THE ROLE OF PLATELETS AND THEIR ASSOCIATED RECRUITMENT MECHANISMS, IN INTESTINAL ISCHAEMIA REPERFUSION INJURY

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Statement of declaration

I, Ian Holyer, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Recent studies have demonstrated a key role for platelets and microthrombi in the pathophysiology of intestinal ischaemia-reperfusion (IR) injury. Mechanistic studies have identified a role for the coagulation pathway in regulating platelet recruitment and activation. In addition, recent evidence suggests a role for the subendothelial matrix in mediating platelet activation in IR injury. Endothelial cells are extremely susceptible to ischaemia and their detachment, as well as damage to the basal lamina, has been demonstrated following IR injury. The research described in this thesis investigates the role of the major platelet glycoprotein receptors in recruiting and activating platelets following intestinal IR injury, namely GPVI, GPIb-IX-V and the integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$. The role of the intracellular protein PLCγ2 was also determined. Remote organ injury to organs such as the liver and lungs is also frequently associated with intestinal IR injury. Therefore the contributory role of platelets in remote microcirculatory injury has also been determined. Intravital microscopy was utilised to monitor individual platelet adhesion and microthrombus formation in anaesthetised mice undergoing intestinal IR injury *in vivo* using a novel dual labelling methodology. This method allowed us to quantitate three platelet events: (i) microthrombus formation (ii) individual platelet adhesion to endothelium and (iii) free flowing platelets. This study focussed on the microcirculation of the mucosal villi as this luminal surface is most susceptible to IR injury. Microthrombi development played a critical role in the pathophysiology of intestinal IR injury through their involvement in microvessel re-occlusion. This event was inhibited by targeting GPIbα, which conferred a short-lived benefit to the gut. However, we further demonstrated that it was necessary to inhibit both microthrombus formation as well as platelet-leukocyte-endothelial interactions in order to ensure longer lasting improvement in gut microcirculation and histology. This was achieved through a dual therapy that targeted both GPIbα and P-selectin. No benefit was observed with blocking GPVI or $\alpha_2\beta_1$ or the $\alpha_{IIb}\beta_3$ integrin. Interestingly, inhibiting PLCγ2 was associated with a decrease in individual platelet adhesion. Overall, the research within this thesis suggests that therapeutic strategies targeting GPIbα and P-selectin may prove beneficial in improving the clinical morbidity associated with gut IR injury.

Acknowledgements

I would like to thank Neena and Steve, my supervisors, for their guidance, advice, critics, sharpening my mind and the opportunity they gave me to work in their lab. The amount of experimental work undertaken, the fascinating results obtained within my thesis and the career opportunities it has lead to for me personally have made it all worthwhile.

Many thanks to all the colleagues on the first floor of the IBR, especially Dean Kavanagh, whose guidance and support, including his horrific taste in music, helped me tremendously throughout my 3 years.

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Abbreviations

ACD – acid citrate dextrose

ALT – alanine aminotransferase

ATP – adenosine triphosphate

COX – cyclo-oxygenase

GPVI – glycoprotein VI

ICAM – intercellular adhesion molecule

Ig – immunoglobulin

IIR – intestinal ischaemia reperfusion

JAM – junction adhesion molecule

mAb – monoclonal antibody

MCEC – murine cardiac endothelial cells

NO – nitric oxide

PBS – phosphate buffered saline

PGI_2 – prostaglandin I_2

PLC – phospholipase C

PSGL-1 – P-selectin glycoprotein ligand – 1

TNFα – tumour necrosis Factor

TxA_2 – thromboxane A_2

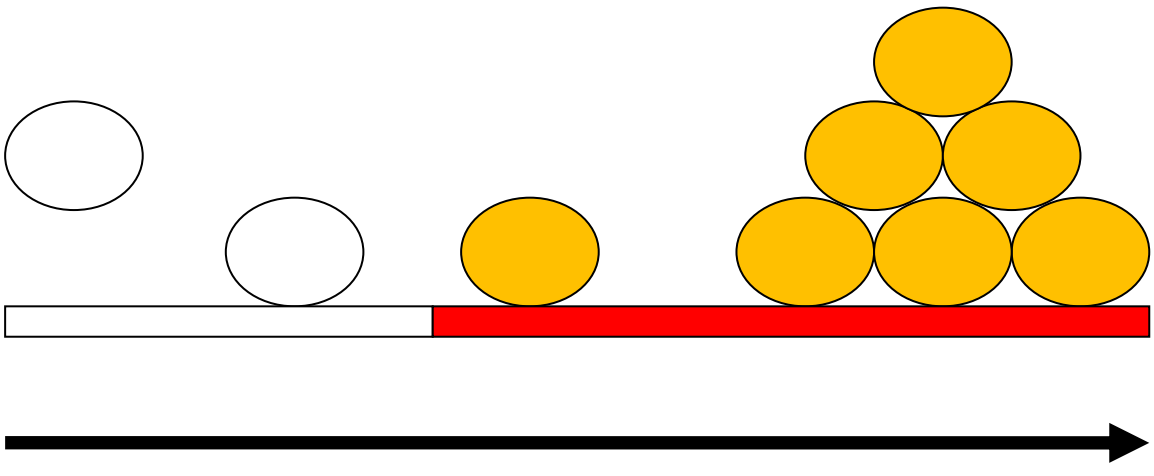
VCAM – Vascular cell adehesion molecule

vWF – von Willebrands Factor

WT – wild type

Chapter 1

Introduction - the role of platelet receptors in intestinal ischaemia-reperfusion injury *in vivo*



1.0. Introduction – the role of platelet receptors in intestinal ischaemia-reperfusion injury *in vivo*

1.1.1 Intestinal ischaemia-reperfusion (IR) injury

Hypoxia refers to any state of reduced oxygen availability. It maybe caused by reduced amounts or saturation of haemoglobin. Ischaemia is the withdrawal of blood flow to a tissue or organ which causes a deprivation of oxygen and nutrients required to keep internal structures in a healthy state. In contrast to hypoxia, during which glycolytic energy production can continue, ischaemia compromises the delivery of substrates for glycolysis. Consequently, in ischaemic tissues, anaerobic energy generation stops after glycolytic substrates are exhausted, such as adenine triphosphate ATP. This inevitably leads to glycolytic function becoming inhibited by the accumulation of metabolites that would have been removed otherwise by blood flow. For this reason, ischaemia tends to injure tissues faster than does hypoxia.

Ischemia-reperfusion (IR) injury is the damage that occurs to tissues after the restoration of blood flow (reperfusion) to an area that had previously experienced deficient or no blood flow (ischaemia). This is the most common type of cell injury in clinical medicine and has been studied extensively in humans and experimental animals

IR injury of the small intestine is associated with several inflammatory pathologies where reduction in gut perfusion is followed by reperfusion e.g. shock, vasculitis, strangulated hernias and neonatal necrotizing enterocolitis (Carden *et al*., 2000; Kong *et al.*, 1998). It also

results from a number of surgical procedures, including thoracoabdominal aorta aneurysm repair and cardiopulmonary bypass (Harward 1996; Kharbanda 2006). Small intestinal IR injury is also an inevitable consequence of the clinically challenging procedure of small bowel transplantation which continues to be beset with high rates of graft loss, rejection and an overall poor prognosis (Fishbein 2009). Although long-term survival of the intestinal transplant is hampered by chronic rejection, it is now becoming apparent that the initial IR injury the graft endures can also adversely influence the short- and long-term survival of the graft. Indeed, studies have demonstrated that early events such as graft preservation and IR-induced tissue damage contribute significantly to the development of the rejection process (Wang *et al*., 2003).

Although rare (1-2 cases in every 1000 hospital admissions) acute intestinal ischaemia is also a clinical problem in its own right due to its associated high mortality rate. Indeed, there have been no convincing reports of any significant improvement in the prognosis of this condition over the last 50 years (Endean *et al.*, 2001). The underlying causes of acute intestinal ischemia are either thrombotic (eg. mesenteric venous thrombosis) or non-thrombotic (eg. sepsis, hypovolemia, strangulated hernia). The treatment for this condition is revascularization and resection of the affected bowel (Foley *et al.*, 2000). However, this is only affective if acute intestinal ischemia is diagnosed within 12 hours from the onset of symptoms with the benefits of surgery decreasing by 70% after 24 hours.

1.1.2. Histological disturbances associated with intestinal IR injury

The intestinal luminal or mucosal surface is folded into numerous microscopic finger-like villi that increase the absorptive surface area to ensure adequate nutrient absorption. Intestinal IR injury not only reduces the overall absorptive capacity, but also disturbs the integrity of the mucosal surface (Chen *et al.*, 2005)**.** This leads to loss of barrier function of the gut lining and thus permits bacterial translocation across the epithelial surface and into the bloodstream (Jones *et al.*, 1991). Bacterial translocation, and the subsequent sepsis that ensues, is a major clinical problem associated with intestinal IR injury with a reported 44% of paediatric patients showing microbiological complications after small bowel transplantation (Cicalese, 2000). Indeed, this event is one of the major causes of the poor morbidity and mortality associated with intestinal IR injury. Therefore, it is essential that the barrier and absorptive function of the mucosal surface is not compromised.

Unfortunately, the intestinal mucosal villi are extremely sensitive to IR injury and even short durations of ischemia can elicit substantial localised tissue injury (Kalia *et al.*, 2002 & 2003 & 2005; Chan *et al.*, 1998**;** Blikslager *et al.,* 2007). The outer submucosal, muscular and serosal layers often undergo minimal damage with injury confined mostly to the villus structures. As the duration of IR injury increases tissue damage becomes apparent, starting at the tips of the villi (superficial mucosal injury) and proceeding to full villous destruction (transmucosal injury). In very severe circumstances, IR injury can lead to the complete destruction of the villi as well as damage to the underlying crypts within the submucosa (transmural injury)

(Haglund, 1994). Intestinal stem cells, that continually replace the villous epithelial cells on a daily basis, are located within the base of these crypts of Lieberkühn. Therefore, although mild mucosal injury can be repaired by these stem cells, severe injury which damages the crypts means the regenerative potential of the mucosa is also lost (Zmora *et al.*, 2005). Evidence suggests that different areas of the small intestine have the ability to cope with IR injury better than others, although it is not clear why this is the case (Boyd *et al.*, 1994). Chan and colleagues demonstrated that after segmental transplantation, healthier mucosal tissue was observed in the more distal ileum rather than the more proximal jejunum (Chan *et al.*, 1998).

1.1.3. Microcirculatory disturbances associated with intestinal IR injury

Within the mucosal villi, a central arteriole passes up to the villus tip and then breaks into a capillary network that flows down the villus as a plexus that can exchange with the artery on the way its way up (Chen *et al*., 2003). However, this peculiar microvascular anatomy makes the villus tip highly susceptible to injury during periods of low blood flow as oxygen diffuses to the venule at the base of the villus before reaching its tip (**Figure 1**). Hence, if blood flow is compromised, as during intestinal IR injury, the mucosal villus is the most susceptible to damage (Haglund 1994; Figueiredo *et al.,* 2002.). This microvascular anatomy suggests that mucosal injury should occur predominately during an ischaemic period. However, early seminal studies demonstrated that the mucosal injury produced by 3 hours of ischaemia followed by 1 hour of reperfusion was more severe than that produced by 4 hours of

ischaemia without reperfusion (Parks and Granger, 1986). This phenomenon, known as intestinal IR injury, clearly demonstrates that restoration of blood flow is paradoxically associated with greater injury than ischaemia alone.

There are a number of microcirculatory disturbances that are generally associated with IR injury and these also occur within the small intestine. The generation of reactive oxygen species (ROS), activation of inflammatory neutrophils and the release of damaging proteolytic enzymes including elastase from them, are amongst the most damaging events to take place during IR injury (**refer to Sections 1.2 and 1.3**).

Due to their close anatomical association with the endothelial lining of the microvessels, it is no surprise that the first structure within the ischaemically-reperfused tissue to undergo injury is the local microcirculation. Lysis of endothelial cells (ECs) and damage to underlying basement membrane leads to vascular albumin leakage (Welbourn *et al*., 1991). This also occurs as a result of surrounding mast cell degranulation and subsequent histamine release, which increases the permeability of the microvessels (Kalia *et al*., 2005). Seminal intravital studies by Kalia and colleagues demonstrated *in vivo* increased vascular leakage of fluorescently labelled albumin from the rat mucosal villous microcirculation following intestinal IR injury, which was directly proportional to the severity of injury (Kalia *et al.*, 2002 & 2003 & 2005). Furthermore, these studies demonstrated that, although a number of pharmacological interventions could confer tissue protection by preventing neutrophil recruitment, it was very difficult to maintain mucosal microvessel integrity as leakage was never completely inhibited. An extensive loss of fluid from the microcirculation leads to

oedema and this is often associated with red blood cell congestion and blood flow stasis. Recruitment of inflammatory cells following IR injury also further contributes to vascular stasis. These effects quickly become pronounced enough in reperfusion to block the microcapillaries entirely and this phenomenon, known as the no-reflow phenomenon, unfortunately further exacerbates the initial ischaemic injury.

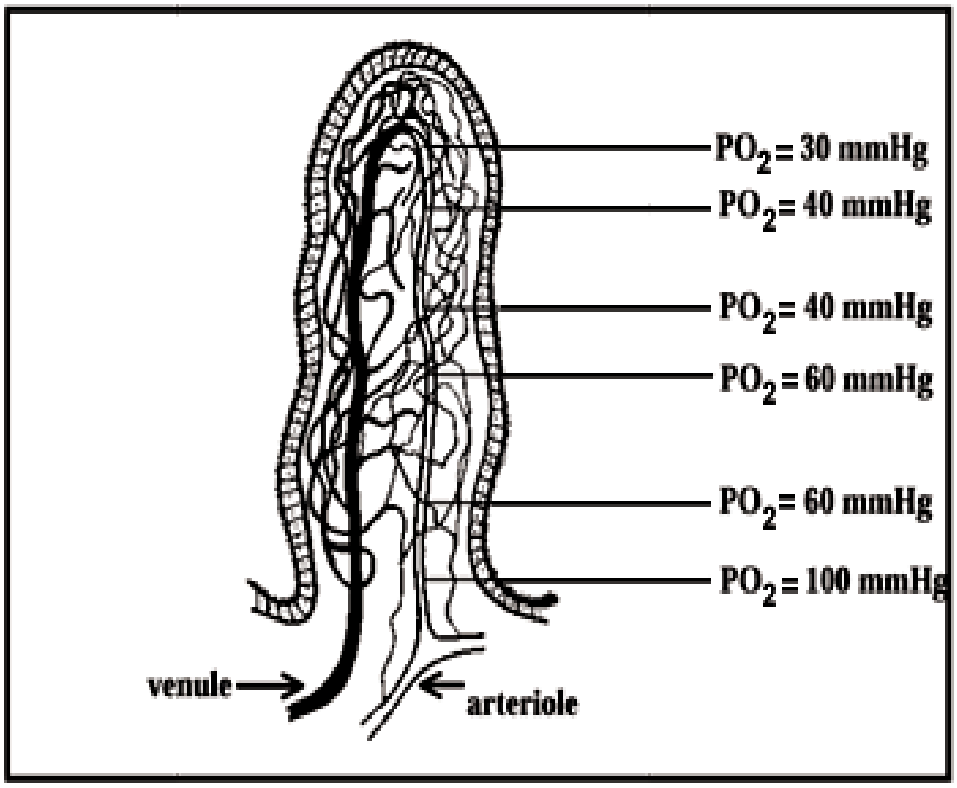


Figure 1.1 Countercurrent exchange of oxygen between the arteriole and venule within the villi, showing the progressive decrease in arteriolar PaO_2. Intestinal mucosal cells are normally under a low oxygen tension, because effective haematocrit within the villi is decreased due to a phenomenon called "plasma skimming". Also, the villi have a peculiar microvascular architecture, characterized by a countercurrent exchange of oxygen from arteriole to adjacent venule along its length. Under normal conditions, this shunting of oxygen is not harmful to the villi. However, in conditions in which blood flow to the gut becomes greatly reduced curtailed, such as in circulatory shock, the oxygen deficit in the tips of the villi can become so severe that they can suffer ischemic death and disintegrate. Taken from de Figueiredo *et al.,* 2002.

1.1.4. Remote organ injury following intestinal IR injury

In addition to localised injury, the intensity of inflammation in post-ischaemic intestinal tissue can be so severe that reperfusion related responses can also manifest in distant organs (Simpson *et al*., 1993). This is a common sequel of intestinal IR injury and is one of the most investigated subjects in experimental surgery as it accounts for a high proportion of the morbidity following intestinal IR injury (Granger 1999). These systemic effects are bought about predominantly by circulating neutrophils that became 'primed' or activated when they circulate through the injured gut microcirculation and are thus able to induce injury in remote sites (Victoni *et al.*, 2010). Increasing evidence has associated intestinal IR injury with the development of remote tissue damage, particularly to the lungs and liver (Chen *et al.*, 2003; Simpson *et al.*, 1993). Simpson and colleagues demonstrated that within 4 hours of intestinal reperfusion, increased neutrophil accumulation within these two remote sites contributed to their damage. A lack of neutrophils decreased the pulmonary microvascular permeability by 80% and the liver injury by 60%. Interestingly, in this study, the local injury to the mucosal villi was unaffected in neutropenic mice, suggesting factors in addition to inflammatory neutrophils could also induce injury (Simpson *et al.*, 1993).

1.2. Reactive oxygen species (ROS) contribute to ischaemia-reperfusion injury

Generally, IR injury starts by affecting cell metabolism and ultimately leads to cell death via apoptosis and necrosis (Wiegele *et al*., 1998). Ischaemia is the withdrawal of blood flow to a tissue or organ which causes a deprivation of oxygen and nutrients but also compromises the

removal of waste products of metabolism. In contrast to hypoxia, during which glycolytic energy production can continue, ischaemia compromises the delivery of substrates for glycolysis and hence adenosine triphosphate (ATP) production decreases. Consequently, in ischaemic tissues, anaerobic energy generation stops after glycolytic substrates are exhausted. During this ischaemic period, ATP is catabolized to adenosine which is further catabolised to yield an increasing amount of hypoxanthine (**Figure 2**). Concomitantly, ischaemia also leads to an influx of calcium ions (Ca^{2+}), which triggers the very rapid and irreversible conversion of xanthine dehydrogenase, highly prevalent within the small intestine, to xanthine oxidase (Engerson *et al.*, 1987; Racasan *et al.*, 2003).

During reperfusion, molecular oxygen is reintroduced into the tissue via the blood. Along with xanthine oxidase, it reacts with hypoxanthine to produce a burst of very toxic reactive oxygen species (ROS), including superoxide anions (O_2^-), hydroxyl radicals (OH^-), hydrogen peroxide (H_2O_2) and nitric oxide-derived peroxynitrite. These highly reactive and toxic ROS initiate lipid peroxidation of cell membrane components (Moore *et al.*, 1995). This is evidenced by the presence of increased intestinal fatty acid binding protein, a marker of cell damage, within circulating blood following IR injury (Derikx *et al.*, 2008). Hence a number of pharmacological agents may be of benefit during IR injury including allopurinol, a xanthine oxidase inhibitor and superoxide dismutase, a free radical scavenger.

Furthermore, a pro-inflammatory environment is also generated by local endothelial up-regulation of adhesion molecules and release of pro-inflammatory mediators. This results in

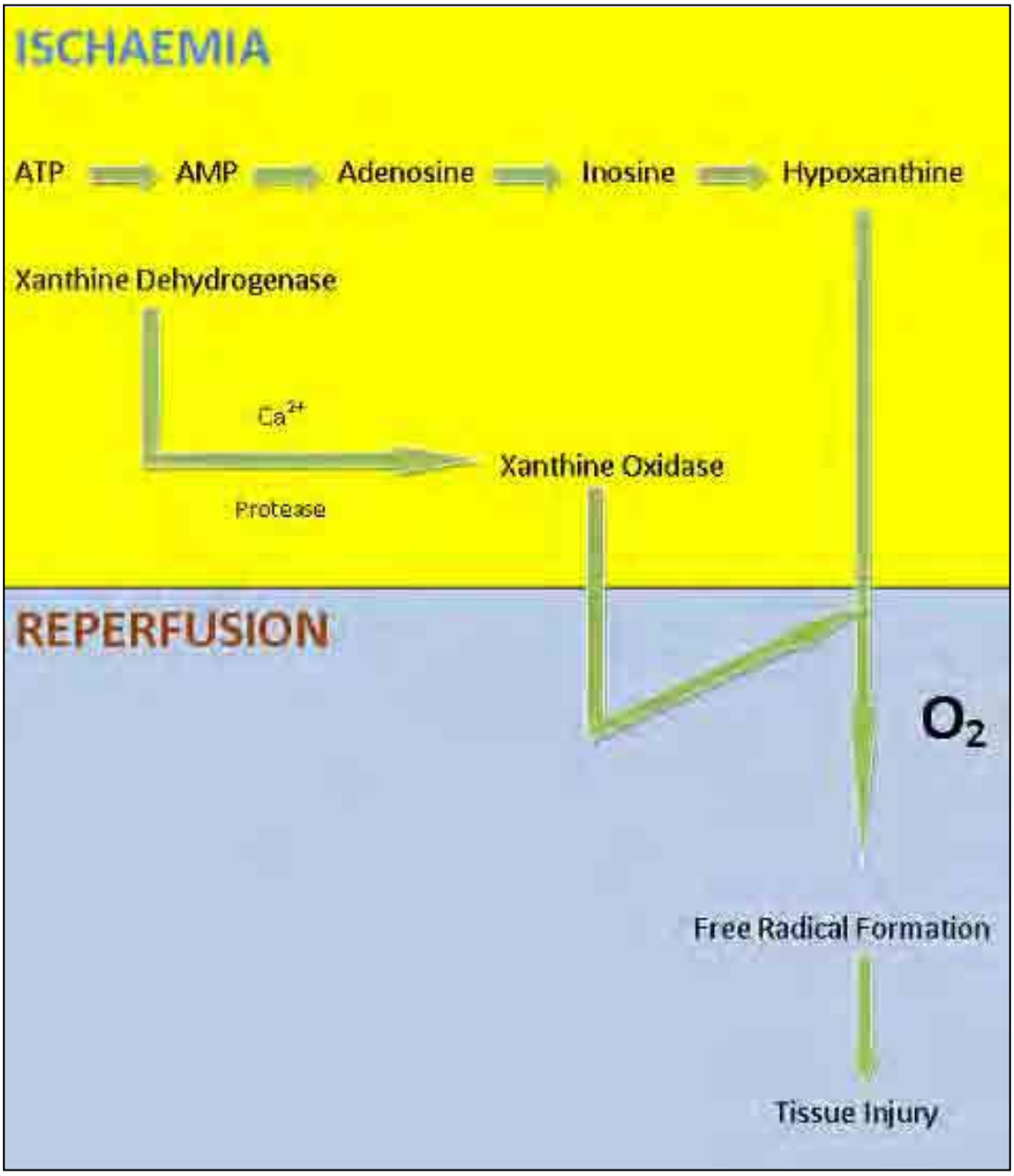


Figure 1.2. Generation of reactive oxygen species (ROS) occurs following events that take place during the ischaemic and reperfusion phases. During ischemia, ATP is degraded to hypoxanthine and xanthine dehydrogenase is converted to xanthine oxidase. If perfusion is re-established, oxidative free radicals are formed. Hence a number of pharmacological agents may be of benefit during IR injury including allopurinol, a xanthine oxidase inhibitor and superoxide dismutatse, a free radical scavenger. ATP, adenosine triphosphate; ADP, adenosine 5'-diphosphate; AMP, adenine monophosphate. Adapted from Mallick *et al.,* 2004.

the attraction, activation and adhesion of leukocytes to the endothelium, which subsequently play a key role in intensifying the initial cell injury caused by ischaemia and cause further EC injury via the release of superoxide and various proteases (Pierro *et al.*, 2004).

1.3. The role of leukocytes in intestinal IR injury

The main function of leukocytes is to protect the body from foreign bodies such as bacteria or parasites. Leukocytes can be separated into the two distinct groups of polymorphonuclear leukocytes (PMN's) or mononuclear leukocytes. PMN's, neutrophils in particular, are reported to be the major player in exacerbation of tissue injury not only following intestinal IR injury, but also in similar injuries of the numerous other organs including the heart, liver, kidney and lungs (Kochanek *et al.*, 1992). Reperfusion is associated with elevated circulating levels of a number of cytokines, particularly tumour necrosis factor-α (TNFα), interleukin 1 (IL-6) and IL-8, the eicosanoids leukotriene B4 (LTB4) and thromboxane B2 (TxB2), endothelin 1 (ET-1) and platelet activating factor (Simpson *et al*., 1993). All of these are powerful chemoattractants and chemoactivators of neutrophils which work by activating the local ECs. Thus the reperfused intestine is associated with a massive influx of neutrophils which contributes to the mucosal and microcirculatory barrier dysfunction and exacerbates the tissue damage from the previous ischaemic episode (Granger *et al.*, 1989; Carmody *et al*., 2004). Furthermore, these soluble factors can also work in concert narrowing the capillary lumen and elevating blood flow resistance to impair perfusion within the microcirculation.

The process of leukocyte recruitment and subsequent migration through activated endothelium is defined by a well characterised adhesion cascade (**Figure 3**). It begins with leukocyte tethering or rolling on the vessel wall using leukocyte L-selectin and/or endothelial P- and E-selectins binding to P-selectin glycoprotein ligand-1 (PSGL-1) and other glycosylated ligands such as E-selectin ligand-1 (Ley *et al*., 2007). This rolling slows leukocyte velocity sufficiently to permit their subsequent firm arrest on activated ECs. This is mediated by the binding of leukocyte β_1 and β_2 integrins such as VLA-4, LFA-1 (CD11a/CD18) and Mac-1 (Cd11b/CD18) with endothelial immunoglobulin superfamily members such as ICAM-1 and VCAM-1. L-selectin has also been shown to be critical in recruiting other leukocytes to the endothelium through leukocyte/leukocyte rolling interactions (Mitchell *et al*, 2000). During inflammation, ECs are activated by inflammatory cytokines to express these adhesion molecules and also synthesise chemokines that are then presented on their luminal surface. Chemokines can modulate the affinity of leukocyte integrins which subsequently leads to firm leukocyte adhesion. This is achieved through surface clustering of integrins, the induction of their transition from a bent low-affinity conformation to an extended high-affinity conformation or from increased expression from endothelial Wiebel-palade bodies (Oynebraten et al, 2004). Leukocyte transmigration through venular endothelial junctions is the least characterised stage of the adhesion cascade and is mediated by endothelial junctional adhesion molecules (JAMs). It is well reported the adhesion molecules ICAM-1, VCAM-1, CD99 and platelet-endothelial cell adhesion molecule-1 (PECAM-1) play a key role in this transmigratory mechanism (Ley *et al*., 2007). Once activated, leukocytes release a

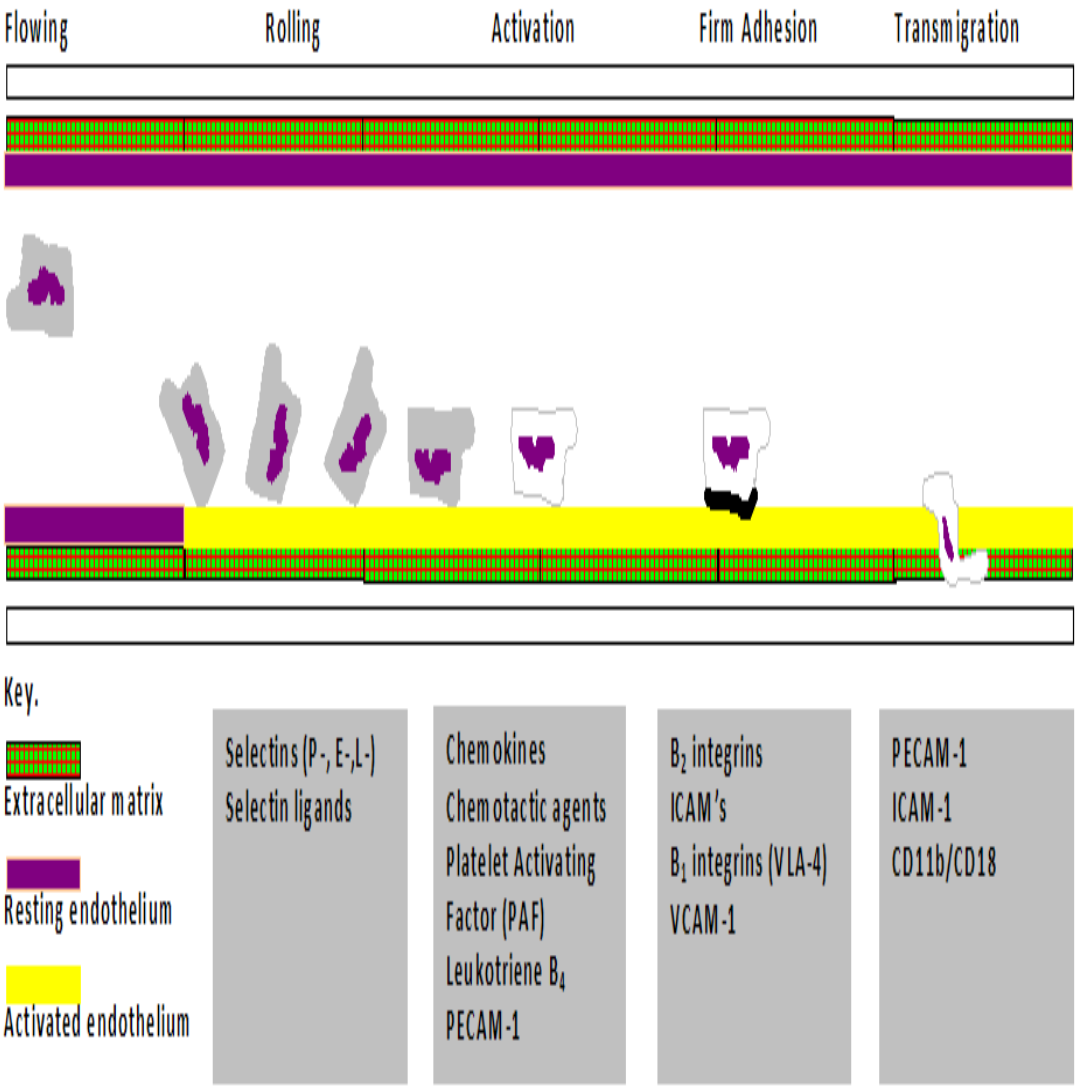


Figure 1.3. Leukocyte recruitment to IR activated endothelium under flow conditions. The leukocyte initially interacts with the endothelium via the selectins and associated ligands. This generates the release of chemokines and the following leukocyte activation**.** Strong adhesion to the endothelium, through integrin interactions, then allows for transmigration of leukocytes into the extracelluar matrix and surrounding tissue. Redrawn from Panes *et al*., 1998

variety of factors that are capable of damaging the surrounding endothelium, extracellular matrix proteins and the tissue parenchyma.

1.4. The role of platelets in intestinal IR injury

1.4.1 Platelets in haemostasis and disease

Platelets are discoid shaped cells with a diameter of between 0.5 and 3µm. They are produced by cytoplasmic fragmentation of precursor megakaryocytes found within the bone marrow. The major physiological role of circulating platelets in the vasculature is prevent blood loss at sites of vessel injury through the formation of platelet plugs or thrombi (Becker *et al.*, 1976). The pathophysiological effects of thrombus formation become apparent when too much or unnecessary platelet activation and recruitment occurs which can lead to the formation of an oversized thrombus that has the ability to fully occlude the injured vessel (Varga-Szabo *et al.*, 2008). This obliterates the flow of blood downstream from the site of injury causing cell and tissue death. This is the typical underlying mechanism leading to the cardiovascular complications of myocardial infarction and stroke (Stoll *et al*., 2008).

As previously stated, it is now well established that reperfusion initiates injury by triggering extensive leukocyte-endothelial interactions following ROS synthesis. In addition, increasing evidence has implicated platelets as potential modulators of IR-induced tissue injury and re-occlusion of microvessels in a variety of tissue beds as a consequence of ROS and pro-inflammatory chemokine release (Gawaz *et al*., 2004; Kupatt *et al*., 2002; Xu *et al*., 2005; Singbartl *et al*., 2001; Khandoga *et al*., 2002 & 2003; Hackert *et al*., 2005; Ishikawa *et al*.,

2003; Salter *et al*., 2001; Kalia *et al*., 2001). Indeed, an important feature of organ failure development following intestinal IR is the abundance of platelet-rich intravascular microthrombi observed in mucosal and submucosal microvessesls (Salter *et al*., 2001; Kalia *et al*., 2001; Massberg *et al*., 1999) (**Figure 4**). Moreover, a beneficial effect of platelet depletion on IR injury pathogenesis has been described (Cooper *et al*., 2003; Kuroda *et al*., 1994 & 1996).

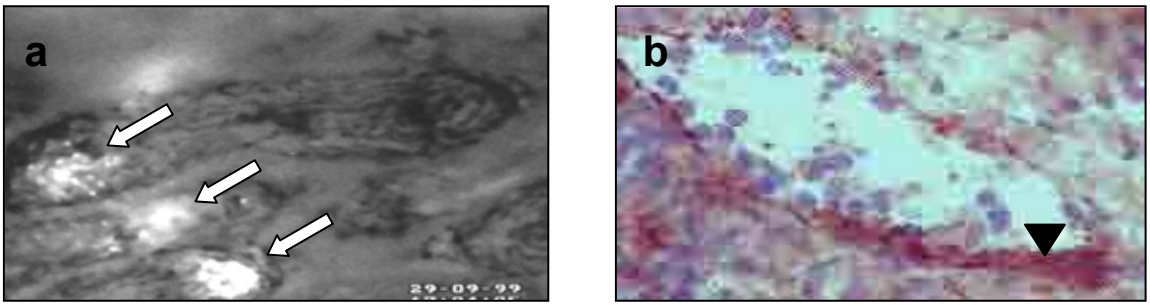


Figure 1.4. Platelet thrombi formation following intestinal IR injury. (a) Formation of large leukocyte-platelet aggregates (arrows) within the base of the villous microcirculation following rat intestinal IR injury – taken from Kalia *et al.,* 2001 **(b)** Formation of platelet aggregates (arrow head), identified by intense staining for P-selectin, within the mouse intestinal venule – taken from Massberg *et al*., 1999.

Intestinal IR injury is associated with significant recruitment of rolling and adherent platelets in post-capillary venules (PCVs; Massberg *et al*. 1998). It is postulated that this may significantly aggravate EC damage and contribute to leukocyte recruitment at the site of injury. Indeed, studies of IR injury in the heart (Gawaz *et al*., 2004; Kupatt *et al*., 2002; Xu *et al*., 2005), kidney (Singbartl *et al*., 2001) and liver (Khandoga *et al*., 2002 & 2003) demonstrate that accumulation of platelets in the microcirculation during reperfusion enhances recruitment of activated leukocytes, thereby exacerbating the intensity of the inflammatory response. Furthermore, IR-induced recruitment of leukocytes may be

dependent on the initial adhesion of platelets to venular endothelium (Massberg *et al*. 1998; Cooper *et al.,* 2004).

1.4.2. Platelets contribute to intestinal IR injury via interactions with intact endothelium

Platelet thrombi are able to form when ECs lining the blood vessels, become damaged following vessel injury. This subsequently exposes underlying pro-thrombogenic extracellular matrix (ECM) proteins to platelets. This process will be described later (**refer to Section 1.5**). However, in recent years it has become increasingly evident that endothelial denudation is not an absolute pre-requisite to allow platelet attachment to the arterial wall (Roberts *et al*., 2004). The intact, non-activated endothelium normally prevents platelet adhesion to underlying ECM and controls platelet reactivity through inhibitory and modulating mechanisms involving nitric oxide, COX-2 and PGI_2. However, damaged or inflamed ECs develop properties that render them adhesive for platelets (Massberg *et al*. 1998 & 1999; Frenette *et al*., 1998; Tull *et al*., 2006, van Gils *et al*., 2009).

The most extensively studied stimulus for platelet adhesion to endothelium is IR injury (Singbartl *et al.*, 2006; Cooper *et al.*, 2003; Kleinschnitz *et al.*, 2007; Takaya *et al.*, 2005) and following intestinal IR injury, rolling and adhesion of platelets in post-PCVs is observed (Massberg *et al*. 1998). Although the adhesion receptors involved in platelet attachment to the subendothelial matrix have been well defined during the past decade (**refer to Sections 1.5.2 and 1.5.3**), the molecular determinants that promote platelet-endothelial interactions

are incompletely understood (**Figure 5**). However, the limited studies to date investigating the role of platelets following intestinal IR injury have focused on platelet interactions with an intact endothelium. Unlike leukocyte-endothelial interactions, which are generally confined to PCV's , platelet-endothelial interactions can also occur in arterioles. Arterioles are the blood supplying vessels of a tissue and so platelets, through arterio-occlusion, can contribute to poor tissue perfusion following IR injury (Roberts *et al.*, 2004). After stimulation with endotoxin, IL-1 or TNFα, ECs synthesize tissue factor and bind soluble fibrinogen on their surface and thereby promote thrombosis (Frenette *et al*., 1998). Alternatively, stimulation of ECs with a combination of TNFα and TGFβ can lead to exposure of von Willebrand factor (vWF) on their surface and thus platelet adhesion (Tull *et al*., 2006). Significantly, increases in these prothrombotic proteins are associated with blood flow stasis and reduced flow, conditions that occur following IR injury (Salter *et al*., 2001; Schoots *et al.*, 2003; Mackman *et al.*, 2003).

1.4.3. Endothelial P-selectin primarily mediates platelet-endothelial interactions following IR injury

Most attention has been devoted to defining the role of P-selectin, an adhesion molecule stored in both platelet α-granules and endothelial Weibel-Palade bodies, in supporting platelet adhesion to ECs. P-selectin is minimally (or not all) expressed on resting platelets. Following platelet activation it can be swiftly transported to the cell surface and play a role in platelet recruitment to injured vessels (Merten *et al.*, 2000)**.** In their activated state there are

approximately 10,000 molecules of P-selectin present on the platelet surface (Hsu-Lin *et al*., 1984). Massberg and colleagues demonstrated increased platelet-endothelial interactions within 5 minutes post-reperfusion in mice undergoing intestinal IR injury which was dependent upon P-selectin (Massberg et al, 1998). It is well documented that endothelial, rather than platelet P-selectin plays a greater role in the initial rolling of platelets to endothelium in vessels of medium and high shear stresses (Michelson 2007). Indeed Massberg and colleagues also demonstrated that endothelial P-selectin was the main mediator since P-selectin-deficient platelets injected into wild-type mice could still adhere but platelet recruitment was reduced when wild-type platelets were injected into P-selectin-deficient mice (Massberg *et al*. 1998).

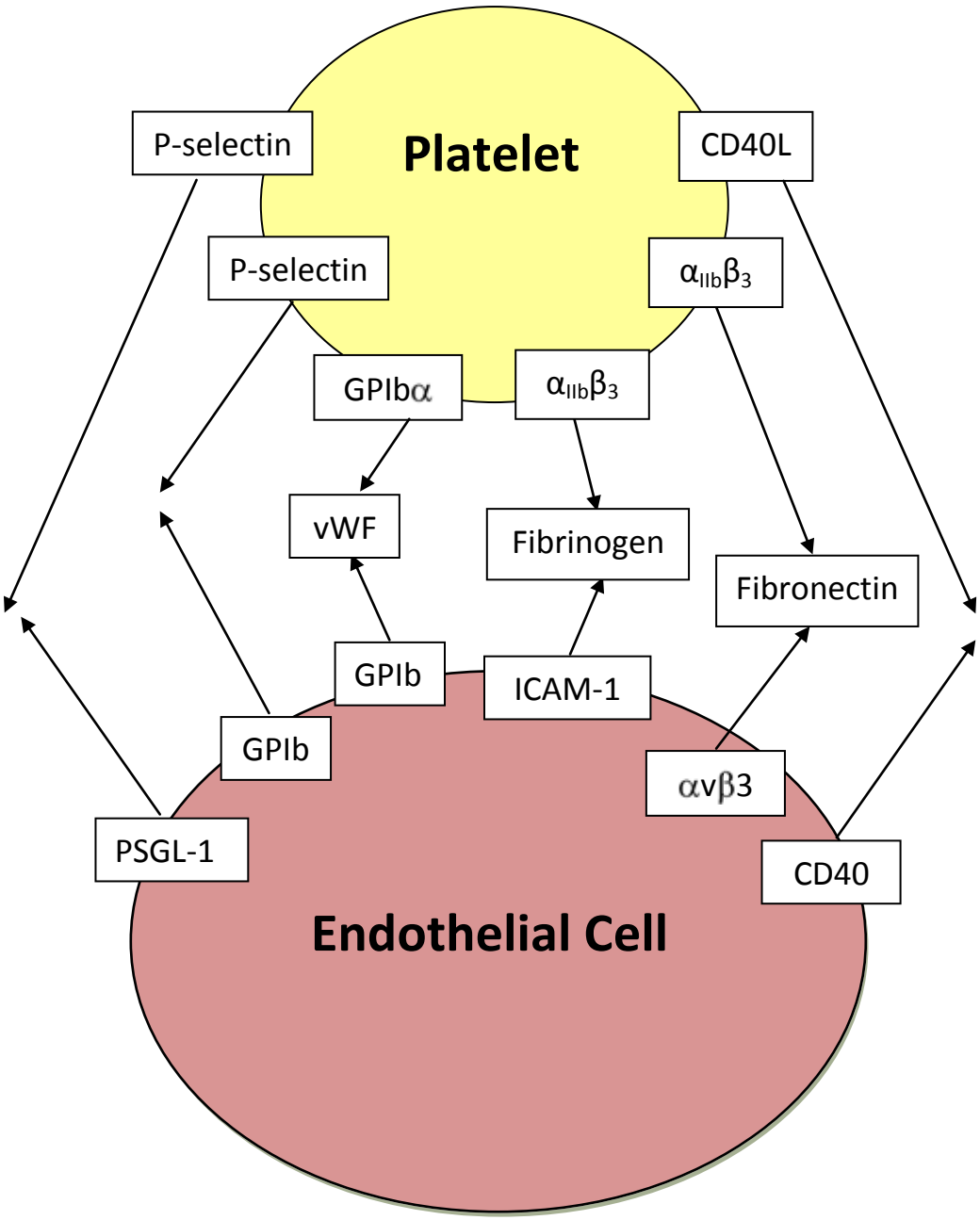


Figure 1.5. Molecular adhesive mechanisms that govern platelet adhesion to endothelium. Platelet adhesion to endothelium primarily involves surface expression of P-selectin on activated platelets and binding to PSGL-1, the counter-receptor present on endothelium. The ligand-receptor combinations P-selectin, PSGL-1 and GPIb are involved in the tethering and rolling of platelets on the EC. Upon activation of integrins, platelets firmly adhere to the endothelium, mainly via the additional bridging molecules fibronectin, fibrinogen and vWF. Upon firm adhesion, also CD40L-CD40 (amongst others) interactions are initiated with this binding inducing an inflammatory response in the EC. Adapted from van Gils *et al.,* 2009.

1.4.4. Platelet integrin $\alpha_{IIb}\beta_3$ can also mediate platelet-endothelial interactions following IR injury

A critical role for the integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) in platelet recruitment to the endothelium was demonstrated by a reduced interaction of platelets from a patient with Glanzmanns disease (which lack functional $\alpha_{IIb}\beta_3$) or in the presence of an antagonist to this integrin (Massberg *et al*., 1999). A role for this integrin in mediating platelet recruitment to the endothelium following IR injury was identified within the venules of post-ischemic gut (Salter *et al.*, 2001) and middle cerebral artery (Ishikawa *et al.*, 2004). IR injury led to deposition of fibrinogen on ECs which interacted with platelet $\alpha_{IIb}\beta_3$. An anti-fibrinogen antibody, that prevented IR-induced fibrinogen deposition, reduced the accumulation of adherent platelets in both arterioles and venules (Tailor *et al.*, 2005).

1.4.5. Platelets can recruit inflammatory leukocytes to sites of IR injury

Activated platelets adherent to the endothelium may significantly aggravate EC damage by subsequently recruiting inflammatory leukocytes to sites of injury. Indeed, studies of IR injury in the heart (Gawaz *et al.,* 2004; Kupatt *et al*., 2002; Xu *et al.*, 2005), kidney (Singbartl *et al*., 2001*)* and liver (Khandoga *et al.,* 2002 & 2003) demonstrate that accumulation of platelets in the microcirculation during reperfusion enhances recruitment of activated leukocytes, thereby exacerbating the intensity of the inflammatory response. Furthermore, some studies have suggested that IR-induced recruitment of leukocytes may be essentially dependent on the initial adhesion of platelets to venular endothelium (Cooper *et al*., 2003 & 2004). These two cells adhere to each other through interactions between platelet P-

selectin and leukocyte PSGL-1 (Chen *et al.*, 2005). The platelet-leukocyte aggregates that form have the capability to block microvessels and interrupt blood flow (Frenette *et al.*, 1998). It is also well documented that small platelet-leukocyte aggregates can form within the circulation due to specific activatory mechanisms of both cell types not requiring pre-vessel wall interaction. Hydrogen peroxide, superoxide dismutase and homocysteine, all of which are expressed during IR injury, play a key role in these associated cell activatory mechanisms. Although these aggregates primarily involve P-selectin- PSGL-1 interactions, other platelet and leukocyte receptors and integrins are also capable of binding these two cell types and are summarised in **Figure 6**.

Platelets can further initiate and exacerbate inflammation within damaged vessels as they can also release cytokines and a variety of inflammatory modulators including interleukin 1β (IL-1β) and CD40L upon activation (Lindemann *et al.*, 2007). CD40L, a member of the TNF family, is not expressed on the resting platelet surface. However, platelets translocate preformed intra-platelet stores of CD40L to the platelet surface within seconds of activation *in vitro* and in the presence of thrombus formation *in vivo* (Henn *et al.*, 1998). Both IL-1β and CD40L induce an inflammatory response within the endothelium by initiating the release of chemoattractants IL-8 and MCP-1 that recruit leukocytes. CD40L and IL-8 also cause increased expression of a variety of adhesion receptors on the surface of the endothelium such as VCAM-1 and $\alpha_v\beta_3$ (Gawaz *et al.*, 2005). Inflammation proceeds with leukocytes, in particular neutrophils and monocytes, being recruited due to the signals generated from the initial platelet-endothelial interactions.

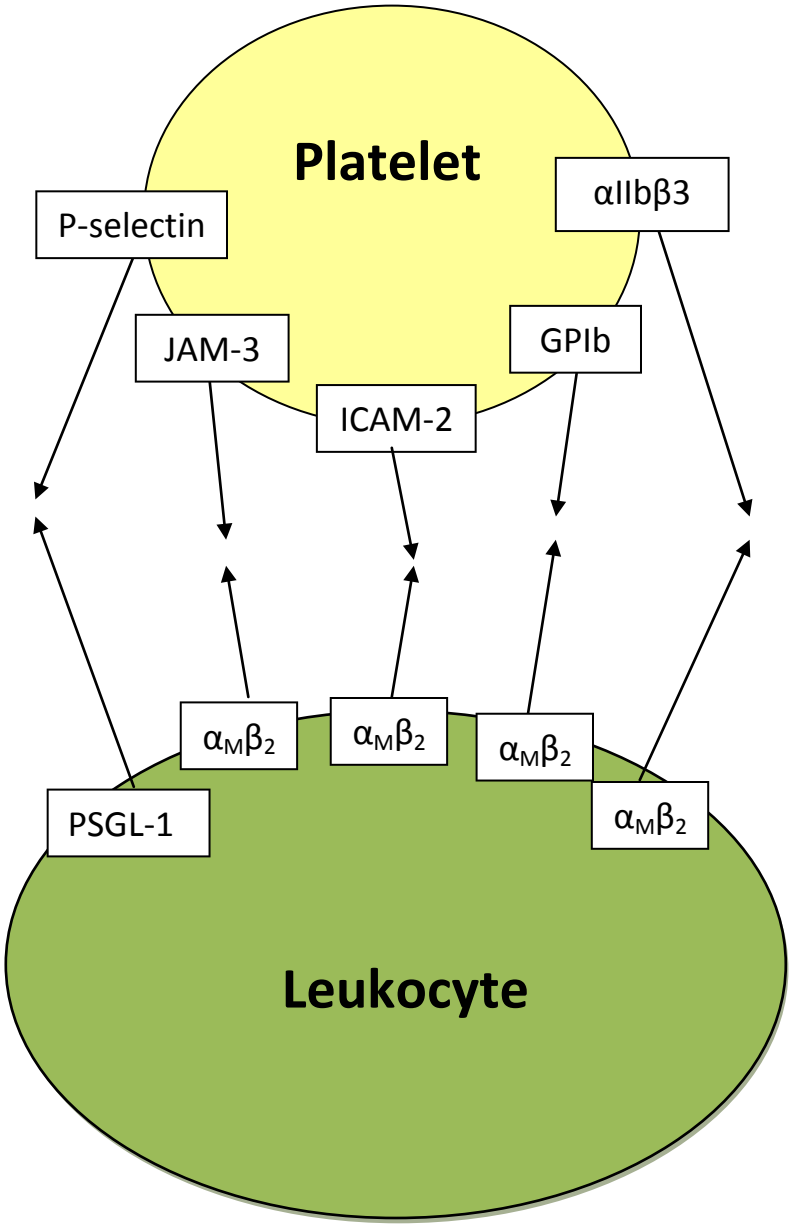


Figure 1.6. Molecular adhesive mechanisms that govern platelet adhesion to leukocytes. Leukocyte adhesion to platelets primarily involves surface expression of P-selectin on activated platelets and binding to PSGL-1, the counter-receptor present on neutrophils and monocytes. In addition to the endothelium, leukocytes can also tether, roll and subsequently rest on activated platelet monolayers via sequential action of platelet P-selectin and ICAM-2 binding to their leukocyte counterreceptors PSGL-1 and $\alpha_M\beta_2$ (CD11b/CD18; MAC-1), respectively. Recent data indicated that platelet GPIb and JAM-3 are also potential counter-receptors for leukocyte $\alpha_M\beta_2$. Furthermore, ICAM-2 and $\alpha_{IIb}\beta_3$-associated fibrinogen have also been proposed to mediate $\alpha_M\beta_2$-dependent platelet-leukocyte adhesion.

