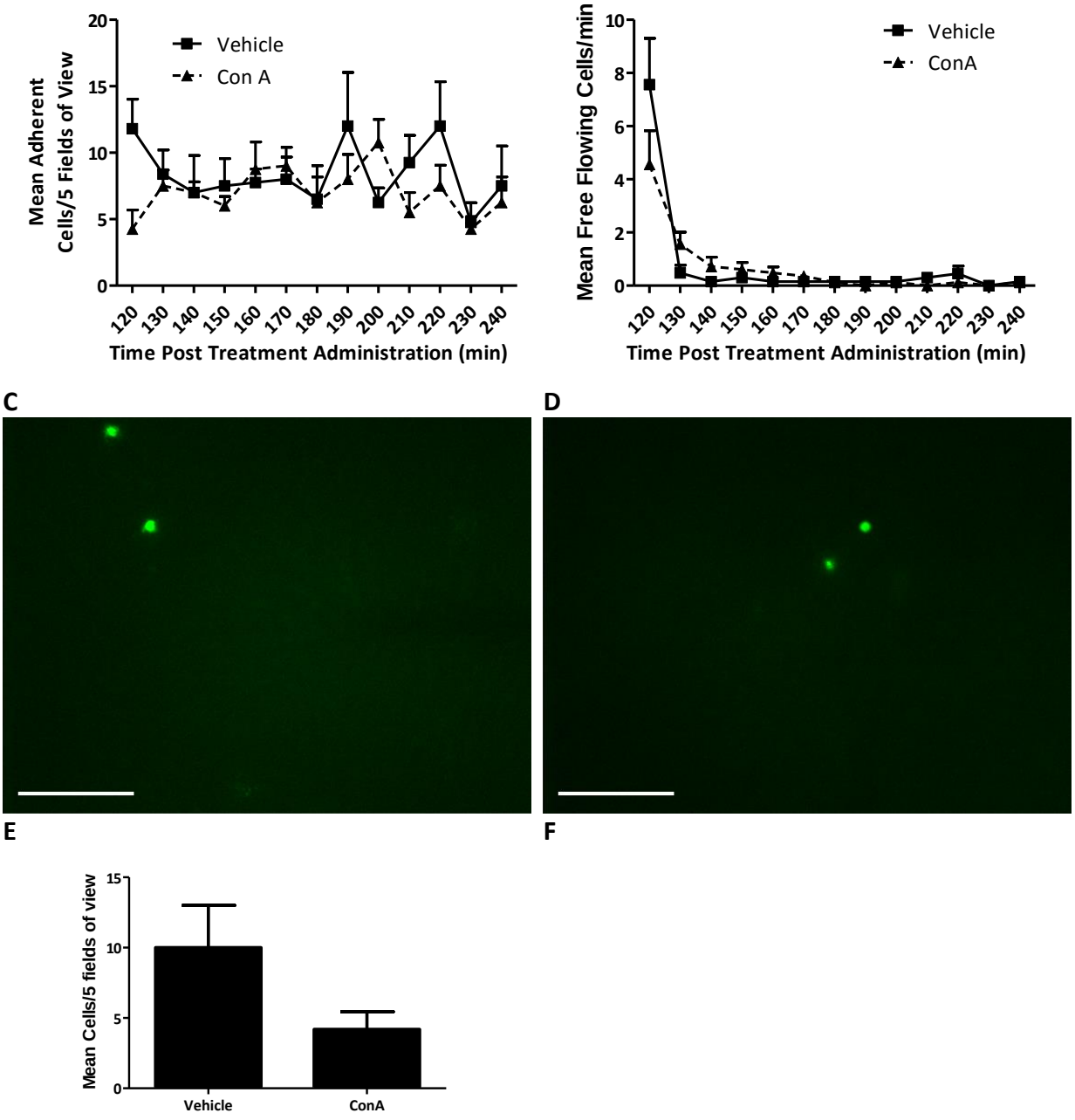


Figure 5:6 There is no significant difference in adhesion of B-cells injected 2 hours post treatment between the control and ConA injured liver. $0.5x10^6$ CFDA-SE stained B-cells were injected into the carotid artery of mice 2 hours after administration of ConA or a vehicle control. Intravital microscopy was used to monitor their trafficking within the liver. Numbers of adherent cells (**A**) and free flowing cells (**B**) in control and ConA injured livers. Images show B-cells (green) in the control liver (**C**) and the ConA injured liver (**D**) at 240 minutes post treatment administration. The numbers of B-cells in cross sections of the liver taken *ex vivo* 4 hours after treatment administration (**E**). Data presented a mean ±SEM, n≥3, (two-way ANOVA (A&B) and t-test (E))) scale bars=100µm.

5.3.5. Platelets Injected at 4 Hours Post Treatment Adhere in Significantly Lower Numbers within the Livers of Mice Treated With ConA Compared to Vehicle Control

As no increase in platelet adhesion was observed at 2-4 hours post ConA treatment, the intravital experiments were repeated at a later time point to determine if a more developed injury would result in increased platelet adhesion. For these experiments CFDA-SE stained platelets were introduced systemically at 4 hours post-ConA or control treatment. Similar results to those seen at 2 hours post ConA administration were obtained. Compared to the controls, platelet adhesion was found to be significantly reduced in the ConA injured liver (*Figure 5.7 A*; $p<0.001$). The difference was most significant at 10 minutes after platelet injection and narrowed as the experiment continued. In *ex vivo* cross sections of the livers there were fewer platelets in ConA injured livers than controls but this difference was not significant (*Figure 5.7 B*; $p>0.05$).

5.3.6. Lymphocytes Injected at 4 hours Post Treatment Adhere in Significantly Lower Numbers within the Livers of Mice Treated with ConA Compared to Vehicle Control

Lymphocyte recruitment at this later time point was also investigated. CMTMR stained T-cells and CFDA-SE stained B-cells were introduced into the circulation 4 hours after ConA or vehicle treatment and their trafficking within the liver was visualised using intravital microscopy. The number of adherent T-cells was significantly reduced in the ConA injured liver compared to the control (*Figure 5.8 A*; $p<0.001$). There was no significant difference in the number of free flowing T-cells between the Injured and control livers (*Figure 5.8 B*; $p>0.05$). The numbers of

T-cells in *ex vivo* cross sections of the livers at 6 hours post treatment were lower in the ConA injured livers than the controls but this difference was not significant (*Figure 5.8 E*; $p>0.05$).

The results for B-cells were similar to those obtained with T-cells. The number of adherent B-cells was quite significantly lower in the ConA injured livers than the controls (*Figure 5.9 A*; $p<0.001$). There was no significant difference in the numbers of free flowing B-cells between the ConA injured livers and the controls (*Figure 5.9 B*; $p>0.05$). The numbers of B-cells in *ex vivo* cross sections of the livers was slightly lower for the ConA injured livers than the controls but this difference did not reach significance (*Figure 5.9 E*; $p>0.05$).

5.3.7. CD4+ T-cell Adhesion is Significantly Higher than CD8a+ T-cell Adhesion within the ConA Injured Liver

CD4+ and CD8+ T-cells are known to play very different roles in inflammation. In order to investigate whether one of these subsets adheres more than the other in ConA injury equal numbers of each subset were infused into the circulation of mice 2 hours after being given a dose of ConA. CD4+ T-cells were found to adhere in significantly greater numbers than CD8a+ T-cells in ConA injured liver (*Figure 5.10 A*; $p<0.05$). *Ex vivo* cross sections of the liver taken at 4 hours post ConA administration also had lower numbers of CD8a+ cells than CD4+ cells but this difference was not significant (*Figure 5.10 E*; $p>0.05$). There was no significant difference between the numbers of free flowing CD4+ and CD8a+ T-cells (*Figure 5.10 B*; $p>0.05$).

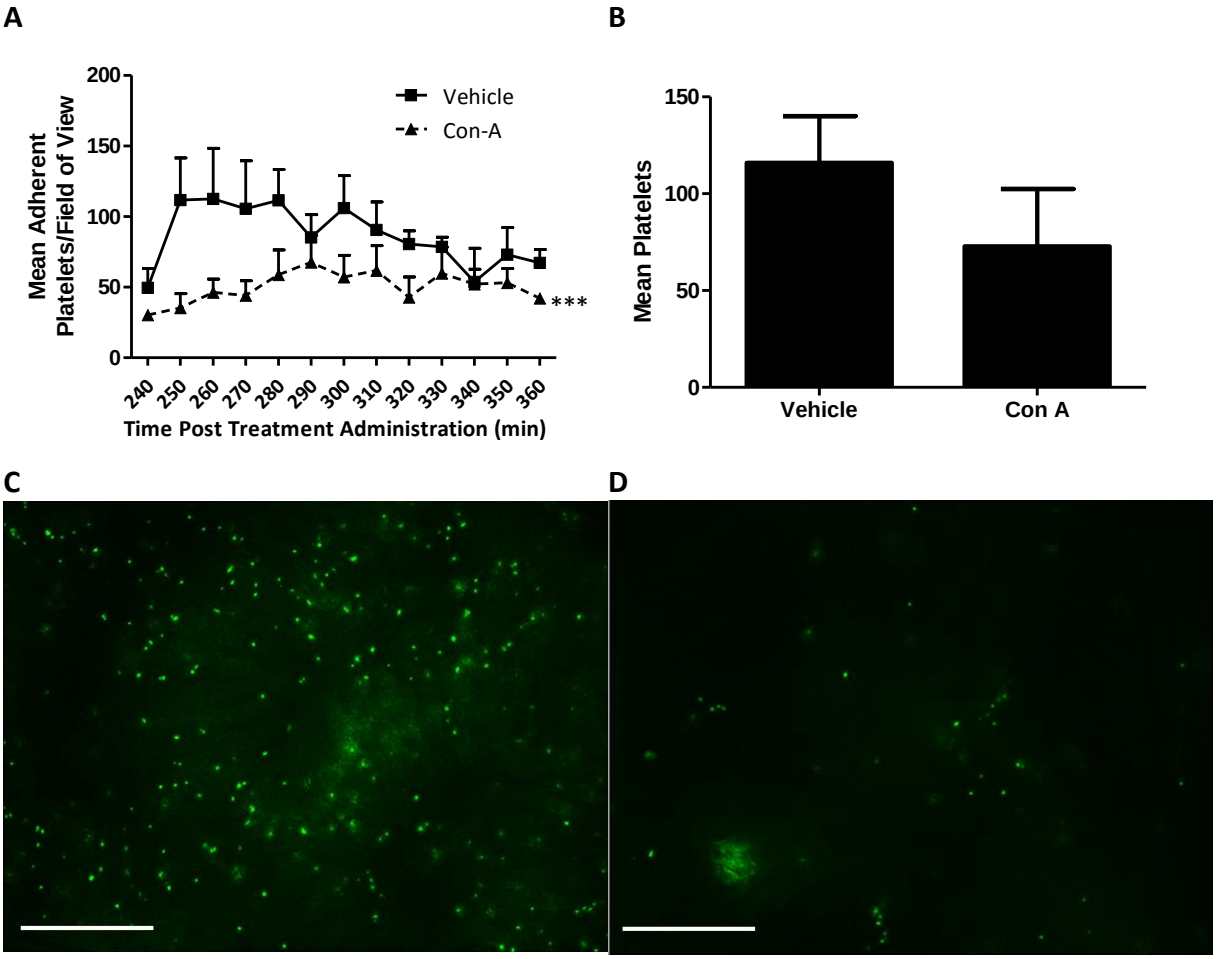


Figure 5:7 Platelets injected at 4 hours post treatment adhere in significantly lower numbers within the livers of mice injected with ConA compared to vehicle control. $1x10^8$ CFDA-SE stained platelets were injected into the carotid artery of mice 4 hours after administration of ConA or a vehicle control. Using intravital microscopy the number of adherent platelets within the hepatic microcirculation was monitored in ConA injured and control livers (**A**). The numbers of platelets in a cross sections of the liver taken *ex vivo* 6 hours after treatment administration (**B**). Images show platelets (green) in the control liver (**C**) and the ConA injured liver (**D**) at 250 minutes post treatment administration. Data presented a mean ±SEM, n≥3, ***p<0.001 (two-way ANOVA (A) and t-test (B)) scale bars=100µm.

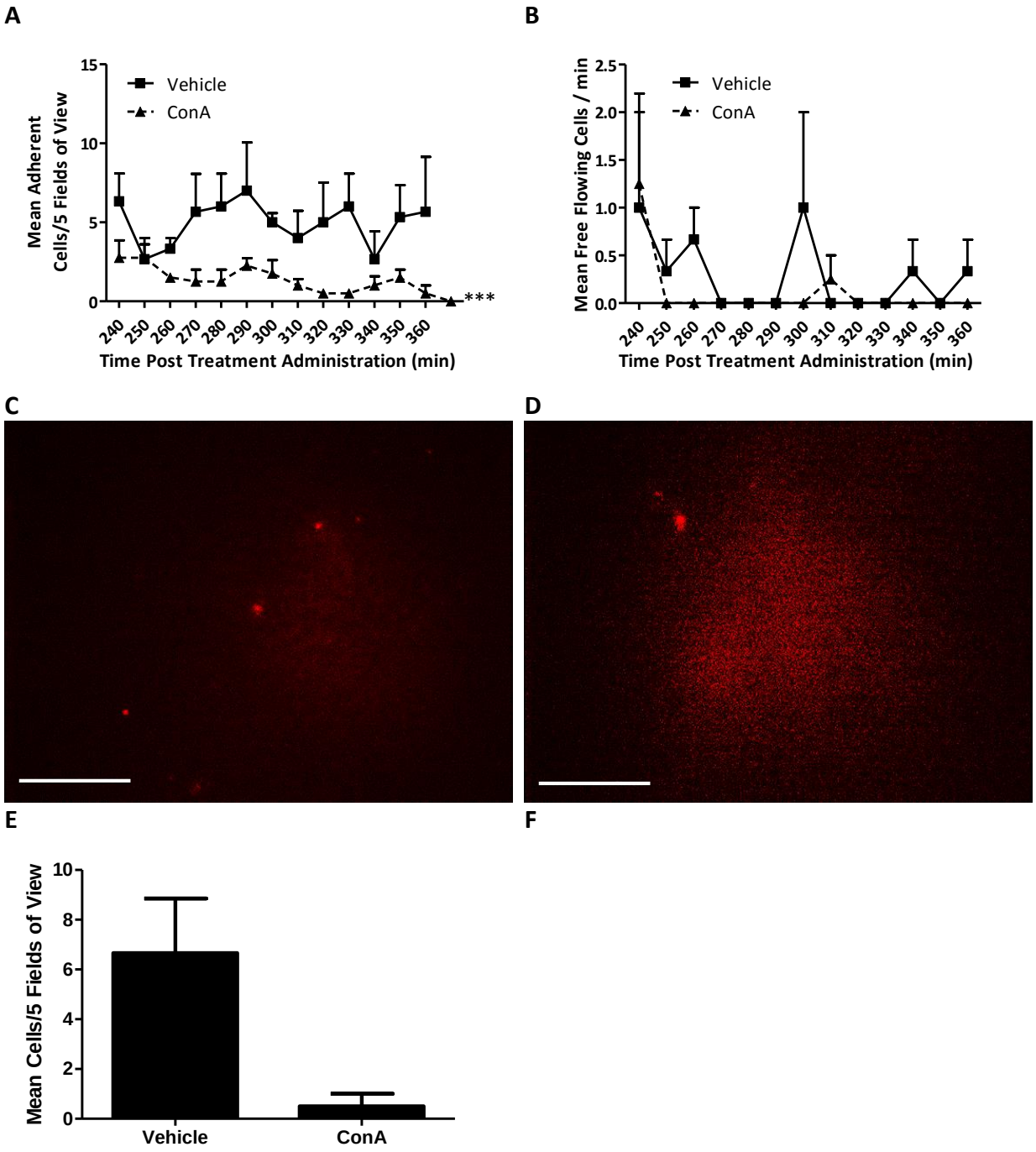


Figure 5:8 T-cells injected at 4 hours post treatment adhere in significantly lower numbers within the livers of mice injected with ConA compared to vehicle control. $0.5x10^6$ CMTMR stained T-cells were injected into the carotid artery of mice 4 hours after administration of ConA or a vehicle control. Using intravital microscopy the number of adherent T-cells within the hepatic microcirculation was monitored in ConA injured and control livers (**A**). The number of free flowing cells within ConA injured and control livers (**B**) Images show T-cells (red) in the control liver (**C**) and the ConA injured liver (**D**) at 330 minutes post treatment administration. The numbers of T-cells in a cross section of the liver taken *ex vivo* 6 hours after treatment administration (**E**). Data presented a mean ±SEM, n≥3, ***p<0.001 (two-way ANOVA (A&B) and t-test (E)) scale bars=100µm.

B

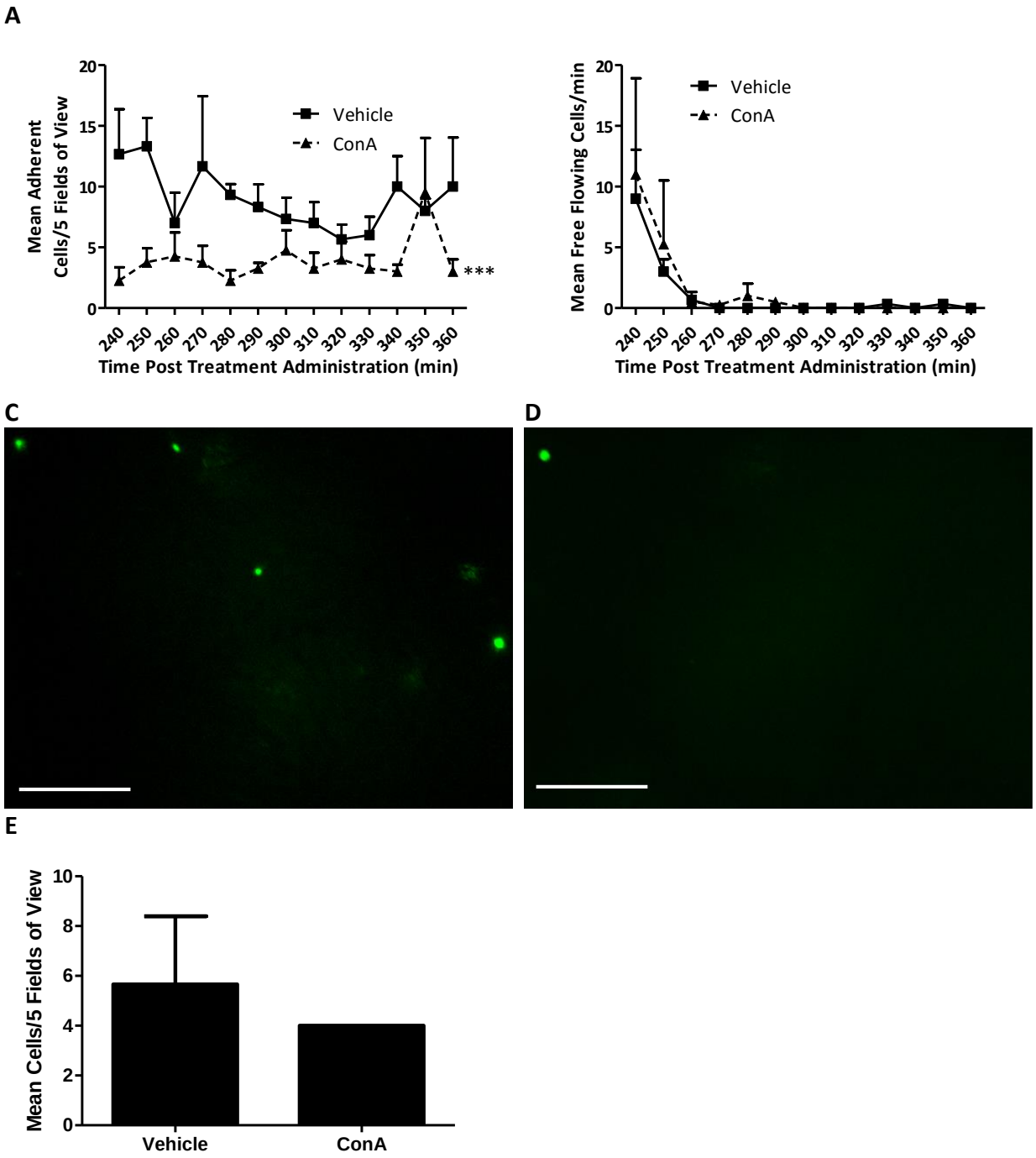


Figure 5:9 B-cells injected at 4 hours post treatment adhere in significantly lower numbers within the livers of mice injected with ConA compared to vehicle control. $0.5x10^6$ CFDA-SE stained B-cells were injected into the carotid artery of mice 4 hours after administration of ConA or a vehicle control. Using intravital microscopy the number of adherent B-cells within the hepatic microcirculation was monitored in ConA injured and control livers (**A**). The number of free flowing cells within ConA injured and control livers (**B**) Images show B-cells (green) in the control liver (**C**) and the ConA injured liver (**D**) at 280 minutes post treatment administration. The numbers of B-cells in a cross section of the liver taken *ex vivo* 6 hours after treatment administration (**E**). Data presented a mean ±SEM, n≥3, ***p<0.001 (two-way ANOVA (A&B) and t-test (E)) scale bars=100µm.

