UNIVERSITY OF BIRMINGHAM

Enhancing Haematopoeitic Stem Cell Recruitment to Injured Murine Colon

By

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Abstract

Haematopoietic stem cells (HSCs) have been described as potential therapeutic agents for the repair of several inflammatory injuries including inflammatory bowel diseases (IBDs). However, their efficacy within clinics has been poor. This has been partially attributed to poor recruitment to sites of injury. Thus identifying the mechanisms by which HSCs are recruited to inflamed bowel, and developing strategies to enhance this recruitment, may increase their clinical efficacy. Critical adhesive mechanisms and several pre-treatment strategies to enhance adhesion to chronically (DSS induced colitis) and acutely (IR) injured murine colon were investigated *in vitro and in vivo*. It was found that recruitment to IR injured colon was mediated by CD49d, whereas recruitment to colitic colon was mediated by both CD18 and CD49d. *In vitro* investigation revealed that both hydrogen peroxide (H_2O_2) and platelet derived pre-treatments, such as coating HSCs with platelet microparticles (PMPs), could enhance adhesion to colon endothelial cells, immobilised endothelial counterligands ICAM-1 and VCAM-1 and frozen tissue sections. Furthermore, pre-treatment of HSCs with PMPs significantly enhanced their adhesion to chronically injured colon *in vivo*. These increases in adhesion were likely to be attributed to the altered distribution of integrins on the HSC surface as determined confocally. Furthermore, electron micrscopy showed that pre-treatment also resulted in overt HSC morphological changes reminiscent of activated macrophages. Overall, these investigations describe a two-component recruitment mechanism for pre-treated cells in which enhanced recruitment is dependent on both activation of stem cells and also vascular endothelium. These studies provide novel evidence that pre-treatment of HSCs can result in their increased recruitment to injured colon, a finding that may allow for stem cell therapy for IBDs to be fully realised.

Publications

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Oral Communications

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Modulating haematopoietic stem cell adhesion to intestinal microvasculature
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Table of Abbreviations

ANOVA	Analysis of variance
APES	3-aminopropyltriethoxysaline
BM	Bone marrow
CAM	Cell adhesion molecule
CD	Cluster of differentiation
CEC	Colon endothelial cell
CFDA-SE	5'6-carboxyfluorescien diacetate succinimidyl ester
CFSE	5'6-carboxyfluorscein succinimidyl ester
CM	Conditioned media
Col	Colon (used as a prefix for conditioned media types to denote tissue source)
Col ICM	Colitis injury conditioned media
CTO	Cell tracker orange
CXCR4	Chemokine CXC motif receptor 4
$DiOC_6$	3,3'-Dihexyloxacarbocyanine iodide
DMEM	Dulbecco's modified eagles media
DSS	Dextran sodium sulfate
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EPC	Endothelial progenitor cell
ESC	Embryonic stem cell
E-selectin	Endothelial selectin
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum (also known as fetal calf serum)
GAG	Glycosaminoglycan
GFP	Green fluorescent protein
GIT	Gastro-intestinal tract
GP	Glycoprotein
H_2O_2	Hydrogen peroxide (chemical notation)
HPC7	Haematopoietic progenitor cell 7
HSCT	Haematopoietic stem cell transplantation
HSCT	Haematopoietic stem cell
IBD	Inflammatory bowel disease(s)
ICAM-1	Intercellular cell adhesion molecule 1
IgG	Immunoglobulin G
Il	Ileum (used as a prefix for conditioned media types to denote tissue source)
Il-	Interleukin-
IR	Ischaemia reperfusion injury
IR ICM	Ischaemia reperfusion injury conditioned media
IVM	Intravital microscopy
Jej	Jejunum (used as a prefix for conditioned media types to denote tissue source)
LFA-1	Lymphocyte function-associated antigen 1
MAdCAM-1	Mucosal addressin cell adhesion molecule 1
MCEC-1	Murine cardiac endothelial cell 1
MP	Microparticle
MSC	Mesenchymal stem cell

PBS	Phosphate buffered saline
PBSA	Phosphate buffered saline with bovine albumin
PECAM-1	Platelet-endothelial cell adhesion molecule 1
PES	Platelet microparticle enriched supernatant
PMP	Platelet microparticle
P-selectin	Platelet selectin
RANTES	Regulated on activation, normal T cell expressed and secreted
ROS	Reactive oxygen species
SC	Stem cell
SCF	Stem cell factor (also known as c-kit ligand)
SCM	Sham injury conditioned media
SDF1α	Stromal cell derived factor 1
SEM	Scanning electron microscopy
SMA	Small mesenteric artery
S-W	Stamper-Woodruff
TNBS	2,4,6-Trinitrobenzenesulfonic acid
TNFα	Tumour necrosis factor α
UC	Ulcerative colitis
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen 4

Chapter 1

INTRODUCTION

1.1 Inflammatory Disorders of the Colon

1.1.1 Inflammatory Bowel Disease

Inflammatory bowel diseases (IBD) are a group of conditions that cause inflammation, particularly of the large bowel or colon. The most common forms of autoimmune chronic IBD are Crohn's disease and ulcerative colitis (UC); however, the aetiology of these life-long diseases still remains unknown. IBD is associated with significant morbidity and mortality as both Crohn's and colitis can develop into gastrointestinal and colon cancers respectively. Within the UK, the incidence of Crohn's and colitis is approximately 1 in 200 people and is predicted to rise (Arnot *et al.*, 2012). In the USA, more than 1 million new cases of IBD are identified annually (Singh *et al.*, 2010). Associated with rising worldwide incidence is the increase in the costs of treatment, with half the costs linked to relapsing patients (Arnot *et al.*, 2012).

Crohn's affects all layers of the entire gastrointestinal tract (GIT) whereas UC primarily affects the lining or mucosa of the colon (Singh *et al.*, 2010). Common symptoms associated with these conditions include diarrhoea, rectal bleeding, urgency and abdominal cramps. The GIT mucosa is lined by a simple epithelial layer which experiences continuous cell loss due to high rates of mechanical attrition, with cells constantly shed into the gut lumen. However, both the mucosal villi of the small intestine and the mucosal crypts of the colon constantly receive new cells and thus have a high turnover rate. This is a highly regulated process involving local stem cells located in the base of the mucosal crypts, referred to as 'intestinal stem cells' (van Leeuwen *et al.*, 2009). These stem cells provide daughter cells which migrate upwards into the intestinal villi or colonic crypts and differentiate either into absorptive (epithelial) or secretory (goblet, Paneth, enteroendocrine) cells. However, following serious tissue injury such as Crohn's disease, UC, ischaemic damage, traumatic injury or necrotising enterocolitis, the auto-regenerative capacity of these local stem cells becomes insufficient to allow complete tissue repair, which therefore leads to long term problems.

1.1.2 Pathology of Colon Colitis Injury

The pathology of colitis, which primarily affects the large bowel or colon, involves oedema, inflammation, ulceration, loss of goblet and crypt cells and decreased mucus production (Koboziev *et al.*, 2010). The many different alterations to the normal intestinal function may be triggered by environmental or genetic susceptibilities, although the exact aetiology of the disease still remains unknown (Cromer *et al.*, 2011). It has also been suggested that, in patients with colitis, the apparent lack of a mucous layer overlying the lining of the colon epithelial cells over-exposes them to large amounts of lumenal bacteria, either host or pathogenic (Cario and Podolsky, 2000). This has been shown to increase the risk of colitis in humans and in mice (Morohoshi *et al.*, 2006, Asquith *et al.*, 2010, Maloy and Powrie, 2011). Receptors on colon epithelial cells, namely toll-like receptors, become activated by lumenal bacteria, which trigger the release of inflammatory cytokines and chemokines. The lack of a protective mucous barrier means bacteria also translocate or gain access between epithelial cells where they can activate resident immune cells in the colon, further promoting cyto/chemokine release from these cells as well (Cario and Podolsky, 2000). Hence the innate and adaptive immune system is activated leading to significant neutrophil and lymphocyte infiltration into the mucosa (Ortega-Cava *et al.*, 2003, Pallone *et al.*, 2003, Singh *et al.*, 2003, Sands and Kaplan, 2007).

Cyto/chemokines are well known to activate cell adhesion molecules (CAMs), including integrins and selectins, on leukocytes and also CAMs on endothelial cells (intercellular adhesion molecule-1 (ICAM-1); vascular cell adhesion molecules (VCAM-1); mucosal addressin cell adhesion molecule-1 (MAdCAM-1)). Interestingly, increased expression of CAMs is associated with increased colitis injury severity and has been demonstrated to occur across several experimental models of UC and in human IBDs (Kawachi *et al.*, 2000, Bardin *et al.*, 2006, Cromer *et al.*, 2011). Soriano *et al.* (2000) further demonstrated that although blockade of both endothelial VCAM-1 and MAdCAM-1 reduced

leukocyte adhesion, only blockade of VCAM-1 attenuated the colitis injury. However, this is not consistent throughout the literature, where several laboratories have identified roles for ICAM-1 in experimental colitis progression as well as VCAM-1 and MAdCAM-1 (Wong *et al.*, 1995, Hamamoto *et al.*, 1999, Farkas *et al.*, 2006, Panes *et al.*, 2007). This variation is likely due to differences between the experimental models used, the severity of the injury or the time point at which inflammation was investigated.

1.1.3 Inflammatory Cells Involved in Colitis Injury

The cellular component of the colitic inflammation is characterised by enhanced leukocyte infiltration into colonic tissue, which is predominantly mononuclear although polymorphonuclear cells are also present in the early stages of the disease (Shimoyama *et al.*, 2001, Koboziev *et al.*, 2010). This cellular component is considered to be a key component in the pathogenesis of colitis. The increased adhesive properties of the injured colon microvasculature triggers substantial recruitment of neutrophils which subsequently leads to tissue degradation and an increase in pro-inflammatory mediators in the surrounding tissue. This subsequently triggers the activation of T cells within the inflamed tissue further exacerbating the immune response (Cromer *et al.*, 2011).

Furthermore, within colitic injured tissue there is often a dysfunction within the antigen presenting components of the tissue leading to poor immune regulation and a resulting heightened immune response (Varol *et al.*, 2009). However, it has also been reported that in colitis tissue, there is not only an increase in activated immune responses but other immunological changes. It has also been identified that in the rat dextran sodium sulfate (DSS)-induced colitis model there was a significant alteration in the immune profile of gut associated lymphoid tissue (GALT), whereby there was an increase in cytotoxic T cell populations with a decrease in helper T cell populations (Lenoir *et al.*, 2011).

In addition to leukocytes, there are over two decades of observations that platelet activity and thrombosis are directly involved in the pathogenesis of colitis, both in experimental animal models and in humans, and is a considerable cause of morbidity (Collins *et al.*, 1994, Collins and Rampton, 1997, Stadnicki, 2012, Tabibian and Streiff, 2012). This is likely to occur in parallel to the increase in coagulation cascade components observed early in IBD (Anthoni *et al.*, 2007, Vowinkel *et al.*, 2007a) as well as an increased thrombogenic phenotype on vascular endothelial cells, as exhibited by an increased platelet-endothelial cell adhesion molecule-1 (PECAM-1) and P-selectin expression (Mori *et al.*, 2005a, Fagerstam and Whiss, 2006). Furthermore, it is interesting to note that people suffering with IBDs have an increased likelihood to having genetic pro-thrombotic disease, whereas those with coagulation disorders rarely develop IBDs (Cromer *et al.*, 2011). However, it is still unknown as to whether platelet activation and coagulation is an initiating event in IBDs or occurs as a consequence of the chronic inflammation (Cromer *et al.*, 2011).

Interestingly, the platelet activation and subsequent adhesion in the microcirculation also results in production of leukocyte-platelet aggregates, which have been suggested to be a potential trigger for a systemic inflammatory response (Irving *et al.*, 2004, Anthoni *et al.*, 2007). Leukocyte recruitment in colitis, as well as an increase in endothelial and immune cell CAMs, has also been shown to be dependent on platelets, which are capable of releasing a host of pro-inflammatory mediators into the injury microenvironment (Mannaioni *et al.*, 1997, Mori *et al.*, 2005a, Vowinkel *et al.*, 2007a, Vowinkel *et al.*, 2007b).

Another consequence of the enhanced platelet activity observed in IBDs is the subsequent increase in the platelet microparticle (PMP) numbers in the blood. Indeed, it has been identified that in patients with IBD there is an increase in circulating PMPs (Andoh *et al.*, 2006, Palkovits *et al.*, 2012) and, interestingly, the increased number has been positively correlated with disease activity (Andoh

et al., 2006). Collectively, these studies highlight the important role platelets play in the pathogenesis of chronic intestinal inflammation.

Additional markers of colitis disease include significant increases in reactive oxygen species (ROS), originating from recruited leukocytes as well as the activated endothelium (Garside, 1999, Egger *et al.*, 2000). Interestingly, it has also been described that platelets isolated from UC patients trigger an increase in ROS production from neutrophils *in vitro* (Suzuki *et al.*, 2001). Damiani *et al.* (2007) investigated the role of oxidative stress and free radical production in the commonly used experimental model of colitis, DSS-induced colitis, in rats. Interestingly, they showed that DSS-induced colitis triggered an increase in ATP-production, which they speculated was the tissue response to greater regenerative requirements, but this concomitantly led to increased ROS production. Furthermore, administration of anti-oxidants and iron chelators, used to prevent production of ROS, substantially reduced colonic mucosal damage (Damiani *et al.*, 2007).

1.1.4 Ischaemia-Reperfusion Injury

In addition to the above mentioned IBDs, ischaemia-reperfusion (IR) injury is also a common inflammatory condition that can affect the small intestine and colon. Intestinal IR injury is associated with high morbidity and mortality in both surgical and trauma patients (Mallick *et al.*, 2004). IR injury occurs as a result of impairment or cessation of blood flow and subsequent restoration of flow to a tissue or organ. This rapid and acute injury is a common occurrence following many surgical procedures, such as organ transplants where the blood supply is inevitably halted, as well as many clinical disorders such as stroke, myocardial infarct and diabetic foot ulcers (Collard and Gelman, 2001). The injury is not just in response to the absence of blood flow, as is described by the term ischaemia (from the Greek *isch* - restriction or thinning, *haema* - blood), but occurs primarily with the return of the blood supply or reperfusion. Although this seems paradoxical in nature, as restoration

of blood flow to an organ is essential to prevent irreversible tissue injury, reperfusion is very highly damaging. For example, Granger and colleagues demonstrated that in a model of intestinal IR, far greater mucosal damage was observed following 3 hours ischaemia and 1 hour reperfusion compared to the damage seen following ischaemia alone (Granger *et al.*, 1986).

However, the damage caused by ischaemia should not be overlooked. With the absence of blood flow, there is the occurrence of hypoxia. This imbalance in oxygen homeostasis leads to many cellular and structural changes. This field has been extensively researched and there are several reviews describing the many outcomes of IR injury (Eltzschig and Collard, 2004, Rodrigues and Granger, 2010). Some of the consequences include changes to vascular endothelium, including cytoskeletal rearrangement leading to cellular contraction and gap formation. Dysregulation of energy metabolism leads to an increase in hypoxanthine production during reperfusion which results in increased ROS and free radical production (Li and Jackson, 2002, Rodrigues and Granger, 2010). More significantly, increases in CAM expression and the production and release of pro-inflammatory cytokines initiates a tissue damaging inflammatory response (Sprague and Khalil, 2009). All of these manifestations contribute to increased leukocyte and platelet recruitment in IR injury, and so similarly to IBD, disturbances in the local microcirculation is a key feature of IR injury (**Figure 1.1**; Rodrigues and Granger, 2010).

Following reperfusion of the previously ischaemic tissue, an exacerbation of the inflammation is observed - a greater increase in the production of ROS, free radicals and other inflammatory molecules (Collard *et al.*, 1999, Li and Jackson, 2002), enhanced adhesion of leukocytes and platelets to endothelia (Vowinkel *et al.*, 2007b, Linfert *et al.*, 2009) and further dysfunction to the vascular endothelium including increased sloughing of endothelium and gap formation (Hernandez *et al.*, 1987, Gautam *et al.*, 2001).

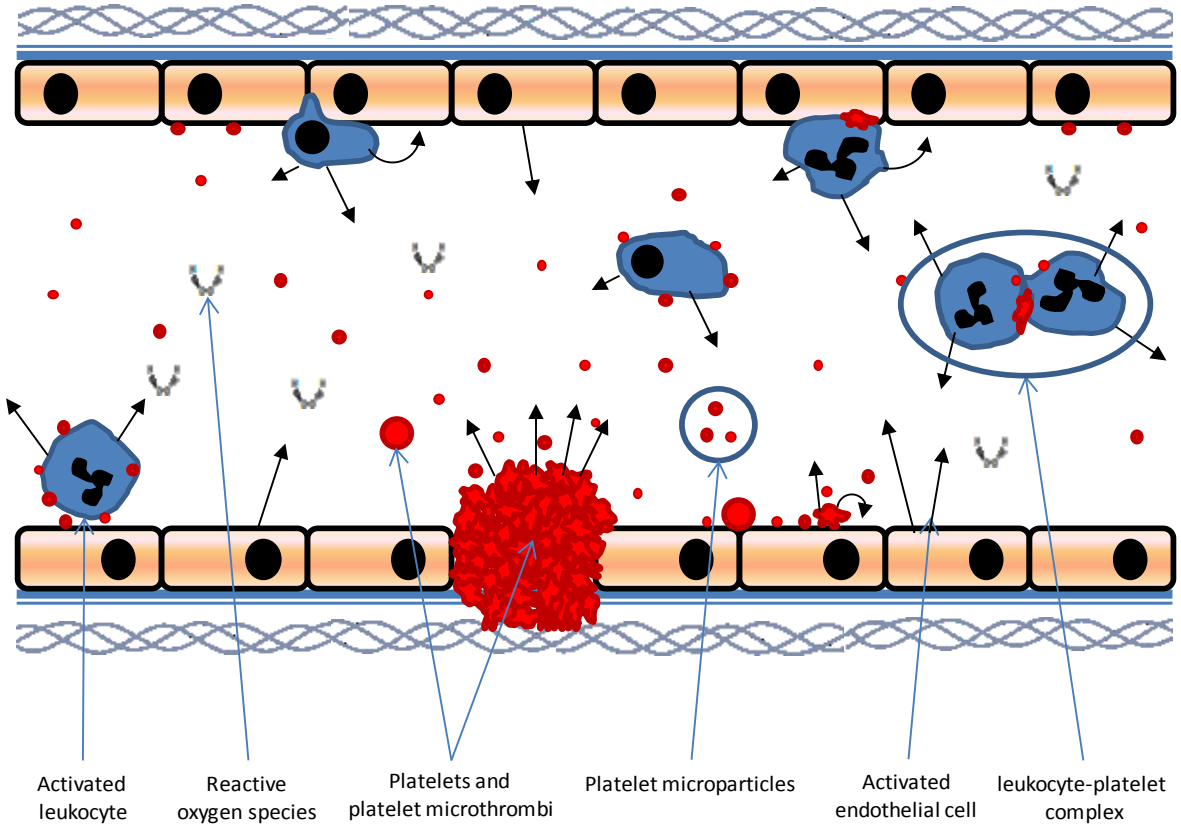

Figure 1.1 Multiple Microcirculatory Effects of Ischaemia-Reperfusion Injury

The IR injury microenvironment is a milieu containing blood cells and a vast repertoire of bioactive molecules. Following colitis and IR stimulus, endothelial cells become activated triggering the recruitment and adhesion of leukocytes, both polymorphic nucleated and mononucleated cells, platelets and microparticles released from many cells, including platelet microparticles. The loss of endothelia and the exposure of the basement membrane trigger the formation of platelet microthrombi. This large body of activated platelets release platelet microparticles that subsequently interact with leukocytes and endothelia triggering further release. However, intact platelets also play a role in neutrophil adhesion to endothelia and also in the formation of leukocyte-platelet aggregates. Furthermore, activated neutrophils release many bioactive molecules such as reactive oxygen species (*e.g.* H_2O_2) and other compounds such as cytokines and chemokines. Similarly, activated endothelium also release a range of bioactive molecules including H_2O_2 and cytokines. Adapted from a figure developed by Dr N Kalia.

IR injury of many organs can lead to severe clinical outcomes such as direct organ failure, multi-organ dysfunction syndrome (MODS) and systemic inflammatory response syndrome (SIRS) (Arumugam *et al.*, 2004, Stallion *et al.*, 2005). However, the most susceptible organ to IR injury is the intestine (Granger *et al.*, 1986, Yamamoto *et al.*, 2001). Intestinal IR injury is associated with several pathologies where reduction in gut perfusion is followed by restoration of flow e.g. shock and vasculitis (Kong *et al.*, 1998, Carden and Granger, 2000). It most commonly results from a number of surgical procedures, including thoracoabdominal aorta aneurysm repair, cardiopulmonary bypass and small bowel transplantation (Banz *et al.*, 2008, Lazaris *et al.*, 2009, Sfyroeras *et al.*, 2010). Furthermore, it has been identified that mortality associated with intestinal IR injury can be as high as 80% (Redaelli *et al.*, 1998).

The small intestinal mucosa undergoes extreme damage following the induction of experimental IR injury, which can be induced by occlusion of the superior mesenteric artery (SMA). Even relatively short durations of ischaemia can elicit substantial localised tissue injury (Souza *et al.*, 2005, Li *et al.*, 2009a, Kavanagh *et al.*, 2010, Santen *et al.*, 2010, Kavanagh *et al.*, 2012). The SMA is the major artery supplying blood to the intestines, from the duodenum (start of small intestine) to the transverse colon. The descending colon, sigmoid colon and part of the rectum are also supplied by the inferior mesenteric artery, thus, occluding the SMA induces IR injury primarily within the ascending and transverse colon (anatomy of colon shown in **Figure 1.2;** taken from: http://www.cedars-sinai.edu). Although it has been experimentally established that SMA occlusion has a lesser injurious effect on the colon compared to the small intestine (Leung *et al.*, 1992), the colon nevertheless still undergoes significant structural and microvascular injury, as evidenced by increased leukocyte recruitment and greater local levels of ROS (Riaz *et al.*, 2002b). The colon is the most prevalent location for the clinical manifestation of intestinal ischaemia and occurs in patients

with IBD as well as other pathologies. Colonic IR injury is associated with hypermotility, abdominal pain, increased mucosal permeability, colonic ulceration and bacterial translocation (Parodi *et al.*, 1987).

1.1.5 Microcirculatory Disturbances Associated with Ischaemia-Reperfusion Injury

IR injury is reported to be a biphasic occurrence and in both phases the pathological changes are primarily mediated through alterations in the vascular endothelium (Rodrigues and Granger, 2010). The first phase of reperfusion injury, which occurs within 1-2 hours, causes vascular endothelium to rapidly up-regulate CAMs such as ICAM-1 and PECAM-1. In the second stage, approximately 3-4 hours post-ischaemia, vascular endothelia has been reported to up-regulate different CAMs such as VCAM-1 and MAdCAM-1 (Shigematsu *et al.*, 2002). The increased number of CAMs results in increased recruitment of leukocytes, further exacerbating injury responses (**Figure 1.1**).

Vascular endothelia also increase levels of soluble inflammatory mediators such as tumour necrosis factor-α (TNFα) and interleukin-1 (IL-1) in most inflammatory responses (Santen *et al.*, 2010). The release of these mediators further contributes to leukocyte recruitment and activation (**Figure 1.1**), which in turn, further exacerbates the inflammation. Furthermore, the increased production of hypoxanthine in endothelial cells following IR leads to the increased production of ROS (Collard and Gelman, 2001). There are several reports demonstrating that the concentration of ROS produced can elicit two responses; direct oxidative damage to lipids and genetic material (Riaz *et al.*, 2002b) and also activation of leukocytes via the phosphorylation of several regulatory pathways (Irani, 2000). Furthermore, ROS, in the presence of transition metals, often form highly oxidative and cytotoxic hydroxyl radicals (Damiani *et al.*, 2007).

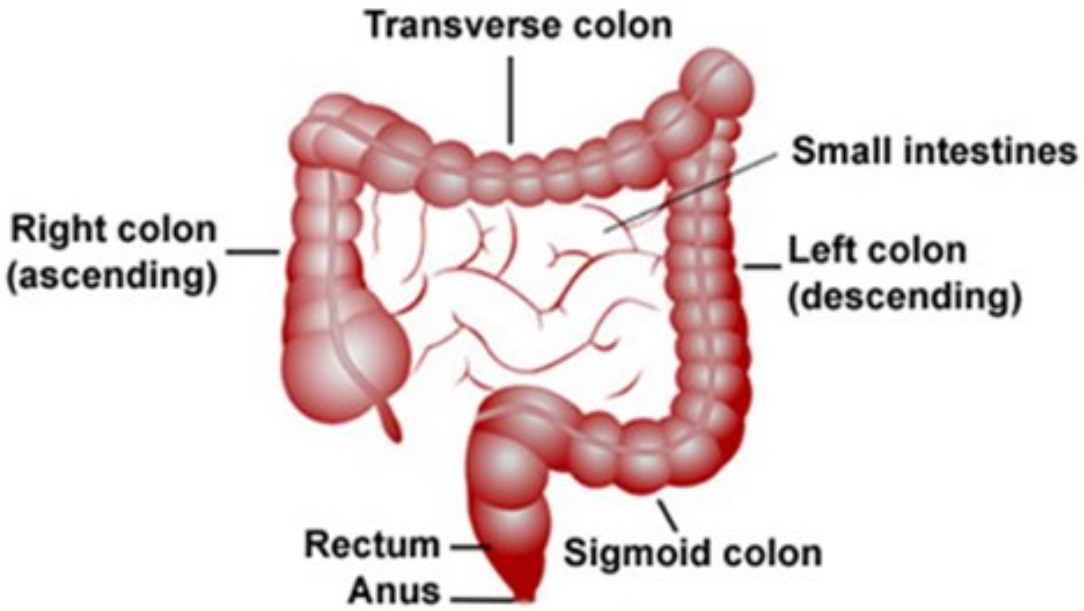

Figure 1.2 Cartoon schematic of the large intestine

The large intestine is comprised of 5 components: the ascending, transverse, descending and sigmoid colon, followed by the rectum and terminating in the anus. The SMA supplies blood to the small intestines and the proximal regions of the colon, namely the ascending and transverse colon. The SMA itself lies beneath the small intestines (thus is not seen in this image) with the mesentery branching throughout the gut. The other regions are supplied by the inferior mesenteric artery. When the SMA is occluded experimentally it results in tissue ischaemia of the small intestine and the proximal regions of the colon, however, the damage is much more severe within the small intestines than the colon. Figure taken from: http://www.cedars-sinai.edu.

Interestingly, the biphasic response is also seen in the neutrophil recruitment pattern, with an initial recruitment shortly after injury, and a second recruitment seen during the latter phase (Victorino *et al.*, 2009). The increased levels of adherent neutrophils lead to further endothelial dysfunction (Victorino *et al.*, 2009). However, neutrophils and other inflammatory leukocytes within the injured environment also produce and release inflammatory mediators into the injury milieu (**Figure 1.1**). Similar to the endothelium releasate, the neutrophils release ROS such as hydrogen peroxide (Lu *et al.*, 2012), cytokines (Sprague and Khalil, 2009) and other inflammatory mediators (Linfert *et al.*, 2009), which in turn, activates endothelium and increases recruitment of inflammatory leukocytes (Heidemann *et al.*, 2007).

The recruitment of leukocytes is often mirrored by that of platelets (Nakano *et al.*, 2008, Rodrigues and Granger, 2010). Platelet activation occurs in many pathological conditions, including IR injury (Yin *et al.*, 2008, Esch *et al.*, 2010), rheumatoid arthritis (Boilard *et al.*, 2010) and colitis injury as described earlier (Andoh *et al.*, 2005, Andoh *et al.*, 2006). The recruitment of platelets exacerbates gut IR injury either through interactions with the intact endothelium or through interactions with sub-endothelial matrix proteins such as collagen that become exposed after endothelial denudation. This latter event is associated with aggregation of platelets and microthrombus formation which often leads to microvessel occlusion and poor blood flow within the reperfused gut (Holyer, 2011). Indeed, the Kalia group has previously shown that inhibiting thrombus formation can attenuate intestinal IR injury and improve perfusion (Holyer, 2011). Furthermore, platelet activation also triggers the release of many bioactive molecules including alpha-granules, cytokines and platelet microparticles (PMPs), which will be discussed later (Thiagarajan and Tait, 1991, Brown and McIntyre, 2011).

1.2 Current Therapies for Inflammatory Bowel Disorders

Current therapies, including anti-inflammatory and immunosuppressive drugs, tend to suppress the symptoms of IBD rather than be curative (Schmidt *et al.*, 2010, Coburn *et al.*, 2012). They remain inadequate primarily due to multiple side-effects, including severe toxicity, with no long-term efficacy observed. Current clinical practice involves using the safest drugs with the least side-effects first, which include the aminosalicyclates and antibodies that target leukocyte sub-populations (Lichtenstein *et al.*, 2009). If these fail to be beneficial, corticosteroids and finally anti-tumour necrosis factor-α (anti-TNFα) treatments are used, but the latter display the greatest side effects (Garcia-Bosch *et al.*, 2010). Although these therapies are effective at inducing and maintaining remission, the occurrence of side effects, varying effectiveness within clinical populations and the loss of effectiveness over time limit their long-term use (Lichtenstein *et al.*, 2009, Singh *et al.*, 2010). For example, corticosteroids may trigger mucosal healing in Crohn's but cannot maintain it (Lichtenstein *et al.*, 2009, Armuzzi *et al.*, 2012, Dave and Loftus, 2012). A review of clinical trials, used to establish guidelines in the management of Crohn's disease, highlighted that many existing treatments, including mesalamine, sulfasalazine and metranidazole, failed to reproducibly provide clinical efficacy (Lichtenstein *et al.*, 2009).

Most IBDs are composed of two pathologies – intestinal tissue damage and immunological dysfunction. Thus far, no drugs used alone or in combination are capable of actively targeting both problems; hence explaining their poor effectiveness and high remission rates (Singh *et al.*, 2010). Thus, alternative therapeutic strategies are urgently needed for patients who do not respond to or are intolerant of currently available treatments. One of the major considerations for treating a whole host of inflammatory and immunological disorders, including IBD, is the use of cellular therapy, particularly exogenously administered stem cells (Garcia-Bosch *et al.*, 2010, Singh *et al.*, 2010).

1.3 Stem Cells for Treatment of Inflammatory Bowel Disease

1.3.1 Bone Marrow-Derived Stem Cells

For a cell to be termed a stem cell it has to be capable of two properties: self-renewal and potency. Self-renewal refers to a cells ability to divide, but with one of the daughter cells retaining the original phenotype of the mother cell. This is essential for maintaining the stem cell population. Potency describes the process in which a cell can differentiate to a more specialised cell type that is distinct from the mother cell. There are a number of stem cells within the adult body which are required for the constant renewal of several tissues.

For example, bone marrow (BM)-residing haematopoietic stem cells (HSCs) constantly replenish the cellular constituents of the blood. Similarly, as described earlier, intestinal stem cells constantly renew the mucosal lining of the GIT. However, an increasing number of studies have demonstrated that some populations of endogenous stem cells, particularly those derived from the BM, can also repair or regenerate injured tissue following a number of ischaemic or degenerative diseases (Komori *et al.*, 2005, Oyama *et al.*, 2005, Khalil *et al.*, 2007, Chen *et al.*, 2008, Lanzoni *et al.*, 2008, Bussolati *et al.*, 2009, Okumura *et al.*, 2009, Schlechta *et al.*, 2010). It is not clear which stem cells may be most useful for regenerative purposes. Although embryonic stem cells (ESCs) would provide a more plastic and thus more flexible therapeutic option, their use is ethically questionable. Furthermore, there is a significant concern of teratoma formation with the use of ESCs (Ratajczak *et al.*, 2012). The use of adult stem cells is more desirable due to ethical considerations, demonstrable clinical and experimental success and relative ease of availability.

Several stem and progenitor cell populations exist with the adult BM, including HSCs, mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs). Accumulating evidence in the past decade or so has demonstrated that, in addition to having their own specialised role, all three of these BM-

derived stem/progenitor cell populations have the ability to contribute to the repair and regeneration of damaged or degenerative tissue. For example, EPCs have been reported to circulate within the peripheral blood and contribute to vasculogenesis and maintenance of the vasculature (Leone *et al.*, 2009). There are also multiple reports indicating that both HSCs and MSCs can confer benefit to a variety to digestive disorders in clinical and experimental studies (Leone *et al.*, 2009, Okumura *et al.*, 2009, Wei *et al.*, 2009).

1.3.2 Haematopoietic Stem and Progenitor Cells

HSCs are essential for homeostasis as their primary role is to constantly replenish the cellular components of the blood (**Figure 1.3**). HSCs were first identified by Till and McCulloch (1961) and were termed "colony forming units (CFUs)". These cells were defined by the ability to give rise to haematopoietic nodules in the spleen post-BM transplantation in irradiated animals (Till and McCulloch, 1961, Till *et al.*, 1964). HSCs are typically identified through their surface marker phenotype with the most common identity being c-kit^{+} Sca-1^{+} Lin^{-}; termed KSL cells (Wognum *et al.*, 2003). CD34 is also commonly used as a marker for human HSC populations (Civin *et al.*, 1984), but it is not a murine HSC marker as ~90% of murine HSCs do not express CD34 (Osawa *et al.*, 1996). However, HSCs have also been shown to play an active role in immune regulation (King and Goodell, 2011) and have also been shown to be capable of extramedullary repair.

1.3.3 Haematopoietic Stem Cells for Inflammatory Bowel Disease

HSCs have been considered a potential source of stem cells for the purpose of regenerative medicine (Herzog *et al.*, 2003, Garcia-Bosch *et al.*, 2010, Kavanagh and Kalia, 2011) and have been shown to be beneficial in several injury states such as colitis, myocardial infarction and renal dysfunction (Lanzoni *et al.*, 2008, Bussolati *et al.*, 2009, Schlechta *et al.*, 2010). The beneficial effects of HSCs for IBD was

initially observed following early case reports of remission in patients undergoing HSC Transplantation (HSCT) for the treatment of haematological malignancies (Oyama *et al.*, 2005, Lanzoni *et al.*, 2008, Glocker *et al.*, 2009, Garcia-Bosch *et al.*, 2010).

Since these initial observations, a number of HSCT clinical trials for IBD have been conducted in which autologous HSCs are mobilised, collected from the patient's peripheral blood and then re-infused following myeloablation (Oyama *et al.*, 2005, Burt *et al.*, 2010). An ongoing clinical trial called ASTIC (Autologous Stem Cell Transplant for Crohn's Disease), headed by Professor Chris Hawkey at the University of Nottingham, has been investigating the use of autologous HSCT for Crohn's disease as an alternative therapy to conventional options with results expected in 2013. Early analyses report a significant reduction in clinical injury index and improved tissue repair in a high number of patients, with several requiring no further drug therapy (Hawkey, 2012). However, they have also revealed that there was a high level of risk with infection occurring frequently. This highlights that HSCT, rather than systemic infusion, may only be a viable option for a limited number of patients (Garcia-Bosch *et al.*, 2010, Hawkey, 2012). There has also been considerable investigation carried out by the Burt laboratory. They have also undertaken HSC therapy clinical trials, and similarly to ASTIC report a significant improvement in Crohn's disease and indeed even induce remission (Oyama *et al.*, 2005, Burt *et al.*, 2010).

However, none of these HSCT trials have identified a cure for IBD as recurrence rates remain high. HSCT is essentially a form of immune suppressive therapy in which drugs are firstly used to ablate or suppress the immune system to remove disease causing immune cells, with HSCs infused to regenerate a new, antigen-naive immune system. However, recent experimental data has demonstrated that exogenous HSCs can themselves confer therapeutic effects even in the absence of myeloablative or immunosuppressive drugs.

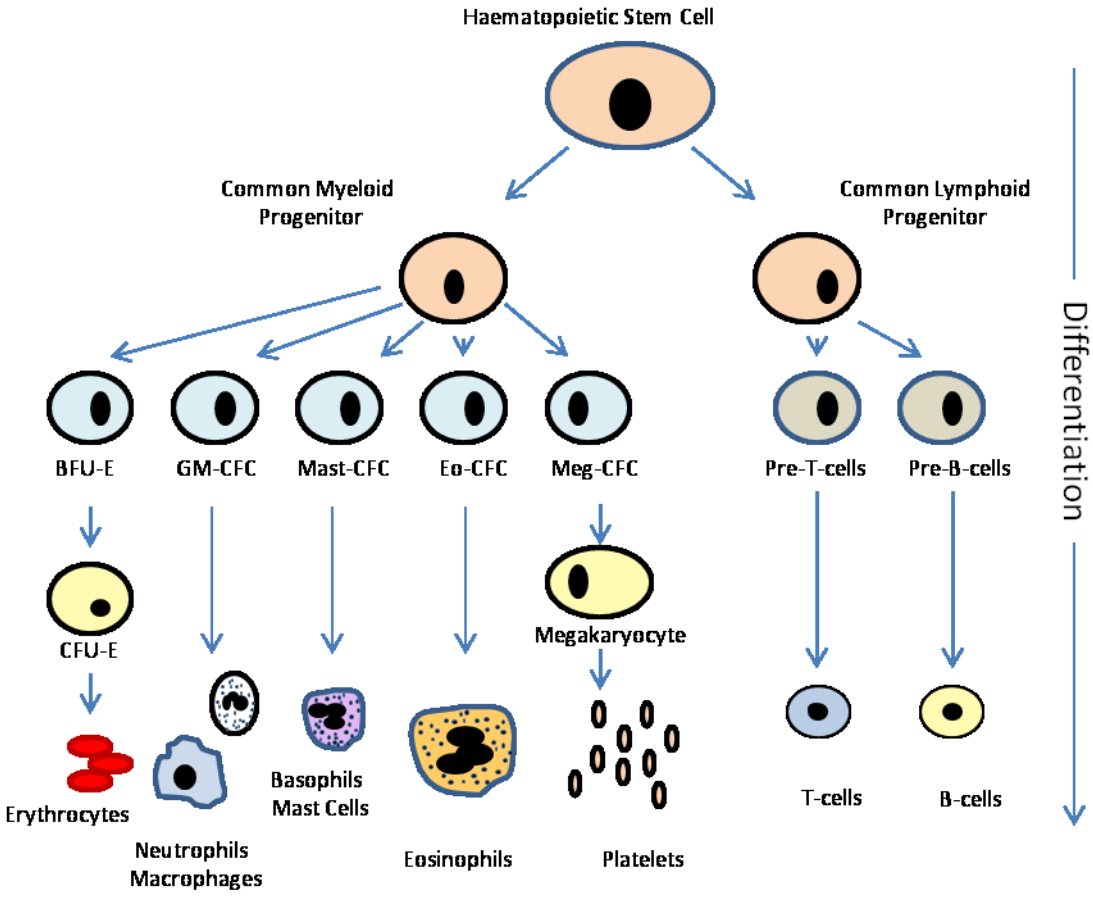

Figure 1.3 Haematopoietic stem cells constantly replenish blood

The haematopoietic or blood system is constantly replenished from HSC populations within the bone marrow. This is achieved through two major differentiation pathways - myeloid or lymphoid routes. The routes are entered via HSC differentiation into either the common myeloid progenitor, giving rise to red blood cells and the cells of the innate immunity, or the common lymphoid progenitor, providing the cells of the adaptive immune system.

1.4 Mechanisms by which Haematopoietic Stem Cells are Beneficial

1.4.1 Direct Repair of Injured Tissues by Haematopoietic Stem Cells

Although it is not fully established how HSCs confer benefit in injured tissues, several potential mechanisms have been described. One mechanism is the differentiation of the HSC into the required host cell. Okamoto and colleagues identified BM-derived HSCs within the GIT of patients undergoing HSCT for the treatment of differing leukaemic and myeloid disorders. They reported that significant numbers of HSCs within the gut had differentiated into epithelial cells (Okamoto *et al.*, 2002). A further role for stem cell differentiation was demonstrated in studies in which HSCs were transplanted into IBD patients also suffering from malignant cancers. Donor HSC-derived epithelial cells were found within the inflamed bowels of these patients, which was associated with a concomitant reduction in their injury scores (Ishikawa *et al.*, 2004, Khalil *et al.*, 2007).

A number of experimental studies have also suggested HSC differentiation as a putative mechanism by which HSCs mediate tissue repair. Indeed, BM-derived cells are recruited to inflamed regions of the colitic mouse colon and contribute to tissue repair by differentiation into vascular smooth muscle cells, endothelial cells, pericytes, or epithelial cells (Brittan *et al.*, 2005). Furthermore, Komori and colleagues showed that GFP-expressing BM cells occupied 37.6% and 4.25% of the rat colonic epithelium at 28 and 56 days respectively after TNBS-induced colitis (Komori *et al.,* 2005). The authors suggested that the BM-derived cells induced regeneration through differentiation into colon cells, but they did not distinguish which population of stem cells within the BM were involved.

Interestingly, Wei and colleagues (2009) demonstrated that exogenously administered HSCs and MSCs could equally populate the colonic mucosa of following TNBS-induced colitis. Furthermore, both cell types were able to improve the gross morphology of the colon with administration of both stem cell types together only improving the morphology slightly more than that of HSC or MSC alone.

Although the reparative mechanisms were not investigated, it was again postulated to be mediated through integration of stem cells into the epithelial layer (Wei *et al.*, 2009).

Another potential mechanism by which HSCs have been reported to aid in repair is through stem cell-host cell fusion. This mechanism involves formation of heterokaryons consisting of the donor stem cell and the host target cell. This phenomenon had been put forward to provide an alternative mechanism to explain the plasticity seen following stem cell repair. It has been reported that *in vitro* cultured stem cells can fuse with differentiated cell types to form tetraploid cells that exhibit cell markers typical of the differentiated cells, whilst maintaining pluripotency markers (Terada *et al.*, 2002, Ying *et al.*, 2002). It has been demonstrated culturing embryonic SCs with B cells and fibroblasts can result in fusion and subsequent reprogramming of the target cells to a pluripotent state (Tsubouchi and Fisher, 2013, Tsubouchi *et al.*, 2013).

However, the mechanism of stem-donor cellular fusion has not been fully elucidated, primarily because it has not been demonstrated to occur *in vivo* with adult stem cells. For example, Okamoto *et al.* extensively studied the GIT following HSC treatment and whilst observing engrafted donor HSCs, they failed to observe any heterokaryons formed by cellular fusion (Okamoto *et al.*, 2002).

Many of these early studies demonstrating differentiation and fusion have not since been reproduced. It is also difficult to conceptualise how altering the phenotype of a very small number of cells within a damaged organ would be capable of exerting such an effect as to enable organ repair, as, due to the limited recruitment of stem cells seen even shortly after infusion, there would simply be too few cells to have a significant effect.

Furthermore, many, but not all, of the observations of stem cell fusion can be explained via horizontal transfer of molecules between cells. As is described below, microparticle production of cells often results in the transfer of genetic, soluble and membranous material, which can explain the presence of cytosolic dyes, donor cell phenotype change through surface markers as well as a change in expression profile (Ratajczak *et al.*, 2012). Indeed, horizontal transfer of surface, mRNA and miRNA molecules has been observed to occur *in vitro* with a detectable frequency (Janowska-Wieczorek *et al.*, 2001, Mause *et al.*, 2005, Meziani *et al.*, 2008, Burnier *et al.*, 2009, Tang *et al.*, 2010).

Overall, the general consensus is that HSCs, and indeed other stem cells such as MSCs, are unlikely to confer therapeutic benefit *in vivo* via cellular fusion or trans-differentiation, however, these mechanisms cannot be ruled out wholly.

1.4.2 Paracrine Effects of Haematopoietic Stem Cells

For inflammatory intestinal disorders, particularly Crohn's disease, UC and IR injury, immune modulation is a key component of their treatment (Lichtenstein *et al.*, 2009, Schmidt *et al.*, 2010, Coburn *et al.*, 2012). More recent evidence suggests that stem cells confer benefit in these disorders through a paracrine effect whereby they release anti-inflammatory or immunomodulatory factors. Stem cells can produce and release an array of cytokines and trophic mediators that can act upon blood and tissue cells (Caplan and Dennis, 2006).

This release of immunomodulators can then allow for the dampening or limiting of pro-inflammatory cell recruitment and promote recruitment of resolution stage leukocytes (Chen *et al.*, 2008). Furthermore, it has been reported that this manner of signalling by stem cells not only effects the cells of the bloodstream and immune system, but may induce the proliferation of endothelial cells to repair damaged vasculature (Aguilar *et al.*, 2009).

It is well documented that MSCs are immunomodulatory (Caplan and Dennis, 2006). They have been shown to impair T-cell priming *in vivo* by limiting interaction of T-cells with dendritic cells (Chiesa *et al.*, 2011), and have also been shown to be capable of modulating leukocyte behaviour *in vivo* (Caplan and Dennis, 2006). Although an anti-inflammatory and immunomodulatory effect has been well described for MSCs, there is little evidence that HSCs also have similar capabilities. This is not likely due to the inability of HSC to immunomodulate, but most likely due to a paucity of investigation within this area. For example, recent data from the Kalia laboratory has demonstrated that within 4 hours of systemic HSC infusion into a mouse undergoing intestinal IR injury, leukocyte infiltration is significantly reduced (Kavanagh *et al.*, 2013).

Emerging evidence suggests stem cells may also undertake a paracrine repair mechanism through the release of bioactive lipid vesicles. Indeed stem cells are capable of, and often do, produce microparticles (Ratajczak *et al.*, 2012). It has been shown that microparticles harvested from stem cell cultures exhibit the same level of repair as the stem cells themselves (Quesenberry *et al.*, 2010, Gatti *et al.*, 2011). Although their role and the mechanism in which stem cell microparticles confer beneficial effects in repair have not been definitively explained, there is evidence that MPs are able to shuttle a variety of membrane molecules, such as S1P and C1P (both of which limit apoptosis whilst stimulating angiogenesis; Ratajczak *et al.,* 2012), mRNA molecules, including those that transcribe for pluripotency markers, tissue specific markers and chemokine transcripts, all of which have been demonstrated to enhance liver and kidney repair (reviewed in Ratajczak *et al,.* 2012).

The observations that HSC can directly mediate tissue repair as well as have indirect paracrine effects suggests stem cells may confer benefit via a two-pronged approach; through immune modulation and through direct repair of tissue by donor derived HSCs, thus making them an attractive therapeutic option for treatment of IBD.

1.5 Stem Cell Homing

As previously mentioned, existing therapies for inflammatory disorders of the bowel are not adequate. Studies suggest adult BM-derived stem cells, including HSCs, may be a useful therapy for treating inflammatory and ischaemic digestive disorders. Clinical studies have been primarily HSCT investigations in which HSCs are delivered following immunoablation. However, experimental studies suggest that simply injecting HSCs either directly into the injured tissue or systemically can confer benefit. Despite evidence that HSCs improve intestinal problems, success is either minor or transitory in both clinical and experimental studies. The rarity of HSCs and MSCs (<0.01% & 0.001% of BM respectively) has partly contributed to their limited clinical utility and success due to their limited availability (Karp and Leng Teo, 2009).