1.4.6. Denudation of the endothelium occurs following intestinal IR injury

The studies summarised above have identified a critical role for platelet-endothelial interactions following IR injury. However, there is currently a paucity of studies investigating the pathophysiological role of platelet-subendothelial ECM protein interactions in mediating platelet recruitment following IR injury. This is despite the fact that the microcirculatory component most damaged by IR injury is the EC itself (Carden and Granger 2000; Granger 1999; Seal and Gewertz *et al*., 2005). Massberg and colleagues demonstrated platelet recruitment in a murine model of intestinal IR injury. Interestingly, their electron microscopy studies confirmed the presence of platelets adherent to intact ECs, with no obvious endothelial denudation detected (Massberg *et al*., 1998 & 1999). However, they induced only segmental intestinal ischemia by isolating and clamping the mesenteric arterial vessels supplying a small (1-2cm) segment of the jejunum. This type of segmental ischemia did not induce severe mucosal injury, remote organ injury or significant morbidity, key features associated with clinical and experimental models of whole gut IR injury. Therefore, it is possible that the injury was not severe enough to lead to endothelial loss and ECM exposure.

Following IR injury, a loss in the integrity of vascular endothelium has been observed which could potentially expose underlying matrix components to circulating platelets (Nievelstein *et al*., 1988). Within the intestinal villous microcirculation, haemorrhage and a significant increase in plasma albumin leakage in response to IR injury has been demonstrated, which

could not be attenuated by a variety of pharmacological manipulations, suggesting gap formation between or denudation of ECs occurs (Kalia *et al.,* 2001; 2002a, 2002b, 2003 & 2005). Morphological studies of post-ischaemic tissue have also demonstrated swelling and detachment of ECs from the basement membrane with a resultant impairment of endothelial barrier function. Degradation of basal lamina and ECM components following IR injury has also been observed (Brodsky *et al*., 2002; Mueller *et al*., 1994). Indeed, Boyle and colleagues suggested migrating neutrophils were responsible for the proteolytic digestion of endothelial basement membranes that led to EC detachment, increased vascular permeability, and microvascular obstruction by these detached cells (Boyle *et al.,* 1996). It therefore seems plausible that exposure of underlying subendothelial ECM following intestinal IR injury may play a critical role in platelet recruitment and activation.

1.5. Platelet adhesion, activation and aggregation

1.5.1. Subendothelial matrix collagen mediates powerful platelet activation

At the site of vascular lesions, ECM proteins and immobilised von Willebrand factor (vWF) are exposed to the blood. Among these constituents of the subendothelial matrix, collagen, the most abundant vessel wall protein, has been proposed to be 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 (Nievelstein *et al.,* 1988; Watson 1999; Nieswandt and Watson 2003). The three stages of thrombus

development induced by platelet-collagen interactions are dependent upon a number of specific platelet glycoproteins (GPs) and integrins (Nievelstein *et al.,* 1988; Clemetson 1999). GPIb-IX-V and integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa; fibrinogen receptor) interact indirectly with collagen via vWF, whereas GPVI and integrin $\alpha_2\beta_1$ (GPIa/IIa) interact directly with collagen (**Figure 7**). Interactions between these molecules are significantly influenced by flow and trigger intracellular signaling events that reinforce adhesion and promote platelet activation. Similarly to the leukocyte adhesion cascade, platelet capture by ECM also involves rolling, activation and firm adhesion but also an additional platelet aggregation stage which leads to thrombus growth. **The role of these individual platelet receptors specifically in IR injury will be introduced in the relevant results chapters. This chapter will simply provide an introduction to their general role in thrombus formation. Suffice it to say, that there are currently limited studies that have investigated the role of platelet collagen receptors in contributing to the pathophysiology associated with IR injury.**

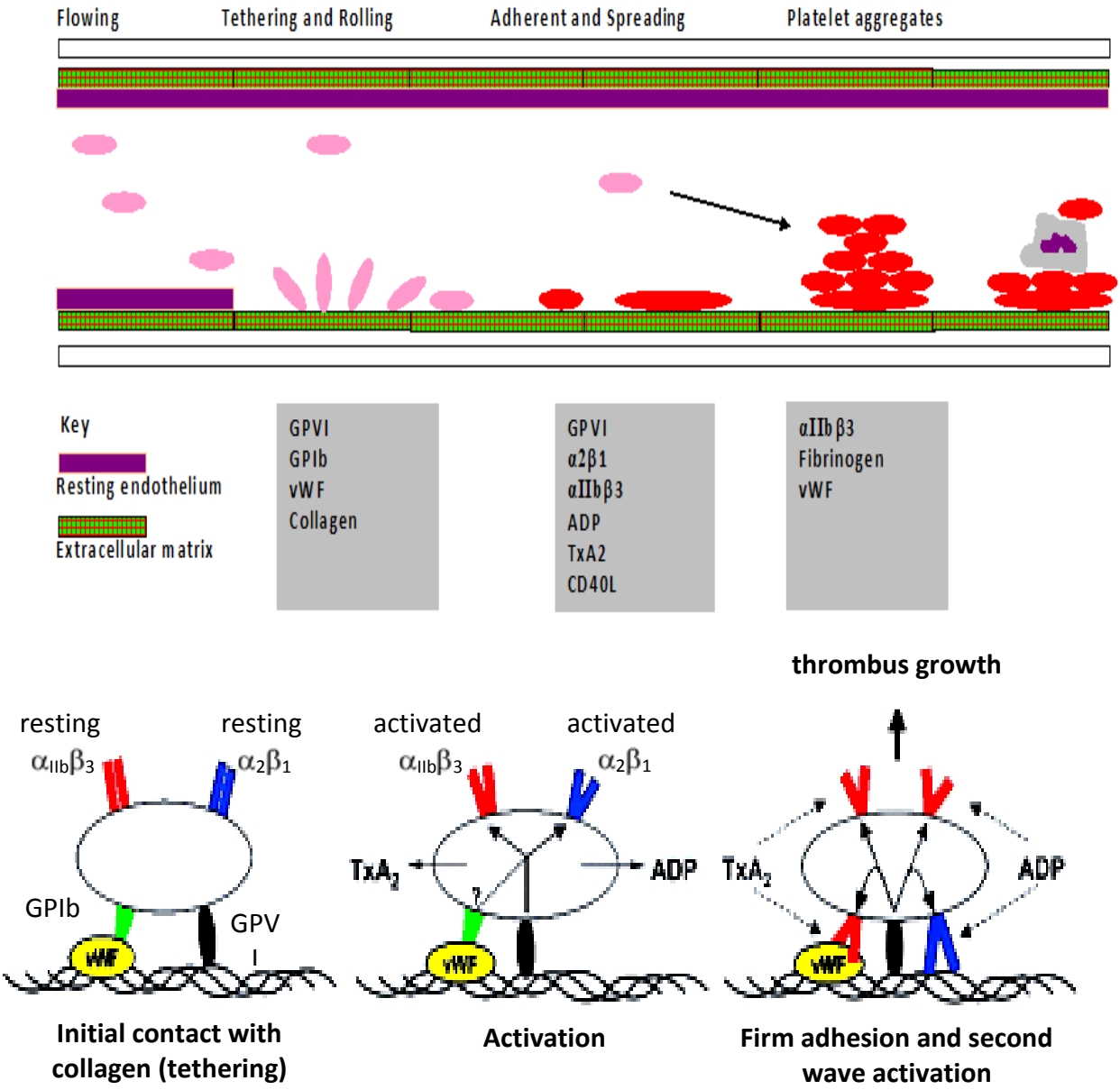


Figure 1.7. Models for platelet adhesion to extracellular matrix collagen. The initial contact and subsequent tethering or rolling of platelets involves the weak interactions of GPIb and GPVI with vWF and collagen respectively. GPVI-collagen interactions initiate cellular activation followed by shifting of integrins to a high-affinity state and release of ADP and TxA_2. Firm platelet adhesion to collagen through activated $\alpha_2\beta_1$ (directly) and $\alpha_{IIb}\beta_3$ (indirectly via vWF) results in sustained GPVI signalling and enhanced agonist release which mediates thrombus growth by activating additional platelets. Adapted from from Gawaz *et al.*, 2005 and Nieswandt & Watson, 2003.

1.5.2. Platelet rolling is mediated primarily by GPIb-von Willebrand interactions

The initial step in platelet recruitment following endothelial denudation is that of platelet rolling or tethering via the interaction of GPIb with immobilised vWF. This occurs through the rapid onset of association and rapid dissociation that occurs between GPIb and vWF. Weak interactions between the platelet surface glycoproteins and other subendothelial matrix proteins, most notably through the immunoglobulin GPVI which binds to collagen, lead to activation of integrin $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$, and firm adhesion (Watson *et al*.,2005). These initial weak interactions allow platelets to remain attached to the vessel wall despite the high shear forces generated within the vascular lumen (Jurk *et al.,* 2003).

There are approximately 25,000 copies of GPIb-IX-V on each platelet (Bergemeier *et al.*, 2007). This glycoprotein receptor is a product of 4 genes: GPIbα (135kDa), GPIbβ (25kDa), GPIX (22kDa) and GPV (82kDa). The N-terminus of GPIbα, between His 1 and Glu 282, is where the binding site for vWF is situated (Canobbi *et al.*, 2004). The GPIbα sub-unit primarily mediates the initial transient tethering or rolling of platelets over vWF bound to collagen. In addition, platelets can be recruited to activated endothelium through interactions between platelet GPIb-IX-V and endothelial P-selectin. However, similarly to platelet-collagen interactions under flow, these interactions also require integrin activation for strong adhesion (Berndt *et al*, 2001)**.** Lack or dysfunction of the GPIb-IX-V receptor complex is associated with the Bernard Soulier syndrome (BSS), a congenital bleeding disorder characterized by macrothrombocytopenia and inability of these platelets to

aggregate in response to the antibiotic ristocetin (Kunishima *et al.*, 1999). As stated earlier, GPIb-IX-V has also been shown to play a role in platelet adhesion to leukocytes via a $\alpha_M\beta_2$ (CD11b/CD18; MAC-1) dependent pathway (Hirahashi *et al*., 2009).

Bacterial translocation from the gut lumen into the systemic circulation is a major complication of intestinal IR injury. This takes place due to a break in the barrier function of the mucosal surface which allows entry of opportunistic indigenous viable bacteria into the bloodstream. Interestingly, translocated bacteria present within the systemic circulation have the ability to bind to platelets through interactions with GPIb. Thereafter, these bacterial-platelet complexes induce platelet activation leading to platelet-endothelial and platelet-platelet interactions which can further exacerbate the initial IR injury (Fitzgerald *et al.,* 2006).

1.5.3. Platelet activation is mediated primarily by GPVI-collagen interactions

Deceleration of platelets through tethering/rolling permits GPVI-collagen interactions which are essential for subsequent platelet activation (Elvers *et al.*, 2010). Platelet activation is a process that mediates the 'inside-out' activation of the $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ integrins from an inactive form to an active form, which is a pre-requisite for firm adhesion. Individual platelets contain approximately between 1500–3000 copies of GPVI (62KDa) on their surface. This receptor requires co-expression with the Fc receptor (FcR) γ-chain to form a functional unit (Watson 1999; Nieswandt *et al.*, 2003; Clemetson 1999). Interaction of collagen with

this receptor complex initiates an intracellular signalling cascade, with the FcR γ-chain being the critical signalling element of the complex (Nieswandt *et al.*, 2003).

1.5.4. Phospholipase Cγ2 plays a critical role in intracellular platelet signaling

Numerous downstream proteins form key elements of the GPVI/FcR γ-chain signaling pathway, which lead to tyrosine phosphorylation and activation of the effector enzyme phospholipase Cγ2 (PLCγ2) by collagen. The signaling pathway of $\alpha_2\beta_1$ also requires PLCγ2 (Michelson 2007). PLCγ2 lies downstream of the receptors GPIb-IX-V, GPVI and $\alpha_2\beta_1$. Activation of Src and Syk family kinases leads to the phosphorylation of PLCγ2. This results in the mobilisation of calcium ions (Ca^{2+}) and activation of protein kinase C (PKC), which are the two main mediators of platelet activation (Leoncini *et al.*, 2007). PLCγ2 mediates a number of events which include platelet degranulation and release of internally stored agonists such as ADP and de novo synthesis of TxA_2. These two agonists then interact with their receptors on the platelet surface. TxA2 binds to TxA2R, whilst ADP binds to both the low affinity P2Y12 receptor and the high affinity P2Y1 receptor.

This leads to a secondary, synergistic platelet activation which activates and increases the expression of the integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ and also increases their affinity for their ligands. Approximately 800-3000 copies of the $\alpha_2\beta_1$ integrin are expressed on the platelet surface. This integrin can also mediate weak platelet activation after which it undergoes a conformational change from a low to high affinity state which enables it to interact with

collagen (Auger *et al.*, 2005). These activated integrins are responsible for the stable adhesion of platelets to collagen. Once this stable adhesion occurs, platelets undergo spreading which is mediated by $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$ and GPVI, and reinforced by release of ADP and TxA2. The formation of filopodia and lamellipodia result from platelet spreading which stabilise and strengthen platelet adhesion to collagen and fibrinogen (Nieswandt & Watson 2003).

1.5.5. Platelet aggregation is mediated primarily by $\alpha_{IIb}\beta_3$-fibrinogen interactions

Following initial platelet adhesion to collagen and subsequent activation, the final event is platelet aggregation. This is mediated by soluble (or released) plasma fibrinogen interacting with $\alpha_{IIb}\beta_3$ on adjacent platelets. This integrin has two major functions. Firstly it forms strong adhesive interactions with the vessel wall that are capable of withstanding the effects of blood flow. Secondly, it assists in platelet to platelet cross-linking to allow for thrombus formation and growth. There are between 40,000-80,000 copies of $\alpha_{IIb}\beta_3$ on the platelet surface making it the most abundant receptor in these anucleate cells. Several agonists, such as collagen and ADP, through 'inside out' signalling, have the capability of activating $\alpha_{IIb}\beta_3$ (Watson 2009). Fibrinogen and vWF are considered the main ligands for this integrin but vitronectin and fibrin are amongst others capable of filling this role. These ligand interactions reinforce platelet activation by the generation of `outside-in' signals (Massberg *et al.*, 2005). Since platelet aggregation is a key stage of thrombus development, $\alpha_{IIb}\beta_3$ antagonists

(commonly called GPIIb/IIIa antagonists) are used both clinically and experimentally to prevent aggregation (Lefkovits *et al.,* 1995).

1.5.6. Limited studies have investigated platelet-subendothelial interactions following IR injury

Despite the importance of ECM proteins such as collagen in inducing thrombus formation physiologically during haemostasis, limited studies have investigated their pathological role following IR injury. Takaya and colleagues demonstrated using scanning electron microscopy (SEM) that murine myocardial IR injury induced platelet aggregation in areas clearly undergoing traumatic EC disruption and basement membrane exposure (Takaya *et al.*, 2005). They further demonstrated that infarct size, numbers of microthrombi and infiltrating neutrophils were significantly smaller in mice deficient in FcRγ-chain (and thus GPVI). The authors concluded that the GPVI / FcR γ-chain complex could be an important therapeutic target for cardiovascular disorders and that new anti-platelet strategies directed against the collagen receptor GPVI might improve cardiac perfusion.

More recently, Kleinschnitz and colleagues demonstrated *in vivo* that interfering with the early steps of platelet-vessel wall interactions mediated by GPIb and GPVI using an antibody approach protected mice from ischemic brain injury in an experimental stroke model. In contrast, blockade of the final common pathway of platelet aggregation with anti-$\alpha_{IIb}\beta_3$ antibodies had no positive effect on infarct size and stroke outcome (Kleinschnitz *et al.*,

2007). Indeed, blocking $\alpha_{IIb}\beta_3$ significantly increased the rate of intracerebral haemorrhage and mortality in a dose-dependent manner (4 out of 7 mice tested died). This study concluded that endothelial denudation associated with stroke could recruit platelets through indirect interactions of GPIb with collagen and that blocking GPIb could open new avenues for acute stroke treatment in humans.

1.6. Summary

A number of studies have demonstrated a key role for platelets in the pathophysiology of intestinal IR injury. Mechanistic studies have identified a role for the activated endothelium in regulating platelet recruitment and activation. Studies on intestinal IR injury have so far only focused on inhibiting platelet-endothelial interactions using $\alpha_{IIb}\beta_3$ receptor antagonists (Salter *et al*., 2001; Massberg *et al.,* 1998 & 1999). However, recent evidence suggests a role for the subendothelial matrix in mediating platelet activation in IR injury (Takaya *et al.*, 2005; Kleinschnitz *et al.*, 2007). ECs are extremely susceptible to ischaemia and their detachment as well as damage to the basal lamina has been demonstrated following IR injury. Platelet-subendothelial matrix interactions, particularly platelet-collagen interactions, are bought about by a range of platelet receptors and integrins which lead to their adhesion, activation and aggregation. Platelet receptors are at the forefront of recent research and major advances have been made in understanding their molecular functions and their downstream signaling pathways.

The use of pharmacological inhibition and knocking out of nearly all known receptors, adhesion molecules, and many signaling molecules have helped to reveal new mechanisms for how the thrombotic and haemorrhagic propensity of platelets is controlled in health and disease. However, the role of the glycoprotein receptors GPVI, GPIb-IX-V and the integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$, which interact directly or indirectly with collagen, in mediating platelet adhesion and activation have not been determined following intestinal (or non-intestinal) IR

injury *in vivo*. Indeed the role of these receptors in experimental models of disease generally is limited. Comparison of the effect of blockade of these glycoprotein receptors would provide important information on the role of the subendothelial collagen in contributing to platelet activation in intestinal IR injury.

1.7. Aims and Hypothesis

In accordance with previously published research, we anticipated endothelial activation to occur as a result of intestinal IR injury. Therefore, we expected to observe platelet-endothelial interactions. However, we further hypothesised that intestinal IR would also lead to localized areas of endothelial detachment and denudation, which would be increased as the duration of the ischaemic insult increased. As a consequence of this, we hypothesized that the resulting interaction with exposed subendothelial collagen would lead to platelet activation and formation of microthrombi, which would contribute significantly to the resulting intestinal pathology. This research therefore aimed to specify the role of subendothelial collagen in supporting formation of microthrombi and subsequent tissue injury *in vivo* using fluorescent intravital microscopy (IVM).

Studies were focussed primarily on the mucosal villous microcirculation due to its susceptibility to IR injury. We initially aimed to determine the time course, kinetics and pattern of platelet events in this region in vivo using fluorescent intravital microscopy (IVM). Thereafter, experiments were conducted in mice deficient in FcR γ-chain (and hence GPVI),

mice-deficient in the $\alpha_{IIb}\beta_3$ or in mice perfused with blocking antibodies to the integrins $\alpha_2\beta_1$, and GPIbα. Additional studies were also conducted in PLCγ2 deficient mice to determine the importance of this intracellular signalling protein.

Specifically the aims of this research were as follows:

1. Develop a viable model of **murine intestinal IR** model and monitor *in vivo* using IVM the recruitment of platelets both spatially and temporally.

2. Monitor whether platelet recruitment varied :
   - with different durations of ischaemic injury
   - in different regions of the gut i.e. proximal jejunum vs the distal ileum
   - in different layers of the gut wall i.e. inner mucosal vs outer serosal layer

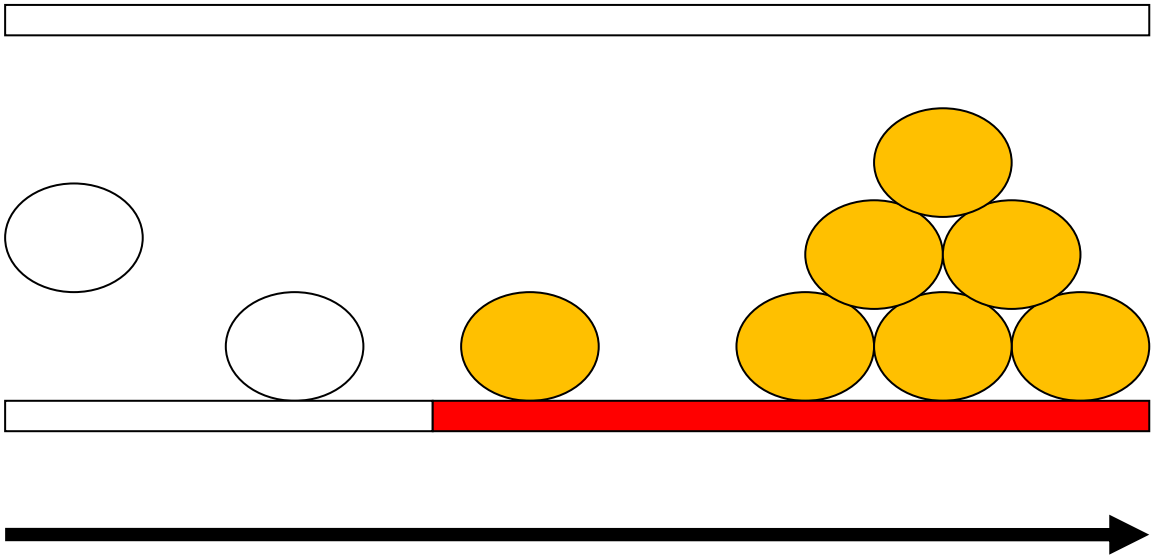
3. Determine whether platelets interacted with intact endothelium and/or exposed subendothelial collagen. In order to conduct these studies, a novel dual labelling method was developed that allowed three platelet events to be monitored within the same animal simultaneously *in vivo,* namely:
   - microthrombi formation
   - adhesion of individual platelets
   - circulation of individual freely flowing platelets

4. Determine the platelet collagen receptors involved in platelet recruitment following intestinal IR injury. Specifically, the role of GPVI, GPIb-IX-V, $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, PLCγ2 and P-selectin was determined using function blocking antibodies or knockout mice.

5. Determine the role of platelets in contributing to the remote organ injury to the liver and lungs which is frequently associated with intestinal IR injury.

Chapter 2

Materials and Methods



2.1. Materials

2.1.1. Antibodies

All antibodies used in this thesis were used at the concentrations detailed in **Table 2.1**.

Antibody	Conj	Clone	Origin	Target	Isotype	Company	Conc^{n}
anti- GPIbα	N/A	Xia. B2	Rat	mouse	IgG2a	Emfret	0.5mg
anti- GPIbα	FITC	Xia.B2	Rat	mouse	IgG2a	Emfret	0.5mg
anti- GPIbα	N/A	Xia. H10	Rat	mouse	IgG2a	Emfret	0.5mg
anti – α2β1	N/A	Sam.C1	Rat	mouse	IgG2b	Emfret	0.5mg
anti – α2β1	N/A	Sam.G4	Rat	mouse	IgG2b	Emfret	0.5mg
anti – α2β1	FITC	Sam.G4	Rat	mouse	IgG2b	Emfret	0.5mg
Alexa 594	N/A	CY3 dye	Goat	Rat	IgG1	Invitrogen	2mg/mL
CD41	FITC	Leo.A1	Rat	Mouse	IgG1	Emfret	0.5mg
CD41	N/A	MWReg30	Rat	Mouse	IgG1	BD Pharmingen	1mg

Table 2.1. Antibodies used in this thesis to investigate the function and mechanisms of recruited platelets at the sites of injury. Antibodies were purchased from Emfret, Invitrogen and BD Pharmingen.

2.1.2 Common materials

A number of materials were used repeatedly in this thesis and are presented in **Table 2.2** for clarity. Materials referred to in the text were obtained from the indicated suppliers unless otherwise stated.

Material	Abbreviation	Manufacturer	Location
Acetone	-	Fisher Scientific	Leicestershire, UK
3-aminopropyltriethoxysaline	APES	Sigma	Poole, UK
Ammonium Hydrochloride	NH_4Cl	Sigma	Poole, UK
Bovine Serum Albumin	BSA	Sigma	Poole, UK
5'6-carboxyfluorescein diacetate succinimidyl ester	CFSE	Molecular Probes (Invitrogen)	Paisley, UK
Endothelial cell growth supplement from neural tissue	EC growth supplement	Sigma	Poole, UK
Heat inactivated Fetal Bovine Serum	FBS	Invitrogen	Paisley, UK
HEPES	-	Sigma	Poole, UK
Gelatin (Type I from porcine skin)	-	Sigma	Poole, UK
Glucose	-	Sigma	Poole, UK
Ketamine Hydrochloride	-	Pharmacia Animal Health	Northampton- shire, UK
L-Glutamine (Glutamax^{TM})	-	Invitrogen PAA Laboratories	Paisley, UK Peterborough, UK
Magnesium Chloride	$MgCl_2$		
2-mercaptoethanol	-	Invitrogen	Paisley, UK
Murine Epidermal Growth Factor	mEGF	Peprotech	London, UK
Sodium Carbonate	$NaHCO_3$		
Sodium Chloride	NaCl	Sigma	Poole, UK
Sodium Phosphate	Na_2HPO_4		
Penicillin	-	Invitrogen PAA Laboratories	Paisley, UK Peterborough, UK
Phosphate Buffered Saline	PBS	Invitrogen	Paisley, UK
Potassium Chloride	KCl		
Sodium ethylene tetraacetic acid	Na.EDTA	Sigma	Poole, UK
Sodium Chloride	NaCl	Sigma	Poole, UK
Streptomycin	-	Invitrogen	Paisley, UK
Trypsin-EDTA	-	Invitrogen	Paisley, UK
Xylazine Hydrochloride	-	Millpledge Vetinary	Nottinghamshire, UK

Table 2.1. Names and purchasing information of the chemicals used in this thesis. Abbreviations (if applicable) are also shown.

2.2. Cell Isolation and preparation

2.2.1. Isolation of mouse platelets

Mouse blood (700-900ul) was drawn from CO_2 asphyxiated mice following isofluorane anaesthesia by descending aorta puncture into 100 µL of acid citrate dextrose (ACD). This was added to an eppendorf containing 200µl of tyrodes solution (129 mM NaCl/0.34 mM Na_2HPO_4/2.9 mM KCl/12 mM $NaHCO_3$/20 mM Hepes/5 mM glucose/1 mM $MgCl_2$) and spun at 2000rpm for 5 minutes in a microfuge. Platelet rich plasma (PRP) and $^1/_3$ of the red blood cells were removed and placed into a separate eppendorf tube. This solution was then centrifuged at 1000rpm for 6 minutes before carefully removing the PRP only and placing into a third tube. To stop platelet aggregation occurring through the final spin, 1µl of prostacyclin (PGI_2) was added to the PRP before centrifugation at 2800rpm for 6 minutes. The supernatant was then removed leaving a platelet pellet in the bottom of the tube, which was resuspended with tyrodes solution to a volume of 200µl. Using a coulter counter (Becton Dickinson, USA), 2.5ul of the PRP solution was pipetted into 10mls of saline to give a platelet count per 1ml. These platelets were then allowed to rest for 30 minutes before use in either *in vivo* or *ex vivo* experimentation.

2.2.2. CFSE labelling of platelets

The initial steps of this process follow the above platelet isolation protocol up to centrifuging the platelets at 2800rpm for 6 minutes. The supernatant was then removed leaving the platelet pellet. This was then resuspended in 500µl of 10µM CFDA-SE solution and incubated

at room temperature for 20 minutes in the dark. CFDA-SE (5'6-carboxyfluorescein diacetate succinimidyl ester) is a non-fluorescent, cell permeable flourescein derivative, which is modified by intracellular esterases to render the molecule brightly fluorescent. Modification by these intracellular enzymes also renders the modified molecule cell impermeant. After this time had elapsed, 1µl of PGI_2 was added to the PRP and spun again at 2800rpm for 6 minutes. The supernatant was then removed leaving a platelet pellet in the bottom of the tube. This pellet was resuspended with tyrodes solution to a volume of 200µl. Using a coulter counter, 2.5µ of the PRP solution was pipetted into 10mls of saline to give a platelet count per 1ml. Dilutions, by the addition of tyrodes, were then undertaken to leave the required platelet count for specific experimentation. These platelets were then allowed to rest for 30 mins before use in *in vivo* or *ex vivo* procedures. For *in vivo* studies, these platelets were injected into a different mouse undergoing IR injury and were hence called donor platelets.

2.2.3. Murine cardiac endothelial cell (MuCEC-1) culture

Isolation of primary murine ECs is typically very difficult and requires elaborate and time consuming purification techniques (Marelli-Berg *et al.,* 2000). Furthermore, overgrowth of contaminating cells often prevents long-term culture of these populations (Marelli-Berg *et al.*, 2000). As a result of the difficulties encountered with primary isolates, an immortalised murine cardiac EC line was used (MuCEC-1) (Bouis *et al.,* 2001). MuCEC-1 cells were obtained locally from Dr. Patricia Lalor (University of Birmingham, UK). Cells were grown to confluence in gelatin coated flasks. MuCEC-1 cells are isolated from H-$2K^b$-tsA58 mice, which express a

thermoliable strain (tsA58) of the SV(40) large T antigen (tsA58 TAg) which allows production of conditionally immortalised cells (Lidington *et al.,* 2002). These ECs are characterised by their phenotypic cobblestone appearance and their ability to form tubes on an artificial extra-cellular matrix. Furthermore, these cells were shown to be positive for ICAM-1 and VCAM-1, with selectin expression neglible (Lidington *et al*., 2002). MuCEC-1 were maintained in DMEM containing 10% FBS, supplemented with 50U/ml penicillin, 50U/ml streptomycin and 2mM L-Glutamine and 10ng/ml mEGF. At confluence, cells were enzymatically dissociated using Trypsin-EDTA, washed in complete media and split 1 in 3. For static adhesion assays, a confluent flask of MuCEC-1 was enzymatically dissociated using Trypsin-EDTA, washed in complete media and one third resuspended in 30ml of complete medium. 1ml of the resulting cell suspension was then added to each well of a 24 well plate containing a pre-treated coverslip (Refer to Sections 2.6.2 and 2.6.3) and allowed to grow to confluence prior to use.

2.3. Monitoring platelet and leukocyte trafficking *in vivo* using intravital microscopy

2.3.1 Animals

Animal experiments were completed with prior approval of the local ethics committee and the United Kingdom Home Office, in accordance with the Animals (Scientific Procedures) Act of 1986 under Project Licence 40/2749 (Dr. Neena Kalia). 10-12 week old male C57Bl/6 wild-type mice were obtained locally (Biomedical Services Unit, University of Birmingham, UK). Some experiments were conducted on P-selectin$^{-/-}$ mice, kindly provided by Professor Paul Hellewell (Sheffield University, UK). P-selectin$^{-/-}$ mice were bred from heterozygotes on a C57Bl/6 background and then transported to Birmingham at least one week before experimentation. P-selectin$^{-/-}$ phenotype was confirmed by flow cytometric analysis of peripheral blood platelets. FcR γ-chain and $\alpha_{IIb}\beta_3$ deficient mice were bred locally in colonies held by Professor Steve Watson (University of Birmingham). Genotyping was undertaken to confirm absence of the knockout gene. All animals had *ad libitum* access to food and water.

2.3.2. Surgical preparation for *in vivo* studies

Anaesthesia was induced by intraperitoneal administration of ketamine (100 mg/kg Vetalar; Amersham Biosciences and Upjohn Ltd., UK) and xylazine hydrochloride (10 mg/kg; Millpledge Pharmaceuticals, UK) delivered in 0.9% saline. The trachea and right common carotid artery was cannulated with polyethylene portex tubing (Smiths Medical, Hythe, UK)

to assist breathing and to allow administration of donor CFSE labelled platelets, function blocking antibodies and additional maintenance anaesthesia as required.

Preliminary *in vivo* experiments were conducted whereby only 100µl of tyrodes solution containing 1 x 10^8 of donor CFSE labelled platelets were injected intra-arterially. However, subsequent experiments combined introducing these donor labelled platelets with fluorescent labelling of all endogenous platelets with a fluorescent dye of a different wavelength. This was achieved by injecting 40µl Alexa Fluor 594 conjugated to goat anti-rat antibody (2 mg/mL; Molecular Probes, Eugene, OR, USA) and 20µl rat anti-murine CD41 (GPIIb) antibody (0.5 mg/mL; BD Biosciences Pharmingen, San Diego, CA, USA) via the carotid cannula. For experiments conducted in GPIIb deficient mice, Alexa 594 was conjugated to a rat anti-muirine GPIb antibiody. All endogenous platelets were labelled as the amount of antibody added per mouse was in excess of the total number of platelets (assuming a platelet concentration of about 1 ×10^9 per mL blood) by around 1,000-fold. The brightness of individual endogenous platelets should be the same for each experiment as the same preparation of labeled antibody, and the same amount of antibody per gram of mouse is introduced. The antibody will dissolve in the blood as expected and since the Alexa-594 fluorophore is strongly conjugated to the primary CD41 antibody, no free fluorescent label should be available to dissolve in the blood (Kalia *et al.,* 2009). Alexa 594/CD41 antibody and/or donor CFSE labelled platelets were introduced into the circulation after neck surgery was performed and given 30 minutes to circulate.

2.3.3. Induction of intestinal IR injury

During this time, the animal was transferred onto a petri-dish and a midline laporotomy was performed by cutting along the linea alba, an avascular fibrous structure found between the left and right rectus abdominis muscles. The proximal jejunum or the distal ileum of the small intestine was gently exteriorised and held in place using moist tissue paper. Tissue paper rather than surgical clips were used to limit the amount of injury. The jejunum was orientated in a way that allowed for some length of the mesenteric arteriole supplying that segment to be positioned flat on the petri-dish surface. Small intestinal ischaemia was induced by application of an atraumatic vascular clamp to the superior mesenteric artery (SMA) for 15, 30, 45 or 60 minutes. This allowed ischaemia to be induced to the lower duodenum, jejunum and ileum, but importantly did not lead to mortality during the 4 hour reperfusion period used for intravital imaging. Visual evidence that ischaemia had been induced could be seen by the palor or change of colour within the intestinal tissue (**Figure 2.1**). The small intestine was reperfused by removal of the clamp and the animal was immediately prepared for intravital monitoring by laying it on its side within the petri-dish.

The mesenteric artery supplying the exteriorised segment of the jejunum or the outer serosal wall microvessels within this region were monitored intravitally in some preliminary experiments. However, the major focus of the research was the microcirculation of the lumenal mucosal villi in the jejunum and ileum. In order to view the mucosa, it was exposed by cauterising 1-1.5cm along the anti-mesenteric border and kept open using moist tissue

paper. Once exposed, the mucosal surface was carefully placed facing downward and wrapped in a single layer of tissue paper to limit peristaltic movement. Throughout the experiment, the preparation was continuously kept most by pipetting bicarbonate-buffered saline solution (36°C, pH 7.4). Control sham mice underwent exactly the same procedure but the SMA was not clamped. The petri-dish holding the mouse was then transferred to the stage of an inverted Olympus intravital microscope.

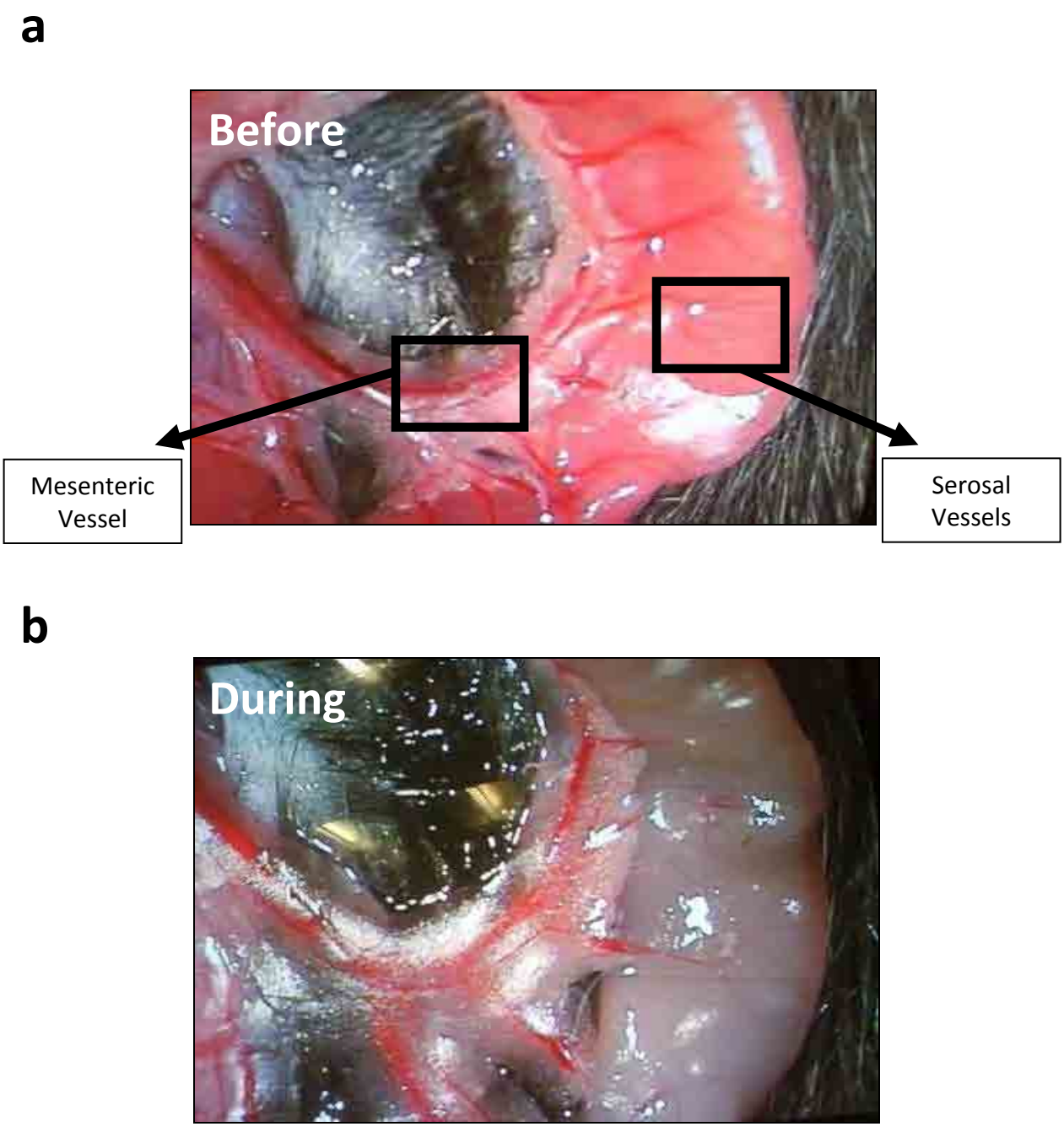


Figure 2.1. Visual evidence of ischaemic episode through SMA clamping. (**a**) A perfused tissue appeared a healthy pink colour. (**b**) However, clamping of the superior mesenteric artery using a non-traumatic clamp led to an obvious tissue palor due to a lack of perfusion within the small intestine.

2.4. Quantification of cell trafficking *in vivo*

2.4.1. Capture of intravital images from the jejunum and ileum

Experiments were conducted using a motorized Olympus BX-61WI microscope (Middlesex, UK). Simultaneous capture of brightfield and fluorescent images was achievable by rapidly alternating the illumination of the specimen between transmission light using a Uniblitz shutter (Vincent Associates, Rochester, NY, USA) and epi-fluorescent light using a Lambda DG-4 high speed wavelength changer (Sutter Instrument Company, Novato, CA, USA). Digital images were captured using a high resolution CCD camera . All these components were controlled by a Dell workstation and imaging software (Slidebook, Intelligent Imaging Innovations, Denver, CO, USA) and stored as permanent digital images for subsequent off-line analysis.

Using the x20 objective, one intestinal serosal field of view or a mucosal field of view from the ileum and jejunum (413µm x 308µm) was pre-selected containing between 6 and 10 individual villi structures in the jejunum or 12 to 16 in the ileum. After 30 minutes reperfusion, 1 minute recordings were taken from the same pre-selected field of view every 30 minutes for a period of 4 hours. A brightfield image and two fluorescent images using the FITC and CY3 filter channels were captured simultaneously. This allowed an image showing the architecture of the microvessels to be captured at the same time as a green image of the donor CFSE labelled platelets and a red image of the Alexa 595 labelled endogenous

platelets. At the end of this monitoring period, an additional six fields of view were taken in order to ensure the events taking place in the pre-selected field of view represented those taking place in other regions of the ileal and jejunal mucosa. These six regions were not randomly selected but followed a strict pre-determined pattern. Six regions were selected relative to the position of the initial field (**Figure 2.2**). Events in the pre-selected region were considered representative if the final level of cell adhesion was within two standard deviations of the mean of the six fields examined following the experiments. Two standard deviations are typically expected to contain around 95% of the statistical population.

2.4.2. Quantification of individual platelets and microthrombi within jejunum and ileum

To quantitate individual green CFSE labelled donor platelet events, each platelet observed during the one minute recording was counted. Individual platelets were deemed either as adherent if situated in the field of view for ≥ 30 seconds or free flowing if ≤ 2 seconds. Red Alexa 594 labelled microthrombi were quantitated according to their size (in pixels) or their fluorescent intensity. Data was presented as the sum of the size or fluorescent intensity of all microthrombi in the field of view. A 'mask' was drawn over the thrombi and imaging software was used to calculate its size and intensity (**Figure 2.3a**). The background fluorescence intensity, predominantly due to freely circulating endogenous platelets, was determined using the image analysis software and subtracted from the fluorescence intensity of the thrombi. This allowed the fluorescent intensity of only the microthrombus to be determined.

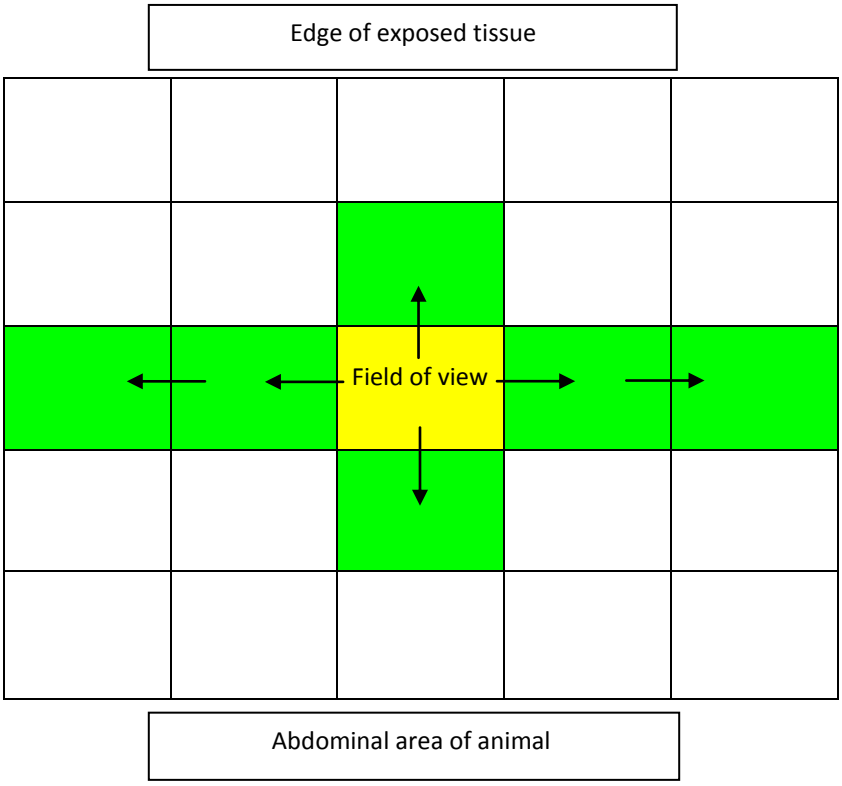


Figure 2.2. Selection criteria for fields of view to monitor for adhesive events intravitally. Six fields of view (green boxes) were selected at the end of the intravital experiment to ensure data obtained from the pre-selected field of view (yellow box) were representative of events across the whole exposed tissue. Events in the pre-selected region were considered representative if the final level of cell adhesion was within two standard deviations of the mean of the six fields examined following the experiments. Two standard deviations are typically expected to contain around 95% of the population.

2.4.3. Quantification of freely flowing platelets within the mesenteric vessels

The number of individual donor CFSE labelled platelets circulating within the mesenteric vessel supplying the exteriorised segment of the jejunum was determined by manually counting them. However, in this large vessel, the numbers of donor platelets that could be visualised and the blood flow velocity was very high and so it was not possible to always distinguish individual platelets as they whizzed past in real time. Hence, manually counting the individual platelets was likely to provide an underestimated value. Therefore, to combat this problem a novel approach of measuring the fluorescent intensity within a 100µm x100µm square drawn over the vessel was determined. The intensity passing through this square at a series of time points, during a 1 minute capture period was averaged automatically by the imaging software to provide a single intensity reading (**Figure 2.3**).

2.4.4. Labeling of endogenous leukocytes and quantification *in vivo*

For some experiments, endogenous leukocytes were labelled *in vivo* using the cell-permeable nucleic dye, Acridine orange (Sigma-Aldrich, Poole, UK). When bound to DNA, Acridine orange displays similar fluorescent spectra to FITC. To visualise leukocytes, animals were injected intra-arterially with 100µl of 200µg/ml Acridine orange. After injection, the dye was allowed to circulate for 5 minutes only before image capture at either 1 hour or 4 hours reperfusion. Adherent leukocytes were quantitated using the same protocol for quantitating adherent platelets (**refer to 2.4.2**).

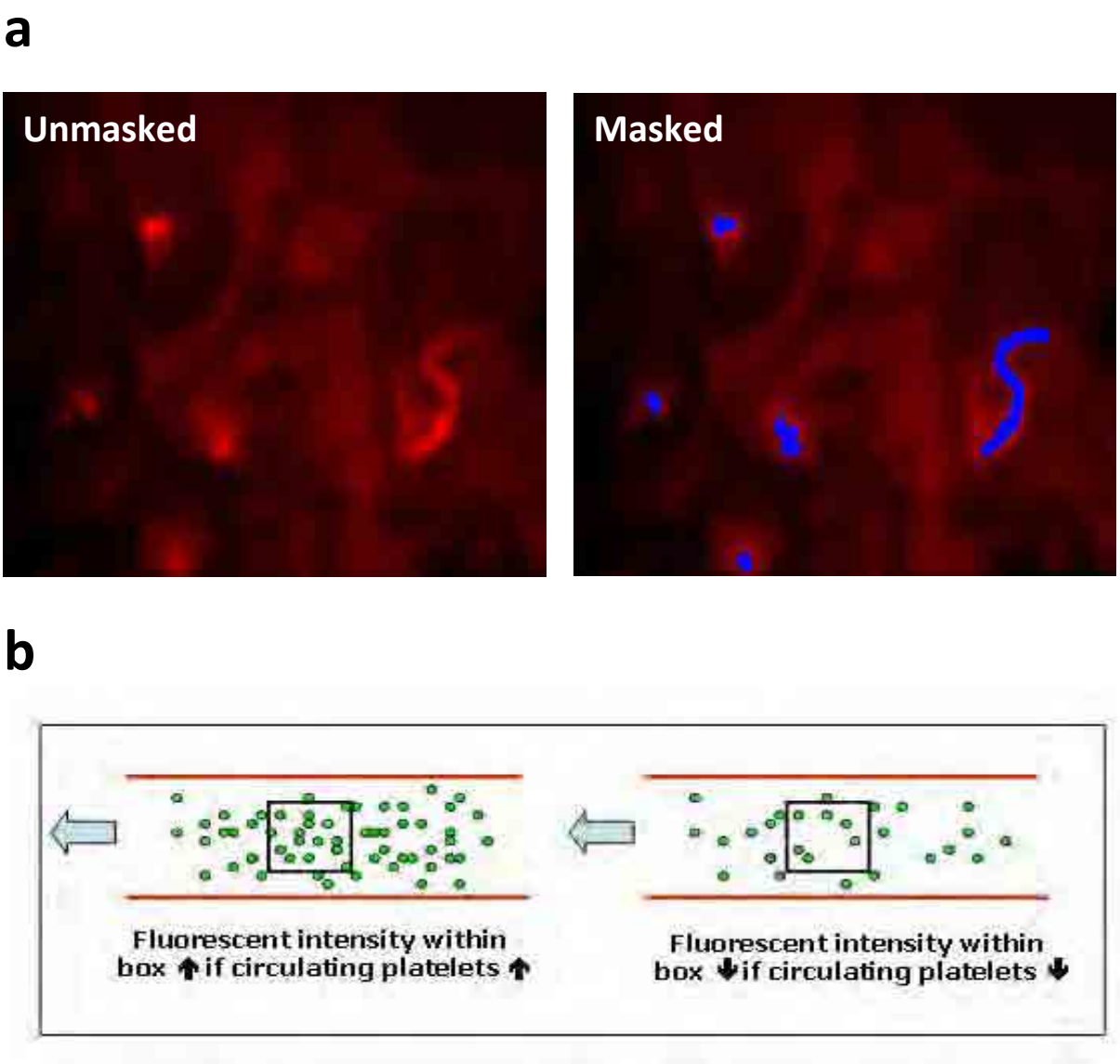


Figure 2.3. Quantification of microthrombi within the jejunum and ileum and freely flowing platelets within the mesenteric vessels. (**a**) Using a masking tool available in the imaging software, it was possible to quantify the sum of the pixel area and fluorescent intensity of a masked region representing the microthrombi in the whole mucosal field of view. (**b**) Free flowing platelets in mesenteric vessels were quantitating by drawing a 100µm x 100µm square over the vessel and getting the software to average the fluorescent intensity within within in over a period of one minute.