A **B**

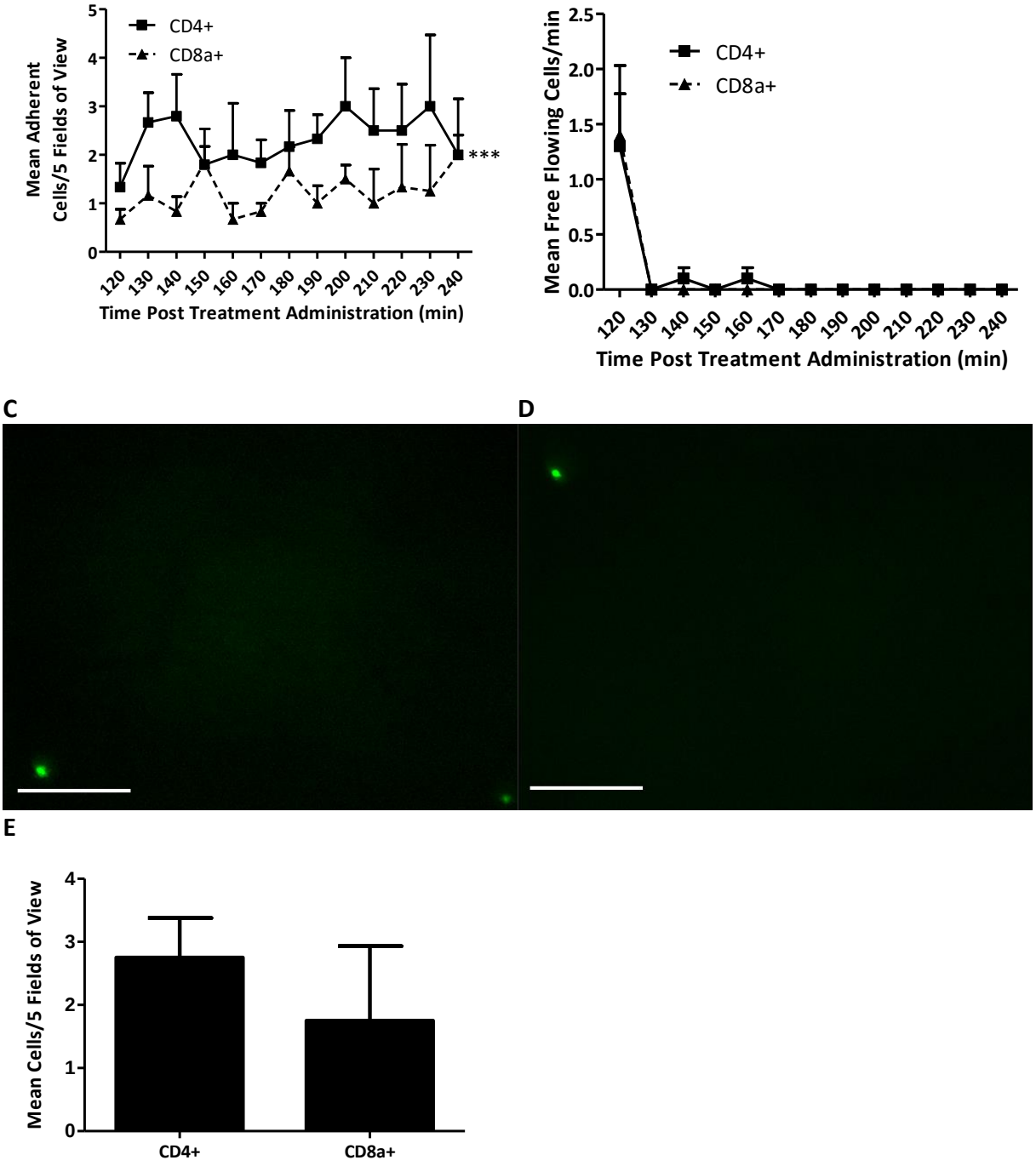


Figure 5:10 CD4+ T-cell adhesion is significantly higher than CD8a+ T-cell adhesion within the ConA injured liver. Equal numbers of CMTMR or CFDA-SE stained CD4+ and CD8a+ were introduced into the circulation of mice 120 minutes after injection of 20mg/kg ConA. Intravital microscopy was used to monitor adhesion of these cells within the hepatic microvasculature (**A**). The number of free flowing CD4+ and CD8a+ T-cells in the ConA injured liver (**B**). Images show CD4+ (**C**) and CD8a+ (**D**) T-cells within the liver at 140 minutes post ConA treatment. Ex vivo analysis of the number of CD4+ and CD8a+ T-cells in a cross section of the liver taken at 240 minutes post ConA treatment (**E**). Data presented a mean ±SEM, n≥4, ***p<0.001 (two-way ANOVA (A&B) and t-test (E)) scale bars=100µm.

5.3.8. Lymphocyte Adhesion within the ConA Injured Liver is Significantly Reduced Following Platelet Depletion

As was shown in the previous chapter, platelet depletion reduces lymphocyte adhesion within the IR injured liver. With the aim of establishing whether the same was true in ConA induced liver injury, platelets were depleted prior to ConA administration using CD42b antibodies. 2 hours after ConA or vehicle control injection, CMTMR stained T-cells and CFDA-SE stained B-cells were infused into the circulation. The number of adherent T-cells in the liver was significantly reduced in platelet depleted mice compared to non-platelet depleted mice (*Figure 5.11 A*; $p<0.001$). Platelet depletion had no significant effect on the number of free flowing T-cells within the liver (*Figure 5.11 B*; $p<0.001$). Platelet depletion also significantly reduced B-cell adhesion within the ConA injured liver (*Figure 5.12 A*; $p<0.05$). Platelet depletion also had no significant effect on the number of free flowing B-cells in the ConA injured liver (*Figure 5.13 B*; $p<0.05$).

5.3.9. Blocking CD162 on T-cells Significantly Reduces there Adhesion within the ConA Injured Liver

As platelets express CD62P in ConA injury, platelet dependent T-cell recruitment may occur through T-cell expressed CD162 interacting with platelet CD62P. T-cell CD162 was blocked using an antibody in order to elucidate its role in T-cell recruitment to the ConA injured liver. Incubation with the CD162 blocking antibody significantly reduced T-cell adhesion compared to an IgG control (*Figure 5.13 A*; $p<0.01$). There was no significant difference in the number of free flowing T-cells between those treated with IgG and those treated with blocking

antibody (*Figure 5.13 B*; p>0.05). *Ex vivo* cross sections showed no significant difference in cell number between the blocked and control cells (*Figure 5.13 E*; p>0.05).

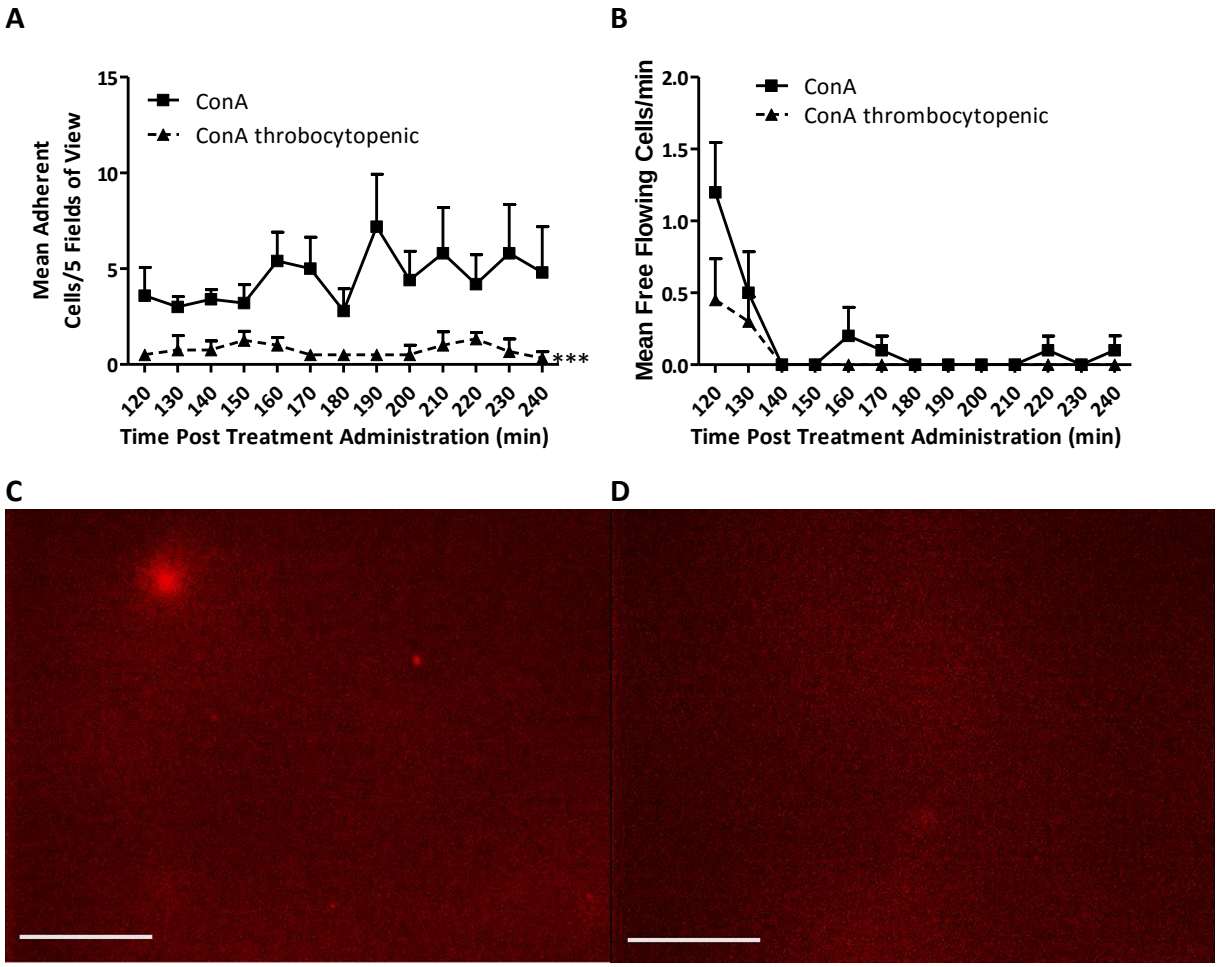


Figure 5:11 T-cell adhesion within the ConA liver is significantly reduced following platelet depletion. Prior to ConA injection mice were depleted of platelets using an antibody. 120 minutes after administration of a 20mg/kg ConA dose $0.5x10^6$ CMTMR stained T-cells were introduced into the circulation. T-cell adhesion (**A**) and free flowing T-cells (**B**) within the injured liver were quantitated in platelet depleted mice. Images show T-cells within liver 190 minutes after ConA injection in non-platelet depleted (**C**) and platelet depleted mice (**D**). Data presented a mean ±SEM, n≥3, ***p<0.001 (two-way ANOVA) scale bars=100µm.

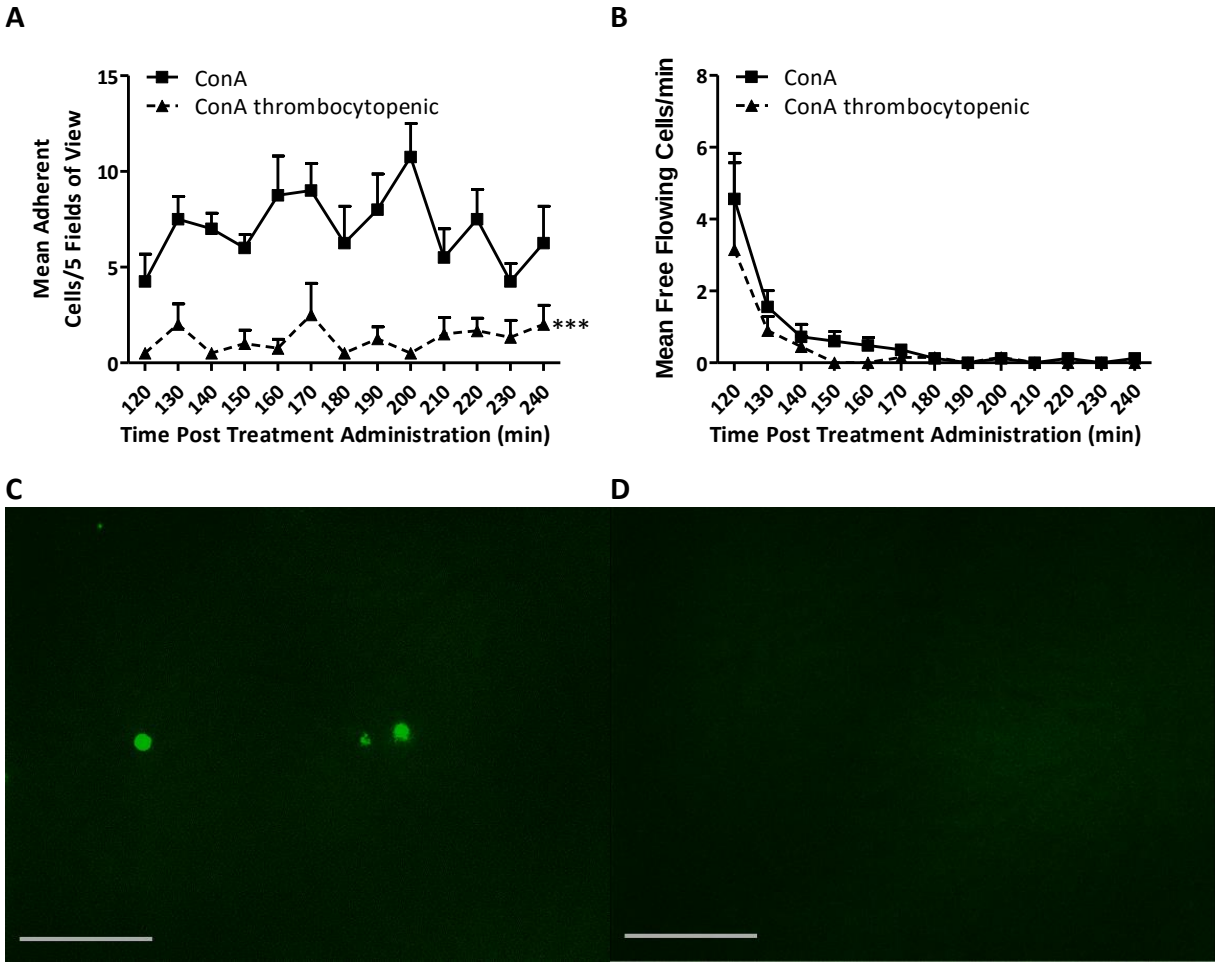


Figure 5:12 B-cell adhesion within the ConA liver is significantly reduced following platelet depletion. Prior to ConA injection mice were depleted of platelets using an antibody. 120 minutes after administration of a 20mg/kg ConA dose $0.5x10^6$ CFDA-SE stained B-cells were introduced into the circulation. B-cell adhesion (**A**) and free flowing B-cells (**B**) were quantitated in the ConA injured livers of platelet depleted mice. Images show B-cells within liver 200 minutes after ConA injection in non-platelet depleted (**C**) and platelet depleted mice (**D**). Data presented a mean ±SEM, n≥3, ***p<0.001 (two-way ANOVA) scale bars=100µm.

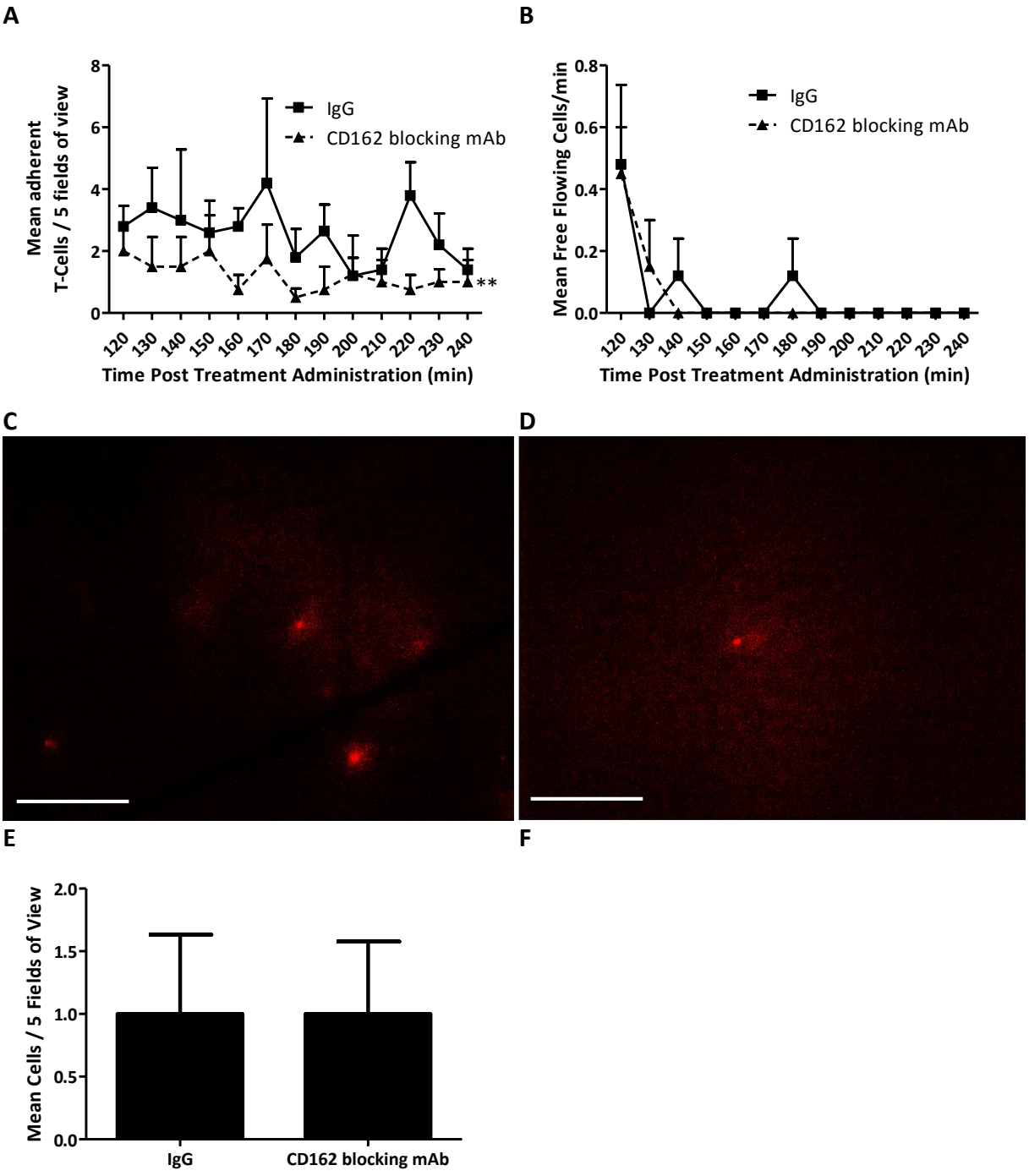


Figure 5:13 Blocking CD162 on T-cells significantly reduces their adhesion within the ConA injured liver. T-cells were incubated for 30 minutes with either CD162 blocking antibody or an IgG control. Their trafficking within the liver 120 minutes after ConA administration was monitored using intravital microscopy. T-cell adhesion (**A**) and free flowing T-cells (**B**) following incubation with either CD162 blocking antibody or IgG control. Images show T-cells incubated with an IgG control (**C**) and CD162 blocking antibody (**D**) in the liver 220 minutes after ConA administration. The number of T-cells in a cross section of the liver taken *ex vivo* at 240 minutes post ConA treatment following incubation with either CD162 blocking antibody or IgG control (**E**). Data presented as mean ±SEM, n≥3, **p<0.01 (two-way ANOVA (A&B) and t-test (E)), scale bars=100µm.

5.4. Discussion

ConA injury is a well-used, clinically relevant model for many forms of hepatitis. When injected intravenously into mice, it triggers a T-cell dependant liver injury (Palacios, 1982, Tiegs *et al*., 1992). This injury appears to be primarily mediated by CD4+ T-cells (Tiegs *et al*., 1992) but a role for NKT cells has also been identified (Kaneko *et al*., 2000, Toyabe *et al*., 1997, Diao *et al*., 2004). High levels of pro-inflammatory cytokines such as TNFα, IL-6, IFNγ and IL-1 is the feature of ConA hepatitis. They are released from resident and infiltrating activated lymphocytes, as well as other hepatic cells, and contribute to disease progression. ConA liver injury was used as a model to study the role of platelets in mediating T-cell recruitment using thrombocytopenic mice – this is novel work and has not previously been explored. Indeed, limited intravital studies have been conducted to monitor the mechanisms of pathophysiology in this model. In the current chapter we demonstrate that ConA has a highly detrimental effect on overall hepatic blood flow and induces significant decreases in the numbers of circulating endogenous red/white blood cells and platelets. Although no increases in injected donor T- or B-cell adhesion was identified in ConA injured mice, their presence was remarkably absent in thrombocytopenic ConA injured mice. The novel results presented in this chapter provide the first evidence that lymphocyte recruitment, including CD4+ T-cells, to the ConA injured liver is platelet dependant.

ConA administration was associated with macroscopically visible injury to the liver and spleen. Previous studies have also shown a dose of 20mg/kg sufficient to induce significant inflammation of the liver within 2-4 hours (Tiegs *et al*., 1992). This was accompanied by a significant decrease in circulating endogenous leukocytes, erythrocytes and platelets. The

large decrease in circulating platelet numbers does not appear to have been described before. It has previously been shown that many circulating cells, including platelets, accumulate within the liver and within the spleen, both of which were noticeably enlarged and engorged in the current study (Massaguer et al., 2002, Miyazawa et al., 1998). Indeed, histological analysis has shown the hepatic sinusoidal lumens to be occluded by agglutinated erythrocytes and by aggregated platelets and leukocytes during ConA injury (Massaguer et al., 2002, Miyazawa et al., 1998). This would explain the decreased full blood count. Splenomegaly is a common feature of liver inflammation and is generally due to congestion of the spleen and/or cellular infiltration. It is frequently accompanied by anaemia and thrombocytopenia (McCormick and Murphy, 2000). Blood counts also showed a significant increase in mean platelet volume following ConA treatment, seen in conjunction with thrombocytopenia this generally indicates destruction of platelets.