It has also been suggested that the poor efficacy of cellular therapy may be a result of limited recruitment of injected stem cells to the target area (Kinnaird *et al.*, 2004). Indeed Khalil and colleagues conducted an experimental study in which HSCs were injected into mice with colitis injury (Khalil *et al.*, 2007). However, they noted limited efficacy, which they and others have postulated is due to limited stem cell numbers found within the tissue following infusion (Jackson *et al.*, 2001, Strauer and Kornowski, 2003, Kinnaird *et al.*, 2004). Therefore, when delivered by the preferred systemic route, poor homing and a subsequent low efficiency of tissue engraftment occurs, processes essential for stem cells to mediate repair (Karp and Leng Teo, 2009, Schulz *et al.*, 2009). Furthermore, the large size of cultured stem cells, particularly MSCs, has meant that post-infusion, many cells are lost to the circulation as they become entrapped within organs such as the lungs. This limits the number of circulating stem cells that are available for recruitment to injured sites (Fischer *et al.*, 2009).

It is therefore likely that increasing the number of exogenously administered stem cells that become adherent within the injured colon microcirculation may improve their efficacy for IBD patients (Kinnaird *et al.*, 2004, Kavanagh and Kalia, 2011). Thus, identifying methods to enhance the stem cell recruitment following systemic infusion would provide an alternative, safer and more economically viable option to HSC transplantation.

Developing methodologies that improve stem cell trafficking/homing and recruitment into injured tissues is a high priority for cellular therapies and could potentially confer more efficient, rapid and longer lasting tissue recovery. Indeed, transfecting plasmid DNA encoding for stromal derived factor-1α (SDF-1α), a potent stem cell chemoattractant, into ischaemic muscle enhances endothelial progenitor cell recruitment (Hiasa *et al.*, 2004). However, delivery of vectors to allow for targeted chemokine expression has been challenging, particularly in the clinical setting. Thus, non-invasive techniques that do not require introduction of genetic material are appealing. To enhance stem cell adhesion and derive a targeted therapy regime for IBD, the adhesive mechanisms governing stem cell recruitment to the injured colon need to firstly be elucidated. However, there are currently no studies that have determined these events in the diseased colon.

1.5.1 Kinetics of Haematopoietic Stem Cell Recruitment

Limited data has identified the adhesive mechanisms utilised by HSCs to home to injured sites. There are multiple reports that HSC can be captured from flow and that stable adhesion is integrin mediated (Frenette *et al.*, 1998, Kavanagh and Kalia, 2011, White *et al.*, 2013). Furthermore, HSCs have been shown to share a similar adhesion molecule phenotype to that of leukocytes (Kobayashi *et al.*, 1994, Turner *et al.*, 1995), suggesting that their recruitment is likely to follow a similar pathway.

The leukocyte adhesion cascade was first described by Cohnheim well over a century ago (Cohnheim, 1877) and has since been substantially developed. The current paradigm was first proposed in 1991 and involves the capture, rolling, firm adhesion and transmigration of leukocytes (Butcher, 1991). The capture stage is involved in the slowing of leukocytes from the vascular flow to enable the initial transient interactions between the leukocyte and the vascular endothelium. The initial interactions are predominantly mediated via selectins and cadherins that are expressed on leukocytes, platelets, endothelia and other cell types such as dendritic cells (Ley, 1996, Vowinkel *et al.*, 2007b). These reversible interactions enable rolling of the leukocytes along the vessel wall so that stable adhesion can subsequently occur. With the many different proteins involved in rolling it enables organ and site-specific interaction through a vast number of combinatorial interactions (**Figure 1.4**; Ley, 1996).

There have been few observations of HSC rolling within tissue microvasculature. Reports from the Kalia laboratory have found that HSC rolling could only be observed within muscle vasculature and not intestinal microvasculature (Kavanagh *et al.*, 2013). Furthermore, unpublished results from the Kalia laboratory have found that HSCs only exhibit stable adhesion in tissues that have low blood shear rates such as the hepatic microcirculation, and do not roll. Again this is similar to leukocyte behaviour, where it has been reported that leukocyte rolling is not a prerequisite for stable adhesion in the injured liver sinusoidal capillaries (Kubes *et al.*, 2002).

Activation of the tethered cell is crucial for the cell to achieve firm adhesion. This is modulated by factors released from the local endothelium and, more importantly, the duration the cell remains in contact with the local endothelium (Jung *et al.*, 1998). The firm adhesion is mediated by CAMs expressed on the circulating cell and its counter-receptor on the endothelial cells, such as the integrin-ligand interaction VLA-4 with VCAM-1 (**Figure 1.4**). This stage of recruitment is essential for many cells. For example, T cells lacking the integrin $\alpha L\beta 2$ (LFA-1) exhibit poor activation and T cell

priming (Kasirer-Friede *et al.*, 2007), as well as poor homing to target tissues. Also, T cells lacking the integrin α4β7 display impaired homing to gut associated lymphoid tissue (Lefrancois *et al.*, 1999, Johansson-Lindbom *et al.*, 2003). It has similarly been hypothesised by several groups, that integrin mediated adhesion is also a necessary pre-requisite for stem cell recruitment (Kumar and Ponnazhagan, 2007, Sordi, 2009, Kavanagh and Kalia, 2011).

1.5.2 Site Specific Mechanisms Govern HSC Recruitment to Injured Tissue

Although much is known regarding leukocyte recruitment, including mechanisms, site specific behaviour and consequences of the adhesion interactions, little data has been generated with regards to stem cells. Early intravital microscopy and histological studies initially described the mechanisms governing HSC recruitment to BM microcirculation.

Similar to that seen with leukocytes, within the BM microcirculation HSCs have been observed to roll prior to firm adhesion (Frenette *et al.*, 1998). HSC rolling has also been identified within cremaster muscle post-capillary venules (Kavanagh *et al.*, 2013). In the BM, firm adhesion was primarily selectin mediated with a major role identified for the cell surface glycoprotein CD44 (Frenette *et al.*, 1998, Avigdor *et al.*, 2004). Interestingly, the VLA-4 / VCAM-1 pathway only appeared to play a role in HSC adhesion when P- and E- selectin adhesion was antibody blocked (Frenette *et al.*, 1998). It was also found during these studies that interfering with the adhesion interactions, on either stem cell or endothelium, was sufficient to inhibit recruitment to these organs (Frenette *et al.*, 1998, Avigdor *et al.*, 2004) highlighting the key role CAMs play in stem cell recruitment. Following the stable adhesion of leukocytes and the subsequent activation of adhesion, leukocytes will often transmigrate (**Figure 1.4**), however, there is limited information regarding the transmigration of HSC. Observations from

within the Kalia laboratory have yet to see any evidence of HSC transmigration within 4 hours of recruitment (*pers. comms.*), but long term studies have seen HSC engraftment within tissue away from the vasculature (Khalil *et al.*, 2007, Tanaka *et al.*, 2008, Wei *et al.*, 2009).

Later studies focussed on the adhesive mechanisms mediating HSCs recruitment to injured non-medullary tissues, with major contributions to the field coming from the Kalia laboratory. Stem cell recruitment to peripheral tissues, such as the injured murine liver, heart and intestines, is primarily integrin mediated (Jackson *et al.*, 2001, Kumar and Ponnazhagan, 2007, Karp and Leng Teo, 2009, Schulz *et al.*, 2009, Kavanagh and Kalia, 2011). Indeed, the integrin VLA-4 (CD49d/CD29, $\alpha_4\beta_1$) and its interaction with endothelial VCAM-1 has shown to be involved in HSC recruitment to the injured mouse liver capillaries or sinusoids (Kavanagh *et al.*, 2010), a similarity shared with leukocyte recruitment to the same organ (Alon *et al.*, 1995).

The concept of site specific mechanisms governing HSC recruitment gains more importance when we note that recruitment to injured murine kidney utilises integrins but also other CAMs such as the glycosaminoglycan (GAG) CD44. Indeed, antibody blockade of CD49d or CD44 results in near total amelioration of HSC recruitment to the kidney (White *et al.*, 2013). Interestingly, CD44 has also been demonstrated to be critical in mediating MSC homing to injured murine liver (Aldridge *et al.*, 2012). Recruitment to the injured small intestine is independent of CD49d, but dependent on the CD18-ICAM pathway (Kavanagh *et al.*, 2013), whereas, adhesion within the cremaster muscle is dependent on both CD18 and CD49d, but with a greater reliance on CD49d (Kavanagh *et al.*, 2013).

Overall, these studies have highlighted that HSC surface adhesion molecules play a critical role in the homing of injected stem cells to not only the BM but also to sites of injury. Therefore, investigation into the adhesive mechanisms utilised by stem cells may allow for the development of strategies to

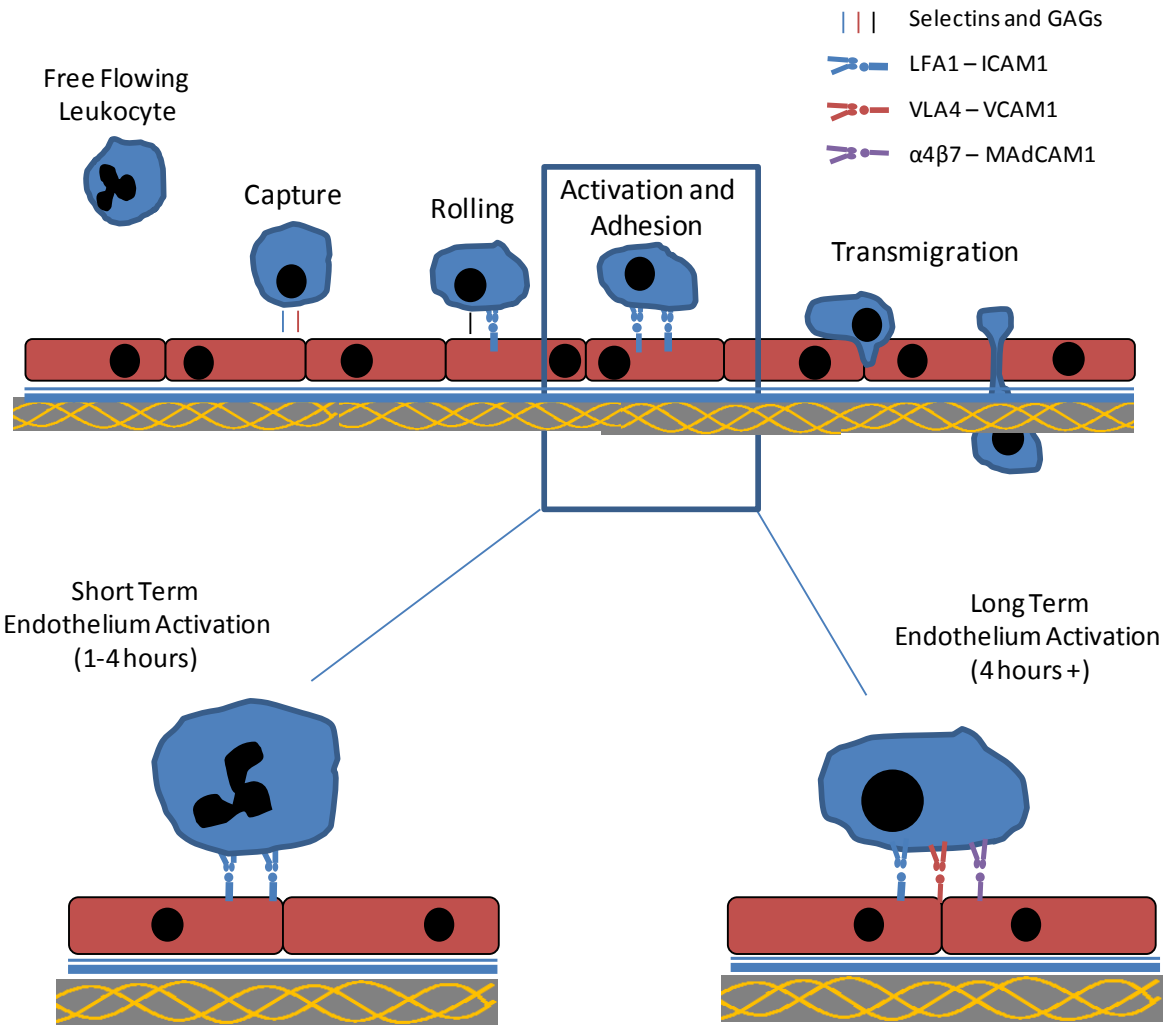

Figure 1.4 Cell Adhesion Cascade

The cell adhesion cascade has been investigated using a range of cell types (predominantly leukocytes) and different injury and stimuli. However, in most physiological settings the principles are the same, but using a variety of differing adhesion molecules and indeed combinations of molecules. The cartoon above demonstrates the initial capture and rolling of leukocytes, predominantly involving selectins. Rolling allows the cell to be slowed and is often a pre-requisite for firm adhesion. It also allows for cells to become activated by increasing the exposure to membrane bound molecules such as cytokines. Firm adhesion requires integrins and their counter-ligands. However, there are tissue specific, injury and temporal differences in the integrin combinations used. For example, depicted is the temporal difference in intestinal endothelial CAM expression. During the early phase of reperfusion, ICAM1 is the major endothelial CAM evident. However, following several hours of reperfusion, intestinal endothelia increase their surface expression of VCAM1 and MAdCAM1, further facilitating leukocyte adhesion.

increase their recruitment and potentially increase their clinical efficacy (Kinnaird *et al.*, 2004, Lanzoni *et al.*, 2008, Kavanagh and Kalia, 2011). It is well established from studies on leukocytes that integrin behaviour, and therefore adhesion, can be modulated via several mechanisms.

1.5.3 Integrin Mediated Circulating Cell Adhesion

Integrins are a protein family of molecules important in cell-cell and cell-extracellular matrix adhesion. 24 integrin heterodimers have been identified, which are made up of 18α and 8β subunits forming heterodimer complexes of non-covalently bound α and β subunits (Springer and Dustin, 2012). Both subunits consist of large extracellular N-terminus domains, a single span transmembrane domain and C-terminus cytoplasmic domains (Springer and Dustin, 2012). The ligand binding domain of integrins is formed of a binding pocket composed of the extracellular domains of the α and β subunits. Some integrins bind ligand primarily with the αI domain, however, some integrins that lack this domain bind primarily with the βI domain (Springer and Dustin, 2012). It is the specificity of these regions for their ligand that provides integrins with their high level of specificity (Springer and Dustin, 2012), but may also go some way to explaining why there is a level of cross-reactivity between some integrin heterodimers – such as that seen between α4β1 (VLA-4) and α4β7 for VCAM-1.

The integrin's primary role is to allow for stable adhesion of cells to substrate, however, some integrins have been shown to be used for cell rolling (Alon *et al.*, 1995). Interestingly, integrins have also been shown to play a key role in cell signalling – both outside-in signalling and inside-out signalling, and furthermore, variations in the transmembrane domains of integrin heterodimers alter the ability to conduct the signalling (Kim and Kim, 2013).

External stimuli result in multiple, highly complex mechanisms of cellular activation, with simultaneous activation of multiple signalling mechanisms that result in an increase in cellular adhesion (Yu *et al.*, 2010). In relation to integrin binding, however, the number of mechanisms a cell can use to increase adhesion are limited. The mechanisms fall into three categories: a change in the level of integrin expression level, a change in integrin conformation and /or a change in integrin arrangement on the cell surface (**Figure 1.4**).

1.5.3.1 Integrin Expression Changes

The change in expression level is often achieved following cell stimulation (Grabovsky *et al.*, 2000, Chigaev *et al.*, 2003). For example treating neutrophils with chemokines such as interleukin-8 (IL-8) triggers an up-regulation of the integrin Mac-1 (CD11b) (Conklyn *et al.*, 1996). Similarly, macrophages can upregulate surface expression of Mac-1 following activation (Takada *et al.*, 1987). However, integrin mediated cell adhesion can be modulated without affecting surface expression of integrins (Yauch *et al.*, 1997, Hart and Greaves, 2010) (**Figure 1.5**).

1.5.3.2 Integrin Conformation Changes

Several models of integrin activation via conformational change have been proposed, however, the most widely accepted model is that of an 'extension and opening' model which is often referred to as an 'activated' state (Reviewed in Springer and Dustin, 2012). It consists of two components: extension of the integrin legs and opening of the integrin head piece (**Figure 1.4**) (Takagi *et al.*, 2002, Wang and Luo, 2010). These conformational changes have been proposed to occur following several occurrences, with ligand binding (Springer and Dustin, 2012), metal ion binding and cytoskeletal torsion (Takagi and Springer, 2002) being common triggers.

Conformational changes have been identified using multiple methods including electron microscopy (Takagi *et al.*, 2002) and X-ray crystallography (Zhu *et al.*, 2008). Furthermore, conformation changes have been assayed through the use of conformation specific antibodies in human. These antibodies have been used to identify extension status of an integrin (Yang *et al.*, 2004) as well as the affinity status of an integrin (Gupta *et al.*, 2007). However, to the author's knowledge, conformation specific antibodies have yet to be raised against murine integrins.

Integrin conformation changes and affinity changes have previously been considered synonymous, but it has been demonstrated that adhesion activating changes consist of three stages – bent closed, extended closed and extended open with each step having differing affinity states. It is most commonly described that the crucial step increasing integrin affinity, which describes how tightly a single monomeric ligand binds to a single heterodimer integrin, is the head piece opening whereby the open conformation increases affinity by 10^{3-4} fold (Springer and Dustin, 2012).

However, whether both extension and head piece opening are required to occur for a high affinity state to be achieved is still contentious, with evidence for the bent conformation with a high affinity state of an integrin in existence (Adair *et al.*, 2005), although there is far more overwhelming and substantial evidence against this (Reviewed in: Wang and Luo, 2010, Springer and Dustin, 2012). Further highlighting this contention, whilst supporting the latter argument, are data reported by Chen *et al.* (2010) that describe that Fab binding could trigger an extended open and an extended closed conformation with a concomitant increase in cellular adhesion, but, a second Fab that induced only the extended closed conformation did not result in increased cellular adhesion. Hence, there is the requirement for both extended and open conformation for an increase in affinity, and that the intermediate stage (extended closed) has no effect on integrin affinity (Chen *et al.*, 2010).

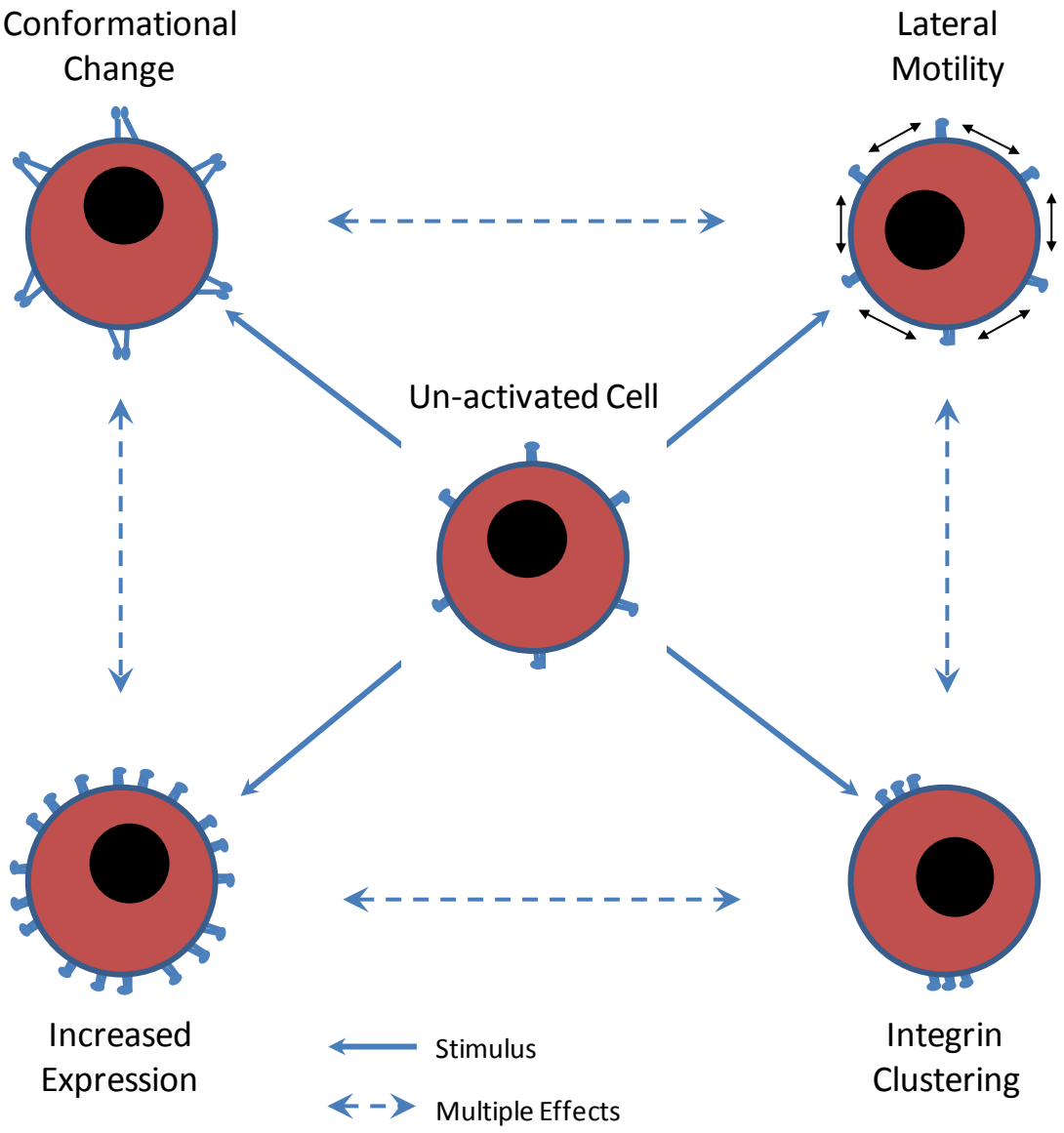

Figure 1.5 Changes in Integrin Activation.

Upon stimulation, cell adhesion can be modulated in a number of manners. These include integrin conformational changes (affinity modulation), increased lateral motility of integrins on a cell surface (avidity modulation), clustering of integrins (avidity modulation) or an increased surface expression of integrins (avidity modulation). It is unlikely that each of these occurs independently in a physiological setting, but it is a combination of some or all of these effects that contribute to increased adhesion.

Conversely, Yu *et al.* (2010) review data that suggests that the high affinity state is not solely dependant on extended and open conformation, as Shi *et al.* (2007) describe a high-affinity state integrin in a bent conformation (Shi *et al.*, 2007). However, Springer & Dustin (2008) have highlighted that many previous studies have poorly assayed for affinity due to the use of multimeric and not monomeric ligand binding. This then is not solely a measure of affinity as avidity is introduced into the system, due to the possibility of multiple integrins involved in the binding process (Springer and Dustin, 2012).

1.5.3.3 Integrin Clustering and Avidity Changes

Avidity modulation is defined as an increase in adhesiveness independent of integrin conformational changes (Yu *et al.*, 2010). Avidity changes may be due to integrins 'clustering' on the surface of the cell, increased rates of movement upon the cell surface or a combination of them both (**Figure 1.5**). However, conformational independent changes in adhesion are less understood than conformational regulated changes in adhesion.

On leukocytes, cellular activation triggers integrin clustering (Wu *et al.*, 2012), but it has been shown that the clustering is not a result of directed movement, but via diffusion following release from cytoskeleton (Sigal *et al.*, 2000, Kucik, 2002, Yu *et al.*, 2010). This is supported through observations that cytochalasin D, a potent actin polymerisation inhibitor, treatment has no effect on integrin clustering (Hart and Greaves, 2010). Furthermore, when integrin α tails are mutated, such that integrins can no longer effectively dissociate from the actin cytoskeleton (due to increased β tail interactions with the cytoskeleton), integrin clustering is inhibited (Yauch *et al.*, 1997).

Regardless of the mechanism, clustering may prove essential physiologically as it provides a rapid and effective increase in adhesion, independent of affinity in 2 ways: first it may allow for movement

of integrins to regions of high ligand density, and secondly, may allow for minute adjustment in orientation, increasing the likelihood for binding interactions to occur (van Kooyk *et al.*, 1999, Kucik, 2002). Furthermore, when integrin clustering is inhibited reduced adhesion is seen as well as a decrease in stable adhesion under shear stresses (Yauch *et al.*, 1997). Conversely, when integrin clustering is triggered, in the absence of further cell stimulation, increased adhesion is seen (Yauch *et al.*, 1997), even if integrin conformation changes are prevented (Yu *et al.*, 2010). Finally, an interesting observation seen recently is that clustering of integrins, artificially via the clustering of ligands upon a surface, resulted in enhanced mesenchymal stem cell differentiation into bone (Petrie *et al.*, 2010), highlighting a role for integrin clustering for cellular signalling, as well as maintaining adhesion. Collectively, these results demonstrate that clustering of integrins upon a cell surface is a critical event in cell adhesion.

Much of the structural understanding on integrin behaviour has been examined through the use of leukocyte sourced materials and integrins. However, this work still has direct applicability with regards to stem cell integrin behaviour. Most recently, Vanderslice and colleagues examined the use of a small molecule agonist against VLA-4 that resulted in enhanced HSC binding to VCAM-1 (Vanderslice *et al.*, 2013). The novel small molecule agonist they had developed was initially a VLA-4 antagonist developed for targeting leukocyte VLA-4, and indeed, any cell expressing VLA-4, however, it was still capable of binding HSC VLA-4 (Vanderslice *et al.*, 2013).

Similarly, results from the Kalia laboratory have demonstrated that stromal cell derived factor-1 (SDF-1α) pre-treatment of HSCs is capable of inducing the clustering of LFA-1 on the cell surface (Kavanagh *et al.*, 2013). This is also seen when primary T cells are stimulated with SDF-1α (Wu *et al.*, 2012). These observations highlight that integrin behaviour, and research of said behaviour, are

directly applicable across fields. However, these observations should be confirmed within HSC to ensure a complete understanding of integrin mediated adhesion and cellular activation of stem cells.

1.6 Enhancing Stem Cell Recruitment to Sites of Injury

Although it has been demonstrated that exogenous HSC transfer might be an effective regenerative or anti-inflammatory regimen for the treatment of colonic injury, their efficacy remains poor. One possible explanation for the limited effectiveness is a result of poor recruitment efficiency once these potentially therapeutic cells are injected systemically. For stem cells to confer benefit more efficiently, they must first be recruited more readily and in greater numbers to sites of injury.

Current attempts to increase stem cell recruitment efficiency involve techniques such as genetic manipulation or modification of the stem cells (Kumar and Ponnazhagan, 2007). However, this in itself could pose a potential risk when used, as it is currently unclear whether these changes to the stem cell may cause potential problems in the long term *in vivo*. For example, genetically modified stem cells may create problems due to the potential misguided insertion of genetic material can lead to oncogene activation and genome instability. Aberrant genomes and gene expression profiles can then lead to uncontrolled proliferation and have the potential to form carcinomas. Instead, identifying a lower risk strategy of improving stem cell recruitment by pre-treatment may provide a safer therapeutic option.

1.6.1 Pre-treatment Strategies to Improve Stem Cell Homing

Pre-treatment of cells is considered a possible therapeutic tool to increase the efficacy of cell based treatments via increased cellular recruitment to the injured organ. This idea was developed by the

observation that stem cell recruitment to many different organs is increased following injury. This suggests local, soluble inflammatory factors activate trafficking stem cells causing them to adhere in a similar manner to that described for leukocytes. From this it could be postulated that pre-exposing stem cells to a mimetic inflammatory environment containing factors known to be released in injured tissue may modulate their activity, either through transcript- and preoteome alterations, changing surface molecule expression, or changing the affinity of adhesion molecules for their counter-ligand may increase cellular recruitment and thus enhance efficacy.

The concept of pre-treating cells to alter behaviour is not novel; indeed many methods exist in cell culture biology. For example the use of thrombopoietin (TPO) to cause HSCs to differentiate into megakaryocytes (Dumon *et al.*, 2006), changing the constitution of culture media to induce MSC into bone, cartilage and fat (Morikawa *et al.*, 2009, Soleimani and Nadri, 2009) and pre-treating leukocytes with hydrogen peroxide (H_2O_2) to increase their rolling on endothelium (Johnston *et al.*, 1996) are all examples of cell pre-treatments. However, investigating their use as a stem cell targeting regime has not yet been fully investigated.

Studies within the Kalia laboratory have identified that the chemokine SDF1α (CXCL12), a known potent chemoattractant, is capable of enhancing HSC adhesion to murine injured small intestine (Kavanagh *et al.*, 2013), injured kidney (White *et al.*, 2013) and can increase HSC rolling in post-capillary venules of the cremaster muscle (Kavanagh *et al.*, 2013); tissue beds in which IR injury results in an increase in stem cell adhesion. Similarly, other groups have demonstrated that exposure of stem cells to SDF1α results in an increase in stem cell homing to injured liver and BM (Kollet *et al.*, 2003, Avigdor *et al.*, 2004).

1.6.1.1 Hydrogen Peroxide (H_2O_2)

Although the aetiology of IBD is unknown, central to diseases such as UC and Crohn's is the presence of both acute and chronic inflammation during times of active disease. Therefore, these pathologies are associated with significant increases in the generation of ROS such as hydrogen peroxide (Harris *et al.*, 1992), generated during the respiratory burst of infiltrating neutrophils (Cook-Mills and McCary, 2010) and from endothelia (Matoba *et al.*, 2000). It is also known that H_2O_2 can modulate the adhesive behaviour of inflammatory leukocytes, as increased rolling and adhesion of pre-treated leukocytes is observed (Fraticelli *et al.*, 1996). ROS are starting to be considered as signalling molecules in their own right (Gough and Cotter, 2011). This idea has developed due to the observation of hyperphosphorylation of signalling components within signalling cascades such as the NFκB pathway and their role as second messengers within these signalling pathways (Anderson *et al.*, 1994, Staal *et al.*, 1994, Baeuerle *et al.*, 1996). Furthermore, Kavanagh *et al.* (2012) have demonstrated that pre-treatment of HSC with H_2O_2 leads to a substantially increased recruitment of HSC within the injured murine gut (Kavanagh *et al.*, 2012). It was found that the increased adhesion was due to an increase in integrin mediated adhesion. Indeed, there are multiple reports that integrin affinity and behaviour are redox regulated that result in enhanced cellular adhesion (Liu *et al.*, 2008, de Rezende *et al.*, 2012, Lim and Hotchin, 2012) (**Figure 1.6**).

Overall these observations suggest that H_2O_2 pre-treatment may be a promising method of enhancing cellular therapy by enhancing stem cell recruitment to target tissues.

1.6.1.2 Platelet Microparticles (PMPs)

In addition to the presence of ROS, numbers of platelet microparticles (PMPs) in the blood have also been found to increase as a result of inflammatory injury. Following IBD, circulating numbers of PMPs in both clinical and experimental studies is significantly increased (Andoh *et al.*, 2005, Andoh *et*

al., 2006, Palkovits *et al.*, 2012). Interestingly, an increased PMP number has also been positively correlated with disease activity (Andoh *et al.*, 2006) suggesting they may be used as biomarkers in IBD as well as other pathologies. PMPs are reportedly increased in several additional acute pathologies such as IR injury (Burnier *et al.*, 2009) and myocardial infarction (Gawaz *et al.*, 1996). Their role in injury and inflammation is not completely clear, however, several models have been proposed.

PMPs are submicrometer vesicles that are released or shed from the platelet surface following platelet activation into the circulation (Perez-Pujol *et al.*, 2007, Morel *et al.*, 2008, Boilard *et al.*, 2010). Although initially considered as cell debris, a central role in haemostasis has been identified for PMPs (Merten *et al.*, 1999, Forlow *et al.*, 2000, Mause *et al.*, 2005). They are now thought to play a significant role in mediating circulating cell adhesion and inflammation. This may be through direct cell interaction (coating or loading) of leukocytes (Mause *et al.*, 2005), through paracrine effects via the release of soluble mediators (Iwamoto *et al.*, 1996) or by acting as a bridge between blood cells and endothelium/extracellular matrix (Forlow *et al.*, 2000). Indeed, Jy and colleagues presented seminal data that PMPs can 'coat' neutrophils and induce their activation, as measured by increased integrin CD11b (α_M) expression and phagocytic activity (Jy *et al.*, 1995). PMPs can also promote platelet interaction with subendothelial matrix proteins exposed after vessel injury (Merten *et al.*, 1999). These submicrometer particles have also been used to enhance EPC recruitment (Mause *et al.*, 2010), HSC recruitment to BM (Janowska-Wieczorek *et al.*, 2001) and are also capable of mediating molecule transfer between cells (Tang *et al.*, 2010, Risitano *et al.*, 2012).

However, their potential for enhancing HSC recruitment to injured extramedullary tissues has not been fully examined, particularly with regards to initial capture of HSC from flow *in vivo*. It is highly likely that they are involved in modulating cellular recruitment considering their extensive existence

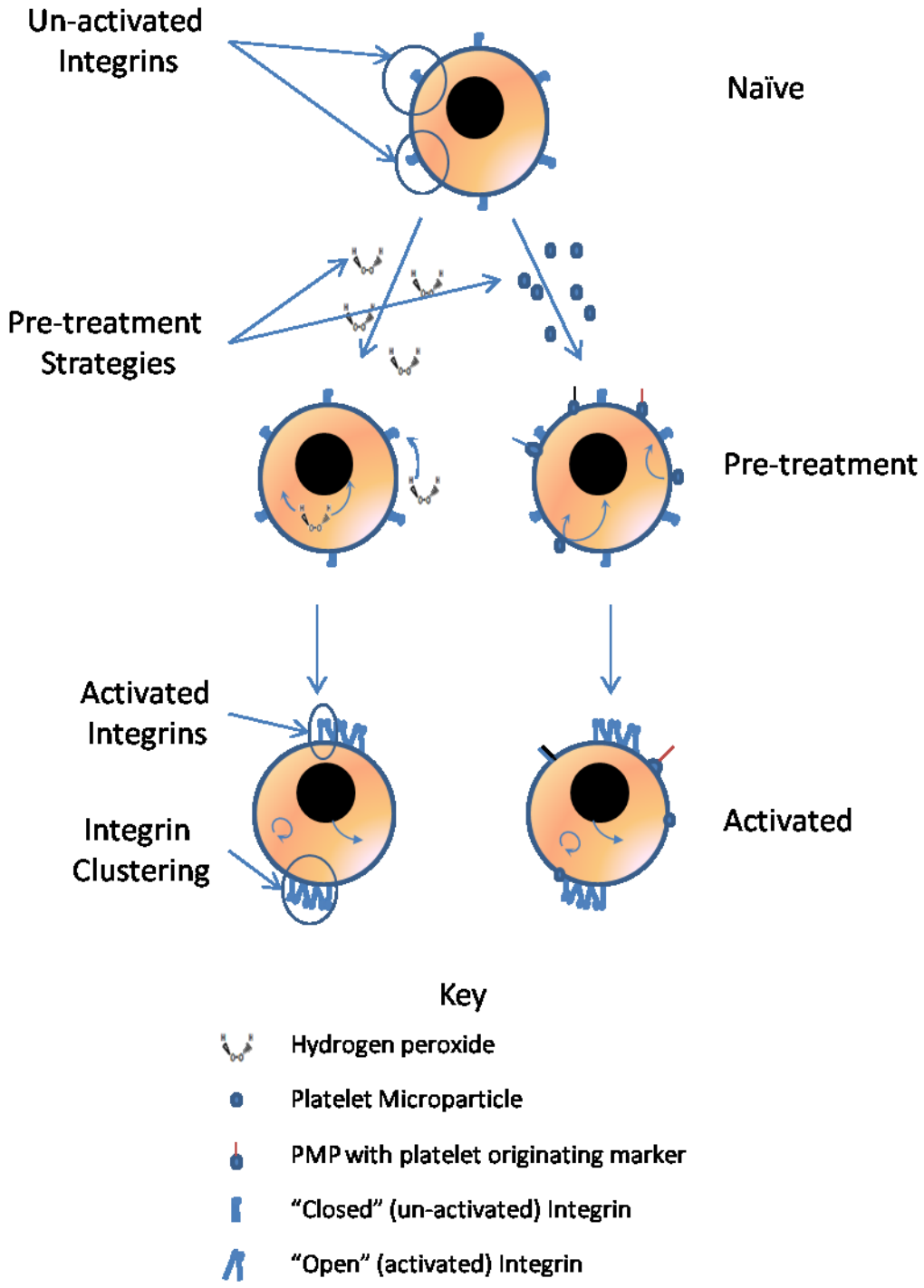

Figure 1.6 Mechanisms by which Cellular Pre-treatment may Improve Adhesion

Stem cell therapies are likely limited by poor recruitment of stem cells to target sites. Pre-treatment of HSCs may be a simple yet effective way to enhance their adhesion. Pre-treatment with H_2O_2 (left side) may have several effects; it may affect integrins directly or have effects on the cytosol and its constituents, resulting in enhanced adhesion. Pre-treatment with platelet microparticles (right side) may have similar effects on cell adhesion. It may trigger integrin modulation, but it may also result in new adhesion molecules present on the cell membrane, either through adhesion of the molecules to the cell, thereby acting as a bridge, or through merging of membranes and depositing surface markers onto the cell. However, it may also have an effect on gene expression and proteome management through the delivery of its cytosolic contents.

within the blood of patients with inflammatory disorders. They may therefore play a role in increasing HSC adhesion to injured tissues, either through activation of HSCs to a state more able to be recruited (*i.e.* activation), or through increased expression of integrins (either through stimulation or potentially through expression of the bound microparticles; **Figure 1.6**). As previously stated, there is evidence to suggest that $CD34^+$ stem cells can be coated with PMPs and that this was therapeutically beneficial as covered cells engrafted the BM of lethally irradiated mice significantly faster that those that had not been covered (Janowska-Wieczorek *et al.*, 2001). This suggests PMPs have the potential to play an important role in the homing of HSCs to sites of injury also which may have clinical implications for the optimisation of cellular therapies.

However, it is poorly understood how these molecules are loaded, and how they are having a beneficial effect on cellular recruitment. It has been identified that PMPs can deliver cell surface molecules such as phosphatidylserine (PS), glycoprotein (GP) Ib, GPIIb/IIIa, P-selectin, CXCR4 and other molecules (Barry *et al.*, 1998, Mause *et al.*, 2005, Mause *et al.*, 2010), but the transfer of integrins VLA-4 and LFA-1 is highly unlikely due to a lack of expression of these on the platelet surface. However, the transfer of other molecules, such as CXCR4 and SDF-1α from the platelets, may allow for the modulation of integrin behaviour (**Figure 1.6**).

In summary, soluble inflammatory molecules such as ROS, as well as inflammatory cells such as platelets, are found in abundance at sites of inflammatory colonic injury. These molecules or cells may activate the HSCs to adhere as they traffick through the colon. As continued observations from the Kalia laboratory highlight a critical role for integrins in HSC adhesion, it was speculated that the bioactive molecules present will enhance integrin mediated adhesion.

1.7 Summary

Overall, inflammatory gut injuries such as Crohn's and colitis are difficult to treat, with treatment regimens rapidly losing effectiveness (Lichtenstein *et al.*, 2009, Singh *et al.*, 2010). Similarly, acute intestinal injuries such as IR injury are also faced with difficulties during treatment. Ultimately, both acute and chronic bowel injuries are faced with inefficient and ineffaceable therapies and result in high morbidity rates. Thus a need for new, effective options are required. Cellular therapy, particularly HSC-based therapy, could provide this needed alternative. It has been demonstrated experimentally and clinically that HSCs are able to directly repair tissue as well as combat the excessive immune responses triggered by these inflammatory injuries. Currently, however, HSC therapies are faced with limited clinical efficacy. This is thought to be attributed partly to limited recruitment of stem cells to the target, injured tissue. It is this need to identify strategies in which stem cell recruitment may be increased that this research is based around.

If stem cells do hold promise in the context of regenerative medicine or for the repair of colon injury, what is clear is that strategies to enhance HSC retention within sites of injury are of utmost importance. Elucidating the molecular adhesive mechanisms governing HSC homing to injured colonic microvessels may have significant clinical consequences as it may allow us to optimise their effective intestinal delivery and improve their therapeutic efficacy. Identifying methods of enhancing HSC engraftment rates *in vivo*, and whether a systemic delivery of HSCs can efficiently deliver cells to intestinal tissues have had limited investigation, with no studies conducted in the colon.

1.8 Aims and Hypotheses

The aims of this thesis were firstly to identify whether HSC adhesion to murine colon microvasculature *in vivo* could be enhanced following two different injuries, namely IR injury and

colitic injury. Furthermore, this project aimed to identify the molecular mechanisms involved in the adhesion of HSC to injured tissue. Following investigations conducted in the Kalia laboratory, it was postulated that pre-exposure of HSCs to a mimetic inflammatory environment may enhance their adhesion within the microcirculation of the murine colon crypts *in vivo*. Therefore, it was investigated whether an injury conditioned media (ICM) containing soluble factors released from injured colon, H_2O_2 or PMPs could be used to enhance HSC adhesion. Furthermore, the mechanisms behind any potential increased adhesion were also investigated.

1.8.1 Specific Aims and Hypotheses

1. Does injury increase HSC adhesion to murine colon microvasculature *in vivo*, and is this pattern seen in both IR and colitic injured tissue?
    - It was hypothesised that, similar to that seen in other tissues, an acute and chronic injury will increase the number of adherent HSCs within colon microvasculature, with possibly greater adhesion observed if the injury is more severe.

2. What are the molecular mechanisms involved in HSC adhesion to the injured colon microvasculature?
    - It was hypothesised that the integrin CD18 (β_2) will play a role in adhesion to IR colon, but not CD49d (α_4), as a similar role for CD18 was seen in IR injured small intestine.
    - Adhesion to colitic colon will be mediated by both CD18 (β_2) and CD49d (α_4). This was hypothesised as the longer duration of the inflammation in colitic tissue may up-regulate endothelial counterligands such as VCAM-1 as well as ICAM-1.

3. Can pre-treating HSCs significantly enhance their adhesion within the injured colon *in vivo*?

- Pre-treatment with either chemical (H_2O_2) and/or biological (platelet derived pre-treatments) pre-treatments will significantly enhance HSC adhesion within both IR and colitis injured colons *in vivo.*

Chapter 2

METHODS AND MATERIALS

2.1 Tissue Culture

2.1.1 CHO K3 Culture

CHO (Chinese hamster ovary) K3 cells, a kind gift from Professor Leif Carlsson (University of Umeå, Sweden), rapidly grow in culture and were used to generate a stem cell factor (SCF) conditioned media. CHO K3 cells generate SCF as a result of a stable transfection. SCF is an essential component of media used for HSC culture and expansion. CHO K3 cells were cultured in Dulbecco's modified Eagles medium (DMEM; Invitrogen, UK) supplemented with 10% FBS (Invitrogen, UK), 50U/ml streptomycin (Invitrogen, UK), 50U/ml penicillin (Invitrogen, UK) and 2mM L-glutamine (PAA Laboratories, UK). CHO K3 cells were grown to confluency in gelatin coated flasks and then split 1 in 4 and grown at 37°C, 5% CO_2. To generate SCF conditioned media, CHO K3 were grown to confluency and left for 48 hours without replacing media. From this point the media was changed to Stem Pro 34 Serum Free Media (SFM) 34 with 0.5% FBS. Cells were kept in this media for 24 hours, then replaced with fresh Stem Pro SFM 34 with 0.5% FBS for a further 48 hours. After this time the media was harvested and filtered through a 0.22µm syringe driven filter unit. The generated SCF media was stored at -20°C in aliquots until required.

2.1.2 Haematopoietic Stem Cell Line Culture

Throughout this body of work the HSC cell line HPC7 has been used for all experiments. The HPC7 cell line was also a gift from Professor Leif Carlsson (University of Umeå, Sweden). Investigating *in vivo* HSC homing intravitally has been hampered by the limited numbers of primary HSCs obtainable from individual mice. Therefore, we utilized an immortalised HSC line, HPC7, generated by transfecting embryonic SCs with the murine LIM-homeobox gene LH2 (Pinto do O *et al.*, 1998). Haematopoietic progenitor cell lines immortalized in this manner display characteristics of primary

HSCs including surface expression of common murine HSC markers (c-kit$^+$, CD34$^-$, Lin$^-$; Pinto do O *et al.*, 1998) and similar behaviour at a molecular level (Wilson *et al.*, 2009). Interestingly HPC cell lines derived in this manner are able to reconstitute haematopoiesis when injected into lethally irradiated mice (Pinto do O *et al.*, 2002). HPC7 cells also express adhesion molecules present on primary HSCs and we have previously used them to model hepatic, renal and intestinal HSC recruitment (Kavanagh *et al.*, 2010, Kavanagh *et al.*, 2012, White *et al.*, 2013).

HPC7s were cultured in Stem Pro SFM 34 (Invitrogen, UK) supplemented with 50U/ml streptomycin, 50U/ml penicillin, 2mM L-glutamine and SCF obtained from conditioned media harvested from CHO K3 (see section 2.1.1) cells at a concentration of 1 in 10. However, the use of CHO-K3 conditioned medium to generate SCF for HPC7 growth was not always reliable. To counter this, HPC7 media was supplemented with 100ng/ml rmSCF (recombinant murine stem cell factor; Invitrogen, UK). This altered method of HPC7 culture was the protocol used for the majority of the work in this thesis.

2.1.3 Labelling HPC7 with Fluorescent Dyes

2.1.3.1 5'6-Carboxyfluorescien Diacetate Succinimidyl Ester (CFDA-SE)

HPC7 were fluorescently labelled using the fluorescein label 5'6-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Invitrogen, UK) to enable their tracking *in vivo* and their identification *in vitro*. CFDA-SE is the cell permeable precursor to the cell impermeable, fluorescent molecule 5'6-carboxyfluorescein succinimidyl ester (CFSE). This change in molecule is achieved by intracellular esterases, enzymes responsible for both the change in fluorescence and retention within the cell. CFSE labelled cells fluoresce under blue light (488nm) and emit green light at a wavelength of 510nm, similar to the properties of fluorescein isothiocyanate (FITC). To label HPC7, 3-6x10^6 HPC7 were isolated from culture and washed with PBS. Cells were then resuspended in 4ml PBS containing 5µM

CFDA-SE and incubated at 37°C for 20 minutes. The staining reaction was stopped by adding 4ml cold Stem Pro. Cells were washed 3 times in warm media, counted by trypan blue exclusion and suspended in complete media at a final density of $2x10^6$ cells/ml until use. HPC7 were used within 4 hours of staining.

2.1.3.2 Cell Tracker Orange

For visualising HPC7 whilst imaging platelet microparticle loading onto the cell surface confocally, it was necessary to label the HPC7 with a different dye, to allow for dual colour fluorescence microscopy. In this instance the commercially available cell labelling kit CellTracker Orange (Invitrogen, UK) was used. The cell tracker dyes are highly cell permeable until intracellular processing renders them cell impermeable. CTO has a peak excitation wavelength of approximately 550nm with a peak emission at approximately 580nm. As this dye is sufficiently separate from FITC (and Alexa488) it was selected as the cellular counter-stain in the dual colour microscopy analysis. HPC7 were labelled with CTO as per manufacturer's instructions. HPC7 were washed by centrifugation with warm PBS and then resuspended in 5µM CTO solution in serum free media. Cells were incubated at 37°C for 45 minutes in the dark. The reaction was stopped by adding 4 ml cold media. Cells were then washed 3 times by centrifugation and counted by trypan blue exclusion. HPC7 were then suspended in complete media at a final density of $2x10^6$ cells/ml until use. HPC7 were used within 4 hours of staining.