2.4.5. FITC-BSA production and quantification of macromolecular leakage

At the end of all intravital experiments, 200μl of FITC-conjugated bovine serum albumin (FITC-BSA) was injected intra-arterially. When introduced systemically, it is retained within the vasculature, thereby facilitating easy visualisation of the vasculature and confirming blood flow (Kalia *et al.,* 2002). However, when the integrity of the vessel is disturbed, it appears as a flare in the surrounding interstitium. 10% FITC on celite was conjugated to bovine serum albumin (both Sigma, Poole, UK) by mixing the FITC, BSA and 10ml of bicarbonate buffer (pH 9.0) overnight at 4°C. The resulting mixture was centrifuged at 10-12,000rpm for 10 minutes and conjugated/unconjugated BSA separated by dialysis (12,000MW cut off; Spectrum Laboratories, CA, USA). Prepared FITC-BSA was frozen and stored until use in eppendorfs. Macromolecular leakage was determined by measuring the fluorescent intensity within the field of view using gray levels. Since a 16-bit camera was used to capture the images, it meant that it was able to distinguish between 65, 536 levels of grey with 0 being black and 65,535 being white and all values in between being different stages of grey. Increasing values are directly proportional to increasing amounts of leakage.

2.5 Non-intravital methods for assessing injury

2.5.1. Haematoxylin and eosin (H&E) tissue staining

Haematoxylin and eosin (H&E) tissue staining was used to examine the severity of tissue damage in intestinal IR injured jejunum, ileum, liver and lungs. Mice were subjected to intestinal IR injury or sham surgery. After 1 or 4 hours reperfusion, the animals were sacrificed and a 0.2cm piece of ileal and jejunal tissue, 1 liver lobe and both lungs were removed and placed into formalin for 12-14 hours. The tissue was then processed through a series of dehydration and fixing steps using IMethylated spirits and xylene solutions. After processing, the tissue was paraffin wax embedded before 10µm sections were cut on to a microslide using a HM 355 motorised microtome (Thermo Scientific, Walldorf, Germany). Sections were allowed to dry in a mini-oven for 10 minutes and then H&E stained using an automated histology stainer and mounted in DPX solution (Sigma, Poole, UK).

2.5.2. Scanning electron microscopy (SEM)

Ileum and jejunum was removed from IR injured or sham animals and pinned out on a corkboard to expose the mucosal villi structures. This was then placed in 2.5% glutaralderhyde to fix overnight. Tissue preparation was kindly undertaken by colleagues within the Department of Metals and Metallurgy, University of Birmingham. This included sectioning the tissue with glass blades and an incubation process that allowed a heavy metal

solution to be absorbed by the tissue allowing for contrast within the images. A Philips 700XL SEM was used to scan and capture images from the mucosal surface.

2.5.3. Liver alanine aminotransferase (ALT) levels following intestinal IR injury

To biochemically confirm the presence of remote hepatic injury subsequent to intestinal IR injury, blood samples were obtained from mice subject to sham or IR procedures at 4 hours reperfusion. Increased levels of serum ALT are directly proportional to the extent of liver injury. Samples were analysed using an Olympus Au400 analyser. The automated analyzer facilitates ALT activity and monitors light absorbance at 340nm which is relative the level of enzyme in the sample. Sampling was performed by the Department of Clinical Chemistry at the Birmingham Women's Healthcare NHS Trust (Birmingham, UK).

2.6. Quantification of platelet adhesion *in vitro*

In addition to monitoring platelet adhesion *in vivo*, their adhesion to ECs (MuCEC-1) and collagen was also determined *in vitro* using a static or flow based adhesion assay respectively.

2.6.1. Static endothelial adhesion assays

24 well plates for static assays were pre-treated with 5% gelatin to aid EC adhesion. MuCEC-1 were cultured to confluence over 2-4 days on the treated surfaces. Media was removed from the endothelial monolayers which were washed twice with 0.5ml warm PBS and then

treated with 10ng/ml TGFβ and 100U/ml TNFα or tyrodes solution for 4 hours. 500µl of activated, or resting, washed IR platelets at a concentration of 1 x 10^7/ml were then incubated with the endothelial cells for 45 mins at 37^{o}C. Proceeding this step, endothelial monolayers were washed with warm 0.1% PBS before fixing with 2% glutaraldehyde in PBS for 15 minutes at 37^{o}C. The fixative was then washed away three times with 0.1% before mounting with medium. Slides were then read within 2 hours for analysis. CFSE labelled platelets were imaged using an Olympus differential interference contrast (DIC) microscope. 6 separate fields were selected from each endothelial monolayer using a set pattern to ensure randomness (**Figure 2.4**). Slides were analysed and cells counted using the slidebook software.

2.6.2. Flow based adhesion assay over collagen

Thin rubber tubing was attached to the three outlets on the valve labelled output, sample and wash. Tubing from the wash and sample outlets were attached to a 20ml syringe, containing tyrodes solution, and a 2.5ml syringe respectively. The input tubing was connected up to the input duct of the glass capillary, which was placed on the stage of an inverted microscope (DM IRB: Leica, Milton Keynes, UK) for imaging. A 50ml glass syringe was fixed securely to the automated pump and a 3-way valve. Attached to this valve was another 20cm long piece of rubber tubing which fed into the output duct of the glass capillary tube to collect flown blood. A 20ml plastic syringe was also attached to this valve to allow for water to enter and fill the output rubber tubing. Careful attention was taken to

allow the whole flow system to be full of water with no apparent air bubbles present. Air bubbles would have affected both flow velocities and the adhesiveness of collagen fibres.

Glass capillary tubes (Camlab, Cambridge, UK) were coated with 100µg/ml type 1 collagen from equine tendon (Horm, Nycomed, munich, Germany) for 1 hour at room temperature. These capillaries were then washed with PBS and left overnight at room temperature. Once the capillary tube was fixed into position, within the flow assay set up, a single field of view was then chosen, to the far left of the slide, as our primary observation area. 500µl of anti-coagulated (40µM P-PACK plus 5U/ml heparin) blood from either sham or IR experiments was then perfused over the chamber at a wall shear rate of 300 s^{-1}. Five additional random images were then taken, following the same imaging protocol as with the endothelial adhesion assay. Images were analyzed off-line using ImageJ software. Platelet adhesion results are expressed as the percentage area covered by platelets.

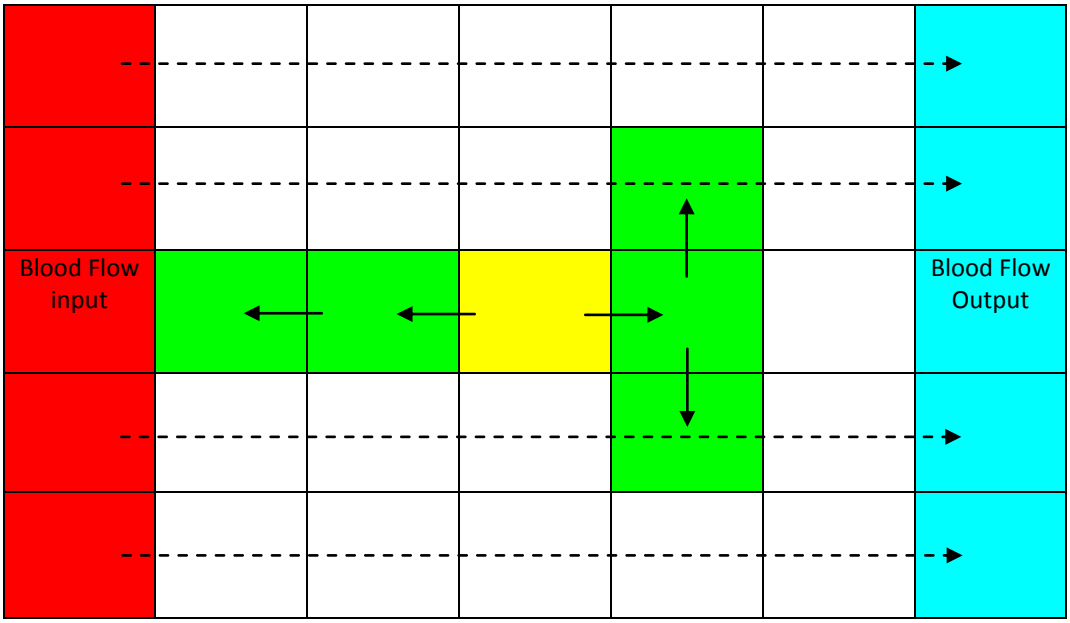


Figure 2.4. Selection criteria for fields of view to monitor for adhesive events *in vitro.* 5 fields of view (green boxes) were selected at the end of the *in vitro* adhesion experiment to ensure data obtained from the preselected field of view (yellow box) were representative of events across the whole thrombogenic surface. Events in the pre-selected region were considered representative if the final level of cell adhesion was within two standard deviations of the mean of the six fields examined following the experiments. Two standard deviations are typically expected to contain around 95% of the population.

2.7. Methodology for receptor blocking studies

2.7.1. Confirmation of adhesion molecule blockade

The role of various platelet receptors in contributing to their adhesion to either activated endothelium or exposed subendothelial matrix proteins *in vitro* and *in vivo* was determined using specific function blocking antibodies. However, prior to antibody use for blocking experiments, FACS based studies were used to confirm complete blockade of the receptor (**Figure 2.5**). 200µl (100µg) of blocking antibody was injected via the cannulated carotid and allowed to flow within the circulation for 30 mins. The murine washed platelet protocol was then followed before dilution of platelets to a concentration of 2×10^7 / ml.

50µl of this solution was then incubated with 5µl of FITC conjugated antibody to the same receptor for 15 mins in the dark. After this incubation period, 450µl of tyrodes was added. Suspensions were loaded onto and analysed on a BD FACSCalibur flow cytometry system. The system has two available excitation lasers (488nm and 635nm) allowing the use of multiple coloured dyes, which can be detected on four different filters (FL1-4). FL1 allows detection of dyes with an emission wavelength of around 530nm including fluorescein isothiocyanate (FITC). Fluorescent intensity was taken as the mean fluorescent intensity of 10,000 platelets.

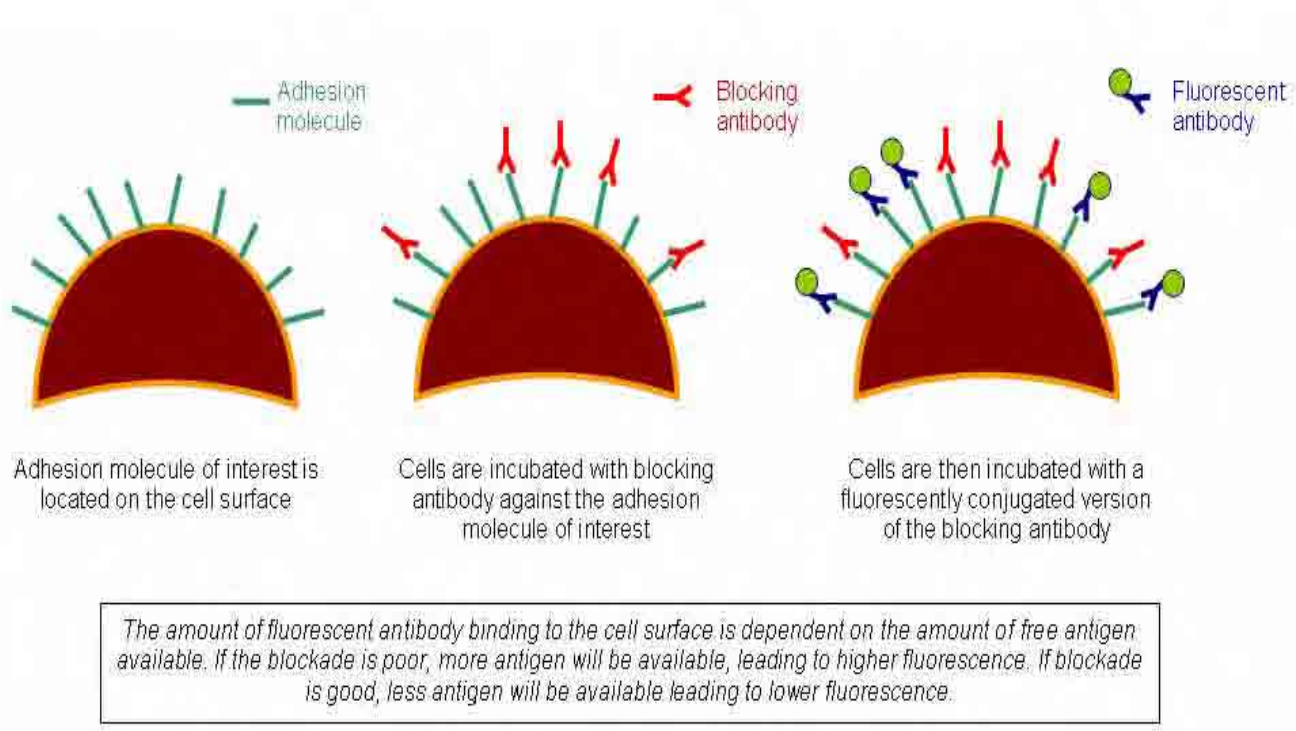


Figure 2.5. Determination of adequate function blocking concentrations for the anti-adhesion molecule antibodies. Platelets were pre-treated with varying concentrations of blocking antibody. Blocked platelets were subsequently treated with a fluorescently conjugated version of the blocking antibody. Fluorescent antibody capture is proportional to free antigen available on the cell surface. *Dr Dean Kavanagh is gratefully acknowledged for the above figure.*

2.7.2. *Ex vivo* platelet receptor blocking

Following the washed platelet protocol, platelets were diluted to 2 x 10^8 in 1ml. 50µl of function blocking antibody was added to this suspension for 30 minutes and then washed by a centrifuge spin of 1000g for 6 minutes. Platelets were then resuspended or 10ml for *in vitro* adhesion and FACS studies. Donor CFSE labelled platelets were resuspended in 100µl of tyrodes solution prior to injection into the recipient mouse for *in vivo* intravital experiments.

2.8. Cell interaction protocols

2.8.1. FACS analysis of platelet-leukocyte interactions

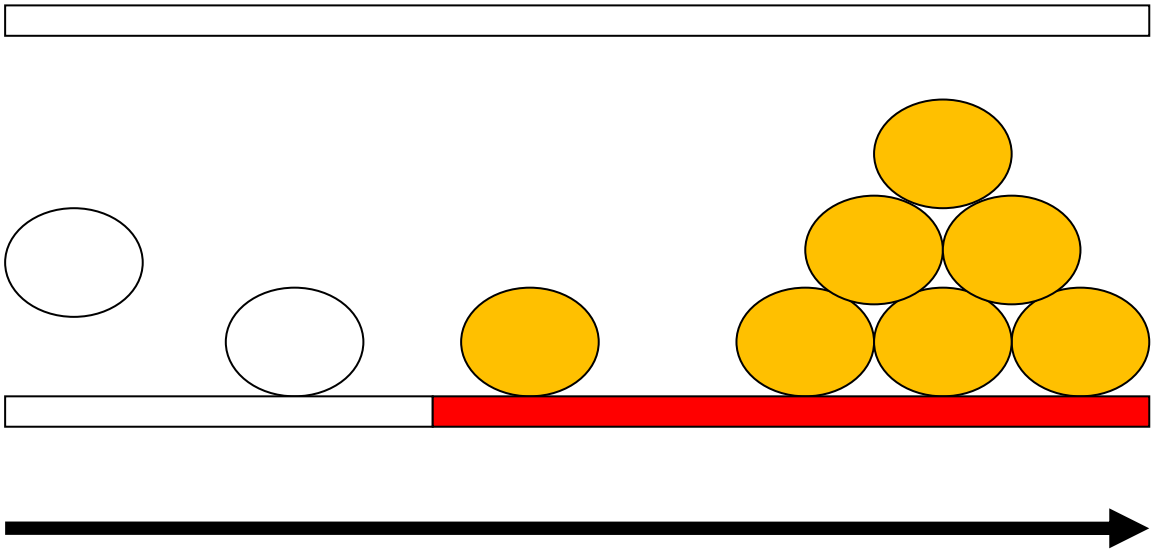
Whole blood was collected from sham or IR injured animals at 2 hours post-reperfusion from the descending aorta into a 1ml syringe containing heparin and 40µM PPAK. 50µl of blood was then placed into a FACS tube and allowed to incubate with 5µl of CD41 FITC-conjugated antibody (Emfret, Germany) for 15 minutes. After this incubation period, whole blood was diluted in 445µl of 37^{o}C tyrodes solution and analysed using a Beckman flow cytometer. Platelet-leukocyte aggregates were studied by evaluating the gated leukocyte population for the binding of platelet specific anti-CD41 mAb, within the first 30,000 events. The percentage of platelet-leukocyte events was concluded by dividing 30,000 by the number of events present in the associated platelet-leukocyte peak.

2.8.2. Platelet aggregations

For platelet aggregations, whole blood from sham or IR injured mice was sampled by uptake into a 1ml syringe containing 100ul of ACD. Preparation of washed mouse platelets was then followed, as stated previously (**refer to Section 2.2.1**). The concentration of platelets was diluted to 2 x 10^8/ml with 400ul of this suspension been placed into an aggregometry tube. A metal stirrer was then placed into the tube and inserted into the 37^{o}C compartment of the Chrono-Log aggregometer (Chrono-Log, Havertown, PA, USA) for 1 minute. After this time elapsed, the tube was placed into the compartment labeled PRP and the 4ul of agonist (collagen or thrombin) was added. Tracers were then observed for 4 minutes. If post platelet counts were analyzed, results were obtained from 3 minutes after the initial stimulation.

Chapter 3

Development and optimisation of an *in vivo* model to monitor platelets following intestinal ischaemia-reperfusion injury



3.0. Development and optimisation of an *in vivo* model to monitor platelets following intestinal ischaemia-reperfusion injury

3.1. Introduction and hypotheses

3.1.1. Introduction

Platelet recruitment has been observed within the microcirculation of several organs following IR injury. Indeed, the beneficial effects of platelet depletion during pancreatic IR injury has been described (Kuroda *et al.*, 1996). Most studies demonstrating a role for platelets in IR injury have characterised the nature and molecular determinants of platelet-endothelial cell interactions (Khandoga *et al.*, 2002; Massberg *et al.*, 1998). Massberg and colleagues provided the first evidence for a role for platelets in murine intestinal IR injury. Intravitally, they observed donor platelets rolling or firmly adherent to post-ischaemic activated endothelium via a mechanism dependent upon endothelial, rather than platelet, P-selectin (Massberg *et al.*, 1998). Furthermore, these studies were conducted within the large mesenteric and submucosal arterioles supplying the SI (Massberg *et al.*, 1998, 2003).

However, despite the intestinal mucosal villi lining the luminal surface of the gut being the most susceptible to IR injury (Kalia *et al.*, 2002), no studies have currently investigated the trafficking, recruitment or pathophysiological role of platelets within this area. Also, recruitment of platelets due to exposure of subendothelial matrix proteins such as collagen, subsequent to endothelial denudation, has not been investigated following intestinal IR

injury. Therefore, it is currently not known whether platelets are recruited to IR injured sites using specific platelet collagen receptors interacting with exposed collagen. Interestingly, blockade of platelet receptors in large arteries has shown great insight into possible future drug targets to decrease vascular complications such as myocardial infarction (Massberg *et al.,* 2002) and stroke (Zoppo 1998).

This chapter therefore aimed to investigate whether murine intestinal IR injury led to exposure of subendothelial ECM and whether platelets subsequently formed thrombi on this surface. Initially, the recruitment of platelets to IR injured SI using the traditional method of fluorescently labelling only donor-derived platelets was investigated. However, donor labelled platelets only represent approximately 5% of a normal murine circulating platelet population when re-introduced into a recipient mouse (Cooper *et al.,* 2002). This methodology allows individual platelet interactions with endothelium and free flowing individual platelets to be identified but does not identify aggregates of platelets or thrombi typically present after ECM exposure. In order to quantitate the latter, all endogenous platelets of the mouse undergoing the ischaemic injury need to be labelled (Falati *et al.,* 2003). We therefore introduced CFSE (green) fluorescently labelled donor platelets into a mouse in which all the α_{IIb} subunit of the endogenous platelets had been labelled with Alexa 594 (red). This methodology allows thrombi, free flowing platelets, and single platelets to be quantitated within the same *in vivo* experiment and thus substantially decreases the number of animals required. The mucosal villi are most susceptible to ischaemic injury and hence

severe microcirculatory damage that will lead to endothelial denudation will most likely occur in this region. Therefore, the studies have focused predominantly on monitoring platelet activity in this region of the gut. Numerous studies have identified inflammatory leukocytes as the main effector cell in IR injury with leukocyte adhesion to vessel walls considered one of the most damaging events taking place during IR injury (Kiss *et al.,* 2008). This chapter therefore compared leukocyte and platelet recruitment within the IR injured mucosal villi intravitally.

3.1.2 Hypothesis

For the work included in this chapter we hypothesized:

1. The recruitment of platelets would be greatest within the mucosal villi due to the high susceptibility of this luminal surface to intestinal IR injury.
2. The recruitment of platelets would be increased as the duration of ischaemia and reperfusion, and thus severity of injury, was increased.
3. Platelet recruitment would manifest itself in the form of microthrombi forming on exposed ECM rather than just adhesion of individual platelets to activated endothelium.
4. Circulating platelets would become activated when trafficking though the injured gut environment and become 'primed' or more likely to adhere to remote sites.

3.2. Methods

The methods used in this chapter are described in detail in Chapter 2 and briefly below.

3.2.1 Does fluorescently labelling donor platelets with CFSE or endogenous platelets with Alexa 594 lead to their inadvertent activation?

Donor platelets were fluorescently labelled with CFSE and endogenous platelets with Alexa 594 for intravital *in vivo* studies. To determine whether labelling with either of these fluorescent dyes could cause inadvertent platelet activation (which would lead to *in vivo* adhesion of platelets to vessel walls), *in vitro* platelet aggregations were undertaken. The aggregatory response to collagen or thrombin of labelled and unlabelled platelets was determined and compared.

3.2.2. Can the duration of ischaemia affect platelet recruitment?

Experimental intestinal IR injury can be fatal and is thus associated with high mortality rates, especially if it involves the entire gut. For this reason, most studies have focussed on segmental intestinal ischaemia whereby the blood vessel supplying a small segment (1-2cm) is occluded. However, this is not a frequent clinical occurrence and in most conditions, ischaemic injury to the whole of the SI occurs. In order to identify a protocol for total IR injury which did not cause mortality within the intravital observation period, we performed experiments on 20-25g C57Bl/6 mice subjected to varying periods of ischaemia followed by 4 hours reperfusion. The aim was to identify a protocol that allowed for significant vascular

damage to occur without leading to mortality. A long post-reperfusion period (4hrs) allowed platelet events to be monitored as the injury became progressively worse. Intestinal ischaemia was achieved by clamping the superior mesenteric artery. Animals were divided into 4 groups as shown below and assessed for survival.

Duration of Ischaemia (mins)	**Duration of Reperfusion (mins)**	**Number of animals**
15	240	5
30	240	5
45	240	5
60	240	3

Long durations of intestinal ischaemia were associated with a high mortality rate and were therefore unsuitable for continuous intravital monitoring. Respiratory distress and mortality was observed in 100% of animals subjected to 60 min ischaemia either during the ischaemic phase or immediately upon reperfusion. Survival throughout the 4 hr reperfusion phase was observed only in animals undergoing 15, 30 and 45min ischaemia and so some data is presented for mice undergoing ischaemia for these durations. **However, the majority of studies within this chapter, and all subsequent experiments within this thesis, were conducted on animals undergoing 45 minutes ischaemia and 4 hours reperfusion.**

3.2.3. Intravital Microscopy

The carotid artery and trachea were cannulated and the animal subjected to either sham or IR treatment. Animals underwent 15, 30 or 45 min ischaemia followed by up to 4 hrs reperfusion. Preliminary experiments were conducted with only CFSE-labeled donor platelets administered via the carotid artery. Thereafter, we developed the methodology and conducted novel experiments combining *ex vivo* administration of CFSE-labeled donor platelets into animals in which endogenous platelets were labeled with Alexa 594. This Alexa dye was systemically administered following conjugation with a non-blocking CY3 antibody that recognises the major platelet integrin CD41 / α_{IIb} (Falati *et al.,* 2004). During reperfusion, a field of view from the proximal jejunum or the terminal ileum was pre-selected for continuous observation and platelet activity was analysed. Recordings were made every 30 minutes. Individual CFSE-labeled donor platelets were defined as adherent if they remained stationary in the field of view for >30 seconds, while remaining cells were classified as free flowing if they were present for <2 seconds. The size and fluorescent intensity of CD41/Alexa 594-labeled microthrombi was determined using image analysis software as previously described (Chapter 2, Section 3.2).

The systemic administration of FITC-BSA allowed vascular macromolecular leakage to be quantitated. Leakage is used as an indicator that the integrity of the vessel wall has been disturbed. Increased fluorescent intensity outside the microvessels, which can be quantitated, is directly proportional to macromolecular leakage. Histological haematoxylin

and eosin (H&E) staining was used to visualise mucosal villi in sham and IR injured small intestine.

3.3 Results

3.3.1. Fluorescently labeling platelets with CFSE or Alexa 594 does not lead to inadvertent activation as determined *in vitro*

In vitro aggregation analysis of collagen and thrombi stimulated CFSE and Alexa 594-labelled platelets showed no significant effect on platelet activatory function in comparison with sham washed platelets **(Figures 3.1a and 3.1b)**. These findings coincide with (Cooper *et al.*, 2003) who showed similar results when labelling with CFSE.

3.3.2 Singular CFSE-labelled platelet adhesion increases and free flowing platelet number decreases in the ileum as intestinal IR injury is intensified

The adhesion of individual CFSE labelled donor platelets increased within the ileal mucosal villi as the ischaemic duration increased from 15 to 45 minutes (**Figure 3.2a)**. At 1 hour reperfusion, significantly increased platelet adhesion was observed with 45 minutes ischaemia when compared to sham controls ($p<0.001$). At 4 hours reperfusion, significantly increased platelet adhesion was observed with 30 ($p<0.001$) and 45 ($p<0.001$) minutes ischaemia when compared to sham controls. Furthermore, the numbers of adherent platelets was greater at 4 hours reperfusion when compared to 1 hour reperfusion ($p<0.001$ for animals undergoing 30 minutes ischaemia).

Numbers of free flowing platelets significantly decreased as ischaemic period increased. At 1 hour reperfusion, significantly decreased numbers were observed only with 45 minutes ischaemia ($p<0.001$) when compared to sham controls. At 4 hours reperfusion, significantly decreased numbers were only observed with 45 minutes ischaemia when compared to sham controls ($p<0.001$). Furthermore, numbers of free flowing platelets demonstrated a trend to decrease at 4 hours reperfusion in mice undergoing 30 and 45 minutes ischaemia compared to 1 hour reperfusion **(Figure 3.2b)**. This decrease could be due to either the increase in platelets adherent to vessel walls limiting the numbers of circulating platelets, or simply due to a decrease in blood flow rates within the injured gut. The lack of adherent platelets witnessed in sham animals provided futher evidence that the CFSE labelling technique did not pre-activate platelets, but also that the surgical manipulation of the gut was insufficient to cause vessel wall activation.

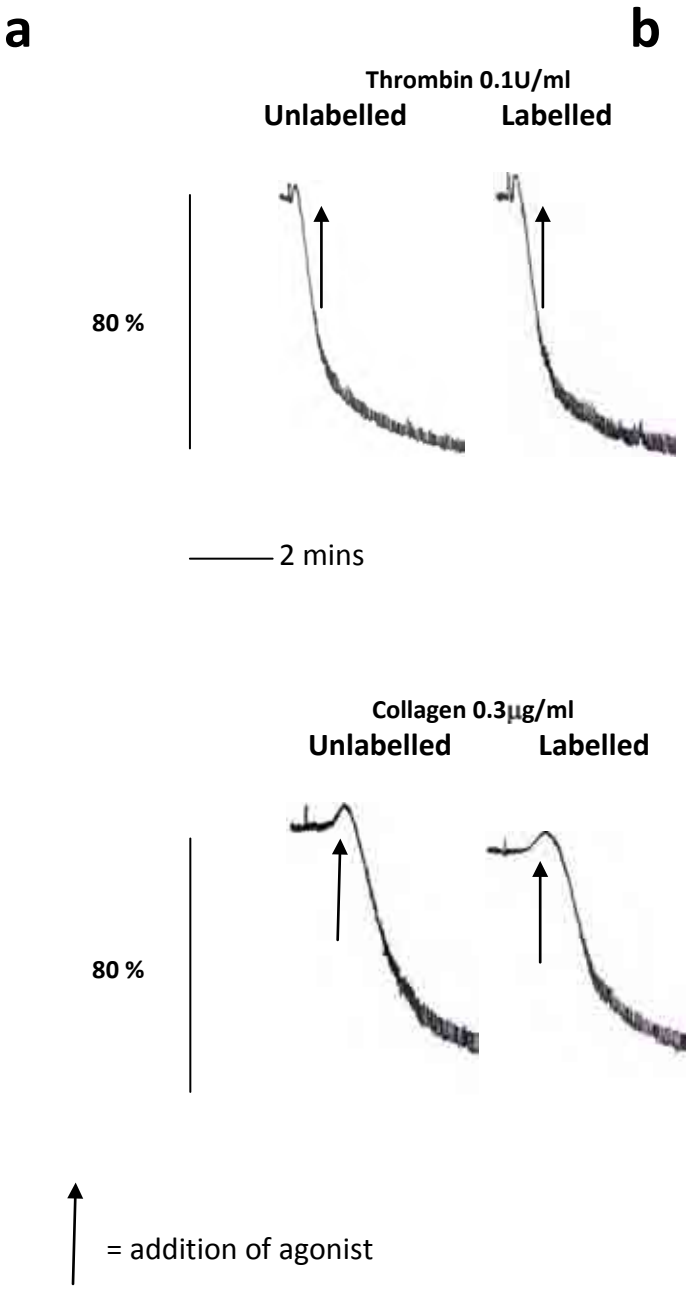


Figure 3.1. Fluorescently labeling platelets with CFSE and Alexa 594 does not lead to inadvertent activation as determined *in vitro*. Washed unlabelled and labeled (both CFSE and CD41/Alexa 594) platelets (2 x10^8/ml) were stimulated with **(a)** 0.1U/ml Thrombin or **(b)** 3μg/ml collagen and the change in optical density indicative of aggregation recorded (arrow head is when agonist was added). The images are from one experiment that is representative of three.

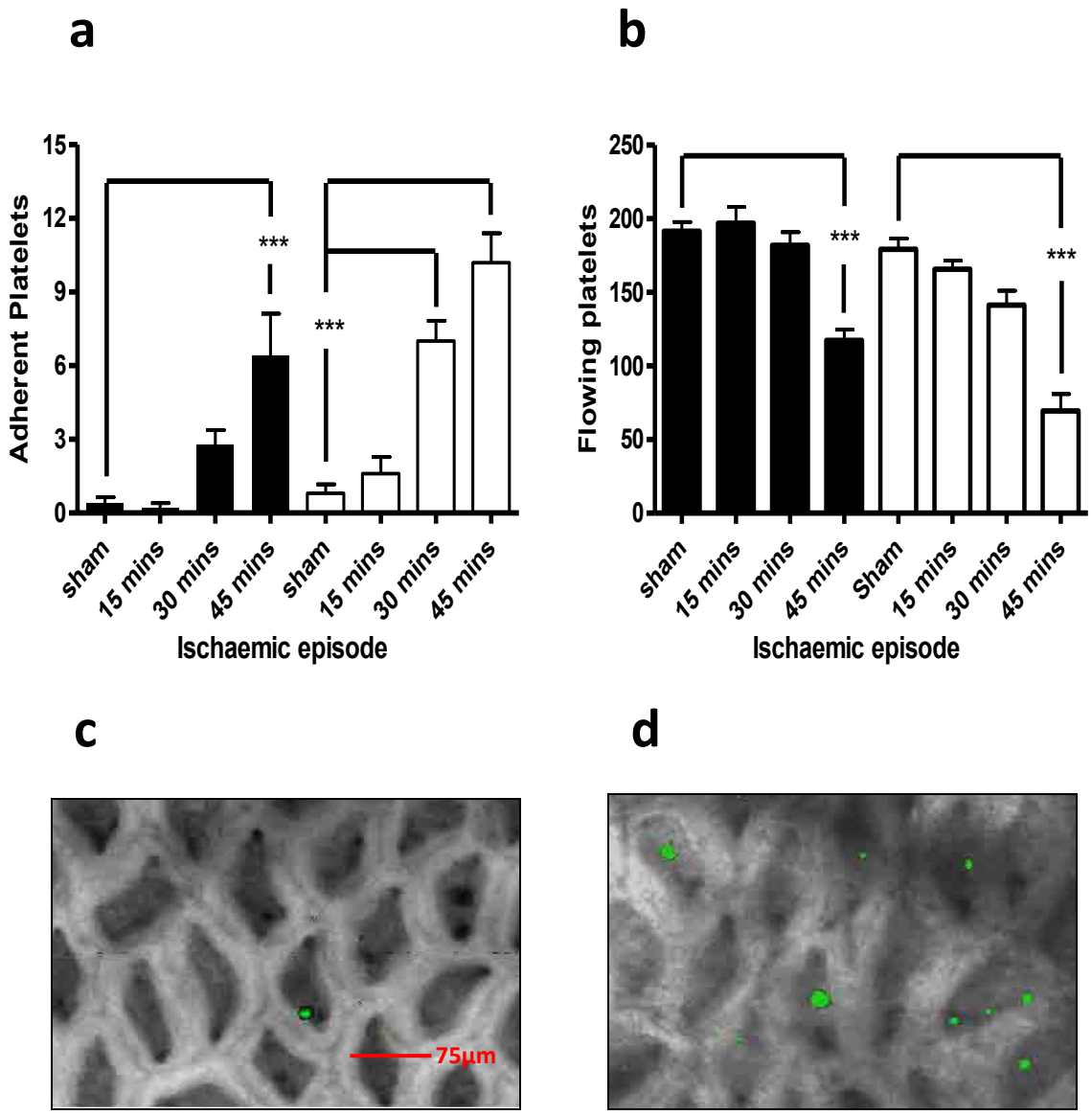


Figure 3.2. Singular CFSE-labelled platelet adhesion increases and free flowing platelet number decreases in the ileum as intestinal IR injury is intensified. Recruitment of CFSE labelled donor platelets to the microcirculation of the ileal mucosal villi was examined intravitally. Platelets (1 x 10^8 platelets, 10µM CFSE) derived from wild-type mice were introduced prior to IR injury. **(a)** Donor platelet adhesion was significantly increased as the ischaemic episode and duration of reperfusion was increased. **(b)** Numbers of free flowing platelets significantly decreased as the ischaemic period and duration of reperfusion increased. Representative images of CFSE labelled donor platelets adherent within ileal villi at 60 minutes post-reperfusion in **(c)** sham and **(d)** 45 minutes ischaemia + reperfusion. Plots in **panels a** and **b** are representative of mean + SEM of at least 5 separate experiments; Black bars = 1 hour reperfusion ; white bars = 4 hours reperfusion. ***p<0.001 when compared using a one way ANOVA followed by Bonferroni post test to determine points of significance.

3.3.3. Singular CFSE-labelled platelet adhesion increases in jejunum more so than in ileum

Interestingly, we observed that intestinal IR injury was associated with a difference in the degree of singular platelet adhesion within different sites of the SI. Numbers of adherent platelets were almost 3 fold higher within jejunal mucosal villi when compared to ileal mucosal villi (**Fig 3.3a**). Within the ileum, more platelets adhered as the reperfusion period increased. However, within the jejunum, there was no significant difference in the numbers of adherent platelets at 1 and 4 hours reperfusion. Interestingly, platelet adhesion within larger serosal and mesenteric vasculature was limited with adherent numbers not exceeding between 1-2 cells throughout reperfusion **(Figure 3.3b).** Free flowing platelets in these larger vessels, assessed either through manually counting (serosa; **Figure 3.4a**) or by measuring their intensity (mesentery; **Figure 3.4b**), significantly decreased at 1 and 4 hours post-reperfusion, indicative of platelet becoming adherent elsewhere. The fate of free flowing platelets within the jejunum after IR injury will be described later in Section 3.2.5.

DUAL LABELLING STUDIES

3.3.4. Alexa 594-labelled microthrombi are observed in the jejunum more so than in the ileum

Since 45 minutes ischaemia followed by 4 hours reperfusion gave rise to the greatest number of CFSE labelled individual platelets adhering within the ileum and jejunum, the formation of microthrombi was investigated in mice undergoing this injury protocol. The total size and

fluorescent intensity of all Alexa 594-labelled microthrombi was determined within the jejunum and ileum. No microthrombi were observed in sham controls in any region. However, significantly increased numbers of microthrombi of high intensity were observed in the jejunum (**Figure 3.5a-b**) relative to the ileum (**Figure 3.5c-d**). Furthermore, they were also much larger.

3.3.5. IR injury of the jejunum is associated with complete cessation of blood flow

As already stated in sections 3.2.2 and 3.2.3, individual CFSE-labelled platelet adhesion was greater in the jejunum compared to the ileum. Figures 3.6a (jejunum) and 3.6c (ileum) present this data again but this time as a continuous graph over the 4 hour reperfusion period (**Figures 3.6a and c**). No free flowing platelets were observed in the jejunum, indicating no blood flow and complete flow occlusion in this region of the small intestine **(Figure 3.6b)**. As already stated in section 3.2.2 the numbers of free flowing CFSE-labelled donor platelets within the ileum was reduced but not abolished suggesting there was still blood flow within the ileum **(Figure 3.6d)**

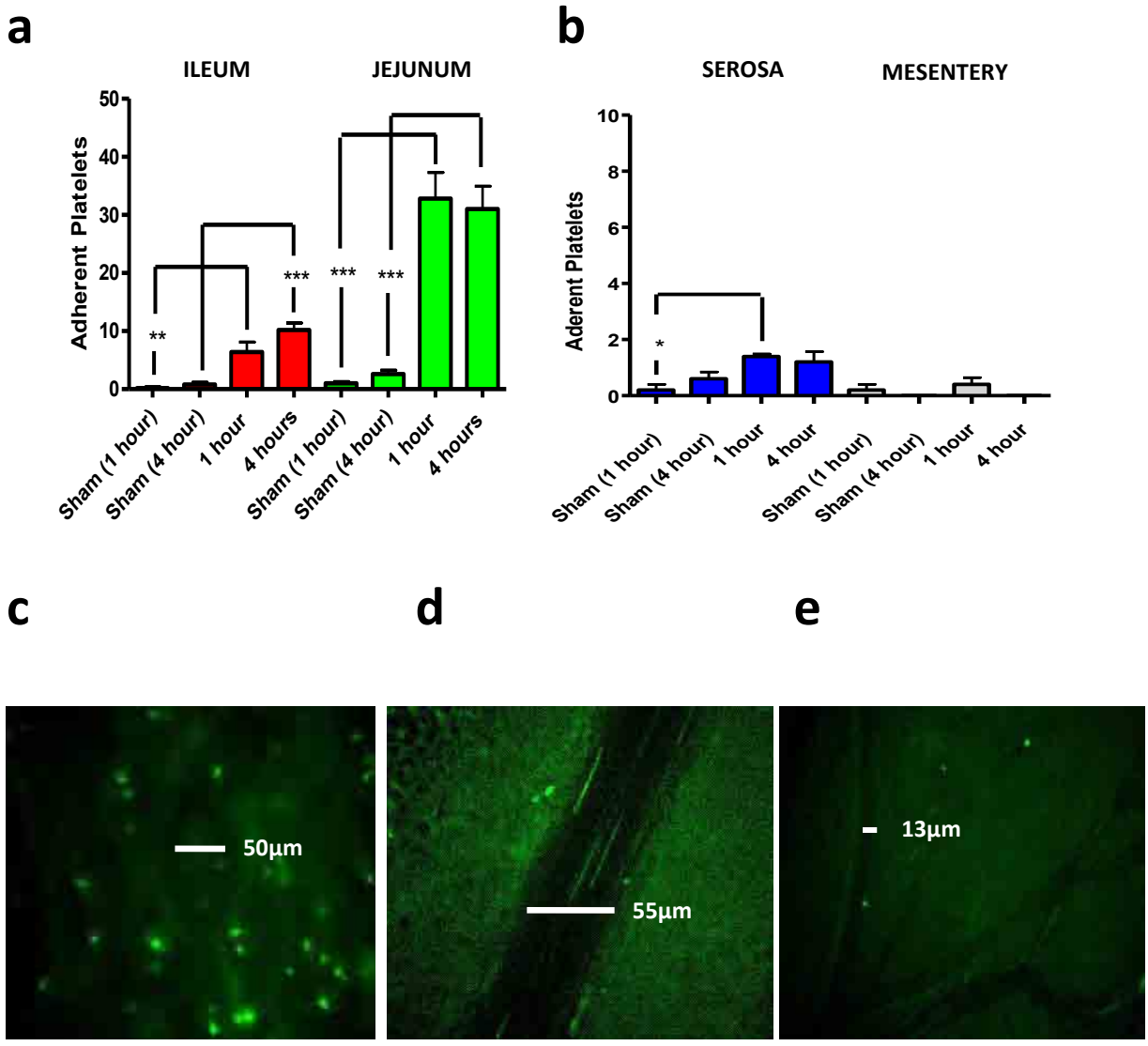


Figure 3.3. Singular CFSE-labelled platelet adhesion increases in the <u>jejunum</u> more so than in the ileum. Donor platelets (1 x 10^8 CFSE labelled platelets) derived from wild-type mice were introduced prior to IR injury. **(a)** Donor platelet adhesion to the microcirculation of <u>ileal villi</u> was significantly increased at 1 and 4 hrs compared to sham**.** Donor platelet adhesion to the microcirculation of the <u>jejunal villi</u> was significantly increased compared to sham. More adherent with jejunum than ileum. **(b)** Only sparse recruitment of 1-2 platelets was observed within the larger vessels of the jejunum serosa and mesentery. Representative images are shown of donor platelet recruitment at 60 minutes post-reperfusion within jejunal villi **(c)** mesentery **(d)** and serosa **(e)**. Plots in **panel a** and **b** are representative of mean + SEM of at least 5 separate experiments, *p<0.05, **p<0.01, ***p<0.001 when compared using a one way ANOVA followed by Bonferroni post test for points of significance.

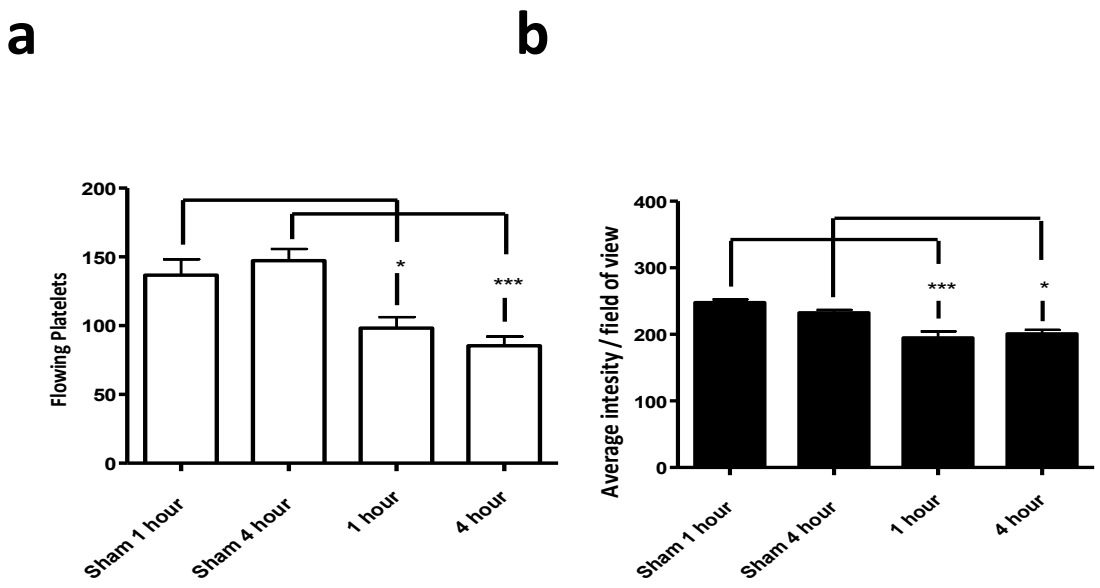


Figure 3.4. Singular CFSE-labelled free flowing platelet number decreases in <u>serosal</u> and <u>mesenteric</u> vessels during intestinal IR injury. Recruitment of CFSE labelled donor platelets to the circulation of the jejunal serosa and mesentery was examined intravitally. Platelets (1 x 10^8 platelets, 10µM CFSE) derived from wild-type mice were introduced prior to IR injury. Numbers of free flowing platelets or the average intensity of slowing platelets significantly decreased during intestinal IR injury in both the **(a)** serosa and **(b)** mesenteric vasculature. Plots in **panels a** and **b** are representative of mean + SEM of at least 5 separate experiments; Black bars = 1 hour reperfusion; white bars = 4 hours reperfusion). *p<0.05, **p<0.01, ***p<0.001 when compared using a one way ANOVA followed by Bonferroni post test to determine points of significance.

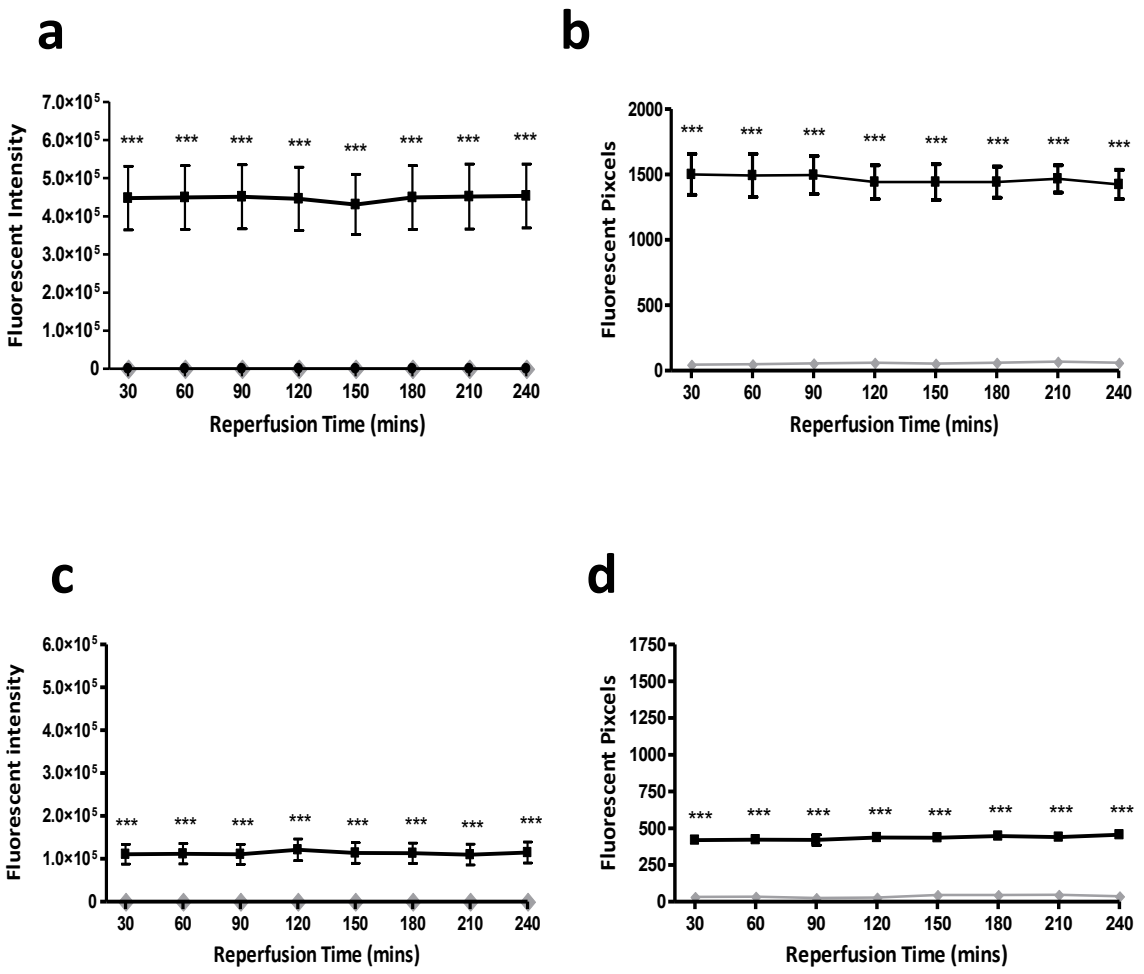


Figure 3.5. Alexa 594-labelled platelet microthrombi are observed in the jejunum more so than in the ileum. The formation of microthrombi (20µl GPIIb ab conjugated to 40 µl Alexa 594 introduced prior to IR injury) within the jejunal and ileal mucosal villi was examined intravitally. The size (represented as pixels) and fluorescent intensity of the microthrombi were significantly increased at all time points in IR mice within both the **(a-b)** jejunum and **(c-d)** ileum when compared to sham controls. For all figures, the grey line = sham controls; black line = IR injured animals. Figures represent mean + SEM of at least 5 separate experiments; ***p<0.001 when compared using a two way ANOVA followed by Bonferroni Post Test for points of significance.