A comparison of the injury suffered by the liver after ConA and IR injury has not previously been described. Here we show that the survival of ConA treated mice was lower than that of IR injured mice despite serum ALT activity showing that the liver injury in ConA treated mice was less severe than that of IR injured mice (almost 4 fold lower). This low survival may be linked to the anaemia seen in the ConA treated animals which is compounded by the mice being under ketamine anaesthetic (Sanford and Colby, 1980).

Similarly to IR injury, ConA resulted in a disruption of hepatic blood flow, as shown intravitally by FITC-BSA injection. Areas of poor blood flow were clearly seen interspersed between areas of good perfusion. This has previously been described histologically as a feature of ConA

induced liver injury and is due to cell aggregation/congestion within the hepatic microvasculature mentioned above (Miyazawa *et al*., 1998). Histologically, it has been shown that at 2 hours post-ConA treatment, almost 20% of the liver sinusoids appear to show intrasinusoidal occlusion ((Massaguer et al., 2002). However, laser Doppler measurements did not detect a decrease in tissue perfusion as was demonstrated in the previous chapter following IR injury. A mild increase in laser Doppler measurements above control values was seen at 60 minutes post-ConA administration which progressively declined by 4 hours - this was not statistically significant. Laser Doppler most likely failed to (statistically) detect an overall decrease in blood flow due to the greatly increased speed of blood flow within the perfused areas. This was assessed by the quantification of the velocity of free flowing donor platelets which was significantly increased between 2 and 4 hours post-ConA. Indeed, the speed at which donor platelets circulated through the perfused regions was remarkably high, often making it difficult to even detect the individual platelet with only a fluorescent streak seen. Such high speed cellular trafficking has not previously been observed by the Kalia lab. This platelet velocity / blood flow remained high for the entire duration of the experiment unlike in IR injury where flow decreased as the injury progressed. The increased flow speed within hepatic capillaries is likely due to vascular congestion in many regions of the liver, which would have resulted in localised increases in blood pressure / blood flow within perfused areas.

Although previous histological studies have shown significant endogenous platelet accumulation within the liver following ConA injury (Massaguer et al., 2002), donor platelet adhesion was significantly reduced in our model when quantitated intravitally. This may be

explained by the reduced hepatic perfusion present at the point of platelet injection. Qualitatively and quantitatively, there was better blood perfusion in the liver at 2 hours than at 4 hours post-ConA treatment, indicating the damage to the liver became progressively worse with time. The importance of adequate hepatic perfusion at the time of platelet injection in order to deliver these cells to the liver is highlighted by the fact that ~50 donor platelets were adherent / field of view when injected at 2 hours rather than ~25-30 platelets adherent when injected at 4 hours post-ConA administration. Furthermore, platelet adhesion was only observed in areas of blood flow. The increased blood velocity described earlier in these areas of flow where platelets could adhere is also likely to affect adhesion as local sinusoidal shear will be elevated. Of course, any increase in shear due to increased velocity may have been at least partially offset by a reduction in blood viscosity as a result of the lower haematocrit of ConA treated animals.

Similarly, ConA injury did not increase either donor T- or B-cell adhesion within the liver. Indeed when lymphocytes were injected at 4 hours post-ConA injection their adhesion was significantly lower than in un-injured controls. The same explanations used above to explain poor platelet adhesion may apply for these cells too. Again, adherent lymphocytes were only present in areas of flow – injection of Alexa 647-BSA to highlight areas of flow showed adherent donor lymphocytes only in perfused areas. These results somewhat contradict previous studies which have shown a significant increase in T-cell infiltration of the liver following ConA injury (Mizuhara *et al*., 1994, Bonder *et al*., 2005). The current intravital observations of limited lymphocyte adhesion does not mean the ConA injury model cannot attract lymphocytes to the liver. The current study assessed donor lymphocyte adhesion, but

it must not be forgotten that endogenous circulating lymhocytes are also simultaneously recruited to the injured liver. Indeed, Massaguer and colleagues demonstrated histologically that ~80 endogenous lymphocytes, identified by their nuclear morphology, were present in liver sinusoids 2 hours post-ConA as opposed to 19.1±0.7 lymphocytes/field in healthy mice(Massaguer et al., 2002). It is therefore possible that the lack of donor lymphocytes adhering could also be explained by them having to compete with endogenous cells for endothelial adhesion molecules. Although at 2 hours the numbers of T- and B-cells adhering is very similar to that observed in control mice, it is important to note that this is still probably biologically significant as this adhesion occurs in a greatly reduced area of blood perfusion. It is also possible that to identify significant lymphocyte presence, we needed to image for longer and inject T-/ B-cells at 6 hours post-ConA. Indeed others have shown histologically that the number of CD4+ T cells in the liver peaked 6 hours post-ConA injection, and remained high for 24 hours with peak ALT levels observed at 12 hours (Higashimoto *et al*., 2013).

Of the T-cells adhering at 2 hours post-ConA, CD4+ T-cells were found to adhere in slightly greater numbers than CD8a+ T-cells. This result is in line with previous work which has shown significantly higher CD4+ T-cell infiltration compared to CD8+ T-cell infiltration in the ConA injured liver (Mizuhara *et al*., 1994). In the current experiments the difference in adhesion between CD4+ and CD8+ T-cells was not as pronounced. This is likely due to injecting equal numbers of each subset. This is in contrast to studies in which peripheral blood lymphocytes infiltration was quantitated where CD4+ T-cells are present in greater numbers than CD8+ T-cells (Chen and Harrison, 2002, Mizuhara *et al*., 1994). Additionally the mice used in this study were male C57BL/6 whereas Mizuhara *et al*. (1994) used female BALB/c mice.

Since T- and B-cell adhesion was observed at 2 hours post-ConA, platelet depletion studies were conducted at this time point. In ConA induced liver injury, as with hepatic IR injury, platelet depletion resulted in a significant decrease in lymphocyte adhesion within the inflamed liver. This is the first study to present evidence that shows a role for platelets in recruitment of lymphocytes to ConA induced liver injury. As platelet depletion did not affect perfusion within the ConA injured liver, this reduction in lymphocyte adhesion does not appear to be due to a reduction in entrapment due to vessel occlusion by platelets. It would have been worthwhile to determine immunohistologically whether endogenous T-cell infiltration was also reduced in thrombocytopenic mice as this would have provided confirmation of the intravital results.

Interestingly, platelet depletion and the subsequent large reduction in lymphocyte adhesion did not result in a concomitant reduction in liver injury. Indeed platelet depletion was associated with a doubling in serum ALT activity following ConA injury. Macroscopically the liver and spleen of platelet depleted animals looked similar to those of normal animals following ConA treatment. These results are somewhat surprising as preventing T-cell recruitment to the liver by depleting circulating T-cells has been shown in a number of studies to reduce the injury caused by ConA (Mizuta *et al*., 1999). One possible explanation for the injury observed in these mice is that increased haemorrhage due to platelet depletion allowed for increased infiltration by inflammatory cells into the hepatic parenchyma. It is also possible there is reduced recruitment of those subsets of lymphocytes that may play anti-inflammatory or protective roles in this injury. Mitogens such as ConA are well known to stimulate T-lymphocyte proliferation *in vitro.* However, interestingly, this *in vitro* proliferation can be

significantly inhibited by adding resting intact platelets to these cultures. Furthermore, co-culture also reduces surface activation markers and cytokine secretion from lymphocytes(Wang and Niu, 2008). This increase may reflect the possibility that platelets could be involved in reducing immune responses in this model.

Although the current study has proposed platelets may mediate lymphocyte recruitment to ConA injured liver, others have highlighted a key role for neutrophils (Bonder *et al*., 2004). Using a similar protocol, Bonder *et al*., showed intravitally that although there was a significant increase in leukocytes interacting and adhering in the postsinusoidal venules, only a small proportion (∼20%) of these cells were GFP-labelled endogenous T lymphocytes, which were found primarily adherent within the sinusoids. Neutrophils appeared to be the main leukocyte infiltrating the liver after ConA damage as determined immunohistologically. They further demonstrated that neutropenic animals had little CD4+ T lymphocyte presence in the liver after ConA. This clearly suggests that the ConA mediated liver injury is a multi-faceted pathology involving many different circulating cells contributing to each other's recruitment and eventually tissue injury.

Following CD162 blockade on T-cells, adhesion of T-cells was significantly reduced. This result is in agreement with previous work showing that T-cell recruitment within the ConA liver is dependent on the CD162 receptor CD62P (Massaguer et al., 2002). As CD62P is expressed on platelets during ConA injury (Massaguer et al., 2002), these results suggest that T-cell CD162 interacting with platelet P-selectin is partially responsible for the role of platelets in T-cell adhesion following ConA injury. However, platelet depletion resulted in a far greater drop in

T-cell recruitment suggesting that other mechanisms are also involved. An increase in shear as evidenced by the increased blood velocity in ConA mice may explain the CD62P dependent nature of ConA injury. Cellular recruitment within the inflamed liver is generally selectin independent due to the low shear stresses within sinusoidal vessels which circumvents the need for 'rolling' molecules on circulating cells (Wong *et al*., 1997).

We have shown here, for the first time a key role for platelets as mediators of lymphocyte recruitment following ConA induced liver injury. In the case of T-cells this appears to occur partially through interactions between platelet CD62P and lymphocyte CD162. However, we have also shown that platelet depletion does not have a protective effect and may even exacerbate the injury. The results presented in this chapter show marked similarities to those obtained with IR injured mice, presented in the previous chapter. Clearly the presence of lymphocytes within the injured liver, be it an acute inflammatory reperfusion injury or an immune T-cell dependent injury, requires platelets. This novel body of research contained within this thesis is the first study to have identified a critical role for platelets in two very different models of liver injury.

CHAPTER 6

GENERAL DISCUSSION

6. GENERAL DISCUSSION

6.1. Summary and Discussion of Main Findings

The rise in deaths related to liver disease, underlines the importance of continued research into the mechanisms which govern liver inflammation (British Liver Trust, 2008). Lymphocytes are known to be important mediators of pathogenesis in a wide range of inflammatory disorders of the liver including viral hepatitis (Chisari, 1997), alcoholic liver disease (Batey and Wang, 2002), autoimmune hepatitis (Eggink *et al*., 1982) and hepatic ischaemia reperfusion injury (Zwacka *et al*., 1997). As such it is important to understand the factors which underlie recruitment of these cells to the inflamed liver. Although the primary role of platelets is in haemostasis, it is becoming increasingly apparent that platelets can modulate inflammatory and immune responses either directly or indirectly. It is perhaps unsurprising that platelets, although small anucleate cells, can interact with and influence the function of other blood cells considering their high circulating number and the diversity of inflammatory mediators released from them. It has long been known that platelets can mediate the adhesion of lymphocytes both *in vitro* (Diacovo *et al*., 1996b, Sheikh *et al*., 2004) and *in vivo* (Diacovo *et al*., 1996c). However, with the exception of work carried out by Iannacone *et al*. (2005), showing a platelet dependent mechanism for T_c cell recruitment to the liver following hepatitis B infection, little is known about the involvement of platelets in recruitment of lymphocytes to the inflamed liver. T-cells, particularly $CD4^+$ T-cells, are key regulators of the immune system and so a better understanding of platelet and T-cell interactions would impact a number of inflammatory and immune conditions, especially of the liver. The primary aim of

this thesis was to determine whether platelets play a role in lymphocyte adhesion in two non-viral models of liver inflammation: hepatic IR injury and ConA induced liver injury.

The major findings of this study are as follows:

- Adhesion of lymphocytes to CD54 and MAdCAM is increased by adherent activated platelets.
- Incubation with naïve platelets increases lymphocyte adhesion to MAdCAM and incubation with the releasate of activated platelets increases lymphocyte adhesion to CD54 and MAdCAM.
- Incubation with platelets or platelet releasate does not significantly increase the adhesion of lymphocytes isolated from the inguinal lymph nodes to colonic endothelial cells *in vitro*. Incubation with activated platelets increases adhesion of splenic T-cells and B-cells to activated colonic endothelium *in vitro*.
- Incubation with PMPs increases lymphocyte adhesion to CD54, CD106 and MAdCAM *in vitro* but does not affect lymphocyte recruitment to the IR injured liver *in vivo.*
- Adhesion of T-cells but not B-cells within the hepatic microvasculature was increased following hepatic IR injury.
- Depletion of platelets reduces T-cell and B-cell adhesion within the IR injured hepatic microvasculature.
- Depletion of platelets reduces B-cell but not T-cell adhesion in the uninjured liver.
- Blocking CD162 on T-cells marginally reduces their adhesion within the IR injured liver.

- Adhesion of T-cells and B-cells within the hepatic microvasculature was not increased following 2 or 4 hours ConA injury.
- Depletion of platelets reduces T-cell and B-cell adhesion within the ConA injured hepatic microvasculature.
- Blocking CD162 on T-cells marginally reduces their adhesion within the ConA injured liver.

In order to image lymphocyte and platelet recruitment in the liver *in vivo*, and characterise the spatial and temporal kinetics of their interactions, the technique of intravital microscopy was utilised. Intravital studies on lymphocyte trafficking have typically involved the injection of fluorescently labelled donor cells (Bonder *et al*., 2005, Gunzer *et al*., 2004, Fujimori *et al*., 2002). In order to minimise the number of mice used we initially aimed to label endogenous lymphocytes and platelets in order to avoid the use of additional donor mice. Also, handling of donor cells *in vitro* and the isolation procedure could perturb cell surface molecules and change the activation status of the cells. To avoid this, we used fluorescent antibodies targeted against cell specific markers on endogenous lymphocytes. However, the results obtained using this technique were found to be unreliable due to high levels of non-specific binding within the liver. Because of this we opted to use the fluorescent intracellular dyes CFDA-SE and CMTMR to label T-cells and B-cells from the spleens of donor mice and platelets form the whole blood. Exogenously labelling cells in this way is advantageous as they fluoresce brightly making them easy to visualise *in vivo*. For future studies the use of transgenic mice which have green fluorescent protein expressing lymphocytes may prove useful in reducing the overall number of mice used (Singbartl *et al*., 2001). Indeed, Singbartl

and colleagues developed a transgenic mouse that expressed enhanced GFP (EGFP) in T cells under a CD2 promoter. These mice continued to express the GFP even when T-cells became activated. They further used this mouse to demonstrate T-cell rolling and adhesion during TNFα-induced inflammation in the cremaster muscle intravitally.

Although the focus of the work conducted in this thesis was to characterise lymphocyte-platelet cross-talk, we also demonstrated that a key feature of both hepatic IR injury and ConA induced liver injury was a major disruption of blood flow within the hepatic microvasculature. These observations are difficult to make without using *in vivo* microcirculatory imaging techniques. Large areas without flow were observed in the liver in both models, which was coupled with an increase in flow rate within the areas of flow although this increase was only transitory in IR injury. These disruptions in flow have major implications for cellular recruitment within the liver. Lack of flow causes localised hypoxia and also prevents invasion of the surrounding tissue by circulating cells. In addition, higher rates of flow result in higher shear rates which may lead to changes in the mechanisms of cellular adhesion. For example selectins are not normally required for leukocyte adhesion within the liver (Wong *et al*., 1997) but may be involved at these higher shears. Furthermore, high shear rates may lead to an inability for adhesive interactions to occur or be sustained. Wall shear stresses confer an anti-adhesive force on circulating cells that that opposes the pro-adhesive forces generated by leukocyte and endothelial adhesion molecules. Therefore, when blood flow increases, less leukocyte adhesion occurs. Indeed, to ensure platelets are able to adhere to blood vessels at high shear, both the GPIb–VWF interactions as well as the $\alpha 2\beta 1$–collagen interaction are used,

possibly serving as a physiologic safeguard if one of these mechanisms is defective (Moroi and Jung, 2007). Therefore, in the current study, the observed lymphocyte adhesion in IR injured mice, although small, is quite remarkable considering the higher blood flow velocity.

Both injury models resulted in an expected increase in serum ALT, a specific biochemical marker of hepatic injury. Interestingly, this increase was lower in ConA treated animals than IR injured animals despite the former having lower survival. This implies that the reduced survival of ConA treated mice is not caused by liver damage *per se* and is most likely the result of severe anaemia that was observed in these animals. It is possible that a lower dose of ConA such as the 13mg/Kg used by Bonder *et al*. (2004) may improve the survival of these animals for future experiments. Whether a change in dose (and hence overall survival) impacts on the lymphocyte and platelet events remains to be elucidated.

We hypothesised that both hepatic IR injury and ConA induced liver injury would result in increased adhesion of both platelets and lymphocytes within the hepatic microvasculature. This however was found not to be the case. In fact the only increase in adhesion observed was that of T-cells following hepatic IR injury. Given the disrupted blood flow observed in our injury models this is perhaps not very surprising. As at the point of cell introduction areas of no flow were already present and so the area in which cells could potentially adhere was greatly reduced. The rate of blood flow within the hepatic sinusoids was also increased at the point of cell injection, which may have reduced the cells ability to form stable interactions with the endothelium.

The work we have presented here clearly shows that adhesion of T-cells and B-cells within the liver is platelet dependent in both hepatic IR injury and ConA induced liver injury. Iannacone *et al*. (2005) previously showed that T_c cell recruitment to the liver following hepatitis B infection was platelet dependent but this is the first time that such a role for platelets has be described in non-viral liver inflammation. It is also the first time that a platelet dependent mechanism for B-cell adhesion in the inflamed liver has been observed. Interestingly although both T-cells and B-cells did adhere within the uninjured liver, platelet depletion only resulted in a small decrease in B-cell adhesion. This suggests the mechanisms of lymphocyte adhesion differ between the inflamed and uninjured liver. Investigation of CD4+ and CD8+ T-cell revealed that both subsets adhered relatively equally within the IR injured liver and within the ConA injured liver although CD4+ T-cell adhesion was slightly higher. Previous studies have shown that CD4+ T-cell infiltration into the liver is significantly higher than CD8+ T-cell infiltration in both injury models (Zwacka *et al*., 1997, Mizuhara *et al*., 1994). These differences in results are likely due to the unequal ratio of CD4+ to CD8+ T-cells in the circulation in contrast to the equal numbers of donor cells injected in our experiments (Chen and Harrison, 2002). As adhesion of T-cells was almost completely abrogated by platelet depletion in both models, and the T-cells used in these experiments were roughly 50% CD4+ and 42% CD8+, it can be concluded that both CD4+ and CD8+ T-cell recruitment is platelet dependent in both of these models.

There are various possible mechanisms by which platelets could mediate lymphocyte adhesion within the inflamed liver. The results of the work presented in this thesis investigating the possible mechanisms involved are summarised in *Figure 6.1*. *In vitro* data

suggests that platelet releasate is a key mediator of lymphocyte adhesion. One component of activated platelet releasate, PMPs, was shown to increase adhesion of lymphocytes to immobilised cell adhesion molecules. These results were not replicated *in vivo* in the IR injured liver, however high levels of PMPs are present in the blood following injury and are likely to mask any effect of pre-incubation with PMPs. The effects of two platelet released cytokines, CCL5 and CXCL12, were also investigated but were found to not significantly alter T-cell adhesion to immobilised cell adhesion molecules *in vitro.* These results suggest that platelet released factors such as PMPs may play critical roles in recruitment of lymphocytes to the inflamed liver - this area certainly requires further study

In addition to platelet released factors, T-cell recruitment in both hepatic IR injury and ConA induced liver injury was shown to be partially dependent on the T-cell expressed CD62P ligand CD162. This is in line with previous studies showing that T-cells roll on immobilised activated platelets in a CD62P dependent manner (Diacovo *et al*., 1996b, Sheikh *et al*., 2004). The B-cells used in this study did not express CD162 and, although expressed on around 30% of circulating B-cells, it appears to be inactive so is unlikely to be involved in B-cell adhesion (Vachino *et al*., 1995). Despite these findings the importance of direct lymphocyte-platelet interaction in recruitment of lymphocytes to liver in the two injury models remains unclear. Although T-cell adhesion was lower in both injury models following CD162 blocking it was still higher than following platelet depletion. Additionally, in our *in vitro* studies, lymphocytes were only very rarely seen in direct contact with platelets. However, a number of possible mechanisms of direct platelet-lymphocyte interactions, including lymphocyte expressed CD11a/CD18 and CD11b/CD18 interactions with platelet CD102 (Sheikh *et al*., 2004, Diacovo *et al*., 1994) and

lymphocyte expressed CD40 interactions with platelet expressed CD154 (Elzey *et al*., 2003), were not investigated here.

Flow following injury was not improved following platelets depletion in either model. Therefore, it can be concluded, that the decrease in stationary (adherent) lymphocytes observed following platelet depletion was not due to a reduction in entrapment of lymphocytes by platelet aggregates occluding the vasculature. These results are comparable to those of Iannacone *et al*. (2005) which showed that recruitment of T_c cells to the liver during acute viral hepatitis is independent of platelet coagulation.

The similarities in the *in vivo* results observed between the two models used in this study are quite surprising as marked differences exist between the pathological mechanisms of the two injuries. Hepatic IR injury is generally believed to be primarily mediated by neutrophils with CD4+ T-cells playing a role in neutrophil recruitment (Zwacka *et al*., 1997, Jaeschke *et al*., 1990). In contrast ConA induced liver injury is primarily mediated by CD4+ T-cells and NKT-cells with neutrophils playing a lesser role (Tiegs *et al*., 1992, Toyabe *et al*., 1997, Bonder *et al*., 2004). Cytokines are important mediators of cell recruitment in both injury models, however the exact cytokines involved differ between the two. For example IL-4 is required for ConA induced liver injury however it elicits anti-inflammatory effects in hepatic ischemia reperfusion injury (Toyabe *et al*., 1997, Kato *et al*., 2000). In addition to cytokines ROS formed due to hypoxia are important in the development of hepatic IR injury (Zhang *et al*., 2007). Production of superoxide by Kupffer cells is also an important mediator of ConA induced liver injury (Nakashima *et al*., 2008). There is also likely to be a hypoxic element to the mechanism

of ConA induced liver injury due to the large areas of non-perfused liver. However, it is likely that there is far less hypoxia in ConA injury than hepatic IR injury therefore it is logical that ROS levels are lower in ConA injury than in hepatic IR injury.