2.1.4 Murine Cardiac Endothelial Cell (MCEC-1) Culture

For some *in vitro* adhesion assays, a murine cardiac endothelial cell line (MCEC-1) was used. These cells were used due to the difficulty and time consuming purification techniques and the high numbers of contaminating cells obtained when culturing primary murine endothelial cells long-term

(Marelli-Berg *et al.*, 2000; Peek *et al.*, 2000). MCEC-1 cells were isolated from H-2k^{b}-tsA58 mice, which express a thermoliable strain (tsA58) of the SV40 large T antigen (tsA58 Tag) to allow the production of conditionally immortalised cell line (Lidington *et al.*, 2002) and were a gift from Professor Leif Carlsson. The endothelial cells are characterised by a phenotypic cobblestone appearance and their ability to form tubes on artificial extra-cellular matrix described by Lidington and colleagues. MCEC-1 are ICAM-1 and VCAM-1 positive, but with negligible selectin expression (Lidington *et al.*, 2002).

MCEC-1 were grown to confluence on 1% gelatin coated tissue culture flasks and maintained in complete media (DMEM supplemented with 10% FBS, 50U/ml penicillin, 50U/ml streptomycin, 2mM L-Glutamine and 10ng/ml mEGF (murine endothelial growth factor, Peprotech, UK)). At confluence, cells were dissociated from tissue flasks with 0.25% Trypsin-EDTA (Invitrogen, UK), washed in MCEC-1 media and split 1 in 3. When used experimentally, MCEC-1 were cultured in 24 well tissue culture flasks. When confluent, wells were washed thoroughly with PBS then some wells were incubated with 100U/ml TNFα (Peprotech, UK) for 4 hours at 37^{o}C. Wells were again washed thoroughly prior to the addition of the HPC7 cells being assayed.

2.1.5 Colon Endothelial Cell Line Culture

Similarly, for some *in vitro* adhesion assays, the colon endothelial cell (CEC) line was used due to the difficulty and time consuming purification techniques of primary colonic endothelial cells. These endothelial cells also offered an attractive and more relevant model than the MCEC-1 cells. CEC cells were a kind gift from Professor Steven Alexander (Louisiana State University, USA), and were isolated from H-2k^{b}-tsA58 mice, which express a thermoliable strain (tsA58) of the SV40 large T antigen (tsA58 Tag) to allow the production of conditionally immortalised cell line (Ando *et al.*, 2005).

CEC were grown to confluence on 1% gelatin coated tissue culture flasks and maintained in growth media (D-Valine MEM (Promocell, UK), 10% FBS, 50U/ml penicillin, 50U/ml streptomycin, 2mM L-Glutamine, 1/100 Non Essential Amino Acids (Sigma, UK), 1/100 Vitamin Mix (Gibco, UK) and 10units/ml mIFNγ (murine interferon gamma (Peprotech, UK)) at 33^{o}C, 5% CO_2. At confluence, cells were dissociated from tissue flasks with 0.25% Trypsin-EDTA, washed in growth media and split 1 in 3. When used for experiments, CEC were grown in 24 well tissue culture flasks at 37^{o}C, in experiment media (450ml Dulbecco's Modified Eagles Medium (4.5g/l glucose) (DMEM); Invitrogen, UK; 10% FBS, 50U/ml penicillin, 50U/ml streptomycin), until confluency and washed thoroughly prior to the addition of 100U/ml TNFα in media to some wells, with some wells left unstimulated. The CEC were incubated for 4 hours at 37^{o}C then washed thoroughly prior to the addition of HPC7 for experiments.

2.2 Animal Experimental Procedures

2.2.1 Animals

Male C57BL/6 mice (Harlan, Oxon, UK) were used. Animals were anaesthetised with an intra-peritoneal injection of ketamine [100mg/kg] and 2% xylazine [10mg/kg]. The trachea was intubated and the left carotid artery cannulated. The carotid cannulation was to allow for the systemic administration of labelled stem cells and to allow the maintenance of anaesthesia with ketamine/xylazine. Throughout all procedures, anaesthetised animals were kept warm with the use of a heating mat and lamp. All intravital procedures were conducted in accordance with the Animals (Scientific Procedures) Act of 1986 (Project Licence: 40/3336; 19b1; Dr Kalia) and had also received local University of Birmingham ethical approval. For some experiments, DSS-induced colitis male C57BL/6 mice were used. These were generated under a separate project licence overseen by Dr Bertus Eksteen (PPL: 40/3226, 19b2a1), until transfer (post-DSS treatment) to PPL 40/3336 for

intravital experimentation. For experiments in which platelet microparticles were generated from donor mice, the bleeding and isolation of platelets from whole blood procedures was performed under a licence overseen by Professor Steve Watson (PPL: 30/2721, 19b2).

2.2.2 Intestinal Ischaemia-Reperfusion Injury Model

To induce ischaemia-reperfusion (IR) injury in the small intestine, a midline laparotomy was performed, following which the superior mesenteric artery (SMA) was isolated and occluded using a non-traumatic artery clamp for 45 minutes. The major portion of the blood supply to the colon is also provided by the SMA in rodents and clamping this vessel reduces microvascular perfusion in the colon by more than 85% (Leung *et al.*, 1992, Riaz *et al.*, 2002b, Santen *et al.*, 2010). Subsequent removal of the clamp initiated the reperfusion injury as blood flow was restored. Control animals underwent sham surgery in which the midline laparotomy and the SMA was isolated but not clamped.

2.2.3 DSS-Induced Colitis Injury Model

Within the mouse there are several well documented models of colitis including oral administration of dextran sodium sulfate (Okayasu *et al.*, 1990), using genetic knockout mice for IL-10 (Singh *et al.*, 2003) in addition to the many other models (Blumberg *et al.*, 1999, Wirtz and Neurath, 2000). All of these models display some of the symptoms experienced by patients with IBDs; however, there are some differences.

For example, within the DSS model, mice exhibit a significant shortening of the colon, whereas the murine IL-$10^{-/-}$ model can display a lengthening of the colon (Kawachi *et al.*, 2000). Interestingly, several groups have identified differences in the chemokine expression of the different models, and

in some cases within models but from different laboratories (Kawachi *et al.*, 2000, Coburn *et al.*, 2012, Yan *et al.*, 2012), further highlighting the complexity of these pathologies. DSS is a sulfated polysaccaride that can exist at a range of molecular masses. The use of sulphated polysaccarides to induce inflammatory bowel disorders was first described in 1969 where carrageenan was used to induce an ulcerative colitis-like model in guinea pigs and rabbits (Marcus and Watt, 1969). In mice, however, Okayasu *et al.,* (1990) first described the use of DSS to induce a colitis-like injury, with a chronic model of colitis that could be induced through cyclic administration of DSS. The feeding of DSS in the drinking water of rodents *ad libitum* induces a colitis-like injury with bloody diarrhoea, ulcerations, histological damage, shortening of the colon and enhanced leukocyte infiltrations of both PMN and mononuclear components (Okayasu *et al.*, 1990).

It is thought that the colitis injury symptoms displayed following DSS administration are induced by disruption of the mucosal barrier, which likely triggers an inflammatory response via activation of the immune system through contact with luminal antigens, similar to that reported in human IBDs. This has been determined by studies in which germ free and antibiotic treated animals were induced with colitis and that the inflammation occurred as a result of luminal antigens derived from the host, and not pathogens (Maloy and Powrie, 2011). Furthermore, it has been demonstrated that the DSS can rapidly degrade the colon mucus leading to an infiltration of bacterial species prior to any signs of inflammation (Johansson *et al.*, 2010) and that DSS is toxic to certain intestinal epithelial cells including paneth cells and goblet cells (Maloy and Powrie, 2011). It has also been suggested that the DSS associated inflammation may also be contributed to by macrophages that have engulfed DSS and subsequently contribute to the local inflammatory response (Okayasu *et al.*, 1990).

To establish a model of colitis in mice the drinking water was changed to 3% dextran sodium sulfate (DSS) water (MW 36–50kDa; MP Biomedicals, UK) and maintained for up to 6 days. Onset of colitis

was defined as the appearance of bloodied stools or 20% weight loss. DSS treatment was typically for 5 days, but did not exceed 6 days. Although there appeared to be a large degree of variation, potentially raising concerns that animals were not injured, Mori *et al.* (2005) described that DSS supplemented drinking water typically caused endothelial dysfunction within 3 days of administration, and indeed the injury scoring measure used allowed for verification of injury (see **Table 2.1**). Animals were maintained at the Biomedical Services Unit (University of Birmingham, UK) until use for intravital experiments.

Although all colitis mice were treated using this protocol, by the nature of the DSS treatment there was a marginal degree of variability exhibited. Indeed, it has been reported that although DSS does induce colitis in rodents, there is a considerable variation in the extent of colitic injury between mice strains, between sources and molecular weights of DSS, between institutions and even between testing groups (Bamba *et al.*, 2012). So to ensure that all colitis mice were injured to the same degree, the extent of injury was scored based on the scoring system from Soriano *et al.* (2000) that relies on investigator interpretation for scoring inflammation, stool consistency, faecal blood presence and ulceration. The results obtained using this scoring system are displayed in **Table 2.1**. As has been previously reported, following the DSS treatment regime, animals underwent significant weight loss, colonic inflammation and shortening of the colon with some associated bleeding from the anus and also contained within the colon (exhibited by presence of blood found within the stool). However, little to no ulceration was exhibited in the colon.

2.2.4 Preparation of the Colon for Intravital Microscopy

2.2.4.1 *Method 1*

Limited studies have imaged the mouse colon intravitally and no studies have previously tracked the homing of stem cell to this organ. Difficulties in imaging the colon lie with the fact that in the mouse

Animal	Colon Inflammation	Blood in stool	Stool Consistency	Anal Bleeding	Ulceration	Δ Body Weight/g	Colon Length/mm
Sham	0	0	0	0	0	0.88±0.12	65.3±2.4
Colitis	2.12±0.07	2.01±0.05	1.55±0.03	1.43±0.06	0.30±0.03	-2.06±0.17	52.5±0.7

Table 2.1 DSS-induced colitis injury scores

This table displays the mean±SEM values from the scoring system based on Soriano *et al.* (2000). Scoring was done as follows: Colon Inflammation: 0 = No observable inflammation, 1 = some swelling and oedema, 2 = Swelling, oedema and some rubor evident, 3 = extensive swelling, oedema, rubor and evidence of bleeding in the tissue. Blood in stool, determined using tissue paper touched to stool and the existence of red colour to the absorbed fluid: 0 = No observable blood in stool, 1 = some blood present, 2 = clear evidence of blood in stool, 3 = clear evidence of blood in stool and blood present within the colon lumen. Stool consistency: 0 = normal stool, 1 = loose stool (not fully formed, but still structure evident), 3 = diarrhoea (no stool formation evident). Anal bleeding: 0 = No evidence of blood around anus, 1 = dried blood around anus, 2 = extensive dried blood around anus and fresh blood observable. Ulceration: 0 = No ulceration evident, 1 = <5 small, isolated ulcers, 2 = extensive ulceration. Δ Body Weight/g (change in body weight in grams): total body weight change as determined on day 0 (start of treatment) subtracted by body weight on day 5 (end of treatment). Colon length/mm: length of entire colon - colon excised from ceacum to anus and measured with a 30cm ruler. Values are mean values across all experiments. Sham N=6, Colitis N=53.

the colon is incredibly short and it is therefore difficult to exteriorise a sufficient length. Therefore, part of the research involved establishing a viable preparation for intravital imaging of the colon mucosa. Following intubation of the trachea, carotid artery cannulation and midline laparotomy the abdominal viscera were moved out of the abdominal cavity to the left of the animal. Using a surgical swab and taking care not to damage any organs or vasculature, the ceacum was located and gently pulled away from the animal taking care not to puncture the organ. A longitudinal incision was made using a cautery to expose the mucosa of the ascending colon. The colon was raised onto a specially designed board (comprising of a 1.5ml eppendorf tube lid that was removed from the eppendorf and fixed into place). This allowed the colon to lie flat without the use of any other implements. However, this preparation proved to be unreliable as the colon persistently slipped from the eppendorf lid causing the field of view to be lost.

2.2.4.2 *Method 2*

Following intubation of the trachea, carotid artery cannulation and midline laparotomy the abdominal viscera were moved out of the abdominal cavity to the left of the animal. Using a surgical swab and taking care not to damage any organs or vasculature, the ceacum was located and gently pulled away from the animal onto a sterile 9cm petri dish, taking care not to puncture the organ. Bent paper-clips were used to hold the ascending colon approximately 2cm from the ceacum (this was as much of the ascending colon that could be removed from the abdominal cavity without damaging it or any of the other abdominal viscera) and to hold it stationary on the petri dish. A longitudinal incision was made using a cautery to expose the mucosa of the colon. The colon was opened flat, mucosal side up, onto the petri dish and held in place using 2 further paper clips (**Figure 2.1**). The mucosa was gently wiped to remove any solid matter and then gently washed with saline. The colonic microcirculation was visualised using an upright widefield fluorescent microscope (motorised BX61WI) with a 10x, 1.4 numerical aperture (NA) air objective (Olympus, UK). A field of

view was randomly pre-selected for intravital observations. Initially, this preparation proved to be unreliable as the signal to noise ratio was very poor. In order to improve image quality, a glass coverslip was placed onto the mucosa and held in place. Due to the excessive mucus produced by the colon, the coverslip had to be removed and the mucosa cleaned gently with a cotton swab every 15 minutes, with care being taken to ensure the pre-selected field of view was not altered. This method was utilised to generate all IVM data within this thesis.

For the laser speckle microscopy, the colon was prepared in the same manner, but the coverslip was removed. To asses blood flow flux values (which are directly proportional to blood flow) were determined within the focussed region of the colon (**Figure 2.1**).

2.2.5 Tracking Stem Cells *In Vivo* in the Colon

In IR injured mice, a single bolus dose of $2x10^6$ CFSE labelled stem cells was injected via the carotid artery cannula at 1 hour reperfusion. The pre-selected field of view was imaged intravitally every 5 minutes for one hour. For tracking cells in the colitic colon, labelled cells were infused once the surgical preparation was completed and had been allowed to stabilise for 30 minutes. Again, HPC7 were systemically infused via the carotid artery. Adherent cells were defined as cells that remained stationary ≥30s. Freeflowing cells were defined as those moving freely through the field of view without remaining stationary for ≥30s. Throughout these experiments, as well as other studies of low shear vasculature, HPC7 did not show any evidence of rolling, nor any sign of extravasation. This can be determined as the vasculature of the colon, and indeed the gut, can be seen as “shadows” or a darker network within the field of view (at 10x). HPC7 adherent in the manner described are considered recruited, as there is evidence to suggest that HPC7, when adherent, are capable of reducing the number of adherent leukocyte and platelets within the microvasculature (Unpublished results from the Kalia Laboratory). To ensure the events in the pre-selected area for IR and colitis

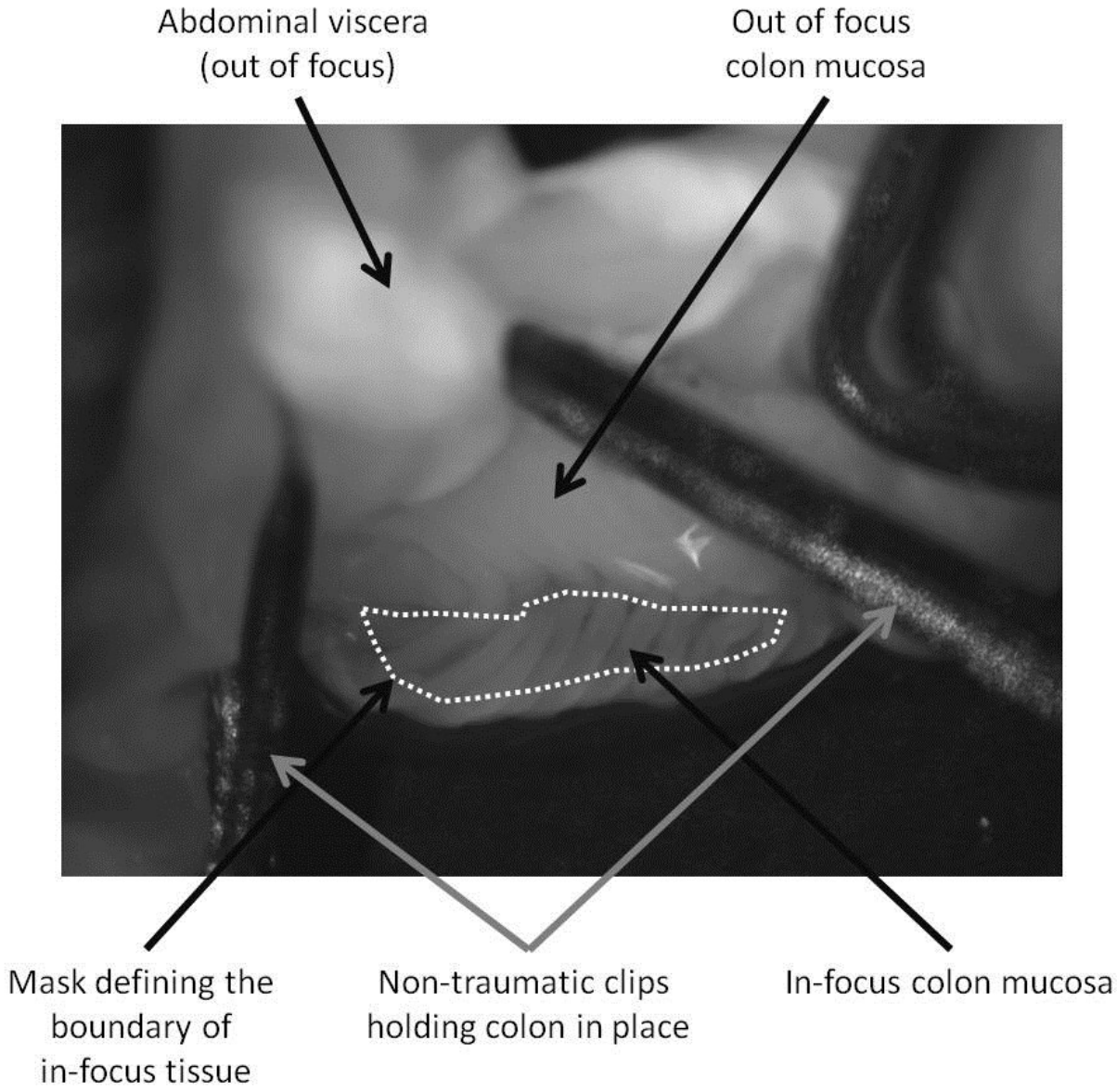

Figure 2.1 Preparation of the mouse colon for intravital imaging

The mouse colon was moved onto a sterile petri dish and mucosa exposed via a longitudinal incision made using a cautery. The colon was then opened flat onto the dish surface and held in place using paper clips. For IVM a glass coverslip was held in place on the mucosal surface (not seen in above image) whilst for laser speckle imaging the glass coverslip was removed. For laser speckle determination of blood flow, flux values were quantitated within the mask defining the in focus colon mucosa (white dotted line).

mice were representative of events taking place in the whole colon, post experiment, the colon was excised and 5 additional fields of view were monitored.

2.2.6 Quantification of HSC Recruitment to Remote sites - *Ex Vivo* Analysis

Post intravital experiment, animals were culled and the lungs and ileum (terminal region of the small intestine) were also excised and imaged to determine stem cell recruitment. The tissues were removed, washed with saline to remove blood and laid flat onto a petri dish. The dish was inverted to allow for imaging using an upright fluorescent microscope (motorised Olympus BX61WI) with a 10x 1.4NA air objective.

2.2.7 Isolation of Washed Mouse Platelets from Whole Blood

Mice were CO_2 narcosed following isofluorane anaesthesia. Following midline laparotomy, blood was collected from the descending aorta into 10% acid citrate dextrose (ACD: 120mM sodium citrate, 110mM glucose, 80mM citric acid). The blood was diluted in 200µl of Tyrode's-HEPES buffer (134mM NaCl, 0.34mM Na_2HPO_4, 2.9mM KCl, 12mM $NaHCO_3$, 20mM HEPES, 5mM glucose, 1mM $MgCl_2$, pH 7.3) and centrifuged at 2000rpm for 5 minutes in a microcentrifuge. The platelet rich plasma (PRP) and the top 33% of erythrocytes were removed and further centrifuged at 200x*g* for 6 minutes. PRP was retained and, to obtain the maximum number of platelets, a further 200µl of Tyrode's-HEPES buffer was added to the remaining erythrocytes, mixed and centrifuged again at 200x*g* for 6 minutes. The PRP was taken and added to the first isolation of PRP, taking care to ensure no erythrocytes were added to the isolated PRP solution. To prevent aggregation 10µg/ml prostacyclin was added to the total PRP and centrifuged at 1000x*g* for 6 minutes. The platelet pellet was resuspended in 200µl Tyrode's-HEPES buffer and counted. These platelets were later used to generate platelet microparticle as described in **Section 2.3.7.**

2.3 *In Vitro* Experiments

2.3.1 Gelatin Coating of Tissue Culture Flasks

To aid endothelial cell adhesion to culture surface, the culture surface was coated with gelatin. This was achieved by incubating tissue culture surface with sterile 1% gelatin Type IIb, from bovine skin (Sigma, UK) for 30 minutes, then aspirating prior to the addition of an endothelial cell suspension. Gelatinised plates, once aspirated and dry, could be stored for up to one week prior to use.

2.3.2 APES Coating of Glass Coverslips for Endothelial Cell Adhesion Assays

To permit adhesion of endothelial cells to 13mm diameter glass coverslips, the coverslips were treated with 3-aminopropyltriethoxysilane (APES) overnight, then treated with 5M nitric acid overnight, 10 washes in 50ml running water, 3 washes with 25ml acetone and 2 further washes with 25ml of 4% APES in acetone. The coverslips were then kept in the dark overnight, washed twice with acetone, followed by a final wash in distilled water. The coverslips were then dried, separated and autoclaved prior to use in *in vitro* endothelial cells adhesion assays.

2.3.3 Lysine Coating of Glass Coverslips for Confocal Microscopy

Poly-lysine polymers are useful molecules to prepare surfaces for cell attachment as they enhance cell adhesion to glass. 13mm diameter glass coverslips were washed in 5M nitric acid overnight, 10 washes in 50ml running water followed by incubation for at least one hour in 10% poly-L-lysine. Lysine solution was aspirated immediately prior to addition of cells for confocal microscopy experiments.

2.3.4 Tissue Preparation and Sectioning for Stamper Woodruff Assay

The Stamper-Woodruff (S-W) assay will be detailed in Section 2.5.1. Briefly, it allows adhesion of cells to be monitored on frozen tissue sections *in vitro*. Tissue samples were harvested from animals' post-intravital experiment and snap frozen in liquid nitrogen within 5 minutes. Organs isolated were the proximal jejunal region of the small intestine, distal ileal region of the small intestine and the ascending colon (2cm). Samples were stored at -80°C until use. Prior to sectioning, intestinal samples were mounted onto a cork board using O.C.T. mountant. Tissue was transversally sectioned into 10µm sections using a cryostat (Bright Instruments, UK), and transferred onto glass microscope slides by heat transfer and fixed in acetone for 5 minutes before wrapping slides in foil and storing at -20°C until ready for use.

2.3.5 Sectioning of Frozen Tissue for the Static Adhesion Assay – Modified Method

As will be explained in detail in the results section of Chapter 3, the above tissue sectioning did not provide the best tissue for the static adhesion assay and was therefore modified. Tissue samples were isolated from the proximal jejunum, distal ileum and the ascending colon from sham, IR and colitis injured animals after the intravital experiment. The intestinal mucosal surface was exposed by cauterising along the anti-mesenteric border and the organ was pinned flat onto cork before being snap frozen in liquid nitrogen. To expose the mucosal microcirculation, small intestinal samples were cryostat sectioned to remove 60µm of the mucosal surface and from the colon 20µm of the mucosal surface was removed. Samples were stored at -80°C until use.

2.3.6 Sham and Injury Conditioned Media Production

2cm of terminal jejunum, 2cm of terminal ileum or 2cm of the ascending colon were removed from sham, IR and colitis injured mice and placed into 3ml Stem Pro and incubated at 37°C for 24 hours.

Tissue was then macerated, centrifuged at 300x*g* for 5 minutes and the filtered through a 0.22µm syringe driven filter unit to remove any remaining particulate matter. The conditioned media was then aliquoted and stored at -20°C until ready for use.

Traditionally, conditioned media's have been considered to be derived from the supernatant taken from defined, cultured cell types. The harvested supernatant then has been conditioned by the cells grown within that media. However, within this thesis conditioned media refers to media in which tissue samples are incubated and macerated to ensure the contents microenvironment were also present – thus the media had been conditioned with a tissue environment. Conditioned media from sham and injured mice was called sham conditioned media (SCM) and injury conditioned media (ICM) respectively. **Table 2.2** provides a full list of the CMs generated and the abbreviations used to describe the CMs in this thesis.

2.3.7 Generation of Platelet Microparticle Pre-treatment Suspensions

One of the responses of activated platelets to certain stimuli is the shedding of microparticles. In order to generate PMPs, mouse platelets were activated with thrombin. In addition to its role in the coagulation pathway, thrombin also promotes platelet activation and aggregation following activation of protease-activated receptors (PAR-1) on the cell membrane (Janowska-Wieczorek *et al.*, 2001). It has routinely been used to generate PMPs from mouse platelets (Janowska-Wieczorek *et al.*, 2001, Mause *et al.*, 2005). Washed platelets were centrifuged at 1000x*g* for 6 minutes and resuspended at 3 x 10^7/ml in Stem Pro SFM 34. To prevent aggregation 10µg/ml prostacyclin was added to the washed platelets prior to centrifugation and rested for 30 minutes prior to experiments. Platelets were activated with 2U/ml of thrombin for 30 minutes at room temperature and then centrifuged at 1000x*g* for 6 minutes. The supernatant generated after centrifugation was aspirated and kept and the pellet discarded. The supernatant was classed as a platelet microparticle enriched

supernatant (PES; **Figure 2.2**). It contained not only the PMPs, but also the soluble factors released by the activated platelets. To generate purified platelet microparticles (PMP), the PES was microcentrifuged at 18,000x*g* for 20 minutes. The supernatant was then removed and the pellet resuspended in an equal volume of Stem Pro as started with. This PMP contained only the microparticles and not the releasate (the total constituents of material released by an activated cell, in this case platelets) from the activated platelets (**Figure 2.2**). PES and PMP suspensions were used within 30 minutes of being generated and not stored prior to use.

2.4 HPC7 Pre-treatment Protocols

2.4.1 Pre-treatment with Tissue SCM/ICM

HPC7 cells were suspended in conditioned media at a density of $2x10^6$/ml with aliquots incubated at 37°C, 5% CO_2 for 30 minutes. The different conditioned medias used are outlined in **Table 2.2**. The cell-CM suspension was then centrifuged at 300x*g* for 5 minutes, then resuspended in un-supplemented Stem Pro at the required volume immediately prior to use.

2.4.2 Pre-treatment with H_2O_2

HPC7 were incubated in Stem Pro supplemented with H_2O_2 (1mM, 100µM or 10µM) at a density of $2x10^6$/ml at 37°C, 5% CO_2 for 1 hour. The cells were then centrifuged at 300x*g* for 5 minutes. HPC7 were then resuspended in un-supplemented Stem Pro at the required volume immediately prior to use in experiment.

Name	Organ Originated	Injury Type
Ileum SCM	Ileum (terminal)	Non-Injured
Ileum IR ICM	Ileum (terminal)	SMA IR*
Ileum Col ICM	Ileum (terminal)	DSS Colitis†
Jejunum SCM	Jejunum (terminal)	Non-Injured
Jejunum IR ICM	Jejunum (terminal)	SMA IR*
Jejunum Col ICM	Jejunum (terminal)	DSS Colitis†
Colon SCM	Colon (ascending)	Non-Injured
Colon IR ICM	Colon (ascending)	SMA IR*
Colon Col ICM	Colon (ascending)	DSS Colitis†

*small mesenteric artery occlusion and release (45mins ischaemia, 120mins reperfusion)

† 3% dextran sodium sulfate induced colitic injury for up to 6 days

Table 2.2 Intestinal Sham and Injury Conditioned Media

This table lists the conditioned Medias used and the tissue of origin and injury type the conditioned media was sourced from. Abbreviations: SCM – sham conditioned media, ICM – injury conditioned media, IR – ischemia-reperfusion, Col – DSS-induced colitis.

2.4.3 Pre-treatment of HPC-7 with Platelet Microparticle Enriched Supernatant (PES) and Purified Platelet Microparticles (PMP)

HPC7 were suspended in either PES or purified PMP at a density of $4x10^6$/ml at 37°C, 5% CO_2 for 1 hour. The HPC7 were then centrifuged at 300x*g* for 5 minutes and resuspended in un-supplemented Stem Pro at the required volume immediately prior to use in experiment.

2.4.4 Antibody Blockade of HPC7 Integrins

To ascertain the roles of critical surface adhesion molecules potentially utilised by HPC7 to adhere within injured tissue, the integrin subunits CD18 (β_2 integrin subunit) and CD49d (α-subunit of the $\alpha_4\beta_1$ integrin) were functionally blocked with monoclonal antibodies. The Kalia laboratory have previously demonstrated an important role for CD18 in small intestinal and CD49d in hepatic stem cell homing (Kavanagh *et al.*, 2010, Kavanagh *et al.*, 2012). $2x10^6$ cells were resuspended in 250µl of PBS supplemented with 0.1% BSA and 80µg/ml of either LEAF (low endotoxin, azide free) rat anti-mouse CD18 (Clone: GAME-46; BD Pharmingen, UK) or rat anti-mouse CD49d (Clone: R1-2; Cambridge Bioscience, UK). Control experiments used the isotype control Rat IgG (Clone: RTK2758; Cambridge Biosciences, UK). Blockade treatments were performed on fluorescently labelled HPC7 for 15 minutes immediately prior to use. Kavanagh and colleagues previously demonstrated that this treatment regime was sufficient to achieve functional blockade of these integrins (Kavanagh, 2009).

2.5 Static Adhesion Assays

2.5.1 Stamper Woodruff Adhesion Assay

The Stamper-Woodruff (SW) frozen tissue adhesion assay used here is a modified form of the assay described by Stamper and Woodruff (Stamper and Woodruff, 1976). Frozen 10µm sections were

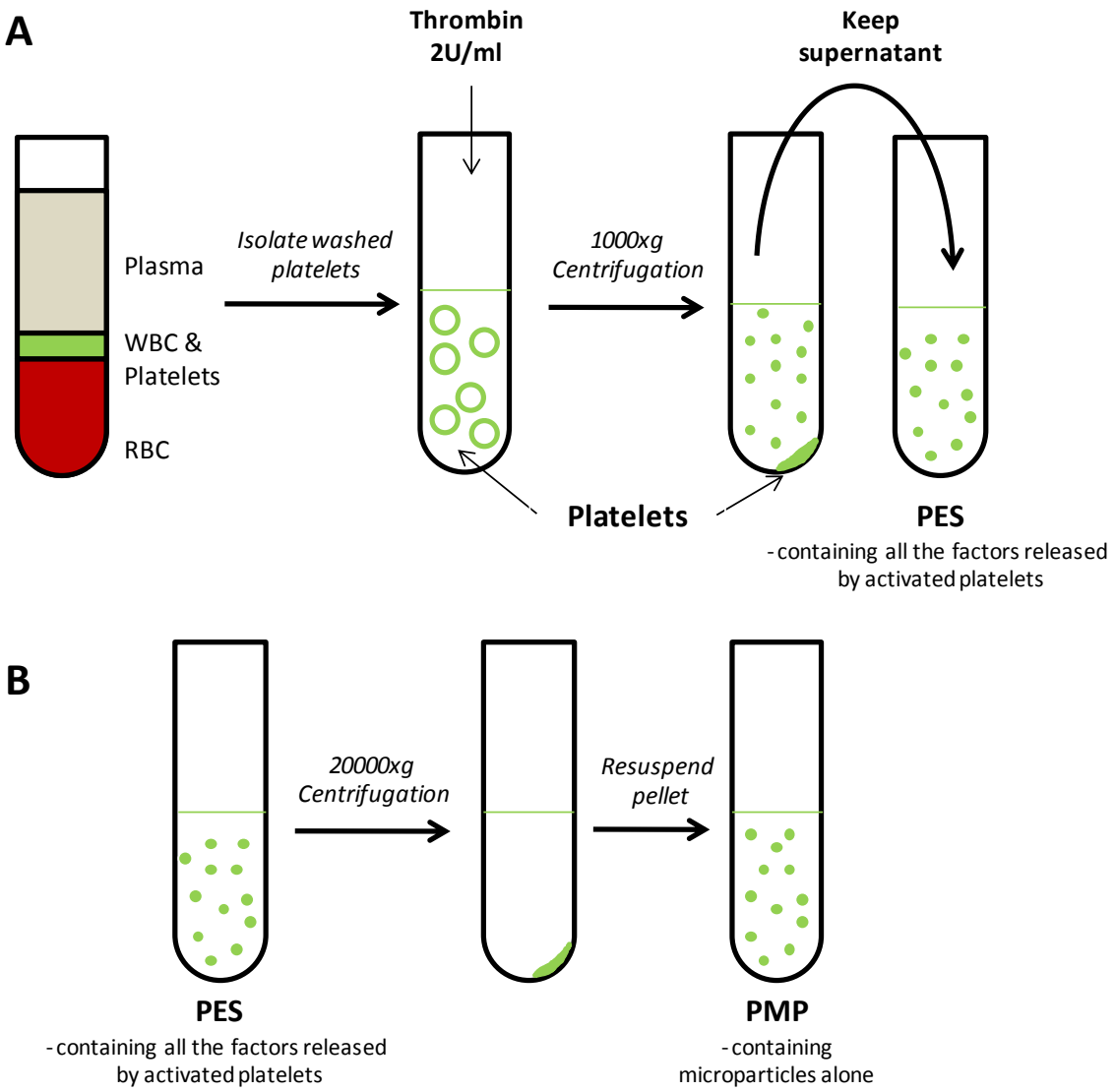

Figure 2.2 Generation and isolation of platelet microparticle enriched supernatant (PES) and purified platelet microparticles (PMP)

(A) Cartoon schematic of the procedure used to generate PES. Washed platelets are stimulated with thrombin for 30minutes and then remaining whole platelets are removed by centrifugation. The supernatant is then kept and the platelet pellet is discarded. To isolate PMP from PES the steps illustrated in (B) are performed. PES is centrifuged at 20000*xg* to pellet platelet microparticles in the solution. The supernatant is discarded and the PMP resuspended in unsupplemented media at the same volume as started with. In both A and B these steps are completed immediately prior to use in experiment.

fixed onto a glass slide and used to quantitate stem cell adhesion *in vitro*. Sections were taken from storage at -20°C and allowed to defrost at room temperature for 10 minutes. Once removed from foil the sections were circled using a wax immunology pen (Immedge, Vector Laboratories, UK) and washed with 1X PBS. 100µl of a fluorescently labelled HPC7 suspension in base Stem Pro SFM 34 (at a density of $1x10^6$ cells/ml) was added to each slide and incubated in a dark humidified tray for 20 minutes. Slides were then immediately fixed in 100% acetone for 5 minutes, washed with PBS, dried and coverslips mounted using Hydromount. Slides were kept in the dark until ready for analysis. Slides were imaged on the upright Olympus microscope using a 10x 1.4 numerical aperture objective air lens, frames captured with Slidebook v.5 (Intelligent Imaging Solutions) which were then exported for off-line analysis. Fluorescent cells were counted blind using in-house software. This SW assay was later modified such that 5-10mm lengths of intestinal frozen sections were used to quantitate stem cell adhesion *in vitro*. Details of this modification are fully described in the results section of Chapter 3.

2.5.2 Static Endothelial Adhesion Assay using Endothelial Cells Grown on Glass Coverslips

Gelatin coated coverslips with a monolayer of MCEC-1 or CEC maintained in a 24-well plate were used to determine stem cell adhesion *in vitro*. Prior to use, some wells were washed with PBS and then 500µl Stem Pro supplemented with 100ng/ml TNFα was added to the endothelium and incubated for 4 hours at 37^oC, 5% CO_2. This pre-treatment was done to stimulate endothelium in order to mimic an inflammatory injury. Some wells remained untreated by incubation with un-supplemented media as controls.

50µl cell suspensions, with cells at a density of $2x10^6$ cells/ml, were treated with sham or injury conditioned media (see section **2.4.1**), washed with Stem Pro and re-suspended in 500µl Stem Pro then immediately added to the endothelium. The cells and endothelium were incubated at 37°C, 5% CO_2 for 20 minutes. Each well was washed twice with PBS and the endothelium was kept in PBS for 10 minutes. The PBS was removed and the endothelium washed once more with PBS, followed by incubation with 2% glutaraldehyde (Sigma, UK) for 15 minutes at 37°C, 5%CO_2. Each well was then washed thoroughly 3 times with PBS. Coverslips were mounted onto glass microscope slides using slide mountant. Slides were stored in the dark until ready for imaging with an inverted Olympus microscope using a 10x 1.4 numerical aperture air objective lens and analysed using in-house cell counting software.

2.5.3 Static Endothelial Adhesion Assay Using Endothelial Cells Grown on Well Base

In order to simplify the adhesion assay, and thus increase throughput and accuracy, endothelial cells were grown on the base of tissue culture flasks (24 well plates) and imaged as above. Cells were handled in the exact same manner, with the exception that were not grown on coverslips and mounted prior to imaging.

2.5.4 Static Adhesion Assays using Immobilized VCAM-1 and ICAM-1

The base of a flat bottomed 96-well plate was coated with 50µl of recombinant murine (rm)VCAM-1 (R&D Systems, UK) or rmICAM-1 (R&D Systems, UK), which are the endothelial counter-ligands for the integrins containing CD49d and CD18 respectively, at a concentration of 10µg/ml for 1 hour at room temperature, or overnight at 4°C. To provide a negative control, some wells were coated with 1% bovine serum albumin in phosphate buffered solution (PBSA) under the same conditions. Protein solutions were then aspirated and wells washed three times with PBS. Any non-coated

plastic was subsequently blocked with heat denatured 1% PBSA for 1 hour at room temperature. Wells were washed 3 times with PBS and the pre-treated HPC-7s were added. HPC7 Fc receptors were simultaneously blocked using low endotoxin axide free (LEAF) CD16/32 blocking antibody (Biolegend, UK) at a dilution of 1:50. Some HPC7 were treated with function blocking monoclonal antibodies against CD18 (LEAF rat anti-mouse CD18 (GAME-46)) and CD49d (LEAF rat anti-mouse CD49d (R1-2)), as described in section 2.4.4, simultaneous to Fc blocking. Cells were incubated in the wells for 20 minutes at 37°C, then aspirated and washed gently 3 times with PBS. Cells were then fixed by incubation 2% glutaraldehyde for 15minutes at 37°C. At this stage, plates could be stored at 4°C until ready for imaging. To image, wells were aspirated and washed gently three times with PBS. 100µl of PBS was subsequently added and cells imaged using an inverted microscope using a 10x, 1.4 numerical aperture air objective.

2.6 Flow Cytometry Protocols

2.6.1 Flow Cytometry Investigation of Cell Surface Markers

Stem cells were removed from media by centrifugation (300x*g*, 5 minutes) and washed in cold PBS. Cells were then resuspended in 300µl PBS supplemented with 5% FBS to block Fc receptors. This was performed to reduce non-specific binding. Following Fc block, cells were incubated with a primary fluorescent antibody at a dilution of 1:250 for 15 minutes in darkness. Antibodies used were rat anti-mouse FITC conjugated anti-CD49d (Clone: R1-2, Cambridge Bioscience, UK), rat anti-mouse FITC conjugated anti-CD18 (Clone: GAME-46, Santa Cruz Biotechnology, US), rat anti-mouse PE conjugated anti-CD44 (Clone: IM7, eBioscience, US). Following antibody incubation, cells were washed three times with 300µl PBS supplemented with 5% FBS. Following washes, cells were resuspended in 300µl PBS supplemented with 5% FBS and supplemented with 1µg/ml propidium iodide, a fluorescent nuclear counter-stain. This was used to identify dead cells from a population as it is not permeable

to viable cells. Once stained, suspensions were loaded onto and analysed using a BD FACSCalibur flow cytometer.

2.6.2 Flow Cytometry Protocol for PMP Coating Verification

To confirm that HPC7s were actually coated with PMPs, flow cytometry was utilised to determine whether the expression of platelet surface markers were increased on the stem cell surface. HPC7 were pre-treated with PMP/PES as previously described and centrifuged at 300x*g* for 5 minutes. HPC7 were fixed in 300µl 1% neutral buffered formalin in FACS buffer (5% FBS in PBS) and incubated for 15 minutes at room temperature. HPC7 were centrifuged at 300x*g* for 5 minutes and subsequently resuspended in 100µl FACS buffer with 2µl conjugate antibody. Antibodies used were FITC-Rat anti-mouse IgG (poly4060; Biolegend, UK), FITC-Rat anti-mouse CD41 (platelet glycoprotein IIb of IIb/IIIa complex; MWReg30; BD Biosciences, UK) and FITC Rat anti-mouse GP42 (platelet GPIbα component of the GPIb-V-IX complex; Clone: Xia.B2, Emfret, Germany). Antibodies were incubated for 20 minutes in the dark at room temperature. HPC7 centrifuged at 300x*g* for 5 minutes and resuspended in 300µl FACS buffer. Samples then run on BD FACS Caliber (BD Biosciences, UK). Data were analysed offline using software detailed below.

2.7 Cell Preparation for Microscopic Analysis

2.7.1 Confocal Investigation of Integrin Clustering

HPC7 were labelled with Cell Tracker Orange (CTO) prior to pre-treatment with either H_2O_2 or PMP pre-treatments as described above. Following pre-treatment, HPC7s were fixed in 5% neutral buffered formalin/1% bovine serum albumin in PBS (PBSA) for 30 minutes at room temperature. Following fixation HPC7 were washed by centrifugation 3 times in 2% PBSA. Following final wash, HPC7 were left in the PBSA for 30 minutes to reduce any un-specific binding. HPC7 were washed and

resuspended in cold blocking antibody rat anti-mouse CD18 (Clone: GAME-46) or rat anti-mouse CD49d (Clone: R1-2) at a 2:25 dilution in 2% PBSA and incubated on ice for 60 minutes. HPC7 were washed 3 times then resuspended in cold Alexa Fluor 488 goat anti-rat IgG in 2% PBSA at 1:100 on ice for 30 minutes. Following incubation HPC7 were washed 3 times, allowed to settle onto poly-L-lysine coated coverslips overnight and then mounted using Hydromount prior to imaging. Slides were imaged using a DM IRE2 confocal microscope (Leica, UK), with a 63x oil immersion lens and images captured using Leica Control Software. Off-line image analysis was conducted using ImageJ software (NIH, US).

2.7.2 Confocal Investigation of PMP Coating

The physical interaction of PMPs with HPC7 cells was further confirmed confocally by labelling the two cells with different colour dyes. HPC7 were pre-treated with the PMP pre-treatments as described above. Following pre-treatment, HPC7 were fixed in 5% neutral buffered formalin/1% bovine serum albumin in PBS (PBSA) for 30 minutes at room temperature. Following fixation HPC7 were washed by centrifugation 3 times in 2% PBSA. Following final wash, HPC7 were left in the PBSA for 30 minutes to reduce any un-specific binding. HPC7 were washed and resuspended in cold blocking antibody Rat anti-mouse CD41 (MWReg30; BD Biosciences, UK) and Rat anti-mouse GPIbα (Clone: Xia.B2, Emfret, Germany) at a 1:50 dilution in 2% PBSA and incubated on ice for 60 minutes. HPC7 were washed 3 times then resuspended in cold Alexa Fluor 488 goat anti-rat IgG in 2% PBSA at 1:100 on ice for 30 minutes. Following incubation HPC7 were washed 3 times, allowed to settle onto poly-L-lysine coated coverslips overnight and then mounted using Hydromount prior to imaging. Slides were imaged using a DM IRE2 confocal microscope (Leica, UK), with a 63x oil immersion lens and images captured using Leica Control Software. Offline analysis conducted using ImageJ (NIH, US).

2.7.3 Scanning Electron Microscopy Analysis of HPC7 Surface Morphology

Samples were kindly prepared for SEM examination by Paul Stanley and Theresa Morris (Centre for Electron Microscopy, University of Birmingham). In brief, samples were fixed and suspended in 2.5% glutaraldehyde in phosphate buffer. Samples were settled onto poly-L-lysine coated coverslips overnight then processed for SEM by dehydrating. Samples were dehydrated through a graded ethanol series (2x15 minutes in 70% ethanol, 2x15 minutes 90% ethanol, 2x15 minutes 100% ethanol, 2x15 minutes Dried 100% ethanol). Samples were then critical point dried and mounted onto SEM stubs. Samples were then coated with evaporated carbon and examined in a Jeol 7000F SEM.

2.8 Software

For all static adhesion assays, cells were counted using in house cell counting software (written and developed by Dr D Kavanagh, University of Birmingham, UK) that allowed blind counting of adherent HPC7 per image. All flow cytometry data were analysed offline using Summit v.4.2 (Dako Cytommation, USA). All statistical analyses performed and graphs compiled using Prism 4 (GraphPad Software, USA). Image capture was completed using Slidebook v.5 (Intelligent Imaging Innovations, US) and Leica Control Software (Leica Microsystems, Germany). Dual colour images and cluster analysis calculated using ImageJ (NIH, US) with the “Find Maxima…” PlugIn.

2.9 Statistics

The statistical tests applied to identify significance have all been detailed within the figure legends for each experiment. In brief, however, two-way analysis of variance (ANOVA) was used to compare multiple treatments between several tissues. One-way ANOVA was used to compare multiple groups with a tissue. Dunnet’s post-hoc test was applied to identify significance within ANOVA groups when

compared to a single group. When this was not appropriate (as some groups were compared to one-another) Bonferroni's correction was used to identify significance. Student's t-test was used to compare two groups to one-another. Results were considered significant if $p<0.05$.