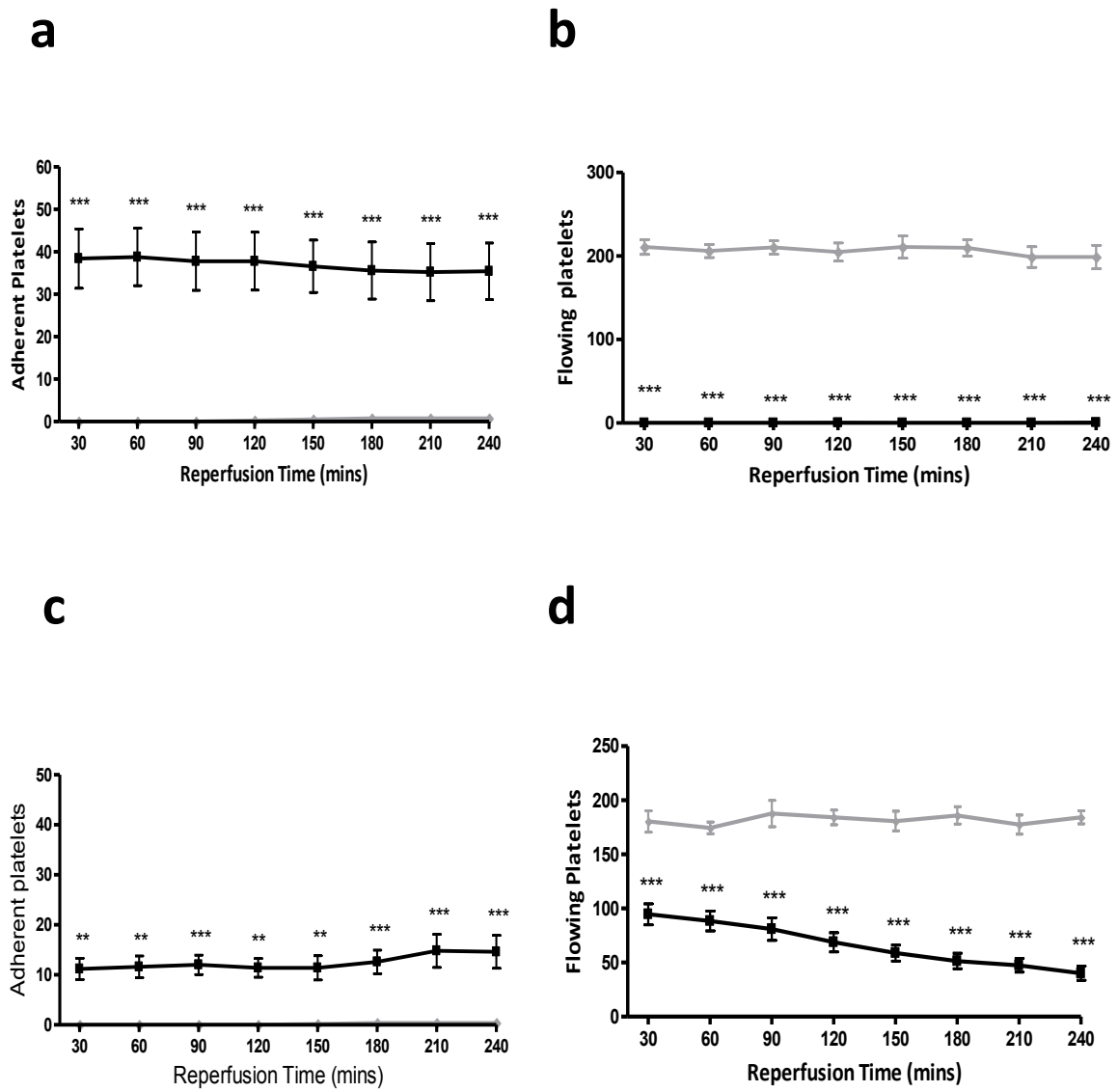


Figure 3.6. Singular CFSE-labelled platelet adhesion increases in the jejunum more so than in the ileum. Furthermore, no free flowing platelets are observed in the jejunum. Donor platelets (1 x 10^8 platelets, 10µM CFSE) derived from wild-type mice were introduced prior to IR injury. **(a)** Donor platelet adhesion to the microcirculation of the jejunal villi was significantly increased compared to sham jejunum. **(b)** No free flowing platelets were observed in the jejunum at any time point indicative of no flow in this region. **(c)** Donor platelet adhesion was also increased within the ileum but less so than the jejunum. **(d)** Numbers of free flowing platelets decreased within the ileum over time. For all figures, the grey line = sham controls; black line = IR injured animals. Figures represent mean + SEM of at least 5 separate experiments; **p<0.01, ***p<0.001 when compared using a two way ANOVA followed by Bonferroni Post Test for points of significance.

3.3.6. Dual labelling allows individual platelets and microthrombi to be observed in the same animal at the same time

The CFSE-labelled individual donor platelets could be seen both adherent within, and thus associated with a microthrombus, but also on their own. When not associated with a thrombus, they appeared green and those out of focus appeared larger than the very small ones in the focal plane. Those associated with a microthrombus appeared yellow due to the mixture of fluorescence from green individual platelets and red microthrombi (**Figures 3.7a-b**). We observe the same sized green fluorescent areas flowing freely through the vasculature. This suggests that these platelets are inactive and consequently of single formation. However, though highly unlikely, it cannot be ruled out that these green fluorescent areas may consist of more than one platelet

3.3.7. The proximal jejunum is more susceptible to intestinal IR injury than the ileum

As described above, greater levels of individual platelet adhesion and microthrombus formation as well as complete cessation of blood flow was observed within the jejunum rather than the ileum. In the ileum, limited microthombus formation was observed and the blood flow to this region of the gut was maintained. These microscopic observations were paralleled by a difference in the degree of macroscopic injury observed within these two regions. The jejunum underwent severe injury, especially with 45 minutes ischaemia, and appeared haemorrhagic, severely congested and swollen upon reperfusion. In contrast, the terminal ileum looked relatively normal grossly **(Figures 3.8a-b)**.

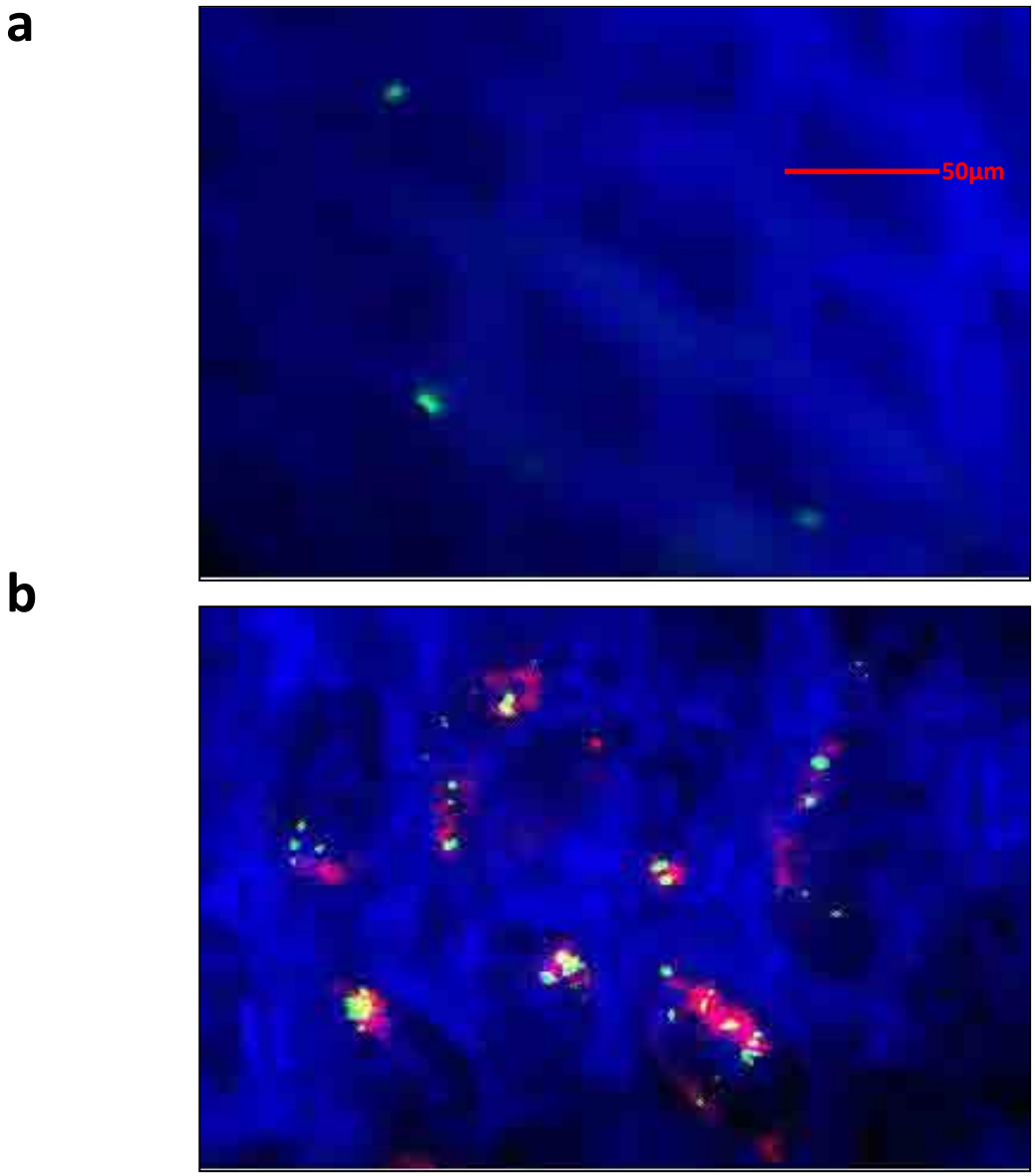


Figure 3.7. Intestinal IR injury was associated with both increased adhesion of singular CFSE-labelled donor platelets as well as the formation of microthrombi within the villous microcirculation. (a) Only singular green CFSE-labelled donor could be observed adherent within both sham controls. **(b)** Singular green CFSE-labelled donor and red Alexa 594-labelled endogenous microthrombi could be observed within the jejunum. Singular platelets were either adherent on their own as illustrated in (a) or contained with a microthrombus, and thus appearing yellow, as illustrated in (b). Representative images are shown of sham and jejunal microcirculation at 60 minutes post reperfusion.

a

b

Figure 3.8. Intestinal IR injury was associated with a difference in the degree of macroscopic injury in the jejunum and the terminal ileum. (a) The ileum appeared relatively healthy after 45 minutes ischaemia and 4 hours reperfusion. **(b)** The proximal jejunum underwent severe injury, especially with 45 minutes ischaemia, and appeared haemorrhagic, severely congested and swollen upon reperfusion. Representative images are shown of IR injured ileal and jejunal segments at 4 hours post-reperfusion.

3.3.8. Increased vascular macromolecular leakage is observed within the jejunal mucosa and not the ileal mucosa.

Significantly ($p<0.001$) increased macromolecular leakage was observed within the jejunal mucosa at 4 hours post-reperfusion when compared to sham jejunum (**Figure 3.9**). This is often used as an indicator to suggest the integrity of the endothelial barrier is disturbed. It was frequently observed that almost the whole field of view appeared fluorescent within the IR injured jejunum. Although there was some increase in macromolecular leakage from the ileal mucosa, this did not reach significance when compared to sham ileum (**Figures 3.9 and 3.10a-d**).

3.3.9. Increased injury, as determined histologically and using SEM, is observed within the jejunum compared to the ileum

The villi from sham jejunal mucosa, sham ileal mucosa and IR injured ileal mucosa were healthy looking finger-like structures (**Figure 3.11a-b**). However, the villi within the IR injured jejunal mucosa after 4 hours reperfusion showed a distinct decrease in jejunal villous length, mucosal sloughing and with areas of inflammation visible around the crypt areas **(Figure 3.11c)**. Scanning electron microscopy (SEM) of this region clearly showed exposure of the ECM through the visualisation of collagen fibres. Platelet microthrombi, apparently adherent to the ECM collagen fibres, were also observed (**Figure 3.11d)**. This was not observed in sham tissue or in IR injured ileal tissue.

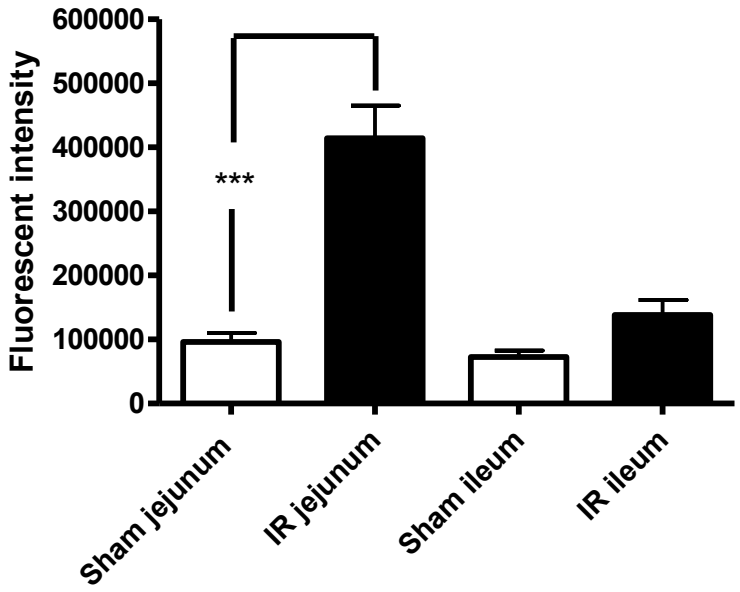


Figure 3.9. Increased vascular macromolecular leakage is observed within the jejunal mucosa and not the ileal mucosa. Leakage was analysed by systemically administering FITC-BSA at 4 hours reperfusion. The fluorescent intensity within a field of view was determined using the slidebook software. A significant increase was seen in fluorescent intensity to the jejunal villi during IR injury. A small and insignificant increase in vascular leakage was observed within the ileum. Plots in are representative of mean + SEM of at least 5 separate experiments, ***$p<0.001$ when compared using a one way ANOVA followed by Bonferroni Post Test for points of significance.

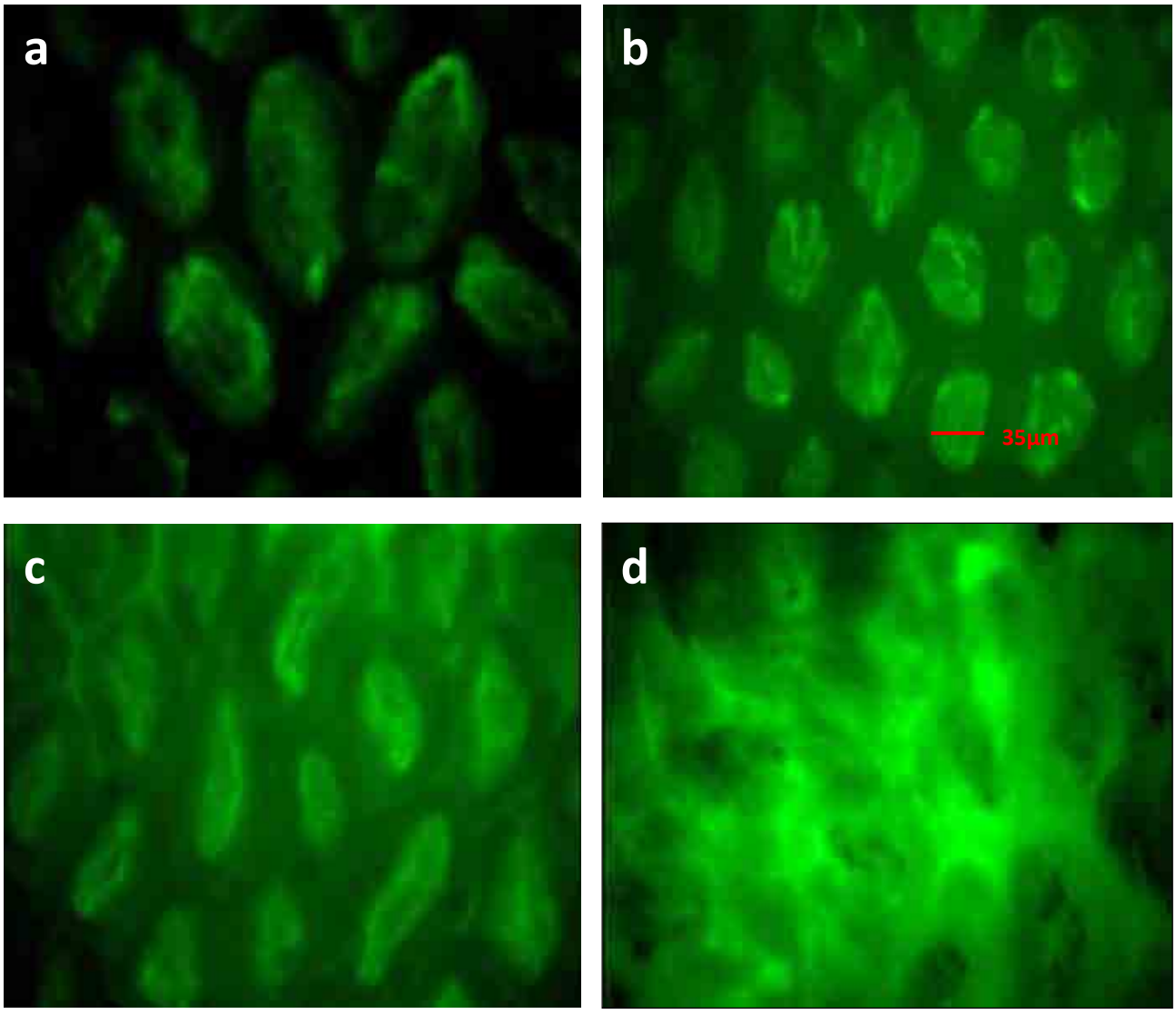


Figure 3.10. Increased vascular macromolecular leakage is observed within the jejunal mucosa and not the ileal mucosa. Leakage was analysed by systemically administering FITC-BSA at 4 hours reperfusion. Representative images are shown of vascular leakage at 4 hours reperfusion within **(a)** sham jejunum **(b)** sham ileum **(c)** IR ileum and **(d)** IR jejunum

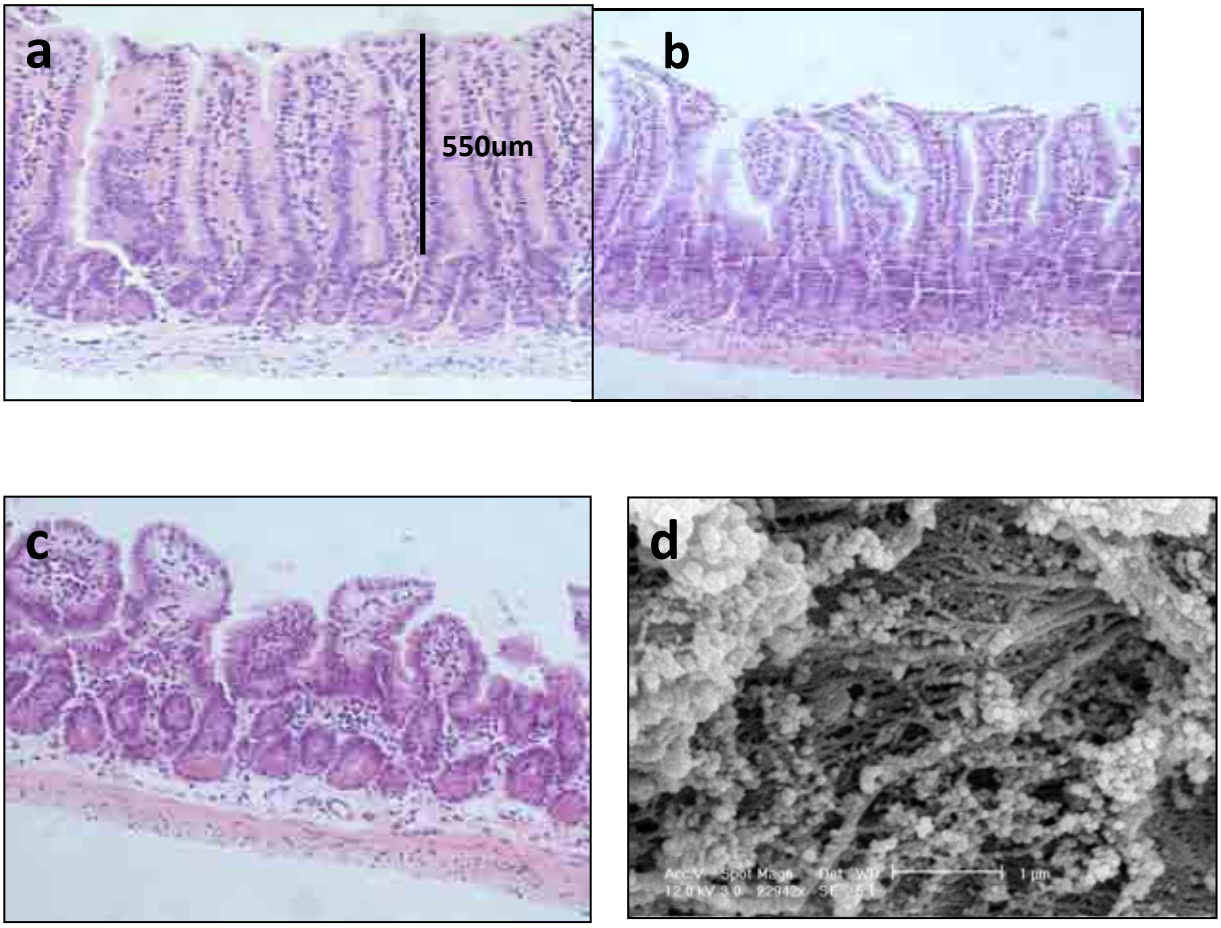


Figure 3.11. Histological damage to jejunal villi in IR injured mice (magnification x200 for light microscopy images and x22942 for SEM). C57BL/6 mice were subjected to small intestinal IR injury. At 4 hours reperfusion, the jejunum was removed for histological analysis and H&E staining. Representative images from **(a)** sham jejunal mucosa and **(b)** ileal mucosa seen in sham and IR. The villous structures are healthy, with no sloughing of epithelial cells observed at the tips of the villi. **(d)** In contrast, severe jejunal mucosal villous damage with inflamed crypts was observed in seen in mice undergoing 45 minutes ischaemia and 4 hours reperfusion. **(d)** Representative SEM image of an IR injured jejunal mucosal villous microcirculation which shown an area where the ECM is exposed and platelet microthrombi are observed on the collagen fibres.

3.3.10. Platelets derived from IR injured mice do not aggregate in response to collagen nor do they aggregate on collagen under *in vitro* flow conditions

Having demonstrated the development of microthrombi within the injured jejunal microcirculation, we postulated whether the circulating platelets that traffic through injured gut, *but do not actually adhere*, were potentially capable of adhering to injured remote extra-digestive sites as a result of becoming activated or 'primed' in the gut. We therefore, removed platelets from sham and IR injured mice and investigated their aggregatory response to collagen and thrombin. Furthermore, their adhesion to collagen in an *in vitro* flow assay was also determined. Sham and IR platelets aggregated in response to the agonist thrombin. However, a significant ($p<0.001$) reduction in the aggregation of IR platelets was observed in response to collagen when compared to sham platelets (**Figure 3.12a**). A significant ($p<0.001$) reduction in the ability of platelets derived from animals 2 hours post-reperfusion to aggregate on collagen coated capillary tubes under flow conditions was also observed compared to platelets derived from sham animals (**Figure 3.12b-d**).

3.3.11. Leukocyte recruitment is only increased within the ileal and not the jejunal villi

Endogenous leukocytes were labelled with acridine orange injected via the carotid artery and observed intravitally. The number of leukocytes significantly increased within the ileal villi after both 1 ($p<0.001$) and 4 ($p<0.001$) hours post-reperfusion when compared to sham controls, with more observed as the duration of reperfusion increased. However, no increase in leukocyte numbers was observed within the jejunum (**Figure 3.13a-e**).

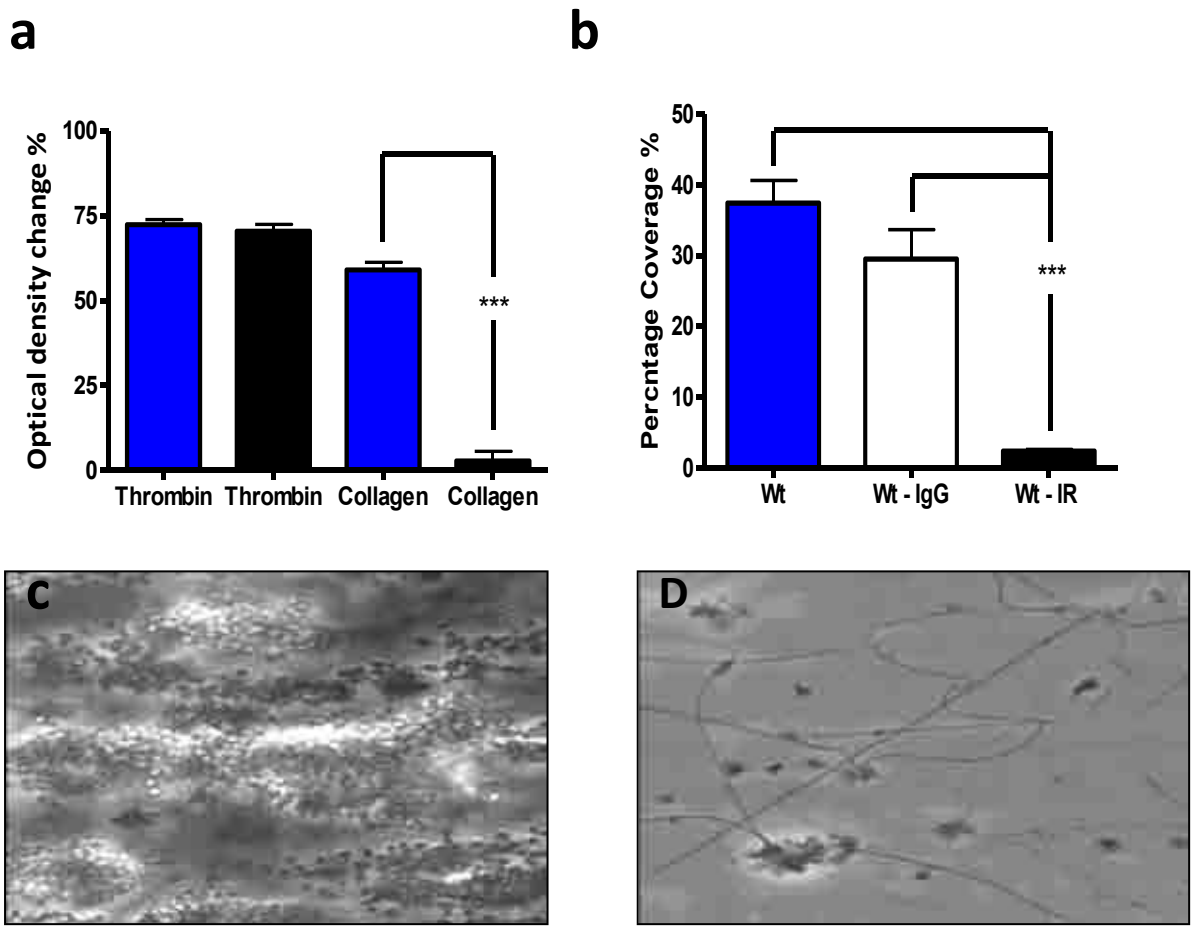


Figure 3.12. Platelets derived from IR injured mice do not aggregate in response to collagen in an aggregometer nor do they aggregate on collagen under *in vitro* flow conditions. (a) Washed sham (blue bars) and IR platelets (black bars) (2 x10^8/ml) were stimulated with 0.1U/ml thrombin or 3ug/ml collagen and the change in optical density indicative of aggregation was analysed at 2 mins post agonist administration. A significant decrease was only witnessed when activating IR platelets with collagen. **(b)** 500ul of mouse blood anti-coagulated with P-Pack and heparin was perfused through collagen coated microslides at a shear rate of 300s-1. After washing for 3 mins, 5 random images were then taken using an Olympus digital camera. Representative images of demonstrating adhesion to collagen of platelets derived from **(c)** sham and **(d)** IR mice are shown. The graphical data represents mean ± SEM of at least 5 separate experiments; ***p<0.001 when compared using a one way ANOVA followed by Bonferroni post-test for points of significance.

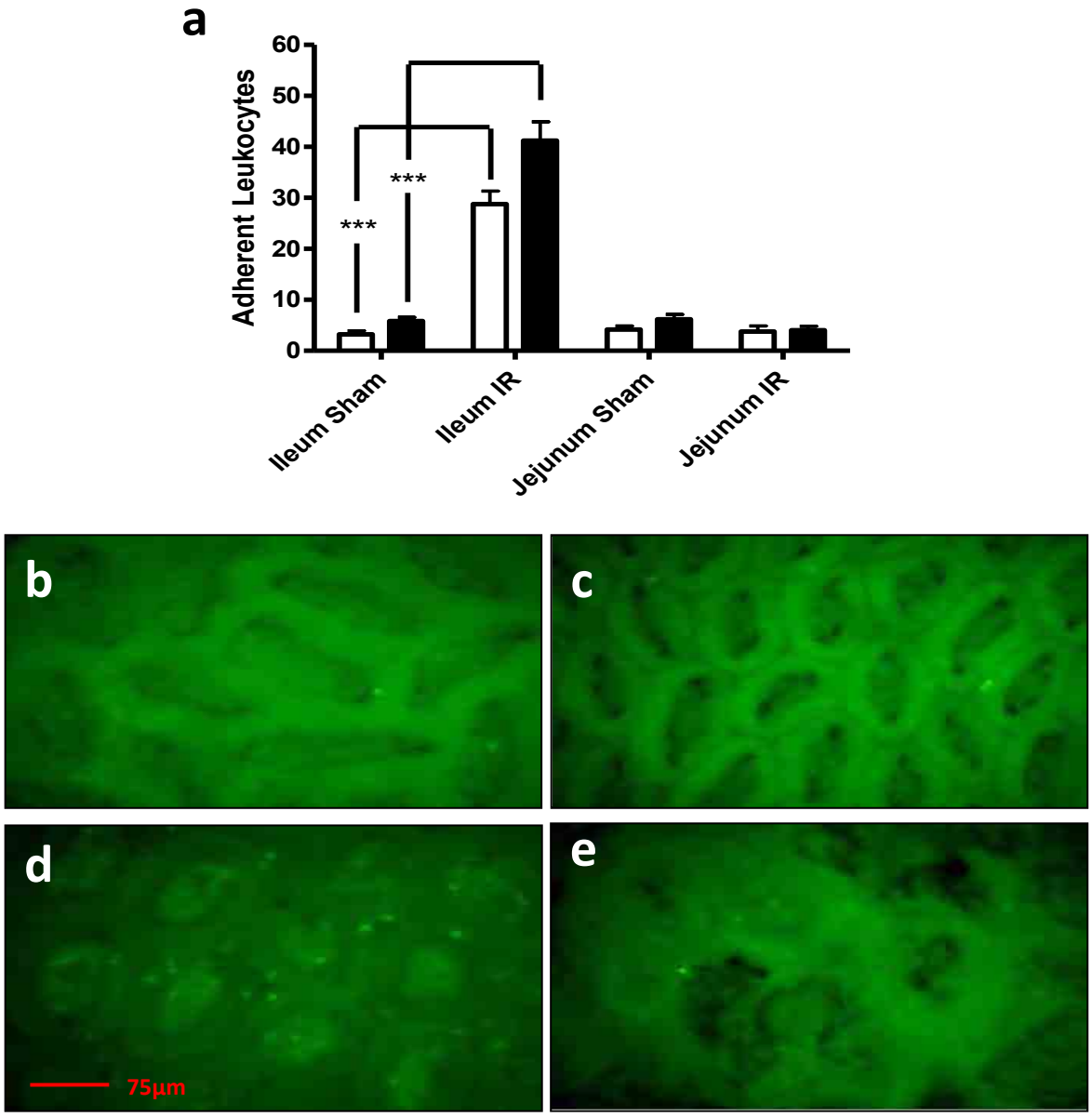


Figure 3.13. Leukocyte recruitment is only significantly increased within the ileal and not the jejunal villi. Recruitment of Acridine orange labelled leukocytes to the microcirculation of the ileal and jejunal mucosal villi was examined intravitally. **(a)** A significant increase was seen in adherent leukocytes to the ileal villi during IR injury. No significant change was observed within the jejunal villi. (white bars = 1 h reperfusion ; black bars = 4 h reperfusion). Representative images are shown of leukocyte recruitment at 4 hours reperfusion within **(b)** sham ileum, **(c)** sham jejunum, **(d)** IR ileum and **(e)** IR jejunum. Note the disturbed architecture of the jejunal villi and the congestion within the villous microcirculation. Data are presented as mean + SEM of at least 5 separate experiments. *** $p < 0.001$ when compared using a two way ANOVA followed by Bonferroni Post Test for points of significance.

3.4. Discussion

The most commonly used procedure for monitoring platelets within the microcirculation *in vivo* has relied on *ex vivo* labeling of platelets from donor mice and injecting them into the experimental mouse. This methodology achieves a labeled fraction in the recipient mouse of approximately 5% of all circulating platelets which can be monitored intravitally. Platelet-endothelial interactions or monolayer formation has been demonstrated in numerous experimental models of IR injury including the retina, liver and lungs using this method (Nishijima *et al.,* 2001; Khandoga *et al.,* 2002). Although in larger diameter vessels undergoing severe $FeCl_3$ injury, thrombus formed has also been observed made up of labelled donor platelets, (Renne *et al*., 2005), we did not observe any microthrombi formed primarily of CFSE-labeled donor platelets. This may be due to the smaller diameters of the villous microvessels studied in which numbers of circulating donor platelets was far smaller than that observed in larger vessels. However, we did demonstrate that intestinal IR injury led to the adhesion of singular platelets within the ileal and the jejunal villous microcirculation, with significantly more in the latter. Furthermore, we were able to demonstrate that numbers of free flowing platelets in the ileum decreased, partly indicative of reduced blood flow in this region. Within the jejunum, we were unable to monitor any free flowing platelets, which was due to complete cessation of blood flow within this area. The activity of platelets within the intestinal mucosal villous microcirculation has not previously been monitored and so this new data demonstrated that platelets can be recruited to this lumenal surface and adhere to activated endothelium.

Unfortunately, this methodology doe not take into account platelet events that may be taking place due to the aggregation of unlabelled endogenous platelets. Therefore, it is possible that previous studies using this methodology have underestimated the role of platelets in IR injury models. Hence we performed additional studies using a novel method of combining the fluorescent labeling of donor platelets with the labeling of all endogenous platelets in the recipient mouse. Considering the sheer numbers of platelets, labelling all of them red made it impossible to identify individual platelets and compromised quantification of freely flowing platelets and their interaction with endothelium. But in combination with introducing green donor platelets, we were able to quantitate three platelet activities – thrombus formation, individual platelet adhesion and circulating platelet numbers. Overall, we have demonstrated that using only a single labelling methodology alone significantly underestimates the role of platelets in intestinal IR injury and it is only when using the dual labelling method have we been able to monitor platelet recruitment to areas of activated endothelium and exposed ECM.

It is possible that the microthrombi observed were simply due to mechanical hindrance of the platelets. Leukocyte retention in capillaries often results from mechanical hindrance of leukocyte transit through narrow capillary segments (Kuebler *et al*., 2000). However, platelets are much smaller discoid cells with diameters of ~2-3 µm. Since capillaries have an approximate diameter of 5-6µm, mechanical hindrance of platelet transit and subsequent

platelet sequestration is unlikely. More importantly, the electron microscopy studies demonstrated the presence of microthrombi within the microvasculature overlying areas of exposed collagen fibres. These observations provide the first evidence to suggest platelet recruitment to IR injured intestinal vasculature could also occur through mechanisms other than interactions with activated endothelium. The microthrombi and exposed collagen fibres were identified only within the jejunal capillaries and central arteriole of villi and not within the ileal, serosal or mesenteric macrovessles. Also, the next chapter of this thesis provides further evidence that thrombosis occurs due to platelet-collagen interactions, as a function blocking antibody to GPIb, which links platelets to collagen via vWF, significantly inhibited microthrombus formation. It is well documented that inhibition of platelet recruitment via interactions with exposed ECM, following IR injury, has positive effects on tissue and vascular damage. However, these studies have taken place within larger vessels such as the cerebral and mesenteric arteries, with little experimentation undertaken to show how platelet inhibition may benefit microvascular beds when ECM is exposed rather than just activated endothelium.

Previous studies have focused only on the role of individual adherent platelets following intestinal IR reperfusion (Cooper *et al.,* 2003; Massberg *et al.,* 1998; Massberg *et al.*, 1999). The numbers of platelets observed adherent in these studies appears to be higher than the numbers identified within the current study. However, it is difficult to make direct comparisons. For example, Massberg and colleagues demonstrated that approximately 400

platelets adhered within the serosa of the jejunum in their study (Massberg *et al.,* 1998; Massberg *et al.*, 1999). However, they induced segmental ischaemia for 1 hour which most likely induced a greater degree of injury to that particular area than our study. Unfortunately, total intestinal ischaemia for this same duration resulted in 100% mortality either during the ischaemic phase or immediately upon reperfusion in our study. Furthermore, the observations of Massberg and colleagues were made in large submucosal arterioles and venules whereas the current observations were made in the relatively smaller villous microcirculation. However, we did not observe such high numbers of platelets adherent within the large mesenteric or serosal vessels, but this could be due to their resistance to IR injury and higher flow rates and wall shear rates.

The involvement of platelets was dependent upon the severity of ischaemic injury and, to a certain extent, the duration of reperfusion. This was demonstrated by the observation that adhesion of individual platelets within the ileal microcirculation significantly increased with increased ischaemia. The numbers of adherent platelets and the development of microthrombi, particularly within the jejunum, occurred immediately upon reperfusion and neither platelet event became significantly more severe with time. Our earliest observation was within 30 minutes of reperfusion. This suggests that endothelial activation (leading to adhesion of individual platelets) and endothelial denudation (leading to microthrombus formation) occurs either during the ischaemic phase or immediately upon reperfusion. This has significant clinical importance when developing therapeutic regimens targeting platelets

during intestinal IR injury.

It is possible that maximal platelet activity takes place early post-reperfusion and actually cannot be increased any further. We demonstrated that the ability of platelets derived from IR injured mice to aggregate in response to collagen, either in an aggregometer or under flow conditions, was lost after 2 hours post-reperfusion i.e. IR injury platelets demonstrated a hypoaggregatory response. Furthermore, Mr Rui Wu (intercalating BMedSci undergraduate project student) demonstrated that platelets derived from mice undergoing intestinal IR injury had normal adherent ability on collagen and endothelial cells *in vitro* under flow conditions at 10 mins post-reperfusion but lost this ability by 2 hours (Wu *et al.*, 2010). Collectively, this may explain why we did not observe increased platelet adhesion with time. It is therefore possible that platelets present within the circulation of IR injured mice become irreversibly activated to a level incapable of undergoing secondary activation through weak to medium strength agonists. Interestingly, IR injured platelets at 2 hours post-reperfusion were still able to aggregate in response to the strongest platelet agonist thrombin. Similarly, Aydemir-Koksoy and colleagues also demonstrated significantly reduced aggregation of platelets isolated from rabbits undergoing intestinal IR injury platelet to collagen whereas platelets taken from animals undergoing only ischaemia were still able to aggregate (Aydemir-Koksoy *et al*., 1999).

Platelets are amongst the most vulnerable cells to oxidative injury due to their low anti-oxidant reserve (Ambrosio *et al.*, 1994). Following IR injury, numerous systemic inflammatory mediators are released including oxygen free radicals (eg. H_2O_2), arachidonic acid products, cytokines and nitric oxide. Platelets can become inactivated by these mediators and become hypoaggregatory in response to agonists such as collagen. Additional mechanisms have been reported to account for this platelet dysfunction, such as platelet membrane damage, alterations in platelet receptors and release of platelet granule contents (Boldt *et al.*, 1996). Indeed, reversible platelet dysfunction with impaired aggregation is a well known phenomenon associated with cardiopulmonary bypass surgery (Jung *et al.,* 1995). Approximately 10% to 20% of patients undergoing cardiac operations exhibit inadequate haemostasis varying in its duration and severity and platelet transfusions are required to stop internal bleeding complications after surgery.

It is interesting that the ileum is better at 'coping' with IR injury than the jejunum as was determined macroscopically, histologically and electron microscopically. Only platelet-endothelial interactions and limited vascular leakage was observed in the ileum even after high ischaemic episodes. This phenomenon has been investigated previously in pigs, in which similar observations to our own were witnessed (Chan *et al*., 1998). The same group went on to show that basal nitric oxide levels are higher in the ileum than the jejunum in rats and decreased upon reperfusion of ischaemic regions (Chan *et al*., 1998(2)). This may provide one explanation for the segmental differences in susceptibility to injury.

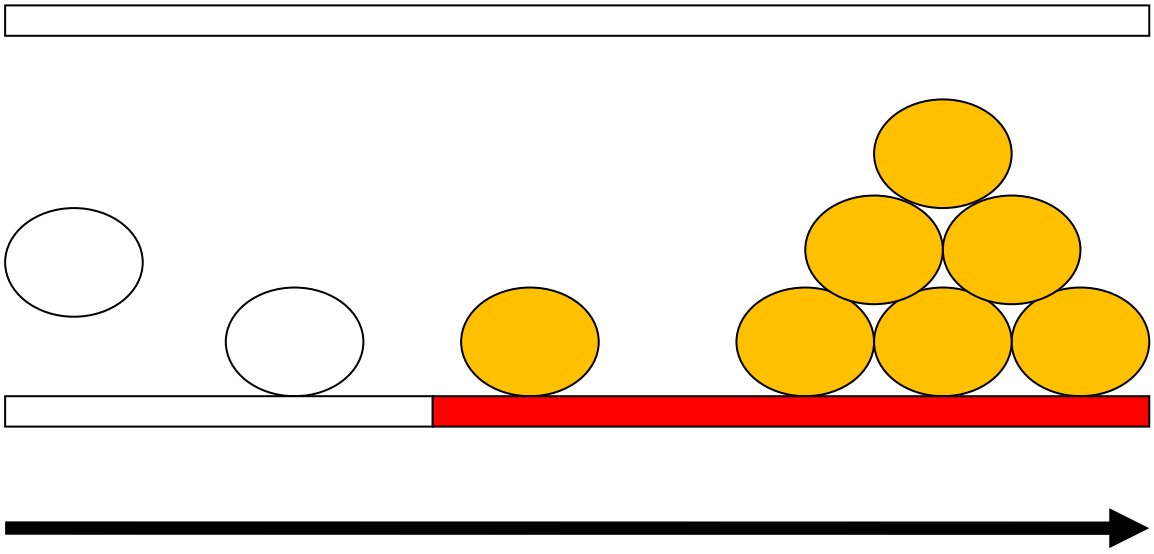
Having identified platelets within the mucosal villous microcirculation, the critical question that remains is whether they have a pathophysiological role in intestinal IR injury. Several groups consider leukocytes as being primarily responsible for injury (Brown *et al*., 1990; Beuk *et al.*, 2008). Indeed, our *in vivo* observations within the ileal vasculature demonstrate a higher number of leukocytes rather than platelets within the ileum. However, adherent individual platelets and microthrombi were predominantly observed in the more severely injured jejunum. Furthermore, less leukocytes were observed within this area when compared to the ileum. These results suggest platelets may be the primary cell responsible for inducing injury within the jejunum microvasculature. In order to determine whether platelets have a pathological role in intestinal IR injury, the remainder of this thesis will identify strategies to inhibit both platelet-endothelial and microthrombus formation and determine whether this confers any macroscopic and microscopic benefit.

In conclusion this chapter has presented data obtained using a novel dual labeling methodology for platelets. This method allows individual, aggregated and freely circulating platelets to be quantitated *in vivo* and will further permit their pathophysiological role to be determined. We have also demonstrated that platelet recruitment varies in different sites of the gut and that recruitment is directly proportional to the severity of injury. This may dictate which parts of the intestine would be better suited to act as a graft for procedures such as small bowel transplantation. The transplanted graft undergoes a period of ischaemia when excised from the donor, and then reperfusion injury upon transplantation. Therefore,

the terminal ileum may be a better choice of graft than the jejunum. Interestingly, intestinal IR injury has a detrimental effect on circulating platelets as evidenced by their inability to aggregate in response to collagen both in an aggregometer and under flow conditions. This may be of beneficial event which could theoretically prevent the adhesion of activated platelets in remote extra-digestive sites.

Chapter 4

Investigating the roles of GPIbα and P-selectin in mediating platelet recruitment to intestinal IR injured microvasculature



4.0. Investigating the roles of GPIbα and P-selectin in mediating platelet recruitment to intestinal IR injured microvasculature

4.1. Introduction and hypotheses

4.1.1. Introduction

The previous chapter presented novel data demonstrating that platelet recruitment within the IR injured gut manifests in two different forms. Individual platelets were observed within the proximal jejunum and the terminal ileum, but less so in the latter region. Furthermore, microthrombi were observed predominantly within the jejunum which also underwent the most severe injury. It is likely that the microthrombi within the villous capillaries exacerbate or contribute to the development of tissue injury within the jejunum through the occlusion of microvessels and recruitment of inflammatory leukocytes. Indeed, congested blood capillaries and lack of blood flow was observed in the jejunal mucosa. This chapter aimed to investigate the molecular adhesive mechanisms governing platelet recruitment to the jejunal and ileal villous microcirculation, and determine whether inhibiting individual platelet recruitment and/or microthrombus formation conferred benefit to the gut.

Most studies that have determined a critical role for platelets in IR injury have identified a critical role for P-selectin in permitting platelet-endothelial interactions (Massberg *et al.*, 1998). Leukocyte adhesion has also been demonstrated to be dependent upon leukocyte PSGL-1 interacting with endothelial and platelet P-selectin (Cooper *et al.,* 2003). The

development of microthrombi has been attributed to the exposure of ECM matrix proteins, especially collagen, which leads to the subsequent adhesion, activation and aggregation of platelets (Vandendries *et al*., 2004). It is therefore likely in our model, that in addition to P-selectin, an additional mechanism is responsible for platelet recruitment and may involve platelet receptors that adhere to and become activated by exposed collagen. Platelet adhesion under hydrodynamic shear stress is mediated by a number of receptors including GPIb-IX-V, GPVI and the integrins $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) and $\alpha_2\beta_1$ (GPIa/IIa). As well as controlling haemostasis after vessel injury, these same receptors also contribute to mediating pathological processes involving platelets such as heart disease and stroke (Kleinschnitz *et al.,* 2007)**.** The contributory role of collagen receptors will be investigated later, but the current chapter will present data obtained with regards the role of GPIb-IX-V, particularly the GPIbα subunit.