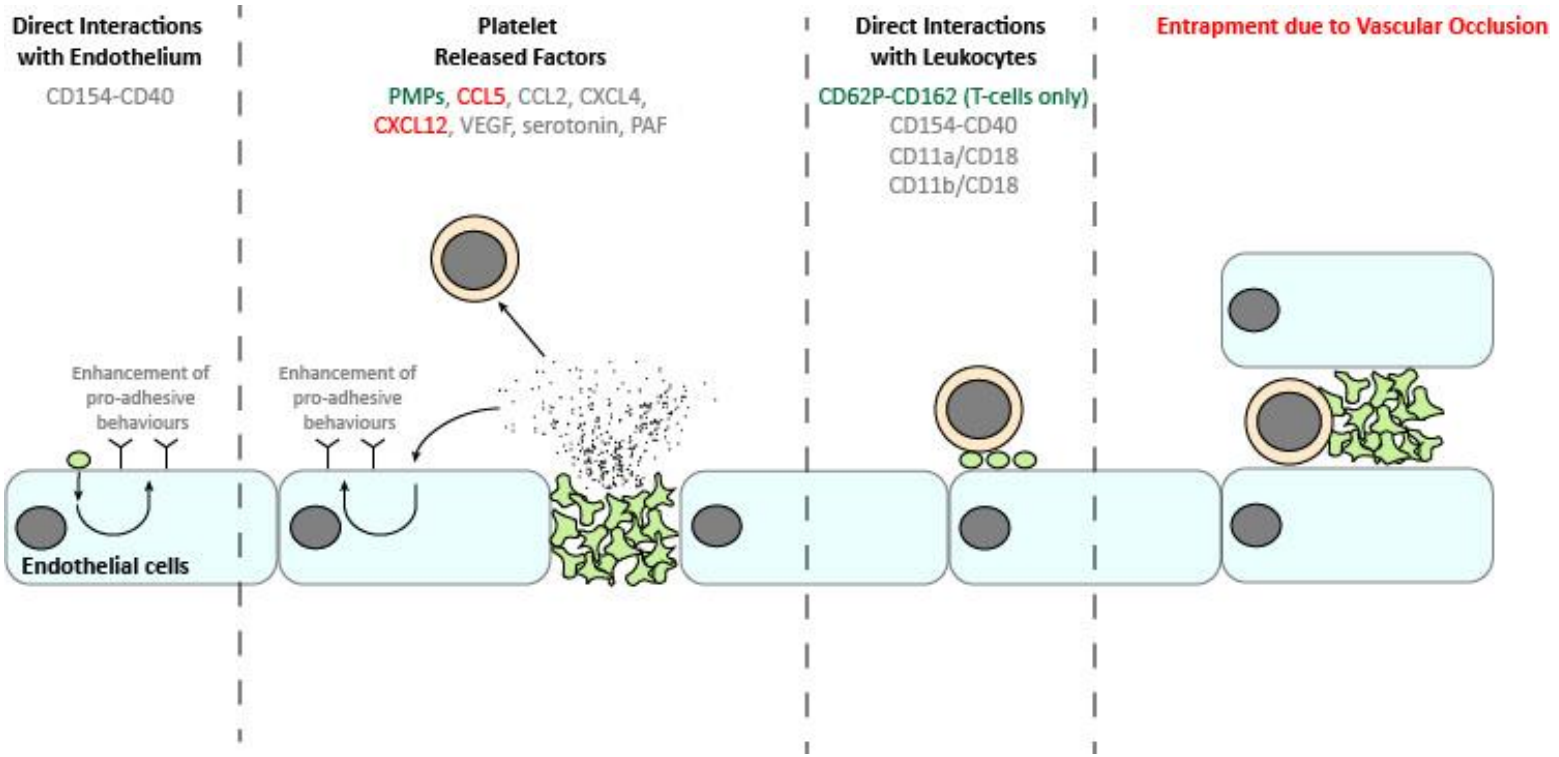


Figure 6:1 Platelet dependent mechanisms of lymphocyte recruitment. In this thesis we have shown that platelets (green) mediate lymphocyte (orange) adhesion following hepatic injury, this could occur through a number of mechanisms. Evidence presented in this study suggests that mechanisms highlighted in green are involved, and that those highlighted in red are not involved, in lymphocyte recruitment to both the IR injured liver and ConA injured liver. Mechanisms written in grey may be involved but were not investigated in this study. PMP, platelet micro-particle; VEGF, vascular endothelial growth factor; PAF, platelet activating factor.

6.2. Future Work

The work presented in this thesis opens a number of possible avenues for further research. We have shown a role for platelets in recruitment of lymphocytes in ConA induced liver injury and hepatic IR injury. However the long-term effects of platelet depletion in these models remains unclear. Platelet derived serotonin has been shown to be important in liver regeneration following IR injury (Nocito *et al*., 2007, Lesurtel *et al*., 2006); however, the effects of platelets on liver regeneration following ConA administration are unknown. Allowing the injury to develop for a longer period would give a better idea of the effects of platelet depletion on injury severity, recovery and inflammatory cell infiltration at later time points. Indeed the peak of injury measured by serum ALT level occurs about 12 hours after ConA administration (Zhou *et al*., 2013) and about 6 hours following hepatic ischaemia (Yadav *et al*., 1999) - far later than the time periods monitored in this study.

Having shown here that platelets are involved in the recruitment of B-cells to the IR and ConA injured livers, it would be interesting to investigate the role of B-cells in these models. This could be achieved using B-cell deficient mice (Kitamura *et al*., 1991) or through depletion of B-cells using antibodies (Uchida *et al*., 2004). It would be particularly interesting to investigate their effect on recovery in these models as they are known to mediate liver fibrosis in CCl_4 induced hepatitis (Novobrantseva *et al*., 2005).

Although our novel studies have unravelled some of the mechanisms involved in platelet recruitment of lymphocytes, further work is required. Interaction between platelet CD154 and lymphocyte expressed CD40 have been suggested as a possible mechanism for

lymphocyte platelet interaction (Elzey *et al*., 2003) but has not been investigated here. This could be investigated through tracking lymphocyte recruitment to the inflamed liver in CD154$^{-/-}$ mice infused with normal or CD154$^{-/-}$ platelets (Elzey *et al*., 2003). Although our *in vitro* studies have shown platelet released factors to increase lymphocyte adhesion their *in vivo* role was not determined. The anti-platelet drug Clopidogrel was used by Nocito *et al*. (2007) to block aggregation but not release of inflammatory mediators by platelets in the IR injured liver. This could potentially be used to either confirm or rule out platelet released factors as mediators of lymphocyte recruitment in our models. The effect of aspirin and warfarin on lymphocyte recruitment in the models used in this thesis would also be of interest. Iannacone et al. (2005) Showed that both Clopidigrel and aspirin, but not warfarin, could reduce recruitment of CD8+ T-cells in models of viral hepatitis.

Another possible mechanism not investigated here is platelet mediated activation of endothelial cells leading to lymphocyte recruitment. Staining for adhesion molecules usually up-regulated following activation of hepatic endothelium, such as CD54 and CD106 (Steinhoff *et al*., 1993), in platelet depleted mice may show if this is important in mediating lymphocyte recruitment.

It would also be of interest to examine the role that hyaluronic acid (HA) plays in these injury models. The lymphocytes used in this thesis do express the HA receptor CD44, as do platelets (Koshiishi et al., 1994). In addition to this high levels of HA expression occur in the liver under both basal and inflammatory conditions (McDonald et al., 2008). Indeed, high levels of HA are observed in the serum during liver fibrosis and may be useful clinical marker (Geramizadeh et

al., 2008). It is possible that HA may act as a bridge between CD44 on platelets and lymphocytes. HA is already known to act as a bridge between lymphocytes and endothelial cells (DeGrendele et al., 1996). Another reason to investigate the role of HA is that platelet released growth factors have previously been shown to upregulate HA expression by fibroblasts and vascular smooth muscle cells (Papakonstantinou et al., 1995, Anitua et al., 2007). HA could easily be rapidly broken down in the injury models used in this thesis through the injection of hyaluronidase (White, 2014). However, this would also effect neutrophil recruitment in these models as CD44-HA interactions have been shown to be important in neutrophil adhesion within the sinusoids (McDonald et al., 2008).

Whilst our studies focused on the liver, platelets may also be required for lymphocyte recruitment to other inflamed organs. There is evidence for platelet dependent lymphocyte recruitment following allergic pulmonary inflammation (Pitchford *et al*., 2004), although it is not known whether platelets are important in recruitment of lymphocytes to other inflamed organs. Lymphocyte mediated inflammation that occurs with high levels of platelet recruitment are also observed in the IR injured kidney and so renal injury may be good models for further investigation (Chintala *et al*., 1994, Awad *et al*., 2006).

6.3. Concluding Remarks

Inflammation of the liver is a common clinical problem with limited treatment options. In order to best treat hepatic inflammation we first require an understanding of the processes involved. The mechanisms underlying recruitment of inflammatory cells to the liver are often poorly understood particularly in non-viral hepatitis.

Platelets have long been known to be involved in the maintenance of haemostasis but it is becoming increasingly clear that they are also important mediators of inflammation. The work presented in this thesis adds to the growing body of evidence that platelets are vital in the recruitment of inflammatory cells to the inflamed liver. Whether anti-platelet strategies can be of use in the treatment of liver inflammation remains to be seen but the work carried out to date makes it an intriguing avenue of research.

7. REFERENCES

ABBASOGLU, O. 2008. Liver transplantation: yesterday, today and tomorrow. *World J Gastroenterol,* 14**,** 3117-22.

AJUEBOR, M. N., ASPINALL, A. I., ZHOU, F., LE, T., YANG, Y., URBANSKI, S. J., SIDOBRE, S., KRONENBERG, M., HOGABOAM, C. M. & SWAIN, M. G. 2005. Lack of chemokine receptor CCR5 promotes murine fulminant liver failure by preventing the apoptosis of activated CD1d-restricted NKT cells. *J Immunol,* 174**,** 8027-37.

AJUEBOR, M. N., HOGABOAM, C. M., LE, T., PROUDFOOT, A. E. I. & SWAIN, M. G. 2004. CCL3/MIP-1α is pro-inflammatory in murine T cell-mediated hepatitis by recruiting CCR1-expressing CD4+ T cells to the liver. *Eur J Immunol,* 34**,** 2907-2918.

ANDO, T., JORDAN, P., WANG, Y., ITOH, M., JOH, T., SASAKI, M., ELROD, J. W., CARPENTER, A., JENNINGS, M. H., MINAGAR, A. & ALEXANDER, J. S. 2005. MAdCAM-1 expression and regulation in murine colonic endothelial cells in vitro. *Inflammatory Bowel Diseases,* 11**,** 258-264.

ANDRE, P., DELANEY, S. M., LAROCCA, T., VINCENT, D., DEGUZMAN, F., JUREK, M., KOLLER, B., PHILLIPS, D. R. & CONLEY, P. B. 2003. P2Y12 regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. *J Clin Invest,* 112**,** 398-406.

ANGELICO, M. 2005. Donor liver steatosis and graft selection for liver transplantation: a short review. *Eur Rev Med Pharmacol Sci,* 9**,** 295-7.

ANITUA, E., SÁNCHEZ, M., NURDEN, A., ZALDUENDO, M., DE LA FUENTE, M., AZOFRA, J. & ANDÍA, I. 2007. Platelet-released growth factors enhance the secretion of hyaluronic acid and induce hepatocyte growth factor production by synovial fibroblasts from arthritic patients. *Rheumatology,* 46**,** 1769-1772.

ANSELMO, D. M., AMERSI, F. F., SHEN, X. D., GAO, F., KATORI, M., LASSMAN, C., KE, B., COITO, A. J., MA, J., BRINKMANN, V., BUSUTTIL, R. W., KUPIEC-WEGLINSKI, J. W. & FARMER, D. G. 2002. FTY720 pretreatment reduces warm hepatic ischemia reperfusion injury through inhibition of T-lymphocyte infiltration. *Am J Transplant,* 2**,** 843-9.

ARBONÉS, M. L., ORD, D. C., LEY, K., RATECH, H., MAYNARD-CURRY, C., OTTEN, G., CAPON, D. J. & TEDDERT, T. F. 1994. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity,* 1**,** 247-260.

ARMSTRONG, M. E., ALEXANDER, H. D., RITCHIE, J. L., MCMILLAN, S. A. & REA, I. M. 2001. Age-related alterations in basal expression and in vitro, tumour necrosis factor alpha mediated, upregulation of CD11b. *Gerontology,* 47**,** 180-5.

AUNE, T. M., GOLDEN, H. W. & MCGRATH, K. M. 1994. Inhibitors of serotonin synthesis and antagonists of serotonin 1A receptors inhibit T lymphocyte function in vitro and cell-mediated immunity in vivo. *The Journal of Immunology,* 153**,** 489-98.

AUSTEN JR, W. G., ZHANG, M., CHAN, R., FRIEND, D., HECHTMAN, H. B., CARROLL, M. C. & MOORE JR, F. D. 2004. Murine hindlimb reperfusion injury can be initiated by a self-reactive monoclonal IgM. *Surgery,* 136**,** 401-406.

AWAD, A. S., HUANG, Y. H., LI, L. L., W., F. F., L., M. T., R., L. K. & D., O. M. 2006. Selective sphingosine 1-phosphate 1 receptor activation reduces ischemia-reperfusion injury in mouse kidney. *American Journal of Physiology - Renal Physiology,* 290**,** F1516-F1524.

BALABAUD, C., BIOULAC-SAGE, P. & DESMOULIÈRE, A. 2004. The role of hepatic stellate cells in liver regeneration. *Journal of hepatology,* 40**,** 1023-1026.

BARCLAY, A. N. 2003. Membrane proteins with immunoglobulin-like domains—a master superfamily of interaction molecules. *Seminars in Immunology,* 15**,** 215-223.

BARON, J. L., GARDINER, L., NISHIMURA, S., SHINKAI, K., LOCKSLEY, R. & GANEM, D. 2002. Activation of a nonclassical NKT cell subset in a transgenic mouse model of hepatitis B virus infection. *Immunity,* 16**,** 583-594.

BATALLER, R. & BRENNER, D. A. Hepatic stellate cells as a target for the treatment of liver fibrosis. Seminars in liver disease, 2001. 437-451.

BATEY, R. G. & WANG, J. 2002. Molecular pathogenesis of T lymphocyte-induced liver injury in alcoholic hepatitis. *Front Biosci,* 7**,** d1662-75.

BERLIN, C., BARGATZE, R. F., CAMPBELL, J. J., VON ANDRIAN, U. H., SZABO, M. C., HASSLEN, S. R., NELSON, R. D., BERG, E. L., ERLANDSEN, S. L. & BUTCHER, E. C. 1995. α4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell,* 80**,** 413-422.

BEUMER, S., HEIJNEN, H. F., MJ, I. J., ORLANDO, E., DE GROOT, P. G. & SIXMA, J. J. 1995. Platelet adhesion to fibronectin in flow: the importance of von Willebrand factor and glycoprotein Ib. *Blood,* 86**,** 3452-60.

BINNERTS, M. E., VAN KOOYK, Y., SIMMONS, D. L. & FIGDOR, C. G. 1994. Distinct binding of T lymphocytes to ICAM-1, -2 or -3 upon activation of LFA-1. *Eur J Immunol,* 24**,** 2155-2160.

BLEUL, C. C., FUHLBRIGGE, R. C., CASASNOVAS, J. M., AIUTI, A. & SPRINGER, T. A. 1996. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J Exp Med,* 184**,** 1101-9.

BLOMBACK, B., HESSEL, B., HOGG, D. & THERKILDSEN, L. 1978. A two-step fibrinogen--fibrin transition in blood coagulation. *Nature,* 275**,** 501-5.

BOMBELI, T., SCHWARTZ, B. R. & HARLAN, J. M. 1998. Adhesion of Activated Platelets to Endothelial Cells: Evidence for a GPIIbIIIa-dependent Bridging Mechanism and Novel Roles for Endothelial Intercellular Adhesion Molecule 1 (ICAM-1), αvβ3 Integrin, and GPIbα. *J Exp Med,* 187**,** 329-339.

BONDER, C. S., AJUEBOR, M. N., ZBYTNUIK, L. D., KUBES, P. & SWAIN, M. G. 2004. Essential Role for Neutrophil Recruitment to the Liver in Concanavalin A-Induced Hepatitis. *The Journal of Immunology,* 172**,** 45-53.

BONDER, C. S., NORMAN, M. U., SWAIN, M. G., ZBYTNUIK, L. D., YAMANOUCHI, J., SANTAMARIA, P., AJUEBOR, M., SALMI, M., JALKANEN, S. & KUBES, P. 2005. Rules of Recruitment for Th1 and Th2 Lymphocytes in Inflamed Liver: A Role for Alpha-4 Integrin and Vascular Adhesion Protein-1. *Immunity,* 23**,** 153-163.

BOYLE, C. C., PAINE, A. J. & MATHER, S. J. 1992. The mechanism of hepatic uptake of a radiolabelled monoclonal antibody. *Int J Cancer,* 50**,** 912-7.

BRITISH LIVER TRUST 2008. Facts about liver disease.

BRUNT, E. M., JANNEY, C. G., DI BISCEGLIE, A. M., NEUSCHWANDER-TETRI, B. A. & BACON, B. R. 1999. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol,* 94**,** 2467-2474.

BURNE-TANEY, M. J., ASCON, D. B., DANIELS, F., RACUSEN, L., BALDWIN, W. & RABB, H. 2003. B cell deficiency confers protection from renal ischemia reperfusion injury. *J Immunol,* 171**,** 3210-5.

CALDWELL, C. C., OKAYA, T., MARTIGNONI, A., HUSTED, T., SCHUSTER, R. & LENTSCH, A. B. 2005. Divergent functions of CD4+ T lymphocytes in acute liver inflammation and injury after ischemia-reperfusion. *Am J Physiol Gastrointest Liver Physiol,* 289**,** G969-76.

CALDWELL, C. C., TSCHOEP, J. & LENTSCH, A. B. 2007. Lymphocyte function during hepatic ischemia/reperfusion injury. *J Leukoc Biol,* 82**,** 457-464.

CARMAN, C. V. & SPRINGER, T. A. 2003. Integrin avidity regulation: are changes in affinity and conformation underemphasized? *Current Opinion in Cell Biology,* 15**,** 547-556.

CARP, R. I., LICURSI, P. C., MERZ, P. A. & MERZ, G. S. 1972. Decreased percentage of polymorphonuclear neutrophils in mouse peripheral blood after inoculation with material from multiple sclerosis patients. *J Exp Med,* 136**,** 618-29.

CARR, M. W., ALON, R. & SPRINGER, T. A. 1996. The C–C Chemokine MCP-1 Differentially Modulates the Avidity of β1 and β2 Integrins on T Lymphocytes. *Immunity,* 4**,** 179-187.

CERADINI, D. J., KULKARNI, A. R., CALLAGHAN, M. J., TEPPER, O. M., BASTIDAS, N., KLEINMAN, M. E., CAPLA, J. M., GALIANO, R. D., LEVINE, J. P. & GURTNER, G. C. 2004. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nature medicine,* 10**,** 858-864.

CHAN, J. R., HYDUK, S. J. & CYBULSKY, M. I. 2000. α4β1 Integrin/VCAM-1 Interaction Activates αLβ2 Integrin-Mediated Adhesion to ICAM-1 in Human T Cells. *The Journal of Immunology,* 164**,** 746-753.

CHEN, J. & HARRISON, D. E. 2002. Quantitative trait loci regulating relative lymphocyte proportions in mouse peripheral blood. *Blood,* 99**,** 561-566.

CHENG, F., LI, Y., FENG, L. & LI, S. Hepatic stellate cell activation and hepatic fibrosis induced by ischemia/reperfusion injury. Transplantation proceedings, 2008. Elsevier, 2167-2170.

CHIGNARD, M., LE COUEDIC, J. P., VARGAFTIG, B. B. & BENVENISTE, J. 1980. Platelet-activating factor (PAF-acether) secretion from platelets: effect of aggregating agents. *Br J Haematol,* 46**,** 455-64.

CHIMEN, M., MCGETTRICK, H. M., APTA, B., KURAVI, S. J., YATES, C. M., KENNEDY, A., ODEDRA, A., ALASSIRI, M., HARRISON, M. & MARTIN, A. 2015. Homeostatic regulation of T cell trafficking by a B cell-derived peptide is impaired in autoimmune and chronic inflammatory disease. *Nature medicine,* 21**,** 467-475.

CHINTALA, M. S., BERNARDINO, V. & CHIU, P. J. 1994. Cyclic GMP but not cyclic AMP prevents renal platelet accumulation after ischemia-reperfusion in anesthetized rats. *J Pharmacol Exp Ther,* 271**,** 1203-8.

CHISARI, F. V. 1997. Cytotoxic T cells and viral hepatitis. *J Clin Invest,* 99**,** 1472-7.

CHTANOVA, T., HAMPTON, H. R., WATERHOUSE, L. A., WOOD, K., TOMURA, M., MIWA, Y., MACKAY, C. R., BRINK, R. & PHAN, T. G. 2014. Real-time interactive two-photon photoconversion of recirculating lymphocytes for discontinuous cell tracking in live adult mice. *Journal of Biophotonics,* 7**,** 425-433.

CLARKSON, A. R., MACDONALD, M. K., FUSTER, V., CASH, J. D. & ROBSON, J. S. 1970. Glomerular coagulation in acute ischaemic renal failure. *Q J Med,* 39**,** 585-99.

CLEVERS, H., ALARCON, B., WILEMAN, T. & TERHORST, C. 1988. The T cell receptor/CD3 complex: a dynamic protein ensemble. *Annu Rev Immunol,* 6**,** 629-662.