Chapter 3

USING ICM TO ENHANCE HPC7 ADHESION

3.0 Introduction

The adhesive mechanisms governing HSC recruitment to the inflamed mouse gut, especially the colon, are poorly known. Furthermore, it is not known whether intestinal HSC recruitment can be enhanced. Work within the Kalia laboratory has previously identified that HSC adhesion within tissue microvasculature *in vitro* and *in vivo* is increased following injury. Indeed, it has been shown that HPC7 are recruited more to the IR injured mouse liver (Kavanagh *et al.*, 2010), IR injured ileum (Kavanagh *et al.*, 2012, Kavanagh *et al.*, 2013) and to the IR injured kidney (White *et al.*, 2013) when compared to non-injured shams. Whilst, the increased adhesion as a result of injury has been identified in several tissues, the Kalia group has also demonstrated that the recruitment to various tissues is site specific, relying on different adhesive mechanisms. In the IR liver the adhesion is mediated by the VCAM-1-VLA-4 pathway (Kavanagh *et al.*, 2010), whereas in the gut the recruitment is predominantly dependent on CD18 (Kavanagh *et al.*, 2013). This chapter therefore firstly aimed to identify whether HPC7 adhesion was enhanced on different regions of the murine gut following an acute inflammatory IR injury and a more chronic colitis injury. Adhesion to the frozen tissue sections of jejunum (proximal region of small intestine), ileum (distal region of small intestine) and colon was investigated *in vitro*. Adhesion to the jejunum was also investigated, as the Kalia lab has previously demonstrated that IR injury induces the greatest injury to this region of the gut. Since recruitment to the colon is the focus of this thesis, recruitment to the IR and colitis injured colon was also determined intravitally. Furthermore, the role of CD18 and CD49d in mediating recruitment to the injured colon was determined both *in vitro* and *in vivo.*

It is well established that a whole host of factors within the injured microenvironment are capable of activating circulating leukocytes and platelets and thus permitting their adhesion to the endothelium (Mori *et al.*, 2005a, Vowinkel *et al.*, 2007b, Sprague and Khalil, 2009, Santen *et al.*, 2010, Cromer *et al.*, 2011). It was therefore hypothesised that similar events would also activate trafficking stem cells.

An injury conditioned media (ICM) was generated, potentially containing a whole host of pro-inflammatory / pro-adhesive factors, and its effects were compared to a sham conditioned media (SCM) generated from healthy tissue. ICM was generated from IR and colitis injured jejunum, ileum and colon and used to pre-treat HPC7s prior to quantitating their adhesion *in vitro* to frozen tissue sections and murine colon ECs. To identify the mechanisms by which the ICM could be enhancing stem cell adhesion, the effects of pre-treatment on HPC7 (i) adhesion molecule expression and (ii) affinity for endothelial counterligands ICAM/VCAM-1 was also determined. Luminex (Invitrogen, UK) was used to identify what was contained within the ICM.

3.1 Methods

3.1.1 Mice and injury models

Experiments were conducted on healthy C57BL/6 wild-type mice or those that had undergone either IR injury (45 minutes ischaemia, 120 minutes reperfusion) or 5 days of 3% DSS induced colitis injury.

3.1.2 Generation of an injury conditioned media production

In brief, conditioned media was derived by incubation of healthy and injured jejunum, ileum and ascending colon tissue for 24 hours in media at 37^{o}C. Tissue was macerated, solid material removed by centrifugation followed by filtration through a 0.22µm filter. HPC7 were incubated in SCM or ICM for 30 minutes at 37^{o}C, washed by centrifugation and resuspended in base media immediately before conducting an experiment.

3.1.3 Stamper Woodruff assay: method development

Conventionally, static cell adhesion assays on frozen tissue sections *in vitro,* also called the Stamper Woodruff (SW) assay, use transverse sections of tissue. However, initial attempts to section intestinal tissue transversely through the entire gut wall, with high reproducibility, proved extremely difficult due to the fragility of the tissue. As a result, the methodology was adapted such that intestinal tissue was firstly cut open longitudinally to expose the lumenal mucosa and then snap frozen. Tissue amounting to 60μm for the small intestinal sections and 20μm for the colon sections was then cryostat sectioned from the lumenal surface to expose the mucosal vasculature (**Figure 3.1**) and mounted onto glass slides lumen face up. Stem cells were incubated with these surfaces and the adhesion was then quantitated.

3.1.4 Endothelial static adhesion Assay

Murine colon or cardiac ECs, cultured in 24 well plates, were used to identify whether tissue specific differences in stem cell adhesion could be identified *in vitro*. Some ECs were treated with TNFα for 4 hours to activate them prior to monitoring adhesion of fluorescently labelled HPC7. Wells were washed and fixed prior to imaging.

3.1.5 Intravital microscopy

Intravital microscopy was performed to determine HPC7 recruitment in the colon microcirculation. Mice were anaesthetised and prepared for surgery as described in Chapter 2. The colon was exteriorised following a mid-line laparotomy and the mucosal surface of the ascending colon was exposed following an incision along the anti-mesenteric border. In mice undergoing IR injury, HPC7s were injected at 60 minutes post-reperfusion. In mice that had been induced with colitis, the colon was prepared for imaging and allowed to rest for 30 minutes prior to infusion of stem cells. A

randomly selected field of view was imaged every 5 minute for 60 minutes with adherent and free flowing cells were quantitated.

3.2 Results

3.2.1 Extensive intestinal tissue destruction following transverse sectioning results in poor reliability when used for the S-W assay

When intestinal tissue was sectioned transversely, much of the tissue became severely fractured, resulting in smaller fragmented intestinal tissue sections. When these sections were subsequently used for the S-W assay, the results were interpreted as the number of adherent HPC7 per 10000μm^2 to account for the extensive damage. Although HPC7 adhesion was increased on IR injured jejunal sections, it did not reach significance (**Figures 3.2 A, C, D**). The number of adherent HPC7 to IR injured ileum was significantly ($p<0.05$) greater than adhesion to sham injured ileum (**Figure 3.2 B, E, F**). Interestingly, previous studies from the Kalia group had demonstrated significantly increased HPC7 in both the IR injured mouse jejunum and ileum *in vivo.* The destruction of the tissue whilst preparing for the SW assay, coupled with variable and inconsistent results compared with those observed *in vivo,* highlight the un-reliable nature of this assay to investigate HPC7 adhesion to injured tissue sections *in vitro*.

3.2.2 HPC7 adhesion increased on IR and colitis injured tissue sections *in vitro*

To reliably investigate HPC7 adhesion *in vitro*, a modified SW assay was performed in which adhesion to the exposed mucosal surface was assessed. Using this modified sectioning method, we demonstrated that HPC7 adhesion was significantly increased to IR injured jejunum ($p<0.001$), ileum ($p<0.01$) and colon ($p<0.01$) when compared to sham controls (**Figure 3.3 A, C, E**). Furthermore,

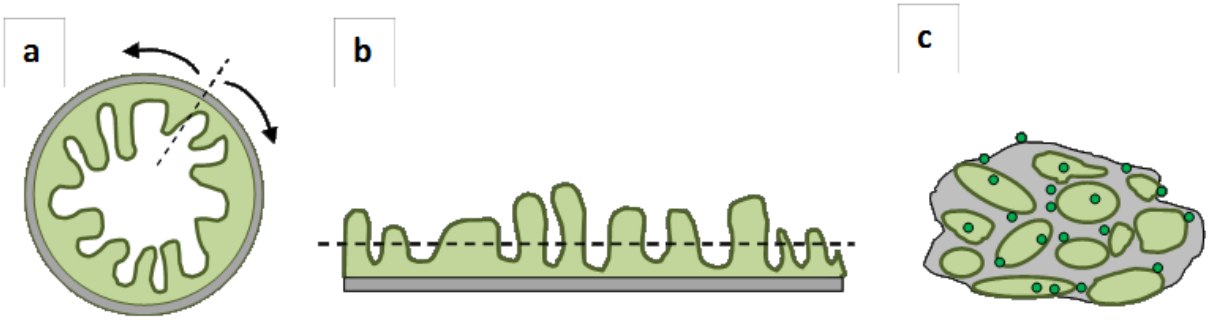

Figure 3.1 Modified Stamper-Woodruff tissue sectioning and assay for use with intestinal tissues

It proved difficult to cryostat section snap frozen mouse small intestine or colon in transverse. The gut was therefore cut open (dotted line) (**a**) and a layer was removed by cryostat sectioning from the lumenal surface (**b**) to expose the microvasculature within the small intestinal villi or the colonic crypts. (**c**) Adhesion of stem cells to this exposed surface was then quantitated with fluorescence microscopy.

HPC7 adhesion was also significantly increased on colitic injured jejunum ($p<0.01$), ileum ($p<0.05$) and colon ($p<0.01$) tissue sections compared to sham controls (**Figure 3.3 B, D, F**). Interestingly, the greatest adhesion was always observed on injured ileal and colon tissue and less on jejunum.

3.2.3 HPC7 recruitment increased in colon following both IR and colitis injury *in vivo*

HPC7 recruitment to IR and colitis injured colon mucosal microcirculation *in vivo* was investigated intravitally. HPC7 adhesion was significantly increased following IR injury ($p<0.01$) and colitis injury ($p<0.05$) when compared to shams (**Figures 3.4 A, B**). Adhesion was increased by approximately 2.5/3-fold throughout the duration of the experiment in both IR and colitis injured colons. The recruited HPC7 remained within the vasculature throughout the duration of the experiment. Free flowing cell number was also significantly increased in IR injured colon when compared to shams ($p<0.05$; **Figure 3.4 C**). However, this was not observed in colitis injured colon (**Figure 3.4 D**). Increased HPC7 adhesion could be the result of more HPC7s being delivered to the tissue due to reactive hyperaemia (increased blood flow), often observed as a consequence of tissue injury. However, blood flow was significantly reduced in both IR ($p<0.01$) and colitis ($p<0.01$) injured colon from the point of HPC7 infusion onwards compared with the sham controls (**Figure 3.4 E, F; Figure 3.5**). Therefore, the increased stem cell number within the injured tissues is not a result of reactive hyperaemia.

3.2.4 HPC7 recruitment in the ileum increased following IR injury - IR and colitis injury have no effect on pulmonary recruitment

To determine HPC7 recruitment to remote organs and to verify the colonic intravital observations, the colon, ileum and lungs were excised following intravital experimentation. *Ex vivo* quantification of HPC7 recruitment was quantitated in 5 random fields. As observed intravitally, HPC7 adhesion

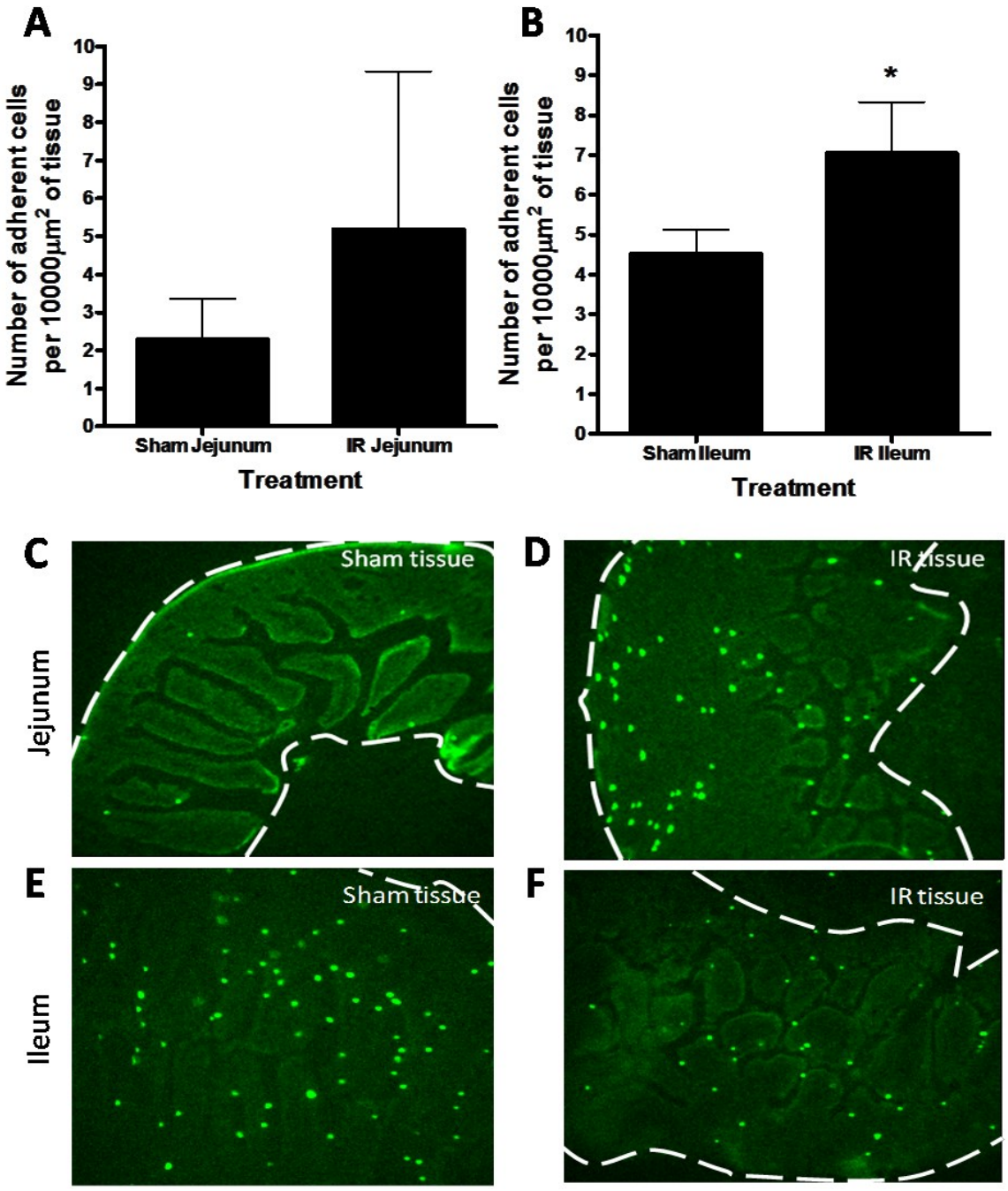

Figure 3.2 More HPC7 cells adhere to IR injured ileum than healthy ileum but similar results are not obtained on the jejunum. These studies were conducted using our original methodology of cutting transverse intestinal sections.

(A) There is no significant difference in adhesion of HPC7 adhesion between sham and IR injured. (B) HPC7 adhere significantly more to IR injured ileum than sham injured ileum. C, D, E and F are representative fluorescence micrographs of adherent HPC7 (brighter dots) to sham jejunum, IR injured jejunum, sham ileum and IR injured ileum respectively. Dotted lines denote tissue boundaries as defined for analysis. Results are presented as mean number of adherent HPC7 per 10000µm^2 ± SEM. Statistical analysis performed using one-way Student's t-test, N≥4 for all groups. *p<0.05.

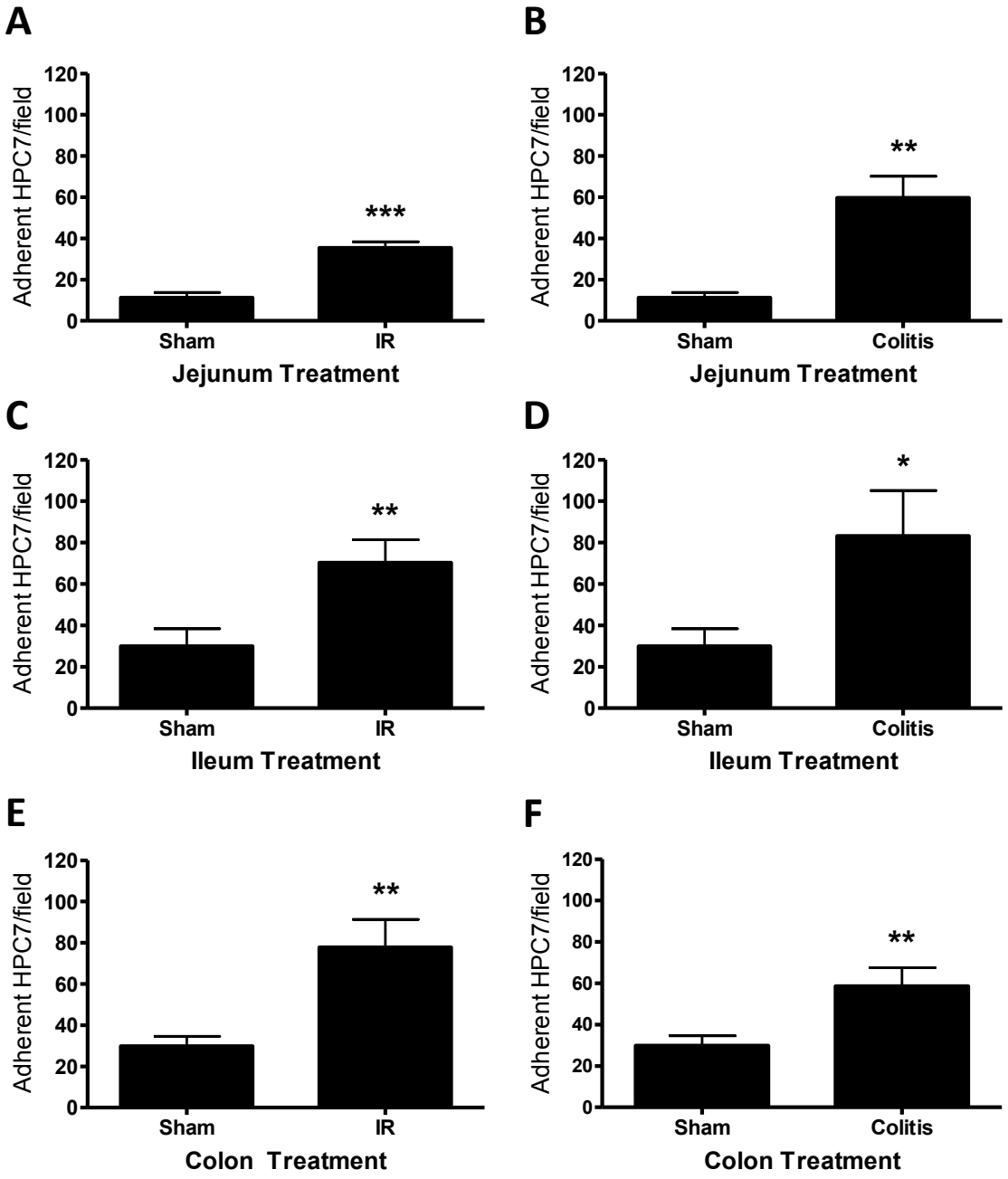

Figure 3.3 HPC7 adhere significantly more to IR and colitis injured snap frozen intestinal tissue sections using the modified methodology of cutting intestinal sections.

HPC7 adhere significantly more to longitudinally sectioned snap frozen IR injured jejunum ($p<0.001$; A) and colitis injured jejunum ($p<0.01$; B) tissue sections compared to vehicle controls. Similarly, HPC7 adhere significantly more to IR ($p<0.01$; C) and colitis ($p<0.05$; D) injured ileum sections compared to vehicle controls. Similarly, HPC7 adhere significantly more to colon tissue sections following IR ($p<0.01$; E) or colitis ($p<0.01$; F) injury compared to vehicle controls. Results presented as mean ± SEM. Statistical analyses performed using one-way Student's t-test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

was significantly increased in both the IR injured colon ($p<0.05$) and the colitis colon ($p<0.01$; **Figure 3.6 A**) when compared to sham controls. Recruitment to the ileum was significantly ($p<0.001$) greater following IR injury compared with the sham control. However, there was no significant difference between the number of adherent cells found in the colitis injured ileum and sham controls (**Figure 3.6 B**). Following both IR and colitis injury, there was no significant difference in recruitment to the lungs (**Figure 3.6 C**).

3.2.5 HPC7 recruitment in IR injured colon microcirculation dependent on CD18 *in vivo*

Use of a function blocking antibody against CD18 (β_2 integrin) resulted in a significant reduction in HPC7 adhesion in IR injured colon (**Figure 3.7 A**). Conversely, blocking CD49d (α_4 integrin) had no effect on HPC7 adhesion to IR injured colon (**Figure 3.7 B**). Blocking either integrin had no effect on the number of free flowing HPC7 observed during the experiment compared with sham controls (**Figure 3.7 C, D**).

3.2.6 HPC7 recruitment to colitis injured colon dependent on CD18 and CD49d *in vivo*

Use of a function blocking antibody against CD18 resulted in a significant reduction in HPC7 adhesion in colitis injured colon (**Figure 3.7 E**). Similarly, blocking CD49d also significantly reduced adhesion in the colitis colon (**Figure 3.7 F**). It is noteworthy that unlike in IR injury, blocking either CD18 or CD49d only partially reduced HPC7 adhesion in the colitis injured colon compared to the basal level of adhesion exhibited in IR following CD18 blockade. Furthermore, blocking CD18 or CD49d did not influence the number of free flowing HPC7 observed in the colitic colon (**Figure 3.7 G, H**).

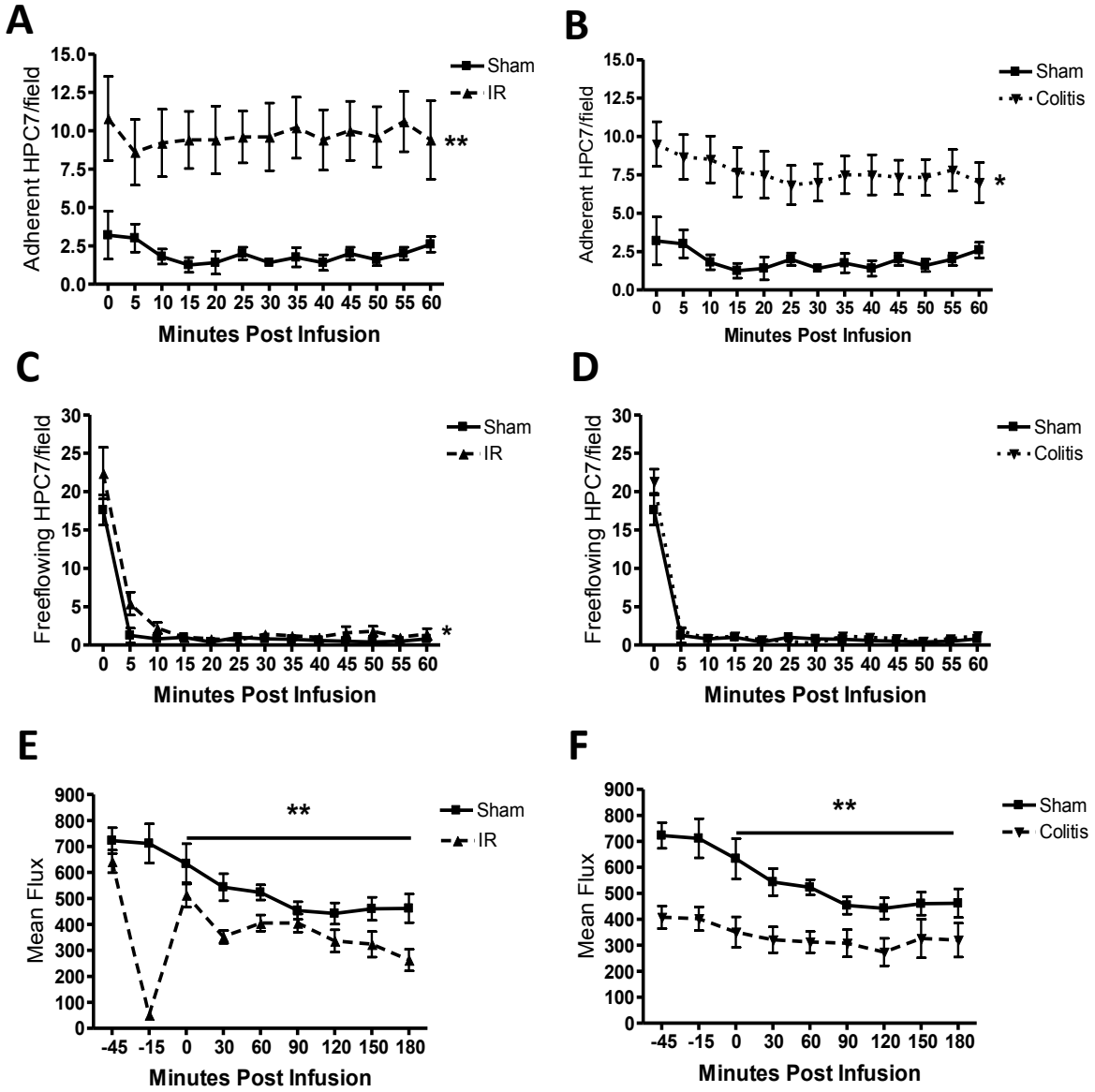

Figure 3.4 HPC7 adhere significantly more to IR and colitis injured tissue in vivo, without an increase in perfusion of the injured colon.

HPC7 adhere significantly more to the IR injured ($p<0.01$; A) and colitis injured ($p<0.05$; B) colon microvasculature *in vivo*. (C) Following IR injury there is a modest, but significant ($p<0.05$), increase in the number of HPC7 freeflowing through the microvasculature compared to that seen in the sham. However, there is no significant difference in the number of freeflowing HPC7 seen in either colitis or sham colon (D). (E) Mean flux values, directly proportional to blood flow, are lower following IR ($p<0.01$; E) and colitis ($p<0.05$; F) injury during the intravital monitoring period. Results are presented as mean ± SEM. Statistical analyses performed using one-way t-tests, analysing the area under the curves, $N\geq 5$ for all groups. *$p<0.05$, **$p<0.01$. In (E) and (F) bar represents area of graph compared for analysis – "infusion period".

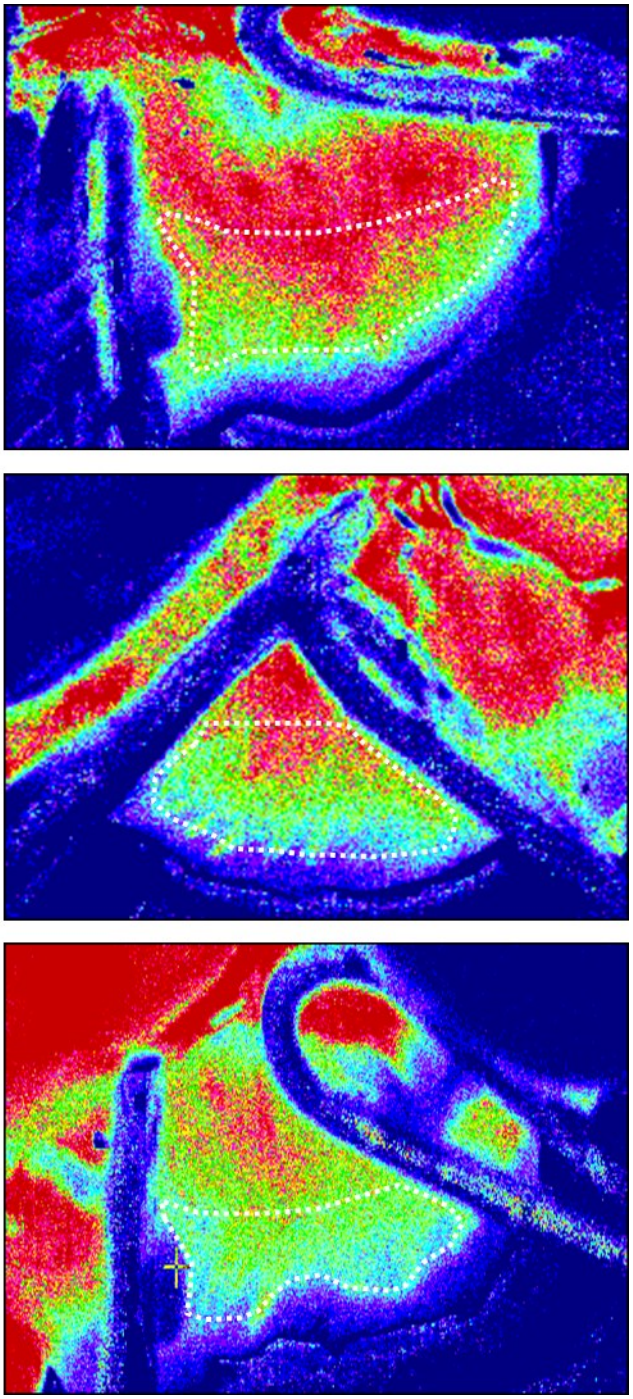

Figure 3.5 IR and colitis injury was not associated with an increase in blood flow to the colon

Mean flux values, directly proportional to blood flow, was lower following IR ($p<0.01$; B) and colitis ($p<0.05$; C) injury when compared to sham controls (A). This suggests increased HPC-7 adhesion in the injured colon is not due to increased blood flow delivering more cells to the organ. These images were obtained using laser speckle microscopy of the colon *in vivo*. (Moor Instruments Ltd). Red indicates areas of high blood flow and blue areas of least blood flow. Dotted lines define mask bounding in focus region in which flux values were obtained from.

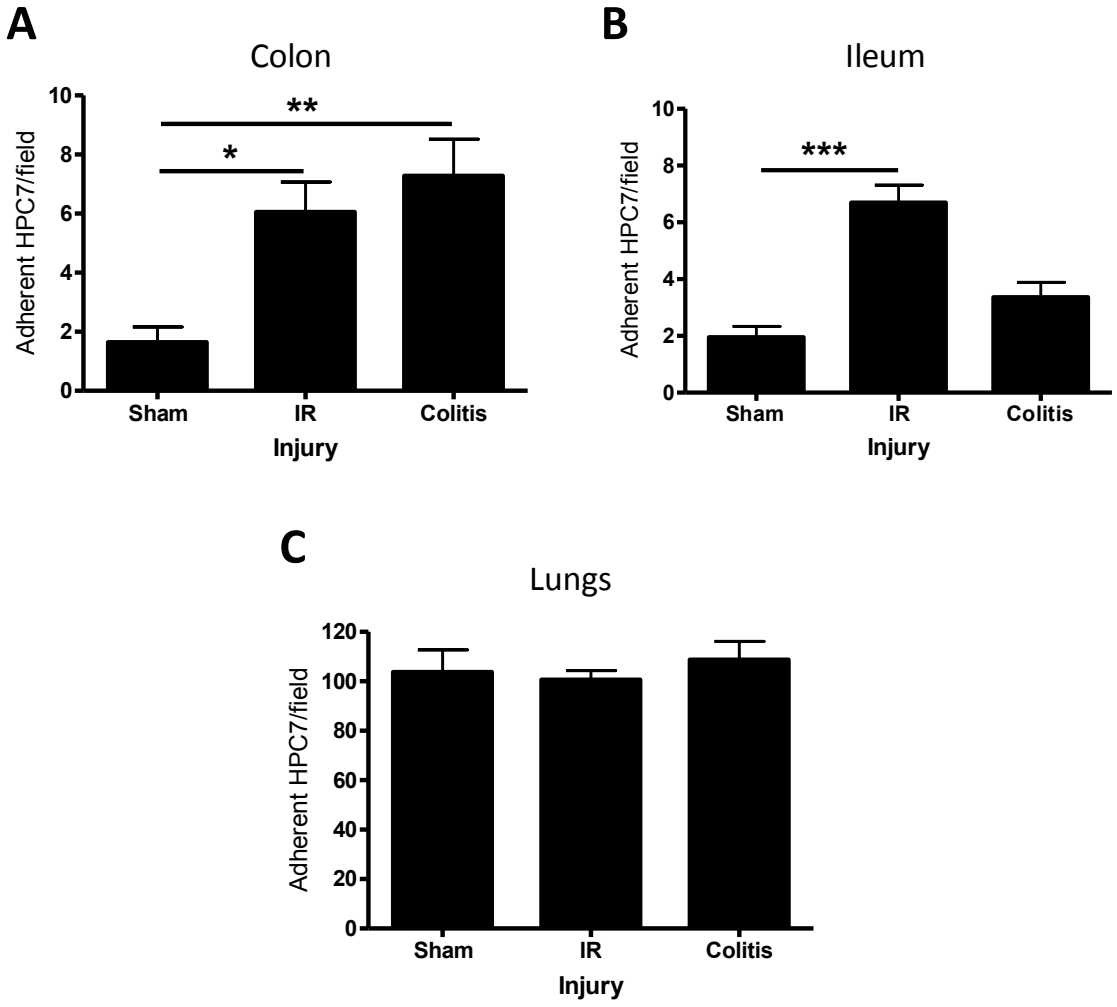

Figure 3.6 IR injury increases HPC-7 adhesion in ileum and colon, colitis injury increases adhesion in colon, and neither injury affects pulmonary adhesion when examined ex vivo

(A) Both IR and colitis injury significantly enhance adhesion within the colon ($p<0.05$, $p<0.01$ respectively). (B) Analysis of the ileum revealed that HPC7 adhesion within the ileum is significantly increased ($p<0.001$) following IR injury, but colitis injury does not enhance ileal recruitment. (C) *Ex vivo* analysis of tissues following IR and colitis intravital experiments reflect that HPC7 adhesion within the lungs is unaffected. Results presented as mean ± SEM. Statistical analysis performed with one-way ANOVA with Dunnet's post hoc testing. N≥5 for all groups. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.2.7 *Ex vivo* tissue analysis confirms the observations seen *in vivo* - CD18 critical in IR injury and both CD18 and CD49d used in colitis injury

As observed intra-vitally, blocking CD18 significantly ($p<0.05$) reduced adhesion within the IR injured colon, whereas blockade of CD49d had no effect (**Figure 3.8 A**). *Ex vivo* analysis of the IR injured ileum also revealed that blocking CD18, but not CD49d, significantly ($p<0.05$) reduced adhesion (**Figure 3.8 B**). Blocking CD18 or CD49d did not affect adhesion within the lungs isolated from an IR injured mice (**Figure 3.8 C**).

As observed intravitally, blocking either CD18 ($p<0.01$) or CD49d ($p<0.01$) significantly reduced adhesion within the colitic colon (**Figure 3.8 D**). *Ex vivo* analysis of the colitis injured ileum showed a trend for adhesion to decrease with blocking of either CD18 or CD49d but this did not reach statistical significance (**Figure 3.8 E**). Again, blocking CD18 or CD49d did not affect adhesion within the lungs isolated from colitis injured mice (**Figure 3.8 F**).

3.2.8 Pre-treatment of HPC7 with IR and colitis ICM increases their adhesion to colon endothelial cells *in vitro*

To investigate whether HPC7 adhesion can be modulated, they were pre-treated with various conditioned medias. The effects on adhesion to a murine colon derived endothelial monolayer was then determined. Interestingly, TNFα activation of colon ECs did not cause an increase in HPC7 adhesion (**Figure 3.9 A-D**). Furthermore, pre-treating HPC-7 with sham conditioned media (SCM) generated from healthy jejunum, ileum or colon did not increase adhesion compared with vehicle controls (**Figure 3.9 A-D**). Jejunum IR injury conditioned media (IR ICM) significantly increased adhesion when compared to vehicle control ($p<0.01$) and the SCM control ($p<0.05$), whereas jejunum colitis injured conditioned media (Col ICM) had no significant effect on enhancing adhesion (**Figure 3.9 A**). Similarly, ileal IR ICM significantly enhanced adhesion compared to vehicle control

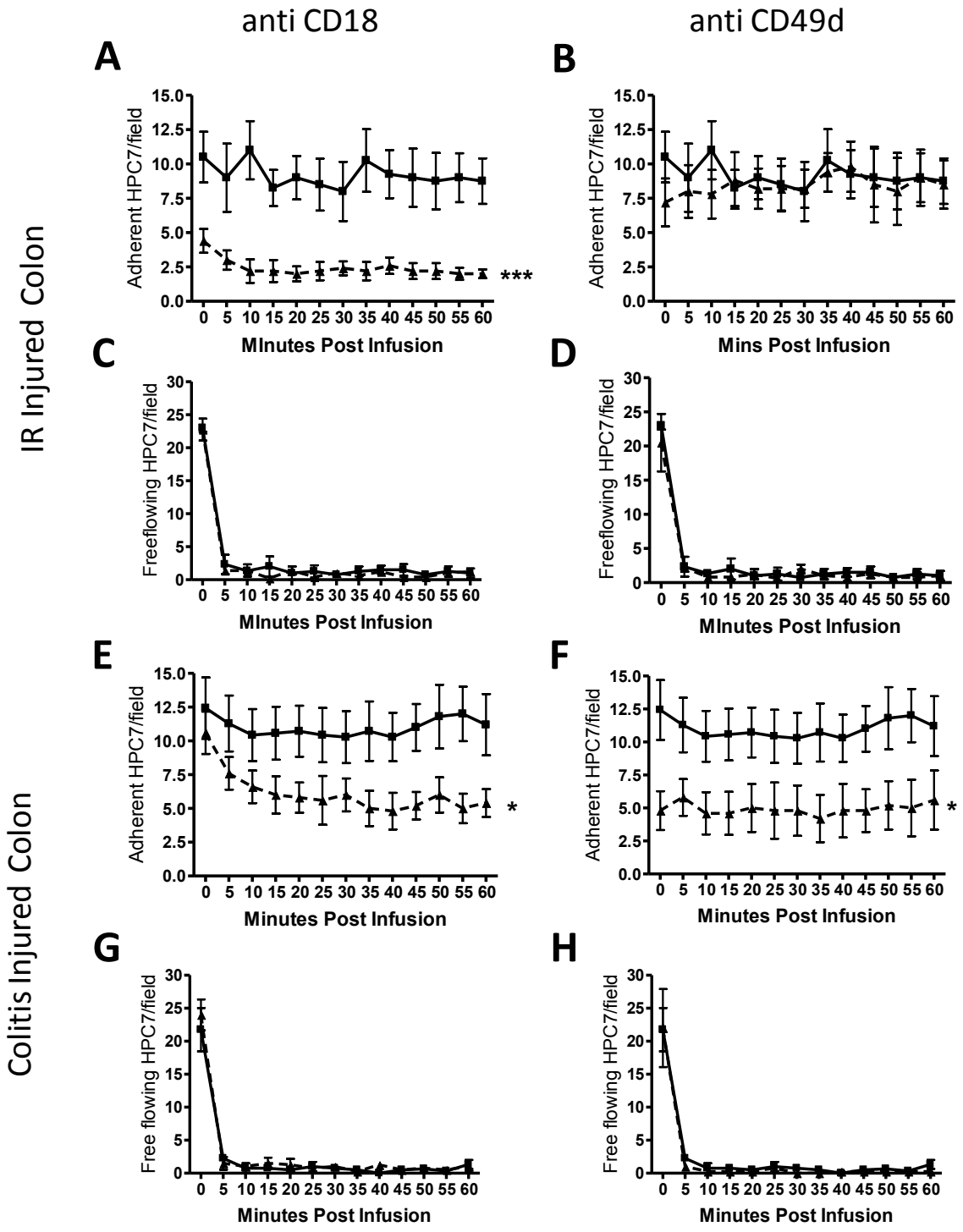

Figure 3.7 HPC7 adhesion to the IR colon is dependent on CD18 and independent of CD49d, whereas HPC7 adhesion to the colitis colon utilises both CD18 and CD49d

Blockade of CD18 significantly reduced adhesion in IR injured colon (A) whereas CD49d had no role (B). In contrast, blocking CD18 (E) or CD49d (F) significantly reduced adhesion in the colitis colon but adhesion was not completely abolished. Blocking integrins CD18 or CD49d did not affect numbers of free flowing HPC7 in IR injured (C-D) or colitis (G-H) colons. Results presented as mean ± SEM. Solid line: IgG control, dotted line anti-integrin group. Statistical analysis performed on area under the curve using Student's t-test. N≥5 for all groups. *p<0.05, ***p<0.001.

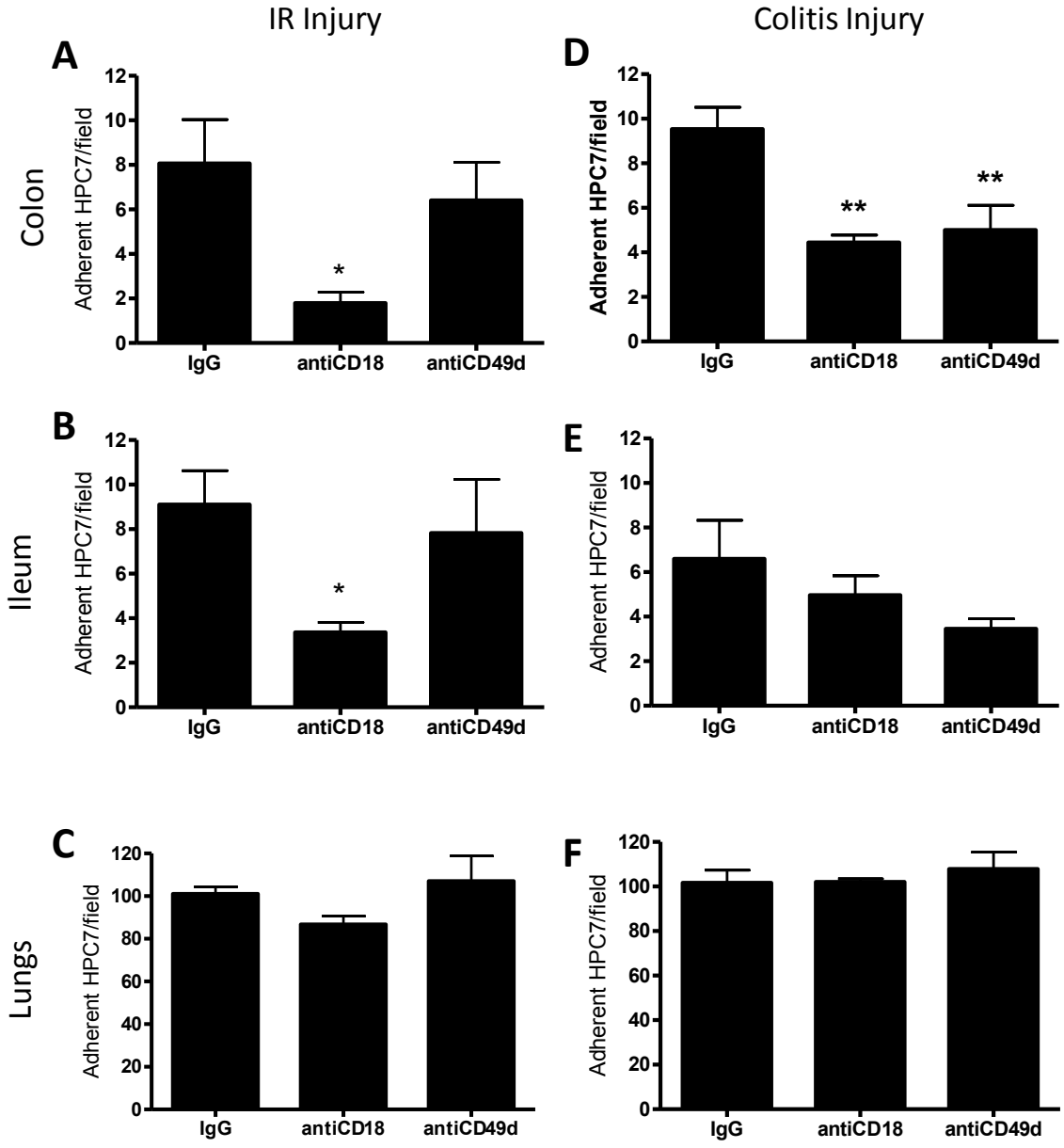

Figure 3.8 Ex vivo analysis confirms the observations seen intravitally following antibody blockade – CD18 critical in IR injury and both CD18 and CE49d used in colitis injury

Recruitment to IR injured colon (A) and ileum (B) was significantly reduced after blocking CD18, but not CD49d. Blocking CD18 or CD49d had no effect on pulmonary recruitment following intestinal IR injury (C). Recruitment to colitis injured colon (D) and ileum (E) was reduced after blocking either CD18 or CD49d, but this was only significant in the colon. Again, blocking CD18 or CD49d had no effect on pulmonary recruitment following intestinal colitis injury (F). Results presented as mean ± SEM. Statistical analysis performed using one-way ANOVA with Dunnet's post-hoc testing. *p<0.05, **p<0.01.

(**Figure 3.9 A**), but ileum Col ICM did not (**Figure 3.9 B**). Again, colon IR ICM significantly ($p<0.05$) increased adhesion to colon ECs compared to vehicle control, but Col ICM had no effect (**Figure 3.9 C**). To determine whether the adhesion enhancing effects of colon ICM were specific for colon ECs only, adhesion was also determined on cardiac ECs. Again, TNFα pre-treatment of cardiac ECs had no effect on adhesion (**Figure 3.9 D**).

To investigate whether pre-treatment of HPC7 with the various conditioned medias affected their survival, cells were pre-treated, washed and re-suspended in normal growth media. The number of viable cells 24 hours later was determined using trypan blue exclusion. No significant differences in cell viability was observed between any of the groups (**Figure 3.9 E)**.

3.2.9 Pre-treatment of HPC7 with conditioned media increases their adhesion to frozen tissue sections *in vitro*

3.2.9.1 Jejunum and jejunal conditioned media

Following the quantitation of HPC7 adhesion to sham and IR injured jejunal tissue following pre-treatment with vehicle, SCM and IR ICM, it was identified that the pre-treatment ($p<0.01$) and the injury state of the tissue ($p<0.001$) influenced HPC7 adhesion. However, there was no significant interaction between these variables. SCM increased adhesion to both sham ($p<0.001$) and IR ($p<0.05$) tissue compared with vehicle controls (**Figure 3.10 A**). Similarly, IR ICM pre-treatment significantly increased adhesion to both sham ($p<0.001$) and IR ($p<0.001$) jejunum sections (**Figure 3.10 A**). However, IR ICM treated cells adhered significantly more ($p<0.05$) to IR injured tissue than to sham tissue (**Figure 3.10 A**).

Again, adhesion of HPC7 to sham and colitic injured jejunum tissue was significantly affected as a result of cellular pre-treatment ($p<0.001$) and the injury status of the tissue ($p<0.01$). Col ICM significantly enhanced adhesion to colitic tissue compared to sham jejunal tissue ($p<0.001$; **Figure 3.10 B**). Furthermore Col ICM enhanced adhesion to colitic tissue more than the vehicle control ($p<0.05$) and SCM ($p<0.01$), whereas SCM did not increase adhesion on colitis tissue (**Figure 3.10 B**).

3.2.9.2 Ileum and ileal conditioned media

Similarly, HPC7 adhesion to sham and IR injured ileum following pre-treatment with vehicle, SCM and IR ICM also displayed that both the pre-treatment ($p<0.001$) and the injury state of the tissue ($p<0.001$) significantly affected HPC7 adhesion. Again, there was no interaction between the two groups. Unlike jejunal tissue, pre-treatment with ileal SCM had no effect on adhesion to either sham or IR injured tissue (**Figure 3.10 C**). IR ICM significantly increased adhesion to IR injured tissue ($p<0.001$), but not to sham injured tissue. Adhesion to IR injured ileum following pre-treatment with IR ICM was significantly ($p<0.001$) greater than the adhesion of IR ICM pre-treated HPC7 to sham injured tissue (**Figure 3.10 C**).

Colitis ICM was more effective at enhancing adhesion on ileal tissue (**Figure 3.10 D**). Again, adhesion to ileum sham and colitis tissue was significantly affected by pre-treatment ($p<0.001$) and injury of the ileal sections ($p<0.001$), but there was no significant interaction between them. Colitis ICM significantly enhanced adhesion to colitis injured tissue when compared to sham ileal tissue (**Figure 3.10 D**) and similarly, within the colitic tissue colitis ICM pre-treatment significantly increased adhesion compared to the vehicle control ($p<0.05$) and SCM ($p<0.001$). However, SCM had no significant effect on adhesion to colitis tissue (**Figure 3.10 D**).