GPIb-IX-V, constitutively expressed on platelets, is a complex of GPIbα disulphide-linked to GPIbβ and non-covalently associated with GPIX and GPV in the ratio of 2:2:2:1 (Sachs *et al.,* 2003). There are approximately 25,000 copies of GPIbα per platelet and it is the major sub-unit which binds to vWF immobilised on exposed collagen at the site of injury. This is commonly accepted as the first step in the formation of a haemostatic plug (Nieswandt *et al.*, 2003). However, GPIbα also plays a key role in the process of platelet tethering or rolling on activated endothelium (Andre *et al.*, 2000; Theilmeier *et al.*, 2002). This allows for their activation and subsequent adhesion. Since both individual platelets and microthrombi were

observed within the IR injured gut, it is likely that the former is due to endothelial activation and the latter collagen exposure. Therefore, GPIbα has the potential to be the major platelet receptor responsible for all types of platelet adhesion within the intestinal vasculature of this disease model. Indeed, GPIbα has been shown to play a key role in platelet recruitment to cardiac and cerebral vessels in murine models of disease (Lowenberg *et al.*, 2010).

4.1.2 Hypothesis

This chapter therefore aimed to investigate the roles of P-selectin and GPIbα in mediating platelet recruitment to the intestinal IR injured microvasculature. Since we observed both platelet events (microthrombus formation and individual platelet adhesion) taking place in the jejunum, we predominantly focused on this region for this study. For the work included in this chapter we hypothesized:

1. Recruitment of individual platelets would be dependent upon P-selectin.
2. Recruitment of microthrombi to the jejunum would be dependent upon GPIbα.
3. Reducing platelet recruitment, either through a P-selectin or a GPIbα dependent mechanism, would confer intravital, histologic and macroscopic benefit to the jejunum.
4. Reducing platelet recruitment, either through a P-selectin or a GPIbα dependent mechanism, would also reduce leukocyte recruitment to the mucosa of the jejunum.

4.2. Methods

Initially, FACS analysis was performed to allow us to demonstrate that the function blocking antibody concentration was sufficient to cause GPIbα receptor blockade of over 90%. Donor platelets and endogenous platelets were blocked separately to increase confidence of GPIb inhibition. Receptor inhibition was not required for P-selectin investigations as knockout mice, with a C57Bl/6 background, were used. These mice were originally from the Jackson labs and the colony they originated from were the C57Bl/6J-*Selp*tm1Bay colony. These mice were a generous gift from Professor Paul Hellewell (University of Sheffield, UK). Fluorescent IVM was performed on male 20-25g wild-type C57Bl/6 mice or P-selectin$^{-/-}$ mice, receiving wild-type or P-selectin-/- donor platelets respectively. All intestinal IR intravital investigations within this chapter used periods of 45 minutes ischaemia followed by 4 hours of reperfusion. The carotid artery and trachea were cannulated and the animal subjected to either sham or IR treatment. During reperfusion, the ileum and jejunum was monitored intravitally. Free flowing platelets, individual adherent platelets and microthrombi were quantitated. The systemic administration of FITC-BSA and Acridine orange allowed vascular macromolecular leakage and leukocyte adhesion to be quantitated. Histological haematoxylin and eosin (H&E) staining was used to visualise mucosal villi. *In vitro* adhesion assays were conducted to determine the role of GPIb and P-selectin in allowing adhesion of platelets to collagen under flow conditions and endothelial cells under static conditions. Flow cytometry was also performed to demonstrate the presence of circulating platelet/leukocyte aggregates.

4.3. Results

4.3.1. Anti-GPIbα antibody does not affect systemic platelet numbers

The first line of critical experimentation was to analyse the percentage receptor occupancy of the GPIbα function blocking antibody and also determine whether it's systemic administration had any detrimental effects on platelet number. Optimum antibody concentrations for complete receptor blockade were determined after injection of C57Bl/6 mice with varying amounts of Xia B2 (anti-GPIbα antibody). The antibody was allowed to circulate in the vasculature for 30 mins, after administration through the cannulated carotid artery. 50µl of blood was then incubated with 5ul of FITC conjugated Xia B2 and flow cytometry was used to detect the minimal amount of the necessary to achieve saturating GPIbα receptor binding *in vivo.* Kleinschnitz and colleagues, who used a similar antibody for *in vivo* studies, used a concentration of 100ug to gain >90% inhibition (Kleinschnitz *et al.,* 2007). Our receptor binding studies also proved that a concentration of 100ug was more than sufficient to achieve receptor occupancy of approximately 95% (Table 4.1). Platelet counts in antibody-treated mice were not statistically different from the rat IgG control (Table 4.1).

	Wt (no antibody)	Rat IgG	Anti-GPIbα IgG
Receptor Occupancy % Platelet Count ($x10^9$)/L	0 1254 ± 123	0 1318 ± 45	>95 1156 ± 110

Table 4.1. The anti-GPIbα antibody achieves receptor occupancy of approximately 95% and does not affect circulating platelet number. Values are mean ± SD with $p<0.05$, unpaired Student t test).

4.3.2. Individual platelet adhesion is not significantly decreased in the ileum of anti-GPIbα mAb pre-treated or P-selectin$^{-/-}$ mice.

Individual platelet recruitment was unaffected in either anti-GPIbα mAb treated or P-selectin$^{-/-}$ mice (**Figure 4.1a**). Free flowing platelets were also unaffected and did not increase in these mice (**Figure 4.1b**). The effects of an IgG control antibody on numbers of free flowing platelets was also determined in the ileum but no difference was observed (**Figure 4.1c.**).

4.3.3. Microthrombus formation, but not individual platelet adhesion, is significantly decreased in the jejunum of anti-GPIbα mAb pre-treated animals. Neither platelet event was reduced in P-selectin$^{-/-}$ mice.

The formation of microthrombi in wild-type animals receiving the anti-GPIbα mAb was significantly ($p<0.001$) reduced when compared to un-treated sham animals undergoing intestinal IR injury (**Figure 4.2a and 4.3b**). This reduction was observed at all time points 4 hours post-reperfusion. No decrease in the numbers of adherent individual platelets was observed, although during the early stages of reperfusion there was an insignificant decrease which reached similar numbers to those seen in wild-type animals 4 hours post-reperfusion **(Figure 4.2b).** In contrast, formation of microthrombi in P-selectin$^{-/-}$ mice was not reduced when compared to un-treated animals undergoing intestinal IR injury (**Figure 4.2a**). Again, no decrease in the numbers of adherent individual platelets was observed in P-selectin$^{-/-}$ **(Figure 4.2b).** Free flowing platelets were only significantly increased in anti-GPIbα pre-

treated animals during the early stages post-reperfusion up to 150 minutes post-reperfusion. However, during the later stages of reperfusion, no free flowing platelets were observed suggesting vascular occlusion was still occurring even though microthrombi formation had been inhibited (**Figure 4.2c)**. Furthermore, the IgG antibody did not have any effects on the formation of microthrombi in the jejunum as determined by quantitating their fluorescent intensity (**Figure 4.2d)**. This evidence, along with other IgG controls stated within several *in vitro* adhesion, aggregatory and FACS experiments throughout this thesis, gives us the confidence that the internal effect of antibody administration in this model is negligible.

4.3.4. Marked macroscopic improvement of the jejunum was observed in anti-GPIbα mAb pre-treated animals during early reperfusion but this effect was not long lasting. No benefit is observed in P-selectin$^{-/-}$ animals.

As stated previously (Chapter 3, Section 3.2.7), the proximal jejunum in un-treated mice underwent severe injury following 45 minutes ischaemia and appeared haemorrhagic, severely congested and swollen upon reperfusion. This area had greater levels of individual platelet adhesion, microthrombus formation and complete cessation of blood flow. However, in anti-GPIbα mAb pre-treated mice, microthrombus formation was significantly reduced and despite significant individual platelet adhesion (**Figure 4.3b**), the jejunum appeared relatively healthy for about 3 hours post-reperfusion (**Figure 4.4b**). However, this remarkable beneficial effect was not observed at 4 hours reperfusion. No macroscopic benefit was observed in P-selectin$^{-/-}$ mice undergoing intestinal IR injury.

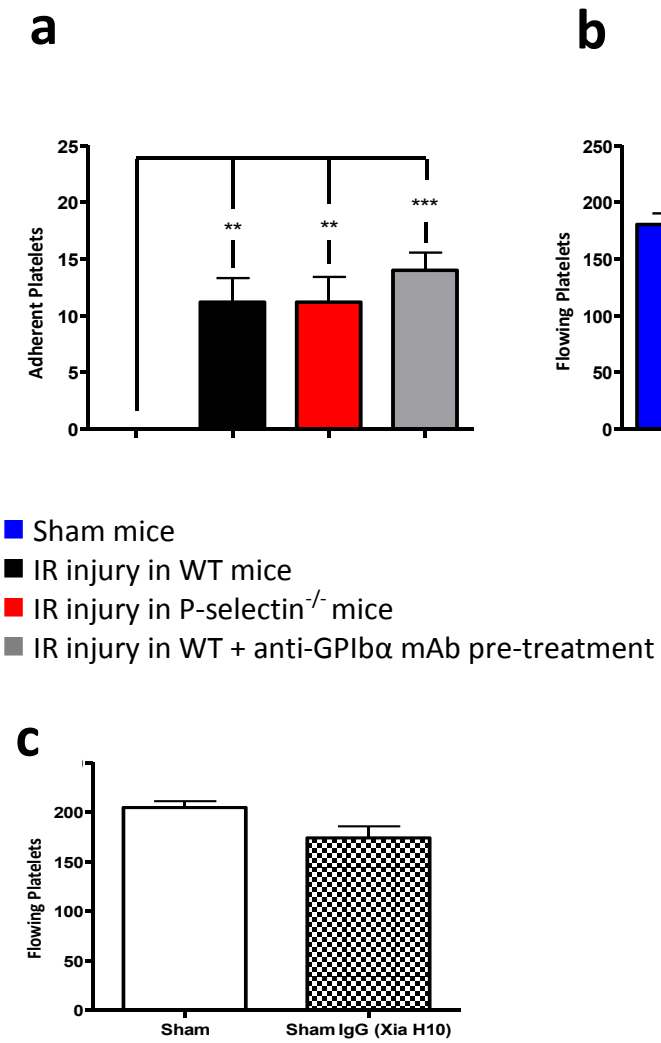


Figure 4.1. Individual platelet adhesion is not significantly decreased in the ileum of anti-GPIbα mAb pre-treated or P-selectin$^{-/-}$ mice at 4 hours reperfusion. Recruitment of CFSE-labelled donor platelets to the microcirculation of ileal mucosal villi in sham, IR injured, GPIbα blocked or P-selectin$^{-/-}$ mice was examined intravitally. Wild-type, GPIbα blocked or P-selectin$^{-/-}$ treated labelled platelets (Donor: 1 x 10^8 platelets, 10μM CFSE) were introduced prior to IR injury. **(a)** CFSE-labelled donor platelet recruitment was not significantly reduced in GPIbα blocked or P-selectin$^{-/-}$ mice. **(b)** Free flowing platelets were not significantly increased in GPIbα blocked or P-selectin$^{-/-}$ mice in comparison to sham animals. **(c)** Free flowing platelets are unaffected by the administration of IgG antibodies in sham animals at 2 hours reperfusion. Plots are representative of mean + SEM of at least 5 separate experiments. **p<0.01, ***p<0.001 when compared using a one way ANOVA followed by Bonferroni Post Test for points of significance.

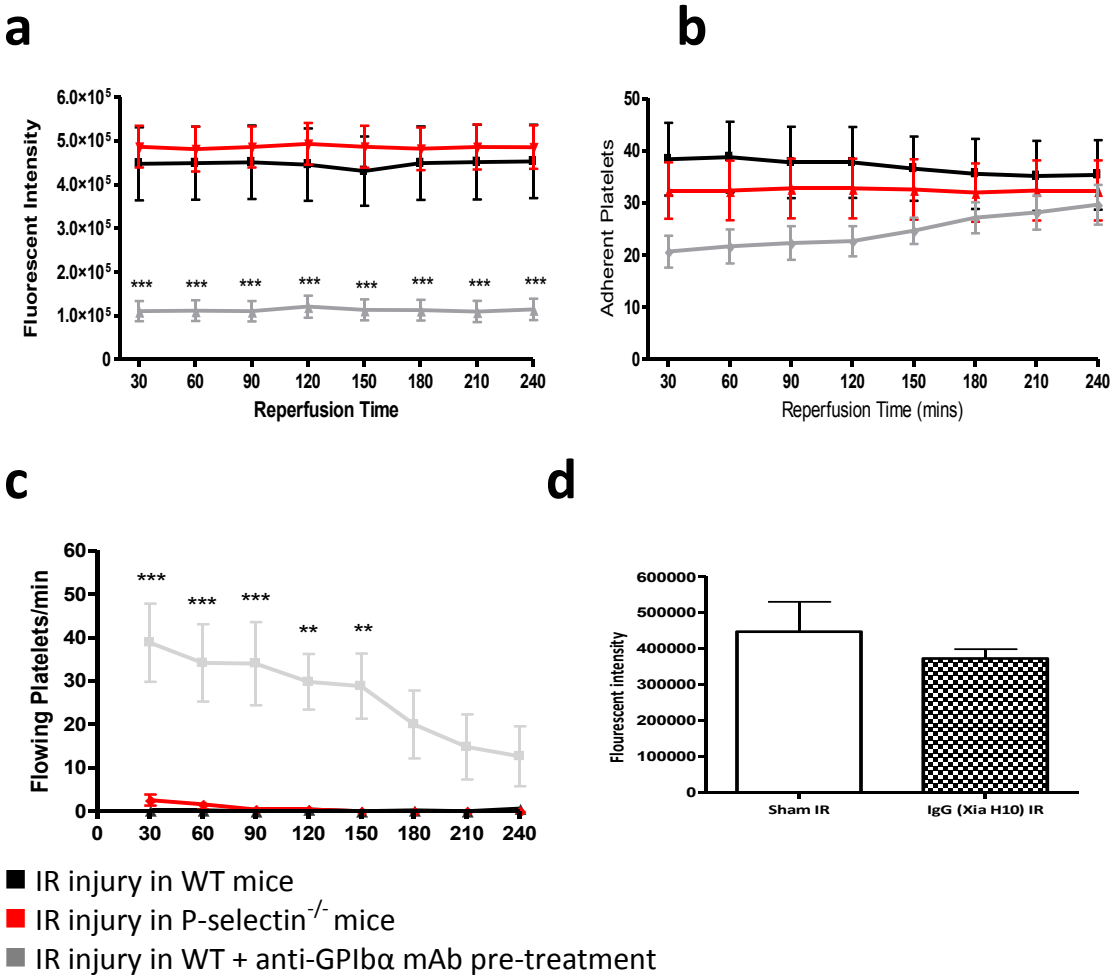


Figure 4.2. Microthrombus formation is significantly decreased in the <u>jejunum</u> of anti-GPIbα mAb pre-treated animals. Neither platelet event was reduced in P-selectin$^{-/-}$ mice. Recruitment of CFSE-labelled donor and Alexa 594-labelled endogenous platelets to microcirculation of jejunal villi in wild-type, GPIbα blocked or P-selectin$^{-/-}$ mice was examined intravitally. Wild-type, GPIbα blocked or P-selectin$^{-/-}$ treated labelled platelets (Donor: 1 x 10^8 platelets, 10µM CFSE; Endogenous: 20 µl GPIIb ab/ 40 µl Alexa 594) were introduced prior to IR injury. **(a)** Alexa 594-labelled endogenous microthrombi formation was only significantly reduced in GPIbα blocked mice. **(b)** CFSE-labeled donor platelet recruitment was not significantly reduced in GPIbα blocked or P-selectin$^{-/-}$ mice. **(c)** Free flowing platelets were significantly increased in GPIbα blocked animals during the early stages of reperfusion. **(d)** Microthrombi intensity was un-affected by the IgG antibody in IR animals at 2 hours reperfusion. Plots are representative of mean + SEM of at least 5 separate experiments. **p<0.01, ***p<0.001 when compared using a one way ANOVA followed by Bonferroni Post Test for points of significance.

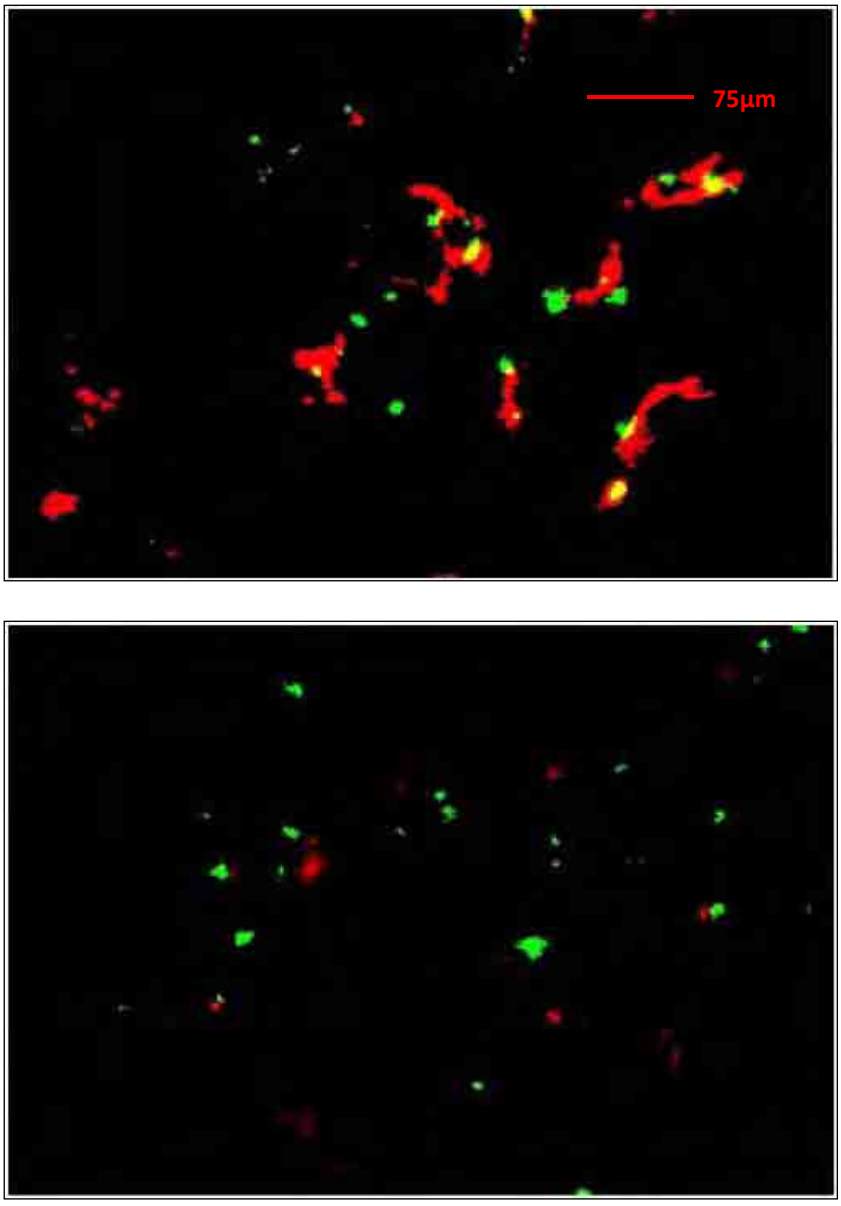


Figure 4.3. Microthrombus formation, but not individual platelet adhesion, within the jejunum is significantly decreased in anti-GPIbα mAb pre-treated animals. (a) Singular green CFSE-labelled donor and red Alexa 594-labelled endogenous microthrombi could be observed within the jejunum of an un-treated animal undergoing IR injury. Singular platelets were either adherent on their own or contained with a microthrombus, and thus appeared yellow. **(b)** Alexa 594-labelled endogenous microthrombi formation was significantly reduced by pre-treatment with an anti-GPIbα mAb but CFSE labelled individual platelets are still observed. Representative images are shown of un-treated and GPIbα treated jejunal microcirculation at 4 hours post reperfusion

a

b

Figure 4.4. Marked macroscopic improvement of the jejunum was observed in anti-GPIbα mAb pre-treated animals during early reperfusion but this effect was not long lasting. No benefit is observed in P-selectin$^{-/-}$ animals. (a) The proximal jejunum underwent severe injury following 45 minutes ischaemia and appeared haemorrhagic, severely congested and swollen upon reperfusion in wild-type animals. A representative image is shown of the jejunum at 4 hours post-reperfusion. **(b)** The jejunum appeared relatively healthy after 45 minutes ischaemia and 1 hour reperfusion in mice receiving the anti-GPIbα mAb. A representative image is shown of the jejunum at 1 hour post-reperfusion. However, this remarkable beneficial effect was not observed at 4 hours reperfusion (not shown).

4.3.5. Macromolecular leakage from the jejunum was significantly reduced in anti-GPIbα mAb pre-treated animals during early reperfusion but this effect was not long lasting. No benefit is observed in P-selectin$^{-/-}$ animals.

The jejunum showed a significant ($p<0.001$) decrease in vascular leakage in anti-GPIbα mAb pre-treated mice at 1 hour reperfusion when compared to wild-type animals undergoing IR injury (**Figure 4.5**). However, this decrease was not observed at 4 hours post-reperfusion at which point similar interstitial fluorescence was recorded to that of IR injured wild-type mice observed. No change in leakage was observed in P-selectin$^{-/-}$ mice at any point post-reperfusion (**Figure 4.5**).

4.3.6. No histological improvement of the jejunal mucosal villi was observed in anti-GPIbα mAb pre-treated animals or in P-selectin$^{-/-}$ at 4 hours post-reperfusion.

No improvement in the histology of the jejunal mucosa was observed in wild-type mice pre-treated with an anti-GPIbα mAb or in P-selectin$^{-/-}$ at 4 hours reperfusion (**Figure 4.6**). Unfortunately, histology was not performed on specimens from animals pretreated with anti-GPIbα mAb at 1 hour reperfusion.

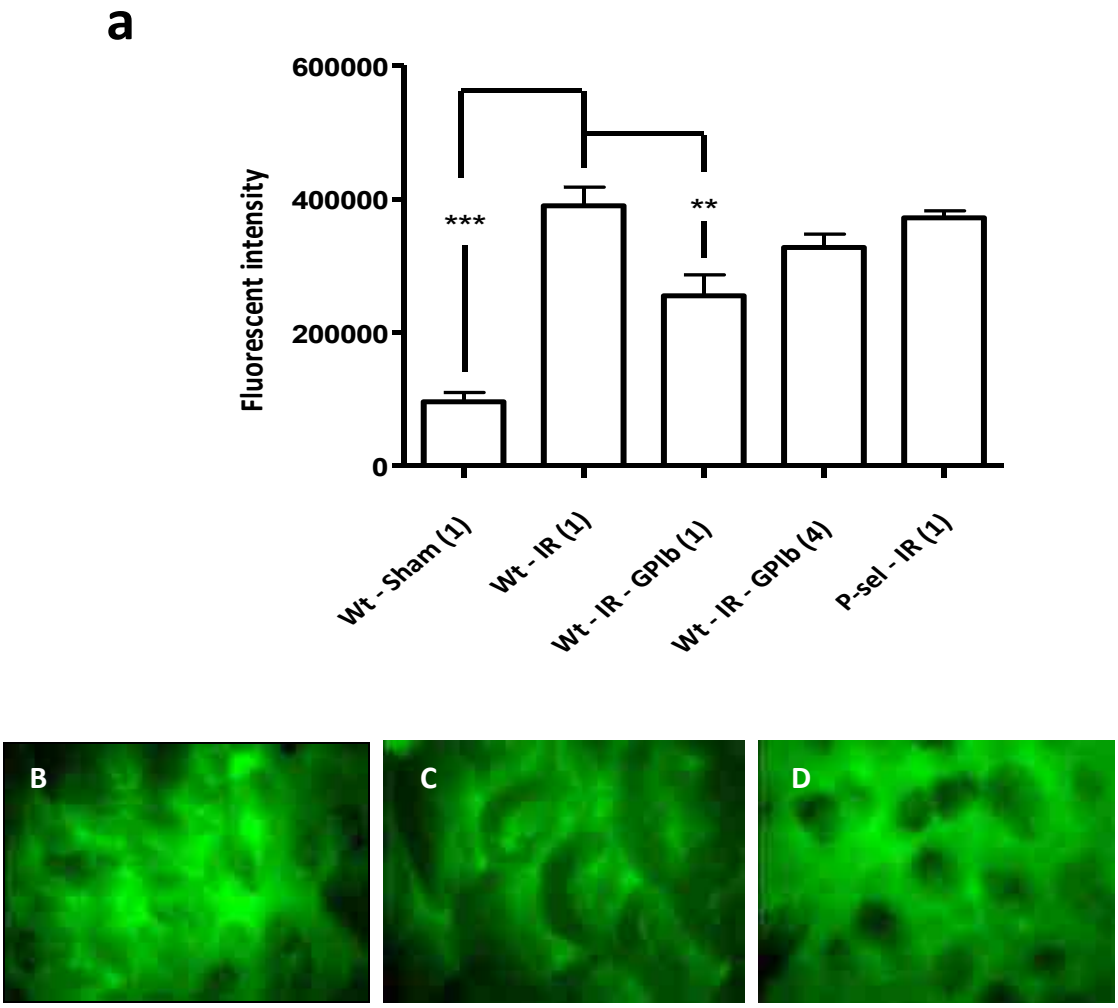


Figure 4.5. Macromolecular leakage from the jejunum was significantly reduced in anti-GPIbα mAb pre-treated animals during early reperfusion but this effect was not long lasting. No benefit is observed in P-selectin$^{-/-}$ animals. Macromolecular leakage was analysed by systemically administering FITC-BSA, at 4 hours post-reperfusion. The increased fluorescence per field of view was monitored intravitally. **(a)** Significant increases in fluorescent intensity to the jejunal villi was observed in wild-type mice after 1 hour post-reperfusion and also in animals receiving the anti-GPIbα mAb at 1 and 4 hours post-reperfusion and in P-selectin$^{-/-}$ mice. Representative images are shown of vascular leakage from **(b)** wild-type animals 4 hours post-reperfusion IR jejunum **(c)** anti-GPIbα mAb pre-treated animals at 1 hour post-reperfusion and **(d)** 4 hours post-reperfusion. Data is representative of mean + SEM of at least 5 separate experiments. **p<0.01, ***p<0.001 when compared using a one way ANOVA followed by Bonferroni post test for points of significance.

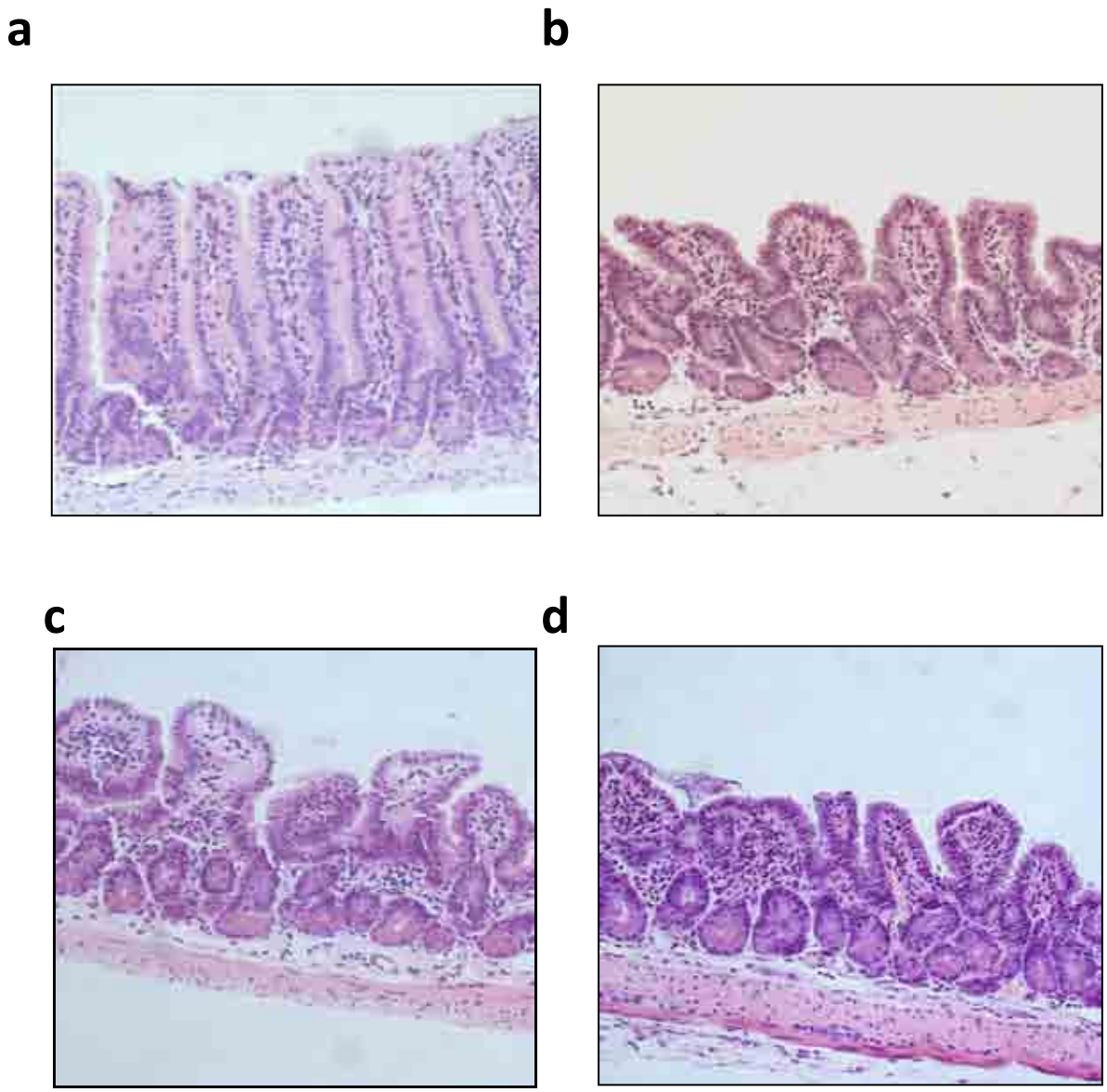


Figure 4.6. No histological improvement of the jejunal mucosal villi was observed in anti-GPIbα mAb pre-treated animals or in P-selectin$^{-/-}$ at 4 hours post-reperfusion (magnification x200). (a) Sections from wild-type shams showed healthy jejunal mucosa. **(b)** Sections from IR injured mice and from those **(c)** receiving the anti-GPIbα mAb demonstrated severe mucosal sloughing and inflamed crypt areas at 4 hours post-reperfusion. **(d)** Similar results were also observed in P-selectin$^{-/-}$ mice at 4 hours post-reperfusion.

4.3.7. Individual platelet adhesion was significantly increased in the jejunum in anti-GPIbα mAb pre-treated animals. This was only demonstratable once the numbers within and outside the microthrombi were analysed.

As mentioned above, individual platelet adhesion did not change in animals receiving the anti-GPIbα mAb. However, on closer inspection, we identified that some of the individual platelets were actually contained within the microthrombi but had been quantitated as singular platelets (appeared yellow – **Figure 4.3a**). We therefore re-counted the numbers of individual CFSE-labelled green platelets within the microthrombi and those located outside the microthrombi at 4 hours post-reperfusion. On doing so, we observed that anti-GPIbα mAb pre-treated mice showed a significant ($p<0.01$) decrease in platelets inside the microthrombi at 4 hours post-reperfusion (**Figure 4.7**). Furthermore, we were also able to demonstrate that individual platelets outside of the thrombus, even though of an increased trend, were statistically unaltered i.e. the 'genuine' singular platelets, in mice receiving the anti-GPIbα mAb (**Figure 4.7).**

4.3.8. Platelets derived from IR injured mice do not adhere to collagen under flow conditions *in vitro* but platelets derived from IR injured mice pre-treated with an anti-GPIbα mAb do adhere.

Murine whole blood containing wild-type platelets, wild-type IR platelets, wild-type platelets + anti-GPIbα mAb or wild-type IR platelets + anti-GPIbα mAb was flowed over collagen coated capillary tubes *in vitro*. Wild-type anti-GPIbα mAb pre-treated platelets showed an

expected significant ($p<0.001$) decrease in adhesion when compared to wild-type controls (**Figure 4.8a**). This decreased adhesion was not as great as the complete inhibition previously described for platelets derived from IR animals (**Figure 4.8a**, but initially described in Section 3.3.10 and Figure 3.12**).** Hence IR injury inhibited the adhesion of platelets to collagen more strongly than the anti-GPIbα mAb. Interestingly, the adhesion of platelets derived from animals undergoing IR injury but receiving an anti-GPIb mAb was not the same as IR animals. Indeed, a significant ($p<0.01$) increase in platelet adhesion was observed in wild-type mice undergoing IR injury pre-treated with an anti-GPIbα mAb when compared to non-treated IR mice (**Figure 4.8a**).

4.3.9. Platelets derived from IR injured mice do not adhere to activated endothelium under static conditions *in vitro* but platelets derived from IR injured mice pre-treated with an anti-GPIbα mAb do adhere.

At 2 hours post-reperfusion, 500ul of mouse blood were incubated on endothelial coated coverslips for 45 minutes. After washing 3x with PBS, coverslips were mounted and 5 random images were then taken using an Olympus digital camera. Treatment of both TGFβ and TNFα induced a significant ($p<0.001$) increase in platelet adhesion (**Figure 4.9a**). This was significantly ($p<0.05$) reduced by pre-treatment with an anti-GPIbα mAb but was not as great as the reduction observed with platelets derived from IR animals**.** Hence IR injury inhibited the adhesion of platelets to endothelium more strongly than the anti-GPIbα mAb. Interestingly, the adhesion of platelets derived from animals undergoing IR injury but

receiving an anti-GPIb mAb was not the same as IR animals. Indeed, a significant ($p<0.01$) increase in platelet adhesion was observed in wild-type mice undergoing IR injury pre-treated with an anti-GPIbα mAb when compared to non-treated IR mice (**Figure 4.9a**).

4.3.10. Leukocyte-endothelial and circulating leukocyte-platelet interactions following IR injury increase significantly in anti-GPIbα mAb pre-treated mice

As previously presented in Chapter 3, leukocyte adhesion after IR injury did not increase in the jejunum when compared to sham controls, and the only leukocyte increase was observed in the ileum (as previously described in **Figure 3.13a**). However, a significant ($p<0.001$) increase in leukocyte adhesion in the jejunum was observed at 4 hours post-reperfusion in IR injured mice pre-treated with an anti-GPIbα antibody when compared to untreated IR injured mice (**Figure 4.10**). We further investigated *in vitro* by FACS analysis, the interaction of circulating leukocytes with platelets. Circulating numbers of platelet-leukocytes aggregates were significantly ($p<0.01$) increased at 4 hours post-reperfusion in IR injured mice pre-treated with a anti-GPIbα mAb when compared to untreated IR injured mice (**Figure 4.11**). Investigation into leukocyte recruitment or circulating platelet-leukocyte aggregates in P-selectin$^{-/-}$ mice undergoing IR injury was not performed as no benefit from IR injury was observed in these mice.

A summary of the major findings from this chapter are presented in **Table 4.2.** The data has been obtained from the jejunum following IR injury as both microthrombus formation and adhesion of individual platelets was observed in this region.

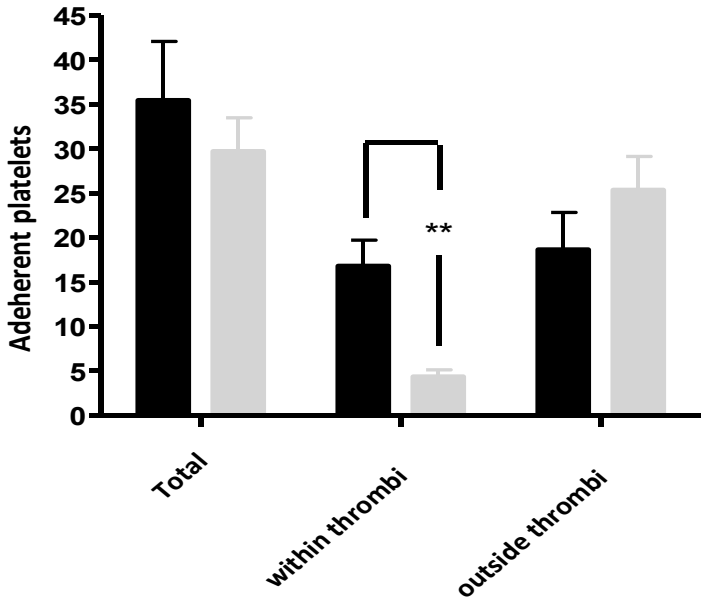


Figure 4.7. Individual platelet adhesion was significantly increased in the jejunum in anti-GPIbα mAb pre-treated animals. This was only demonstratable once the numbers within and outside the microthrombi were analysed. Black bars = IR injury in wild-type mice; grey bar = IR injury in wild-type type mice with receiving the anti-GPIbα mAb. Data is representative of mean + SEM of at least 5 separate experiments, **p<0.01 when compared using a one way ANOVA followed by Bonferroni post test for points of significance.

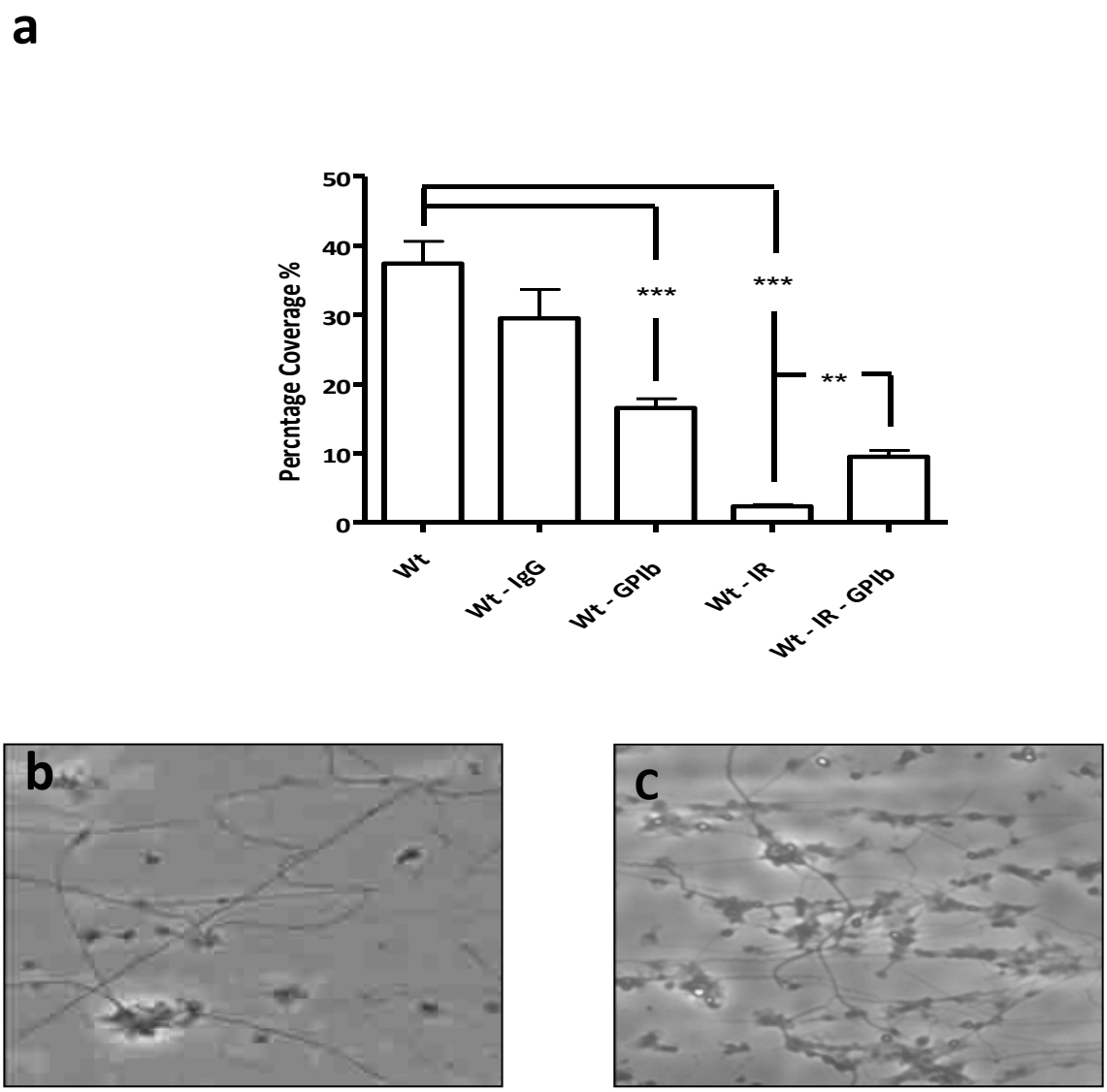
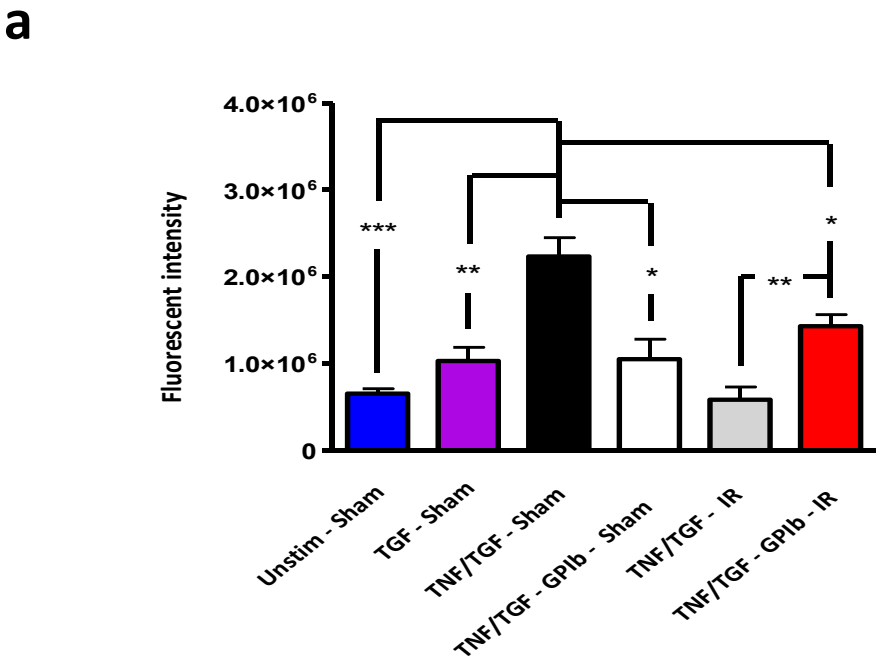


Figure 4.8. Platelets derived from IR injured mice do not adhere to collagen under flow conditions *in vitro* but platelets derived from IR injured mice pre-treated with an anti-GPIbα mAb do adhere. At 2 hours reperfusion, 500ul of mouse blood, anti-coagulated with P-Pack and heparin, was perfused through collagen coated microslides at a shear rate of $300s^{-1}$. After washing for 3 mins, 5 random images were then taken using an Olympus digital camera. **(a)** A significant increase in platelet adhesion was observed in wild-type mice undergoing IR injury pre-treated with an anti-GPIbα mAb when compared to non-treated IR mice. Representative images of adhesion to collagen of platelets derived from **(b)** IR injured wild-type mice and **(c)** IR injured wild-type mice pre-treated with anti-GPIbα mAb. Data is representative of mean + SEM of at least 3 separate experiments. **p<0.01, ***p<0.001 when compared using a one way ANOVA followed by Bonferroni Post Test for points of significance.



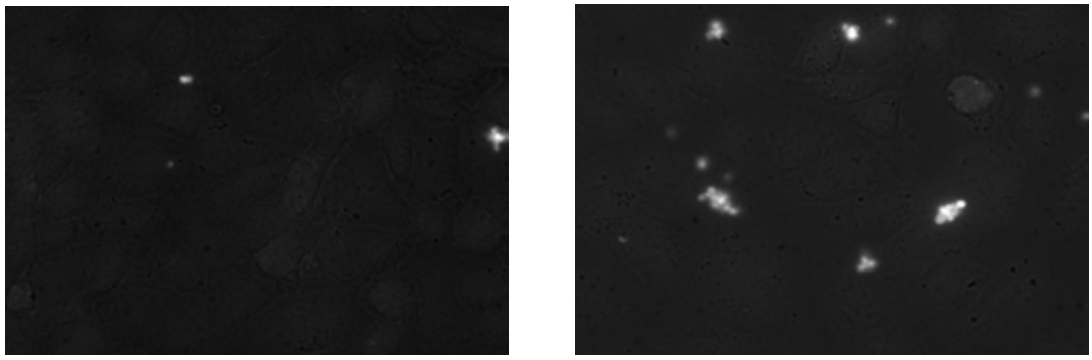


Figure 4.9. Platelets derived from IR injured mice do not adhere to activated endothelium under static conditions *in vitro* but platelets derived from IR injured mice pre-treated with an anti-GPIbα mAb do adhere. At 2 hours reperfusion, 500ul of mouse blood were incubated on endothelial coated coverslips for 45 minutes. After washing 3x with PBS, coverslips were mounted and 5 random images were then taken using an Olympus digital camera. **(a)** A significant increase in platelet adhesion was observed with platelets derived from in wild-type mice undergoing IR injury pre-treated with an anti-GPIbα mAb when compared to platelets derived from non-treated IR mice. Representative images of platelet adhesion to endothelium derived from **(b)** IR injured wild-type mice and **(c)** IR injured wild-type mice pre-treated with GPIbα. Data is representative of mean + SEM of at least 3 separate experiments. **p<0.01 when compared using a one way ANOVA followed by Bonferroni Post Test for points of significance.

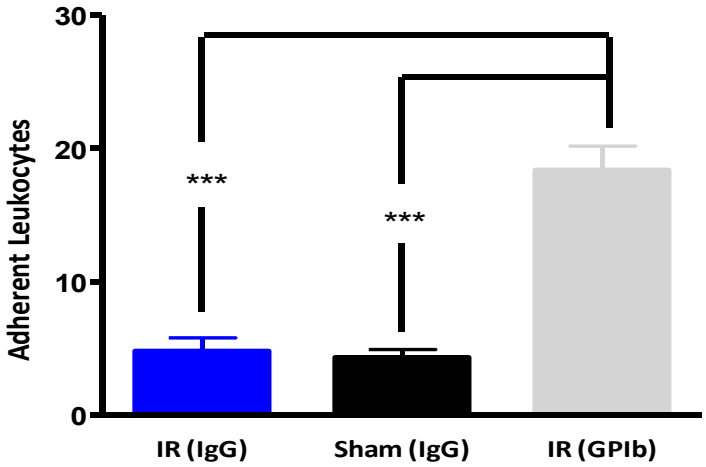


Figure 4.10. Leukocyte-endothelial interactions following IR injury increase significantly in the jejunum of anti-GPIbα mAb pre-treated mice. Leukocyte adhesion did not increase in the jejunum when compared to sham controls when observed intravitally. However, in IR injured mice pre-treated with the anti-GPIbα mAb, a significant increase in leukocyte adhesion was observed. Data is representative of mean + SEM of at least 5 separate experiments. ***p<0.001 when compared using a one way ANOVA followed by Bonferroni Post Test for points of significance.

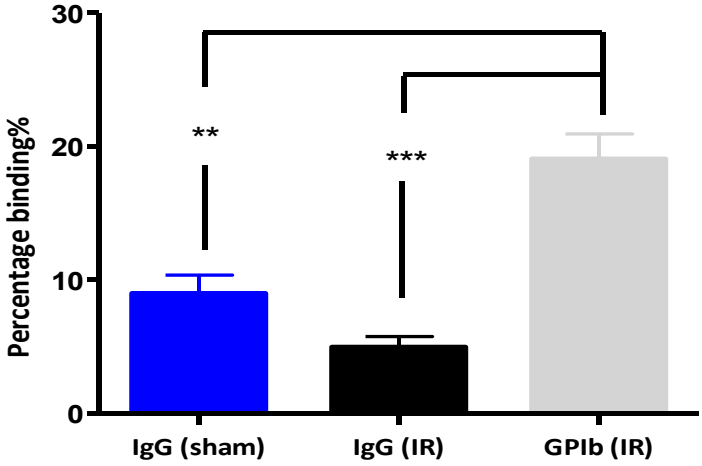


Figure 4.11. Circulating leukocyte-platelet interactions following IR injury increase significantly in anti-GPIbα mAb pre-treated mice. FACS analysis was performed on 50ul blood samples taken from IR injured mice at 2 hours reperfusion. Platelet-leukocyte interactions were not increased in wild-type mice undergoing IR injury compared to sham controls. However, in IR injured mice pre-treated with the anti-GPIbα mAb, a significant increase in leukocyte-platelet interactions were observed. Data is representative of mean + SEM of at least 5 separate experiments. **p<0.01, ***p<0.001 when using a one way ANOVA followed by Bonferroni Post Test for points of significance.