CONKLYN, M. J., NEOTE, K. & SHOWELL, H. J. 1996. Chemokine-Dependent Upregulation of CD11b on Specific Leukocyte Subpopulations in Human Whole Blood: Effect of Anticoagulant on Rantes and MIP-1β Stimulation. *Cytokine,* 8**,** 762-766.

CONSTANTIN, G., MAJEED, M., GIAGULLI, C., PICCIO, L., KIM, J. Y., BUTCHER, E. C. & LAUDANNA, C. 2000. Chemokines trigger immediate β2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity,* 13**,** 759-769.

COOK, M. J. 1965. *The anatomy of the laboratory mouse*, Academic Press.

COUGHLIN, S. R. 2000. Thrombin signalling and protease-activated receptors. *Nature,* 407**,** 258-64.

COUVELARD, A., SCOAZEC, J. Y. & FELDMANN, G. 1993. Expression of cell-cell and cell-matrix adhesion proteins by sinusoidal endothelial cells in the normal and cirrhotic human liver. *Am J Pathol,* 143**,** 738-52.

CRISPE, I. N., DAO, T., KLUGEWITZ, K., MEHAL, W. Z. & METZ, D. P. 2000. The liver as a site of T-cell apoptosis: graveyard, or killing field? *Immunological reviews,* 174**,** 47-62.

CYWES, R., PACKHAM, M. A., TIETZE, L., SANABRIA, J. R., HARVEY, P. R. C., PHILLIPS, M. J. & STRASBERG, S. M. 1993. Role of platelets in hepatic allograft preservation injury in the rat. *Hepatology,* 18**,** 635-647.

DANESE, S., DE LA MOTTE, C., STURM, A., VOGEL, J. D., WEST, G. A., STRONG, S. A., KATZ, J. A. & FIOCCHI, C. 2003. Platelets trigger a CD40-dependent inflammatory response in the microvasculature of inflammatory bowel disease patients. *Gastroenterology,* 124**,** 1249-1264.

DE FOUGEROLLES, A. R., SPRAGUE, A. G., NICKERSON-NUTTER, C. L., CHI-ROSSO, G., RENNERT, P. D., GARDNER, H., GOTWALS, P. J., LOBB, R. R. & KOTELIANSKY, V. E. 2000. Regulation of inflammation by collagen-binding integrins α1β1 and α2β1 in models of hypersensitivity and arthritis. *J Clin Invest,* 105**,** 721-729.

DE LALLA, C., GALLI, G., ALDRIGHETTI, L., ROMEO, R., MARIANI, M., MONNO, A., NUTI, S., COLOMBO, M., CALLEA, F. & PORCELLI, S. A. 2004. Production of profibrotic cytokines by invariant NKT cells characterizes cirrhosis progression in chronic viral hepatitis. *The Journal of Immunology,* 173**,** 1417-1425.

DE MARCO, L., GIROLAMI, A., ZIMMERMAN, T. S. & RUGGERI, Z. M. 1986. von Willebrand factor interaction with the glycoprotein IIb/IIa complex. Its role in platelet function as demonstrated in patients with congenital afibrinogenemia. *J Clin Invest,* 77**,** 1272-7.

DE PERROT, M., YOUNG, K., IMAI, Y., LIU, M., WADDELL, T. K., FISCHER, S., ZHANG, L. & KESHAVJEE, S. 2003. Recipient T cells mediate reperfusion injury after lung transplantation in the rat. *The Journal of Immunology,* 171**,** 4995-5002.

DEGRENDELE, H. C., ESTESS, P., PICKER, L. J. & SIEGELMAN, M. H. 1996. CD44 and its ligand hyaluronate mediate rolling under physiologic flow: a novel lymphocyte-endothelial cell primary adhesion pathway. *The Journal of experimental medicine,* 183**,** 1119-1130.

DIACOVO, T., ROTH, S., BUCCOLA, J., BAINTON, D. & SPRINGER, T. 1996a. Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. *Blood,* 88**,** 146-157.

DIACOVO, T., ROTH, S., MORITA, C., ROSAT, J., BRENNER, M. & SPRINGER, T. 1996b. Interactions of human alpha/beta and gamma/delta T lymphocyte subsets in shear flow with E-selectin and P-selectin. *J Exp Med,* 183**,** 1193-1203.

DIACOVO, T. G., DEFOUGEROLLES, A. R., BAINTON, D. F. & SPRINGER, T. A. 1994. A functional integrin ligand on the surface of platelets: intercellular adhesion molecule-2. *J Clin Invest,* 94**,** 1243-51.

DIACOVO, T. G., PURI, K. D., WARNOCK, R. A., SPRINGER, T. A. & VON ANDRIAN, U. H. 1996c. Platelet-mediated lymphocyte delivery to high endothelial venules. *Science,* 273**,** 252-5.

DIAMANT, M., TUSHUIZEN, M. E., STURK, A. & NIEUWLAND, R. 2004. Cellular microparticles: new players in the field of vascular disease? *European Journal of Clinical Investigation,* 34**,** 392-401.

DIAO, H., KON, S., IWABUCHI, K., KIMURA, C., MORIMOTO, J., ITO, D., SEGAWA, T., MAEDA, M., HAMURO, J., NAKAYAMA, T., TANIGUCHI, M., YAGITA, H., VAN KAER, L., ONÓE, K., DENHARDT, D., RITTLING, S. & UEDE, T. 2004. Osteopontin as a Mediator of NKT Cell Function in T Cell-Mediated Liver Diseases. *Immunity,* 21**,** 539-550.

DOMÍNGUEZ-GERPE, L. & REY-MÉNDEZ, M. 2001. Alterations induced by chronic stress in lymphocyte subsets of blood and primary and secondary immune organs of mice. *BMC immunology,* 2**,** 7.

DRAKE, T. A., MORRISSEY, J. H. & EDGINGTON, T. S. 1989. Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis. *Am J Pathol,* 134**,** 1087-97.

DUBOIS, C., PANICOT-DUBOIS, L., GAINOR, J. F., FURIE, B. C. & FURIE, B. 2007. Thrombin-initiated platelet activation in vivo is vWF independent during thrombus formation in a laser injury model. *J Clin Invest,* 117**,** 953-60.

DUHEN, T., GEIGER, R., JARROSSAY, D., LANZAVECCHIA, A. & SALLUSTO, F. 2009. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat Immunol,* 10**,** 857-63.

DULKANCHAINUN, T. S., GOSS, J. A., IMAGAWA, D. K., SHAW, G. D., ANSELMO, D. M., KALDAS, F., WANG, T., ZHAO, D., BUSUTTIL, A. A., KATO, H., MURRAY, N. G., KUPIEC-WEGLINSKI, J. W. & BUSUTTIL, R. W. 1998. Reduction of hepatic ischemia/reperfusion injury by a soluble P-selectin glycoprotein ligand-1. *Ann Surg,* 227**,** 832-40.

DUNNE, J. L., BALLANTYNE, C. M., BEAUDET, A. L. & LEY, K. 2002. Control of leukocyte rolling velocity in TNF-alpha-induced inflammation by LFA-1 and Mac-1. *Blood,* 99**,** 336-41.

DUSSEAUX, M., MARTIN, E., SERRIARI, N., PÉGUILLET, I., PREMEL, V., LOUIS, D., MILDER, M., LE BOURHIS, L., SOUDAIS, C. & TREINER, E. 2011. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17–secreting T cells. *Blood,* 117**,** 1250-1259.

DUSTIN, M. L. & SPRINGER, T. A. 1988. Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *The Journal of Cell Biology,* 107**,** 321-331.

EGGINK, H., HOUTHOFF, H., HUITEMA, S., GIPS, C. & POPPEMA, S. 1982. Cellular and humoral immune reactions in chronic active liver disease. I. Lymphocyte subsets in liver biopsies of patients with untreated idiopathic autoimmune hepatitis, chronic active hepatitis B and primary biliary cirrhosis. *Clinical and experimental immunology,* 50**,** 17.

EIPEL, C., BORDEL, R., NICKELS, R. M., MENGER, M. D. & VOLLMAR, B. 2004. Impact of leukocytes and platelets in mediating hepatocyte apoptosis in a rat model of systemic endotoxemia. *American Journal of Physiology - Gastrointestinal and Liver Physiology,* 286**,** G769-G776.

ELTZSCHIG, H. K. & COLLARD, C. D. 2004. Vascular ischaemia and reperfusion injury. *British Medical Bulletin,* 70**,** 71-86.

ELZEY, B. D., TIAN, J., JENSEN, R. J., SWANSON, A. K., LEES, J. R., LENTZ, S. R., STEIN, C. S., NIESWANDT, B., WANG, Y., DAVIDSON, B. L. & RATLIFF, T. L. 2003. Platelet-Mediated Modulation of Adaptive Immunity: A Communication Link between Innate and Adaptive Immune Compartments. *Immunity,* 19**,** 9-19.

EMOTO, M., ZERRAHN, J., MIYAMOTO, M., PÉRARNAU, B. & KAUFMANN, S. H. E. 2000. Phenotypic characterization of CD8+NKT cells. *Eur J Immunol,* 30**,** 2300-2311.

ENGELHARDT, B., KEMPE, B., MERFELD-CLAUSS, S., LASCHINGER, M., FURIE, B., WILD, M. K. & VESTWEBER, D. 2005. P-Selectin Glycoprotein Ligand 1 Is Not Required for the Development

of Experimental Autoimmune Encephalomyelitis in SJL and C57BL/6 Mice. *The Journal of Immunology,* 175**,** 1267-1275.

ESCH, J. S. A., JURK, K., KNOEFEL, W. T., ROEDER, G., VOSS, H., TUSTAS, R. Y., SCHMELZLE, M., KRIEG, A., EISENBERGER, C. F., TOPP, S., ROGIERS, X., FISCHER, L., AKEN, H. V. & KEHREL, B. E. 2010. Platelet activation and increased tissue factor expression on monocytes in reperfusion injury following orthotopic liver transplantation. *Platelets,* 21**,** 348-359.

FAURIAT, C., LONG, E. O., LJUNGGREN, H.-G. & BRYCESON, Y. T. 2010. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood,* 115**,** 2167-2176.

FENG, D., NAGY, J. A., PYNE, K., DVORAK, H. F. & DVORAK, A. M. 1998. Neutrophils emigrate from venules by a transendothelial cell pathway in response to FMLP. *J Exp Med,* 187**,** 903-15.

FONDEVILA, C., BUSUTTIL, R. W. & KUPIEC-WEGLINSKI, J. W. 2003. Hepatic ischemia/reperfusion injury—a fresh look. *Experimental and molecular pathology,* 74**,** 86-93.

FORLOW, S. B., MCEVER, R. P. & NOLLERT, M. U. 2000. Leukocyte-leukocyte interactions mediated by platelet microparticles under flow. *Blood,* 95**,** 1317-1323.

FREEMAN, C. M., QUILLIN III, R. C., WILSON, G. C., NOJIMA, H., JOHNSON III, B. L., SUTTON, J. M., SCHUSTER, R. M., BLANCHARD, J., EDWARDS, M. J. & CALDWELL, C. C. 2014. Characterization of microparticles after hepatic ischemia-reperfusion injury. *PloS one,* 9**,** e97945.

FRENETTE, P. S., DENIS, C. V., WEISS, L., JURK, K., SUBBARAO, S., KEHREL, B., HARTWIG, J. H., VESTWEBER, D. & WAGNER, D. D. 2000. P-Selectin Glycoprotein Ligand 1 (Psgl-1) Is Expressed on Platelets and Can Mediate Platelet–Endothelial Interactions in Vivo. *J Exp Med,* 191**,** 1413-1422.

FRENETTE, P. S., MOYNA, C., HARTWELL, D. W., LOWE, J. B., HYNES, R. O. & WAGNER, D. D. 1998. Platelet-endothelial interactions in inflamed mesenteric venules. *Blood,* 91**,** 1318-1324.

FUJIMORI, H., MIURA, S., KOSEKI, S., HOKARI, R., KOMOTO, S., HARA, Y., HACHIMURA, S., KAMINOGAWA, S. & ISHII, H. 2002. Intravital observation of adhesion of lamina propria lymphocytes to microvessels of small intestine in mice. *Gastroenterology,* 122**,** 734-744.

FUJIMURA, Y., TITANI, K., HOLLAND, L. Z., ROBERTS, J. R., KOSTEL, P., RUGGERI, Z. M. & ZIMMERMAN, T. S. 1987. A heparin-binding domain of human von Willebrand factor. Characterization and localization to a tryptic fragment extending from amino acid residue Val-449 to Lys-728. *J Biol Chem,* 262**,** 1734-9.

FURIE, B. & FURIE, B. C. 2008. Mechanisms of Thrombus Formation. *New England Journal of Medicine,* 359**,** 938-949.

FURUZAWA-CARBALLEDA, J., VARGAS-ROJAS, M. I. & CABRAL, A. R. 2007. Autoimmune inflammation from the Th17 perspective. *Autoimmunity Reviews,* 6**,** 169-175.

GABOURY, J. & KUBES, P. 1994. Reductions in physiologic shear rates lead to CD11/CD18-dependent, selectin-independent leukocyte rolling in vivo. *Blood,* 83**,** 345-350.

GACHET, C. 2001. ADP receptors of platelets and their inhibition. *Thromb Haemost,* 86**,** 222-32.

GANTNER, F., LEIST, M., LOHSE, A. W., GERMANN, P. G. & TIEGS, G. 1995. Concanavalin A—induced T-cell—mediated hepatic injury in mice: The role of tumor necrosis factor. *Hepatology,* 21**,** 190-198.

GARCIA-CRIADO, F. J., TOLEDO-PEREYRA, L. H., LOPEZ-NEBLINA, F., PHILLIPS, M. L., PAEZ-ROLLYS, A. & MISAWA, K. 1995. Role of P-selectin in total hepatic ischemia and reperfusion. *J Am Coll Surg,* 181**,** 327-34.

GARCÍA-MONZÓN, C., JARA, P., FERNÁNDEZ-BERMEJO, M., HIERRO, L., FRAUCA, E., CAMARENA, C., DÍAZ, C., DE LA VEGA, A., LARRAURI, J., GARCÍA-IGLESIAS, C., BORQUE, M. J., SANZ, P., GARCÍA-BUEY, L., MORENO-MONTEAGUDO, J. A. & MORENO-OTERO, R. 1998. Chronic hepatitis C in children: A clinical and immunohistochemical comparative study with adult patients. *Hepatology,* 28**,** 1696-1701.

GERAMIZADEH, B., JANFESHAN, K. & SABERFIROOZI, M. 2008. Serum hyaluronic acid as a noninvasive marker of hepatic fibrosis in chronic hepatitis B. *Saudi journal of gastroenterology: official journal of the Saudi Gastroenterology Association,* 14**,** 174.

GERMANOV, E., VEINOTTE, L., CULLEN, R., CHAMBERLAIN, E., BUTCHER, E. C. & JOHNSTON, B. 2008. Critical role for the chemokine receptor CXCR6 in homeostasis and activation of CD1d-restricted NKT cells. *The Journal of Immunology,* 181**,** 81-91.

GIRMA, J. P., KALAFATIS, M., PIETU, G., LAVERGNE, J. M., CHOPEK, M. W., EDGINGTON, T. S. & MEYER, D. 1986. Mapping of distinct von Willebrand factor domains interacting with platelet GPIb and GPIIb/IIIa and with collagen using monoclonal antibodies. *Blood,* 67**,** 1356-1366.

GODFREY, D. I., HAMMOND, K. J. L., POULTON, L. D., SMYTH, M. J. & BAXTER, A. G. 2000. NKT cells: facts, functions and fallacies. *Immunology Today,* 21**,** 573-583.

GODFREY, D. I., MACDONALD, H. R., KRONENBERG, M., SMYTH, M. J. & VAN KAER, L. 2004. NKT cells: what's in a name? *Nature Reviews Immunology,* 4**,** 231-237.

GOEBEL, S. H. M. D. W. C. J. M. S. T. J. A. J. S. K. C. G. 2006. VEGF-A stimulation of leukocyte adhesion to colonic microvascular endothelium: implications for inflammatory bowel disease. *American Journal of Physiology - Gastrointestinal and Liver Physiology,* 290**,** G648-G654.

GOTO, S., IKEDA, Y., SALDIVAR, E. & RUGGERI, Z. M. 1998. Distinct mechanisms of platelet aggregation as a consequence of different shearing flow conditions. *J Clin Invest,* 101**,** 479-86.

GRABOVSKY, V., FEIGELSON, S., CHEN, C., BLEIJS, D. A., PELED, A., CINAMON, G., BALEUX, F., ARENZANA-SEISDEDOS, F., LAPIDOT, T., VAN KOOYK, Y., LOBB, R. R. & ALON, R. 2000. Subsecond induction of alpha4 integrin clustering by immobilized chemokines stimulates leukocyte tethering and rolling on endothelial vascular cell adhesion molecule 1 under flow conditions. *J Exp Med,* 192**,** 495-506.

GRANT, A. J., LALOR, P. F., HÜBSCHER, S. G., BRISKIN, M. & ADAMS, D. H. 2001. MAdCAM-1 expressed in chronic inflammatory liver disease supports mucosal lymphocyte adhesion to hepatic endothelium (MAdCAM-1 in chronic inflammatory liver disease). *Hepatology,* 33**,** 1065-1072.

GROOTJANS, J., LENAERTS, K., DERIKX, J. P., MATTHIJSEN, R. A., DE BRUINE, A. P., VAN BIJNEN, A. A., VAN DAM, R. M., DEJONG, C. H. & BUURMAN, W. A. 2010. Human intestinal ischemia-

reperfusion-induced inflammation characterized: experiences from a new translational model. *Am J Pathol,* 176**,** 2283-91.

GUNZER, M., WEISHAUPT, C., HILLMER, A., BASOGLU, Y., FRIEDL, P., DITTMAR, K. E., KOLANUS, W., VARGA, G. & GRABBE, S. 2004. A spectrum of biophysical interaction modes between T cells and different antigen-presenting cells during priming in 3-D collagen and in vivo. *Blood,* 104**,** 2801-2809.

GUYER, D. A., MOORE, K. L., LYNAM, E. B., SCHAMMEL, C., ROGELJ, S., MCEVER, R. P. & SKLAR, L. A. 1996. P-selectin glycoprotein ligand-1 (PSGL-1) is a ligand for L-selectin in neutrophil aggregation. *Blood,* 88**,** 2415-2421.

HAAS, A., ZIMMERMANN, K. & OXENIUS, A. 2011. Antigen-Dependent and -Independent Mechanisms of T and B Cell Hyperactivation during Chronic HIV-1 Infection. *Journal of Virology,* 85**,** 12102-12113.

HADA, M., KAMINSKI, M., BOCKENSTEDT, P. & MCDONAGH, J. 1986. Covalent crosslinking of von Willebrand factor to fibrin. *Blood,* 68**,** 95-101.

HAMANN, A., ANDREW, D. P., JABLONSKI-WESTRICH, D., HOLZMANN, B. & BUTCHER, E. C. 1994. Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo. *The Journal of Immunology,* 152**,** 3282-93.

HAMZE, M., DESMETZ, C. & GUGLIELMI, P. 2013. B cell-derived cytokines in disease. *European cytokine network,* 24**,** 20-26.

HANSCHEN, M., ZAHLER, S., KROMBACH, F. & KHANDOGA, A. 2008. Reciprocal activation between CD4+ T cells and Kupffer cells during hepatic ischemia-reperfusion. *Transplantation,* 86**,** 710-8.

HANTGAN, R., HINDRIKS, G., TAYLOR, R., SIXMA, J. & DE GROOT, P. 1990. Glycoprotein Ib, von Willebrand factor, and glycoprotein IIb:IIIa are all involved in platelet adhesion to fibrin in flowing whole blood. *Blood,* 76**,** 345-353.

HARLAN, J. M. & WINN, R. K. 2002. Leukocyte-endothelial interactions: clinical trials of anti-adhesion therapy. *Critical care medicine,* 30**,** S214-S219.

HELLSTRAND, K. & HERMODSSON, S. 1993. Serotonergic 5-HT1A receptors regulate a cell contact-mediated interaction between natural killer cells and monocytes. *Scand J Immunol,* 37**,** 7-18.

HENN, V., SLUPSKY, J. R., GRAFE, M., ANAGNOSTOPOULOS, I., FORSTER, R., MULLER-BERGHAUS, G. & KROCZEK, R. A. 1998. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature,* 391**,** 591-594.

HEYDTMANN, M. 2009. Macrophages in Hepatitis B and Hepatitis C Virus Infections. *Journal of Virology,* 83**,** 2796-2802.

HFRSHKOVIZ, R., GREENSPOON, N., MFKORI, Y. A., HADARI, R., ALON, R., KAPUSTINA, G. & LIDER, O. 1994. Inhibition of CD4+ T lymphocyte binding to fibronectin and immune-cell accumulation in inflammatory sites by non-peptidic mimetics of Arg-Gly-Asp. *Clinical & Experimental Immunology,* 95**,** 270-276.

HIGASHIMOTO, M., SAKAI, Y., TAKAMURA, M., USUI, S., NASTI, A., YOSHIDA, K., SEKI, A., KOMURA, T., HONDA, M. & WADA, T. 2013. Adipose tissue derived stromal stem cell therapy in murine ConA-derived hepatitis is dependent on myeloid-lineage and CD4+ T-cell suppression. *European journal of immunology,* 43**,** 2956-2968.

HOFFMEISTER, K. M., FELBINGER, T. W., FALET, H., DENIS, C. V., BERGMEIER, W., MAYADAS, T. N., VON ANDRIAN, U. H., WAGNER, D. D., STOSSEL, T. P. & HARTWIG, J. H. 2003. The Clearance Mechanism of Chilled Blood Platelets. *Cell,* 112**,** 87-97.