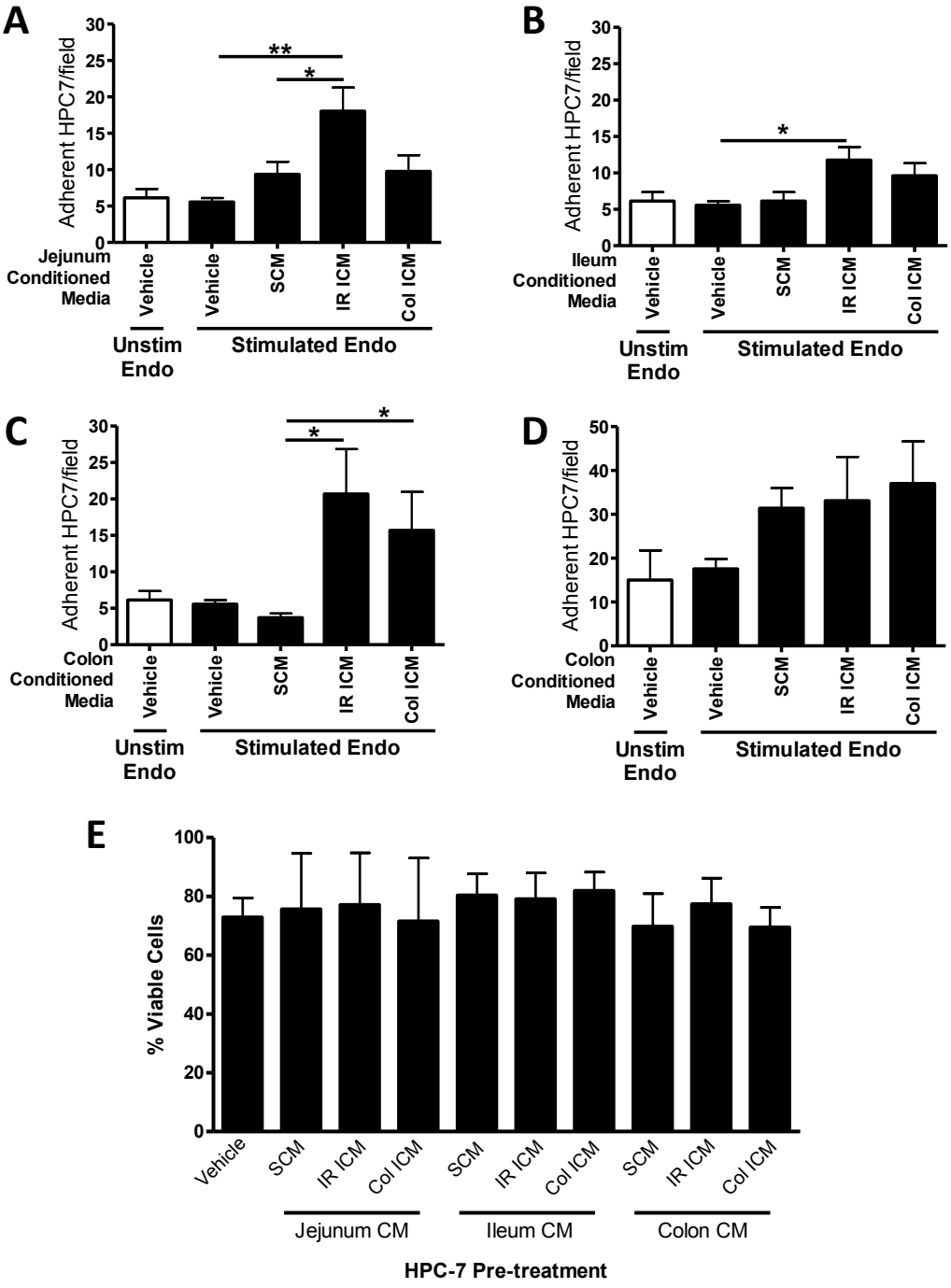

Figure 3.9 Pre-treating HPC7 can enhance adhesion to colon endothelial cells in vitro, but does not affect cell viability

(A) Pre-treatment with jejunum IR injury conditioned media (IR ICM) significantly enhanced adhesion to colon endothelium compared to sham conditioned media (SCM; $p<0.05$) and vehicle controls ($p<0.01$). (B) Similarly, ileum IR ICM significantly enhances adhesion ($p<0.05$) compared to vehicle control. (C) Both IR and colitis colon ICM significantly ($p<0.05$) increase adhesion compared to SCM controls. (D) Pre-treatment of HPC7 with CM had no significant effect on HPC7 adhesion to murine cardiac endothelium. (E) Pre-treatment of HPC7 has no effect on cellular viability as determined by trypan blue exclusion. Results presented as mean ± SEM. Statistical analysis performed using one-way ANOVA with Dunnet's post-hoc testing. *$p<0.05$, **$p<0.01$.

3.2.9.3 Colon and colonic conditioned media

HPC7 adhesion to sham and IR injured colon sections was significantly affected by both the pre-treatment ($p<0.01$) and the injury status of the tissue ($p<0.001$) but there was no significant interaction observed between them. Similarly to the jejunum, HPC7 pre-treated with colon SCM significantly increased adhesion to both sham ($p<0.05$) and IR colon ($p<0.05$) sections (**Figure 3.10 E**). However, colon IR ICM only increased adhesion to IR injured colon sections ($p<0.05$) compared to vehicle control and to SCM treated HPC7 (**Figure 3.10 E**). Again, HPC7 adhesion following colon IR ICM pre-treatment was significantly ($p<0.001$) greater to IR tissue than that seen on sham tissue following pre-treatment (**Figure 3.10 E**).

Colitis conditioned media was more effective at enhancing adhesion to sham as well as injured tissue (**Figure 3.10 F**). Again, adhesion was significantly affected by pre-treatment ($p<0.001$) and by injury to the colon sections ($p<0.001$) but there was no significant interaction between them. Colitis ICM significantly enhanced adhesion to both sham ($p<0.001$) and colitis tissue ($p<0.001$). Furthermore, the increase in adhesion following pre-treatment was greater following colitis injury when compared to sham controls ($p<0.001$). Interestingly, SCM resulted in an increase in adhesion both on sham tissue ($p<0.05$) and colitis tissue ($p<0.05$), but in both cases the increase was lower than that seen when treated with colitis ICM.

3.2.10 Pre-treatment of HPC7 with conditioned media does not increase surface expression of CD18 or CD49d

Flow cytometry was performed to identify whether pre-treating HPC7 with conditioned media had any effect on the surface expression of the CAMs CD18, CD49d or CD44. Pre-treatment with jejunal SCM, IR ICM or Col ICM had no effect on CD18 or CD49d expression (**Figure 3.11 A**). Col ICM

appeared to cause a reduction in CD44 expression, but these decreases did not reach significance (**Figure 3.11 A**).

Similarly, Ileal SCM, IR ICM and Col ICM had no effect on CD18 or CD49d expression (**Figure 3.11 B**), but Col ICM did significantly ($p<0.05$) reduce the surface expression of CD44 (**Figure 3.11 B**).

Colon conditioned media also had no effect on the surface expression of CD18 and CD44, but Col ICM does appear to show an increase in CD49d expression (**Figure 3.11 C**) but this increase does not reach significance.

3.2.11 Pre-treatment of HPC7 with injury conditioned media increases their adhesion to ICAM-1 and VCAM-1

3.2.11.1 Adhesion to ICAM-1

There was a significant effect of pre-treating HPC7 ($p<0.001$) on their adhesion to ICAM-1 and also a significant difference ($p<0.05$) between tissue of origin of the conditioned media and the effect on adhesion, however, there was no interaction between the two variables. Only pre-treatment with jejunal IR ICM significantly increased adhesion to ICAM-1 when compared to vehicle ($p<0.01$) and SCM ($p<0.01$; **Figure 3.12 A**). Ileal IR ICM and Col ICM pre-treatment significantly increased adhesion to ICAM-1 when compared to vehicle controls (IR ICM $p<0.001$, Col ICM $p<0.05$) and SCM (IR ICM $p<0.001$, Col ICM $p<0.05$; **Figure 3.12 A**). Colon SCM, IR ICM and Col ICM pre-treatment significantly increased adhesion to ICAM-1 when compared to vehicle controls (SCM $p<0.05$, IR ICM $p<0.05$, Col ICM $p<0.05$; **Figure 3.12 A**). Colon IR ICM and Col ICM pre-treatment also significantly increased adhesion when compared to SCM (IR ICM $p<0.01$, Col ICM $p<0.05$; **Figure 3.12 A**).

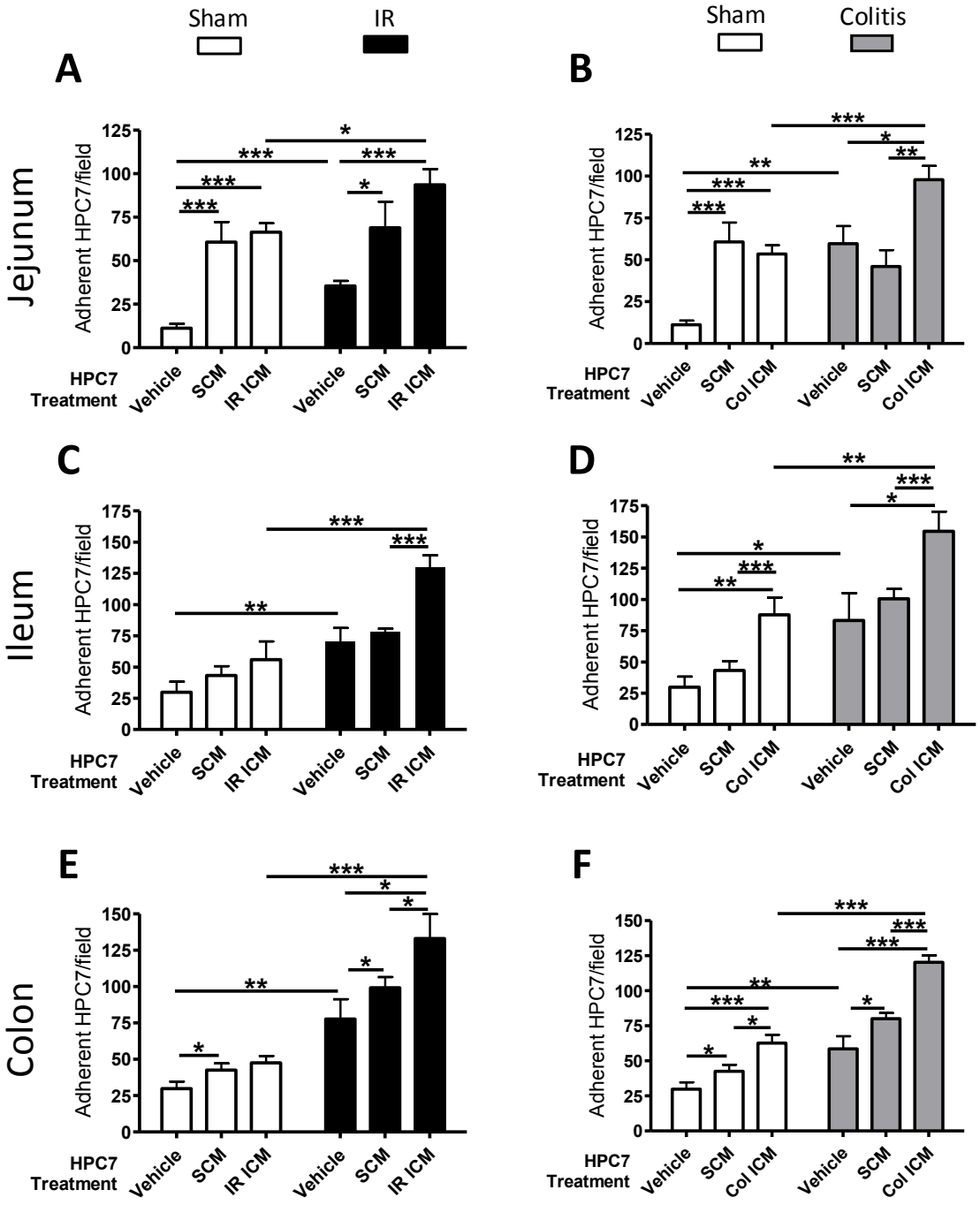

Figure 3.10 Pre-treatment of HPC7 significantly enhances adhesion to intestinal tissue sections with the greatest adhesion seen to injured tissues compared to sham tissues

(A-B) Adhesion to jejunal sham, IR injured tissue and colitis tissue following various pre-treatments is presented. Interestingly, even SCM could influence adhesion to healthy jejunal tissue. (C-D). Adhesion to ileal sham, IR injured tissue and colitis tissue following various pre-treatments is presented. (E-F) Adhesion to colon sham, IR injured tissue and colitis tissue following various pre-treatments is presented. Interestingly, even SCM could influence adhesion to healthy colon tissue. Results presented as mean ± SEM. Statistical analysis performed with two-way ANOVA, with Student's t-test performed post-hoc. *p<0.05, **p<0.01, ***p<0.001. Abbreviations SCM- sham conditioned media, IR ICM- IR injury conditioned media, Col ICM- colitis conditioned media.

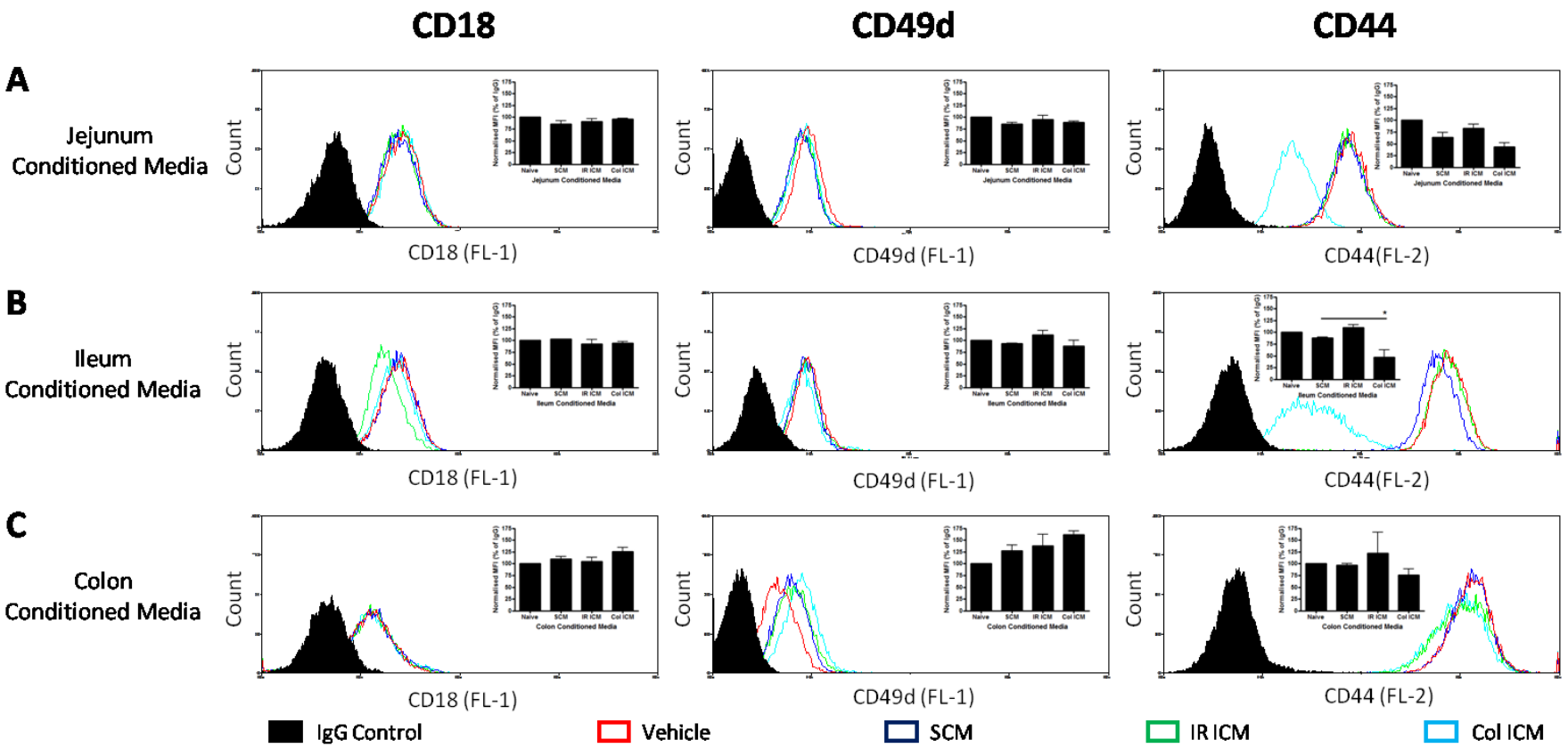

Figure 3.11 Pre-treatment of HPC7 with conditioned media has no effect on CD18 or CD49d expression, but small bowel Col ICM reduces expression of CD44

(A) Pre-treatment with jejunum SCM, IR ICM and Col ICM has no effect on CD18 or CD49d expression, however, Col ICM reduces CD44 expression but the decrease is not significant. (B) Pre-treatment with ileum SCM, IR ICM and Col ICM has no effect on CD18 or CD49d expression, but there is a significant decrease in CD44 expression ($p<0.05$). (C) Colon SCM, IR ICM and Col ICM have no effect on CD18, CD49d and CD44 expression. Data presented as fluorescence histographs. Inset: bar graphs with geometric mean fluorescence intensity values for each group. Statistical analysis performed with one-way ANOVA with Dunnet's post-hoc testing.

3.2.11.2 Adhesion to VCAM-1

There was a significant effect of pre-treating HPC7 ($p<0.001$) on their adhesion to VCAM-1 but there was no significant differences due to the origins of the conditioned media on adhesion and furthermore, there was also no interaction between the two variables. Pre-treatment of HPC7 with jejunal IR ICM and Col ICM significantly increased adhesion to VCAM-1 when compared to vehicle (IR ICM $p<0.001$, Col ICM $p<0.05$) and SCM (IR ICM $p<0.01$, Col ICM $p<0.05$; **Figure 3.12 B**). Ileal SCM pre-treatment also caused a significant ($p<0.05$) increase when compared with vehicle (**Figure 3.12 B**). Similarly, ileum IR ICM and Col ICM significantly increased adhesion when compared to vehicle (IR ICM $p<0.05$, Col ICM $p<0.01$) and SCM (IR ICM $p<0.05$, Col ICM $p<0.05$; **Figure 3.12 B**). Colon SCM significantly ($p<0.05$) increased adhesion as did colon IR ICM ($p<0.05$) and Col ICM ($p<0.05$) when compared to vehicle. Colon IR ICM ($p<0.05$) and Col ICM ($p<0.05$) also significantly increased adhesion when compared to SCM (**Figure 3.12 B**).

3.2.12 Luminex analysis of conditioned media did not identify any cytokines or chemokines

To determine what bioactive molecules may be present within the conditioned media, the samples were investigated using a Luminex assay (Invitrogen, UK) targeted against a (20mer plex) wide screen of cytokines and chemokines including IFNγ, IL1β and KC. However, no cytokines or chemokines were within the conditioned media at a detectable level (≈pg/ml sensitivity).

3.3 Discussion

In this chapter, we have developed a murine model of colon IR and colitis injury and also successfully imaged stem cell trafficking intravitally within the mucosal microcirculation. We present novel *in*

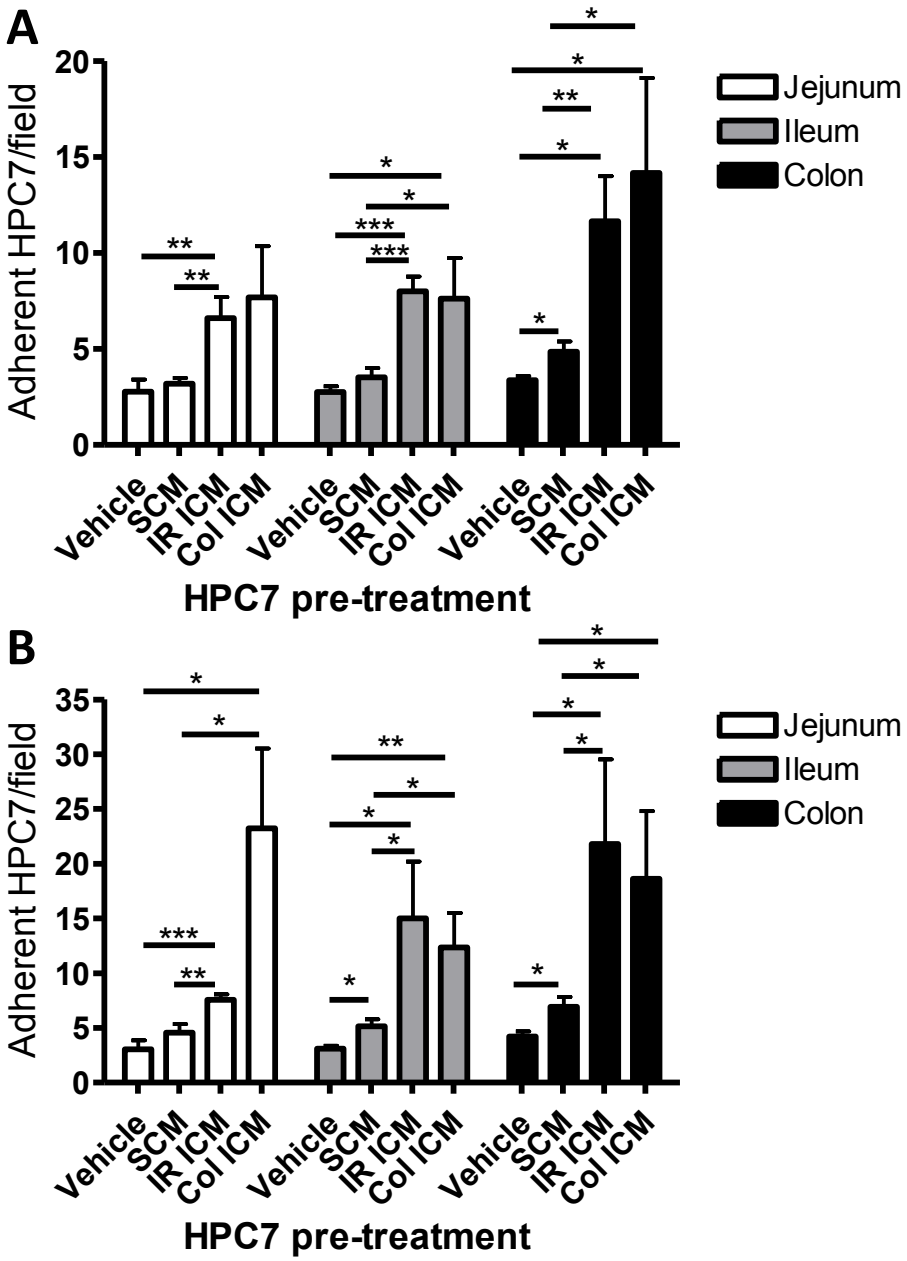

Figure 3.12 Pre-treatment with intestinal conditioned media significantly enhances adhesion to ICAM-1 and VCAM-1

(A) Pre-treatment with jejunal, ileal and colonic IR ICM significantly enhances adhesion to ICAM-1 surface compared to vehicle controls ($p<0.05$, $p<0.001$, $p<0.01$ respectively). However, only ileal and colonic Col ICM significantly enhanced adhesion to ICAM-1 compared to vehicle controls ($p<0.05$, $p<0.05$ respectively. (B) Pre-treatment of HPC7 with jejunal, ileal and colonic IR ICM significantly enhanced adhesion to VCAM-1 coated surface compared to vehicle controls ($p<0.001$, $p<0.05$, $p< 0.05$ respectively. Similarly, pre-treatment with jejunal, ileal and colonic Col ICM also significantly enhanced adhesion to VCAM-1 coated surface ($p<0.05$, $p<0.01$, $p<0.05$ respectively). Results presented as mean ± SEM. Statistical analysis performed with two-way ANOVA, with Student's t-test performed post-hoc. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Abbreviations SCM- sham conditioned media, IR ICM- IR injury conditioned media, Col ICM- colitis conditioned media.

vitro and *in vivo* evidence that HSCs are recruited to IR and DSS injured colon more readily than to healthy controls. It was interesting that the degree of adhesion in both acute and chronic injuries was similar, initially suggesting a recruitment maximum for HPC7 cells within injured colon. However, pre-treating HPC7s prior to their infusion with soluble factors found within the injury milieu enhanced their recruitment. This suggests that recruitment to the injured colon is actually a modulatable event. A summary of the data can be seen in **Table 3.1**. Importantly, this data provided the first evidence that colonic recruitment achieved as a result of the injury was not maximal.

The Kalia group previously published data that HPC7 adhesion within IR injured ileum was greater than non-injured ileum *in vivo* (Kavanagh *et al.*, 2012). However, our initial attempts at the S-W assay did not confirm these observations, most likely due to tissue destruction upon sectioning. Furthermore, transverse sectioning meant that only a small area of mucosa was available for the assay. Since this region is most susceptible to ischaemic and colitis injury (and also the region that is viewed intravitally), we developed a new methodology that allowed the mucosa to be monitored with minimal tissue destruction when sectioned. Cryosectioning to remove either the microvillous tips of the small intestine or the crypt structure of the colon exposed the mucosal vasculature providing a greater area of 'substrate' for HSC adhesion. Using this modified method, we demonstrated that HPC7 adhesion was indeed significantly increased following intestinal IR and colitis injury, in both the small intestine and colon. Although limited studies have monitored leukocyte adhesion in the murine colon (Soriano *et al.*, 2000, Panes *et al.*, 2007, Vowinkel *et al.*, 2007b, Santen *et al.*, 2010), no studies to date have directly monitored stem cell trafficking in the mouse colon following either of these different inflammatory insults. We demonstrated that HPC7 adhesion was also increased within the injured colon *in vivo*. This could potentially be attributed to an increased blood supply to the colon (reactive hyperaemia) as has been observed after inflammatory injuries (Cobellis *et al.*, 2010) This would increase the delivery of the HPC7s to the.

	HPC7 Pre-treatment	Colon Endothelial Cells	Immobilised Ligands		Frozen Tissue Sections									Flow Cytometry Experiments			*In vivo* experiments					
					Jejunum			Ileum			Colon			Surface Expression Changes			IR Injured			Colitis Injured		
			ICAM-1	VCAM-1	Sham	IR Injury	Colitis Injury	Sham	IR Injury	Colitis Injury	Sham	IR Injury	Colitis Injury	CD18	CD48d	CD44	Lungs	Ileum	Colon	Lungs	Ileum	Colon
	Naïve				Control	+++	++	Control	++	+	Control	++	++				±	+++	++	±	±	+
Jejunum	SCM	±	±	±	+++	+	±							±	±	±						
Jejunum	IR ICM	++	++	+++	+++	+++								±	±	±						
Jejunum	Col ICM	±	±	+	+++		+							±	±	-						
Ileum	SCM	±	±	+				±	±	±				±	±	±						
Ileum	IR ICM	+	+++	+				±	+++					±	±	±						
Ileum	Col ICM	±	+	++				++		+				±	±	-						
Colon	SCM	±	+	+							+	+	+	±	±	±						
Colon	IR ICM	+	+	+							+	+		±	±	±						
Colon	Col ICM	+	+	+							+		+++	±	±	±						

Compared to relevant controls

Table 3.1 Summary of experiments investigating the effect of conditioned media pre-treatment on HPC7 adhesion to injured tissue

The results table summarises significant increases and decreases in the number of HPC7 adherent to either: colon endothelial cells, immobilised ligands, frozen tissue sections or *in vivo* investigations. Data displayed are significant differences against relevant controls. +++ - $p<0.001$ increase in HPC7 adhesion, ++ - $p<0.01$ increase in HPC7 adhesion, + - $p<0.05$ increase in HPC7 adhesion, ± - No significant difference in HPC7 adhesion, **-** - $p<0.05$ decrease in HPC7 adhesion

colon and the potential for recruitment. However, laser Dopple speckle microscopy demonstrated that blood flow to injured colon was actually decreased.

The reduction seen in IR injured colon may have been caused by the no-reflow phenomenon. This is when, upon reperfusion, activated leukocytes and platelets obstruct microvessels. Vasoconstriction induced by vasoactive factors such Endothelin-1 and the loss of nitric oxide also contributes to no-reflow. Reduced blood flow has also been demonstrated within the colitis injured colon microvasculature (Harris *et al.*, 2011). However, it is also possible that reduced blood flow promotes HPC7 adhesion, as lower perfusion rates would lower vessel shear rates. This is more conducive to circulating cell adhesion and also the maintenance of adhesion than at higher shear rates. Interestingly, no rolling HSCs were observed within the colon either in sham or injured tissues. This is likely due to the abrogated need for rolling in tissues in which there is low-shear flow in the blood vessels (Sanz and Kubes, 2012).

An increase in the number of free flowing HPC7s was also seen immediately upon infusion in IR injured colons only. Again this may be due to the lowered blood flow as it may be possible to actually see more free flowing cells – at high flow rates it is difficult to accurately count cells as they flow too fast to be captured intravitally. Alternatively, the establishment of a chemokine gradient following IR injury may prevent the cells from being cleared from the microcirculation (McDonald *et al.*, 2011). However, the increase was only observed at 5 minutes post-infusion, and there was only 1 free-flowing cell more than was seen in sham tissue, and thus is not likely to have much physiological impact.

Immediately following systemic administration of HPC7s, numbers of free-flowing cells rapidly decreased to plateau at approximately 2 cells/minute in both sham and injured tissue. This was

despite the fact that a single bolus dose of $2x10^6$ cells were injected. However, a significant proportion of HPC7s were observed within the pulmonary vasculature when examined *ex vivo*. This suggests that most of the injected cells become lodged immediately within vascular beds remote to the site of injury. The number of HPC7s in the lungs did not vary regardless of whether they were removed from mice undergoing gut injury or not. This was somewhat surprising as it has been reported that extensive intestinal IR injury can trigger systemic inflammation which leads to remote injury in multiple organs including the lungs (Kalia *et al.*, 2002, Kalia *et al.*, 2005). However, it is possible that the acute timeframe of the IR experiments meant pulmonary injury was not evident, with the large number of HPC7 within the lungs most likely due to their physical entrapment within the small pulmonary capillaries. Indeed, numerous studies investigating exogenous SC infusion report very high pulmonary entrapment (Aguilar *et al.*, 2009, Kavanagh, 2009, Loebinger *et al.*, 2009, Sordi, 2009, Kavanagh *et al.*, 2012). Identifying strategies that reduce this phenomenon may be a potential mechanism for improving SC therapy efficacy.

With the little variation seen in the number of free-flowing cells and the reduced blood flow, it suggests that the enhanced HPC7 adhesion is most likely due to an active process and not merely a result of increased delivery. This is supported by the *in vivo* observations that blocking integrins on the SCs surface reduced HPC7 numbers within injured colon and also suggests adhesion was not due to their physical entrapment within colonic microvessels. Blocking CD18 reduced adhesion within the IR colon, to the extent of lowering that adhesion to baseline, whereas, blocking CD49d did not influence adhesion at all. This suggests that there is no redundancy within the adhesion mechanisms involved in mediating HPC7s to IR injured colon. The lack of a role for CD49d is perhaps not all that surprising as there would probably be no endothelial VCAM-1 up-regulation at this early stage of IR injury. Others have previously demonstrated VCAM-1 to be upregulated after several hours reperfusion (Esposito *et al.*, 2007).

A critical role for CD18 has also been demonstrated by the Kalia group for mediating HPC7 recruitment to the similarly injured small intestine (Kavanagh *et al.*, 2013). However, further studies from our group have demonstrated that differing tissues utilise different adhesive mechanisms. For example, in the IR injured hepatic microcirculation, CD49d-VCAM-1 interactions are critical. Knowledge of the local adhesive events involved is particularly important when trying to develop strategies to enhance recruitment in specific sites.

Interestingly, in the colitis injured colon, blockade of either CD18 or CD49d reduced adhesion to a similar level with neither blockade lowering SC numbers equivalent to that seen in sham experiments. This is contrary to HPC7 recruitment seen in other tissues and injuries (Kavanagh *et al.*, 2010, Kavanagh and Kalia, 2011, Kavanagh *et al.*, 2012, Kavanagh *et al.*, 2013, White *et al.*, 2013), where recruitment can be ameliorated following blockade of one specific integrin or its endothelial counterligand. The colitis colon therefore appears to be using multiple mechanisms with a degree of redundancy as adhesion is merely reduced and not completely inhibited. This again may not be surprising, as the chronically injured tissue will have had sufficient time to up-regulate other endothelial cell adhesion molecules in response to the injury (Soriano *et al.*, 2000, Farkas *et al.*, 2006, Vowinkel *et al.*, 2007a, Vowinkel *et al.*, 2007b). Overall, the function blocking data demonstrates that SC adhesion is governed not only by the tissue type but also the nature and extent of the inflammatory injury as well. Of interest was also the observation that blocking either CD18 or CD49d did not decrease the HPC7 number in the lungs. This suggests that pulmonary recruitment is not an active process and that entrapment is the most likely explanation for their presence.

The next aim of this chapter was to identify whether HPC7 recruitment to the colon could be enhanced by exposing them to a conditioned media derived from injured tissue. As there were

inherent differences between the recruitment mechanisms observed between IR and colitis tissue, it was decided that the pre-treatment was to be tailored specific to the injury and tissue of interest. Overall, these novel studies investigating pre-treatment with ICMs demonstrated for the first time that colon adhesion was a modulatable event with most pronounced *in vitro* adhesion observed when HPC7 were pre-treated with an ICM generated from colitis colon. It is interesting that the nature of the injury and the tissue from which ICM was generated differently modulated adhesion. The enhanced adhesion was not due to any detrimental or cytotoxic effects on the HPC7 cells as the pre-treatment followed by 24 hours of culture did not affect the survival or proliferation of the cells in culture.

The work in this chapter, and indeed the work of others, describes how HSC adhesion to injured tissue is mediated by integrins (Kavanagh *et al.*, 2010, Kavanagh and Kalia, 2011, White *et al.*, 2013). It is known (Hart and Greaves, 2010, Yu *et al.*, 2010) that integrin mediated cellular adhesion can be modulated in three ways: (i) changing adhesion molecule number expressed on the cell surface, (ii) changing the affinity of the integrin for its endothelial counterligand and (iii) changing the distribution of integrins on the cell surface. In the current study it was demonstrated using flow cytometry that the ICM did not enhance HPC7 adhesion by changing the surface expression of either CD18 or CD49d. However, the enhanced adhesion mediated by ICM could be due to an increased affinity of CD18 and CD49d for their endothelial counterligands ICAM-1 and VCAM-1 respectively, although this was not investigated directly. What was observed was an increased adhesion to an immobilised ICAM-1 and VCAM-1 surface, a result that can be explained via a change in integrin avidity and/or a change in affinity for integrins and their counterligands. These events may have been brought about by clustering integrins on the stem cell surface – however this was not investigated in this current chapter (see later results chapters).

Another potent mediator of cell adhesion is the non-integrin glycosaminoglycan CD44 (Turner *et al.*, 1995, Avigdor *et al.*, 2004, Aldridge *et al.*, 2012). CD44 is primarily recruited to endothelial hylauronan, but also has other ligands (Avigdor *et al.*, 2004, Garton *et al.*, 2006, White *et al.*, 2013). Interestingly, pre-treatment of HPC7s with an ICM generated from the colitis ileum decreased CD44 surface expression. This could be as a result of CD44 shedding. This process has been described in several inflammatory situations and has been suggested to play a role in signalling as well as cell adhesion (Garton *et al.*, 2006). Indeed, it has been suggested that soluble proteases such as ADAM10 are capable of dissociating the extracellular portion of CD44 from leukocytes to allow for their detachment and subsequent transmigration (Garton *et al.*, 2006).

To improve the efficacy of stem cell therapies for inflammatory bowel disorders, it has been suggested that cellular recruitment levels must be increased. However, for therapies to be readily available within a clinic they must be safe, effective and efficient. Hence, the use of a conditioned media is not a readily viable option therapeutically. However, it has enabled the observation that recruitment mediated by the presence of the injury alone is not maximal. Furthermore, identification of the active soluble component(s) in the ICM could lead to more 'clean' pre-treatment strategies. It was hypothesised that the conditioned media would contain a variety of inflammatory cytokines and chemokines that could activate stem cells. Following Luminex analysis, a highly sensitive technique capable of detecting pg/ml of protein within a solution it was found that none of the samples contained any of the 20 cytokines or chemokines assayed for. It is not clear why this assay was not successful – it may be that during the production of conditioned media, any cytokines and chemokines were broken down or degraded. In the following chapter data are presented on how hydrogen peroxide, a free radical produced in inflamed tissue, and most likely contained within the ICM and thus the injury milieu, can influence HPC7 recruitment to the injured colon.

Chapter 4

USING H_2O_2 TO ENHANCE HPC7 ADHESION

4.0 Introduction

The previous chapter presented novel data demonstrating that HPC7 recruitment to IR and colitis injured colon could be enhanced using soluble factors released in an ICM. This was demonstrated *in vitro* on frozen tissue sections and also on mouse colon ECs. This was the first demonstration that a pre-treatment strategy, that did not involve genetic modification of stem cells, could enhance their adhesion in mouse colon. The use of a conditioned media cannot be considered a clinically viable pre-treatment option – it would be difficult to obtain, it is time consuming and difficult to reproducibly develop. Therefore, identifying bioactive molecules within the conditioned media may unveil another pre-treatment option which has the potential to be used for therapeutic purposes clinically.

A potent modulator of circulating cell behaviour, identified in the inflammatory milieu, is the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2). This molecule is released from a wide array of cells including vascular endothelium and inflammatory leukocytes such as neutrophils (Matoba *et al.*, 2000, Li and Jackson, 2002). It is widely known that H_2O_2 synthesis increases in many inflammatory situations including IR injury, colitis and other non-digestive inflammatory disorders such as rheumatoid arthritis (Lopes *et al.*, 2011). It has been previously shown that H_2O_2 has many roles within the inflammatory environment and is capable of establishing a gradient such as to recruit leukocytes (Niethammer *et al.*, 2009), enhancing neutrophil rolling and increasing cellular recruitment (Fraticelli *et al.*, 1996, Johnston *et al.*, 1996). As a result of this, it is capable of influencing the fate of many cells via modulation of the action of redox sensitive second messengers, which in turn can affect signalling pathways such as NF-κB, a key pathway in regulating cell survival (Anderson *et al.*, 1994, Baeuerle *et al.*, 1996).

The Kalia group previously developed a method of pre-treating murine HPC7s with H_2O_2 (100μM; 1hr), which significantly increased their adhesion to murine cardiac ECs and frozen sections of murine IR injured gut *in vitro*. Furthermore, when injected systemically, a three-fold increase in H_2O_2 pre-treated HSC adhesion within IR injured intestinal villous microcirculation *in vivo* was observed (Kavanagh *et al.,* 2012). It is not known whether pre-treatment of HPC7s with H_2O_2 is a universal means of enhancing stem cell recruitment within all injured tissues or whether its effects are site and injury specific. We therefore aimed, in this chapter, to determine whether H_2O_2 pre-treatment could enhance HPC7 adhesion *in vitro* and *in vivo* within IR and colitis injured colon microcirculation. Furthermore, a mechanism by which enhanced adhesion could occur was identified, focussing primarily on effects on stem surface integrins.

4.1 Materials and Methods

4.1.1 Mice and injury models

Experiments were conducted on healthy C57BL/6 wild-type mice or those that had undergone either IR injury (45 minutes ischaemia, 120 minutes reperfusion) or 5 days of 3% DSS induced colitis injury.

4.1.2 H_2O_2 concentration measurement and pre-treatment protocol

Preliminary studies were conducted looking at endothelial adhesion of HPC7s pre-treated with different H_2O_2 concentrations to determine the most effective concentration and it was found that 100μM was most pro-adhesive and so all subsequent experiments were conducted using this dose. HPC7 were pre-treated in media supplemented with H_2O_2 to establish the required concentration and incubated at 37°C, 5% CO_2 for 1 hour. Cells were then washed by centrifugation and resuspended in base Stem Pro SFM34 media for *in vitro* assays, and PBS for *in vivo* assays. H_2O_2

concentration was measured using PeroXOquant Quantitative Peroxide Assay Kit (ThermoScientific, UK) as per manufacturer's instructions.

4.1.3 Cell Adhesion Assays

Murine colon ECs, cultured in 24 well plates, were used to identify whether tissue specific differences in stem cell adhesion could be identified *in vitro*. Some ECs were treated with TNFα for 4 hours to activate them prior to monitoring adhesion of fluorescently labelled HPC7. Wells were washed and fixed prior to imaging.

Immobilised ICAM-1 and VCAM-1 surfaces were also used to investigate cell adhesion. Flat bottomed 96-well plates were incubated with 50µl at 10µg/ml protein solution and subsequently blocked using 1% PBSA. Pre-treated HSC were incubated on the plate surface, wells were subsequently washed and fixed prior to imaging.

4.1.4 Immunofluorescence and Scanning Electron Microscopy Imaging

HPC7 were labelled with Cell Tracker Orange as described in Chapter 2 and then pre-treated with H_2O_2 as described above. Following pre-treatment, HPC7s were fixed in 5% neutral buffered formalin/1% bovine albumin in PBS (PBSA) for 30 minutes at room temperature. Following fixation HPC7 were washed by centrifugation 3 times in 2% PBSA. Following final wash, HPC7 were left in the PBSA for 30 minutes to reduce any un-specific binding. HPC7 were washed and resuspended in cold integrin blocking antibody (LEAF rat anti-mouse CD18 (GAME-46), LEAF rat anti-mouse CD49d (R1-2)) at a 1:50 dilution in 2% PBSA, incubated on ice for 60 minutes. HPC7 were washed 3 times then resuspended in Alexa Fluor 488 goat anti-rat IgG in 2% PBSA at 1:100 on ice for 30 minutes. Following incubation HPC7 were washed 3 times allowed to settle onto poly-L-lysine coated

coverslips overnight and then mounted using Hydromount prior to imaging. Cells were imaged using a DM IRE2 confocal microscope (Leica, UK), with a 63x oil immersion lens and images captured using Leica Control Software. Offline analysis conducted using ImageJ (NIH, US).

For SEM samples were kindly prepared for imaging by Paul Stanley and team at University of Birmingham Centre for EM. In brief, pre-treated cells were fixed and washed as described in Chapter 2. HPC7 were mounted onto poly-L-lysine coverslips and dehydrated using a graded ethanol series. Samples were then critical point dried, mounted onto SEM stubs and coated with evaporated carbon. Samples were examined in a Jeol 7000F SEM.

4.2 Results

4.2.1 There is a greater concentration of H_2O_2 in injured tissue conditioned media

It is known that H_2O_2 is common product of inflammation, and so it was probable that it would be found in the injury microenvironment and thus the ICMs used in Chapter 3. However, this was confirmed using a commercial kit. It was identified that in both IR ICM and Col ICM, the concentration of H_2O_2 was significantly greater than that observed in the respective SCM across all tissues (jejunum IR ICM *vs.* SCM, $p<0.05$; Col ICM *vs.* SCM, $p<0.01$; ileum IR ICM *vs.* SCM, $p<0.001$; Col ICM *vs.* SCM, $p<0.05$; colon IR ICM *vs.* SCM, $p<0.05$; Col ICM *vs.* SCM, $p<0.05$; **Figure 4.1**). Interestingly, out of all the ICMs, the least H_2O_2 was found in colitis ICM generated from the colon. Furthermore, two-way ANOVA analysis identified that there were significant differences between the tissue of origin ($p<0.001$) and the injury status of the tissue ($p<0.001$), however, there was no interaction between the two groups.

4.2.2 Greater intensity of anti-8-oxo-2'-deoxyguanosine staining demonstrates increased oxidative damage in IR and colitis injured colon

We further assayed for H_2O_2 indirectly, in the injured colon tissue by determining the presence of 8-oxo-2'-deoxyguanosine (8-oxodG). 8-oxodG is one of the major products of oxidative damage to DNA and its concentration within a cell is an indicator of oxidative stress after triggered by exposure to ROS such as H_2O_2. Interestingly, some 8-oxodG was observed within the sham colon, suggesting a basal level of oxidative damage occurring within the colon (**Figure 4.2 A**). However, a greater intensity and more widespread labelling of 8-oxodG was observed following injury, indicating there were signs of oxidative damage in both IR and colitis injured colon (**Figure 4.2 B** and **C**), with the labelling being focussed predominantly in the mucosal region.

4.2.3 H_2O_2 pre-treatment significantly enhances HPC7 adhesion to colon endothelial cells and is mediated by the integrins CD18 and CD49d

Pre-treated cells were incubated with colon ECs and adhesion quantitated *in vitro*. Only pre-treatment with 100µM H_2O_2 significantly ($p<0.01$) enhanced adhesion compared to pre-treatment with the vehicle control (**Figure 4.3**). To determine whether the enhanced adhesion following pre-treatment with 100µM H_2O_2 was integrin mediated, CD18 and CD49d activity on HPC7s was functionally blocked with monoclonal antibodies. Blocking either CD18 ($p<0.01$) and CD49d ($p<0.001$) significantly reduced endothelial adhesion compared to the IgG control (**Figure 4.3**).

4.2.4 H_2O_2 pre-treatment enhances adhesion to all sham and IR intestinal tissues, but only enhances adhesion to the colitic ileum and colon

The effect of 100µM pre-treatment on HPC7 adhesion to tissue sections was also evaluated. Interestingly, H_2O_2 pre-treatment significantly ($p<0.05$) increased HPC7 adhesion to healthy jejunal sham tissue when compared to vehicle pre-treated cells (**Figure 4.4 A**). H_2O_2 pre-treatment also

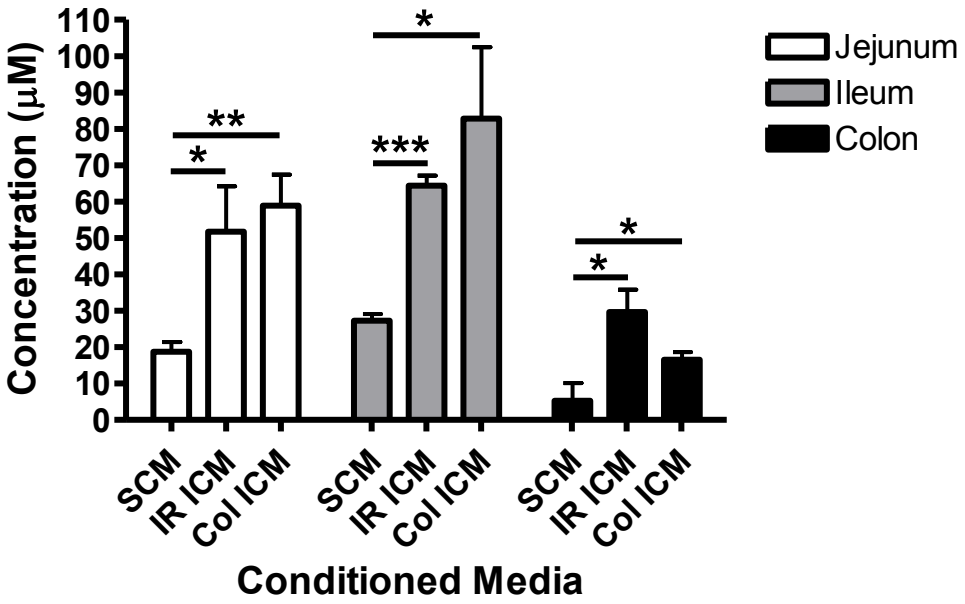

Figure 4.1 H_2O_2 concentration is greater in injury conditioned medias than sham conditioned medias

H_2O_2 concentration is greater in jejunal, ileal and colonic IR and colitis ICM when compared to the respective SCM. Results are presented as mean±SEM. Statistical analysis performed with 2-way ANOVA and Student's t-test for post hoc analysis. Abbreviations: SCM = sham conditioned media, IR ICM = IR injury conditioned media, Col ICM = colitis injury conditioned media. N≥3 across all groups. *p<0.05, **p<0.01, ***p<0.001.

significantly ($p<0.01$) increased adhesion to IR injured jejunum compared to vehicle pre-treated cells. Importantly, this was significantly ($p<0.01$) higher than that observed on healthy sham tissue (**Figure 4.4 A**). However, H_2O_2 pre-treatment did not induce any increases in HPC-7 adhesion on jejunal tissue following colitis injury (**Figure 4.4 B**).