	IR injury alone	**IR injury in anti-GPIbα mAb pre-treated mice**	**IR injury in P-selectin$^{-/-}$ mice**
Microthrombus formation	↑↑↑	↓↓↓	No change
Individual platelet adhesion	↑↑↑	Initially goes down. No change by 4 hrs	No change
Free flowing platelets	↓↓↓	Initially goes up. No change by 4 hrs	No change
Macromolecular leakage	↑↑↑	Initially goes down. No change by 4 hrs	No change
Macroscopic and histological appearance	Very poor	Initially improves. No change by 4 hrs	No change
Platelet adhesion to collagen under flow conditions *in vitro*	Poor	Significant improvement in ability to adhere	Not investigated
Platelet adhesion to activated endothelium under static conditions *in vitro*	↓↓↓	Significant improvement in ability to adhere	Not investigated
Leukocyte recruitment to endothelium	Not much (more in ileum)	Significantly increased	Not investigated
Platelet-Leukocyte aggregates	None	Significantly increased	Not investigated

Table 4.2. Summary of the effects of inducing intestinal IR injury within wild-type mice, mice pre-treated with the GPIbα antibody and in P-selectin$^{-/-}$ mice. Although the major benefits were observed in GPIbα pre-treated mice, these effects were short-lived and were not observed at 4 hours post-reperfusion.

4.4. Discussion

Platelet recruitment to sites of vascular injury is mediated predominantly by exposure of ECM proteins such as collagen and also by adhesion to activated endothelium. We initially described in Chapter 3 that both events takes place in the small intestine following IR injury with both microthrombi as well as individual adherent platelets being observed. In the current chapter, we have demonstrated that the formation of microthrombi are dependent upon the platelet receptor GPIbα. However, the adhesion of individual platelets was not dependent upon GPIbα as their adhesion within the jejunum and ileum did not decrease on blockade of this receptor during IIR injury. It was anticipated that the adhesion of individual platelets would be inhibited in the P-selectin$^{-/-}$ mice. However, quite surprisingly, we did not observe any difference in platelet activity when IR injury was induced in these knockout mice. By preventing the formation of microthrombi in anti-GPIbα mAb pre-treated mice, we were also able to reduce the macromolecular leakage from the villous microcirculation. This suggests that the integrity of the microvessels is intact in these mice. Furthermore, GPIbα pre-treatment also conferred macroscopic and histological benefit. We demonstrated that blood flow within the jejunum improved in anti-GPIbα mAb, as evidenced by the presence of free flowing platelets trafficking through this region. However, it was interesting to observe that although these positive effects were elicited, they were short-lived. By 4 hours post-reperfusion, blood flow decreased and macroscopic and histological injury became apparent again. Enhanced leukocyte adhesion and the formation of circulating leukocyte-platelet aggregrates in IR injured mice pre-treated with the GPIbα antibody was also observed.

It was surprising that no *in vivo* platelet activity, especially individual platelet adhesion, was reduced in P-selectin$^{-/-}$ mice. P-selectin has been shown to be involved in key mechanisms allowing for platelet adhesion to occur at sites of vessel wall injury as well as stabilizing thrombus formation (Yokoyama *et al*., 2005). It allows for platelets to initially role on the endothelium leading to subsequent activation and adhesion (Gawaz *et al.*, 2005). P-selectin has also been shown to play a key role in several models of IR injury, including intestinal IR injury but these observations were undertaken within the mesenteric vessels rather than those in the more vulnerable mucosa. Indeed, preliminary data from the Kalia lab has demonstrated that the adhesion of activated platelets to activated endothelium can be inhibited under static and flow conditions using a P-selectin antibody (unpublished data). However, it is possible that due to the lower shear stresses associated with mucosal villous microcirculation a role for P-selectin in platelet rolling and activation may not be required. The presence of rolling leukocytes may also hinder the free flow of platelets. Although we did not anticipate P-selectin to have a role in preventing microthrombus formation, previous findings have suggested that platelet P-selectin is also involved in the maintaining the stability of a developing thrombus. However, these studies are inconclusive, with publications showing both a significant decrease in thrombi formation (Yokoyama *et al*., 2005) to negligible differences on inhibition of this receptor (Patel *et al.,* 2008)**.**

P-selectin is located within the Weibel-Palade bodies of endothelial cells as well as in the alpha granules of activated platelets. However, P-selectin deficient mice do not express this

receptor on either the endothelium or the platelets. In the current study, donor platelets were derived from P-selectin KO mice, and so these would also have been deficient in P-selectin. Had a role for P-selectin been identified, further studies would have been conducted to determine whether endothelial P-selectin or platelet P-selectin, was important by assessing interaction of wild-type donor platelets in P-selectin deficient mice and interaction of P-selectin-deficient donor platelets in wild-type mice. Although most studies have strongly implicated platelet P-selectin, Frenette and colleagues were the first to demonstrate that platelet-endothelial interactions could also be dependent upon endothelial P-selectin (Frenette *et al.,* 1995). Indeed, an important role for endothelial P-selectin in governing platelet-endothelial cell interactions within hepatic pre-sinusoidal arterioles and post-sinusoidal venule following IR injury has been demonstrated (Khandoga *et al.,* 2002). Platelet-endothelial cell interactions were nearly absent in arterioles and venules of mice lacking endothelial P-selectin and sinusoidal perfusion failure was ameliorated.

The blocking of GPIbα inhibited the formation of microthrombi throughout the 4 hour reperfusion duration and most likely contributed to the inhibition of vessel occlusion during the early stages of reperfusion. GPIbα predominantly plays a critical role in aiding the initial tethering/rolling of platelets on collagen exposed in injured vessels at high shear rates as observed in large arterioles. Therefore, blocking GPIbα abolishes platelet adhesion to sites of vascular damage and could explain the lack of microthrombus formation. However, the shear rates within the microvessesls of the villi are not high and therefore it was surprising to

observe a critical role for GPIbα. The involvement of GPIbα in promoting platelet tethering at low shear appears to contradict previous findings demonstrating that thrombus growth on type 1 fibrillar collagen under low shear conditions occurs independent of both vWf and GPIbα (Wu *et al.,* 1996). However, Kulkarni and colleagues were also able to identify a critical role for the GPIbα-vWF interaction in mediating platelet capture from blood at arteriole (high) and venular (low) shear rates, suggesting it is not implausible for GPIb to play a role in low shear vessels such as the intestinal villous microvessels (Kulkarni *et al.,* 2000).

GPIbα can also bind platelets to vWF expressed on activated endothelium (Clemetson *et al.,* 2008). It is therefore possible that the microthrombi we have observed are just aggregates of platelets attached to intact endothelium, not exposed collagen, or formed due to platelet stagnation within the small capillary vessels. Indeed, we did demonstrated that blocking GPIbα could significantly reduce the adhesion of platelets to TNFα/TGFβ stimulated endothelial cells *in vitro*. However, *in vivo* we did not observe a decrease in individual platelet adhesion, only microthrombus formation. Quite surprisingly, the former was increased in anti-GPIbα antibody pre-treated mice (though not to a significant level). Furthermore, SEM images also provided evidence that within the jejunum, microthrombi were present on collagen surfaces within the mucosal vasculature. This suggests that the microthrombi that were formed, were most likely due to platelet interactions with subendothelial collagen.

It is also possible that the lack of thrombus formation in the current study is not just due to inhibition of platelet tethering and capture, but also due to the inability of platelets to actually aggregate following introduction of an antibody to GPIbα. Indeed, recent evidence has supported the possibility that GPIbα can also support the platelet-platelet interactions required for thrombus formation**.** Bergmeier and colleagues studied thrombus formation in transgenic mice in which the extracellular domain of GPIbα was replaced by that of human IL-4 receptor (IL4Rα/GPIbα-tg mice). They demonstrated that platelet aggregration, as well as adhesion to ferric chloride-treated mesenteric arterioles, was completely inhibited in the IL4Rα/GPIbα-tg mice when compared to wild-type mice (Bergmeier *et al.*, 2006). Indeed, the authors stated that this defect was the strongest the group had observed in this injury model and even more severe than that of mice deficient in $\alpha_{IIb}\beta_3$ (GPIIb-IIIa), the major integrin mediating platelet aggregation. This finding was consistent with *in vitro* studies demonstrating that GPIb-vWF interactions also played a role in platelet thrombus formation ie. the adhesion of platelets not just to subendothelial collagen but also to already adherent platelets (Kulkarni *et al.*, 2000)

Although GPIbα had a beneficial effect during early reperfusion as evidenced by the lack of thrombus formation, decreased macromolecular leakage, improved histological and macroscopic appearance, these events did not persist throughout reperfusion and by 4 hours, tissue injury was again apparent. These results suggest inhibiting the mechanisms responsible for platelet-collagen (and possibly platelet-platelet) interactions are only

sufficient to prevent injury during early reperfusion. The adhesion of individual platelets was not affected and indeed increased in animals pre-treated with the anti-GPIbα antibody and these increased numbers of platelets may be responsible for recruitment of inflammatory leukocytes. This is evidenced by the observation that leukocyte adhesion and circulating numbers of platelet-leukocyte aggregates increased in GPIbα blocked mice undergoing IR injury. It is also possible, that since flow is initially restored, more circulating leukocytes and platelets gain entry to the jejunum and thus adhere within the microvessels.

Furthermore, we previously observed in Chapter 3 that platelets isolated from animals that had undergone intestinal IR injury were unable to adhere to collagen *in vitro* under flow conditions. In the current chapter we described a similar phenomenon on activated endothelial cells. Fascinatingly, platelets derived from IR animals pre-treated with an anti-GPIbα antibody were now able to adhere to collagen and to activated endothelium. This may also partly contribute to the enhanced adhesion of individual platelets observed throughout the 4 hours reperfusion. It is possible that IR injury leads to some phenotypic or functional damage to platelets which is why circulating ones derived from IR injured mice were unable to adhere to collagen *in vitro.* Several mechanisms may cause decreased platelet aggregation. Porte and colleagues reported that following liver transplantation, platelet activation occurred upon reperfusion, and these platelets lacked most of their secretory granules and microtubules (Porte *et al.*, 1994). Because platelets became non-functional after losing their granules, the finding of agranular platelets was compatible with decreased

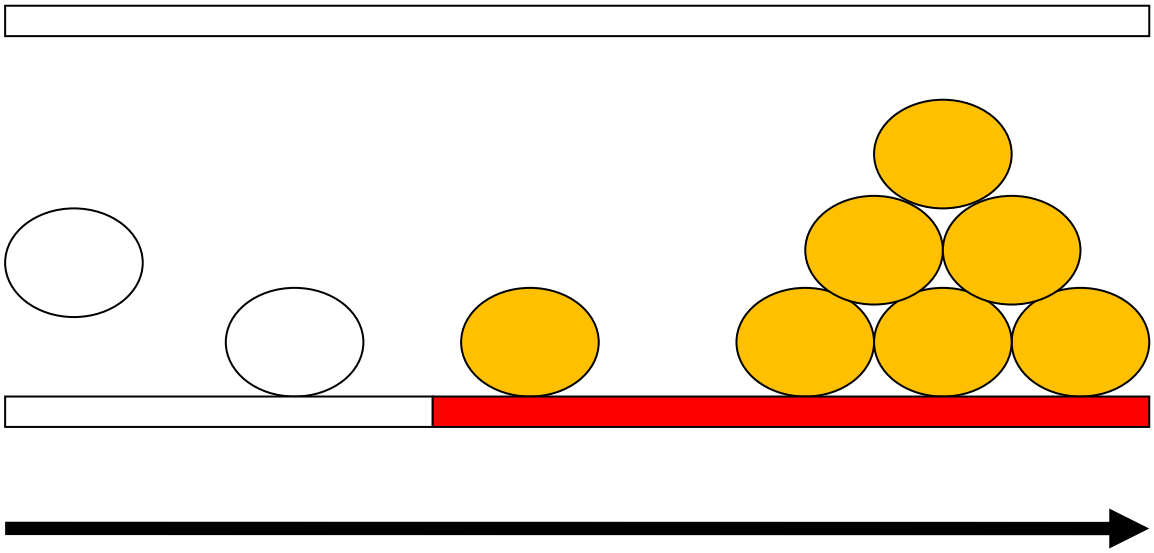
platelet aggregation. However, in the current studies where reduced IR damage was observed as a result of anti-GPIbα antibody pre-treatment, we suggest that less platelet activation occurs and this explains why these platelets retain their aggregatory ability. Indeed, Himmelreich and colleagues also demonstrated that intra-operative infusion of prostaglandin E1 not only attenuated liver IR injury but also reduced the associated impairment in platelet aggregation (Himmelreich *et al*., 1993).

Overall, these novel results demonstrate that platelets play a key role in induction of microcirculatory and tissue damage following intestinal IR injury. The inhibition of microthrombus formation decreased both microvasculature occlusion and vessel leakage during the early stages of reperfusion. These results do raise the possibility of using GPIb antagonists or antibodies therapeutically to limit the tissue or transplanted graft injury that takes place immediately following reperfusion. $\alpha_{IIb}\beta_3$ / GPIIbIIIa antagonists are currently used extensively in the clinic (their role in this injury model will be investigated later) (Scarborough *et al.*, 2008). Although effective pharmacologically at inhibiting platelet aggregation, they are not able to prevent platelet adhesion and activation because both of these processes occur earlier than aggregation. Since GPIbα receptors allow the initial capture of platelets from flow at sites of damaged vasculature, their blockade is a potential mechanism to block platelet adhesion and activation upstream of aggregation. Indeed, a monoclonal antibody directed against GPIbα has been used in baboons effectively to reduce injured femoral artery injury without increasing bleeding time (Fontayne *et al.,* 2008).

Unfortunately, in the current study, the initial benefit of blocking platelet recruitment through inhibiting GPIbα, decreases as reperfusion prolongs towards 4 hours. Indeed, macroscopic, histological and FITC-BSA leakage analysis showed no improvement at 4 hours reperfusion. It is possible that enhanced leukocyte and platelet recruitment in this scenario may play a vital role in contributing to this secondary injury. It therefore seems that a dual therapy that can prevent both microthrombus formation and prevent the recruitment of potentially detrimental individual platelet and leukocyte recruitment may confer more longer lasting protection within the gut following intestinal IR injury. This will be investigated in the next chapter.

Chapter 5

Effects of preventing platelet and leukocyte recruitment to intestinal IR injured microvasculature by dual blockade of GPIbα and P-selectin



5.0. Effects of preventing platelet and leukocyte recruitment to intestinal IR injured microvasculature by dual blockade of GPIbα and P-selectin

5.1. Introduction and hypotheses

5.1.1. Introduction

In Chapter 4 we demonstrated a beneficial effect following intestinal IR injury in anti-GPIbα antibody pre-treated mice and, quite surprisingly, no benefit in P-selectin$^{-/-}$ mice. However, inhibiting microthrombus formation alone was not sufficient to permit sustained blood flow within the most severely affected area of the small intestine, throughout the 4 hour reperfusion period. One of the most interesting and potentially important consequences of anti-GPIbα antibody pre-treatment was the significant increase in the number of leukocytes that were observed intravitally. It is possible microthrombi adhering to either endothelial cells or subendothelial matrix proteins occupy or cover receptors that would normally sustain the adhesion of leukocytes (or individual platelets). Therefore, in the absence of microthrombi, more surface area of endothelial receptors becomes available for circulating leukocytes. Similar observations were made by Vowinkel and colleagues who demonstrated that neutropaenia led to a remarkable increase in the number of platelets that could directly bind to venular endothelial cells in an experimental murine model of colitis (Vowinkel *et al.,* 2007). This study, and ours, suggests that inhibiting the adhesion of one cell type enhances the adhesion of the other.

We also observed a remarkable increase in the number of circulating platelet-leukocyte aggregates in anti-GPIbα antibody pre-treated mice. The presence of circulating platelet-leukocyte aggregates has been identified as a more sensitive marker of *in vivo* platelet activation than platelet surface P-selectin expression (Michelson *et al.*, 2001). These aggregates form when platelets are activated and undergo degranulation, after which they adhere to circulating leukocytes. Platelets and leukocytes do not normally interact with each other in the circulation. However, enhanced platelet-leukocyte interactions occur *in vivo* in inflammatory processes, such as unstable angina (Ott *et al.,* 1995) acute myocardial infarction, stroke (Zellar *et al.,* 2005), and diabetes (Jawahar *et al.,* 1992*)* as well as during vascular surgical procedures such as cardiopulmonary bypass (Faraday *et al.*, 2001) and aortic valve surgery (Leguyader *et al.*, 2006). Upon platelet activation**,** P-selectin is released and expressed on its surface. It is probable that platelet activation, within our intestinal IR injury model, is caused by the associated changes in homeostatic conditions within the vasculature. Decreases in nitric oxide and prostaglandin production, as described previously, are frequently associated with IR injury and allow for platelet activation within injured vessels. Binding of P-selectin to its ligand PSGL-1, constitutively expressed on all leukocytes, mediates the formation of platelet-leukocyte aggregates in the circulation and on damaged vascular surfaces (Yokoyama *et al.*, 2005). This association leads to increased expression of CD11b/CD18 (Mac-1) on leukocytes (Neumann *et al.,* 1999) which itself supports interactions with platelets, possibly through fibrinogen linking this integrin with platelet $\alpha_{IIb}\beta_3$ (GPIIb/IIIa; Gawaz *et al.,* 1991).

The formation of platelet-leukocyte aggregates plays an important role in the initiation of inflammation (and also thrombosis by causing microembolism in capillaries). Adhesion of platelets to leukocytes has been shown to regulate various leukocyte actions such as inducing monocyte cytokine (Neumann *et al.*, 1997) and chemokine synthesis (Weyrich *et al.*, 1996). In light of the data obtained from Chapter 4, it is therefore likely that leukocytes, potentially recruited by individually attached or activated platelets, could be mediating the 'secondary' vessel occlusion and the subsequent tissue damage observed in anti-GPIbα antibody pre-treated mice during late reperfusion. Since the main interaction binding platelets to leukocytes is P-selectin/PSGL-1 dependent, we undertook studies to identify the beneficial effects of combining anti-GPIbα antibody pre-treatment in P-selectin knockout mice.

5.1.2. Hypotheses

This chapter therefore aimed to investigate the effects of inhibiting GPIbα in P-selectin$^{-/-}$ mice on platelet, leukocyte and platelet-leukocyte interactions. We predominantly focused on the jejunum for this study, although some additional studies were also conducted in the ileum. For the work included in this chapter we hypothesized:

1. Recruitment of individual platelets, leukocytes, microthrombus formation and platelet-leukocyte interactions in the jejunum would be decreased in P-selectin$^{-/-}$ mice pre-treated with an anti-GPIbα antibody.
2. Reducing all these events would confer intravital, histologic and macroscopic benefit

even at 4 hours post-reperfusion, within the jejunum.

3. Any remote organ damage, particularly to the liver and lungs, associated with intestinal IR injury would be inhibited in P-selectin$^{-/-}$ mice pre-treated with an anti-GPIbα antibody.

5.2. Methods

Donor platelets, from P-selectin$^{-/-}$ mice, and endogenous platelets were both blocked with the anti-GPIbα antibody introduced into P-selectin$^{-/-}$ mice. Fluorescent IVM was performed as described previously following 45 minutes ischaemia followed by 4 hours of reperfusion. The carotid artery and trachea were cannulated and the animal subjected to either sham or IR treatment. During reperfusion, the jejunum and ileum was monitored intravitally. Free flowing platelets, individual adherent platelets and microthrombi were quantitated. The systemic administration of FITC-BSA and Acridine orange allowed vascular macromolecular leakage and leukocyte adhesion to be quantitated. Histological haematoxylin and eosin (H&E) staining was used to visualise mucosal villi. *In vitro* adhesion assays were conducted to determine the role of GPIb and P-selectin in allowing adhesion of platelets to collagen under flow conditions and endothelial cells under static conditions. Flow cytometry was performed to demonstrate the presence of circulating platelet/leukocyte aggregates.

5.3. Results

5.3.1. Leukocyte adhesion in the jejunum and circulating leukocyte-platelet aggregates significantly decrease following IR injury in P-selectin$^{-/-}$ mice pre-treated with an anti-GPIbα mAb at 4 hours post-reperfusion

Leukocyte recruitment in the jejunum was significantly ($p<0.05$) reduced in IR injured P-selectin$^{-/-}$ + anti-GPIbα antibody mice when compared to IR injured wild-type + anti-GPIbα mAb mice at 4 hours post-reperfusion (**Figures 5.1a and 5.2**). However, it was interesting to observe that the adhesion of individual platelets within the jejunum was not reduced although there was a slight trend for this number to fall (**Figure 5.1b**). Furthermore, blood sustained flow was apparent within the jejunum in IR injured P-selectin$^{-/-}$ + anti-GPIbα mAb mice as demonstrated by a significant ($p<0.05$) increase in the number of freely flowing donor platelets when compared to IR injured wild-type + anti-GPIbα antibody mice (**Figure 5.1c**). This dual blockade was the only combination that led to the observation of free flowing platelets at 4 hours post-reperfusion.

We further investigated, by FACS analysis, the interaction of circulating platelets with leukocytes. Platelet-leukocyte interactions were significantly ($p<0.05$) reduced in IR injured P-selectin$^{-/-}$ + anti-GPIbα mAb mice when compared to IR injured wild-type + anti-GPIbα mAb mice (**Figure 5.3**).

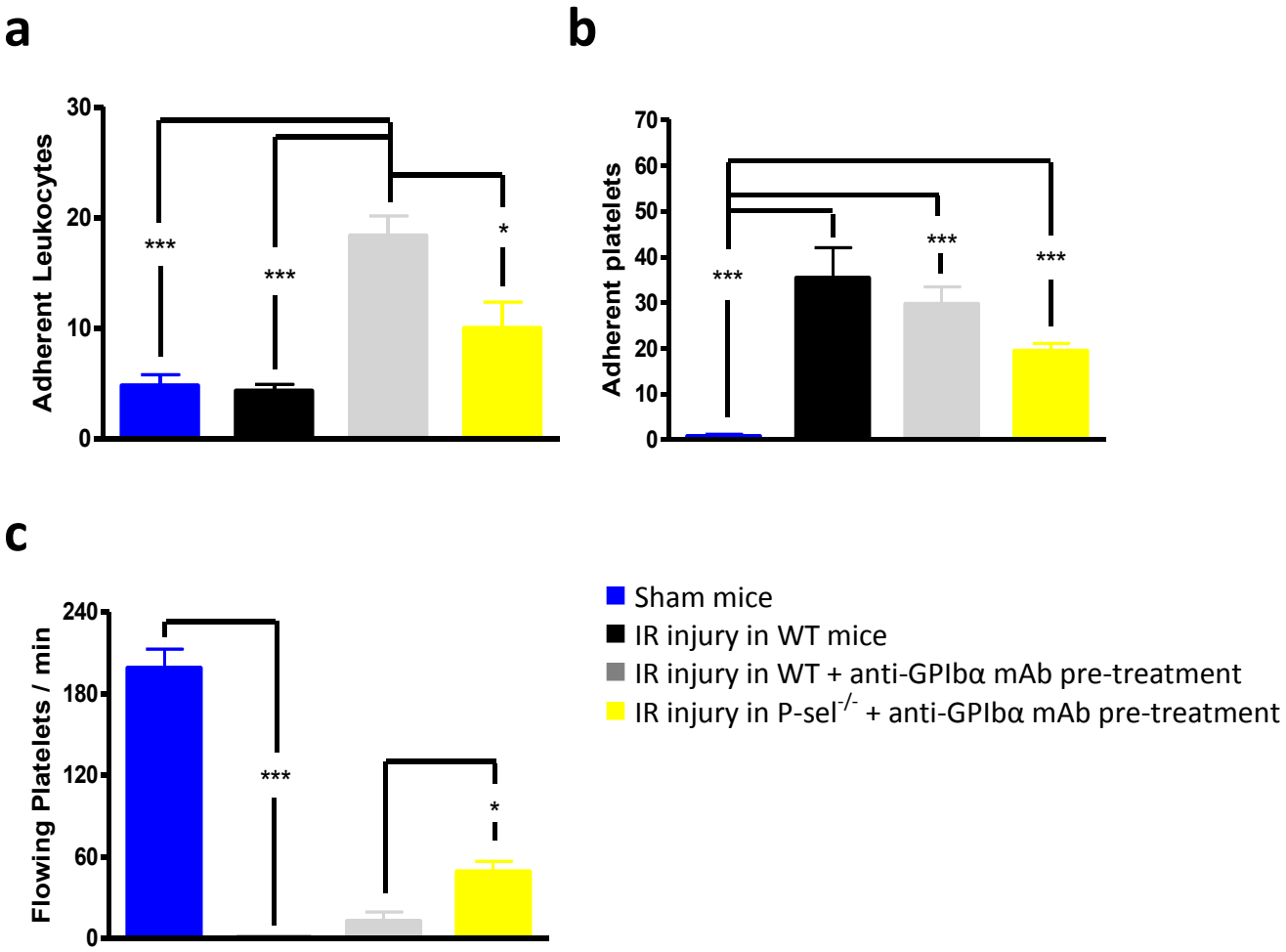


Figure 5.1. Leukocyte adhesion significantly decreased in the jejunum of IR injured P-selectin$^{-/-}$ mice pre-treated with an anti-GPIbα antibody at 4 hours reperfusion. Recruitment of leukocytes and platelets to the jejunal microcirculation were examined intravitally. **(a)** Leukocyte recruitment was significantly reduced in IR injured P-selectin$^{-/-}$ + anti-GPIbα antibody mice when compared to IR injured wild-type + anti-GPIbα antibody mice. **(b)** Individual platelet recruitment is also reduced under these conditions but not to a significant level. **(c)** Furthermore, a significant increase in the number of freely flowing platelets (1 x 10^8 platelets, 10μM CFSE) was observed in IR injured P-selectin$^{-/-}$ + anti-GPIbα antibody mice compared to IR injured wild-type + anti-GPIbα antibody mice. Data represents mean + SEM of at least 5 separate experiments. *p<0.05, *** p<0.001 when compared using a one way ANOVA followed by Bonferroni post test for points of significance. *Only yellow bars represent new data – blue, black and grey bars are presented in Chapter 4.*

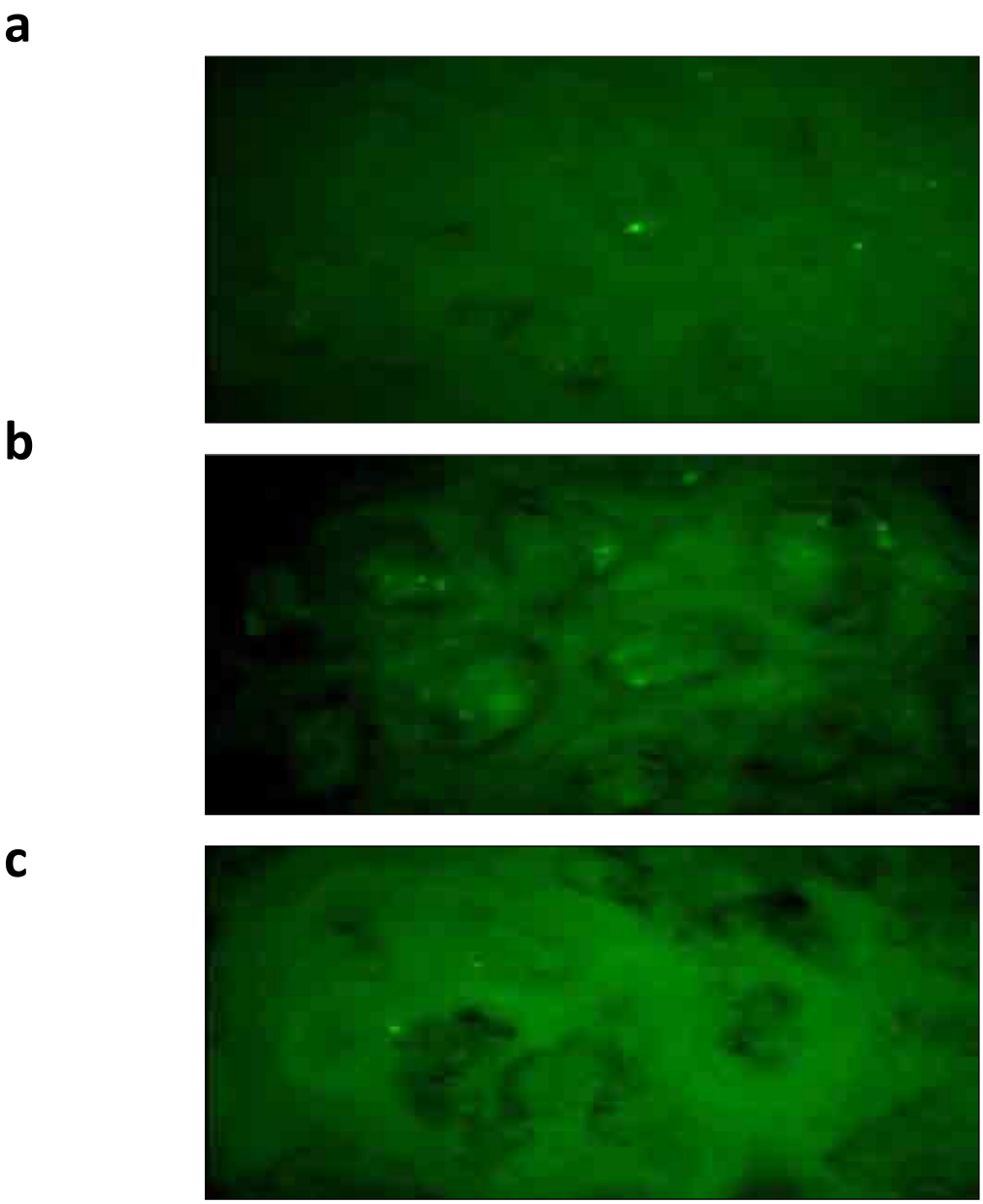
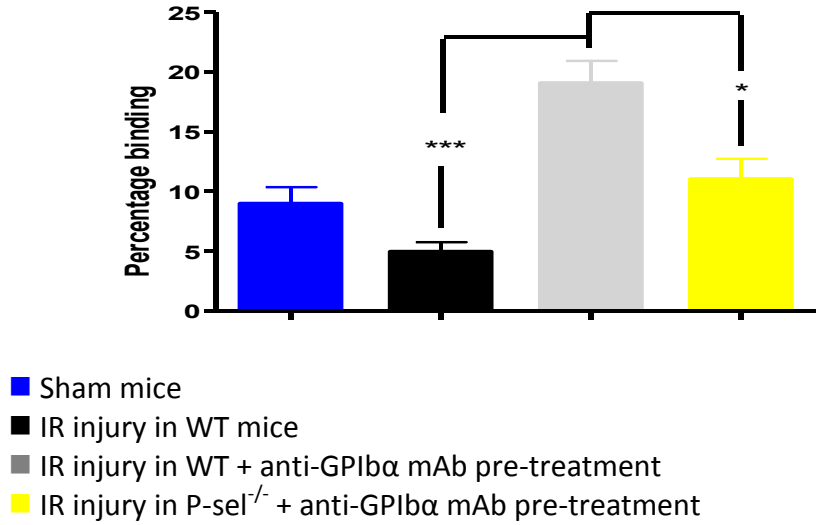


Figure 5.2. Leukocyte adhesion significantly decreased in the jejunum of IR injured P-selectin$^{-/-}$ mice pre-treated with an anti-GPIbα mAb at 4 hours reperfusion. Individual platelet recruitment is also reduced under these conditions but not to a significant level. Representative images of leukocyte adhesion in **(a)** sham wild-type **(b)** IR injured wild-type and **(c)** IR injured P-selectin$^{-/-}$ + anti-GPIbα mAb mice are presented.

a



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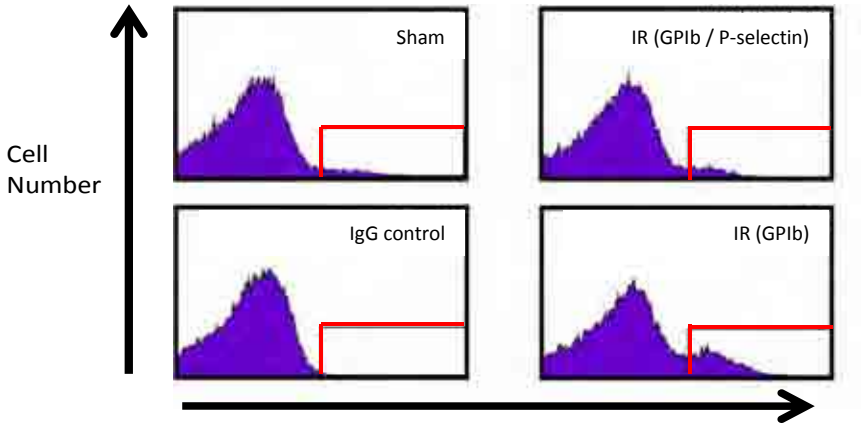


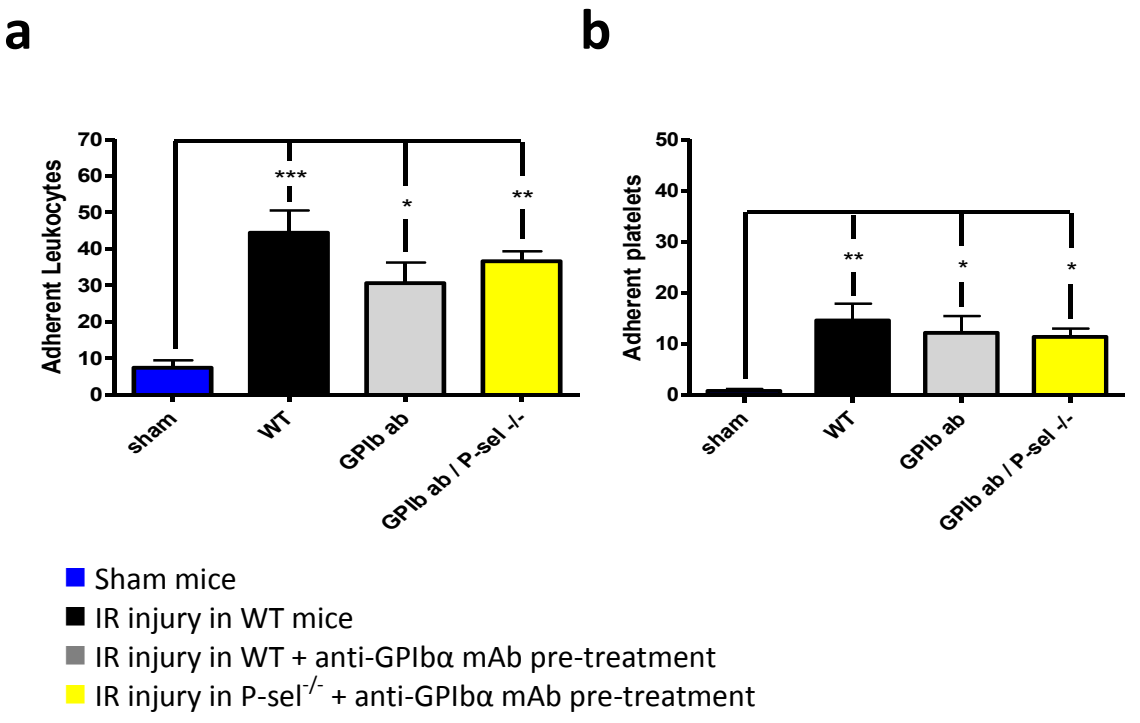
Figure 5.3. Circulating leukocyte-platelet interactions significantly decreased in IR injured P-selectin$^{-/-}$ mice pre-treated with an anti-GPIbα mAb at 4 hours reperfusion. FACS analysis was performed on 50ul blood samples, taken from IR injured mice at 4 hours reperfusion. Platelet-leukocyte interactions were significantly reduced in IR injured P-selectin$^{-/-}$ + anti-GPIbα mAb mice when compared to IR injured wild-type + anti-GPIbα antibody mice (**5.3a**). Data represent mean + SEM of at least 5 separate experiments. Representative FACS plots are also presented with regions outlined in red indicating platelet-leukocyte aggregates (**5.3b**). *p<0.05, ***p<0.001 when compared using a one way ANOVA followed by Bonferroni post test for points of significance. *Only yellow bars represent new data – blue, black and grey bars are presented in Chapter 4.*

5.3.2. Leukocyte and individual platelet adhesion did not decrease in the ileum of IR injured P-selectin$^{-/-}$ mice pre-treated with an anti-GPIbα mAb at 4 hours post-reperfusion

It was interesting to observe that although leukocyte and platelet adhesion decreased in the jejunum in P-selectin$^{-/-}$ + anti-GPIbα mAb mice, similar events were not observed within the terminal ileum when compared to IR injured wild-type + anti-GPIbα mAb mice (**Figure 5.4**).

5.3.3. Marked macroscopic, microscopic and histological improvement as well as a decrease in macromolecular leakage was observed in the jejunum of IR injured P-selectin$^{-/-}$ mice pre-treated with an anti-GPIbα mAb at 4 hours post-reperfusion

The proximal jejunum appeared healthy when observed grossly throughout the 4 hour duration of reperfusion (**Figure 5.5**). H&E staining revealed a distinct improvement to villus length and the apparent lack of an inflammatory response around the crypt areas (**Figure 5.6**). Furthermore, a significant ($p<0.001$) decrease in vascular leakage of FITC-BSA was observed in IR injured P-selectin$^{-/-}$ + anti-GPIbα mAb mice compared to IR injured wild-type + anti-GPIbα mAb mice at 4 hours post-reperfusion in the jejunum (**Figure 5.7**). These results suggest that dual receptor blockade is required to significantly decrease microvasculature injury caused by platelet and inflammatory cell recruitment during intestinal IR injury.



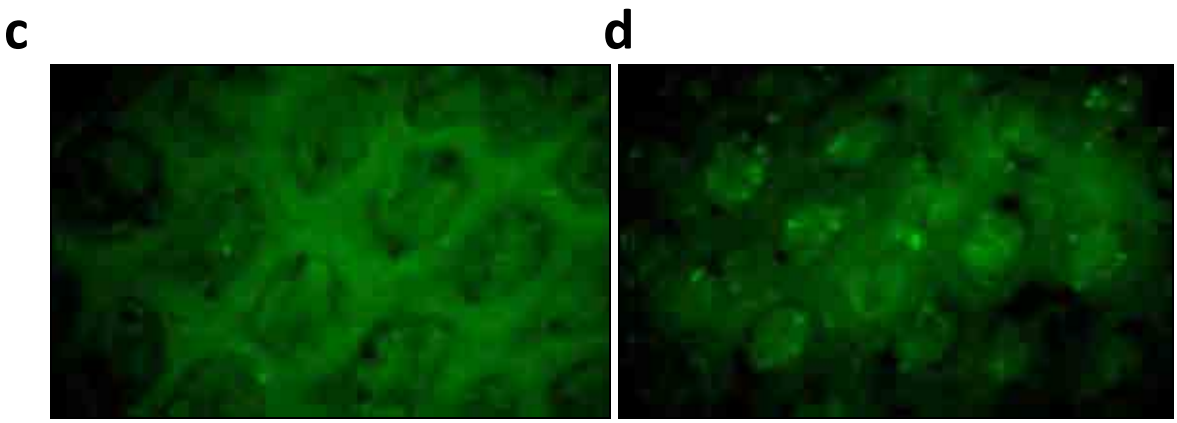
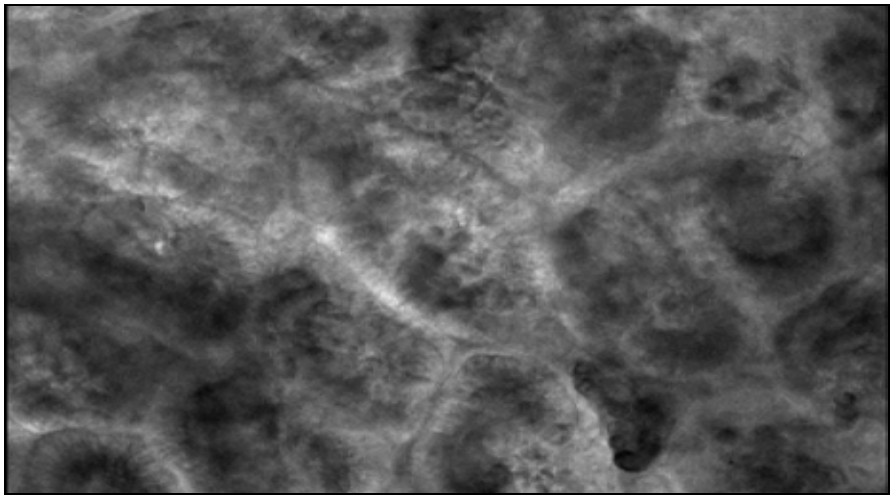


Figure 5.4. Leukocyte and individual platelet adhesion did not decrease in the ileum of IR injured P-selectin$^{-/-}$ mice pre-treated with an anti-GPIbα mAb at 4 hours post-reperfusion. Recruitment of leukocytes and platelets to the ileal microcirculation was examined intravitally. **(a)** Leukocyte recruitment was not significantly reduced in IR injured P-selectin$^{-/-}$ + anti-GPIbα mAb mice. **(b)** Individual platelet recruitment was also not reduced under these conditions. Representative images of ileal leukocyte adhesion from **(c)** sham wild type **(d)** IR injured wild type. Data represents mean + SEM of at least 5 separate experiments. $p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ when compared using a one way ANOVA followed by Bonferroni post test for points of significance. *Only the yellow bars represent new data – blue, black and grey bars are presented in Chapter 4.*

a



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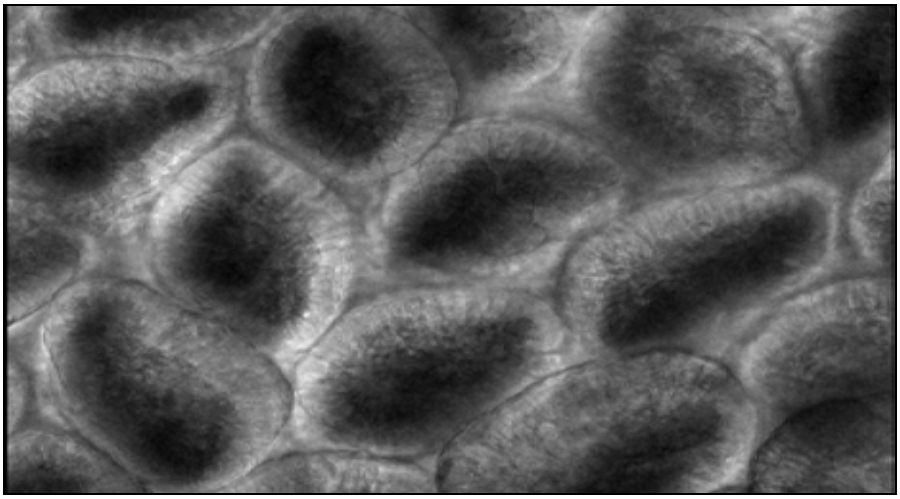


Figure 5.5. Marked microscopic improvement of the <u>jejunum</u> was observed in IR injured P-selectin$^{-/-}$ + anti-GPIbα mice compared to wild-type + anti-GPIbα mAb mice at 4 hours post-reperfusion in the jejunum when viewed using brightfield intravital imaging. (a) The proximal jejunal mucosa appeared haemorrhagic, severely congested and swollen after 4 hours reperfusion in IR injured wild-type + anti-GPIbα mAb mice**. (b)** The jejunum remained healthy in IR injured P-selectin$^{-/-}$ + anti-GPIbα mAb mice throughout the 4 hour reperfusion duration. Representative images are shown of the jejunum at 4 hours post-reperfusion.

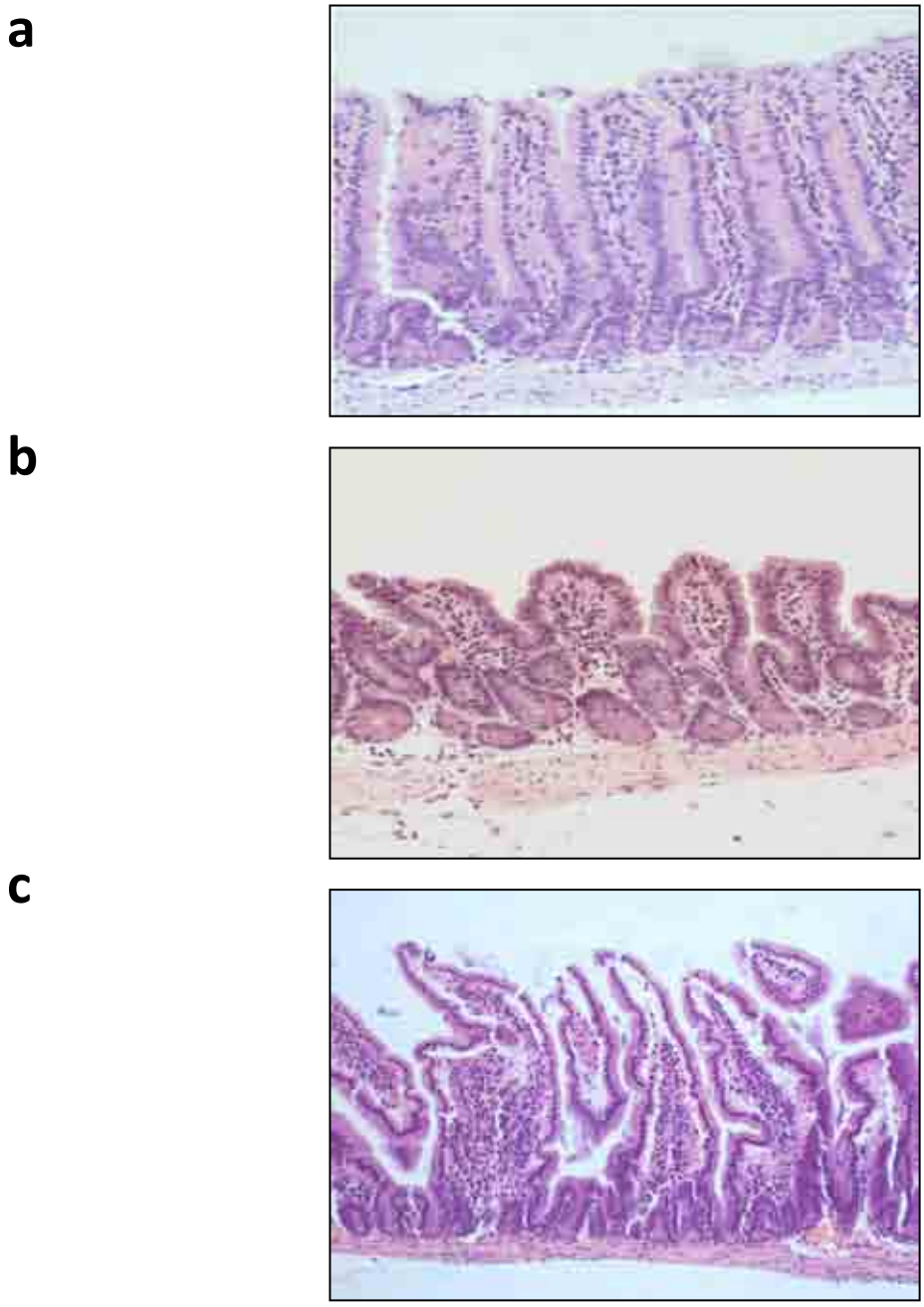


Figure 5.6. Histological improvement of the jejunal villi in IR injured P-selectin$^{-/-}$ mice pre-treated with an anti-GPIbα mAb was observed at 4 hours reperfusion (magnification x200). **(a)** No sloughing and healthy villi structures were observed in sham models. **(b)** Severe mucosal sloughing and inflamed crypt areas were seen in IR injured mice. **(c)** An increase in villous length and very little inflammation to the lower mucosa was observed in P-selectin$^{-/-}$ mice + anti-GPIbα mAb.