HOKARI, R., MIURA, S., FUJIMORI, H., KOSEKI, S., TSUZUKI, Y., KIMURA, H., HIGUCHI, H., SERIZAWA, H., GRANGER, D. N. & ISHII, H. 1999. Altered migration of gut-derived T lymphocytes after activation with concanavalin A. *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 277**,** G763-G772.

HOLMSEN, H. & WEISS, H. J. 1979. Secretable storage pools in platelets. *Annual review of medicine,* 30**,** 119-134.

HOLYER, I. 2010. *The Role of Platelets and Their Associated Recruitment Mechanisms, in Itestinal Ischemia Reperfusion Injury.* Ph.D., The University of Birmingham.

HOUDIJK, W. & SIXMA, J. 1985. Fibronectin in artery subendothelium is important for platelet adhesion. *Blood,* 65**,** 598-604.

HU, H., ZHU, L., HUANG, Z., JI, Q., CHATTERJEE, M., ZHANG, W. & LI, N. 2010. Platelets enhance lymphocyte adhesion and infiltration into arterial thrombus. *Thrombosis & Haemostasis,* 104**,** 1184.

HUIZINGA, E. G., TSUJI, S., ROMIJN, R. A. P., SCHIPHORST, M. E., DE GROOT, P. G., SIXMA, J. J. & GROS, P. 2002. Structures of glycoprotein Ibα and its complex with von Willebrand factor A1 domain. *Science,* 297**,** 1176-1179.

HULSTAERT, F., HANNET, I., DENEYS, V., MUNHYESHULI, V., REICHERT, T., DE BRUYÈRE, M. & STRAUSS, K. 1994. Age-related changes in human blood lymphocyte subpopulations: II. Varying kinetics of percentage and absolute count measurements. *Clinical immunology and immunopathology,* 70**,** 152-158.

IANNACONE, M., SITIA, G., ISOGAWA, M., MARCHESE, P., CASTRO, M. G., LOWENSTEIN, P. R., CHISARI, F. V., RUGGERI, Z. M. & GUIDOTTI, L. G. 2005. Platelets mediate cytotoxic T lymphocyte-induced liver damage. *Nat Med,* 11**,** 1167-9.

IANNACONE, M., SITIA, G., NARVAIZA, I., RUGGERI, Z. M. & GUIDOTTI, L. G. 2007. Antiplatelet drug therapy moderates immune-mediated liver disease and inhibits viral clearance in mice infected with a replication-deficient adenovirus. *Clin Vaccine Immunol,* 14**,** 1532-5.

IKEN, K., CHHENG, S., FARGIN, A., GOULET, A.-C. & KOUASSI, E. 1995. Serotonin Upregulates Mitogen-Stimulated B Lymphocyte Proliferation through 5-HT1AReceptors. *Cell Immunol,* 163**,** 1-9.

INOUE, O., SUZUKI-INOUE, K., DEAN, W. L., FRAMPTON, J. & WATSON, S. P. 2003. Integrin α2β1 mediates outside-in regulation of platelet spreading on collagen through activation of Src kinases and PLCγ2. *The Journal of Cell Biology,* 160**,** 769-780.

IVANOV, K. P., KALININA, M. K. & LEVKOVICH, Y. I. 1985. Microcirculation velocity changes under hypoxia in brain, muscles, liver, and their physiological significance. *Microvascular Research,* 30**,** 10-18.

JACKSON, S. P. & SCHOENWAELDER, S. M. 2003. Antiplatelet therapy: in search of the'magic bullet'. *Nature Reviews Drug Discovery,* 2**,** 775-789.

JAESCHKE, H. 2003. Anti-selectin therapy against hepatic ischemia-reperfusion injury. *Hepatology,* 37**,** 220-222.

JAESCHKE, H. 2006. Mechanisms of Liver Injury. II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions. *Am J Physiol Gastrointest Liver Physiol,* 290**,** G1083-8.

JAESCHKE, H., FARHOOD, A. & SMITH, C. W. 1990. Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. *FASEB J,* 4**,** 3355-9.

JAESCHKE, H., FARHOOD, A. & SMITH, C. W. 1991. Neutrophil-induced liver cell injury in endotoxin shock is a CD11b/CD18-dependent mechanism. *American Journal of Physiology - Gastrointestinal and Liver Physiology,* 261**,** G1051-G1056.

JAESCHKE, H. & SMITH, C. W. 1997. Mechanisms of neutrophil-induced parenchymal cell injury. *J Leukoc Biol,* 61**,** 647-53.

JANG, H. R., GANDOLFO, M. T., KO, G. J., SATPUTE, S. R., RACUSEN, L. & RABB, H. 2010. B Cells Limit Repair after Ischemic Acute Kidney Injury. *Journal of the American Society of Nephrology,* 21**,** 654-665.

JANOWSKA-WIECZOREK, A., MAJKA, M., KIJOWSKI, J., BAJ-KRZYWORZEKA, M., RECA, R., TURNER, A. R., RATAJCZAK, J., EMERSON, S. G., KOWALSKA, M. A. & RATAJCZAK, M. Z. 2001. Platelet-derived microparticles bind to hematopoietic stem/progenitor cells and enhance their engraftment. *Blood,* 98**,** 3143-3149.

JARUGA, B., HONG, F., KIM, W. H. & GAO, B. 2004. IFN-gamma/STAT1 acts as a proinflammatory signal in T cell-mediated hepatitis via induction of multiple chemokines and adhesion molecules: a critical role of IRF-1. *Am J Physiol Gastrointest Liver Physiol,* 287**,** G1044-52.

JEONG, W. I., PARK, O. & GAO, B. 2008. Abrogation of the antifibrotic effects of natural killer cells/interferon-γ contributes to alcohol acceleration of liver fibrosis. *Gastroenterology,* 134**,** 248-258.

JIANG, Y., BELLER, D. I., FRENDL, G. & GRAVES, D. T. 1992. Monocyte chemoattractant protein-1 regulates adhesion molecule expression and cytokine production in human monocytes. *The Journal of Immunology,* 148**,** 2423-8.

JOENSUU, H., RISTAMAKI, R., KLEMI, P. J. & JALKANEN, S. 1993. Lymphocyte homing receptor (CD44) expression is associated with poor prognosis in gastrointestinal lymphoma. *Br J Cancer,* 68**,** 428-432.

JOHNSTON, B. K. S. K. P. 1996. Hydrogen peroxide induces leukocyte rolling: modulation by endogenous antioxidant mechanisms including NO. *American Journal of Physiology - Heart and Circulatory Physiology,* 271**,** H614-H621.

JONES, T. R., SHIRASUGI, N., ADAMS, A. B., PEARSON, T. C. & LARSEN, C. P. 2003. Intravital microscopy identifies selectins that regulate T cell traffic into allografts. *J Clin Invest,* 112**,** 1714-1723.

JY, W., MAO, W.-W., HORSTMAN, L. L., TAO, J. & AHN, Y. S. 1995. Platelet Microparticles Bind, Activate and Aggregate Neutrophils In Vitro. *Blood Cells, Molecules, and Diseases,* 21**,** 217-231.

KALIA, N., POCKLEY, A. G., WOOD, R. F. & BROWN, N. J. 2002. Effects of hypothermia and rewarming on the mucosal villus microcirculation and survival after rat intestinal ischemia-reperfusion injury. *Ann Surg,* 236**,** 67-74.

KAMEYOSHI, Y., DORSCHNER, A., MALLET, A. I., CHRISTOPHERS, E. & SCHRODER, J. M. 1992. Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J Exp Med,* 176**,** 587-92.

KAMIIKE, W., WATANABE, F., HASHIMOTO, T., TAGAWA, K., IKEDA, Y., NAKAO, K. & KAWASHIMA, Y. 1982. Changes in cellular levels of ATP and its catabolites in ischemic rat liver. *Journal of biochemistry,* 91**,** 1349-1356.

KANEKO, Y., HARADA, M., KAWANO, T., YAMASHITA, M., SHIBATA, Y., GEJYO, F., NAKAYAMA, T. & TANIGUCHI, M. 2000. Augmentation of Vα14 Nkt Cell–Mediated Cytotoxicity by Interleukin 4 in an Autocrine Mechanism Resulting in the Development of Concanavalin a–Induced Hepatitis. *J Exp Med,* 191**,** 105-114.

KANSAS, G. 1996. Selectins and their ligands: current concepts and controversies. *Blood,* 88**,** 3259-3287.

KAPLAN, M. H. 2013. Th9 cells: differentiation and disease. *Immunological Reviews,* 252**,** 104-115.

KAPLANSKI, G., MARIN, V., MONTERO-JULIAN, F., MANTOVANI, A. & FARNARIER, C. 2003. IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. *Trends in Immunology,* 24**,** 25-29.

KATO, A., YOSHIDOME, H., EDWARDS, M. J. & LENTSCH, A. B. 2000. Reduced hepatic ischemia/reperfusion injury by IL-4: potential anti-inflammatory role of STAT6. *Inflamm Res,* 49**,** 275-9.

KAVANAGH, D. 2009. *Molecular Events Governing Hematopoietic Stem Cell Recruitment in vivo in Murine Liver Following Ischaemia-Reperfusion Injury* Ph.D, University of Birmingham.

KAWASUJI, A., HASEGAWA, M., HORIKAWA, M., FUJITA, T., MATSUSHITA, Y., MATSUSHITA, T., FUJIMOTO, M., STEEBER, D. A., TEDDER, T. F., TAKEHARA, K. & SATO, S. 2006. L-selectin and intercellular adhesion molecule-1 regulate the development of Concanavalin A-induced liver injury. *J Leukoc Biol,* 79**,** 696-705.

KEHREL, B., WIERWILLE, S., CLEMETSON, K. J., ANDERS, O., STEINER, M., GRAHAM KNIGHT, C., FARNDALE, R. W., OKUMA, M. & BARNES, M. J. 1998. Glycoprotein VI Is a Major Collagen Receptor for Platelet Activation: It Recognizes the Platelet-Activating Quaternary Structure of

Collagen, Whereas CD36, Glycoprotein IIb/IIIa, and von Willebrand Factor Do Not. *Blood,* 91**,** 491-499.

KHANDOGA, A., BIBERTHALER, P., MESSMER, K. & KROMBACH, F. 2003. Platelet–endothelial cell interactions during hepatic ischemia–reperfusion in vivo: a systematic analysis. *Microvascular research,* 65**,** 71-77.

KHANDOGA, A., HANSCHEN, M., KESSLER, J. S. & KROMBACH, F. 2006a. CD4+ T cells contribute to postischemic liver injury in mice by interacting with sinusoidal endothelium and platelets. *Hepatology,* 43**,** 306-15.

KHANDOGA, A., KESSLER, J. S., HANSCHEN, M., KHANDOGA, A. G., BURGGRAF, D., REICHEL, C., HAMANN, G. F., ENDERS, G. & KROMBACH, F. 2006b. Matrix metalloproteinase-9 promotes neutrophil and T cell recruitment and migration in the postischemic liver. *J Leukoc Biol,* 79**,** 1295-305.

KIM, H. K., SONG, K. S., PARK, Y. S., KANG, Y. H., LEE, Y. J., LEE, K. R., RYU, K. W., BAE, J. M. & KIM, S. 2003. Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in patients with gastric cancer: possible role of a metastasis predictor. *European Journal of Cancer,* 39**,** 184-191.

KING, C., TANGYE, S. G. & MACKAY, C. R. 2008. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu Rev Immunol,* 26**,** 741-66.

KITAMURA, D., ROES, J., KUHN, R. & RAJEWSKY, K. 1991. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin [mu] chain gene. *Nature,* 350**,** 423-426.

KNIJFF-DUTMER, E. A. J., KOERTS, J., NIEUWLAND, R., KALSBEEK-BATENBURG, E. M. & VAN DE LAAR, M. A. F. J. 2002. Elevated levels of platelet microparticles are associated with disease activity in rheumatoid arthritis. *Arthritis & Rheumatism,* 46**,** 1498-1503.

KNOLLE, P. A., GERKEN, G., LOSER, E., DIENES, H. P., GANTNER, F., TIEGS, G., MEYER ZUM BUSCHENFELDE, K. H. & LOHSE, A. W. 1996. Role of sinusoidal endothelial cells of the liver in concanavalin A-induced hepatic injury in mice. *Hepatology,* 24**,** 824-9.

KOLIOS, G., VALATAS, V. & KOUROUMALIS, E. 2006. Role of Kupffer cells in the pathogenesis of liver disease. *World Journal of Gastroenterology,* 12**,** 7413.

KONSTANTINIDES, S., WARE, J., MARCHESE, P., ALMUS-JACOBS, F., LOSKUTOFF, D. J. & RUGGERI, Z. M. 2006. Distinct antithrombotic consequences of platelet glycoprotein Ibalpha and VI deficiency in a mouse model of arterial thrombosis. *J Thromb Haemost,* 4**,** 2014-21.

KOSHIISHI, I., SHIZARI, M. & UNDERHILL, C. 1994. CD44 can mediate the adhesion of platelets to hyaluronan. *Blood,* 84**,** 390-396.

KRÄMER, B., KÖRNER, C., KEBSCHULL, M., GLÄSSNER, A., EISENHARDT, M., NISCHALKE, H. D., ALEXANDER, M., SAUERBRUCH, T., SPENGLER, U. & NATTERMANN, J. 2012. Natural killer p46High expression defines a natural killer cell subset that is potentially involved in control of hepatitis C virus replication and modulation of liver fibrosis. *Hepatology,* 56**,** 1201-1213.

KROLL, M. H., HARRIS, T. S., MOAKE, J. L., HANDIN, R. I. & SCHAFER, A. I. 1991. von Willebrand factor binding to platelet GpIb initiates signals for platelet activation. *J Clin Invest,* 88**,** 1568-73.

KUBOKI, S., SAKAI, N., TSCHÖP, J., EDWARDS, M. J., LENTSCH, A. B. & CALDWELL, C. C. 2009. Distinct contributions of CD4+ T cell subsets in hepatic ischemia/reperfusion injury. *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 296**,** G1054-G1059.

KUNKEL, E. J. & LEY, K. 1996. Distinct Phenotype of E-Selectin–Deficient Mice: E-Selectin Is Required for Slow Leukocyte Rolling In Vivo. *Circ Res,* 79**,** 1196-1204.

LALOR, P., LAI, W., CURBISHLEY, S., SHETTY, S. & ADAMS, D. 2006. Human hepatic sinusoidal endothelial cells can be distinguished by expression of phenotypic markers related to their specialised functions in vivo. *World journal of gastroenterology: WJG,* 12**,** 5429-5439.

LALOR, P. F., CLEMENTS, J. M., PIGOTT, R., HUMPHRIES, M. J., SPRAGG, J. H. & NASH, G. B. 1997. Association between receptor density, cellular activation, and transformation of adhesive behavior of flowing lymphocytes binding to VCAM-1. *European journal of immunology,* 27**,** 1422-1426.

LALOR, P. F., SHIELDS, P., GRANT, A. & ADAMS, D. H. 2002a. Recruitment of lymphocytes to the human liver. *Immunol Cell Biol,* 80**,** 52-64.

LALOR, P. F., SHIELDS, P., GRANT, A. J. & ADAMS, D. H. 2002b. Recruitment of lymphocytes to the human liver. *Immunology and cell biology,* 80**,** 52-64.

LANG, P. A., CONTALDO, C., GEORGIEV, P., EL-BADRY, A. M., RECHER, M., KURRER, M., CERVANTES-BARRAGAN, L., LUDEWIG, B., CALZASCIA, T., BOLINGER, B., MERKLER, D., ODERMATT, B., BADER, M., GRAF, R., CLAVIEN, P.-A., HEGAZY, A. N., LOHNING, M., HARRIS, N. L., OHASHI, P. S., HENGARTNER, H., ZINKERNAGEL, R. M. & LANG, K. S. 2008. Aggravation of viral hepatitis by platelet-derived serotonin. *Nat Med,* 14**,** 756-761.

LE BOURHIS, L., MBURU, Y. K. & LANTZ, O. 2013. MAIT cells, surveyors of a new class of antigen: development and functions. *Current opinion in immunology,* 25**,** 174-180.

LE MOINE, O., LOUIS, H., DEMOLS, A., DESALLE, F., DEMOOR, F., QUERTINMONT, E., GOLDMAN, M. & DEVIÈRE, J. 2000. Cold liver ischemia-reperfusion injury critically depends on liver T cells and is improved by donor pretreatment with interleukin 10 in mice. *Hepatology,* 31**,** 1266-1274.

LEE, R. G. 1989. Nonalcoholic steatohepatitis: A study of 49 patients. *Human Pathology,* 20**,** 594-598.

LESURTEL, M., GRAF, R., ALEIL, B., WALTHER, D. J., TIAN, Y., JOCHUM, W., GACHET, C., BADER, M. & CLAVIEN, P.-A. 2006. Platelet-derived serotonin mediates liver regeneration. *Science,* 312**,** 104-107.

LEY, K., LAUDANNA, C., CYBULSKY, M. I. & NOURSHARGH, S. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol,* 7**,** 678-689.

LI, N., GOODALL, A. H. & HJEMDAHL, P. 1999. Efficient flow cytometric assay for platelet-leukocyte aggregates in whole blood using fluorescence signal triggering. *Cytometry,* 35**,** 154-161.

LI, N., JI, Q. & HJEMDAHL, P. 2006. Platelet–lymphocyte conjugation differs between lymphocyte subpopulations. *Journal of Thrombosis and Haemostasis,* 4**,** 874-881.

LIASKOU, E. 2010. *MAdCAM-1 Expression and Function in Human Liver.* University of Birmingham.

LINFERT, D., CHOWDHRY, T. & RABB, H. 2009. Lymphocytes and ischemia-reperfusion injury. *Transplant Rev (Orlando),* 23**,** 1-10.

LIU, C. Y., BATTAGLIA, M., LEE, S. H., SUN, Q.-H., ASTER, R. H. & VISENTIN, G. P. 2005. Platelet Factor 4 Differentially Modulates CD4+CD25+ (Regulatory) versus CD4+CD25− (Nonregulatory) T Cells. *The Journal of Immunology,* 174**,** 2680-2686.

LONGHI, M. S., MITRY, R. R., SAMYN, M., SCALORI, A., HUSSAIN, M. J., QUAGLIA, A., MIELI-VERGANI, G., MA, Y. & VERGANI, D. 2009. Vigorous activation of monocytes in juvenile autoimmune liver disease escapes the control of regulatory T-cells. *Hepatology,* 50**,** 130-142.

LOUIS, H., LE MOINE, O., PENY, M. O., QUERTINMONT, E., FOKAN, D., GOLDMAN, M. & DEVIERE, J. 1997. Production and role of interleukin-10 in concanavalin A-induced hepatitis in mice. *Hepatology,* 25**,** 1382-9.

LUETTIG, B., PAPE, L., BODE, U., BELL, E. B., SPARSHOTT, S. M., WAGNER, S. & WESTERMANN, J. 1999. Naive and Memory T Lymphocytes Migrate in Comparable Numbers Through Normal Rat Liver: Activated T Cells Accumulate in the Periportal Field. *The Journal of Immunology,* 163**,** 4300-4307.

LUND, F. E. 2008. Cytokine-producing B lymphocytes-key regulators of immunity. *Curr Opin Immunol,* 20**,** 332-8.

MAHAUT-SMITH, M. P., JONES, S. & EVANS, R. J. 2011. The P2X1 receptor and platelet function. *Purinergic Signal,* 7**,** 341-56.

MARCH, S., GARCIA-PAGAN, J. C., MASSAGUER, A., PIZCUETA, P., PANES, J., ENGEL, P. & BOSCH, J. 2005. P-selectin mediates leukocyte rolling in concanavalin-A-induced hepatitis. *Liver Int,* 25**,** 1053-60.

MARMUR, J. D., ROSSIKHINA, M., GUHA, A., FYFE, B., FRIEDRICH, V., MENDLOWITZ, M., NEMERSON, Y. & TAUBMAN, M. B. 1993. Tissue factor is rapidly induced in arterial smooth muscle after balloon injury. *J Clin Invest,* 91**,** 2253-9.

MARTÍ, R., MÚRIO, E., VARELA, E., BILBAO, I., PASCUAL, C., MARGARIT, C. & SEGURA, R. M. 2004. Xanthine oxidoreductase and preservation injury in human liver transplantation. *Transplantation,* 77**,** 1239-1245.

MARTIN, M., MORY, C., PRESCHER, A., WITTEKIND, C., FIEDLER, M. & UHLMANN, D. 2010. Protective effects of early CD4+ T cell reduction in hepatic ischemia/reperfusion injury. *Journal of Gastrointestinal Surgery,* 14**,** 511-519.

MARTINO, G., GROHOVAZ, F., BRAMBILLA, E., CODAZZI, F., CONSIGLIO, A., CLEMENTI, E., FILIPPI, M., COMI, G. & GRIMALDI, L. M. E. 1998. Proinflammatory cytokines regulate antigen-independent T-cell Activation by two separate calcium-signaling pathways in multiple sclerosis patients. *Annals of Neurology,* 43**,** 340-349.

MASSAGUER, A., PEREZ-DEL-PULGAR, S., ENGEL, P., SERRATOSA, J., BOSCH, J. & PIZCUETA, P. 2002. Concanavalin-A-induced liver injury is severely impaired in mice deficient in P-selectin. *J Leukoc Biol,* 72**,** 262-270.

MASSBERG, S., ENDERS, G., DE MELO MATOS, F. C., TOMIC, L. I. D., LEIDERER, R., EISENMENGER, S., MESSMER, K. & KROMBACH, F. 1999. Fibrinogen deposition at the postischemic vessel wall promotes platelet adhesion during ischemia-reperfusion in vivo. *Blood,* 94**,** 3829-3838.

MASSBERG, S., ENDERS, G., LEIDERER, R., EISENMENGER, S., VESTWEBER, D., KROMBACH, F. & MESSMER, K. 1998. Platelet-endothelial cell interactions during ischemia/reperfusion: the role of P-selectin. *Blood,* 92**,** 507-515.