Similarly, H_2O_2 pre-treatment significantly ($p<0.01$) increased HPC7 adhesion to healthy ileal sham tissue when compared to vehicle pre-treated cells (**Figure 4.4 C**). H_2O_2 pre-treatment also significantly ($p<0.05$) increased adhesion to IR injured ileum compared to vehicle pre-treated cells. Importantly, this was significantly ($p<0.05$) higher than that observed on healthy sham tissue (**Figure 4.4 C**). However, unlike the jejunum, H_2O_2 pre-treatment did significantly ($p<0.01$) enhance adhesion to ileal colitic injured tissue when compared to healthy sham tissue (**Figure 4.4 D**). However, this increase was not significantly greater compared to the pre-treatment control (**Figure 4.3 D**).

Again, H_2O_2 pre-treatment significantly ($p<0.001$) increased HPC7 adhesion to healthy colon sham tissue when compared to vehicle pre-treated cells (**Figure 4.4 E**). Increased adhesion to IR injured colon was also observed when compared to vehicle pre-treated cells ($p<0.01$) and importantly, this was significantly higher than that observed on healthy sham tissue ($p<0.01$; **Figure 4.4 E**). However, H_2O_2 pre-treatment did not induce any increases in HPC-7 adhesion on colon tissue following colitis injury (**Figure 4.4 F**).

4.2.5 H_2O_2 pre-treatment enhances adhesion to the colitis colon, but not to the IR injured colon *in vivo*

Interestingly, H_2O_2 did not enhance HPC7 recruitment within the IR colon or have any effect on numbers of free flowing HPC7 (**Figure 4.5 A** and **B**). Conversely, H_2O_2 pre-treatment significantly

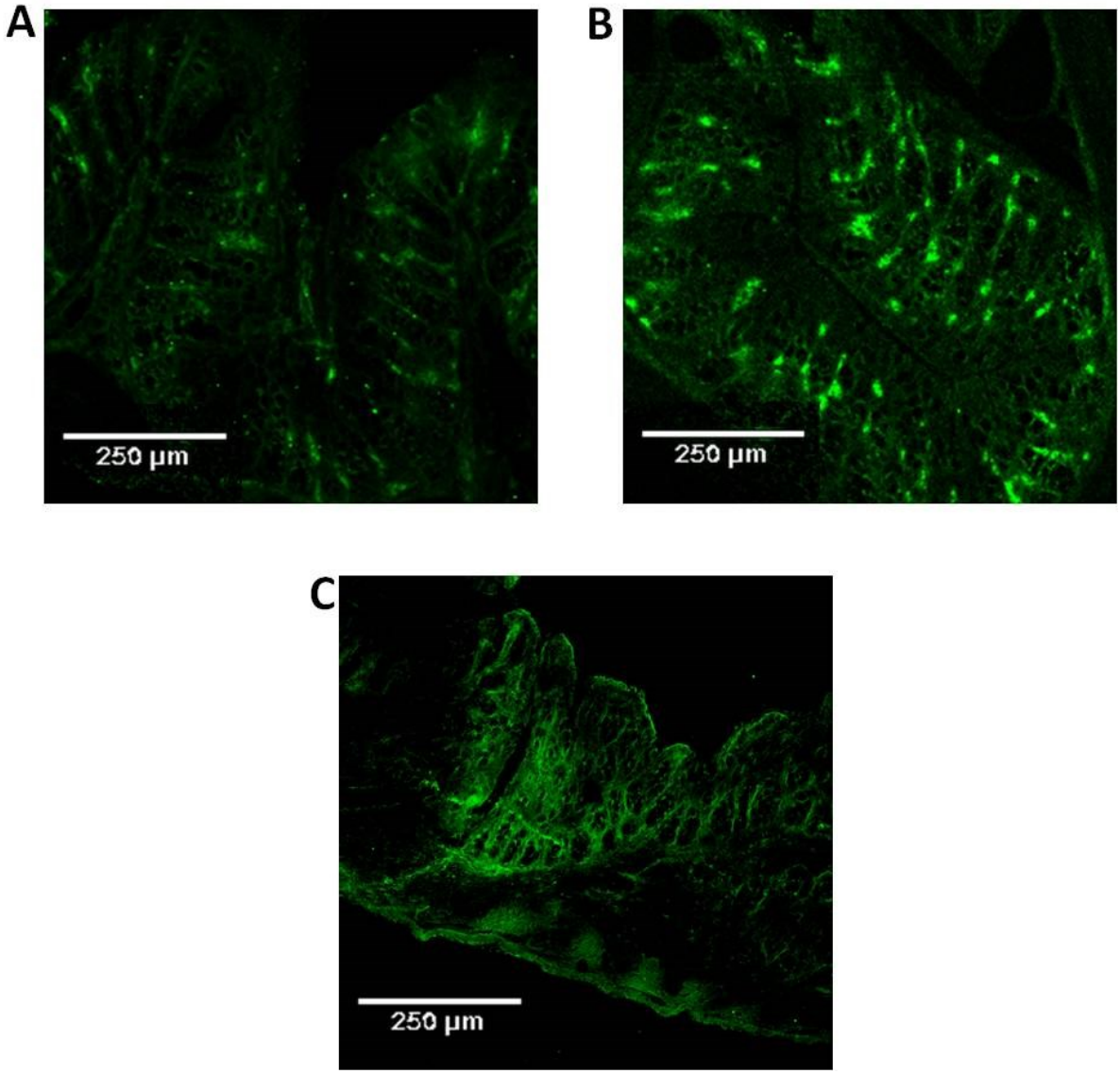

Figure 4.2 8-oxo-2'-deoxyguanosine staining reveals oxidative damage in colon tissues, with more extensive damage observed in IR and colitis tissue

(A) Sham colon tissue displays a basal level of oxidative damage occurring within the colon. (B) Greater intensity and more widespread staining of 8-oxodG is indicative of greater levels of oxidative damage in the IR colon. (C) Similar to the IR injured colon, the colitis injured colon displays more 8-oxodG staining than the sham colon. Representative images of 3 colons from each injury group over 2 separate experiments.

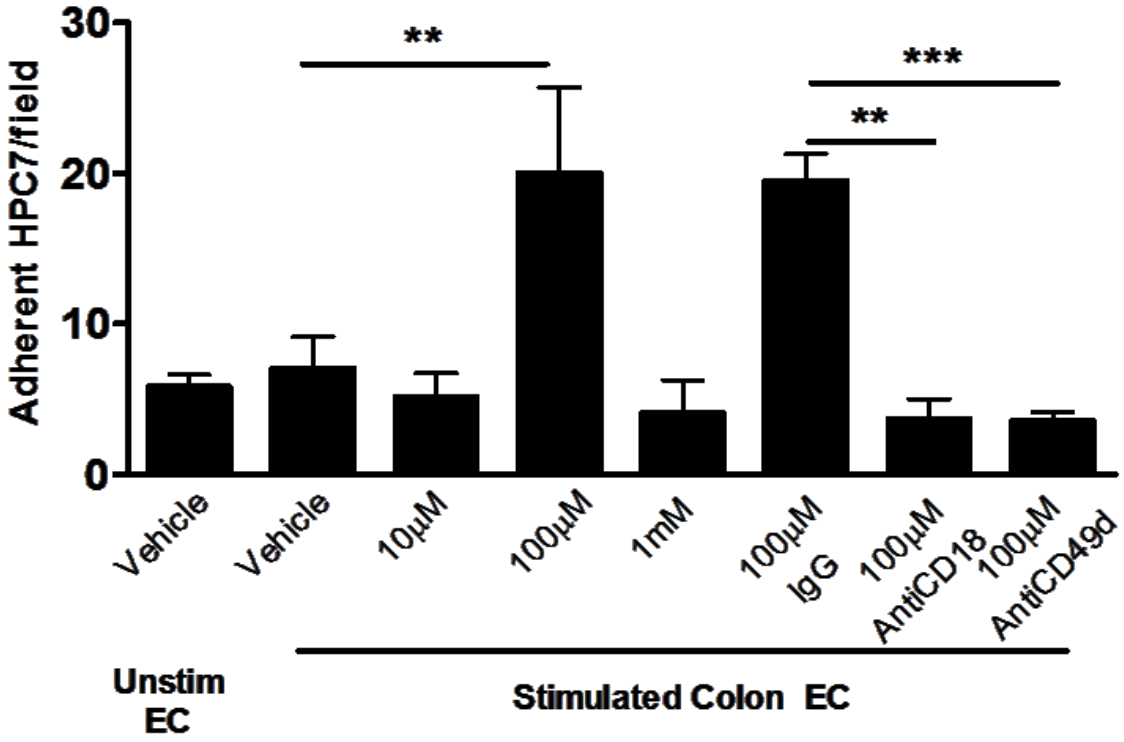

Figure 4.3 Pre-treating HPC-7s with 100μM H_2O_2 significantly enhances adhesion to colon endothelial cells and this is integrin mediated

Only pre-treatment with 100μM H_2O_2 enhances adhesion to colon endothelial cells. This adhesion is ameliorated following functional blockade of either CD18 (100μM antiCD18 mAb) or CD49d (100μM antiCD49d mAb) compared to an IgG control. Results are presented as mean±SEM. Statistical analysis performed using 1-way ANOVA, with Bonferroni multiple comparisons test. N≥3 across all groups. **p<0.01, ***p<0.001.

enhanced adhesion within the colitis colon ($p<0.05$). Again it had no effect on numbers of free flowing HPC7 (**Figure 4.5 C** and **D**).

4.2.6 H_2O_2 pre-treatment has no effect on recruitment to the lungs following IR or colitis injury, but does enhance adhesion to the IR ileum and the colitis colon

Ex vivo analysis revealed that H_2O_2 pre-treatment had no effect on pulmonary recruitment following IR or colitis injury (**Figure 4.6 A** and **B** respectively). However, it did enhance adhesion to the IR ileum ($p<0.05$; **Figure 4.6 C**). Adhesion within the colitis ileum was increased following H_2O_2 pre-treatment, but this increase failed to reach statistical significance (**Figure 4.6 D**). As observed intravitally, H_2O_2 recruitment had no effect on adhesion to the IR colon (**Figure 4.6 E**), but adhesion to the colitic colon was significantly increased ($p<0.05$; **Figure 4.6 F**).

4.2.7 H_2O_2 pre-treatment results in ruffling and cellular projections from HPC7

Scanning electron microscopy identified phenotypic changes in HPC7 membrane organisation following H_2O_2 pre-treatment. Extended cellular projections and ruffling, similar to that seen in activated leukocytes, was observed indicative of an activated HPC7 phenotype (**Figure 4.7**).

4.2.8 H_2O_2 pre-treatment enhances adhesion to ICAM-1 and VCAM-1 coated surfaces

To identify whether H_2O_2 pre-treatment modulated integrin based adhesion, pre-treated cells were incubated on an ICAM-1 and VCAM-1 surfaces. HPC7 adhesion to ICAM-1 was significantly increased following pre-treatment ($p<0.01$; **Figure 4.8 A**). Furthermore, following functional blockade of CD18, adhesion was ameliorated ($p<0.001$; **Figure 4.8 A**). Similarly, adhesion was significantly increased to VCAM-1 surface ($p<0.001$; **Figure 4.8 B**) and this adhesion was mediated solely by CD49d, as demonstrated by reduced adhesion following functional integrin blockade ($p<0.01$; **Figure 4.8 B**).

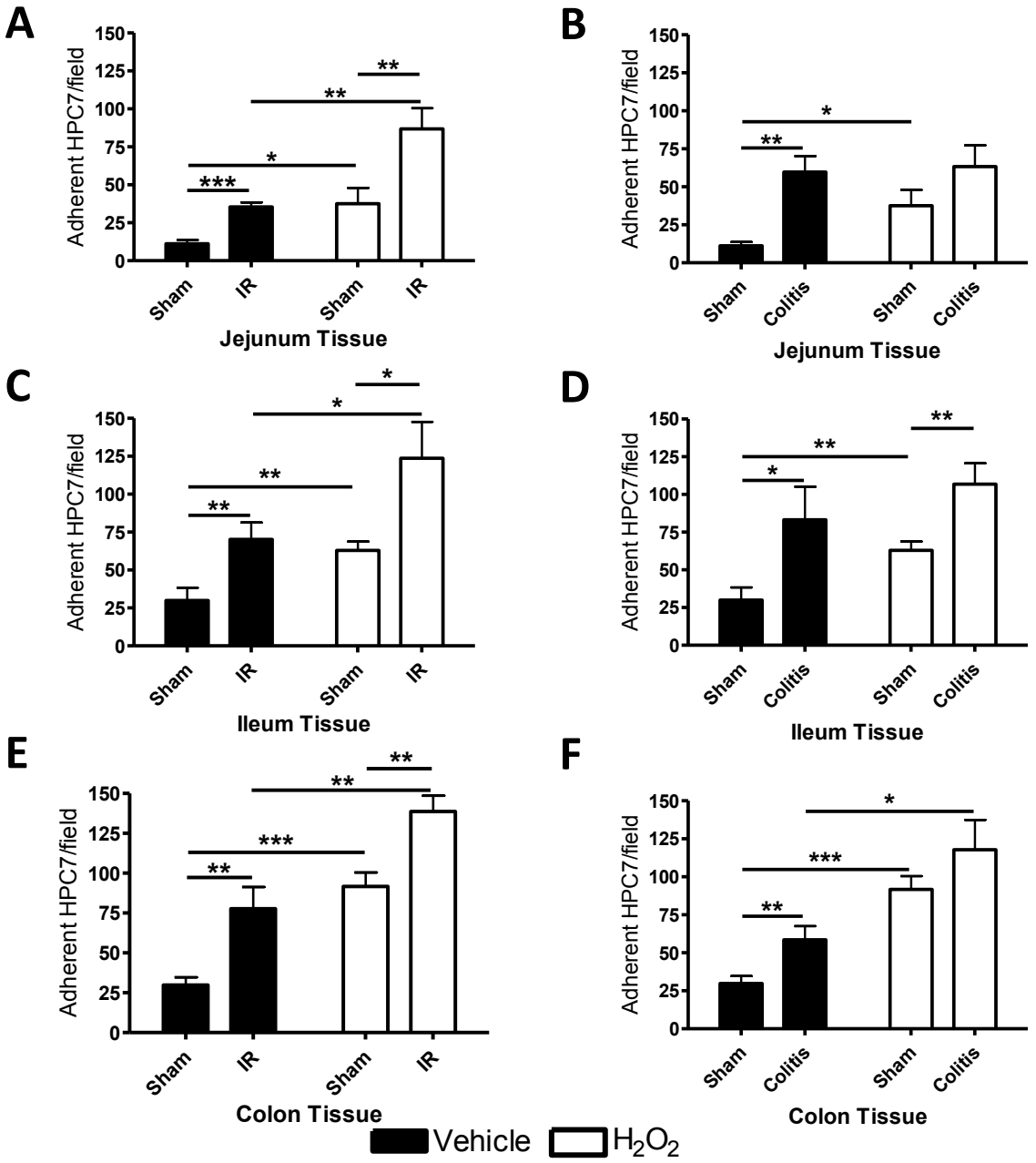

Figure 4.4 H_2O_2 pre-treatment of HPC7 significantly increases adhesion IR injured jejunal, ileal and colon tissue sections, but only increases adhesion to colitis injured ileum and colon.

(A) H_2O_2 pre-treatment significantly enhances adhesion to sham and IR jejunal tissue, with the greatest effect seen on IR tissue. (B) H_2O_2 pre-treatment does not further enhance adhesion to colitic jejunal tissue. (C) H_2O_2 pre-treatment significantly enhances adhesion to sham and IR ileal tissue sections, with the greatest effect seen on IR injured tissue. (D) H_2O_2 pre-treatment significantly enhances adhesion compared to colitis injury control, but not to the vehicle control. (E) H_2O_2 pre-treatment significantly enhances adhesion to sham and IR colon tissue, with the greatest effect seen on IR injured colon. (E) Similar to the sham and IR tissue, H_2O_2 pre-treatment significantly enhances adhesion to colon tissue, with the greatest effect seen with colitis injured tissue. Results presented as mean+SEM. Analysis performed using 2-way ANOVA with Student's t-test post hoc analysis. N≥6 across all groups. *p<0.05, **p<0.01, ***p<0.001.

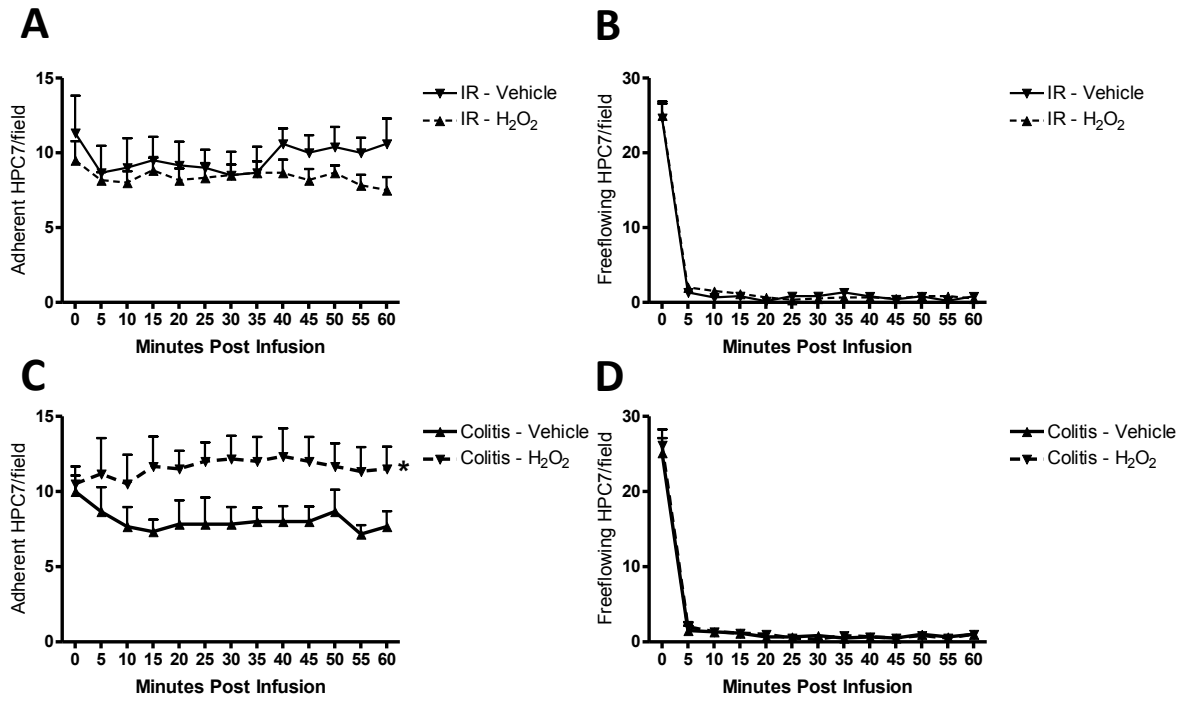

Figure 4.5 H_2O_2 pre-treatment significantly enhances adhesion within the colitis colon, but not the IR colon.

H_2O_2 pre-treatment has no effect on HPC7 adhesion (A) or the number of free flowing (B) HPC7 within the IR colon. However, pre-treatment significantly increases adhesion within the colitis colon (C) without affecting the number of free flowing (D) HPC7. Results presented as mean+SEM. Statistical analysis performed on area under the curve data using Student's t-test. N≥5 for all groups. *p<0.05.

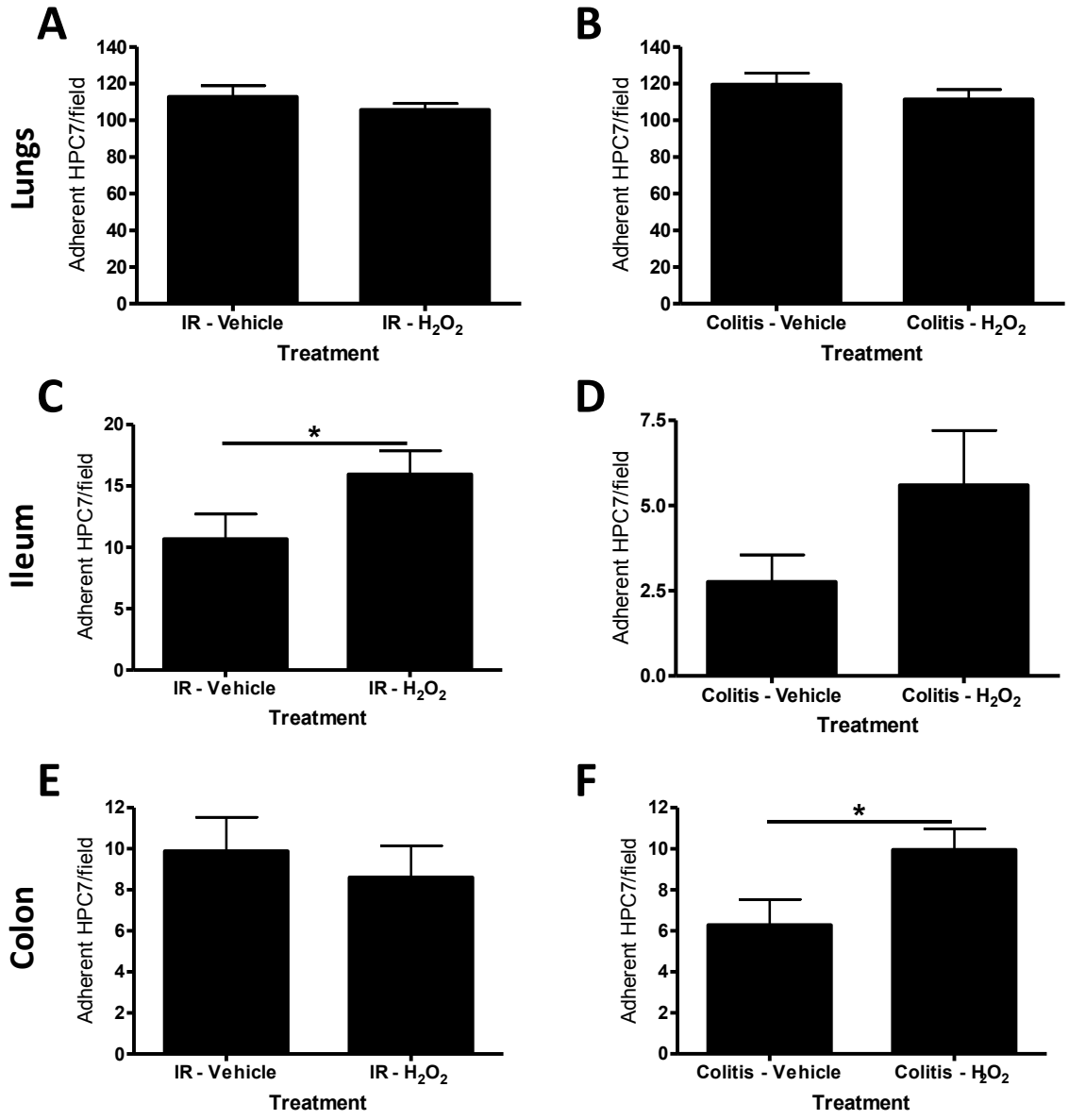

Figure 4.6 H_2O_2 pre-treatment has no effect on lung recruitment following injury but increases adhesion within the IR injured ileum and colitis injured colon

Recruitment to the lungs following IR (A) and colitis (B) injury is unaffected by H_2O_2 pre-treatment. Recruitment to the IR (C), but not the colitis (D), ileum was increased following H_2O_2 pre-treatment. Recruitment within the colon was increased in the colitis colon (F), but not the IR colon (E). Results presented as mean+SEM. Statistical analysis performed using Student's t-test. N≥4 for all groups. *$p<0.05$.

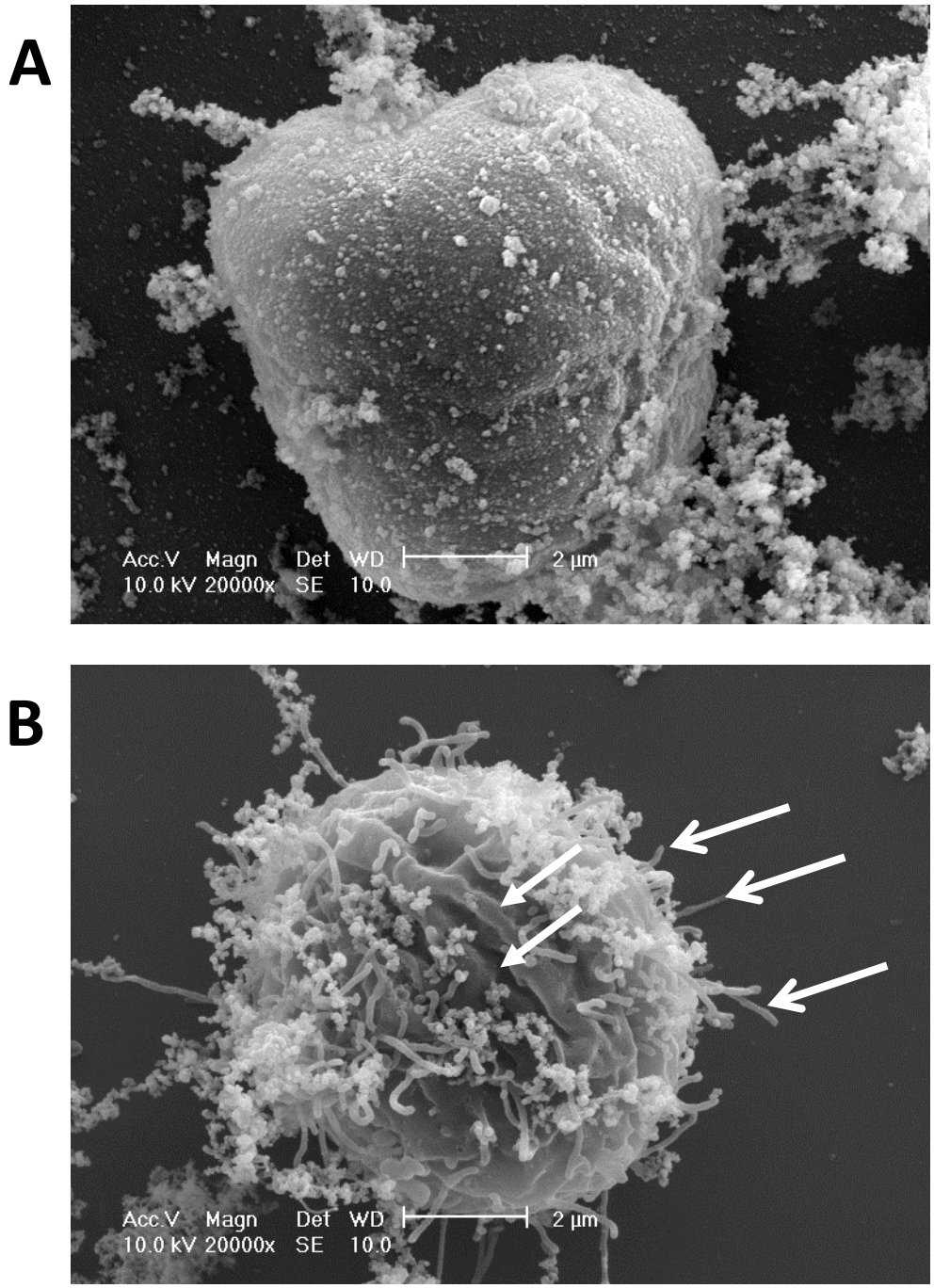

Figure 4.7 H_2O_2 pre-treatment induces ruffling and cellular protrusions in the HPC7 membrane

Scanning electron micrographs of vehicle (A) and H_2O_2 (B) pre-treated HPC7. H_2O_2 induces cellular protrusions (open arrows, seen on edge of cell) from the cell surface and ruffling of the membrane (closed arrows, seen in the centre of the cell) indicative of cellular activation. Representative images of N=5 cells per group.

4.2.9 H_2O_2 pre-treatment increases the number of CD18 clusters, without affecting size, and increases CD49d cluster size, without affecting cluster number

The increased adhesion following pre-treatment observed on ICAM-1 and VCAM-1 suggests direct modulation of integrin adhesion mechanisms. It was identified by Kavanagh *et al.* (2012) that pre-treatment of HPC7 had no effect on cell adhesion molecule expression. Hence, an alternative mechanism was investigated. It was found that following pre-treatment with H_2O_2, CD18 clustered on the cell surface, compared to the less clustered, more diffuse distribution observed on vehicle treated cells ($p<0.05$; **Figure 4.9 A**). However, it was found that pre-treatment had no effect on the area of the clusters found on the cell surface (**Figure 4.9 A)**. Interestingly, H_2O_2 had an opposite effect on the clustering of CD49d; pre-treatment had no effect on CD49d cluster number (**Figure 4.9 B**) but significantly increased the area of the integrin clusters ($p<0.05$; **Figure 4.9 B**)**.**

4.3 Discussion

Although Chapter 3 demonstrated that an ICM could enhance HSC adhesion, it is not an attractive option as an adjuvant pre-treatment for clinical purposes. However, the data obtained revealed that adhesion was a modulatable event, and that factors found within the injury microenvironment could enhance HSC delivery to sites of injury. H_2O_2 was hypothesised to be a potential factor within the conditioned media and this was confirmed in the current chapter. The concentration of H_2O_2 in ICM was approximately 70μM which, importantly, is similar to the concentration found physiologically (Johnston *et al.*, 1996). H_2O_2 is also generated during inflammation by endothelial cells, smooth muscle and activated macrophages and neutrophils. Indeed, resident neutrophils can generate local concentrations of 200-500μM H_2O_2 upon stimulation (Johnston *et al.*, 1996). Hence it is likely that trafficking HSCs will be exposed to similar concentrations of H_2O_2 in IR injured colon. Novel data generated in this chapter demonstrated that pre-treating HPC7 with H_2O_2 significantly enhanced

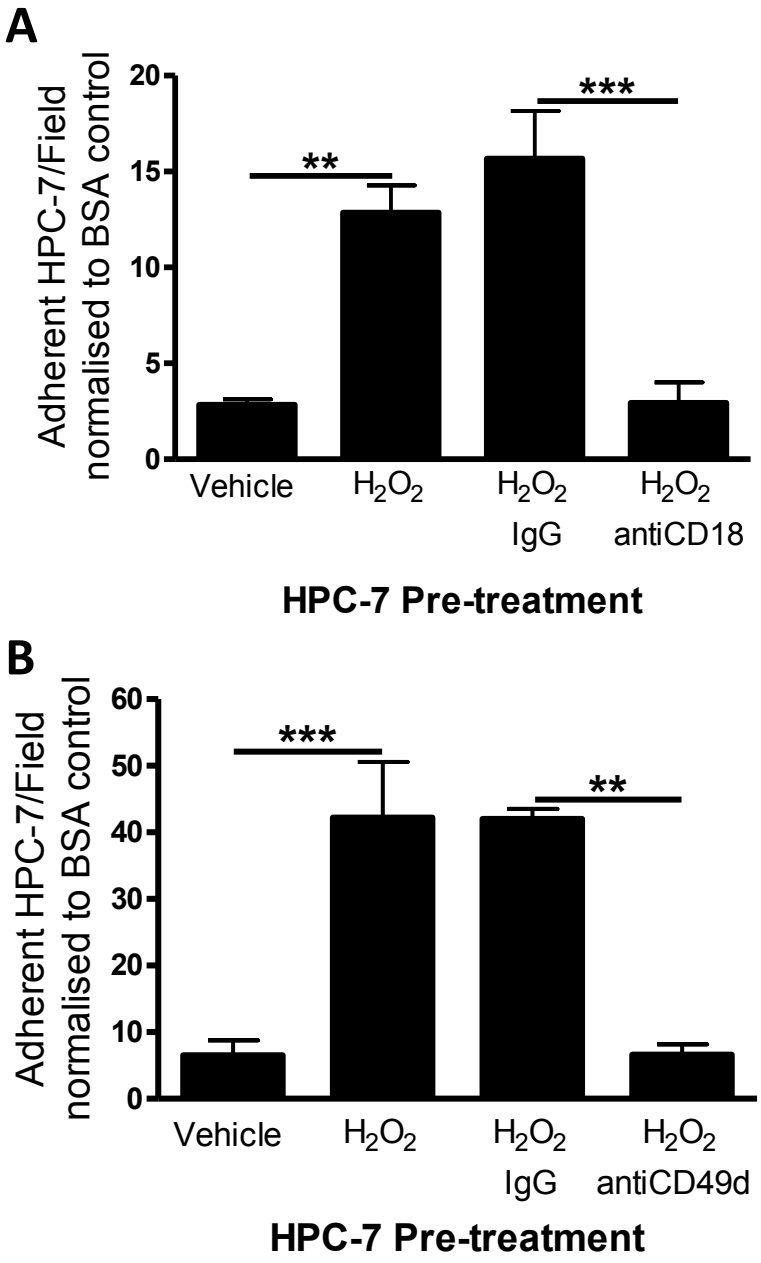

Figure 4.8 H_2O_2 pre-treatment significantly enhances adhesion to ICAM-1 and VCAM-1 coated surfaces

(A) Pre-treatment of HPC7 with H_2O_2 significantly enhances adhesion to and ICAM-1 coated surface and is dependent on CD18. (B) Similarly, H_2O_2 pre-treatment results in enhanced adhesion to VCAM-1 coated surface and is dependent on CD49d. IgG= IgG Isotype control incubation group, antiCD18= function blocking antibody against CD18 incubation group, antiCD49d= function blocking antibody against CD49d incubation group. Results presented as mean±SEM. Statistical analysis performed using 1-way ANOVA with Bonferroni multiple comparisons test. N≥3 across all groups. **p<0.01, ***p<0.001.

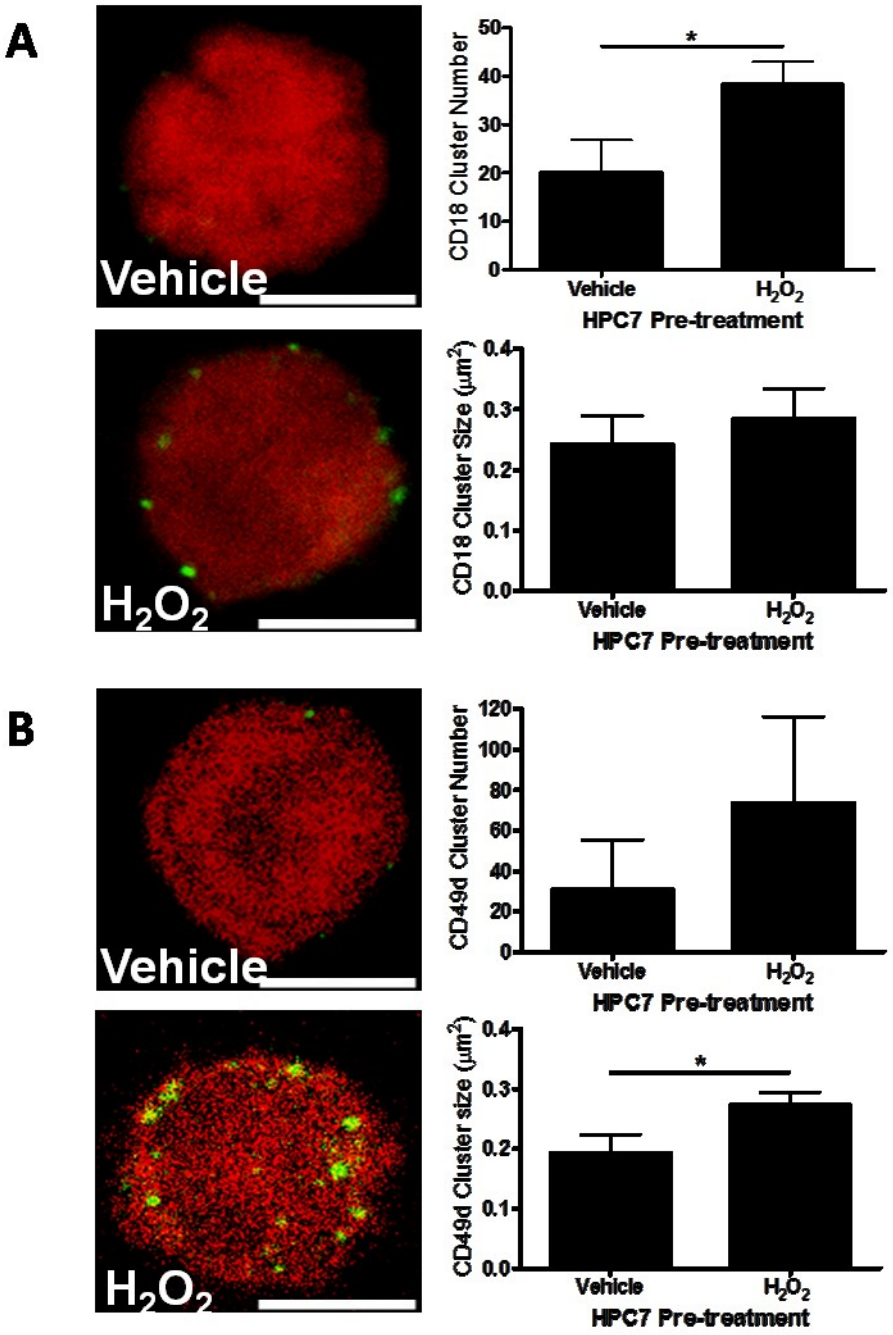

Figure 4.9 H_2O_2 pre-treatment induces greater cluster number of CD18, without affecting cluster size, but does not alter CD49d cluster number, but increases cluster size.

H_2O_2 pre-treatment increases the number of CD18 clusters, without affecting cluster size (A). However, H_2O_2 increases CD49d cluster size without significantly affecting cluster number (B). Fluorescence micrographs representative of N≥5 cells over 3 independent experiments, scale bars = 5μm. Red= cytoplasmic cell tracker orange labelled HPC7, Green= Alexa 488 labelled integrin clusters. Results presented as mean+SEM. Statistical analysis performed using Student's t-test. N≥5 cells over 3 independent experiments. *$p<0.05$.

their adhesion both *in vitro* and *in vivo*. The most effective dose was 100µM, with lower and higher concentrations having no effect on cellular adhesion. It is interesting that this effective concentration is very similar to that seen *in vivo* in the injured environment and to that observed within the ICM. This suggests that the changes induced by pre-treatment may be similar to the actions of the injury microenvironment on trafficking HPC7s *in vivo.*

As expected, IR and colitis ICM contained greater concentrations of H_2O_2 than observed in sham tissue. Interestingly, H_2O_2 concentrations were greater in the SI (jejunum and ileum) than in the colon. This may be due to greater damage occurring in the SI or that it undergoes greater oxidative stress than the colon, either during rest or injury, or that the colon is more capable of managing oxidative stress than the SI. Certainly, the SI is one of the most susceptible organs to ischaemic injury. The presence of H_2O_2 even within SCM media was interesting, suggesting a baseline level of H_2O_2 even in an un-inflamed, relatively healthy environment. This could suggest a role for H_2O_2 in intestinal homeostasis, or may simply reflect the nature of intestinal tissue whereby constant challenges to the immune system result in an active state (Maloy and Powrie, 2011). This is further supported by the 8-oxodG staining results, where sham colon showed a residual level of oxidative damage. However, the IR colon, and to a lesser degree, the colitis colon showed a greater level of oxidative damage. Interestingly, Lee and colleagues reported that a basal level of mutations occurred even within healthy mouse intestinal epithelium, which they postulated could be linked to oxidative stress induced DNA damage (Lee *et al.*, 2006).

The adhesion studies conducted on frozen tissue demonstrated that H_2O_2 pre-treatment could enhance HPC7 adhesion to sham jejunum/ileum/colon, IR injured jejunum/ileum/colon and colitis injured ileum/colon. No increase was observed on colitis injured jejunum but this may be due to the

fact that the DSS colitis injury model predominantly affects the terminal ileum and colon rather than the more proximal jejunum, thus not altering the phenotype of the jejunal vascular endothelium.

The enhanced adhesion to sham tissues suggests H_2O_2 pre-treatment increases HPC7 adhesion by modulating stem cell surface adhesion molecules i.e. H_2O_2 leads to increased adhesion to counterligands that are already present, such as ICAM-1 which is constitutively expressed on even healthy endothelium. This may be brought about by increased integrin/adhesion molecule expression, affinity for endothelial counterligands or enhanced avidity/clustering. The further enhanced adhesion to injured tissues suggests increased HPC7 recruitment is mediated by injury increasing the expression of endothelial cell counterligands as well as the direct effects of H_2O_2 on stem cell surface adhesion molecules. Indeed, collective results from this chapter and from Chapter 3, suggest that HPC7 are recruited more readily to injured (activated) endothelium rather than to sham (un-activated) endothelium. However, when these two processes are combined i.e. when pre-treated stem cells traffick through an injured environment, a greater propensity for adhesion occurs.

Interestingly, colonic intravital studies demonstrated that enhanced HPC7 adhesion following H_2O_2 pre-treatment was only observed within the colitis colon and not the IR injured colon. Inducing IR injury within the colon involves occlusion of the superior mesenteric artery. However, since this artery also feeds the SI as well as the colon, both small and large bowel injury occurs upon reperfusion. Therefore, when naïve HPC7s are injected, they become adherent within the SI as well as the colon. Thus, pre-treated HPC7s also adhere with greater efficiency with the SI, which most likely reduces the number of HPC7 available to bind within IR injured colon. This phenomenon is not observed following colitis injury, as the ileum is not injured to a greater extent than the colon; in which the damage is most severe. Interestingly, the adhesion of H_2O_2 pre-treated cells within colitis

colon appears to be sustained throughout the duration of the experiment. This adhesion pattern is different to the adhesion of naïve HPC7 which is initially high and then falls to a lower plateau.

4.3.1 Mechanisms by which H_2O_2 mediates increased adhesion

We further sought to determine the mechanisms by which H_2O_2 could enhance stem cell adhesion. After electron microscopic examination of the cell surface, it was revealed that H_2O_2 induced ruffling of the cell membrane and the extension of cellular protrusions, reminiscent of pseudopodia and filopodia. However, we did not determine exactly what these protrusions were, how they were formed and the mechanism behind their formation. Regardless of the make-up of these phenotypic changes, they are often features of cellular activation. Indeed, macrophages have been shown to 'ruffle' following activation with lipopolysaccharide, but prior to antigen binding, suggesting that this is a direct cellular effect during activation (Patel and Harrison, 2008). Unlike other ROS mediators such as hydroxyl radicals and superoxide anions, H_2O_2 readily diffuses across plasma membranes and can thus act as a potent intracellular signalling messenger (Enesa *et al.*, 2008). This may explain why H_2O_2 was able to induce morphological changes. Since cell migration requires the coordinated formation of filopodia at the cell front, the development of such structures prior to administration may enable HPC7 to increase their migratory responses to local chemokines and thus improve their subsequent adhesion. Ruffling could also be a means by which cells potentially regulate the clustering and positioning of cell surface receptors so as to enhance ligand binding (Lim and Hotchin, 2012). This may also contribute to the enhanced adhesion of HPC7 following H_2O_2 pre-treatment. However, further work needs to be undertaken to investigate how integrin and other cell adhesion molecules localise within the ruffles, and indeed how the H_2O_2 triggers the formation of ruffles and filopodia.

To identify specifically which surface integrins could be modulated by H_2O_2 pre-treatment, HPC-7 adhesion to immobilised ICAM-1 and VCAM-1 surfaces was determined *in vitro*. Pre-treatment significantly enhanced adhesion to both these surfaces and was dependent on CD18 and CD49d respectively. Similarly, when function blocking antibodies were used to investigate the roles of integrins in adhesion to colon endothelium *in vitro* and intravitally *in vivo*, blockade of either CD18 or CD49d ameliorated adhesion (See Chapter 3). These data demonstrate that H_2O_2 enhanced adhesion is likely modulated by changes in α_4 or β_2 integrin affinity/avidity changes for endothelial counterligands. This dependence on integrin activity, and the complete amelioration of adhesion following blockade, could infer that H_2O_2 directly acts upon the integrins CD18 and CD49d themselves. This is perhaps not all that surprising as there is substantial evidence that integrin conformational changes and integrin-ligand interactions are mediated by the redox status of the environment and the redox modulation of peptide domains of the integrins and cytoskeletal structure (de Rezende *et al.*, 2012, Fiaschi *et al.*, 2012).

Furthermore, evidence has been presented here suggesting H_2O_2, through its effects on integrin clustering, was also able to modulate integrin avidity, the increased adhesiveness of a cell independent of integrin conformational changes. Further evidence that demonstrates that dynamic reorganisation of integrins into microclusters, through F-actin polymerisation, is a major mechanism regulating integrin binding strength and is a prerequisite for their activation and ligand binding (van Kooyk *et al.*, 1999, van Kooyk and Figdor, 2000). Changes in levels of cytoskeletal elements and integrin clustering may play a major role in H_2O_2 mediated HPC7 adhesion. With regards to CD18, H_2O_2 caused an increase in the number of clusters upon the cell surface, whereas CD49d cluster number did not increase, but the clusters themselves became greater in size. We have previously demonstrated using flow cytometry that pre-treatment of HPC7 with H_2O_2 has no effect on surface adhesion molecule expression (Kavanagh *et al.,* 2012). The observed changes in integrin cluster size

or number were not detected by flow cytometry. This may be due to how dense the distribution of integrins on the cell surface are – under a resting state the diffuse integrins are unable to be seen using confocal microscopy, but are still labelled with a fluorescent antibody and so can be detected with a flow cytometer. However, once clustered, hence at a more dense distribution, the collective integrins can be identified using confocal microscopy, but without increasing copy number, as they have merely moved laterally across the cell membrane. Thus this phenomenon is a result of receptor re-distribution and not copy number change. Importantly, changes in integrin cluster number and cluster size can both represent an increase in avidity – either through enhanced interaction with their endothelial counter-ligands or through increased probability of interaction with their counter-ligands. Therefore, the increased avidity can explain the enhanced adhesion observed in our *in vitro* and *in vivo* studies.

It is also likely that H_2O_2 pre-treatment directly affects the affinity state, or the physical conformation, of integrins. However, this is difficult to determine directly in murine samples. In human samples, monoclonal antibodies have been developed to specifically detect conformational changes in integrins, but we are unaware of any such antibodies for murine samples. However, Yu *et al.* (2010) described a method to specifically identify integrin affinity changes indirectly in mice that would exclude avidity alterations. They demonstrated that the Mn^{2+} ion, when bound to the metal ion dependent adhesion site (MIDAS) on integrins, specifically triggered a conformational change in their structure and hence changed their affinity state (Yu *et al.*, 2010). To determine whether H_2O_2 directly affected integrin affinity, we could have conducted an additional experiment pre-treating HPC7 with Mn^{2+}. If the enhanced adhesion observed following H_2O_2 pre-treatment was even further increased in the presence of Mn^{2+}, we would have been able to state that H_2O_2 increased adhesion by predominantly changing integrin avidity/clustering with a limited affinity change. Unfortunately, we did not perform these eloquent and informative experiments.