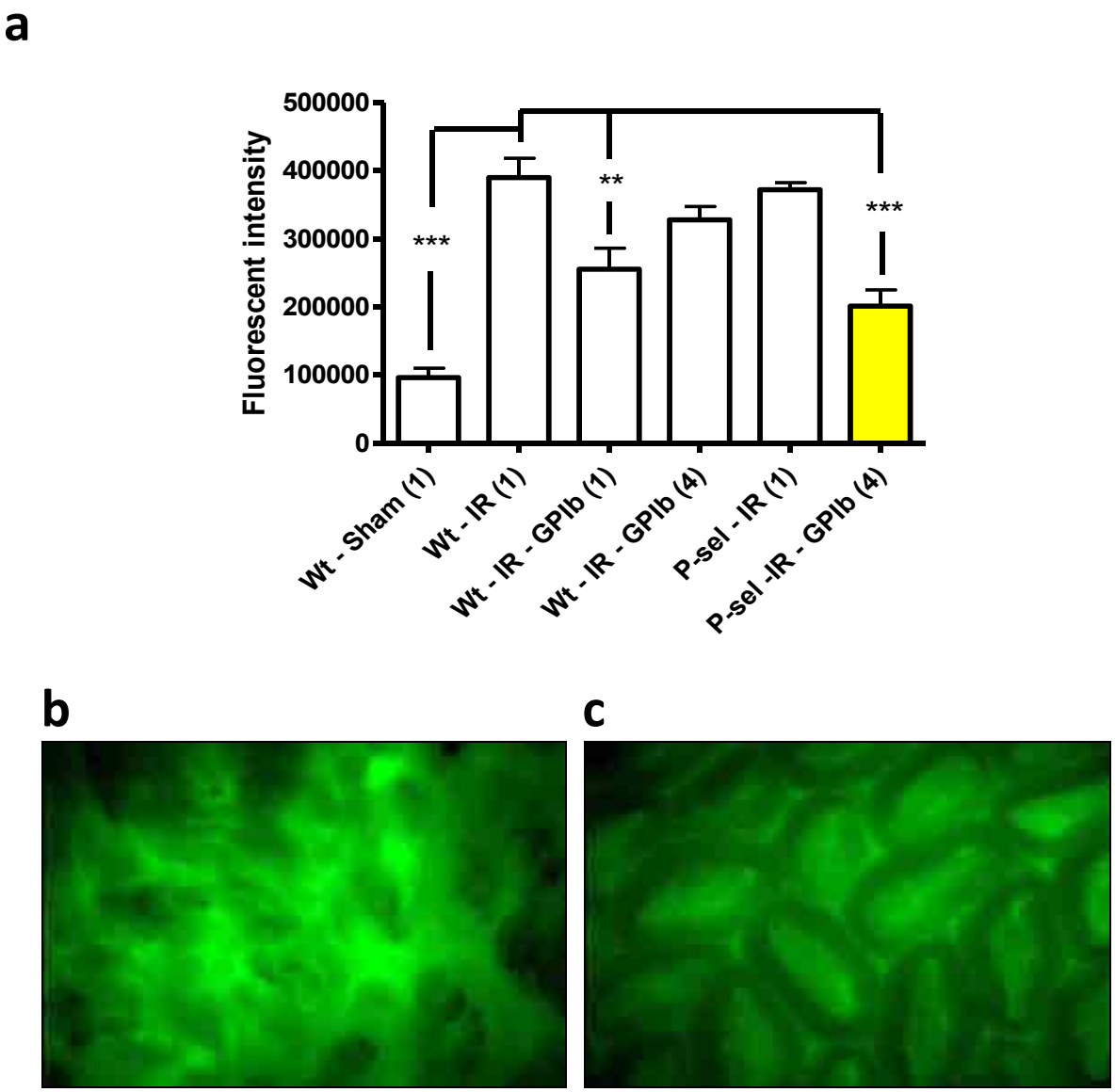


Figure 5.7. Vascular leakage significantly decreased <u>jejunally</u> in IR injured P-selectin$^{-/-}$ + anti-GPIbα mAb mice when compared to wild-type + anti-GPIbα antibody mice at 4 hours post-reperfusion. Macromolecular leakage was analysed by systemically administering FITC-BSA, at 4 hours reperfusion, and observing increased fluorescence intravitally. **(a)** A significant decrease in fluorescent intensity was observed in P-selectin$^{-/-}$ + anti-GPIbα mAb mice compared to IR injured mice. Representative jejunal images are shown of vascular leakage from IR injured **(b)** wild-type mice and **(c)** P-selectin$^{-/-}$ + anti-GPIbα mAb mice at 4 hours post-reperfusion. Data is representative of mean + SEM of at least 5 separate experiments. **p<0.01, ***p<0.001 when compared using a one way ANOVA followed by Bonferroni post test for points of significance. *<u>Only the yellow bar represents new data – other bars are presented in Chapter 4.</u>*

5.3.4. Platelets derived from IR injured mice do not adhere to collagen under flow conditions but platelets derived from P-selectin$^{-/-}$ + anti-GPIbα mAb mice do

Platelets from IR injured P-selectin$^{-/-}$ + GPIbα mAb mice demonstrated a significant ($p<0.05$) increase in their ability to adhere to collagen under flow conditions when compared to IR injured wild-type platelets (**Figure 5.8**). However, the extent of the adhesion was not different to that observed with IR injured + GPIbα mAb mice.

5.3.5. Reduced remote organ injury to the liver and lungs was observed in IR injured P-selectin$^{-/-}$ + anti-GPIbα mAb mice at 4 hours post-reperfusion

Since this dual therapy markedly improved the gut, the effects on remote organ injury was also assessed. Histological analysis of lung tissue from animals undergoing intestinal IR injury revealed collapsed alveoli, thickened interstitial walls and a dense neutrophilic infiltrate **(Figure 5.9).** Capillaries were often congested with red blood cells and a marked neutrophilic infiltrate was apparent. Occasionally, neutrophils and red blood cells were observed within the alveolar spaces, indicating haemorrhage. However, relatively small areas of cell infiltrate were present in aerated and distended lungs of IR injured P-selectin$^{-/-}$ + anti-GPIbα mAb mice which appeared similar to control lungs (**Figure 5.10**). Hepatic histology revealed very little in the way of tissue damage in animals undergoing intestinal IR injury. However, biochemical analysis of plasma alanine transferase (ALT) levels demonstrated a significant increase in ALT levels at 4 hours reperfusion. This was significantly ($p<0.05$) reduced in IR injured P-selectin$^{-/-}$ + anti-GPIbα mAb mice and similar to levels observed in sham mice (**Figure 5.10**).

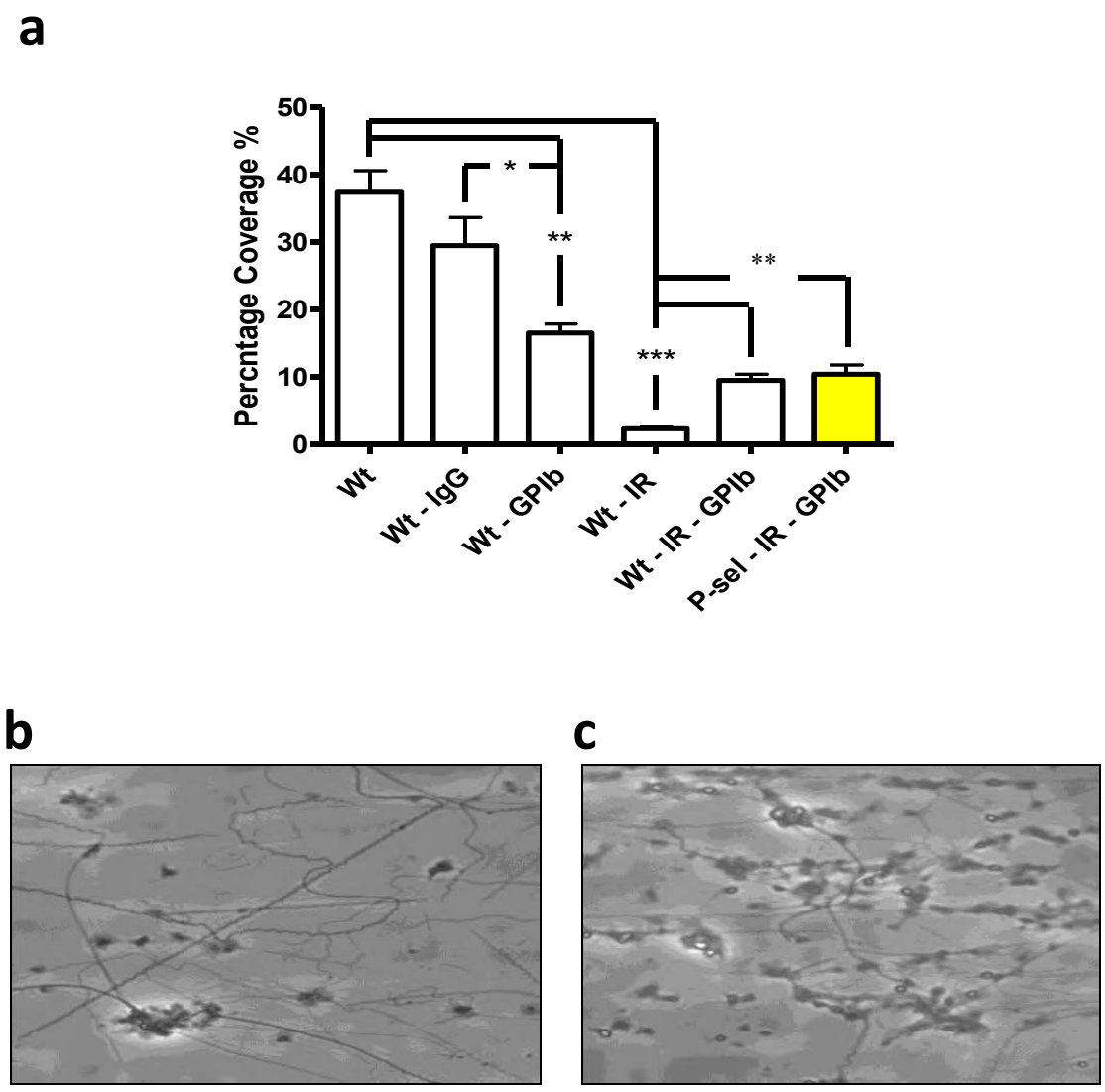


Figure 5.8. Platelets derived from IR injured mice do not adhere to collagen under flow conditions *in vitro* but platelets derived from P-selectin$^{-/-}$ mice pre-treated with an anti-GPIbα mAb do adhere. At 2 hours reperfusion, 500ul of mouse blood anticoagulated with P-Pack and heparin was perfused through collagen coated microslides at a shear rate of 300s-1. After washing for 3 mins, 5 random images were then taken using an Olympus digital camera. Although not much of an increase was observed when IR injured P-selectin$^{-/-}$ + anti-GPIbα mAb mice were compared to IR injured mice + anti-GPIbα mAb mice, a significant increase was observed when compared to IR injured mice. Representative images of adhesion to collagen of platelets derived from **(b)** IR injured wild-type and **(c)** IR injured + GPIbα mAb mice. Data represents mean + SEM of at least 3 separate experiments. *p<0.05, **p<0.01 when compared using an unpaired student t test for points of significance. *Only the yellow bar represents new data – other bars are presented in Chapter 4.*

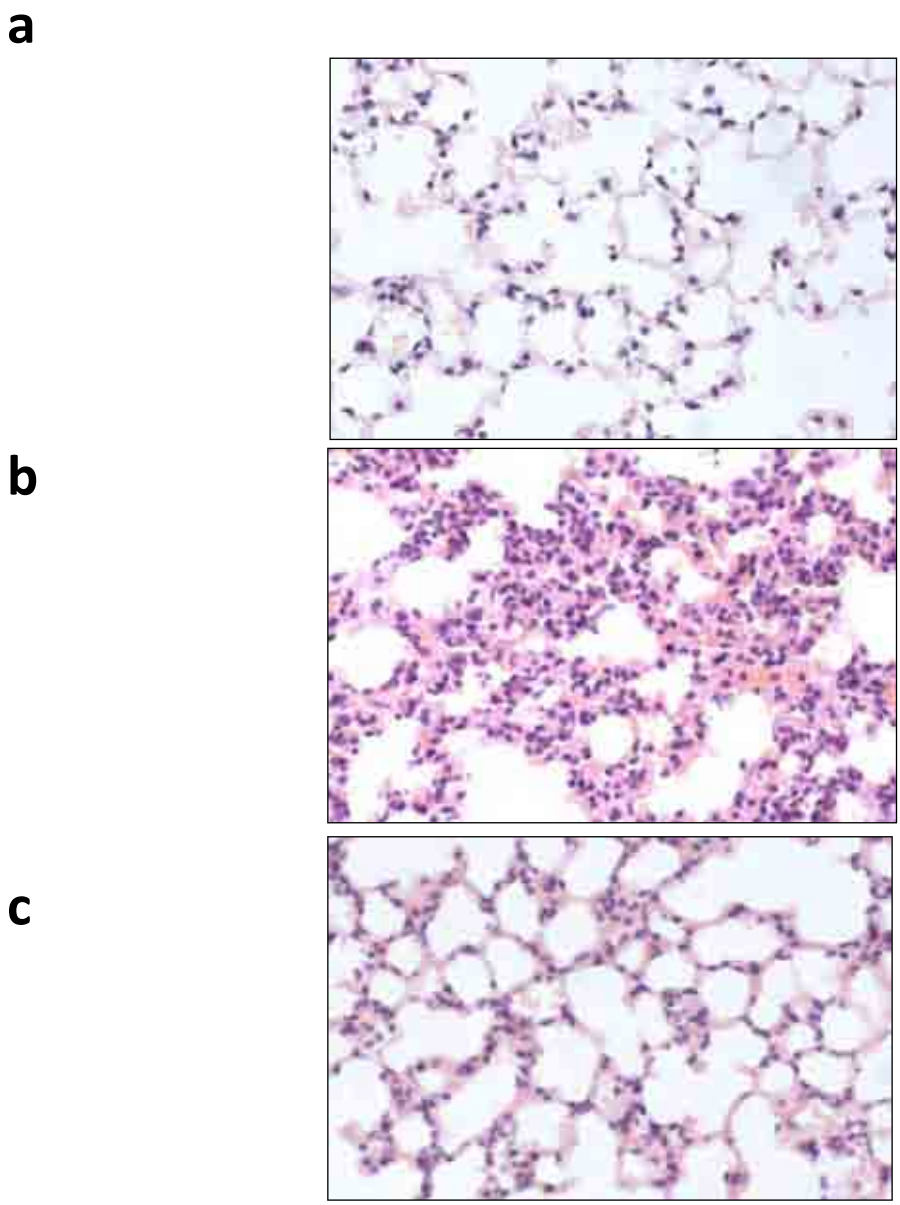


Figure 5.9. Histological improvement to lung tissue in IR injured P-selectin$^{-/-}$ mice pre-treated with an anti-GPIbα mAb (magnification x200). At 4 hours reperfusion, the lungs were removed for histological analysis and H&E stained. **(a)** Healthy structures were observed in sham mice. **(b)** Collapsed alveoli, thickened interstitial walls and a dense neutrophilic infiltrate were observed in intestinal IR injured mice animals. **(c)** However, relatively normal histology, with numerous aerated alveoli and thin alveolar walls, was observed in IR injured P-selectin$^{-/-}$ + anti-GPIbα mAb mice.

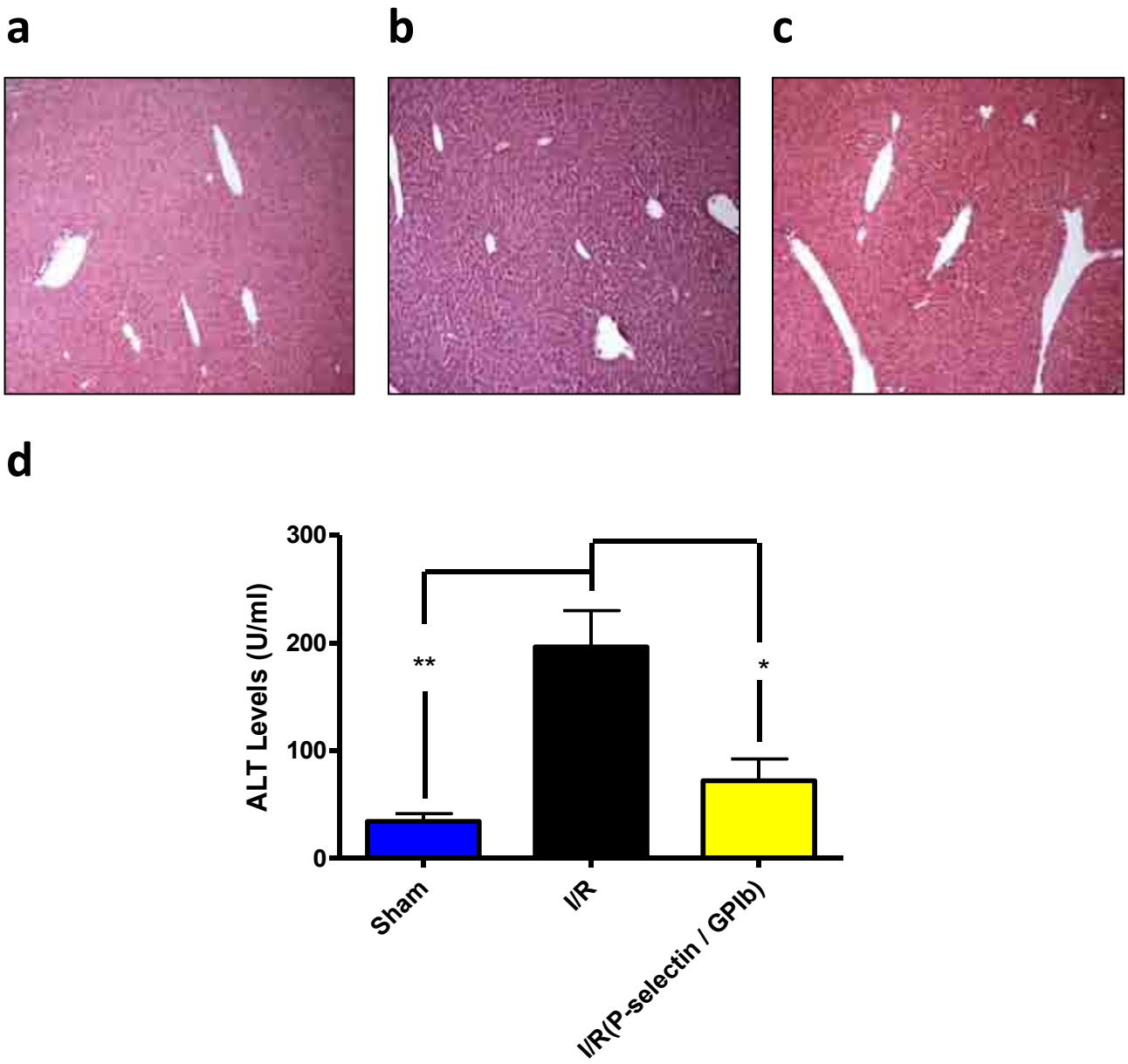


Figure 5.10. Although the livers of mice undergoing intestinal IR injury did not demonstrate histological injury, plasma levels of ALT suggested damage occurred at the cellular level. This was significantly decreased in IR injured P-selectin$^{-/-}$ mice pre-treated with an anti-GPIbα antibody. Healthy hepatic tissue was observed in **(a)** sham mice **(b)** IR injured mice and **(c)** IR injured P-selectin$^{-/-}$ + anti-GPIbα antibody mice. After 4 hours reperfusion 100ul of blood was removed from each mouse and placed in a heparin coated haematological screening tube. Samples were then sent to the haematology department at the Queen Elizabeth Hospital, Birmingham, for enzyme analysis **(d)**. Data represents mean + SEM of at least 3 separate experiments. *p<0.05, **p<0.01 when compared using an unpaired student test for points of significance.

5.4. Discussion

As demonstrated in Chapter 4, the use of anti-GPIbα mAb therapy was associated with an improvement in tissue and microcirculatory viability during early reperfusion. However, this was not long lasting and could possibly be linked to the concomitant significant increase in leukocyte adhesion and circulating platelet-leukocyte interactions and the trend for individual platelet adhesion to increase. In the current chapter we provide novel data demonstrating that inhibition of both leukocyte adhesion and thrombosis formation is essential in order to preserve tissue and microcirculatory integrity for longer periods of time. This was achieved by a 'dual therapy' relying on the absence of P-selectin and GPIbα. We did not conduct detailed studies to determine whether platelet and/or endothelial P-selectin was important and this would be worthy of future investigation. Furthermore, remote as well as local tissue integrity was preserved. Previous pharmacological studies have demonstrated that it is difficult to completely preserve microvessel integrity following intestinal IR injury, as evidenced by persistence of macromolecular leakage of labelled albumin (Kalia *et al.,* 2005). However, by inhibiting platelet and leukocyte recruitment, it was also possible to improve this microcirculatory disturbance.

We initially anticipated that the individual platelet adhesion observed in the ileum and jejunum of IR mice would be decreased in the P-selectin knockout mice. This is because platelet P-selectin binds via its high-affinity counter-receptor PSGL-1 on endothelium and mediates platelet-endothelial interactions during thrombo-inflammatory reactions.

However, as demonstrated in Chapter 4, no effect on individual platelet adhesion was observed in the P-selectin deficient mice. Nor were these interactions decreased in the dual therapy mice. However, we did observe a significant decrease in leukocyte adhesion and circulating leukocyte-platelet interactions in the latter mice. This suggests that although individual platelet adhesion is not P-selectin dependent, leukocyte adhesion to platelets or endothelium is. Hence, leukocytes must be interacting via PSGL-1 expressed on their surface with endothelial or platelet P-selectin. Interestingly, the antagonism of P-selectin has previously been observed to accelerate thrombolysis and enhance the anti-aggregatory effects of $\alpha_{IIb}\beta_3$ (GPIIb/III)a inhibitors (Theoret *et al.,* 2006). Theoret and colleagues demonstrated that P-selectin antagonism using rPSGL-Ig delayed the aggregation process, which was more efficiently inhibited with dual antagonism of P-selectin and $\alpha_{IIb}\beta_3$. However, no previous studies have demonstrated that in combination with anti-GPIbα therapy, microthrombus formation, leukocyte adhesion and platelet-leukocyte interactions can be significantly inhibited.

It was interesting to note that although leukocyte adhesion (and to a lesser extent individual platelet adhesion), decreased within the jejunum in dual therapy mice, this response was not mirrored to the ileum. This suggests that different molecular adhesive mechanisms operate to recruit circulating cells within the *severely* injured jejunum compared to the *mildly* injured ileum, with neither leukocyte or individual platelet recruitment to ileal endothelium being GPIbα or P-selectin dependent. Leukocyte recruitment can be bought about by a number of

alternative and well characterised mechanisms. Activated endothelium, leading to leukocyte recruitment can be initiated by several stimuli including cytokines such as TNFα. Once endothelium is activated, leukocytes are able to firstly roll on this surface through selectin mediated processes including E and P-selectin. Subsequent leukocyte activation occurs via two distinct mechanisms. The first mechanism involves β1 and β2 activation, initiated by cytokine release, which allows for these integrins to bind with their associated endothelial ligands ICAM-1/2 and VCAM-1. The second involves the selectins, associated with the initial leukocyte rolling, which independently activate the β2 integrins. Firm adhesion and transmigration of leukocytes through the activated endothelium completes the recruitment process. (Ravi *et al.*, 2007). It is possible that these mechanisms may be involved in mediating leukocyte recruitment in the ileum in the current study.

Bombeli and colleagues conducted one of the earliest studies investigating the various mechanisms by which platelets could adhere to intact endothelium (HUVECs) *in vitro*. They demonstrated no role for endothelial VCAM-1, E-selectin, or P-selectin. However, a significant decrease in platelet binding was observed when HUVECs were treated with monoclonal antibodies to ICAM-1, $\alpha_v\beta_3$ integrin, as well as GPIbα, or when platelets were treated with an anti-$\alpha_{IIb}\beta_3$ antibody. (Bombeli *et al.,* 1998). The greatest inhibition (50%) was found when HUVECs were treated with the mAb 2D5, which blocks the fibrinogen-binding site of ICAM-1. It was noteworthy that in their study they also demonstrated that platelet adhesion was not affected by blockade of platelet P-selectin. It is therefore possible that

one of these alternative mechanisms may also contribute to the recruitment of platelets to the activated endothelium of ileal and jejunal villi.

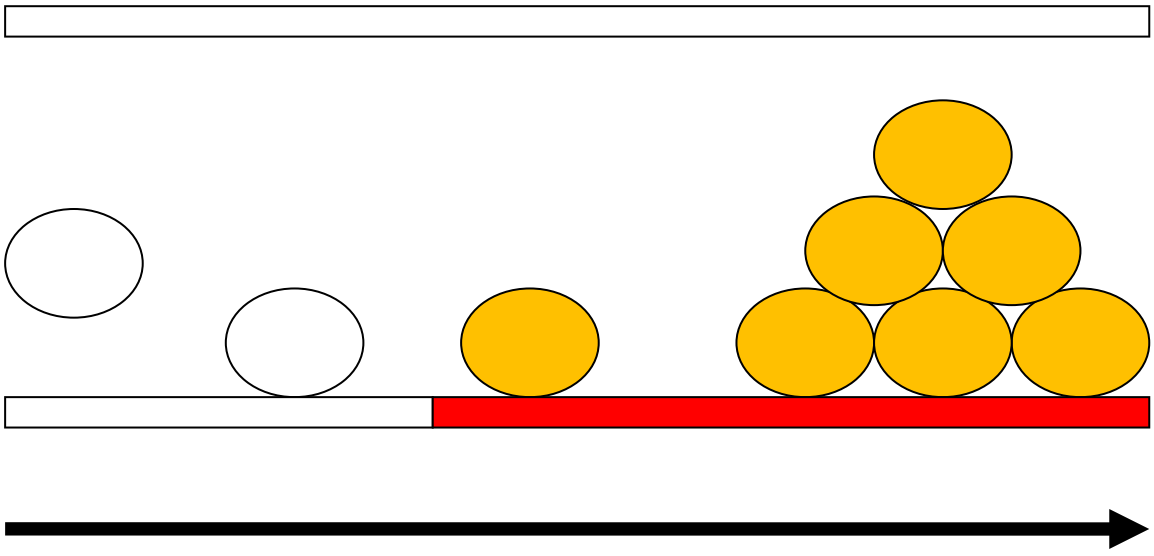
The benefits of the dual therapy used in the current chapter were not just confined to the intestine. Indeed, the lungs and liver also demonstrated a structural or biochemical improvement. Damage to remote extra-digestive sites is a common sequel of intestinal IR injury. Intestinal IR injury is associated with high levels of cytokines, activated complement fragments (Gibbs *et al*, 1996) eicosanoids, neutrophils (*Simpson et al*, 1993) and endotoxin within the systemic circulation (Grootjans *et al*, 2010). The liver is the first port of call for blood from the small intestine, which carries with it all the soluble inflammatory mediators released by the IR injured gut. Hence neutrophil sequestration, reduced bile (Nakamura *et al.*, 2001) and hepatic blood flow rates and impaired hepatocyte metabolism are often observed in such livers (Savas *et al.*, 2003). Furthermore, cardiac contractile dysfunction and acute lung injury characterized by neutrophil sequestration, microvascular dysfunction, and ultrastructural evidence of endothelial cell injury has been reported previously (Cui *et al.,* 2009)**.** However, our histological studies demonstrated an apparent decrease in pulmonary damage in IR injured mice undergoing dual therapy. Although in our studies, the liver showed very little histological damage in IR untreated mice, in comparison to sham samples, we still demonstrated that injury was present at the cellular level and that this could also be reduced in dual therapy mice.

In summary, this chapter highlights the importance of targeting both the platelet and the

leukocyte in order to attain longer lasting benefits from intestinal IR injury. The importance of targeting both of these cell types was also demonstrated by Ishikawa and colleagues in a murine model of stroke induced by middle cerebral artery occlusion followed by reperfusion (Ishikawa *et al.*, 2004). Previous studies had demonstrated that ICAM-1-bound fibrinogen on endothelial cells recruited platelets via $\alpha_{IIb}\beta_3$ and endothelial P-selectin recruited leukocytes via ICAM-1. Hence Ishikawa and colleagues hypothesised that $\alpha_{IIb}\beta_3$ blockade would be effective in reducing the IR-induced recruitment of platelets and the blockade of either endothelial or platelet-associated P-selectin would blunt both leukocyte and platelet recruitment responses to IR injury. Indeed, this is what they observed. Although the latter observations differ slightly from our own, certainly this study highlights the importance of blocking the interactions of both of these cell types for effective treatment of ischaemic disorders. Although this chapter has identified an effective strategy to treat intestinal IR injury, we further compared the benefits observed with targeting additional platelet receptors. This will be discussed in Chapter 6.

Chapter 6

Investigating the roles of GPVI, $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$ and PLCγ2 in mediating platelet recruitment to intestinal IR injured microvasculature



6.0. Investigating the roles of GPVI, $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$ and PLCγ2 in mediating platelet recruitment to intestinal IR injured microvasculature

6.1. Introduction and hypotheses

6.1.1. Introduction

This chapter will look at the possibilities of further inhibiting platelet recruitment to IR injured vessel walls through alternative mechanisms dependent upon the inhibition of GPVI, $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$. It is possible that a single therapy targeting one platelet receptor may be as effective as the dual therapy described in Chapter 5. Amongst the numerous collagen receptors expressed in platelets, GPVI is of central importance for platelet activation and subsequent firm arrest. Its inhibition in several *in vivo* experimental models of thrombosis, involving physical and chemical vessel damage, has proved to be of great benefit. Furthermore, a critical role has been identified for GPVI in experimental models of myocardial infarction (Kleinschnitz *et al*., 2007). Takaya and colleagues demonstrated that the myocardial infarct size was significantly smaller in FcRγ$^{-/-}$ mice (therefore GPVI deficient) subjected to occlusion and reperfusion of the coronary artery than in control FcRγ$^{+/+}$ mice (Takaya *et al.,* 2005).

The same beneficial effects have been observed for the highly expressed receptor $\alpha_{IIb}\beta_3$ (GPIIb/IIIa). Binding of platelets to soluble fibrinogen is the terminal step in platelet

aggregation, but this integrin also plays a key role in platelet adherence to both collagen and endothelium as demonstrated *in vitro* using flow assays (Auger *et al*., 2005; Tull *et al*., 2006). Adhesion to endothelium can occur via interactions with $\alpha_v\beta_5$ and also ICAM-1. Therefore, $\alpha_{IIb}\beta_3$ presents a highly likely candidate for investigation following intestinal IR injury where both collagen is exposed and endothelium is activated in the jejunum or ileum. However, post-vessel injury bleeding has been a constant problem associated with targeting this receptor. Nevertheless, it has been observed to be beneficial in murine models of cerebral ischemia when blockers are used at a low dose that does not produce clinically significant haemorrhage (Abumiya *et al*., 2000).

Inhibiting the integrin $\alpha_2\beta_1$ (GPIa/IIa) may also show positive effects in the IR injured gut as it has a critical role in maintaining thrombus stabilisation. It is the major collagen receptor on platelets that mediates stable adhesion on collagen following platelet activation. However, previous *in vitro* studies have shown great variability in the effectiveness of blocking this integrin and *in vivo* studies investigating the role of this integrin in thrombosis have been limited. He and colleagues were the first to generate a knockout mouse in which the expression of the $\alpha_2\beta_1$ integrin was eliminated, and demonstrated that a delay in thrombus formation was observed following carotid artery injury *in vivo* (He *et al.*, 2003).

In addition to surface proteins, this chapter will also investigate the role of intracellular PLCγ2 following intestinal IR injury. PLCγ2-dependent signalling plays an essential part in platelet

activation and could represent an attractive target for treatment of thrombotic diseases. It lies downstream of adhesion receptors such as integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$, GPIb-V-IX and GPVI, with impaired activation observed to vWF and collagen in PLCγ2 deficient platelets. Currently, no *in vivo* studies have investigated the role of PLCγ2 in a pathological injury such as intestinal IR injury. However, an interesting *in vitro* study by Leoncini and colleagues showed platelets could be activated by oxygen free radicals and also homocysteine, an amino acid found in blood, with PLCγ2 phosphorylation playing a key role in this process (Leoncini *et al.,* 2007). Since free radicals and homocysteine increase following intestinal IR injury, PLCγ2 may play a critical role in activating platelets in this situation (Gradman *et al.,* 2001). A study by Nonne *et al* showed that PLCγ2 played a key role in thrombus formation in mild laser-induced lesions within mesenteric vessels, but interestingly, this specific phospholipase played no significant role as the severity of injury increased (Nonne *et al.,* 2005).

6.1.2. Hypothesis.

The roles of these four platelet surface and intracellular proteins have not previously been investigated in intestinal disease or even in many other models of ischemic disorders. Since GPVI and $\alpha_2\beta_1$ have been associated with platelet recruitment to collagen, their role was investigated within the jejunum where collagen exposure and thrombus formation was observed. The roles of $\alpha_{IIb}\beta_3$ and PLCγ2 were investigated within the jejunal and ileal mucosa. For the work included in this chapter we hypothesized:

1. Microthrombus formation and individual platelet recruitment in the jejunum would be decreased in FcR γ-chain$^{-/-}$ (non-functioning GPVI) and $\alpha_{IIb}\beta_3^{-/-}$ mice and in mice administered an anti-$\alpha_2\beta_1$ antibody.
2. Individual platelet recruitment in the jejunum would be decreased when using PLCγ2$^{-/-}$ donor platelets.
3. Individual platelet recruitment in the ileum would be decreased when using $\alpha_{IIb}^{-/-}$ or PLCγ2$^{-/-}$ donor platelets.

6.2 Methods

Initially, FACS analysis was performed to allow us to demonstrate that the function blocking antibody (SAM.G4 – Emfret) concentration was sufficient to cause blockade of over 90% of the α_2 subunit (CD49d/GPIa) of the α2β1 integrin. Donor and endogenous platelets were blocked separately to increase confidence of inhibition. Receptor inhibition was not required for $\alpha_{IIb}\beta_3$ investigations as α_{IIb} deficient mice were used. To determine the role of GPVI, FcR γ-chain$^{-/-}$ mice were used. The FcR γ-chain is the critical signaling element of the GPVI-FcR γ-chain complex and is also essential for GPVI expression on the platelet surface. Therefore lack of it essentially creates a non-functioning GPVI mouse. To investigate the role of PLCγ2, donor platelets were derived from PLCγ2$^{-/-}$ mice and administered into a wild-type mouse, which allowed individual platelets to be quantitated. Unfortunately, we did not have sufficient knockout mice to monitor the role of PLCγ2 in the development of microthrombi,

so all endogenous platelets in mice undergoing IR injury were still PLCγ2$^{+/+}$ (i.e. wild-type mice platelets). Since previous studies (Nonne *et al.*, 2005) had demonstrated that a role for PLCγ2 was dependent upon the severity of injury, we investigated its role in the ileum and the jejunum where varying degrees of injury severity were observed. There was no difference in circulating platelet counts in the knockouts that were used when compared to controls (data not presented). Some experiments were conducted on thrombocytopenic mice which were obtained by injecting 2ug/g body of IgG platelet depletion antibody (Emfret, R300) through the carotid artery cannula. After one hour, full blood counts were performed on 120ul of blood and IR injury was induced thereafter. Fluorescent IVM was performed on male 20-25g wild-type C57Bl/6, knockout or thrombocytopenic mice. All intestinal IR intravital investigations within this chapter used periods of 45 minutes ischaemia followed by 4 hours of reperfusion. The carotid artery and trachea were cannulated and the animal was subjected to either sham or IR treatment. During reperfusion, the ileum and jejunum was monitored intravitally. Free flowing platelets, individual adherent platelets and microthrombi were quantitated.

6.3. Results

6.3.1 Anti-α_2 antibody does not affect systemic platelet numbers

Optimum antibody concentrations for complete receptor blockade were determined after injection of C57Bl/6 mice with varying amounts of SAM.G4. The antibody was allowed to circulate in the vasculature for 30 mins, after administration through the cannulated carotid artery. 50µl of blood were then incubated with 5ul of FITC conjugated SAM.G4 (1:10 dilution) and flow cytometry was used to detect the minimal amount of the necessary to achieve saturating α_2 binding *in vivo.* Our receptor binding studies demonstrated that a concentration of 100ug / 200µl was more than sufficient to achieve receptor occupancy of approximately 95%. Also, platelet counts in SAM.G4 antibody-treated mice were not statistically different from the rat IgG control (Table 6.1). However, using the anti-GPIb antibody caused a significant depletion in platelet number and thus generated the thrombocytopenic mice (**Table 6.1**).

	Wt (no ab)	Rat IgG	$\alpha_2\beta_1$ IgG	IgG (Depletion)
Receptor Occupancy %	0	0	>95	n/a
Platelet Count ($x10^9$)/L	1254 ± 123	1318 ± 45	1039 ± 140	2.9* ± 3.1

Table 6.1. The anti-α_2 antibody achieves receptor occupancy of approximately 95% and does not affect circulating platelet number. The anti-GPIb antibody depletes circulating platelet numbers and thus generated the thrombocytopenic mice. Values are mean ± SD with *p<0.05 when compared using an unpaired Student t test.

6.3.2. Microthrombus formation and individual platelet adhesion was not significantly decreased in the jejunum of IR injured FcR γ-chain$^{-/-}$ and anti-$\alpha_2\beta_1$ antibody pre-treated mice.

The formation of microthrombi or the adhesion of individual platelets in FcR γ-chain$^{-/-}$ animals or in wild-type animals receiving the anti-$\alpha_2\beta_1$ mAb was not significantly reduced when compared to un-treated animals undergoing intestinal IR injury (**Figure 6.1a and 6.1b**). Furthermore, free flowing platelet numbers did not increase either indicating that blood flow was not apparent in the jejunal microcirculation of these mice (**Figure 6.1c**).

6.3.3. Microthrombus formation and individual platelet adhesion was significantly decreased in the jejunum of IR injured $\alpha_{IIb}^{-/-}$ mice.

A significant decrease in microthrombus formation ($p<0.001$) and individual platelet adhesion ($p<0.01$) was observed within the jejunal mucosa of IR injured $\alpha_{IIb}^{-/-}$ mice when compared to un-treated sham animals undergoing intestinal IR injury (**Figures 6.2a-b**). The antibody used to label endogenous platelets was usually a CD41 (GPIIb) derivative. To observe the effect of this integrins inhibition this was obviously an unsuitable option. Platelets were instead labelled with the Alexa dye and a CD42b (GPIb) which showed no difference in intensity measuresurements. Furthermore, free flowing platelets were present within the observed field of view, indicating blood flow was also maintained within the jejunum of these mice. However, an increased bleeding from the mucosal surface (see later) was also observed in these mice and this made it technically difficult to analyse this event quantitatively.

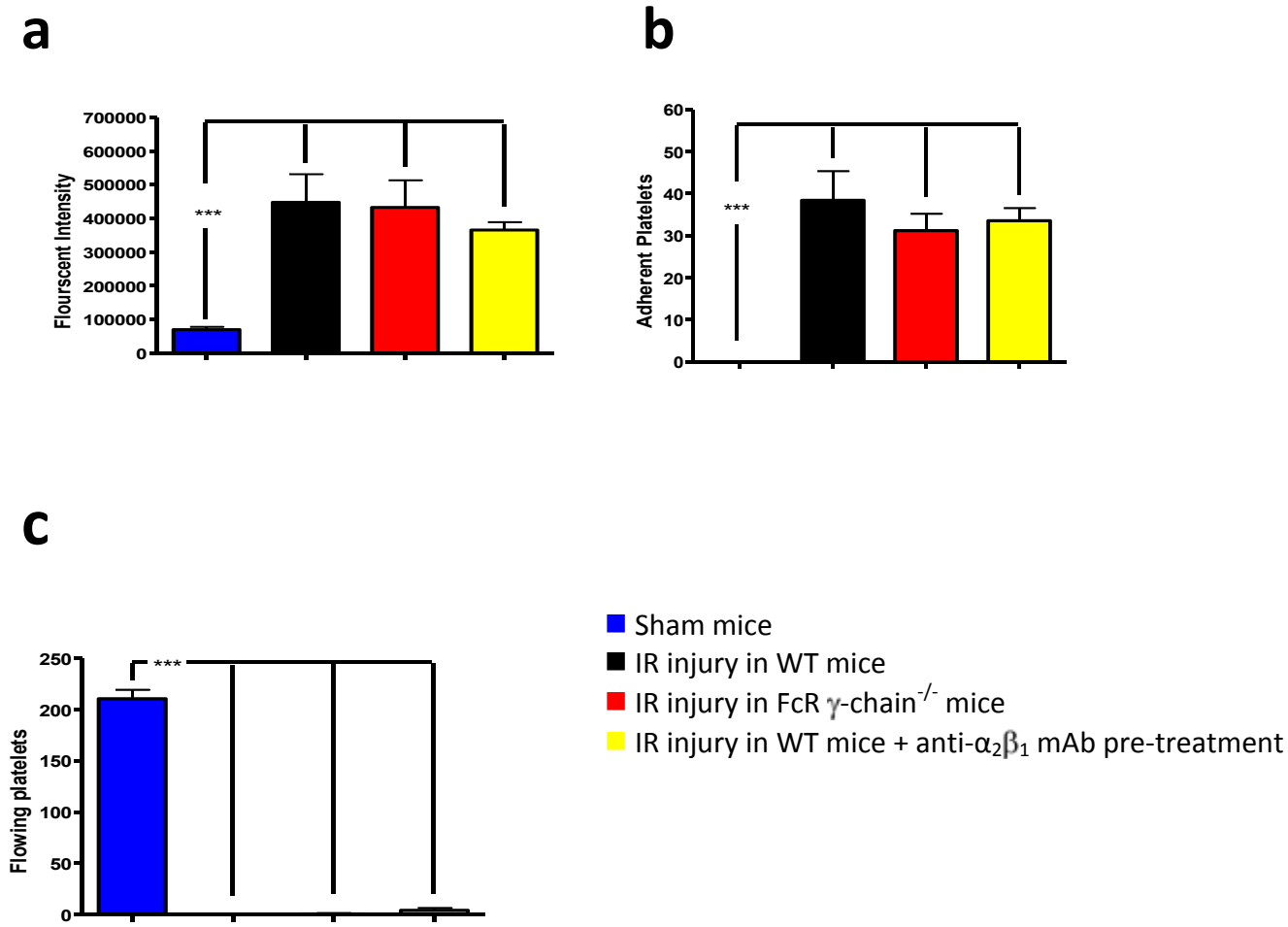


Figure 6.1. Microthrombus formation and individual platelet adhesion was not significantly reduced in the <u>jejunum</u> of IR injured FcR γ-chain$^{-/-}$ or anti-$\alpha_2\beta_1$ mAb pre-treated mice. Recruitment of endogenous (20 µl GPIIb ab + 40 µl Alexa 594) and donor (1 x 10^8 platelets, 10µM CFSE) platelets to the microcirculation of the jejunal mucosal villi in wild-type, $\alpha_2\beta_1$ blocked and FcR γ-chain$^{-/-}$ mice was examined using intravital microscopy. Wild-type, FcR γ-chain$^{-/-}$ or anti-$\alpha_2\beta_1$ mAb pre-treated donor platelets were introduced prior to IR injury. **(a)** Microthrombus formation and **(b)** individual adherent platelets were not significantly reduced in any of the above knockout or antibody treated mice. **(c)** Numbers of free flowing platelets did not increase indicating there was no improvement on flow either. All data are representative of mean + SEM of at least 5 separate experiments. ***p<0.001 when compared using a one way ANOVA followed by Bonferroni Post Test for points of significance.

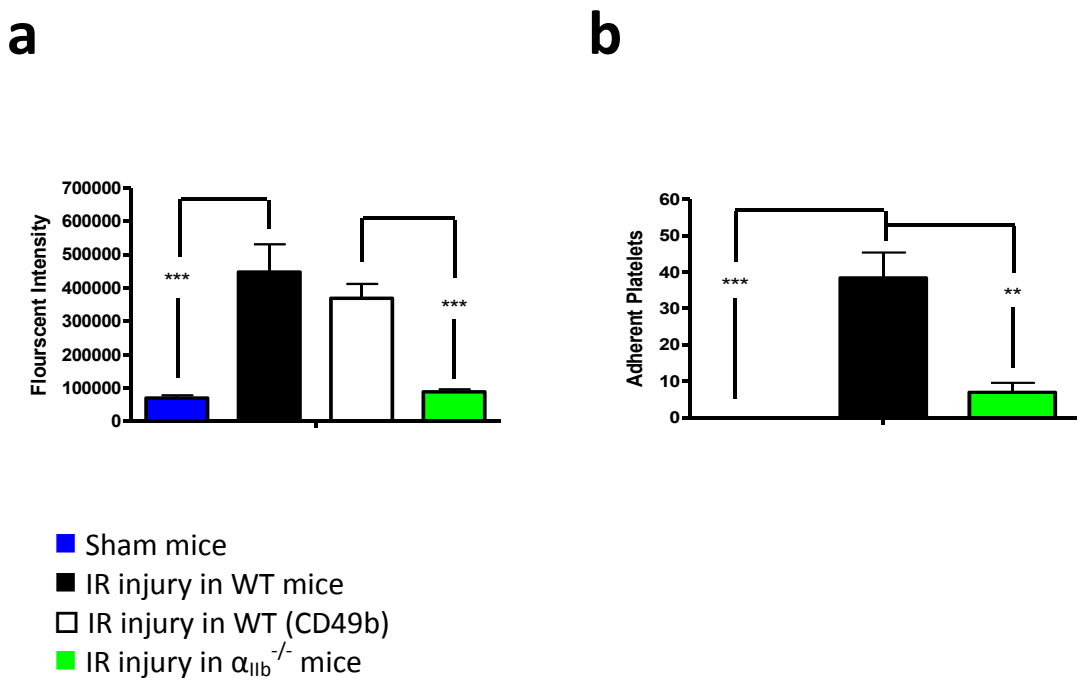


Figure 6.2. Microthrombus formation and individual platelet adhesion was significantly decreased in the <u>jejunum</u> of IR injured $\alpha_{IIb}^{-/-}$ mice. Recruitment of endogenous (20 µl GPIIb ab + 40 µl Alexa 594) and donor (1 x 10^8 platelets, 10µM CFSE) platelets to the microcirculation of the jejunal mucosal villi in wild-type or $\alpha_{IIb}^{-/-}$ mice was examined using intravital microscopy. Wild-type or $\alpha_{IIb}^{-/-}$ donor platelets were introduced prior to IR injury. **(a)** Microthrombus formation and **(b)** numbers of individual adherent platelets was significantly reduced in the $\alpha_{IIb}^{-/-}$ mice. All data are representative of mean + SEM of at least 5 separate experiments. **p<0.01, ***p<0.001 when compared using a one way ANOVA followed by Bonferroni Post Test for points of significance.

6.3.4. Individual platelet adhesion was also significantly decreased in ileum of IR injured $\alpha_{IIb}^{-/-}$ mice.

Since the severity of injury varied between the jejunum and ileum, the effects of the absence of $\alpha_{IIb}\beta_3$ on individual platelet adhesion within the ileum was also determined. Individual platelet adhesion was significantly ($p<0.05$) decreased in ileal villi in $\alpha_{IIb}^{-/-}$ mice when compared to wild-type animals undergoing intestinal IR injury (**Figure 6.3a**). Furthermore, freely flowing platelets could also be observed at 30 minutes post-reperfusion, indicating that blood flow was still apparent, although this was not significantly increased (**Figure 6.3b**).

6.3.5. No histological improvement in FcR γ-chain$^{-/-}$, anti-$\alpha_2\beta_1$ antibody pre-treated or $\alpha_{IIb}^{-/-}$ mice after IR injury.

No improvement in histological appearance was observed in the FcR γ-chain$^{-/-}$ or anti-$\alpha_2\beta_1$ antibody pre-treated mice after IR injury when compared to wild-type mice undergoing IR injury. Despite a reduction in microthrombus formation and individual platelet adhesion in the $\alpha_{IIb}^{-/-}$ mice, interestingly, no histological improvement was observed in these mice (**Figure 6.4**). In fact, it did seem as if the injury was worse in these mice after IR injury with villous structures often missing from the jejunal mucosa. Furthermore, increased bleeding from the mucosal surface was also quite visible which led to mice undergoing distress as bleed loss increased (**Figure 6.4**). IR injured thrombocytopenic mice also demonstrated similar histological and macroscopic disturbances as those observed in the $\alpha_{IIb}^{-/-}$ mice.

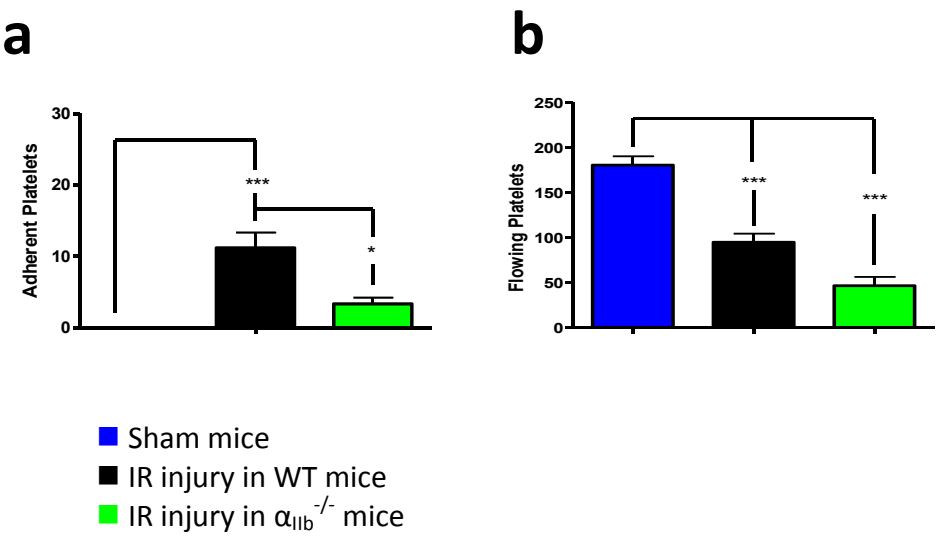


Figure 6.3. Individual platelet adhesion was also significantly decreased in the ileum of IR injured $\alpha_{IIb}^{-/-}$ mice. Recruitment of donor (1 x 10^8 platelets, 10μM CFSE) platelets to the microcirculation of the ileal mucosal villi in wild-type or $\alpha_{IIb}^{-/-}$ mice was examined using intravital microscopy. Wild-type or $\alpha_{IIb}^{-/-}$ donor platelets were introduced prior to IR injury. **(a)** Numbers of individual adherent platelets was significantly reduced in the $\alpha_{IIb}^{-/-}$ mice. **(b)** There was still sufficiently high enough numbers of freely flowing platelets observed in this region suggesting blood flow was still maintained. All data are representative of mean + SEM of at least 5 separate experiments. *p<0.05, **p<0.01, ***p<0.001 when compared using a one way ANOVA followed by Bonferroni Post Test for points of significance.