MASSBERG, S., KONRAD, I., SCHURZINGER, K., LORENZ, M., SCHNEIDER, S., ZOHLNHOEFER, D., HOPPE, K., SCHIEMANN, M., KENNERKNECHT, E., SAUER, S., SCHULZ, C., KERSTAN, S., RUDELIUS, M., SEIDL, S., SORGE, F., LANGER, H., PELUSO, M., GOYAL, P., VESTWEBER, D., EMAMBOKUS, N. R., BUSCH, D. H., FRAMPTON, J. & GAWAZ, M. 2006. Platelets secrete stromal cell-derived factor 1alpha and recruit bone marrow-derived progenitor cells to arterial thrombi in vivo. *J Exp Med,* 203**,** 1221-33.

MATZDORFF, A. C., KUHNEL, G., KEMKES-MATTHES, B. & PRALLE, H. 1998. Quantitative assessment of platelets, platelet microparticles, and platelet aggregates with flow cytometry. *Journal of Laboratory and Clinical Medicine,* 131**,** 507-517.

MAURI, C. & BOSMA, A. 2012. Immune regulatory function of B cells. *Annu Rev Immunol,* 30**,** 221-41.

MAUSE, S. F., VON HUNDELSHAUSEN, P., ZERNECKE, A., KOENEN, R. R. & WEBER, C. 2005. Platelet Microparticles: A Transcellular Delivery System for RANTES Promoting Monocyte Recruitment on Endothelium. *Arterioscler Thromb Vasc Biol,* 25**,** 1512-1518.

MCCLAIN, C. J. & COHEN, D. A. 1989. Increased tumor necrosis factor production by monocytes in alcoholic hepatitis. *Hepatology,* 9**,** 349-351.

MCCORMICK, P. A. & MURPHY, K. M. 2000. Splenomegaly, hypersplenism and coagulation abnormalities in liver disease. *Baillieres Best Pract Res Clin Gastroenterol,* 14**,** 1009-31.

MCCUSKEY, R. S. 2000. Morphological mechanisms for regulating blood flow through hepatic sinusoids. *Liver,* 20**,** 3-7.

MCDONALD, B., MCAVOY, E. F., LAM, F., GILL, V., DE LA MOTTE, C., SAVANI, R. C. & KUBES, P. 2008. Interaction of CD44 and hyaluronan is the dominant mechanism for neutrophil sequestration in inflamed liver sinusoids. *J Exp Med,* 205**,** 915-927.

MCFADDEN, R. G., BISHOP, M. A., CAVENEY, A. N. & FRAHER, L. J. 1995. Effect of platelet activating factor (PAF) on the migration of human lymphocytes. *Thorax,* 50**,** 265-9.

MCNICOL, A. & ISRAELS, S. J. 2008. Beyond hemostasis: the role of platelets in inflammation, malignancy and infection. *Cardiovascular & Haematological Disorders-Drug Targets (Formerly Current Drug Targets-Cardiovascular & Hematological Disorders),* 8**,** 99-117.

MEHAL, W. Z., JUEDES, A. E. & CRISPE, I. N. 1999. Selective Retention of Activated CD8+ T Cells by the Normal Liver. *The Journal of Immunology,* 163**,** 3202-3210.

MELHEM, A., MUHANNA, N., BISHARA, A., ALVAREZ, C. E., ILAN, Y., BISHARA, T., HORANI, A., NASSAR, M., FRIEDMAN, S. L. & SAFADI, R. 2006. Anti-fibrotic activity of NK cells in experimental liver injury through killing of activated HSC. *Journal of hepatology,* 45**,** 60-71.

MIDDLETON, J., PATTERSON, A. M., GARDNER, L., SCHMUTZ, C. & ASHTON, B. A. 2002. Leukocyte extravasation: chemokine transport and presentation by the endothelium. *Blood,* 100**,** 3853-3860.

MIELE, L. F., TURHAN, A., LEE, G. S., LIN, M., RAVNIC, D., TSUDA, A., KONERDING, M. A. & MENTZER, S. J. 2009. Blood Flow Patterns Spatially Associated with Platelet Aggregates in Murine Colitis. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology,* 292**,** 1143-1153.

MILINKOVIC, M., ANTIN, J. H., HERGRUETER, C. A., UNDERHILL, C. B. & SACKSTEIN, R. 2004. CD44-hyaluronic acid interactions mediate shear-resistant binding of lymphocytes to dermal endothelium in acute cutaneous GVHD. *Blood,* 103**,** 740-2.

MILLAN, J., HEWLETT, L., GLYN, M., TOOMRE, D., CLARK, P. & RIDLEY, A. J. 2006. Lymphocyte transcellular migration occurs through recruitment of endothelial ICAM-1 to caveola- and F-actin-rich domains. *Nat Cell Biol,* 8**,** 113-123.

MIYAZAWA, Y., TSUTSUI, H., MIZUHARA, H., FUJIWARA, H. & KANEDA, K. 1998. Involvement of intrasinusoidal hemostasis in the development of concanavalin A-induced hepatic injury in mice. *Hepatology,* 27**,** 497-506.

MIZUHARA, H., O'NEILL, E., SEKI, N., OGAWA, T., KUSUNOKI, C., OTSUKA, K., SATOH, S., NIWA, M., SENOH, H. & FUJIWARA, H. 1994. T cell activation-associated hepatic injury: mediation by tumor necrosis factors and protection by interleukin 6. *J Exp Med,* 179**,** 1529-37.

MIZUTA, K., OHMORI, M., MIYASHITA, F., KITOH, Y., FUJIMURA, A., MORI, M., KANNO, T., HASHIZUME, K. & KOBAYASHI, E. 1999. Effect of Pretreatment with FTY720 and Cyclosporin on Ischaemia-Reperfusion Injury of the Liver in Rats. *Journal of Pharmacy and Pharmacology,* 51**,** 1423-1428.

MONTECUCCO, F., STEFFENS, S., BURGER, F., DA COSTA, A., BIANCHI, G., BERTOLOTTO, M., MACH, F., DALLEGRI, F. & OTTONELLO, L. 2008. Tumor necrosis factor-alpha (TNF-α) induces integrin CD11b/CD18 (Mac-1) up-regulation and migration to the CC chemokine CCL3 (MIP-1α) on human neutrophils through defined signalling pathways. *Cellular Signalling,* 20**,** 557-568.

MOORE, J. W., BEATTIE, L., DALTON, J. E., OWENS, B. M., MAROOF, A., COLES, M. C. & KAYE, P. M. 2012. B cell: T cell interactions occur within hepatic granulomas during experimental visceral leishmaniasis. *PloS one,* 7**,** e34143.

MOROI, M. & JUNG, S. 2007. A mechanism to safeguard platelet adhesion under high shear flow: von Willebrand factor–glycoprotein Ib and integrin α2β1–collagen interactions make complementary, collagen-type-specific contributions to adhesion. *Journal of Thrombosis and Haemostasis,* 5**,** 797-803.

MOSMANN, T. R., LI, L. & SAD, S. 1997. Functions of CD8 T-cell subsets secreting different cytokine patterns. *Semin Immunol,* 9**,** 87-92.

MULIVOR, A. W. & LIPOWSKY, H. H. 2002. Role of glycocalyx in leukocyte-endothelial cell adhesion. *Am J Physiol Heart Circ Physiol,* 283**,** H1282-91.

MULIVOR, A. W. & LIPOWSKY, H. H. 2004. Inflammation-and ischemia-induced shedding of venular glycocalyx. *American Journal of Physiology-Heart and Circulatory Physiology,* 55**,** H1672.

MURAI, M., YONEYAMA, H., HARADA, A., YI, Z., VESTERGAARD, C., GUO, B., SUZUKI, K., ASAKURA, H. & MATSUSHIMA, K. 1999. Active participation of CCR5+ CD8+ T lymphocytes in the pathogenesis of liver injury in graft-versus-host disease. *Journal of Clinical Investigation,* 104**,** 49.

NAGORE, N. & SCHEUER, P. J. 1988. The pathology of diabetic hepatitis. *The Journal of Pathology,* 156**,** 155-160.

NAKAMURA, T., KAMBAYASHI, J.-I., OKUMA, M. & TANDON, N. N. 1999. Activation of the GP IIb-IIIa Complex Induced by Platelet Adhesion to Collagen Is Mediated by Both α2β1 Integrin and GP VI. *Journal of Biological Chemistry,* 274**,** 11897-11903.

NAKANO, Y., KONDO, T., MATSUO, R., HASHIMOTO, I., KAWASAKI, T., KOHNO, K., MYRONOVYCH, A., TADANO, S., HISAKURA, K., IKEDA, O., WATANABE, M., MURATA, S., FUKUNAGA, K. & OHKOHCHI, N. 2008. Platelet dynamics in the early phase of postischemic liver in vivo. *J Surg Res,* 149**,** 192-8.

NAKASHIMA, H., KINOSHITA, M., NAKASHIMA, M., HABU, Y., SHONO, S., UCHIDA, T., SHINOMIYA, N. & SEKI, S. 2008. Superoxide produced by Kupffer cells is an essential effector in concanavalin A–induced hepatitis in mice. *Hepatology,* 48**,** 1979-1988.

NANDI, A., ESTESS, P. & SIEGELMAN, M. 2004. Bimolecular complex between rolling and firm adhesion receptors required for cell arrest; CD44 association with VLA-4 in T cell extravasation. *Immunity,* 20**,** 455-65.

NANJI, A. A. 2002. Role of Kupffer cells in alcoholic hepatitis. *Alcohol,* 27**,** 13-15.

NI, H., YUEN, P. S. T., PAPALIA, J. M., TREVITHICK, J. E., SAKAI, T., FÄSSLER, R., HYNES, R. O. & WAGNER, D. D. 2003. Plasma fibronectin promotes thrombus growth and stability in injured arterioles. *Proceedings of the National Academy of Sciences,* 100**,** 2415-2419.

NIESWANDT, B., BERGMEIER, W., RACKEBRANDT, K., GESSNER, J. E. & ZIRNGIBL, H. 2000. Identification of critical antigen-specific mechanisms in the development of immune thrombocytopenic purpura in mice. *Blood,* 96**,** 2520-7.

NIESWANDT, B. & WATSON, S. P. 2003. Platelet-collagen interaction: is GPVI the central receptor? *Blood,* 102**,** 449-461.

NIEUWDORP, M., VAN HAEFTEN, T. W., GOUVERNEUR, M. C. L. G., MOOIJ, H. L., VAN LIESHOUT, M. H. P., LEVI, M., MEIJERS, J. C. M., HOLLEMAN, F., HOEKSTRA, J. B. L., VINK, H., KASTELEIN, J. J. P. & STROES, E. S. G. 2006. Loss of Endothelial Glycocalyx During Acute Hyperglycemia Coincides With Endothelial Dysfunction and Coagulation Activation In Vivo. *Diabetes,* 55**,** 480-486.

NIEVELSTEIN, P., D'ALESSIO, P. & SIXMA, J. 1988. Fibronectin in platelet adhesion to human collagen types I and III. Use of nonfibrillar and fibrillar collagen in flowing blood studies. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 8**,** 200-206.

NOCITO, A., GEORGIEV, P., DAHM, F., JOCHUM, W., BADER, M., GRAF, R. & CLAVIEN, P.-A. 2007. Platelets and platelet-derived serotonin promote tissue repair after normothermic hepatic ischemia in mice. *Hepatology,* 45**,** 369-376.

NOMURA, S., IMAMURA, A., OKUNO, M., KAMIYAMA, Y., FUJIMURA, Y., IKEDA, Y. & FUKUHARA, S. 2000. Platelet-Derived Microparticles in Patients with Arteriosclerosis Obliterans: Enhancement of High Shear-Induced Microparticle Generation by Cytokines. *Thrombosis Research,* 98**,** 257-268.

NORMAN, K. E., MOORE, K. L., MCEVER, R. P. & LEY, K. 1995. Leukocyte rolling in vivo is mediated by P-selectin glycoprotein ligand- 1. *Blood,* 86**,** 4417-4421.

NOVOBRANTSEVA, T. I., MAJEAU, G. R., AMATUCCI, A., KOGAN, S., BRENNER, I., CASOLA, S., SHLOMCHIK, M. J., KOTELIANSKY, V., HOCHMAN, P. S. & IBRAGHIMOV, A. 2005. Attenuated liver fibrosis in the absence of B cells. *J Clin Invest,* 115**,** 3072-82.

OGAWA, K., KONDO, T., TAMURA, T., MATSUMURA, H., FUKUNAGA, K., ODA, T. & OHKOHCHI, N. 2013. Influence of Kupffer cells and platelets on ischemia-reperfusion injury in mild steatotic liver. *World journal of gastroenterology: WJG,* 19**,** 1396.

OGINO, T., MIURA, S., KOMOTO, S., HARA, Y., HOKARI, R., TSUZUKI, Y., WATANABE, C., KOSEKI, S., NAGATA, H., HACHIMURA, S., KAMINOGAWA, S. & ISHII, H. 2004. Senescence-associated decline of lymphocyte migration in gut-associated lymphoid tissues of rat small intestine. *Mechanisms of Ageing and Development,* 125**,** 191-199.

OLSON, J. P., MILLER, L. L. & TROUP, S. B. 1966. Synthesis of clotting factors by the isolated perfused rat liver. *J Clin Invest,* 45**,** 690-701.

PAK, S., KONDO, T., NAKANO, Y., MURATA, S., FUKUNAGA, K., ODA, T., SASAKI, R. & OHKOHCHI, N. 2010. Platelet adhesion in the sinusoid caused hepatic injury by neutrophils after hepatic ischemia reperfusion. *Platelets,* 21**,** 282-288.

PALACIOS, R. 1982. Concanavalin A triggers T lymphocytes by directly interacting with their receptors for activation. *The Journal of Immunology,* 128**,** 337-42.

PANES, J., PERRY, M., ANDERSON, D., MANNING, A., LEONE, B., CEPINSKAS, G., ROSENBLOOM, C., MIYASAKA, M., KVIETYS, P. & GRANGER, D. 1995. Regional differences in constitutive and induced ICAM-1 expression in vivo. *American Journal of Physiology-Heart and Circulatory Physiology,* 269**,** H1955-H1964.

PAPAKONSTANTINOU, E., KARAKIULAKIS, G., ROTH, M. & BLOCK, L. 1995. Platelet-derived growth factor stimulates the secretion of hyaluronic acid by proliferating human vascular smooth muscle cells. *Proceedings of the National Academy of Sciences,* 92**,** 9881-9885.

PELLICCI, D. G., HAMMOND, K. J. L., ULDRICH, A. P., BAXTER, A. G., SMYTH, M. J. & GODFREY, D. I. 2002. A Natural Killer T (NKT) Cell Developmental Pathway Involving a Thymus-dependent NK1.1−CD4+ CD1d-dependent Precursor Stage. *J Exp Med,* 195**,** 835-844.

PETERS, M. J., DIXON, G., KOTOWICZ, K. T., HATCH, D. J., HEYDERMAN, R. S. & KLEIN, N. J. 1999. Circulating platelet–neutrophil complexes represent a subpopulation of activated neutrophils primed for adhesion, phagocytosis and intracellular killing. *Br J Haematol,* 106**,** 391-399.

PHIPPS, R. P. 2000. Atherosclerosis: The emerging role of inflammation and the CD40–CD40 ligand system. *Proceedings of the National Academy of Sciences,* 97**,** 6930-6932.

PITCHFORD, S. C., MOMI, S., GIANNINI, S., CASALI, L., SPINA, D., PAGE, C. P. & GRESELE, P. 2004. Platelet P-selectin is required for pulmonary eosinophil and lymphocyte recruitment in a murine model of allergic inflammation. *Blood,* 105**,** 2074-2081.

PLEIMAN, C. M., D'AMBROSIO, D. & CAMBIER, J. C. 1994. The B-cell antigen receptor complex: structure and signal transduction. *Immunology Today,* 15**,** 393-399.

RACANELLI, V. & REHERMANN, B. 2006. The liver as an immunological organ. *Hepatology,* 43**,** S54-S62.

RAND, J. H., GLANVILLE, R. W., WU, X. X., ROSS, J. M., ZANGARI, M., GORDON, R. E., SCHWARTZ, E. & POTTER, B. J. 1997. The significance of subendothelial von Willebrand factor. *Thromb Haemost,* 78**,** 445-50.

RENNER, B., STRASSHEIM, D., AMURA, C. R., KULIK, L., LJUBANOVIC, D., GLOGOWSKA, M. J., TAKAHASHI, K., CARROLL, M. C., HOLERS, V. M. & THURMAN, J. M. 2010. B Cell Subsets Contribute to Renal Injury and Renal Protection after Ischemia/Reperfusion. *The Journal of Immunology,* 185**,** 4393-4400.

ROBERTS, D. D., WILLIAMS, S. B., GRALNICK, H. R. & GINSBURG, V. 1986. von Willebrand factor binds specifically to sulfated glycolipids. *J Biol Chem,* 261**,** 3306-9.

ROCKEY, D. C. & WEISIGER, R. A. 1996. Endothelin induced contractility of stellate cells from normal and cirrhotic rat liver: implications for regulation of portal pressure and resistance. *Hepatology,* 24**,** 233-240.

RODRIGUEZ, R. M., PITZALIS, C., KINGSLEY, G. H., HENDERSON, E., HUMPHRIES, M. J. & PANAYI, G. S. 1992. T lymphocyte adhesion to fibronectin (FN): a possible mechanism for T cell accumulation in the rheumatoid joint. *Clinical & Experimental Immunology,* 89**,** 439-445.

ROMAGNANI, S. 1999. Th1/Th2 cells. *Inflamm Bowel Dis,* 5**,** 285-94.

RUGGERI, Z. M. & MENDOLICCHIO, G. L. 2007. Adhesion mechanisms in platelet function. *Circ Res,* 100**,** 1673-85.

RUMJANTSEVA, V., GREWAL, P. K., WANDALL, H. H., JOSEFSSON, E. C., SØRENSEN, A. L., LARSON, G., MARTH, J. D., HARTWIG, J. H. & HOFFMEISTER, K. M. 2009. Dual roles for hepatic lectin receptors in the clearance of chilled platelets. *Nature medicine,* 15**,** 1273-1280.

SAELMAN, E. U., NIEUWENHUIS, H. K., HESE, K. M., DE GROOT, P. G., HEIJNEN, H. F., SAGE, E. H., WILLIAMS, S., MCKEOWN, L., GRALNICK, H. R. & SIXMA, J. J. 1994. Platelet adhesion to collagen types I through VIII under conditions of stasis and flow is mediated by GPIa/IIa (alpha 2 beta 1-integrin). *Blood,* 83**,** 1244-50.

ŠAFAŘíK, I. & ŠAFAŘíKOVÁ, M. 1999. Use of magnetic techniques for the isolation of cells. *Journal of Chromatography B: Biomedical Sciences and Applications,* 722**,** 33-53.

SANFORD, T. D. & COLBY, E. D. 1980. Effect of xylazine and ketamine on blood pressure, heart rate and respiratory rate in rabbits. *Laboratory animal science,* 30**,** 519-523.

SARRATT, K. L., CHEN, H., ZUTTER, M. M., SANTORO, S. A., HAMMER, D. A. & KAHN, M. L. 2005. GPVI and α2β1 play independent critical roles during platelet adhesion and aggregate formation to collagen under flow. *Blood,* 106**,** 1268-1277.

SASS, G., HEINLEIN, S., AGLI, A., BANG, R., SCHÜMANN, J. & TIEGS, G. 2002. CYTOKINE EXPRESSION IN THREE MOUSE MODELS OF EXPERIMENTAL HEPATITIS. *Cytokine,* 19**,** 115-120.

SAVAGE, B., ALMUS-JACOBS, F. & RUGGERI, Z. M. 1998. Specific Synergy of Multiple Substrate–Receptor Interactions in Platelet Thrombus Formation under Flow. *Cell,* 94**,** 657-666.

SAVAGE, B., SALDÍVAR, E. & RUGGERI, Z. M. 1996. Initiation of Platelet Adhesion by Arrest onto Fibrinogen or Translocation on von Willebrand Factor. *Cell,* 84**,** 289-297.

SAVAGE, B., SIXMA, J. J. & RUGGERI, Z. M. 2002. Functional self-association of von Willebrand factor during platelet adhesion under flow. *Proc Natl Acad Sci U S A,* 99**,** 425-30.

SAWAYA, D. E. J., ZIBAR, G. B., MINARDI, A., BILTON, B., BURNEY, D., GRANGER, D. N., MCDONALD, J. C. & BROWN, M. 1999. P-Selectin Contributes to the Initial Recruitment of Rolling and Adherent Leukocytes in Hepatic Venules After Ischemia/Reperfusion. *Shock,* 12**,** 227-232.

SCAPINI, P., LAPINET-VERA, J. A., GASPERINI, S., CALZETTI, F., BAZZONI, F. & CASSATELLA, M. A. 2000. The neutrophil as a cellular source of chemokines. *Immunological Reviews,* 177**,** 195-203.

SCHALL, T. J., BACON, K., TOY, K. J. & GOEDDEL, D. V. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature,* 347**,** 669-71.

SCHUMANN, J., WOLF, D., PAHL, A., BRUNE, K., PAPADOPOULOS, T., VAN ROOIJEN, N. & TIEGS, G. 2000. Importance of Kupffer cells for T-cell-dependent liver injury in mice. *Am J Pathol,* 157**,** 1671-83.

SCOAZEC, J. Y., RACINE, L., COUVELARD, A., FLEJOU, J. F. & FELDMANN, G. 1994. Endothelial cell heterogeneity in the normal human liver acinus: in situ immunohistochemical demonstration. *Liver,* 14**,** 113-23.

SERRACINO-INGLOTT, F., HABIB, N. A. & MATHIE, R. T. 2001. Hepatic ischemia-reperfusion injury. *The American Journal of Surgery,* 181**,** 160-166.