Similar to results described in Chapter 3, naïve and H_2O_2 pre-treated HPC7 recruitment within the lungs was unaltered following either colitis or IR injury. This again suggests pulmonary recruitment is either at a maximum capacity, not influenced by intestinal injury or is not integrin mediated. Pre-treatment did not enhance HPC7 recruitment to the lungs which from a clinical perspective is highly desirable. Respiratory distress following systemic stem cell transfusion has been proposed as a stumbling block for therapy – by not exacerbating this problem, whilst increasing recruitment to injured organs, highlights that H_2O_2 pre-treatment may be a viable therapeutic option.

To conclude, we have demonstrated that H_2O_2 pre-treatment increases HPC7 adhesion with no concomitant negative effect on cell viability. This enhanced adhesion is achieved through changes in integrin avidity, and potentially affinity, without altering integrin copy number. Hence, pre-treating HSCs with H_2O_2, at physiologically relevant concentrations, could therapeutically enhance retention within acutely and chronically injured colon, effectively 'priming' them for maximal adhesion prior to their systemic delivery. Interestingly, MSCs are protected from apoptosis following oxidative preconditioning, suggesting that H_2O_2 pre-treatment may also aid in enhancing stem cell survival in ischaemic tissues (Li *et al.*, 2009b).

Chapter 5

USING PMP TO ENHANCE HPC7 ADHESION

5.0 Introduction

Microparticles are defined as vesicles that bud off cells, that are lacking a nucleus, contain a membrane skeleton and are defined by size (<1µm diameter) (Johnstone, 2006, Perez-Pujol *et al.*, 2007). They are further defined by the originating cell and the subsequent surface antigens presented (Burnier *et al.*, 2009, Thom *et al.*, 2011). They have been identified to originate from a large variety of cell types including red blood cells, leukocytes (in both myeloid and lymphoid lineages), endothelial cells and platelets (Leroyer *et al.*, 2007, Gelderman and Simak, 2008, Flaumenhaft *et al.*, 2009).

Not only have microparticles been identified from a plethora of cell types, the rate at which they are produced, and indeed the phenotype that they possess, is highly dependant on the disease state of the body. For example, it has been identified that hypertension (Preston *et al.*, 2003), atherosclerosis (Boulanger *et al.*, 2006, Leroyer *et al.*, 2007), Crohn's disease (Chamouard *et al.*, 2005) and many other disease conditions exhibit increased microparticle release. However, it appears that the biggest source of circulating microparticles as a result of disease is from platelets (See Table 2 from Burnier *et al.*, 2009 for detailed list), released as a result of platelet activation (Flaumenhaft, 2006). However, it has been shown that even at rest, platelet microparticles (PMP) form the largest constituent of the microvesicles found in the blood (Lacroix *et al.*, 2010).

The roles of PMPs are widely varied and have ranged from highly reactive procoagulation bodies (Wolf, 1967, Merten *et al.*, 1999), to playing a role in the transfer of cytokines and chemokines (Barry *et al.*, 1997, Mause *et al.*, 2005), transcellular delivery of mRNA (Risitano *et al.*, 2012), as a method of transferring surface receptors such as CXCR4 and the platelet integrin αIIb (Janowska-Wieczorek *et al.*, 2001) and also as a bridging molecule for enhancing leukocyte adhesion to vessel walls (Forlow *et al.*, 2000).

It is these cellular modulation properties that have been recently investigated to see if PMPs can be utilised as a delivery vehicle (Faille *et al.*, 2012) and also as a donor cell modulator (Mause *et al.*, 2005). PMPs have been reported to enhance HSC engraftment in bone marrow (Janowska-Wieczorek *et al.*, 2001), and enhance progenitor cell repair efficiency (Mause *et al.*, 2010), highlighting their potential in modulating HSC recruitment. However, previous studies have not investigated whether short term pre-treatment (≤1 hour) of HSCs with PMPs improves their recruitment to extramedullary sites of injury such as the microcirculation of injured intestinal tissues *in vivo*. Furthermore, the potential mechanisms by which PMPs may increase HSC recruitment have not been identified. The previous chapter has demonstrated that HSC adhesion within the injured gut could indeed be significantly improved using H_2O_2. Since this pre-treatment strategy utilises a ROS chemical approach to modify HSC trafficking, it may raise concerns for translational applicability. The use of PMPs to improve adhesion offers a more biological approach to modify HSC trafficking and this will be investigated in this chapter.

Initial studies aimed to confirm whether thrombin stimulation of mouse platelets could generate PMPs *in vitro* and also to determine whether the generated PMPs could be purified. Two forms of platelet generated microparticles were to be investigated. The total platelet releasate – supernatant from activated platelets that would be enriched in PMPs, thrombin, and other factors released by thrombin stimulated platelets termed PMP enriched supernatant (PES). The other pre-treatment to be investigated were purified PMPs (PMP). The purified PMP were to be isolated from PES by high speed centrifugation and resuspended into empty media. From both PES and PMP pre-treatment it was hypothesised that HPC7 cells could be coated with PMPs following a short-term co-incubation with them and that this pre-treatment would enhance their subsequent adhesion. This was explored via a range of *in vitro* and *in vivo* adhesion assays. Furthermore, some of the potential mechanisms

involved in the predicted increase in adhesion, such as integrin clustering and surface expression changes of CAMs, were also investigated.

5.1 Materials and Methods

5.1.1 Mice and injury models

Experiments were conducted on healthy C57BL/6 wild-type mice or those that had undergone either IR injury (45 minutes ischaemia, 120 minutes reperfusion) or colitis injury (5 days of 3% DSS in the drinking water).

5.1.2 Isolation of murine platelets and PMP generation

Blood was taken from the descending aorta of C57BL/6 wild type mice, platelets isolated, washed by centrifugation and resuspended in Stem Pro SFM34 at $3x10^7$/ml. Platelets were then stimulated with thrombin (30 minutes, 37^oC). Platelet microparticle enriched supernatant (PES) was derived by removing intact platelets by centrifugation from the stimulated platelet releasate. Purified PMPs were isolated by high speed centrifugation of PES, discarding the supernatant and resuspending the PMP pellet with the same volume of Stem Pro as was initially centrifuged. HPC7 were resuspended in either PES or PMP solution at $4x10^6$ cells/ml for 60 minutes for use experimentally immediately following pre-treatment.

5.1.3 Fluorescent Labelling of Cells

Work within this chapter utilises a range of fluorescent dyes to facilitate microscopy studies. In order to visualise PMPs, a platelet rich plasma (PRP; obtained during the process of washing and isolating platelets) was incubated with $DiOC_6$ at a final concentration of 4μg/ml and mixed gently by inversion

(in the dark, room temperature, 30 minutes) and then washed. $DiOC_6$ (3,3'-dihexyloxacarbocyanine iodide) is a cell permeant, green fluorescent, lipophilic dye used for staining membranes.

For this chapter, HPC7 were labelled with two dyes, CFDA-SE or CellTracker Orange (CTO; Invitrogen, UK). CFDA-SE labelling was performed as described in other chapters. HPC7 were labelled with CTO as per the manufacturer's instructions. In brief, HPC7 were incubated at $4x10^6$ cells/ml in 5µM dye in base Stem Pro media (30 minutes, 37°C), washed by centrifugation and kept in base media (within 2 hours) until ready for use.

5.1.4 Endothelial Static Adhesion Assay

Murine colon ECs, cultured in 12 well plates, were used to identify whether differences in stem cell adhesion following pre-treatment with PES or PMP could be identified *in vitro*. Some ECs were treated with TNFα for 4 hours to activate them prior to monitoring adhesion of fluorescently labelled HPC7. Wells were washed and fixed prior to imaging.

5.1.5 Intravital microscopy

Fluorescent IVM was performed to determine HPC7 recruitment in the murine colon microcirculation *in vivo*. Mice were anaesthetised and prepared for surgery as described in Chapter 2. The colon was exteriorised following a mid-line laparotomy and the mucosal surface of the ascending colon was exposed following an incision along the anti-mesenteric border. In mice undergoing IR injury, HPC7s were injected at 60 minutes post-reperfusion. A randomly selected field of view was imaged every 5 minute for 60 minutes. Adherent and free flowing cells were quantitated.

5.2 Results

5.2.1 Pre-treatment of platelets with 2U/ml thrombin generates platelet microparticles

In order to determine whether PMPs could be generated *in vitro* using thrombin, freshly isolated (<2 hours) murine platelets were isolated, washed, labelled with $DiOC_6$ and finally resuspended in Stem Pro and then stimulated with thrombin. The whole solution containing the stimulated platelets, releasate and any microparticles released were mounted onto glass slides and imaged. **Figure 5.1** depicts a fluorescently labelled platelet surrounded by small circular fluorescently labelled particles approximately 0.2-1µm in diameter. These labelled smaller structures are highly likely to be microparticles generated from thrombin stimulated murine platelets.

5.2.2 Incubation of platelet microparticle enriched supernatant and purified platelet microparticle coats HPC7 with microparticles

HPC7 were pre-treated with either PMPs or PES and flow cytometry was used to identify whether the platelet markers glycoprotein (GP) IIb and GPIb could be found on the HSC surface. Interestingly, but not unexpectedly, GPIIb was found to already be expressed on the HPC7 surface (**Figure 5.2 A**). Although GPIIb is most often described as a platelet marker, it is also found on early haematopoietic progenitor cells such as HSCs (Janowska-Wieczorek *et al.*, 2001). However, following pre-treatment with both PES and PMP, surface expression of both GPIIb and GPIb were increased on HPC7s (**Figure 5.2 A**), providing evidence that these microparticles can indeed bind or coat HPC7 cells.

To further confirm interactions between PES and PMP pre-treatments with HPC7s, labelled microparticles were incubated with HPC7 and imaged using confocal microscopy. The upper panels of **Figure 5.2 B** are representative Z projections of unlabelled HPC7 incubated with PMPs labelled with the green membrane dye $DiOC_6$ (**Figure 5.2 B**). This membrane dye is considered to be a leaky

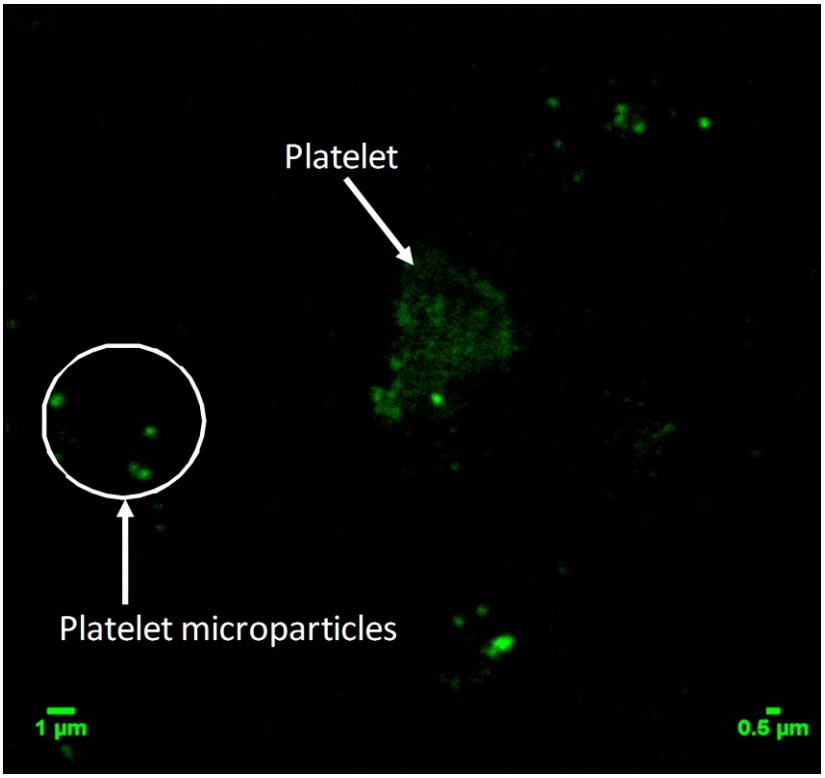

Figure 5.1 Platelet stimulation with thrombin induces PMP expression

Fluorescence micrograph of DiOC6 labelled platelet that has been stimulated with 2U/ml thrombin. Both the stimulated platelet (labelled with an arrow) and released microparticles surrounding (circled) are clearly labelled. As described in the literature, the PMP seen here are small, sub-micrometer particles.

dye and can leach out and diffuse into neighbouring membranes. This 'artefact' highlighted very effectively the binding of PMPs from both pre-treatments, as the intensity of the HSC themselves (the dimmer larger objects within the frame) was greater in the groups pre-treated with PES and PMP compared to the thrombin controls. Interestingly, there are no clear particle shapes found to be bound to the HPC7 in the PMP group. This could potentially be due to membrane fusion of the microparticles as has been previously described (Mause *et al.*, 2010).

The lower panels of **Figure 5.2 B** presents images again confirming PMP interactions with HPC7s but using a different labelling protocol. GPIb from the microparticles are labelled green, via an Alexa 488 antibody against anti-GPIb (**Figure 5.2 B**) and the HPC7 are labelled red with CTO. The increased expression of GPIb on the PES and PMP pre-treated HPC7 cells was clearly evident. It is interesting that the GPIb remains highly clustered in the PMP pre-treated cells, although their membranes diffuse and merge with the HSC membrane. This may occur as a result of the hierarchal architecture of the membrane (Kusumi *et al.*, 2011), whereby transmembrane proteins, such as GPIb are known to diffuse slower (Yu *et al.*, 2010), and thus would remain clustered, potentially due to interaction with the cell cytoskeleton or trapping within the picket fence regions (Kusumi *et al.*, 2011).

As the number of GPIb and GPIIb on the PMP surface could not be quantified, and as a result of the $DiOC_6$ leaking, the number of PMP coating each HPC7 could not be identified, however, the confocal imaging of the cells highlights that a large proportion of HPC7 exhibit coating following pre-treatment. However, the flow cytometry data highlights that the number coating is potentially very low in a large population and also highly heterogeneous in nature.

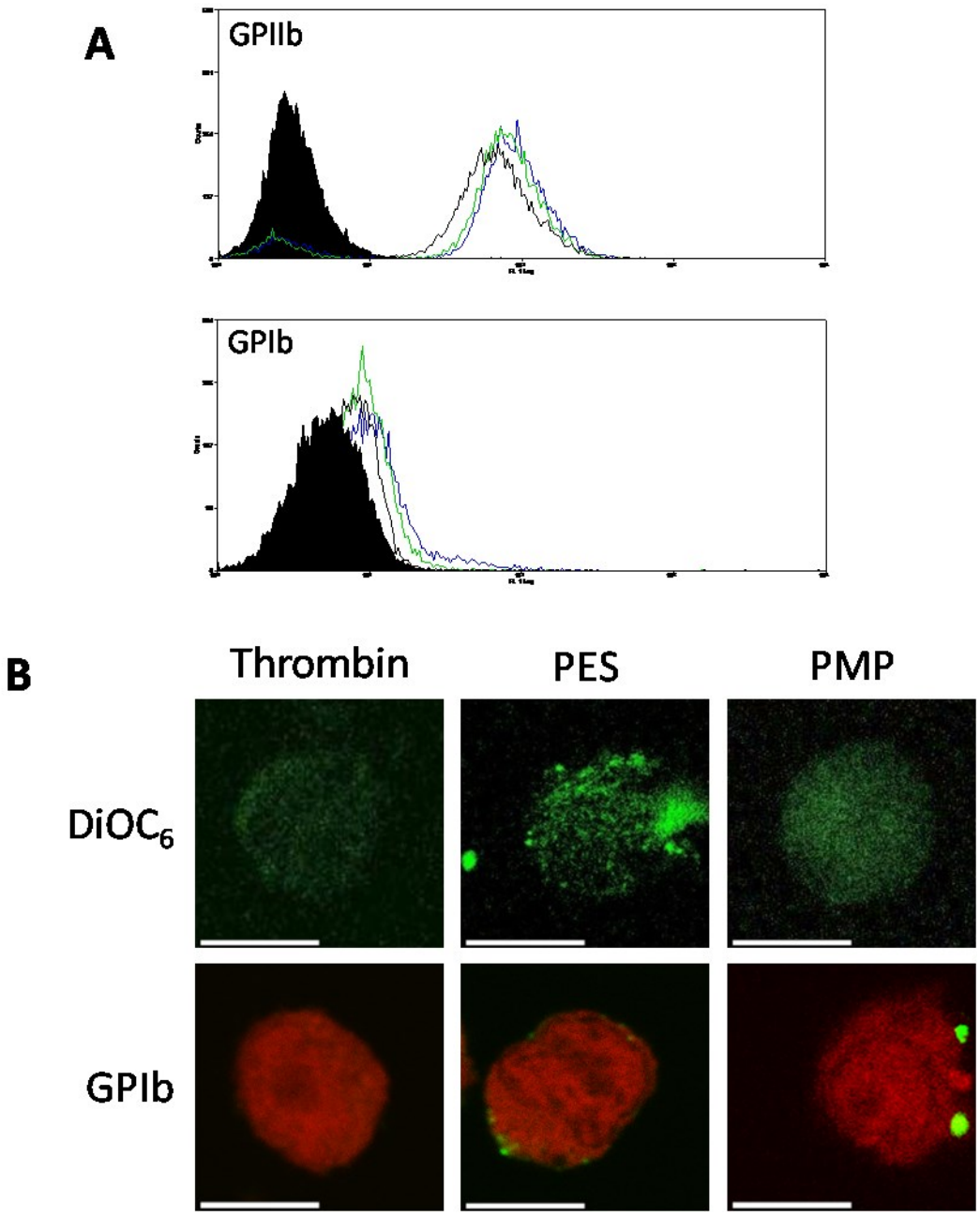

Figure 5.2 Pre-treatment of HPC7 with PES and PMP results in transfer of the platelet markers GPIb and GPIIb, which can be visualised by flow cytometry and microscopically on the surface of HPC7

(A) Pre-treatment of HPC7 with PES (blue line) and PMP (green line) increases their surface expression of glycoproteins GPIb and GPIIb compared to thrombin controls (black line). Plots representative of 3 experiments; Isotype IgG control represented by filled black peak. (B) Confocal microscopy reveals that pre-treating HPC7 with PES and PMP results in microparticle binding to HPC7. Upper panels are Z projections of HPC7 pre-treated with microparticles labelled with $DiOC_6$. In the lower panels Cell Tracker Orange labelled HPC7 (red) are pre-treated with PES and PMP, with the microparticles labelled with an anti-GPIb monoclonal antibody and counterstained with Alexa Fluor-488 antibody (green). Scale bar = 5µm, images representative of 3 experiments.

5.2.3 PES and PMP pre-treatment of HPC7 significantly enhances HPC7 adhesion to colon endothelial cells

To investigate the effects on HP7 adhesion following pre-treatment with PES and PMP, pre-treated fluorescently labelled HPC7 were incubated on a TNFα stimulated colon endothelial cell (CEC) monolayer, washed and adhesion quantitated using fluorescence microscopy. ANOVA statistical analysis revealed that there was a significant difference between the groups ($p<0.001$) with Dunnet's post testing utilised to identify differences between the groups. Pre-treatment with PES significantly ($p<0.05$) increased the number of HPC7 adherent to stimulated CEC compared to the thrombin control whereas PMP pre-treatment had no significant effect on HPC7 adhesion compared to the thrombin control (**Figure 5.3 A and B**).

5.2.4 PES and PMP pre-treatment of HPC7 had no effect on the number of freeflowing cells in either IR or colitis injured colon *in vivo*

Neither pre-treatment strategy had any effect on the numbers of freeflowing HPC7 within the IR injured colon *in vivo* (**Figure 5.4 A**). Similarly, within the colitis colon there was no effect on the number of freeflowing cells (**Figure 5.4 A**).

5.2.5 PES and PMP pre-treatment of HPC7 significantly enhances HPC7 adhesion to colitis injured but not to IR injured colon microvasculature *in vivo*

In the IR injured colon, neither pre-treatment significantly raised HPC7 adhesion (**Figure 5.4A**). However, within the colitic colon there was a significant ($p<0.05$) effect on adhesion following pre-treatment. Dunnet's post testing identified that only PES pre-treatment significantly ($p<0.05$) increased adhesion compared to thrombin controls, whilst, PMP pre-treatment again failed to elicit a significant increase in adhesion (**Figure 5.4 B**). As described in earlier chapters, all adherent cells were observed intravascularly with no indication of extravasation.

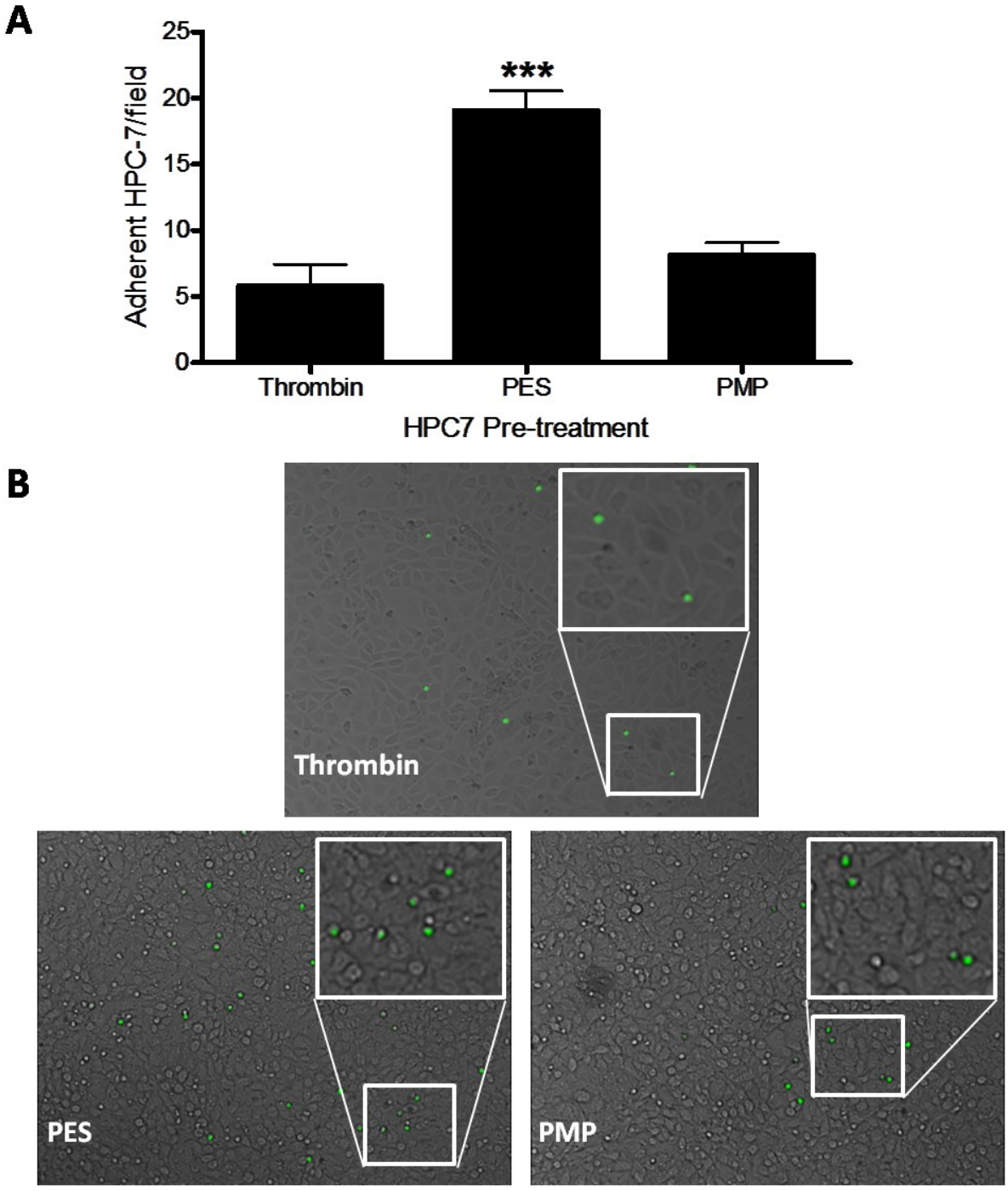

Figure 5.3 Pre-treatment of HPC7 with PES, but not PMP, enhances adhesion to TNFα stimulated colon endothelial cells

(A) Pre-treatment of HPC7 with PES significantly ($p<0.001$) enhances HPC7 adhesion to colon endothelial cells. However, PMP pre-treatment has no effect on HPC7 adhesion compared to thrombin controls. (B) Representative micrographs display a brightfield CEC monolayer with fluorescently labelled HPC7 adherent to the surface. Inset images are 2x electronic magnification of boxed region. Data are presented as mean+SEM. Statistical analysis performed with one way ANOVA with Dunnet's post testing - results from the Dunnet's test are displayed. Abbreviations: PES – platelet microparticle enriched supernatant, PMP – purified platelet microparticles, ***$p<0.001$.

Ex vivo analysis of the colon tissue from both IR and colitis colons revealed a similar pattern to that observed in the pre-selected region of interest *in vivo*. There were no significant differences within the IR colon (**Figure 5.5 A**). Similarly, the colitis *ex vivo* data again demonstrated that PES significantly ($p<0.05$) enhanced HPC7 adhesion compared to thrombin pre-treatment, whereas PMP did not (**Figure 5.5 A**).

5.2.6 PES pre-treatment of HPC7 significantly enhances their adhesion within the lungs and ileum of mice undergoing colonic IR injury

Following intravital experiments the lungs and ileum were excised from mice undergoing intestinal IR injury and also microscopically imaged *ex vivo* for adherent labelled HPC7 cells. Within the lungs, only PES pre-treatment significantly ($p<0.01$) enhanced adhesion when compared to the thrombin controls (**Figure 5.5 B**). Similarly, within the ileum of IR injured mice, it was found that only PES pre-treatment significantly ($p<0.05$) enhanced adhesion (**Figure 5.5 C**). Overall, the greatest number of HPC7s was observed within the pulmonary tissue, with around 100 cells identified per field of view quantitated. This is more than 10x the number of cells observed in the ileum and more than 20x the number of cells observed per field of view in the colon.

5.2.7 PES pre-treatment significantly lowers HPC7 adhesion within the lungs of a colitis mouse, but has no significant effect on the colitis injured ileum *in vivo*

Following intravital experiments the lungs and small intestinal ileum were also excised from mice undergoing colitis injury and imaged *ex vivo* for adherent labelled HPC7 cells. Interestingly, within the lungs, PES pre-treatment significantly ($p<0.05$) reduced HPC7 adhesion when compared to thrombin controls (**Figure 5.5 B**). However, PMP pre-treatment had no effect compared to thrombin controls (**Figure 5.5 B**). Within the ileum derived from mice undergoing colitic injury, neither PES or

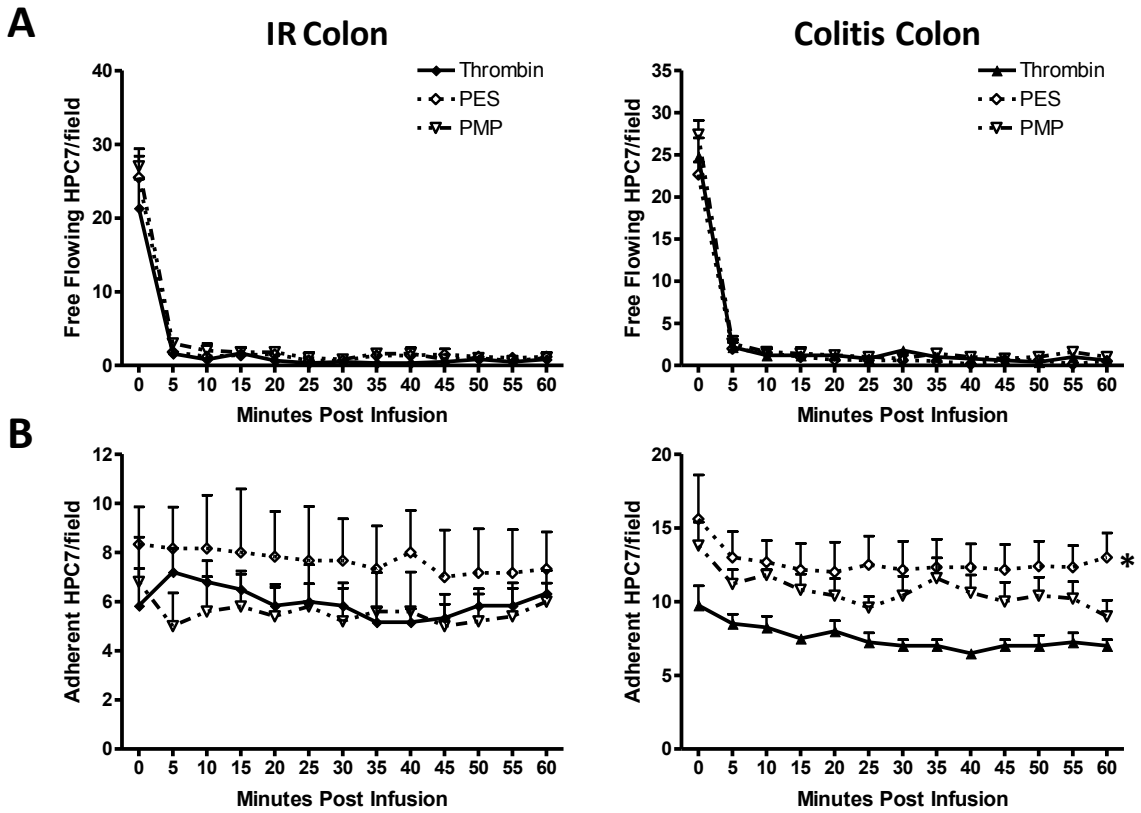

Figure 5.4 Neither PES nor PMP pre-treatment affects the number of freeflowing HPC7 within injured colon, but PES enhances adhesion to colitis colon in vivo.

(A) Neither PES nor PMP pre-treated changed the number of freeflowing HPC7 within the IR or colitis injured colon (B) Similarly, neither PES nor PMP pre-treatment enhanced HPC7 adhesion to IR colon, although there was a trend for the PES pre-treated cells to exhibit greater adhesion. However within the colitis colon, HPC7 adhesion was significantly increased following PES pre-treatment ($p<0.05$) with a trend towards increased adhesion also seen with PMP pre-treatment. Data are presented as Mean HPC7/field+SEM. Statistical analysis performed using One-way ANOVA with Dunnet's post-test and calculated using AUC data. Abbreviations: PES – platelet microparticle enriched supernatant, PMP – purified platelet microparticles.

PMP pre-treatment significantly affected adhesion when compared to the thrombin controls, although there was a trend for adhesion to increase with PES pre-treatment (**Figure 5.5 C**).

5.2.8 The increased adhesion of HPC7 following pre-treatment is not as a result of increased integrin copy number

Increased cellular adhesion can occur as a result of many different components. One such factor is the copy number of the integrins and other CAMs on the cell surface following stimulation of the cells. Therefore, to investigate whether HPC7 pre-treatment had any effect on CAM expression on the cell surface flow cytometry was applied to determine if the expression levels were altered.

Figure 5.6 A displays a representative flow cytometry histogram of CD18 expression following pre-treatment with either thrombin, PES or PMP. As can be clearly seen, there was no difference between each pre-treatment (**Figure 5.6 A**). Similarly, there was no significant difference in CD49d expression following pre-treatment with PES and PMP compared to thrombin controls (**Figure 5.6 B**). Finally, the cell adhesion molecule CD44 was also investigated, but again there was no significant difference of the surface expression levels (**Figure 5.6 C**).

5.2.9 Pre-treatment of HPC7 with PES and PMP significantly increases adhesion to VCAM-1, whereas adhesion to ICAM-1 is only increased following pre-treatment with PES

To begin to determine the mechanism by which PES and PMP pre-treatments enhance adhesion of HPC7, their adhesion to an ICAM-1 or VCAM-1 surface was quantitated by light microscopy. One way ANOVA analysis reveals that pre-treatment of HPC7 has a significant effect on HPC7 adhesion to ICAM-1 ($p<0.01$; **Figure 5.7 A**). Further analysis revealed that adhesion to ICAM-1 following pre-treatment with PES significantly ($p<0.05$) enhanced adhesion, however, PMP pre-treatment had no significant effect on adhesion compared to thrombin controls (**Figure 5.7 A**).

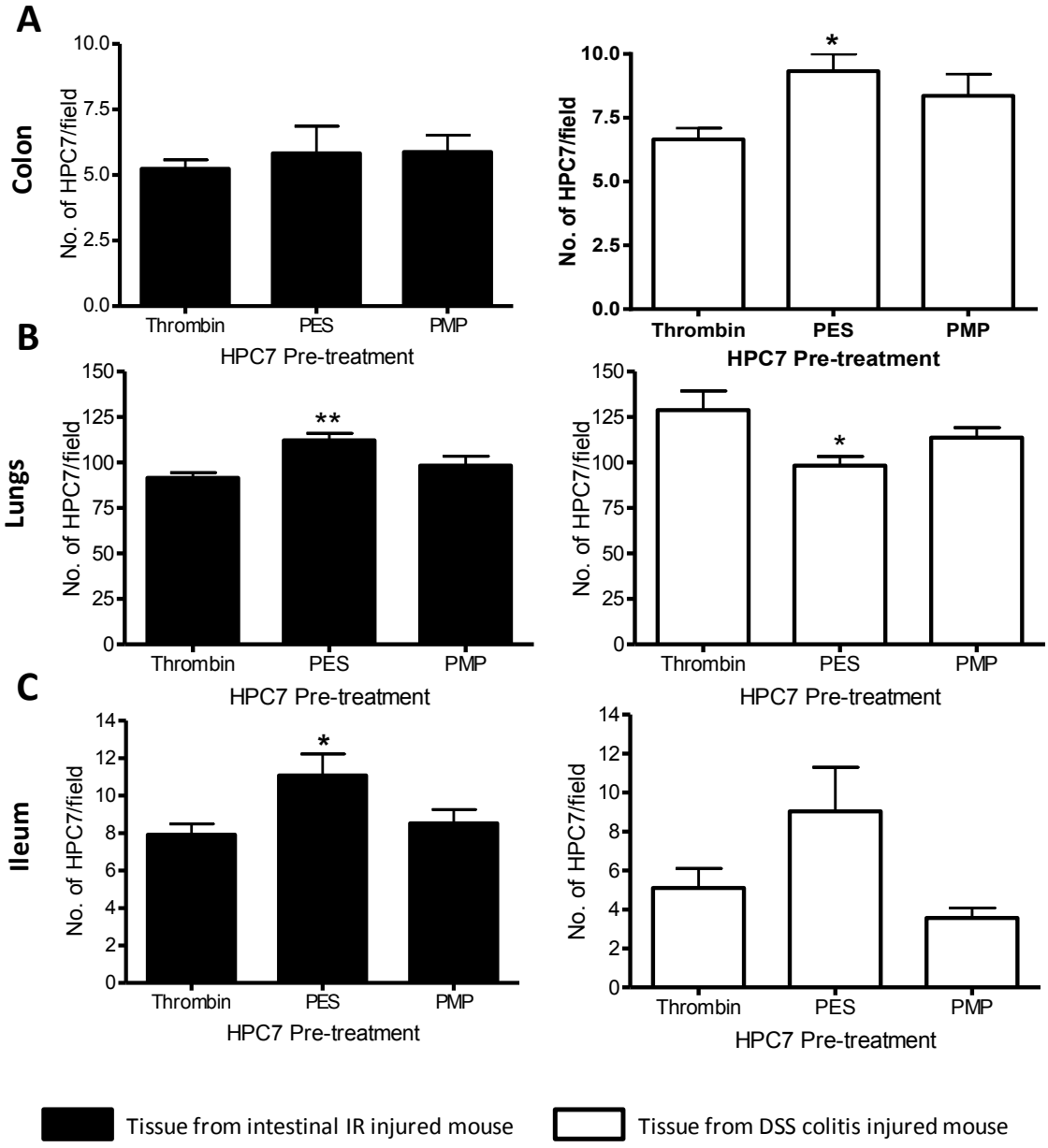

Figure 5.5 Ex vivo analysis of lung, ileal and colon tissue demonstrated that PES pre-treatment enhances HPC7 adhesion to injured tissue

(A) *Ex vivo* analysis of the excised colon from the IR and colitis injured mice confirmed what was seen *in vivo.* Within the IR colon (left panel), PES and PMP pre-treatment did not significantly enhance recruitment, but within the colitis colon (right panel) only PES pre-treatment significantly ($p<0.05$) enhanced adhesion. (B) PES pre-treatment significantly ($p<0.01$) increased HPC7 adhesion within lungs derived from intestinal IR injured mice (left panel), whereas, it significantly ($p<0.05$) reduced adhesion within lungs from colitis injured mice (right panel). In both IR and colitic lung tissue, PMP did not enhance adhesion. (C) Analysis of ileum derived from IR injured mice (left panel) demonstrated that only PES pre-treatment significantly ($p<0.05$) enhanced HPC7 adhesion. Within the colitic ileum (right panel) neither PES nor PMP pre-treatment significantly enhanced HPC7 adhesion. Data presented as mean+SEM, with statistical analysis performed using one way ANOVA and Dunnet's post test. Abbreviations: PES – platelet microparticle enriched supernatant, PMP – purified platelet microparticles.

5.2.10 Pre-treatment of HPC7 with PES and PMP trigger clustering of the integrins CD18 and CD49d and membrane ruffling

As mentioned above PES and PMP pre-treatment did not alter integrin expression. However, enhanced adhesion to the counterligands ICAM-1 and VCAM-1 was observed. Thus it was investigated whether pre-treatment of HPC7s affected integrin distribution upon the cell surface. Confocal microscopy was used to quantify the extent of clustering of the integrin sub-units CD18 and CD49d.

The analysis identified that both PES and PMP pre-treatment resulted in a significantly ($p<0.05$) increased number of CD18 clusters compared to the thrombin control ($p<0.05$; **Figure 5.8 A**). However, there was no significant difference in the size of the CD18 clusters regardless of pre-treatment (**Figure 5.8 A**). Similarly, PES significantly ($p<0.05$) increased integrin subunit CD49d cluster number, although PMP pre-treatment did not when compared to thrombin controls (**Figure 5.8 B**). Again, neither PES nor PMP pre-treatment had any significant effect on the size of the CD49d clusters (**Figure 5.8 B**). Interestingly, unlike the clustering observed following H_2O_2 pre-treatment, PES and PMP pre-treatments resulted in some internalisation of integrin clusters (**Figure 5.8**; yellow colour on fluorescence micrographs).

Further to investigating integrin clustering effects, the morphology of the cell was also studied. Scanning electron microscopy was employed to identify any overt morphological changes of the HPC7 following pre-treatment with PES or PMP. It revealed that following exposure to PES and PMP the membrane of the HPC7 ruffles (**Figure 5.9 B and C**) – a phenomenon often seen in activated macrophages (Patel and Harrison, 2008) but this is not seen in the thrombin treated HPC7 (**Figure 5.9 A**). Furthermore, cells pre-treated with PES (**Figure 5.9 B**) also appear to have extended pseudopodia, far more than that seen with the thrombin treated (**Figure 5.9 A**) control or the PMP

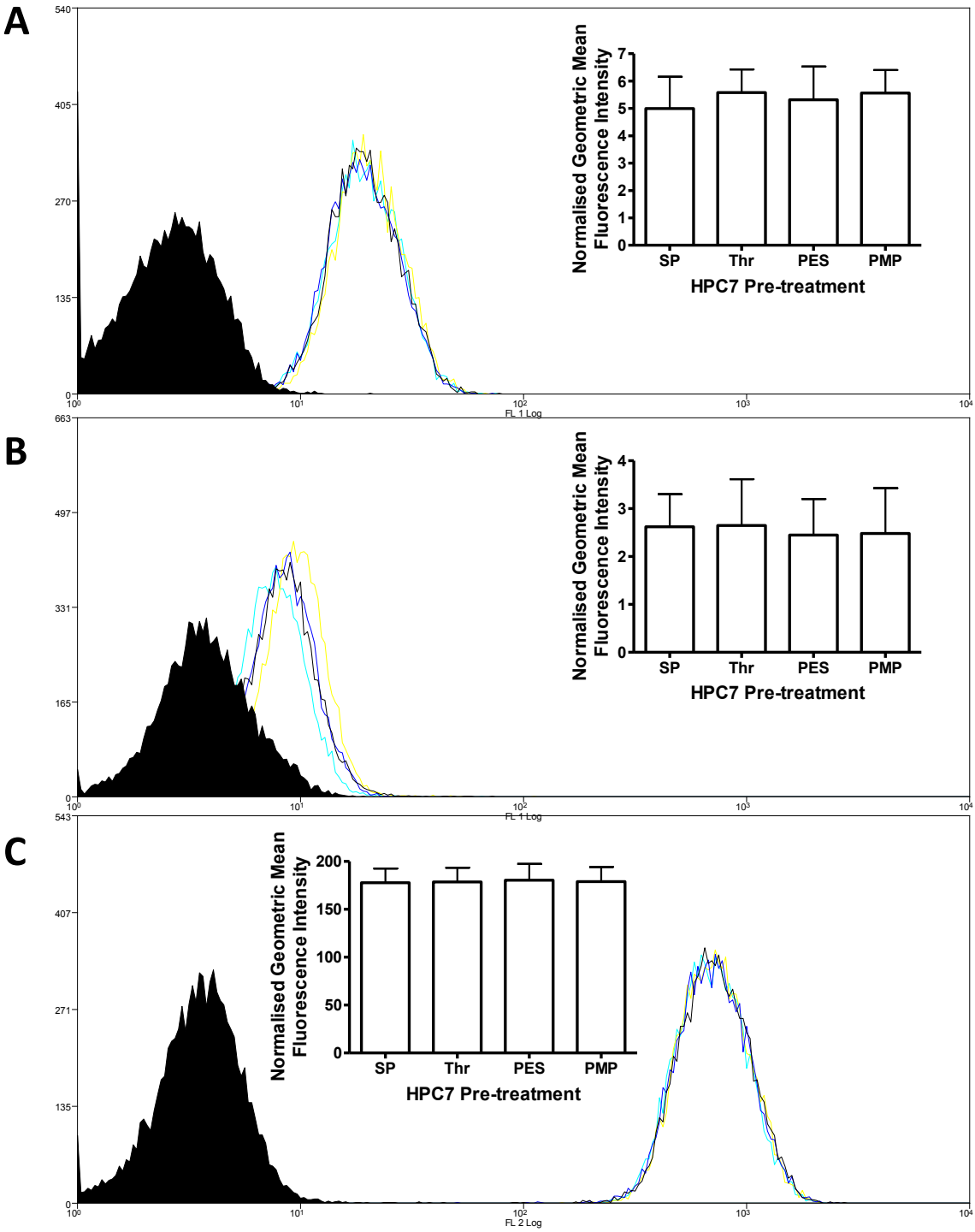

Figure 5.6 Enhanced HPC7 adhesion following pre-treatment is not due to increased cell adhesion molecules on the cell surface

Pre-treatment of HPC7 with PES or PMP had no effect on the surface expression of CD18 (A), CD49d (B) or CD44 (C). Inset graphs mean±SEM of geometric mean normalised against the IgG controls. Black line = Stem Pro control, yellow line = thrombin controls, light blue line = PES group, dark blue line = PMP group. Abbreviations: SP – Stem Pro, Thr – thrombin, PES – platelet microparticle enriched supernatant, PMP – purified platelet microparticles.

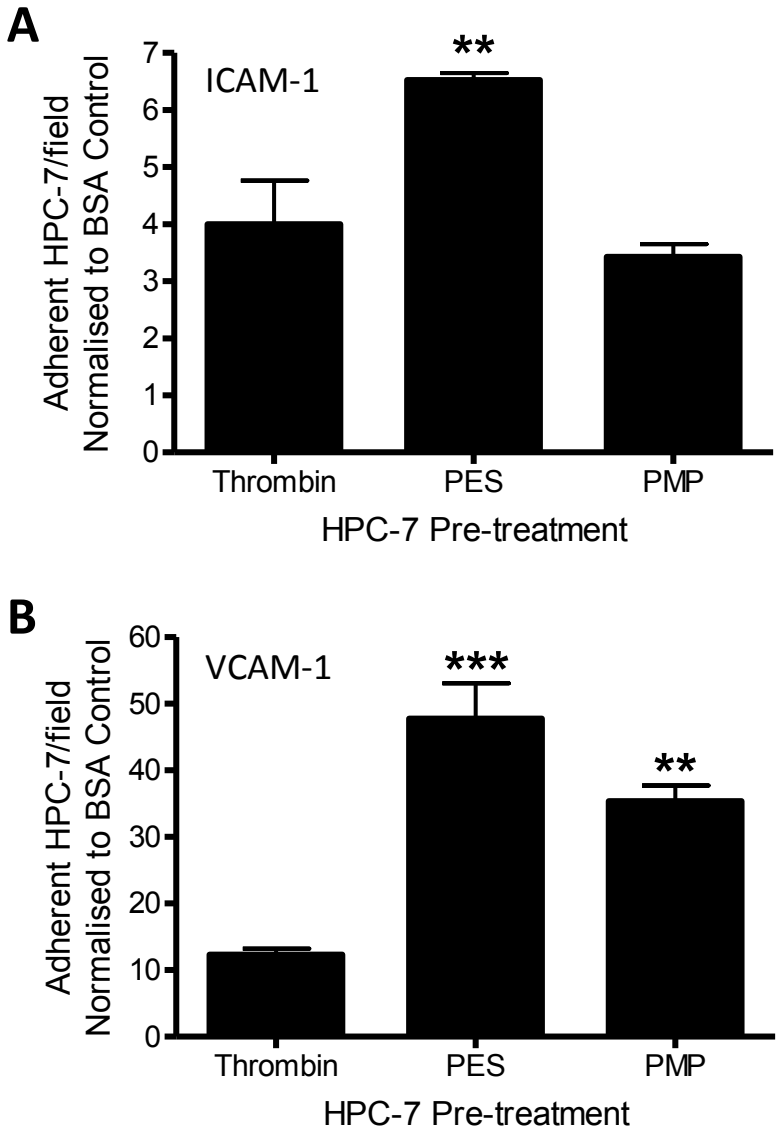

Figure 5.7 PES pre-treatment enhanced HPC7 adhesion to ICAM-1 and VCAM-1, but PMP pre-treatment only increased adhesion to ICAM-1

(A) Pre-treatment of HPC7 with PES resulted in a significant ($p<0.01$) increase in adhesion to an ICAM-1 coated surface, whereas, PMP pre-treatment had no effect on HPC7 adhesion to ICAM-1. (B) Pre-treatment of HPC7 with PES and PMP significantly ($p<0.001$, $p<0.01$ respectively) enhanced adhesion to an immobilised VCAM-1 surface. Data presented as mean (normalised to BSA protein control)+SEM. Statistical analysis performed using one way ANOVA with Dunnet's post test, with the Dunnet's test results displayed. Abbreviations: PES – platelet microparticle enriched supernatant, PMP – purified platelet microparticles.

treated (**Figure 5.9 C**) HPC7, but it is interesting to note that both of the latter two groups do seem to have extended some pseudopodia unlike the PBS treated cells in **Figure 4.7**.