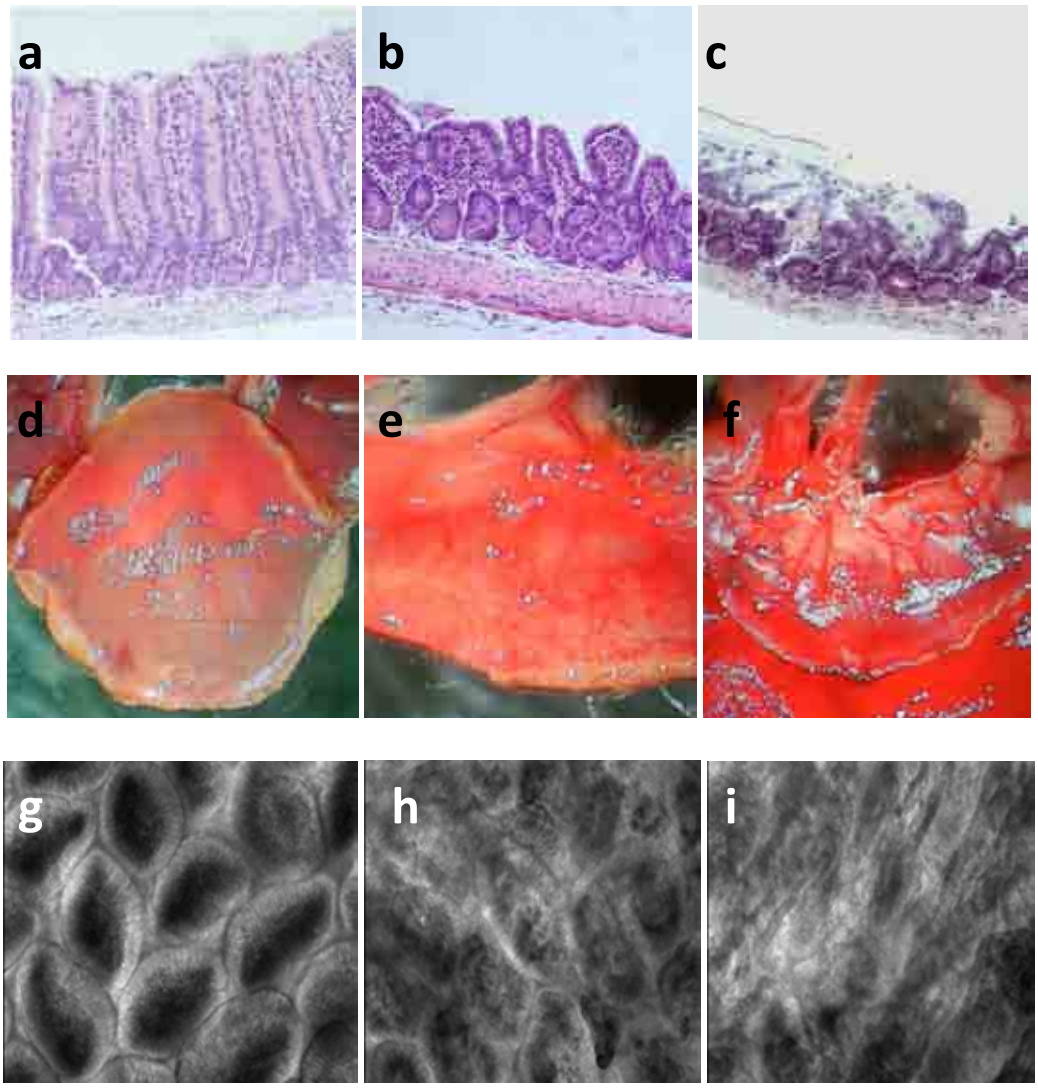


Figure 6.4. No histological improvement in FcR γ-chain$^{-/-}$, anti-$\alpha_2\beta_1$ antibody pre-treated or $\alpha_{IIb}^{-/-}$ mice was observed after intestinal IR injury (magnification x200). (a/d/g) Wild-type shams showed healthy jejunal mucosa both histologically, grossly and when viewed intravitally. **(b/e/h)** IR injured mice and **(c/f/i)** $\alpha_{IIb}^{-/-}$ (and also thrombocytopenic) mice demonstrated severe mucosal sloughing and inflamed crypt areas at 4 hours post-reperfusion histologically. Indeed, within the $\alpha_{IIb}^{-/-}$ mice, villous structures were often missing from the mucosa of the jejunum. Excessive bleeding was also witnessed from the mucosal surface in $\alpha_{IIb}^{-/-}$ (and thrombocytopenic) mice.

6.3.6. Individual PLCγ2$^{-/-}$ donor platelet adhesion was significantly decreased in the ileum of IR injured wild-type mice. No difference was observed in the jejunum.

In these experiments, only the donor platelets were PLCγ2$^{-/-}$. We did not have sufficient knockout mice for the recipient IR injured mice to also be PLCγ2 deficient. Therefore, the small intestine in these animals still manifested the detrimental microcirculatory effects associated with ischemia and subsequent reperfusion. However, by using donor platelets derived from PLCγ2$^{-/-}$ mice, we were able to demonstrate a significant ($p<0.01$) reduction in the adhesion of individual platelets in the ileum when compared to wild-type platelets being monitored in an IR injured wild-type mouse (**Figure 6.5a**). Since microthrombus development still occurred in the jejunum, it was no surprise that individual platelet adhesion of PLCγ2$^{-/-}$ donor platelets was unaffected in this region (**Figure 6.5b**). Evidently, free flowing platelets did not significantly increase either (**Figure 6.5c**).

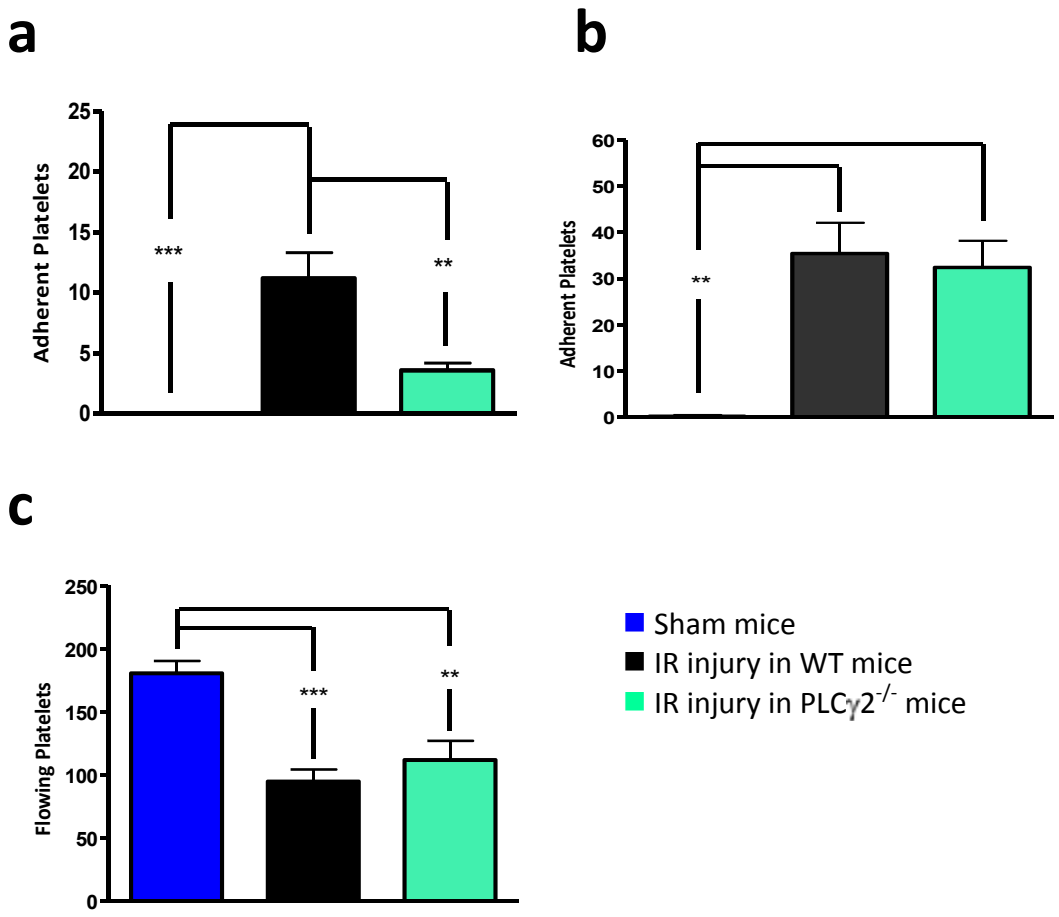


Figure 6.5. Individual PLCγ2$^{-/-}$ donor platelet adhesion was significantly decreased in the <u>ileum</u> of IR injured wild-type mice. No difference was observed in the jejunum. Recruitment of PLCγ2$^{-/-}$ donor (1 x 10^8 platelets, 10μM CFSE) platelets to the microcirculation of the ileal and jejunal mucosal villi in wild-type mice was examined using intravital microscopy. Wild-type or PLCγ2$^{-/-}$ donor platelets were introduced prior to IR injury. **(a)** Numbers of individual adherent platelets was significantly reduced in the ileum but not in the **(b)** jejunum. **(c)** Free flowing platelets were observed in the ileum but were not significantly increased above IR injured mice. All data is are representative of mean + SEM of at least 5 separate experiments. **p<0.01, ***p<0.001 when compared using a one way ANOVA followed by Bonferroni Post Test for points of significance.

6.4 Discussion

In this chapter, the most effective strategy at preventing both microthrombus formation and the adhesion of individual platelets was demonstrated by using mice deficient in the platelet α_{IIb} sub-unit. In these knockout mice, a significant reduction in microthrombi was observed in the jejunum and individual platelet adhesion was reduced in both the jejunum and the ileum. Furthermore, blood flow was apparent within the jejunum and ileum of these mice. The use of the α_{IIb} deficient mice was far more effective than the anti-GPIb antibody used in P-selectin$^{-/-}$ mice as described in Chapter 5. However, despite these striking anti-platelet effects, induction of intestinal IR injury in α_{IIb} deficient mice was associated with significant bleeding from the injured gut mucosa, as well as no improvement in tissue morphology as observed histologically. We did not pursue further experiments to quantitate macromolecular leakage or leukocyte adhesion in these mice as the sustained bleeding from the mucosal surface made it impossible to obtain sufficiently clear intravital images. Furthermore, this mucosal bleeding was associated with increased distress to the animal as blood loss increased, which also limited further experimentation. Interestingly, despite observing collagen exposure electron microscopically, as described in Chapter 3, no anti-platelet or beneficial effect was observed in FcR γ-chain$^{-/-}$ mice (lacking GPVI) or in mice pre-treated with an anti-$\alpha_2\beta_1$ antibody.

The results from this chapter and Chapter 5 are somewhat conflicting. We demonstrated that by using an anti-GPIα strategy we could reduce the platelet recruitment at sites of of

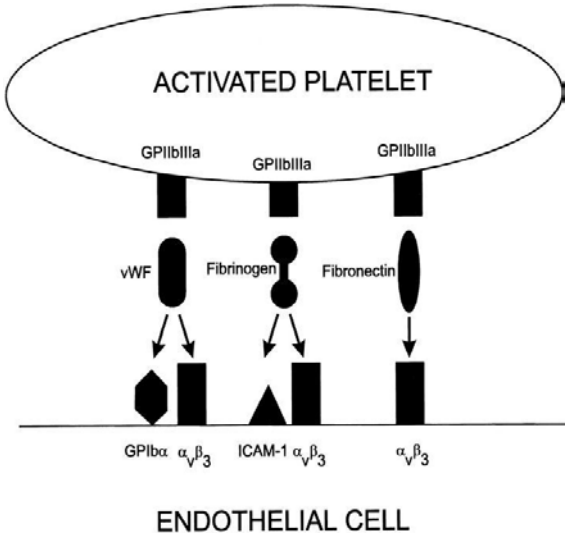
microvessel injury and that this was beneficial. However, using a far more effective anti-platelet strategy, through inhibiting α_{IIb}, caused increased bleeding. The importance of having some platelet recruitment was also demonstrated by the increased bleeding and poor histological improvement observed following IR injury in the thrombocytopenic mice (achieved by administration of an anti-mouse depleting GPIbα antibody). This phenomenon of increased bleeding in response to a lack $\alpha_{IIb}\beta_3$ or platelets has not previously been demonstrated in the gut following intestinal IR injury, most likely because previous studies have not focussed on the events taking place in the mucosal surface. However, Kleinschnitz and colleagues also witnessed similar bleeding events in their *in vivo* model of experimental stroke whereby cerebral haemorrhage was a frequent observation of $\alpha_{IIb}\beta_3$ inhibition (Kleinschnitz *et al.,* 2007). They also demonstrated that blockade of GPIb could decrease infarct size to a greater extent than GPVI – these results again mirror those presented in the current study.

These collective observations suggest that some platelets are required in ischemic sites to prevent bleeding (i.e. to cause haemostasis), but the complete blockage of blood flow that occurs as a result of platelet-platelet interactions during reperfusion is not required. Indeed, $\alpha_{IIb}\beta_3$ antagonists were developed on exactly this basis with the hypothesis that their lack of effect on other platelet adhesion receptors would theoretically allow a single layer of platelets to contribute to haemostasis, whereas the anti-$\alpha_{IIb}\beta_3$ effect would greatly diminish platelet thrombus formation (Coller *et al*, 2008).

To further demonstrate that some level of platelet recruitment is necessary in IR injured gut, further studies were conducted in thrombocytopenic mice. Again, a severe bleeding phenomenon was observed highlighting the importance of some platelet recruitment being necessary. Recent research from the Wagner group in Boston investigated the effects of thrombocytopenia in models of dermatitis (irritant contact and immune complex–mediated), lung inflammation (intranasal endotoxin) and following stroke (Goerge *et al.,* 2008). In all injury models, massive bleeding into the skin, lungs and brain tissue was visible but only in the thrombocytopenic mice. In platelet-depleted mice undergoing skin injury, the first microscopic appearance of bleeding or petechiae was visible at 20 minutes post-injury with bleeding increasing by 2 hours and still present after 6 hours. This mirrors the observations within the current study in the gut where animals began to demonstrate serious distress in response to the sustained bleeding. Collectively, these experiments indicate that platelets secure the integrity of the injured microcirculation during inflammatory challenge. However, interestingly, this group further demonstrated that the actual adhesion and of the platelets themselves was not required to prevent the hemorrhage following injury. They speculated that platelet storage granules were important in the prevention of inflammatory haemorrhage through release of an array of vasoactive molecules that could regulate vascular junctions, permeability and endothelial migration such as serotonin, sphingosine-1P and angiopoietin-1. Hence, platelets could be required to deliver such agents to the site of inflammation and, in this way, secure vascular integrity rather than through haemostasis

dependent mechanisms.

We anticipated $\alpha_{IIb}\beta_3$ to play a critical role in platelet-platelet interactions and therefore thrombosis and this was indeed the case. However, we also demonstrated that $\alpha_{IIb}\beta_3$ played an important role in mediating platelet-endothelial interactions, as single platelet adhesion was reduced in the jejunum and, also for the first time, in the ileum. A number of previous studies have demonstrated a critical role for $\alpha_{IIb}\beta_3$ in supporting platelet adhesion to activated endothelium. Li and colleagues demonstrated that thrombin, found in high concentrations at sites of injury, was able to significantly enhance platelet adhesion to endothelial cells *in vitro* (Li *et al*., 1996). Bombeli *et al* further provided a more detailed model (see diagram) to explain the pathways by which platelet $\alpha_{IIb}\beta_3$ could lead to the interaction of platelets with activated endothelial cells via fibrinogen, vWF, and fibronectin (Bombeli et al., 1998). These proteins are either present in plasma or secreted by the platelets themselves. Furthermore they demonstrated that the endothelial counter-receptors responsible were $\alpha_v\beta_3$ integrin, (binding fibronectin, fibrinogen or vWF), ICAM-1 (binding fibrinogen) or endothelial GPIbα (binding vWF). These same endothelial counter-receptors are known to be increased following IR injury and so it is likely that these mechanisms may also explain the adhesion of platelets to endothelial cells in the current study.



Interestingly, we did not observe any beneficial role for GPVI in the current study. This is despite the fact that collagen exposure within the mucosal microcirculation was demonstrated in Chapter 3. The mechanism of collagen-platelet interactions is complex. It involves direct or indirect binding of collagen to several platelet receptors including the GPIb-IX-V, $\alpha 2\beta 1$, GPIV and GPVI which leads to both adhesion and activation steps. Initially platelet adhesion at high shear occurs via GPIb-IX-V binding to collagen-bound vWF. Platelets become fully arrested by direct interaction between collagen and $\alpha_2\beta_1$ with strengthening of platelet attachment occurring after platelet activation predominantly via GPVI. Therefore, the platelet collagen receptor GPVI has been considered an attractive therapeutic target to treat cardiovascular diseases. However, Mangin and colleagues were amongst the first to demonstrate that loss of GPVI did not produce the anticipated dramatic effect when thrombosis was induced *in vivo* within arterioles in mice (Mangin *et al.,* 2006). They demonstrated a functional overlap between thrombin and GPVI in which reduction in the platelet activating properties of one mechanism can be partially compensated by the other. Their study suggested a greater functional redundancy for GPVI in thrombogenesis than previously recognized and speculated that the full anti-thrombotic potential of GPVI inhibitors *in vivo* would require concurrent administration of anti-coagulant agents. Furthermore, mice deficient in the GPVI only show a minor increase in bleeding times (Nieswandt *et al.,* 2001), which again suggests that other matrix proteins play a role in promoting thrombus formation and can thereby compensate for the absence of GPVI.

The integrin $\alpha_2\beta_1$ is a major collagen receptor predominantly activated after GPVI-collagen interactions to a high-affinity state via "inside-out" signals, enabling the platelet to establish firm adhesion that resists shear forces in the bloodstream. Since no role for GPVI was observed following intestinal IR injury, initially the role of $\alpha_2\beta_1$ was not going to be investigated. However, studies have demonstrated that it can also be activated by G protein–coupled receptor agonists independently of GPVI. Indeed, the intracellular signaling cascade used by $\alpha_2\beta_1$ shares many of the features of the GPVI signaling cascade, including participation of Src kinases and PLCγ2 (Inoue *et al.,* 2003) Grüner and colleagues also used intravital fluorescence microscopy to examine the dynamic process of platelet accumulation at sites of vascular injury in the carotid artery of α_2- and β_1-deficient mice (Grüner *et al.,* 2003).They also found that platelet adhesion and thrombus growth on the exposed extracellular matrix of the arterial wall were unaffected in the absence of $\alpha_2\beta_1$ or indeed all β_1 integrins on platelets. They concluded that multiple integrin-ligand interactions could synergize shear-resistant platelet adhesion at sites of arterial injury *in vivo* and that there was a high degree of functional redundancy amongst them**.** Interestingly, they did state that this integrin could have a significant function in more pathophysiologic processes or disease models. However, in the current model of intestinal IR injury, no role for $\alpha_2\beta_1$ was demonstrated, but its role in other experimental models of disease is still currently lacking.

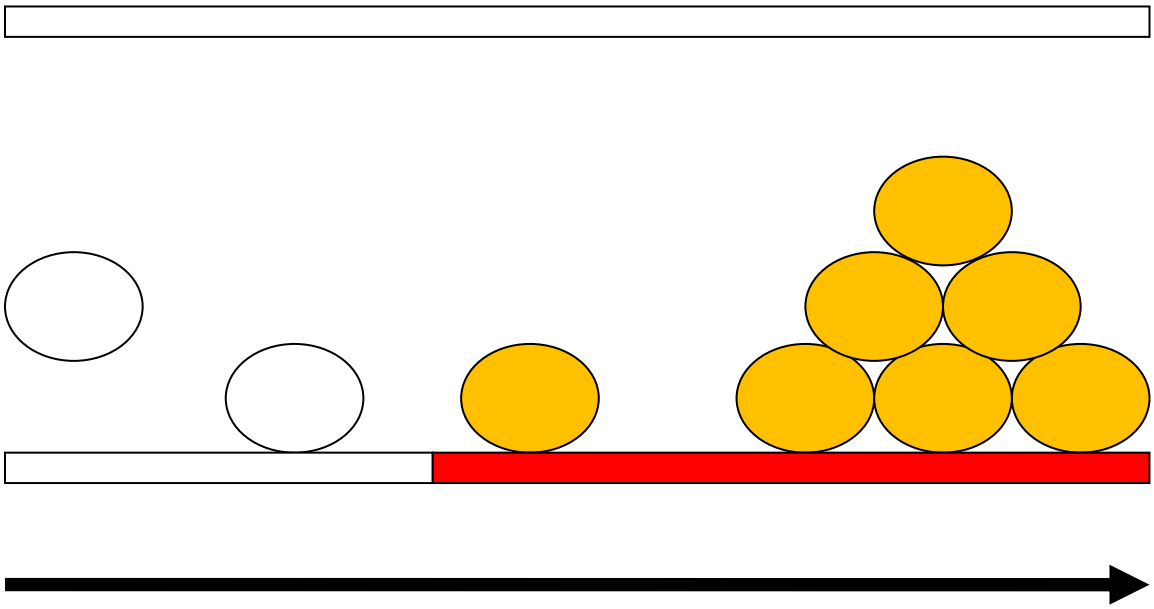
PLCγ2 also appears to be a particularly good target following IR injury in that it lies downstream of the integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ and GPVI. Furthermore, Nonne and colleagues demonstrated a severe impairment of thrombus formation in PLCγ2$^{-/-}$ mice *in vivo* after a superficial and mild laser-induced lesion in mesenteric arteries but no role following a severe, occlusive injury (Nonne *et al.,* 2005). Unfortunately, due to a shortage of PLCγ2 deficient mice, we were unable to determine whether it also had an important role in mediating jejunal thrombosis following intestinal IR injury. Interestingly, PLCγ2$^{-/-}$ mice are often accompanied by spontaneous gastrointestinal and intraperitoneal bleeding, which may have made it difficult to monitor the gut intravitally. However, the finding that lack of intracellular PLCγ2 could affect individual platelet interactions with endothelium was both novel and exciting. The surface expression of platelet receptors such as GPVI, GPIbα, $\alpha_2\beta_1$ and in particular $\alpha_{IIb}\beta_3$ is not altered on the donor PLCγ2$^{-/-}$ platelets and so decreased endothelial interactions cannot be explained by this (Mangin *et al.,* 2003). A role for PLCγ2 in mediating platelet-endothelial interactions has not previously been investigated and is certainly worthy of further investigation both *in vivo* and *in vitro*.

In summary, we have demonstrated that while platelets may plug the flow of blood in the mucosal villous microcirculation and also promote recruitment of inflammatory cells, they are also essential in preventing the deleterious consequences of inflammation on the vasculature that lead to haemorrhage and subsequent tissue damage. Although $\alpha_{IIb}\beta_3$ had the most strongest anti-platelet effect in this model of intestinal IR injury, it was not tissue

protective. However, a role for anti-thrombotic agents targeting this integrin should not be ruled out completely for intestinal IR injury, as it could be possible to identify a lower dose of an antagonist which may be beneficial.

Chapter 7

General Discussion



7.0. General Discussion

7.1. Summary of the main findings

Among the internal organs, the small intestine is probably the most sensitive to IR injury (Granger *et al.,* 1986; Yamamoto *et al.,* 2001). Unfortunately, intestinal IR is a potentially life-threatening condition, quite often leading to a systemic inflammatory response and the potential development of the multiple organ dysfunction syndrome. It can develop in both surgical and trauma patients when there is interruption of blood flow to the gut. Surgically, abdominal aortic aneurysm repair, cardiopulmonary bypass, strangulated hernias, neonatal necrotizing enterocolitis and intestinal transplantation are associated with IR injury but it also occurs in septic and hypovolemic shock (Mallick *et al.*, 2004). Despite several promising interventions both pharmacological and physical in nature, including anti-oxidant therapy, hypothermia and pre-conditioning, there are currently no clearly established methods available to prevent intestinal IR injury (Kalia *et al*., 2002; Mallick *et al*., 2004). Increasing our understanding of the complex process of ischaemia and reperfusion will allow us to find novel targets to develop new strategies to combat this serious injury.

Although recent studies have begun to implicate platelets in IR injury, their pathophysiological role in IR injury of the gut is not fully understood, particularly their role in contributing to mucosal injury. Platelet adhesion and aggregation at sites of vascular injury is essential for the cessation of bleeding. However, excessive accumulation of platelets at sites of injuries can lead to severe pathologies. Indeed,

after atherosclerotic plaque rupture, arterial thrombotic occlusion can lead to acute myocardial infarction and stroke (McCullagh *et al.,* 1980). Collagen is among the most powerful activators of platelets and plays a major role in promoting platelet thrombus formation *in vivo*. Recently, there has been increased focus on collagen receptors as potential anti-thrombotic targets. This is due not only to the fact that collagen plays an important role in atherothrombosis, but also because inhibitors targeting platelet collagen receptors can produce an anti-thrombotic effect *in vivo* (Nieswandt *et al.,* 2001; Massberg *et al.*, 2003; Massberg *et al*., 2004). Furthermore, platelet collagen receptor deficiency only leads to a relatively mild bleeding tendency (Arai *et al.,* 1996). Therefore, the role of direct and indirect platelet collagen receptors in mediating platelet recruitment following intestinal IR injury was investigated.

This thesis initially presented data that platelet recruitment does indeed occur following intestinal IR injury, but this is confined specifically to the villous microcirculation with very little recruitment in the larger vessels of the serosal wall or the mesentery. This recruitment varied not only in its nature but also in its location. Individual platelets were primarily observed within the lesser injured ileum and microthrombus formation observed within the severely injured jejunum. It was only possible to identify these two different types of platelet events within the small intestine by using a novel dual platelet-labelling methodology. Simultaneous, real-time capture of different populations of platelets labeled with dyes of distinct excitation wavelengths was only possible using a rapid wavelength filter switcher attached to the intravital set-up. Previous studies have only demonstrated platelet-

endothelial interactions following IR injury. Therefore, an important aspect of the current research has been the development of a novel methodology that allows individual adherent and free flowing platelet events to be quantitated as well as thrombus formation. Using this methodology, we were able to highlight that the traditional method for monitoring platelet trafficking *in vivo,* using only donor injected platelets, quite significantly underestimates the involvement of platelets in this injury model.

The subsequent studies, presented in Chapter 4-6, demonstrated that the formation of microthrombi was predominantly dependent upon platelet GPIbα and $\alpha_{IIb}\beta_3$. Inhibiting thrombus formation was associated with a significant improvement in tissue and microcirculatory integrity. However, inhibition of the $\alpha_{IIb}\beta_3$ integrin was associated with increased bleeding from the mucosal surface, despite it's anti-thrombotic effects, which may limit its therapeutic use clinically. Lack of $\alpha_{IIb}\beta_3$ was also associated with a reduction in the adhesion of individual platelets within the jejunum and ileum. However, an interesting finding from this study was that the intracellular enzyme, PLCγ2, also appeared to have a role in contributing to platelet-endothelial interactions, a mechanism of platelet adhesion which so far has been sparsely investigated. Finally, this thesis demonstrated that targeting only the platelets following intestinal IR injury did not confer a sustained benefit to the jejunum despite restoration of blood flow to this region. Although platelets seemed to be the 'first on the scene' and the primary instigators of tissue damage, inhibiting their adhesion did not prevent subsequent leukocyte recruitment which contributed to a secondary injury to the gut. Indeed, enhanced leukocyte recruitment was

observed in animals receiving the anti-GPIbα antibody and some explanation as to why this may have happened will be provided later on (**refer to Section 7.4 and Figure 7.1)**. A longer lasting protection was only observed in animals in which both platelet and leukocyte recruitment was inhibited by blocking GPIbα in P-selectin deficient mice. Future studies on recovery animals are required in order to determine how lasting the benefit of targeting these two cells types is following acute intestinal IR injury and whether this therapeutic strategy has any potential for use in animals undergoing more chronic intestinal pathologies.

7.2. The nature of platelet recruitment is dependent upon the regional severity of intestinal IR injury

The mucosal surface is most susceptible to ischaemic injury. There is substantial evidence that the mucosa of the intestine becomes the site for the production of various acute-phase proteins (Molmenti *et al*., 1993; Wang *et al.*, 1998), gut hormones (Zamir *et al.,* 1992) and cytokines (Mester *et al.,* 1993; Meyer *et al.,* 1995) following injury. Indeed, the enterocyte itself, or the epithelial cells of the villi, produce increased amounts of cytokines during IR injury. These not only influence the intestine but also affect the function and integrity of distant organs. Maintaining the integrity of this mucosal surface is essential in order to prevent bacterial translocation into the bloodstream. Indeed, up to 55% of small bowel transplant recipients die from sepsis-related complications due to a breakdown in the mucosal integrity of the graft lining (Browne *et al.,* 1992). However, it is surprising that all studies to date investigating vascular disturbances and the role of circulating cells in contributing to IR injury have focussed on the larger outer wall or mesenteric vessels

of the gut (Massberg et al., 1998; Copper et al., 2003). The current study is the first to demonstrate the substantial recruitment of platelets within the mucosal villous microcirculation.

Thrombosis, blood flow occlusion and subsequent injury was confined to the jejunal mucosa during the 4 hour duration of the experiment. From a transplantation point of view, this is interesting data as it suggests that this region may not be best suited for use as a graft and the ileum may be more appropriate. However, it is important to identify strategies that can improve the outcome of the of jejunum as it is damage to this area that seems to most affect morbidity and mortality following intestinal IR injury in non-transplant related scenarios. Furthermore, longer durations of ischaemia and / or longer periods of reperfusion affect the ileum, with the injury dependent upon similar platelet events to those observed in the jejunum. Hence strategies that are beneficial in the jejunum may also have benefit in a more severely compromised ileum.

7.3. Platelets lead to tissue damage in the jejunum following their aggregation on exposed subendothelial matrix

Platelets can be recruited to sites of vascular injury either by activated endothelium or following exposure of ECM collagen, with the latter event taking place as the severity of vessel damage increases. However, previous studies have demonstrated intestinal IR injury is associated with platelet adhesion to activated endothelium only, primarily via P-selection / PSGL-1 interactions (Massberg et al., 1998; Copper et al., 2003). There are two reasons why these previous studies have never

demonstrated thrombosis formation dependent upon platelet-subendothelial interactions. Firstly, the injury sustained by the larger vessels following intestinal IR injury is not as great as that observed in the the villous microcirculation and this latter region has not been the focus of previous studies *in vivo*. Secondly, the recruitment of endogenous platelets has not been easy to investigate.

We clearly demonstrated that ECM collagen, underlying the villous microvessels becomes exposed only in the jejunum following IR injury. This supports the suggestion that the formation of microthrombi are probably not due to aggregates forming on activated endothelial cells and that platelet GPIbα most likely interacts with vWF immobilised on collagen. These microthrombi contribute to the occlusion of the vasculature within the jejunum which leads to no-reflow and resultant tissue damage. However, recent evidence has supported the possibility that the vWF ligand GPIbα not only plays a critical role in the initial rolling to injured vessels but also mediates the platelet-platelet interactions required for thrombus (Bergmeier *et al.,* 2006). It is therefore possible that the key role of GPIbα within our IR model is either to mediate the initial rolling of platelets to exposed collagen or lead to secondary activation and subsequent aggregation of platelets, or both. Regardless, inhibiting GPIbα has beneficial effects in this model. It is possible the shear rates associated with the villous microvasculature may pre-empt the requirement for platelet rolling i.e. this event may not be as critical for platelet capture as it is in the faster flowing, larger vasculatures. Hence the predominant role of this receptor at this injury site may be in promoting thrombus growth. It is also possible that the indirect collagen receptor, GPIbα is mediating platelet-endothelial interactions

rather than platelet-collagen or platelet-platelet interactions (Andre *et al.,* 2000; Theilmeier *et al.,* 2002). However, if this was the case, we would have anticipated all platelet events to have been inhibited and not just thrombus formation in the anti-GPIbα antibody pre-treated mice. Interestingly, individual platelet adhesion demonstrated a trend to increase and not decrease following inhibition of GPIbα.

The importance of platelet collagen receptors in mediating platelet recruitment in clinically relevant models of ischaemic and non-ischaemic injury is gaining interest. (Meyer et al,) recently presented data that binding of vWF to both collagen and GPIb were mandatory steps in stroke development. Furthermore they demonstrated that vWF-$\alpha_{IIb}\beta_3$ interactions were not critical which could explain the ineffectiveness of $\alpha_{IIb}\beta_3$ blockade in experimental and clinical models of stroke (De Meyer *et al.,* 2010). They further suggested that inhibition of vWF-mediated platelet adhesion could provide a novel therapeutic option in stroke management. It is encouraging that several anti-thrombotic compounds blocking vWF-collagen or vWF-GPIbα interactions are currently being developed (De Mayer *et al.*, 2009; Vanhoorelbeeke *et al.*, 2007), which may prove to have beneficial effects in the treatment of intestinal IR injury.

7.4. Inhibiting GPIbα alone does not afford long lasting tissue protection - platelet and leukocyte inhibition is critical

Historically, IR in various organs has been attributed to the recruitment of inflammatory leukocytes to the activated endothelium, with neutrophils which accumulate when the tissue is reperfused demonstrated to be the primary instigator

of the injury (Welbourn *et al.,* 1991; Carden and Granger, 2000). Once adherent to endothelium, neutrophils mediate damage by secretion of additional reactive oxygen species as well as proteolytic enzymes, in particular elastase. However, in the current study, only small numbers of leukocytes were observed adherent following IR injury in wild-type mice, both in the jejunum and ileum, with the most immediate event being thrombus formation.

Leukocyte recruitment did increase after microthrombus development was inhibited using the anti-GPIbα antibody. It was hypothesised that recruitment of these inflammatory cells may be responsible for mediating the secondary injury, which interestingly only took place in the jejunum and not in the ileum. One possible explanation for this increased leukocyte adhesion in GPIbα blocked mice may be the fact that by preventing microthrombus formation and enabling blood flow within the jejunal microvessels, leukocytes were able to to gain entry and become adherent to activated endothelium. This is supported by the observation that inhibiting microthrombus formation using an anti-GPIb antibody in P-selectin deficient mice, was able to inhibit thrombus formation and prevent the adhesion of leukocytes that gained entry in the jejunal microcirculation (**Figure 7.1**). Only by decreasing adhesion of both cell types was vessel occlusion inhibited, blood flow maintained and tissue histology improved throughout the 4 hour duration of the experiment. Although inhibition of both platelet and leukocyte events seems to confer the longest lasting benefit to the gut, there are obvious limitations associated with developing therapies that target both the inflammatory and haemostatic cells.

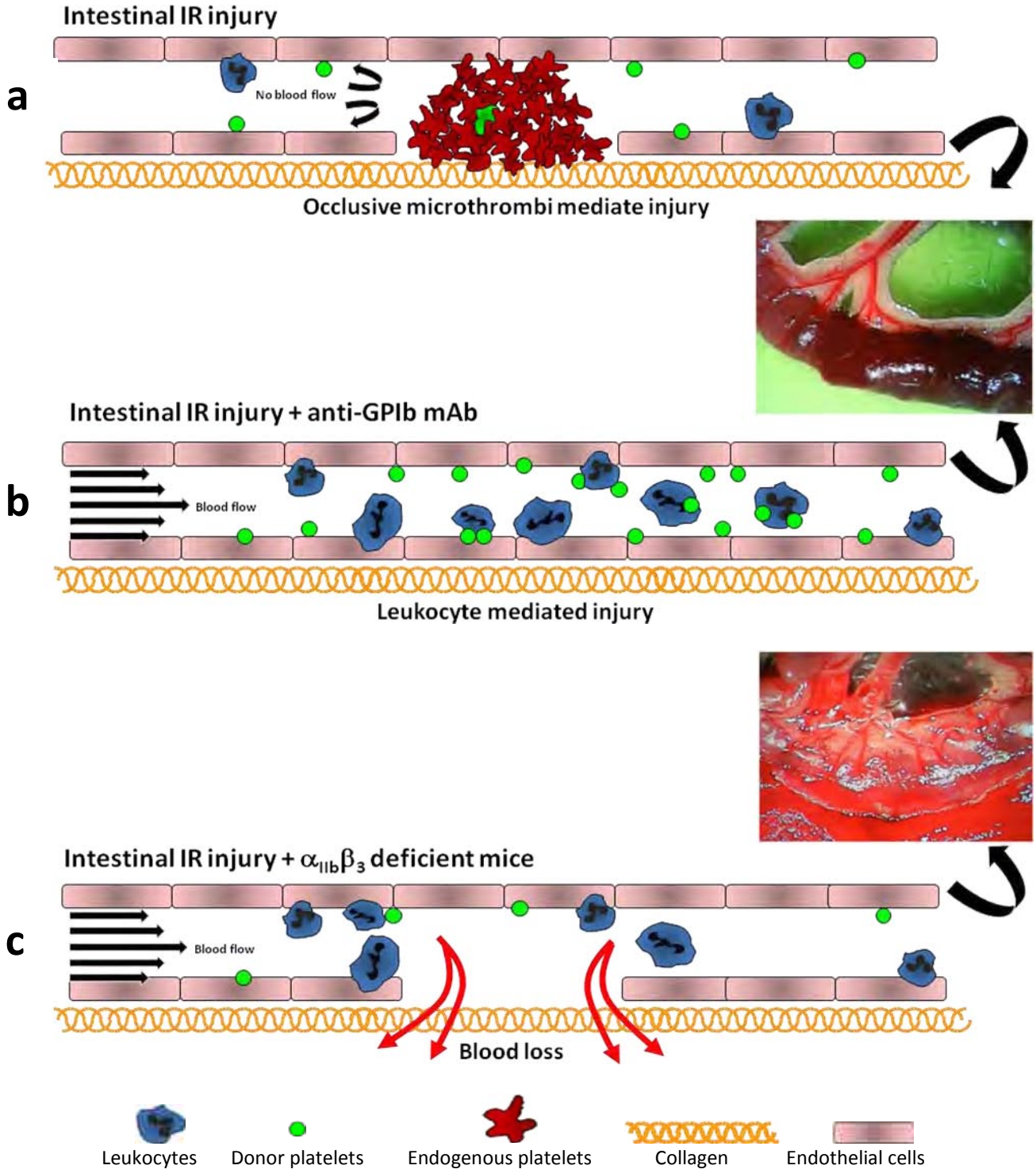


Figure 7.1. Proposed mechanisms by which platelets and leukocyte could confer injury within the jejunum following intestinal IR injury in wild-type mice, mice pre-treated with an anti-GPIbα antibody and in $\alpha_{IIb}\beta_3^{-/-}$ mice. (a) IR injury in wild-type mice was associated with endothelial denudation and subsequent microthrombus formation. This led to blood flow occlusion within the microvessels and subsequent tissue injury. Minimal leukocyte adhesion was observed due to the lack of blood flow limiting their entry to this site. **(b)** Pre-treating mice with an anti-GPIb mAb prevented microthrombus formation, and so blood flow was restored upon reperfusion. However, this meant leukocytes could gain entry to the activated endothelium and either adhere to it directly or via adherent platelets. This also lead to tissue injury. **(c)** Induction of IR injury in $\alpha_{IIb}\beta_3$ deficient mice was a very effective anti-platelet strategy but was associated with blood loss from the mucosal surface suggesting some platelet recruitment was essential.

7.5. Some platelet recruitment is necessary to prevent mucosal bleeding following intestinal IR injury

The data obtained from the use of α_{IIb} deficient mice and from conducting studies in thrombocytopenic mice was very interesting. In the former, no microthrombus formation or individual platelet adhesion was observed either in the jejunum or the ileum, indicating that this was a very effective anti-platelet strategy. However, progressive bleeding was observed throughout late stage ischaemia and reperfusion in both α_{IIb} deficient and thrombocytopenic mice. These results lead us to the conclusion that some degree of platelet recruitment is required at sites of vessel injury to counteract bleeding related problems. (**Figure 7.1**). The risk of excessive bleeding associated with an effective anti-platelet therapy needs to be balanced against the risk of thrombosis and vessel occlusion associated with insufficient anti-platelet therapy. This is true not only following intestinal IR injury, but for almost all diseases where platelets are pathological (Yan *et al*., 2006).

Several anti-platelet therapies have been shown to be effective in reducing thrombosis and vessel occlusion in complications such as myocardial infarction and other vascular ischaemic injuries. Blockade of $\alpha_{IIb}\beta_3$ by abciximab or integrillin (which inhibits the associated peptide) have been shown to have a positive effect on decreasing thrombotic incidents. However, the side effects of internal bleeding, haemorrhage and thrombocytopenia suggest that blockade of this integrin may not be the best strategy clinically (Suleiman *et al*, 2003; Kleinschnitz *et al*, 2007). Clopidogrel is an antagonist of the P2Y12 / ADP receptor which is essential for $\alpha_{IIb}\beta_3$ activation. It is administered mostly in patients with coronary artery disease as it is

proven to decrease the risk of cardiovascular death by 20%. However, incidents of gastrointestinal haemorrhage and thrombocytopenia have also been reported with use of clopidigrel but the numbers are particularly low (Caruana, 2007).

Interestingly, Nocito and colleagues similarly demonstrated that neither inhibition of platelet function nor platelet depletion led to a reduction of hepatic IR injury. However, liver regeneration and repair were significantly impaired in platelet-depleted animals, suggesting platelets mediate tissue repair and liver regeneration. Furthermore, platelet-derived serotonin was identified as the mediator of hepatocyte proliferation in the post-ischaemic liver (Nocito *et al.,* 2007). The role of platelets in tissue repair and regeneration is an area of research that will most likely grow as increasing evidence suggests these cells are sources of valuable growth factors and could be instrumental in guiding stem cells to sites of injury (de Boer *et al.,* 2006; Andrae *et al.,* 2008).

7.6. Major collagen receptors do not have a role to play in intestinal IR injury

Considerable progress has been made over the last few years in elucidating the roles of the two major collagen receptors, glycoprotein VI (GPVI) and integrin $\alpha_2\beta_1$, in promoting platelet adhesion and thrombus growth (Nieswandt and Watson 2003). The evidence that GPVI is of central importance for collagen activation of platelets is strong and recent studies have suggested that $\alpha_2\beta_1$ also plays a key role in promoting primary platelet adhesion on collagen. However, in the current study no important role for either of these direct collagen receptors was identified. This suggests that although an important role for these proteins has been identified in animal models

of thrombosis *in vivo*, it does not follow that they will also have a critical role in clinically relevant models of disease. Localised injuries such as the laser injury or the $FeCl_3$ injury, used extensively to monitor thrombosis *in vivo*, do not mimic the environment that surrounds tissues that have undergone IR injury or perturb the microcirculation in a similar manner (Dubois *et al.,* 2006). Furthermore, *in vivo* models of thrombosis most often revolve around inducing injury to a large arteriole or venule such as mesenteric or cremasteric arterioles or the carotid artery. However, the shear stresses the platelets are subject to in these vessels are considerably different to those found within the villous microcirculation. It is therefore unsurprising that the mechanisms of platelet recruitment and thrombus formation rely on different platelet receptors in models of thrombosis compared to IR injury.

7.7. Future Directions

Even though the body of literature on platelet recruitment to injured or diseased tissues is expanding, there are still questions which remain to be answered. Therefore the work detailed in this thesis could be expanded in a number of ways.

It would be interesting to determine whether the tissue protection afforded to the gut by an anti-GPIbα and P-selectin strategy lasts longer than 4 hours. We did not monitor the bowel beyond 4 hrs and so it is unclear whether other mechanisms come into play to recruit platelets or leukocytes beyond this time point. Mortality can occur within 48 hrs of experimental intestinal IR injury in rats and mice due to respiratory distress or through loss of gut barrier function. It is also not clear

whether internal bleeding complications could arise in these dual therapy treated mice after several days, so this would require further investigation.

Similar studies could be conducted in different organ beds to determine the role of platelets, and specifically platelet collagen receptors, in mediating injury. It would be interesting to determine whether a similar dual receptor blockade is beneficial. It may be that GPIbα is effective in the villous microcirculation because of the shear stresses and blood flow kinetics specific to this microcirculatory bed. The liver sinusoidal capillaries, for example, have much lower rates of blood flow and so the recruitment of platelets in this tissue may vary when compared to the gut. Certainly no studies to date have compared the mechanisms involved in recruitment of platelets and microthrombus formation in different microcirculatory beds undergoing clinically relevant acute or chronic injuries. Furthermore, the role of platelets in other disease models or organs may be limited to activating the inflammatory cascade and recruiting other nuclear cells such as neutrophils and lymphocytes rather than occlusion of blood flow through microthrombus formation.

Due to the shortage of PLCγ2 deficient mice, it was not possible to determine whether this enzyme had a role in microthrombus formation within the jejunum, but this is worthy of future experimentation. Also, the interesting observation that platelet-endothelial interactions could be inhibited in PLC$\gamma2^{-/-}$ mice needs to be developed further as this has never been previously described *in vitro* or *in vivo.*

It is well accepted that platelets can recruit inflammatory leukocytes following IR injury. However, recent evidence demonstrates that surface-adherent platelets can also support circulating stem cell recruitment *in vivo* via P-selectin and α_{IIb} dependent mechanisms (de Boer et al., 2006; Massberg et al., 2006). Stellos and colleagues presented data showing that platelet-derived SDF-1α recruited $CD34^+$ progenitor cells in murine small intestinal microcirculation following IR injury (Stellos *et al.,* 2008). Preliminary data from the Kalia lab has also demonstrated platelet-haematopoietic stem cells interactions intravitally following arteriolar laser-induced injury. Therefore, platelets within injured vessels may be a means of guiding stem cells to appropriate sites and may accelerate healing through recruitment of bone marrow-derived stem/progenitor cells. It would be interesting to monitor whether the individual platelets that adhere despite GPIbα inhibition can recruit potentially therapeutic stem cells to sites of injury.

7.8. Concluding Remarks

The primary aim of this thesis was to clarify whether platelets played a pathophysiological role in intestinal IR injury and identify the mechanisms governing their recruitment. A more detailed understanding of the mechanisms governing platelet recruitment to the mucosal villi was only achieved after development of a novel methodology that allowed individual and aggregated platelets to be identified. We demonstrated that there was more than one cellular contributor to intestinal IR injury which meant it was impossible to achieve sustained tissue protection by targeting only platelets. In this IR injury model, GPIbα was effective, but it may be that a chronic injury or an injury in a different organ may rely on different platelet

receptors. Therefore, despite the general excitement about the development of new anti-platelet drugs, a major lack of understanding of how platelets contribute to the pathophysiology of injuries in specific tissues may hinder their overall success. A move towards investigating the mechanisms governing platelet recruitment in clinically relevant experimental models is therefore a must rather than, or in addition to, investigating their role in animal models of thrombosis.

Chapter 8

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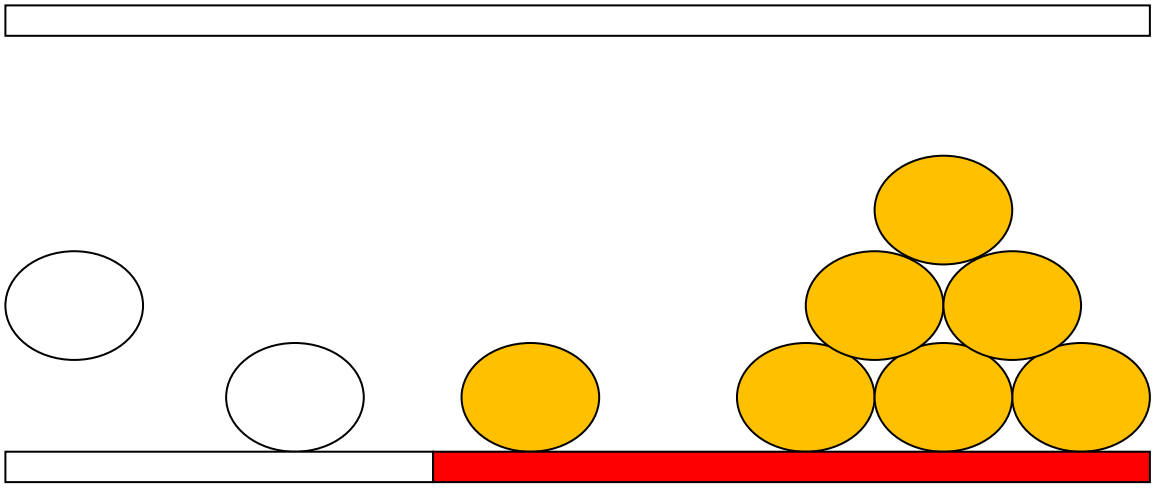

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