SHAO, J.-Y., YU, Y. & DUSTIN, M. 2005. A Model for CD2/CD58-Mediated Adhesion Strengthening. *Annals of Biomedical Engineering,* 33**,** 483-493.

SHARKEY, R. M., NATALE, A., GOLDENBERG, D. M. & MATTES, M. J. 1991. Rapid blood clearance of immunoglobulin G2a and immunoglobulin G2b in nude mice. *Cancer research,* 51**,** 3102-3107.

SHEIKH, S., PARHAR, R. S., BAKHEET, R., SALEH, S., COLLISON, K. & AL-MOHANNA, F. 2004. Immobilization of rolling NK cells on platelet-borne P-selectin under flow by proinflammatory stimuli, interleukin-12, and leukotriene B4. *J Leukoc Biol,* 76**,** 603-8.

SHIELDS, P. L., MORLAND, C. M., SALMON, M., QIN, S., HUBSCHER, S. G. & ADAMS, D. H. 1999. Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver. *The Journal of Immunology,* 163**,** 6236-6243.

SHIRAISHI, M., HIROYASU, S., NAGAHAMA, M., MIYAGUNI, T., HIGA, T., TOMORI, H., OKUHAMA, Y., KUSANO, T. & MUTO, Y. 1997. Role of Exogenousl-Arginine in Hepatic Ischemia–Reperfusion Injury. *Journal of Surgical Research,* 69**,** 429-434.

SHULMAN, Z., SHINDER, V., KLEIN, E., GRABOVSKY, V., YEGER, O., GERON, E., MONTRESOR, A., BOLOMINI-VITTORI, M., FEIGELSON, S. W., KIRCHHAUSEN, T., LAUDANNA, C., SHAKHAR, G. & ALON, R. 2009. Lymphocyte crawling and transendothelial migration require chemokine triggering of high-affinity LFA-1 integrin. *Immunity,* 30**,** 384-96.

SINDRAM, D., PORTE, R. J., HOFFMAN, M. R., BENTLEY, R. C. & CLAVIEN, P. A. 2000. Platelets induce sinusoidal endothelial cell apoptosis upon reperfusion of the cold ischemic rat liver. *Gastroenterology,* 118**,** 183-191.

SINGBARTL, K., THATTE, J., SMITH, M. L., WETHMAR, K., DAY, K. & LEY, K. 2001. A CD2-Green Fluorescence Protein-Transgenic Mouse Reveals Very Late Antigen-4-Dependent CD8+ Lymphocyte Rolling in Inflamed Venules. *The Journal of Immunology,* 166**,** 7520-7526.

SITIA, G., AIOLFI, R., DI LUCIA, P., MAINETTI, M., FIOCCHI, A., MINGOZZI, F., ESPOSITO, A., RUGGERI, Z. M., CHISARI, F. V. & IANNACONE, M. 2012. Antiplatelet therapy prevents hepatocellular carcinoma and improves survival in a mouse model of chronic hepatitis B. *Proceedings of the National Academy of Sciences,* 109**,** E2165-E2172.

SMITH, J. A. 1994. Neutrophils, host defense, and inflammation: a double-edged sword. *J Leukoc Biol,* 56**,** 672-86.

STEINHOFF, G., BEHREND, M., SCHRADER, B., DUIJVESTIJN, A. M. & WONIGEIT, K. 1993. Expression patterns of leukocyte adhesion ligand molecules on human liver endothelia. Lack of ELAM-1 and CD62 inducibility on sinusoidal endothelia and distinct distribution of VCAM-1, ICAM-1, ICAM-2, and LFA-3. *Am J Pathol,* 142**,** 481-8.

STOOP, R., GÁL, I., GLANT, T. T., MCNEISH, J. D. & MIKECZ, K. 2002. Trafficking of CD44-deficient murine lymphocytes under normal and inflammatory conditions. *Eur J Immunol,* 32**,** 2532-2542.

SUMAGIN, R. & SARELIUS, I. H. 2007. A role for ICAM-1 in maintenance of leukocyte-endothelial cell rolling interactions in inflamed arterioles. *American Journal of Physiology - Heart and Circulatory Physiology,* 293**,** H2786-H2798.

SZABO, M. C., BUTCHER, E. C., MCINTYRE, B. W., SCHALL, T. J. & BACON, K. B. 1997. RANTES stimulation of T lymphocyte adhesion and activation: role for LFA-1 and ICAM-3. *Eur J Immunol,* 27**,** 1061-8.

SZEKANECZ, Z., HUMPHRIES, M. J. & AGER, A. 1992. Lymphocyte adhesion to high endothelium is mediated by two beta 1 integrin receptors for fibronectin, alpha 4 beta 1 and alpha 5 beta 1. *J Cell Sci,* 101 (Pt 4)**,** 885-94.

TAGAWA, Y., SEKIKAWA, K. & IWAKURA, Y. 1997. Suppression of concanavalin A-induced hepatitis in IFN-gamma(-/-) mice, but not in TNF-alpha(-/-) mice: role for IFN-gamma in activating apoptosis of hepatocytes. *The Journal of Immunology,* 159**,** 1418-28.

TAÏEB, J., MATHURIN, P., ELBIM, C., CLUZEL, P., ARCE-VICIOSO, M., BERNARD, B., OPOLON, P., GOUGEROT-POCIDALO, M. A., POYNARD, T. & CHOLLET-MARTIN, S. 2000. Blood neutrophil

functions and cytokine release in severe alcoholic hepatitis: effect of corticosteroids. *Journal of Hepatology,* 32**,** 579-586.

TAMURA, T., KONDO, T., PAK, S., NAKANO, Y., MURATA, S., FUKUNAGA, K. & OHKOHCHI, N. 2012. Interaction between Kupffer cells and platelets in the early period of hepatic ischemia–reperfusion injury—An in vivo study. *Journal of surgical research,* 178**,** 443-451.

TANG, X.-Z., JO, J., TAN, A. T., SANDALOVA, E., CHIA, A., TAN, K. C., LEE, K. H., GEHRING, A. J., DE LIBERO, G. & BERTOLETTI, A. 2013. IL-7 licenses activation of human liver intrasinusoidal mucosal-associated invariant T cells. *The Journal of Immunology,* 190**,** 3142-3152.

THIMME, R., WIELAND, S., STEIGER, C., GHRAYEB, J., REIMANN, K. A., PURCELL, R. H. & CHISARI, F. V. 2003. CD8+ T Cells Mediate Viral Clearance and Disease Pathogenesis during Acute Hepatitis B Virus Infection. *Journal of Virology,* 77**,** 68-76.

THORNHILL, M. & HASKARD, D. 1990. IL-4 regulates endothelial cell activation by IL-1, tumor necrosis factor, or IFN-gamma. *The Journal of Immunology,* 145**,** 865-872.

TIAN, Z., CHEN, Y. & GAO, B. 2013. Natural killer cells in liver disease. *Hepatology,* 57**,** 1654-1662.

TIEGS, G., HENTSCHEL, J. & WENDEL, A. 1992. A T cell-dependent experimental liver injury in mice inducible by concanavalin A. *J Clin Invest,* 90**,** 196-203.

TOLEDO-PEREYRA, L. H. & SUZUKI, S. 1994. Neutrophils, cytokines, and adhesion molecules in hepatic ischemia and reperfusion injury. *Journal of the American College of Surgeons,* 179**,** 758.

TOYABE, S., SEKI, S., IIAI, T., TAKEDA, K., SHIRAI, K., WATANABE, H., HIRAIDE, H., UCHIYAMA, M. & ABO, T. 1997. Requirement of IL-4 and liver NK1+ T cells for concanavalin A-induced hepatic injury in mice. *The Journal of Immunology,* 159**,** 1537-42.

TRAPANI, J. A. 1995. Target cell apoptosis induced by cytotoxic T cells and natural killer cells involves synergy between the pore-forming protein, perforin, and the serine protease, granzyme B. *Aust N Z J Med,* 25**,** 793-9.

TRAUTWEIN, C., RAKEMANN, T., BRENNER, D. A., STREETZ, K., LICATO, L., MANNS, M. P. & TIEGS, G. 1998. Concanavalin A-induced liver cell damage: activation of intracellular pathways triggered by tumor necrosis factor in mice. *Gastroenterology,* 114**,** 1035-45.

TREINER, E., DUBAN, L., BAHRAM, S., RADOSAVLJEVIC, M., WANNER, V., TILLOY, F., AFFATICATI, P., GILFILLAN, S. & LANTZ, O. 2003. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature,* 422**,** 164-169.

TREINER, E., DUBAN, L., MOURA, I. C., HANSEN, T., GILFILLAN, S. & LANTZ, O. 2005. Mucosal-associated invariant T (MAIT) cells: an evolutionarily conserved T cell subset. *Microbes and Infection,* 7**,** 552-559.

TSUJII, S., OKABAYASHI, T., SHIGA, M., TAKEZAKI, Y., SUGIMOTO, T., KOBAYASHI, M. & HANAZAKI, K. 2012. The effect of the neutrophil elastase inhibitor sivelestat on early injury after liver resection. *World journal of surgery,* 36**,** 1122-1127.

TURNER, L., WARD, S. G. & WESTWICK, J. 1995. RANTES-activated human T lymphocytes. A role for phosphoinositide 3-kinase. *The Journal of Immunology,* 155**,** 2437-44.

TUTTLE, H. A., DAVIS-GORMAN, G., GOLDMAN, S., COPELAND, J. G. & MCDONAGH, P. F. 2003. Platelet-neutrophil conjugate formation is increased in diabetic women with cardiovascular disease. *Cardiovascular diabetology,* 2**,** 12.

UCHIDA, J., HAMAGUCHI, Y., OLIVER, J. A., RAVETCH, J. V., POE, J. C., HAAS, K. M. & TEDDER, T. F. 2004. The Innate Mononuclear Phagocyte Network Depletes B Lymphocytes through Fc Receptor–dependent Mechanisms during Anti-CD20 Antibody Immunotherapy. *The Journal of Experimental Medicine,* 199**,** 1659-1669.

UEDA, S., TAKAHARA, M., TOHTANI, T., YOSHIZAKI, T., KISHIBE, K. & HARABUCHI, Y. 2010. Up-regulation of ss1 integrin on tonsillar T cells and its induction by in vitro stimulation with alpha-streptococci in patients with pustulosis Palmaris et Plantaris. *J Clin Immunol,* 30**,** 861-71.

UHLMANN, D., UHLMANN, S. & SPIEGEL, H. U. 2000. Endothelin/Nitric Oxide Balance Influences Hepatic Ischemia-Reperfusion Injury. *Journal of Cardiovascular Pharmacology,* 36**,** S212&hyhen;S214.

VACHINO, G., CHANG, X.-J., VELDMAN, G. M., KUMAR, R., SAKO, D., FOUSER, L. A., BERNDT, M. C. & CUMMING, D. A. 1995. P-selectin Glycoprotein Ligand-1 Is the Major Counter-receptor for P-selectin on Stimulated T Cells and Is Widely Distributed in Non-functional Form on Many Lymphocytic Cells. *Journal of Biological Chemistry,* 270**,** 21966-21974.

VADDI, K. & NEWTON, R. C. 1994. Regulation of monocyte integrin expression by beta-family chemokines. *The Journal of Immunology,* 153**,** 4721-32.

VAN DE WIEL-VAN KEMENADE, E., VAN KOOYK, Y., DE BOER, A., HUIJBENS, R., WEDER, P., VAN DE KASTEELE, W., MELIEF, C. & FIGDOR, C. 1992. Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the beta subunit of VLA. *The Journal of Cell Biology,* 117**,** 461-470.

VAN DER MERWE, P. A., BARCLAY, A. N., MASON, D. W., DAVIES, E. A., MORGAN, B. P., TONE, M., KRISHNAM, A. K. C., IANELLI, C. & DAVIS, S. J. 1994. Human Cell-Adhesion Molecule CD2 Binds CD58 (LFA-3) with a Very Low Affinity and an Extremely Fast Dissociation Rate but Does Not Bind CD48 or CD59. *Biochemistry,* 33**,** 10149-10160.

VAN DINTHER-JANSSEN, A. C., HORST, E., KOOPMAN, G., NEWMANN, W., SCHEPER, R. J., MEIJER, C. J. & PALS, S. T. 1991. The VLA-4/VCAM-1 pathway is involved in lymphocyte adhesion to endothelium in rheumatoid synovium. *The Journal of Immunology,* 147**,** 4207-10.

VANWIJK, M. J., VANBAVEL, E., STURK, A. & NIEUWLAND, R. 2003. Microparticles in cardiovascular diseases. *Cardiovascular Research,* 59**,** 277-287.

VARGA-SZABO, D., PLEINES, I. & NIESWANDT, B. 2008. Cell adhesion mechanisms in platelets. *Arteriosclerosis, thrombosis, and vascular biology,* 28**,** 403-412.

VIÑAS, O., BATALLER, R., SANCHO-BRU, P., GINÈS, P., BERENGUER, C., ENRICH, C., NICOLÁS, J. M., ERCILLA, G., GALLART, T. & VIVES, J. 2003. Human hepatic stellate cells show features of antigen-presenting cells and stimulate lymphocyte proliferation. *Hepatology,* 38**,** 919-929.

VOLLMAR, B. & MENGER, M. D. 2009. The hepatic microcirculation: mechanistic contributions and therapeutic targets in liver injury and repair. *Physiological reviews,* 89**,** 1269-1339.

VOLPES, R., VAN DEN OORD, J. J. & DESMET, V. J. 1990. Immunohistochemical study of adhesion molecules in liver inflammation. *Hepatology,* 12**,** 59-65.

WAGNER, D. D. & BURGER, P. C. 2003. Platelets in inflammation and thrombosis. *Arterioscler Thromb Vasc Biol,* 23**,** 2131-7.

WANG, L., FUSTER, M., SRIRAMARAO, P. & ESKO, J. D. 2005. Endothelial heparan sulfate deficiency impairs L-selectin- and chemokine-mediated neutrophil trafficking during inflammatory responses. *Nat Immunol,* 6**,** 902-910.

WANG, Y. & NIU, J. 2008. Platelets inhibit in vitro response of lymphocytes to mitogens. *Immunology letters,* 119**,** 57-61.

WARTIOVAARA, U., SALVEN, P., MIKKOLA, H., LASSILA, R., KAUKONEN, J., JOUKOV, V., ORPANA, A., RISTIMÄKI, A., HEIKINHEIMO, M., JOENSUU, H., ALITALO, K. & PALOTIE, A. 1998. Peripheral Blood Platelets Express VEGF-C and VEGF which Are Released during Platelet Activation. *Thromb Haemost,* 80**,** 171-175.

WATSON, S. P. 1999. Collagen receptor signaling in platelets and megakaryocytes. *Thromb Haemost,* 82**,** 365-376.

WEBB, N. J., BOTTOMLEY, M. J., WATSON, C. J. & BRENCHLEY, P. E. 1998. Vascular endothelial growth factor (VEGF) is released from platelets during blood clotting: implications for measurement of circulating VEGF levels in clinical disease. *Clin Sci (Lond),* 94**,** 395-404.

WEBER, C. & SPRINGER, T. A. 1997. Neutrophil accumulation on activated, surface-adherent platelets in flow is mediated by interaction of Mac-1 with fibrinogen bound to alphaIIbbeta3 and stimulated by platelet-activating factor. *J Clin Invest,* 100**,** 2085-93.

WEINBAUM, S., TARBELL, J. M. & DAMIANO, E. R. 2007. The Structure and Function of the Endothelial Glycocalyx Layer. *Annual Review of Biomedical Engineering,* 9**,** 121-167.

WHITE, R. 2014. *The recruitment mechanisms and beneficial roles of haematopoietic stem cells in murine acute kidney injury.* Ph.D., University of Birmingham.

WING, K. & SAKAGUCHI, S. 2010. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat Immunol,* 11**,** 7-13.

WONG, C. H. Y., JENNE, C. N., PETRI, B., CHROBOK, N. L. & KUBES, P. 2013. Nucleation of platelets with blood-borne pathogens on Kupffer cells precedes other innate immunity and contributes to bacterial clearance. *Nat Immunol,* 14**,** 785-792.

WONG, J., JOHNSTON, B., LEE, S. S., BULLARD, D. C., SMITH, C. W., BEAUDET, A. L. & KUBES, P. 1997. A minimal role for selectins in the recruitment of leukocytes into the inflamed liver microvasculature. *J Clin Invest,* 99**,** 2782-90.

WORBS, T., MEMPEL, T. R., BÖLTER, J., VON ANDRIAN, U. H. & FÖRSTER, R. 2007. CCR7 ligands stimulate the intranodal motility of T lymphocytes in vivo. *J Exp Med,* 204**,** 489-495.

YADAV, S. S., HOWELL, D. N., STEEBER, D. A., HARLAND, R. C., TEDDER, T. F. & CLAVIEN, P.-A. 1999. P-selectin mediates reperfusion injury through neutrophil and platelet sequestration in the warm ischemic mouse liver. *Hepatology,* 29**,** 1494-1502.

YAMAMOTO, T., TADA, T., BRODSKY, S. V., TANAKA, H., NOIRI, E., KAJIYA, F. & GOLIGORSKY, M. S. 2002. Intravital videomicroscopy of peritubular capillaries in renal ischemia. *American Journal of Physiology - Renal Physiology,* 282**,** F1150-F1155.

YAMANAKA, A., HAMANO, S., MIYAZAKI, Y., ISHII, K., TAKEDA, A., MAK, T. W., HIMENO, K., YOSHIMURA, A. & YOSHIDA, H. 2004. Hyperproduction of proinflammatory cytokines by WSX-1-deficient NKT cells in concanavalin A-induced hepatitis. *The Journal of Immunology,* 172**,** 3590-3596.

YANG, Z., DAY, Y.-J., TOUFEKTSIAN, M.-C., XU, Y., RAMOS, S. I., MARSHALL, M. A., FRENCH, B. A. & LINDEN, J. 2006. Myocardial Infarct–Sparing Effect of Adenosine A2A Receptor Activation Is due to Its Action on CD4+ T Lymphocytes. *Circulation,* 114**,** 2056-2064.

YEMM, A. I. 2013. *Enhancing Haematopoetic Stem Cell Recuitment to Injured Murine Colon.* PhD, University of Birmingham.

YILMAZ, G., ARUMUGAM, T. V., STOKES, K. Y. & GRANGER, D. N. 2006. Role of T lymphocytes and interferon-γ in ischemic stroke. *Circulation,* 113**,** 2105-2112.

ZARBOCK, A., POLANOWSKA-GRABOWSKA, R. K. & LEY, K. 2007. Platelet-neutrophil-interactions: Linking hemostasis and inflammation. *Blood Reviews,* 21**,** 99-111.

ZHAI, Y., SHEN, X.-D., HANCOCK, W. W., GAO, F., QIAO, B., LASSMAN, C., BELPERIO, J. A., STRIETER, R. M., BUSUTTIL, R. W. & KUPIEC-WEGLINSKI, J. W. 2006. CXCR3+CD4+ T Cells Mediate Innate Immune Function in the Pathophysiology of Liver Ischemia/Reperfusion Injury. *The Journal of Immunology,* 176**,** 6313-6322.

ZHANG, H., STOLZ, D. B., CHALASANI, G. & THOMSON, A. W. 2013. Hepatic B cells are readily activated by Toll-like receptor-4 ligation and secrete less interleukin-10 than lymphoid tissue B cells. *Clinical & Experimental Immunology,* 173**,** 473-479.

ZHANG, J., SILVA, T., YAROVINSKY, T., MANES, T. D., TAVAKOLI, S., NIE, L., TELLIDES, G., POBER, J. S., BENDER, J. R. & SADEGHI, M. M. 2010. VEGF blockade inhibits lymphocyte recruitment and ameliorates immune-mediated vascular remodeling. *Circ Res,* 107**,** 408-17.

ZHANG, M., AUSTEN, W. G., CHIU, I., ALICOT, E. M., HUNG, R., MA, M., VERNA, N., XU, M., HECHTMAN, H. B., MOORE, F. D. & CARROLL, M. C. 2004. Identification of a specific self-reactive IgM antibody that initiates intestinal ischemia/reperfusion injury. *Proc Natl Acad Sci U S A,* 101**,** 3886-3891.

ZHANG, M., MICHAEL, L. H., GROSJEAN, S. A., KELLY, R. A., CARROLL, M. C. & ENTMAN, M. L. 2006. The role of natural IgM in myocardial ischemia–reperfusion injury. *J Mol Cell Cardiol,* 41**,** 62-67.

ZHANG, W., WANG, M., XIE, H. Y., ZHOU, L., MENG, X. Q., SHI, J. & ZHENG, S. 2007. Role of Reactive Oxygen Species in Mediating Hepatic Ischemia-Reperfusion Injury and Its Therapeutic Applications in Liver Transplantation. *Transplant Proc,* 39**,** 1332-1337.

ZHOU, Y., DAI, W., LIN, C., WANG, F., HE, L., SHEN, M., CHEN, P., WANG, C., LU, J., XU, L., XU, X. & GUO, C. 2013. Protective Effects of Necrostatin-1 against Concanavalin A-Induced Acute Hepatic Injury in Mice. *Mediators of Inflammation,* 2013**,** 15.

ZUCKER, S., MIRZA, H., CONNER, C. E., LORENZ, A. F., DREWS, M. H., BAHOU, W. F. & JESTY, J. 1998. Vascular endothelial groth factor induces tissue factor and matrix metalloproteinase production in endothelial cells: Conversion of prothrombin to thrombin results in progelatininase a activation and cell proliferation. *International Journal of Cancer,* 75**,** 780-786.

ZWACKA, R. M., ZHANG, Y., HALLDORSON, J., SCHLOSSBERG, H., DUDUS, L. & ENGELHARDT, J. F. 1997. CD4(+) T-lymphocytes mediate ischemia/reperfusion-induced inflammatory responses in mouse liver. *J Clin Invest,* 100**,** 279-89.