5.3 Discussion

The data presented in this chapter provides novel evidence that thrombin generated PMPs can coat HSCs and that this process results in an enhanced intestinal recruitment potential as demonstrated both *in vitro* and *in vivo.* This phenomenon appeared to be dependent upon the tissue type and the injury status of the tissue. Furthermore, we demonstrated that co-incubation of HPC7s with PMPs in a supernatant also containing the activated platelet releasate was even more effective at enhancing adhesion. Similar to the enhanced adhesion observed with H_2O_2, the mechanisms primarily involved increasing the affinity of stem cell surface integrins for endothelial counterligands. Although this approach offers a more biological strategy for improving stem trafficking to the colon, to be fully realised clinically there are several areas that require further in-depth investigation and refinement.

The data demonstrating that a high dose of thrombin can trigger the production of PMPs, corroborates previous work published (Jy *et al.*, 1995, Perez-Pujol *et al.*, 2007). We further demonstrated that platelet surface markers, such as GPIIb (CD41; $\alpha_{IIb}\beta_3$) and GPIb (CD42), could be transferred to the stem cell via the PMPs. Interestingly, it was identified that this transfer was not seen to occur to all of the incubated HPC7. This raises a few questions, many of which warrant further investigation. For example, it is not clear whether the variability in coating is due to heterogeneity within the PMP population or within the HSC population. It could be that there are subsets within either the target cell or the microparticle population that have the correct phenotype to facilitate binding. It could be a more technical problem in that the duration of the incubation process needs to be increased or the generation of PMPs needs to be better optimised.

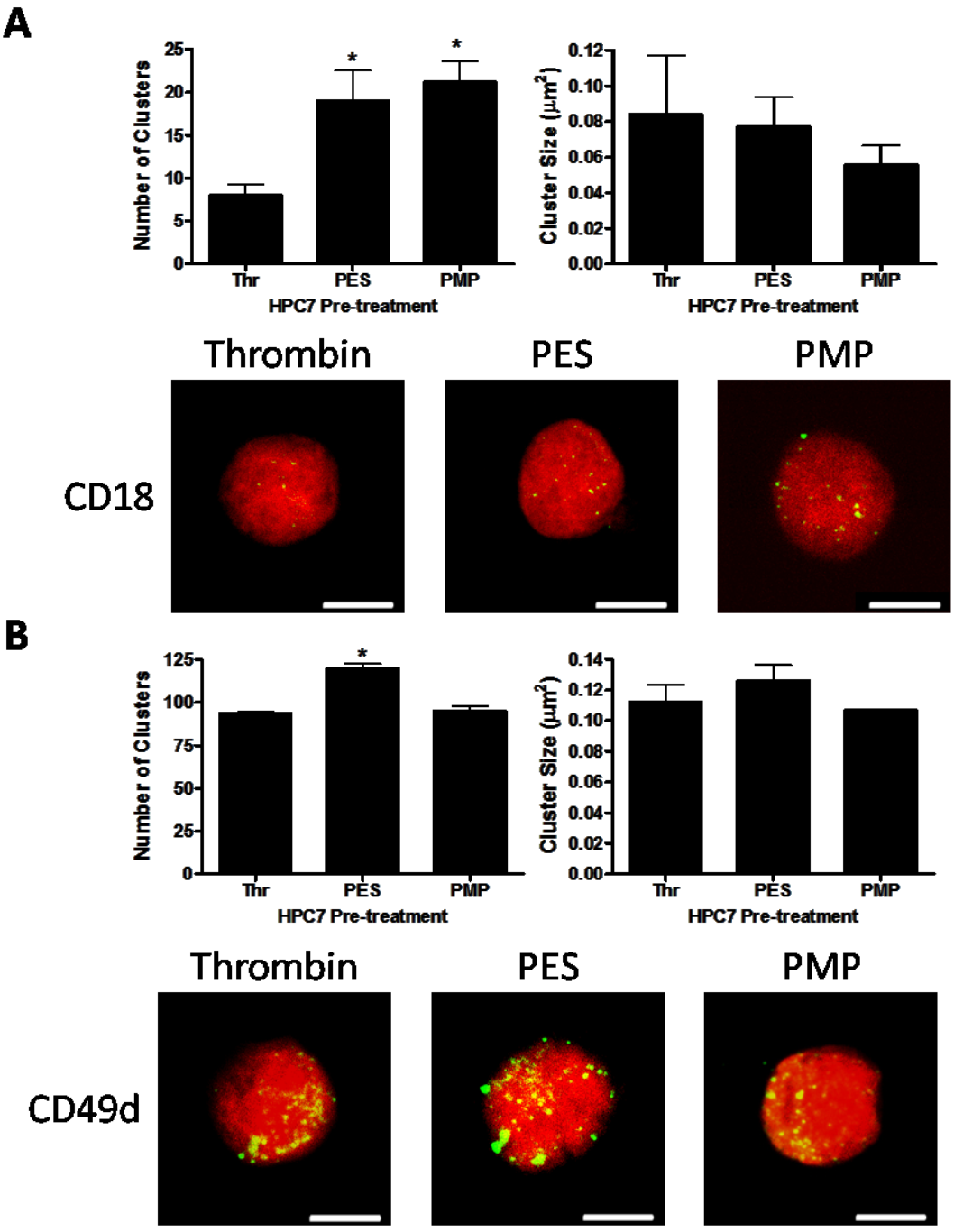

Figure 5.8 PES and PMP pre-treatment result in enhanced clustering of the integrin subunit CD18, but only PES pre-treatment results in enhanced clustering of CD49d

(A) Both PES and PMP significantly ($p<0.05$) enhances CD18 cluster number. However, neither pre-treatment had a significant effect on the size of integrin clusters. The lower three panels are representative Z-projections of HPC7 (red) with CD18 clusters (green) following pre-treatment. (B) CD49d clustering only significantly ($P<0.05$) increased following PES pre-treatment. Again no change in cluster size was observed. Below are representative Z-projections of HPC7 (red), the integrin clusters (green) and internalised clusters (yellow) following pre-treatments and the thrombin control. Data are presented as mean±SEM and statistically analysed using one way ANOVA and Dunnet's post test, with the Dunnet's results displayed. Scale bars = 5μm. Abbreviations: Thr – Thrombin, PES – platelet microparticle enriched supernatant, PMP – purified platelet microparticles.

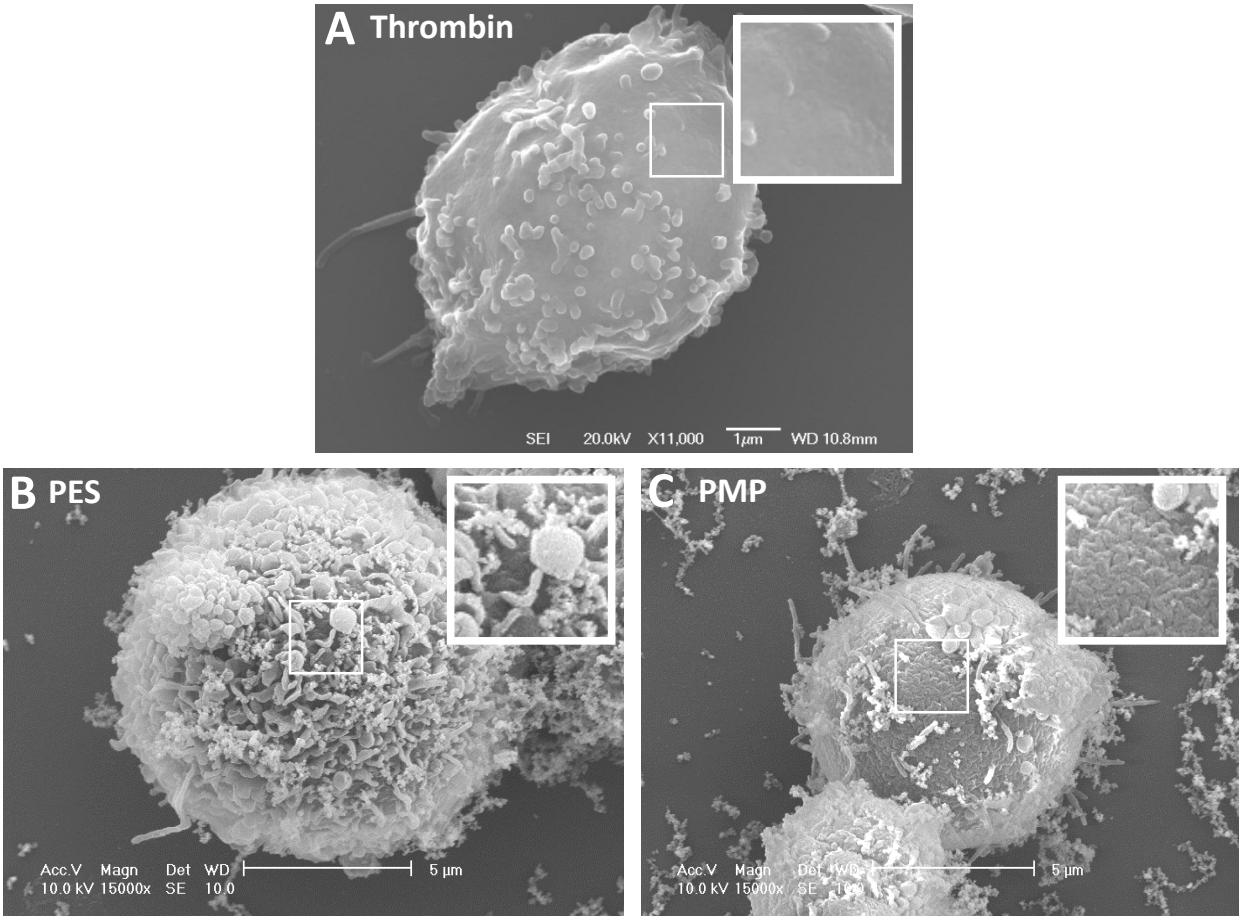

Figure 5.9 PES and PMP pre-treatment result in membrane ruffling and more pseudopodia extension

(A) Scanning electron micrograph of thrombin treated HPC7 displaying little membrane ruffling (insert box: 2x zoom of highlighted area) with some extended pseudopodia, whereas, following PES pre-treatment (B) HPC7 exhibit increased membrane ruffling (insert box: 2x zoom of highlighted area) with much greater numbers of pseudopodia extended. (C) PMP pre-treatment displays extensive membrane ruffling (insert box: 2x zoom of highlighted area), with pseudopodia extension comparable to that seen with the thrombin controls. Abbreviations: PES – platelet microparticle enriched supernatant, PMP – purified platelet microparticles.

Indeed, a variety of agonists have been used to generate mouse PMPs other than thrombin including collagen, calcium ionophores and combinations of these stimuli (Janowska-Wieczorek *et al.*, 2001, Mause *et al.*, 2005, Perez-Pujol *et al.*, 2007). However, to date there is yet to be a comprehensive study comparing the effects of the different stimuli. The generation of a higher concentration or yield of PMPs may lead to more effective coating of stem cells and potentially lead to even better recruitment both experimentally and therapeutically.

There is a paucity in the current literature regarding PMP binding to any cell type. Probably the most characterised interaction is between PMPs and neutrophils. PMPs expose platelet-specific antigens on their surface and can adhere to leukocytes in a P-selectin/PSGL-1 dependent manner (Barry and FitzGerald, 1999). Also, more recent studies have demonstrated that platelet GPIb may also play a role in PMP binding to neutrophil CD11/CD18 and that the glycoproteins IIb/IIIa are not involved (Lo *et al.*, 2006) Furthermore, both P-selectin/PSGL-1 and GPIb-CD11/CD18 interactions can also subsequently activate the neutrophil and modify its adhesion. To the author's knowledge, such detailed investigations have not been carried out with regards to stem cells, and furthermore, it has not clear what proportion of platelet derived microparticles are GPIb positive, or indeed, if GPIb is involved in PMP coating of HPC7.

The observed increase of $DiOC_6$ labelling and GPIb and GPIIb surface expression on HPC7 can be explained by a number of options including the possibility that (i) the microparticles are bound as separate entities on the stem cell surface, (ii) they become endocytosed (or possibly phagocytosed), with a subsequent transfer of cell surface markers to the stem cell or (iii), the two membranes become fused in a novel mechanism. Interestingly, we did not note any obvious microparticles bound to the HPC7 surface when observed using electron microscopy. However, this may be due to

our inability to differentiate between the microparticles and the highly activated HPC7 membrane surface.

There have also been several reports of microparticles delivering their intermembranous contents into the target cell, which also partially negates the possibility of them remaining as separate entities (Mause *et al.*, 2005, Faille *et al.*, 2012). Indeed, Faille and colleagues demonstrated that one of the ways PMPs are cleared from the circulation is by becoming internalised by ECs. They further demonstrated that PMP internalisation by ECs did not involve the fusion of PMP lipids with the endothelial plasma membrane but, rather, an uptake of PMP material with subsequent localization in intracellular vesicles (Faille *et al.*, 2012). Further investigations into the mechanisms by which microparticles may deposit themselves or their contents is essential as this could be a keystone for the use of microparticles for cellular transformation (Faille *et al.*, 2012), clinical applications (such as the one investigated here) or as novel targeted drug delivery vehicles (van Dommelen *et al.*, 2012).

Regardless of the mechanisms involved in PMP coating, it is likely that the method employed to coat cells is capable of further optimisation. This is demonstrated by the observation that PES pre-treatment was more capable of enhancing HPC7 adhesion than purified PMPs both *in vitro* and *in vivo* (**Table 5.1**). In fact, PMPs only raised adhesion to the colitis colon *in vivo*, but not significantly. Co-incubation with PES also appeared to have the highest level of HPC7 coating, as determined by confocal microscopy and flow cytometry. This may be due to the loss of PMPs during the high speed centrifugation step involved in PMP generation. However, it is also possible that other constituents within the PES may play an additive role in facilitating PMP binding. Indeed, Coppinger and colleagues, who also stimulated platelets with thrombin for 3 minutes to achieve maximum release of all granule contents (α, dense, and lysosomal), identified using a proteomics approach more than 300 proteins in the releasate (Coppinger *et al.*, 2004). Many of these are well known to have pro-

adhesive effects on inflammatory cells (IL-8, SDF-1α, RANTES, etc.) and in combination with the PMPs themselves may have a greater stimulatory effect on stem cells. **Table 5.1** summarises how PES was a far more effective pre-treatment than PMPs at increasing HPC7 adhesion in the various *in vitro* and *in vivo* assays used in this chapter.

The enhanced adhesion to the injured colon of HPC7 following PES pre-treatment was to a certain extent dependent on the type of colonic injury. PES pre-treatment only significantly enhanced HPC7 adhesion to the colitis colon, with only a trend for increased adhesion observed in the IR injured colon. As previously mentioned in Chapter 4, inducing IR injury within the colon involves occlusion of the superior mesenteric artery. This artery also feeds the small intestine (SI), as well as the colon, and so both small and large bowel injury occurs upon reperfusion. Indeed, it is well known that following intestinal IR injury, significantly more damage takes place in the ileum than the ascending colon. Therefore, when naïve HPC7s are injected, they become adherent within the SI as well as the colon. Therefore, PES pre-treated HPC7s also adhere with greater efficiency with the SI, which most likely reduces the pool of circulating HPC7s available to bind within IR injured colon. PES most likely would have significantly increased HPC7 adhesion in the IR injured colon had it not been for the fact that most of the injected cells probably adhered in the simultaneously injured SI - this is particularly true since PES significantly increased adhesion in all the assays used (**Table 5.1**).

Interestingly, PMP pre-treatment failed to significantly enhance adhesion to the colon *in vivo* regardless of the severity of the injury. The reasons for a lack of enhanced adhesion in the IR injured colon may be similar to the reason given earlier for PES. The absence of adhesion in lungs derived from colitis mice may be due to the fact that there is little evidence demonstrating any injuries to the pulmonary system following DSS induced colitis injury. However, within the colitis colon there was a trend for the PMP pre-treated cells to enhance adhesion, but this did not reach significance. This

again calls into question whether this is due to insufficient coating of HPC7s with PMPs during co-incubation, or whether the mode of action of PES and PMPs vary considerably. However, it is worth noting that the adhesion of PMP-coated HPC7s was greatly enhanced on the immobilised endothelial counterligand ICAM-1. Interactions between the β2 integrin (CD11/CD18; LFA-1) and ICAM-1 are critical in mediating stable leukocyte adhesion to the colonic endothelium during IR injury (Riaz *et al.*, 2002a). It is therefore possible that PMP coating may show some benefit in improving stem cell adhesion in other injured organs where expression of ICAM-1 is significantly increased.

The mechanisms of enhanced adhesion were also investigated in this chapter. One mechanism that can be excluded to explain increased adhesion is that of increased surface expression of CAMs. Neither pre-treatment with PES or PMP increased surface expression of CD18, CD44 or CD49d. This is not surprising as various attempts within the Kalia lab, using a whole host of pre-treatment strategies including H_2O_2 described in Chapter 4, have failed to induce any increase in expression of CAMs. Platelets also have no CD18, CD49d or CD44 which could be transferred to the HPC7s. However, the potential transfer of known platelet surface markers to stem cells may be one of the main mediators of increased adhesion. During haemostasis, platelet-platelet interactions or their aggregation primarily involves bridging of GPIIb-IIIa receptors by the adhesive protein fibrinogen (Kasirer-Friede *et al.*, 2007). Therefore, the increased presence of GPIIb on the HPC7 surface may allow similar interactions of the stem cell to take place with platelet aggregates or monolayers already adherent in the injured microcirculation. Indeed, both intestinal and DSS-induced colitis injuries are associated with the presence of platelet microthrombi (Anthoni *et al.*, 2007). Platelet GPIb is a component of the GPIb-V-IX complex which binds von Willebrand factor immobilised on the endothelium, permitting platelet capture at high shear (Kasirer-Friede *et al.*, 2007). Therefore, the transfer of GPIb to the stem cell surface may also provide a means of increasing stem cell-endothelial interactions.

	In vitro Experiments			Intravital Experiments		Lungs - *ex vivo*		Ileum - *ex vivo*	
	Colon ECs	ICAM-1	VCAM-1	IR Injured Colon	Colitis Colon	Intestinal IR	Colitis injury	Intesinal IR	Colitis Injury
PES	↑ ***	↑ **	↑ ***	↑ NS	↑ *	↑ **	↓ *	↑ *	↑ NS
PMP	–	–	↑ **	–	↑ NS	–	–	–	–

Table 5.1 Summary of the pro-adhesive effects of PES and PMP pre-treatment when investigated in vitro and in vivo.

PES most likely would have significantly increased HPC7 adhesion in the IR injured colon had it not been for the fact that most of the injected cells probably adhered in the simultaneously injured small intestine. Up arrows = Increase, down arrows = decrease, line = no change. *p<0.05; **p <0.01; ***p<0.001; NS = not significant.

Only PES pre-treatment enhanced HPC7 adhesion to an ICAM-1 coated surface *in vitro*. This was surprising, as despite the absence of an increase in CAMs, both PES and PMP pre-treatments clustered CD18 on the stem cell surface. However, the observed CD18 clustering may not be 'effective'. It has previously been demonstrated that the neutrophil integrins can interact with platelet GPIIb via a fibrinogen cross-bridge (Patko *et al.*, 2012). Hence, the leukocyte integrin CD11/CD18 can serve as a receptor for fibrinogen (Loike *et al.*, 1991). It is well established that the binding of a ligand to the cell surface can induce triggering of surface integrins (Takagi and Springer, 2002, Yu *et al.*, 2010). Therefore the observed CD18 clustering in our HPC7s may be the result of residual soluble fibrinogen on an adherent microparticle surface that was not fully removed during washing and isolation of the platelets. The use of a fibrinogen bridge to link the PMP to the HPC7 may not always be necessary. The assumption is based on the observations made by others that PMP can actively deliver their contents to target cells – a process that would require membrane fusion.

Unlike with ICAM-1, pre-treatment with both PES and PMP increased adhesion to VCAM-1 surface. This was a surprising result when the clustering data was considered as only PES pre-treatment significantly enhanced CD49d clustering. Therefore, PES enhanced adhesion is most likely due to the increased clustering of CD49d, with the clustering being induced by some component of the platelet releasate rather than the PMPs themselves. This may be SDF-1α, a potent chemokine released in large quantities by stimulated platelets and thus likely to be a large component of the PES. We have recently demonstrated that SDF-1α also results in a significant increase in HPC7 adhesion to a VCAM-1 surface, which again may be a result of alteration in integrin distribution. Interestingly, however, SDF1α pre-treatment had no effect on CD49d cluster number/cell, but instead resulted in an increased cluster size (Kavanagh *et al.*, 2013). These seemingly juxtaposed results (in terms of the effects on integrin distribution) are likely due to other contributing factors altering integrin behaviour

in a highly complex and intricate pathway. Interestingly, PMPs were also able to enhance adhesion to VCAM-1 surface. In the absence of PMPs increasing CD49d expression or its clustering, it is likely to be either another platelet receptor that is capable of binding to VCAM-1, such as αVβ1 (Barry *et al.*, 2000), being transferred to the HPC7 surface, or it may be that a component of the microparticles (surface bound or internal content) is capable of altering integrin structure and hence its affinity state.

Another phenotypic change exhibited by HPC7 following pre-treatment with PES and PMP was the membrane ruffling and pseudopodia extension. Both of these occurrences have been demonstrated to be markers of cellular activation (Bretscher, 2008, Patel and Harrison, 2008, Hoon *et al.*, 2012). What is noticeable, however, is that the extension of the pseudopodia occurred even following thrombin stimulation, although not to the same extent as that observed with PES pre-treatment. This suggests HPC7 are capable of responding to thrombin, but no subsequent effect on adhesion molecule expression, clustering or stem cell adhesion was observed. Interestingly, the membrane ruffling only appeared to be exhibited on the PES and PMP pre-treated cells. It is unknown why this would occur, although as described in **Chapter 4**, ruffling has been linked with re-distribution of integrins and other cellular processes.

Overall, it has been demonstrated that HSC recruitment to injured tissue can be enhanced by pre-treatment with PES – the platelet releasate. The recruitment appears to be predominantly enhanced at sites of greatest damage. This is the key aim of enhancing recruitment of infused stem cells in regenerative medicine in an effort to improve clinical efficacy. However, it is yet to be determined whether enhanced cellular recruitment, by pre-treatment with PES, will also improve the therapeutic repair efficacy of stem cells. Furthermore, it will be interesting to determine whether pre-treatment will have other effects on repair efficiency, other than that of enhancing recruitment.

Chapter 6

GENERAL DISCUSSION

6.0 Summary of the Main Findings

HSC transplantation has been used routinely over the past 50 years for the treatment of various diseases such as cancers, numerous anaemias and various inborn errors of metabolism (Copelan, 2006). The safety and efficacy of this procedure is well documented in both autologous and allogenic transplantation (Gatti *et al.*, 1968, Copelan, 2006). When the first studies suggested BM-derived cells could reconstitute injured intestinal and non-intestinal tissue, the relatively routine nature of the technique fuelled much hope in the field of regenerative medicine. However, their limited homing means that in order for HSCs to be beneficial in the clinic for disorders of the colon, a better understanding of the molecular adhesive factors regulating their engraftment is essential in order to develop strategies to enhance recruitment. Therefore, this thesis elucidated the adhesion mechanisms involved in the recruitment of HSCs to injured colonic microvasculature. A summary of the major findings from the thesis have been presented in **Table 6.1**.

The body of work contained in this thesis firstly presented novel data that inflammatory injuries of the colon do indeed result in increased adhesion of exogenously administered HPC7 cells, a cell line considered a very good model of haematopoietic stem cells. The increased adhesion of stem cells following injury is similarly seen in other organs investigated by our group, including the liver (Kavanagh *et al.*, 2010), kidney (White *et al.*, 2013), ileum (Kavanagh *et al.*, 2012) and the cremaster muscle (Kavanagh *et al.*, 2013). It is likely, given the diversity of tissues examined, that this phenomenon is common to all tissues following injury. However, there are several key differences between homing to different tissue types that may allow for targeted cellular treatment, and furthermore, there are also differences within the same tissue that is dependent upon the type of injury. Novel data within this thesis demonstrate that colonic recruitment following an acute IR injury was dependent on the integrin subunit CD18, whereas, within the chronically inflamed colitis

	HPC7 Pre-treatment	Colon Endothelial Cells	Immobilised Ligands		Frozen Tissue Sections									*In vivo* experiments					
					Jejunum			Ileum			Colon			IR Injured			Colitis Injured		
			ICAM-1	VCAM-1	Sham	IR Injury	Colitis Injury	Sham	IR Injury	Colitis Injury	Sham	IR Injury	Colitis Injury	Lungs	Ileum	Colon	Lungs	Ileum	Colon
	Naïve	0	0	0	0	+++	++	0	++	+	0	++	++	±	+++	++	±	±	+
Jejunum	SCM	±	±	±	+++	+	±	0	0	0	0	0	0	0	0	0	0	0	0
Jejunum	IR ICM	++	++	+++	+++	+++	0	0	0	0	0	0	0	0	0	0	0	0	0
Jejunum	Col ICM	±	±	+	+++	0	+	0	0	0	0	0	0	0	0	0	0	0	0
Ileum	SCM	±	±	+	0	0	0	±	±	±	0	0	0	0	0	0	0	0	0
Ileum	IR ICM	+	+++	+	0	0	0	±	+++	0	0	0	0	0	0	0	0	0	0
Ileum	Col ICM	±	+	++	0	0	0	++	0	+	0	0	0	0	0	0	0	0	0
Colon	SCM	±	+	+	0	0	0	0	0	0	+	+	+	0	0	0	0	0	0
Colon	IR ICM	+	+	+	0	0	0	0	0	0	+	+	0	0	0	0	0	0	0
Colon	Col ICM	+	+	+	0	0	0	0	0	0	+	0	+++	0	0	0	0	0	0
	H_2O_2	++	++	+++	+	++	±	++	+	++	+++	++	+	±	+	±	±	±	+
	PES	+++	++	+++	0	0	0	0	0	0	0	0	0	±	+	±	-	±	+
	PMP	±	±	++	0	0	0	0	0	0	0	0	0	±	±	±	±	±	±

Table 6.1 Summary results table displaying HPC7 adhesion changes following pre-treatment strategies

The results table summarises significant increases and decreases in the number of HPC7 adherent to either: colon endothelial cells, immobilised ligands, frozen tissue sections or *in vivo* investigations. Data displayed are significant differences against relevant controls and obtained from results chapters. 0 – Not investigated, +++ - $p<0.001$ increase in HPC7 adhesion, ++ - $p<0.01$ increase in HPC7 adhesion, + - $p<0.05$ increase in HPC7 adhesion, ± - No significant difference in HPC7 adhesion, **-** - $p<0.05$ decrease in HPC7 adhesion.

colon, both CD18 and CD49d were required for HPC7 adhesion. Interestingly, within IR injured small intestine, adhesion was also solely dependent on CD18, whereas in IR injured kidney additional and multiple adhesion molecules, including CD49d and CD44, are involved (White *et al.*, 2013). Overall, these studies demonstrate that the kinetics of HSC recruitment follows a similar adhesion cascade to that well described for inflammatory neutrophils. This has very important implications from a clinical perspective because one of the major areas of debate clinically for cellular therapy is on the best and most effective route for delivery. This current study (and others from our group) demonstrates that these cells can be delivered systemically, they are capable of circulating in the bloodstream and they can home to and be captured by inflamed colonic vasculature.

Interestingly, increased adhesion within injured colon was not due to a reactive hyperaemia, an increased blood perfusion, often associated with tissue injury. In fact, perfusion rates within the injured colon mucosal microcirculation were actually reduced compared to healthy sham controls. It is not clear whether adhesion would have been better if perfusion was not altered. In similar experimental models of DSS-induced colitis in mice, a modest decrease in blood flow within the resistance arterioles of the colon submucosa has been demonstrated at days 4 and 6 post-DSS (Mori *et al.*, 2005b). However, the current study is the first to demonstrate that the mucosal lining of injured colon also undergoes a significant reduction in blood flow. Preservation of the lining of the gut is essential. Therefore, it is reassuring to observe that circulating stem cells can still be recruited to the mucosa despite the poor perfusion in this region. This has important clinical implications as many inflamed organs suffer from poor blood flow. Indeed, in human subjects with ulcerative colitis, a reduction in flow is often detected in the chronically inflamed and remodelled colon (Hulten *et al.*, 1977). It is possible that this reduced blood flow in injured sites is more conducive to adhesive interactions of stem and endothelial cells taking place, whereas it may limit the local bioavailability of systemically administered drugs. To achieve successful colonic delivery, oral drugs need to be

designed that are protected from absorption in the upper GIT and are released into the proximal colon. Therefore, systemically delivered stem cells may have advantages over pharmacological drugs for treatment of diseases of the colon such as Crohn's disease and ulcerative colitis.

Regardless of the molecular mechanisms utilised for stem cell adhesion within injured tissue, it is commonly reported that the clinical efficacy and success of cellular therapies is either minor or transitory. This may be due to the limited number of stem cells recruited, when delivered by the preferred systemic route, as a result of poor homing and a subsequent low efficiency of tissue engraftment, processes essential for stem cells to mediate repair. Developing methodologies that improve trafficking and recruitment into injured tissues is a high priority for cellular therapies and could potentially confer more efficient, rapid and longer lasting tissue recovery. Indeed, transfecting plasmid DNA encoding for SDF-1α into ischaemic muscle enhances endothelial progenitor cell recruitment (Hiasa *et al.*, 2004). However, delivery of vectors to allow for targeted chemokine expression has been challenging, particularly in the clinical setting. Thus, non-invasive (and relatively easier and cheaper) techniques that do not require introduction of genetic material are appealing. The gained understanding of the mechanisms governing SC homing to injured colonic microvessels may have significant clinical consequences as it may allow us to optimise their effective colonic delivery and improve their therapeutic efficacy**.**

Hence, the next stage of the research was to identify strategies that enhanced stem cell colonic recruitment *in vivo*. With the observation that trafficking through injured tissue resulted in stem cell adhesion, it was hypothesised that exposing stem cells to soluble factors, commonly identified within the inflammatory microenvironment, would prime the cells and increase their ability to interact with inflamed endothelium. Initially, we demonstrated that pre-treating cells with media that had been conditioned by incubation with injured tissue resulted in increased adhesion *in vitro*. Increased

adhesion was observed on endothelial cells, injured tissue sections and immobilised endothelial counter-ligand coated surfaces. These proof of concept experiments clearly demonstrated HSC adhesion to injured colon could indeed be modified. However, this strategy does not have the potential to be applied clinically, and so more translational approaches were identified.

One factor identified within ICM was the reactive oxygen species H_2O_2. Within injured gut, particularly the small intestine, a significantly higher concentration of H_2O_2 was present compared to healthy tissue. With earlier studies demonstrating that H_2O_2 increased leukocyte rolling and chemotaxis (Fraticelli *et al.*, 1996), it was investigated whether pre-treatment of HPC7 with H_2O_2 also influenced their adhesion. Pre-treatment of cells with 100µM H_2O_2, a concentration reported to be similar to that seen *in vivo* (Johnston *et al.*, 1996), resulted in a significant increase in HPC7 adhesion *in vitro*. Furthermore, through the use of antibody blockade of CD18 and CD49d, it appeared that the increased adhesion was an active process and integrin mediated, when investigated *in vitro*. Integrin clustering may provide a mechanism by which stem cell adhesion could be improved.

H_2O_2 also increased adhesion *in* vivo but only under certain circumstances, with the increased adhesion *in vivo* occurring only within colitic colon, not IR colon. This surprising result can be explained due to greatly increased adhesion within the IR ileum, likely resulting in a reduced pool of circulating cells available to be recruited within the IR colon.

These studies present the first evidence that colonic stem cell homing can be modified using a relatively cheap, effective and easy protocol. These studies also highlight how chemically manipulating stem cell surface integrins can modify their adhesion. One very important therapeutic implication of improving or targeting delivery to a particular site is that non-specific retention in healthy tissues is avoided. Indeed, we have previously demonstrated that improving HSC

recruitment in the small intestine reduces non-specific entrapment in the pulmonary tissue (Kavanagh *et al.*, 2012).

Overt morphological changes were also observed following H_2O_2 pre-treatment, namely an increase in membrane ruffling and cellular protrusions that resembled filopodia. Although we can only speculate that these morphological changes are not detrimental to the cells ability to function therapeutically, this clearly would need further investigation. However, similar morphological occurrences have been described when LPS activated macrophages were examined (Patel and Harrison, 2008) and it was suggested that the ruffling allowed for directed cellular motility (Stupack *et al.*, 2000, Bretscher, 2008), phagocytosis (Patel and Harrison, 2008), adhesion (Arjonen *et al.*, 2011) and receptor re-distribution (Hoon *et al.*, 2012) and so could be considered a marker of cellular activation. Furthermore, it should be noted that there are several classes of membrane ruffling that are described to play many diverging roles. Hence, further investigation into the nature and biochemical make-up of these ruffles and extensions may reveal deeper insight into the cellular effects following H_2O_2 pre-treatment.

The advantages of pre-treating stem cells with H_2O_2 may not just be limited to their ability to improve adhesion. Li and colleagues demonstrated that the ability of MSCs to survive in an ischaemic environment could be improved by pre-exposure to H_2O_2. They demonstrated that preconditioning MSCs with 20μM H_2O_2 significantly protected them against apoptosis induced by higher concentrations of H_2O_2 (Li *et al.*, 2009b). More recently, Pendergrass *et al.*, demonstrated that left ventricular cardiac function, when assessed 28 days after IR injury, was greatly improved when cardiac progenitor cells pre-treated for 2 days with 100μM H_2O_2 were used as opposed to naïve ones (Pendergrass *et al.*, 2013). Furthermore, cardiac fibrosis was significantly decreased and endothelial cell density significantly increased when using pre-conditioned cells. Although our study focussed on

improving stem cell retention, we may not have fully discovered any additional therapeutic effects this pre-treatment strategy may have. Recovery studies assessing tissue and vasculoprotective effects of naïve versus H_2O_2 pre-treated HSCs are therefore essential future experiments.

Another component of the injury milieu are PMPs, released as a result of platelet activation and microthrombus formation. It has been prior demonstrated that PMP can interact with cells and alter behaviour to enhance activity and homing (Barry *et al.*, 1998, Forlow *et al.*, 2000, Janowska-Wieczorek *et al.*, 2001, Mause *et al.*, 2005). Thus, their ability to improve stem cell retention was investigated as this was considered an alternative and more biological approach, when compared to the H_2O_2 chemical approach, which may have resulted in further benefits due to the complexity of the PMP contents and their make up. Both purified PMPs and PMPs contained within the activated platelet releasate (PES) allowed for reproducible coating of HPC7s, as demonstrated by transfer of platelet glycoproteins to the HPC7 surface.

It is not clear whether the transfer of platelet adhesion molecules such as GPIb, an integrin that allows platelets to tether to immobilised von Willebrand factor, aids in stem cell recruitment. This would need to be determined using an anti-GPIb antibody. If transfer of GPIb is useful, improving the protocol to generate a greater number of PMPs or improving the coating of stem cells with PMPs may be a worthwhile pursuit.

At present the likely mechanism of enhanced adhesion is via the redistribution of existing integrins on the stem surface as changes in integrin cluster size or number were observed confocally. This parallels what was demonstrated with H_2O_2. Similarly, the increased membrane ruffling and cellular protrusions were observed following pre-treatment, as was observed following H_2O_2 pre-treatment.

Interestingly, only the PES pre-treatment significantly increased adhesion *in vivo*, and that the increased adhesion was only observed in the colitis injured, not IR injured, colon. The lack of an effect following PMP pre-treatment was a somewhat unexpected result as both PES and PMP pre-treatment did result in increased adhesion to some surfaces investigated with *in vitro* assays. This suggests activated platelets release important factors that significantly modulate the adhesion of stem cells, but in a potentially site-specific manner.

It has previously been demonstrated that platelets, often found adherent in abundance in ischaemic and inflammatory environments, may guide the homing of stem cells to sites of injury (de Boer *et al.*, 2006). Further characterization of the chemical and cellular factors in platelet related homing, platelet releasate effects on target cells and the processes of PMP generation and their interactions may contribute to the development of innovative strategies to augment stem cell mediated tissue repair for colon pathophysiological conditions.

6.1 The two-component enhanced recruitment model

This project has shown that injury promotes HPC7 adhesion and that pre-treatment with known inflammatory mediators enhances adhesion. Interestingly, it was also found that pre-treated HPC7 displayed enhanced adhesion to sham or healthy tissue. It is from these observations that a potential model with regards to stem cell adhesion can be postulated. This model suggests stem cell recruitment, and the enhancement of this event, requires at least two components.

The first component is tissue injury, with the concomitant increase in expression of endothelial CAMs and generation of the inflammatory releasate (**Figure 6.1 B**). The second component is cellular activation, achieved pathophysiologically by exposure to environment (such as the inflammatory

releasome; **Figure 6.1 B**), but this can also be achieved by pre-treatment in the absence (**Figure 6.1 C**) or presence of tissue injury (**Figure 6.1 D**). It appears that each component can act independently of each other, but when the actions are combined, the greatest level of adhesion is observed (**Figure 6.1 D**). Hence, maximal adhesion requires activation of the stem cells, but also activation of the tissue. Evidence supporting all of these stages has been presented in this thesis. For example, the *in vitro* SW assay performed with H_2O_2 pre-treatment demonstrates this very well (See **Figure 4.4** and **Table 6.1**). When pre-treated cells were incubated on the sham tissue there was a significant increase in adhesion, but when pre-treated cells were incubated on IR injured tissue there was a greater increase.

Further investigation and understanding of this mechanism may allow for improved pre-treatment strategies to be developed that may be used for future therapeutic purposes. Our observations that certain pre-treatments are effective only in certain organs or in diseases are novel as it highlights how a single universal approach may be difficult to identify. Careful consideration will need to be given to identifying which pre-treatment generates the correct cellular response for tissue type or injury.

6.2 Future directions

Although these data have demonstrated that phenotypic alterations of the cell modify their adhesion, it is yet to be fully investigated whether enhancing the local presence of stem cells improves tissue repair and regeneration above and beyond that observed when they are recruited by injury alone. Furthermore, it needs to be determined whether the identified pre-treatment strategies have therapeutic benefits independent to their effects on adhesion. Recent work undertaken by the Kalia group has demonstrated that although HPC7s home to IR injured small

intestine, they were only able to significantly reduce the presence of inflammatory neutrophils (within 4 hours post-injury) if their local presence was enhanced using SDF-1α (Kavanagh *et al.*, 2013).It is yet to be determined whether the dampened neutrophil recruitment was a result of increased stem cell presence, or whether the pre-treatment exerted some, as yet unidentified, effect on the HPC7s to improve/introduce immunomodulatory effects. The fact that SDF-1α can modify stem cell adhesion is interesting in light of our PES/PMP data as this chemokine can be released by platelets.

Identification of additional chemical and biological factors within the ICM pre-treatment would be interesting as they may be more potent modulators of stem cell behaviour than H_2O_2/PMPs. Luminex analysis failed to identify any likely factors in the form of cytokines and chemokines, and furthermore, MALDI-TOF spectroscopic analysis of CM performed (data not presented within this thesis) reinforced this absence of chemo/cytokines but did detect complement and leukotrienes within these ICMs. Although, these many other potent cellular activators, such as leukotrienes and complement, may have an effect on the HPC7, they were not investigated. Indeed, such factors may be unique to tissue or injury type and may offer the potential to further refine the targeting process. Although this concept is seemingly far-fetched, the data from the H_2O_2 experiments reveal that the tissues that contained the greatest concentration of H_2O_2, namely the IR injured ileum and jejunum, also had more HPC7 adhering within them following H_2O_2 pre-treatment.

It has been demonstrated by several groups that altering the redox balance within the cytosol can have many different effects from protein kinase activation (Staal *et al.*, 1994), phosphatase inhibition (Heffetz *et al.*, 1990, Reyes *et al.*, 2005), transcription factor localisation (Los *et al.*, 1995), enhanced affinity of integrins (Liu *et al.*, 2008, de Rezende *et al.*, 2012) and integrin re-distribution, as demonstrated here. But, with regards to stem cell behaviour and regenerative potential, the multit-

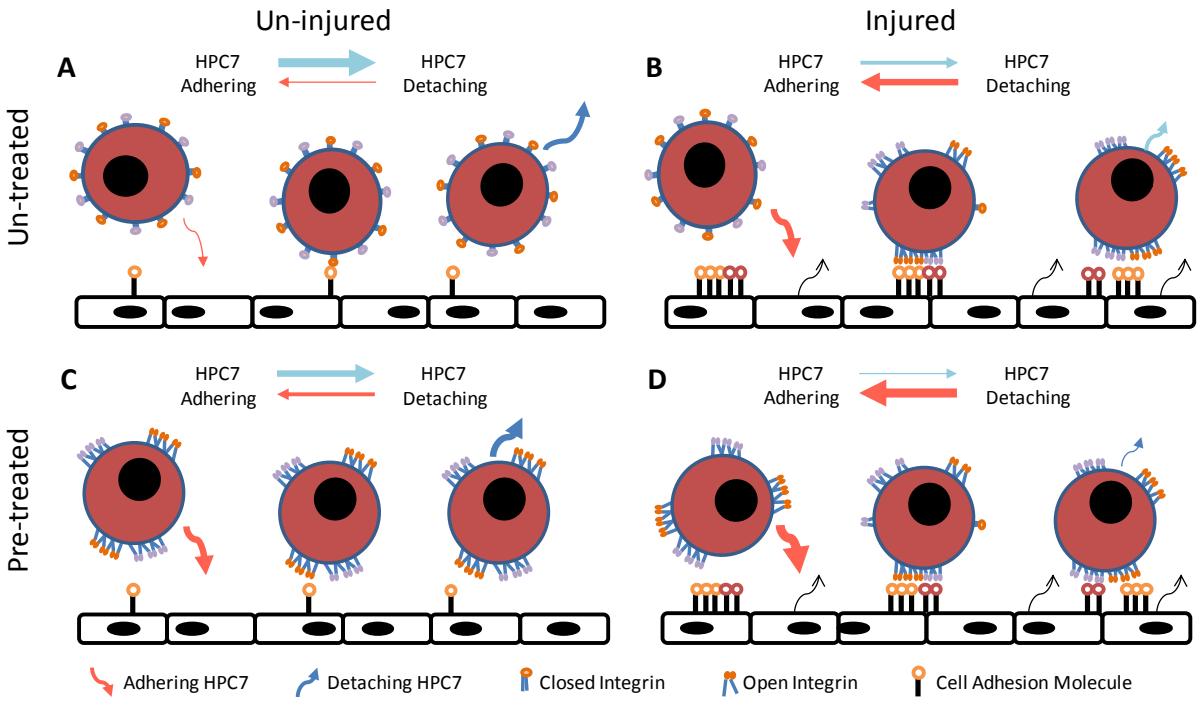

Figure 6.1 The two-component enhanced recruitment model

(A) HPC7 flowing through uninjured microvasculature will only adhere in very low numbers, the few that do adhere are likely to become detached over time. This is probably due to 'un-opened' integrins (those in an inactivated state) and low copy number of cell adhesion molecules (CAMs). However, when HPC7 are flowing through injured microvasculature (B) they are exposed to the inflammatory factors released by endothelium (black arrows). This in turn activates the HPC7 causing integrins to cluster and probably achieve an 'open' or activated conformation. This will result in greater HPC7 numbers adhering to the activated endothelium (now exhibiting increased CAM on the surface) with fewer detaching. (C) When HPC7 are pre-treated then exposed to uninjured tissues, their integrins are clustered and activated and so can interact more readily with the limited numbers of CAMs on the vascular surface. This results in a marginal increase in HPC7 adhesion, with a reduced rate of detachment. However, pre-treated HPC7 flowing through an injured environment (D) are primed (that is their integrins are clustered and open) to bind to the increased number of CAMs on the injured surface resulting in an even greater adhesion than seen in B, with a reduced rate of detachment.

- udes of effects of H_2O_2 pre-treatment are yet to be investigated, but hold considerable promise.

Another intriguing question is how the PMPs physically interact with the stem cell surface. Although several studies have investigated this for other cell types, there remains no overwhelming consensus on the surface receptors used (although it is highly likely that differing cell types, and indeed MP types, will utilise differing markers) or the mechanisms involved in PMP loading. Suggestions that have been made include that they are endocytosed or phagocytosed with their receptors recycled (Faille *et al.*, 2012), whilst others have proposed membrane assimilation for PMP loading and incorporation, however, the mechanisms for this remain unclear and somewhat unsubstantiated (Mause *et al.*, 2010). Increased studies would be highly informative considering the increasing role of many types of microparticles, including those from platelets, in the aetiology of many diseases (Boulanger *et al.*, 2006, Burnier *et al.*, 2009), as well as them potentially being powerful therapeutic agents in their own right (van Dommelen *et al.*, 2012).

A further consideration to be made regarding PMPs is the nature of the generated population. It has been demonstrated that depending on the source of stimulation platelet microparticles display differing phenotypes (Perez-Pujol *et al.*, 2007), but this needs to be fully explored to determine the extent of variation between stimuli. Furthermore, it needs to be determined whether the PMP populations are heterogeneous in receptor repertoire as well as size, even when derived from a single source. More importantly, with regards to the application made here, does one sub-population alter SC behaviour in a more beneficial manner than another – that is, does a certain phenotype of microparticle further improve engraftment, does another improve the regenerative capacity of microparticles? These are fairly sizeable holes in MP research that need to be investigated to allow their use as research tools as well as therapeutically.

6.3 Implications and closing remarks

Overall, this thesis has described several methods of enhancing SC recruitment within both IR and colitic injured intestinal tissue. This may allow for improved stem cell therapeutics by increasing SC presence in the most damaged and affected areas, thus improving the reported poor efficacy of current SC treatments. This requirement for improved efficacy is emphasised further when the poor prognosis for patients with intestinal IR injury and the loss of effective treatments for those with IBDs over time is taken into consideration.

Increasing our knowledge of stem cell homing may have benefits beyond those associated with their regenerative potential. The fact that stem cells home specifically to certain sites may provide an important clinical application – namely targeted drug delivery. There is current excitement that MSCs, may be an attractive cellular vehicle for anti-cancer drugs (Loebinger *et al.*, 2009, Ciavarella *et al.*, 2011), but HSC have also been considered potential tools for targeted drug and gene delivery (Coleman and Steptoe, 2012, Kiem *et al.*, 2012, Freistadt *et al.*, 2013). Furthermore, it is possible that these pre-treatment strategies may also be employed within the bone marrow transplantation field. It has already been demonstrated that crude PMP pre-treatment strategies can enhance HPC recruitment to bone marrow (Janowska-Wieczorek *et al.*, 2001), thus refining the process is more likely to allow PMP pre-treatment to enter therapeutic protocols and increase effectiveness and efficacy of treatment.

It is hoped that these promising pre-treatments can be explored further with regards to both how they are working and, most importantly, whether they are increasing SC therapeutic efficacy. With the solution to these questions it can be hoped that they lead to further development of the field of regenerative medicine.

Chapter 7

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