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Yemm, Adrian; Adams, David; Kalia, Neena

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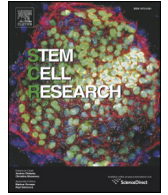
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Targeting the delivery of systemically administered haematopoietic stem/progenitor cells to the inflamed colon using hydrogen peroxide and platelet microparticle pre-treatment strategies



Adrian Yemm^a, David Adams^{a,b}, Neena Kalia^{a,*}

^a Centre for Cardiovascular Sciences, Institute of Biomedical Research, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

^b School of Immunity and Infection, Institute of Biomedical Research, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

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ABSTRACT

Haematopoietic stem and progenitor cell (HSC) therapy may be promising for the treatment of inflammatory bowel disorders (IBDs). However, clinical success remains poor, partly explained by limited HSC recruitment following systemic delivery. The mechanisms governing HSC adhesion within inflamed colon, and whether this event can be enhanced, are not known. An immortalised HSC-like line (HPC7) was pre-treated with hydrogen peroxide (H₂O₂), activated platelet releasate enriched supernatant (PES) or platelet microparticles (PMPs). Subsequent adhesion was monitored using adhesion assays or *in vivo* ischaemia–reperfusion (IR) and colitis injured mouse colon intravitaly. Integrin clustering was determined confocally and cell morphology using scanning electron microscopy. Both injuries resulted in increased HPC7 adhesion within colonic mucosal microcirculation. H₂O₂ and PES significantly enhanced adhesion *in vitro* and in the colitis, but not IR injured, colon. PMPs had no effect on adhesion. PES and PMPs induced clustering of integrins on the HPC7 surface, but did not alter their expression. Adhesion to the colon is modulated by injury but only in colitis injury can this recruitment be enhanced. The enhanced adhesion induced by PES is likely through integrin distribution changes on the HPC7 surface. Improving local HSC presence in injured colon may result in better therapeutic efficacy for treatment of IBD.

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1. Introduction

Inflammatory and ischaemic bowel injuries such as Crohn's and colitis are difficult to treat, with current treatments rapidly losing effectiveness. Hence there is a pressing need for new, effective options to be identified. Evidence suggests that haematopoietic stem cell (HSC) therapy may be an effective treatment option for inflammatory gastrointestinal disorders (Singh et al., 2010; Lanzoni et al., 2008; Garcia-Bosch et al., 2010). HSCs confer benefit in experimental models of colitis and in Crohn's disease patients. This was originally thought to be due to direct repair of tissue by HSCs transdifferentiating into non-haematologic cells of the gut, but is now considered to be primarily due to combatting the excessive immune responses triggered by these inflammatory injuries through paracrine immunomodulatory mechanisms (Oyama et al., 2005; Wei et al., 2009). Administration of adult CD34⁺ stem cells (SCs) improves clinical scores and significantly reduces mortality in dextran sodium sulphate (DSS)-induced murine colitis

(Khalil et al., 2007). Furthermore, coeliac patients have increased circulating CD34⁺ cells when compared with healthy controls (Mastrandrea et al., 2008). There have also been several promising trials utilising HSCs as a therapy for Crohn's disease, with initial reports suggesting some improvement although recurrence rates remained high (Burt et al., 2010; Hawkey 2012).

Despite ongoing trials, it is clear that clinical efficacy of cellular therapies is either minor or transitory. Poor success has been partially explained by a limited number of systemically transplanted SCs interacting with local microvessels (Karp and Teo, 2009; Kavanagh and Kalia, 2011). Considering the extension of the inflamed intestine, systemic infusion of cellular therapy offers a less invasive mode of delivery than multiple localised injections directly into the bowel and thus requires less HSCs (Karp and Teo, 2009). Furthermore, this preferred route of SC administration most closely mimics how endogenous bone marrow-derived HSCs may participate physiologically in tissue regeneration. Capture of exogenously administered circulating HSCs by injured tissue microvasculature is therefore a prerequisite event for successful therapy, regardless of the subsequent mechanisms underlying HSC-mediated repair. However, systemic injection is associated with low levels of SC retention in injured tissues. Indeed,

* Corresponding author.

E-mail address: n.kalia@bham.ac.uk (N. Kalia).

within infarcted heart, endothelial progenitor cell retention is <5%, with most cells not demonstrating sustained engraftment (Aicher et al., 2003). Furthermore, difficulties remain in obtaining sufficiently large quantities of high purity HSCs using existing culture conditions. Therefore, identification of methodologies that improve or enhance SC recruitment to injured tissue microcirculation is a current high priority for cellular therapies (Karp and Teo, 2009; Kavanagh and Kalia, 2011). Although attempts have been made to enhance stem/progenitor cell recruitment using genetically modified cells or by increasing the local concentration of potent SC chemoattractants within injured tissue, non-invasive techniques, not requiring genetic manipulation, is more appealing.

It is well established that a host of factors within the injured microenvironment are capable of activating circulating leukocytes and platelets and thus permitting their endothelial adhesion during inflammatory processes (Mori et al., 2005a; Vowinkel et al., 2007; Sprague and Khalil, 2009; Santen et al., 2010). A potent leukocyte pro-adhesive modulator found within many inflammatory environments, particularly the ischaemic and colitis gut, is the free radical hydrogen peroxide (H_2O_2), generated locally by endothelium and infiltrating neutrophils (Fracicelli et al., 1996; Damiani et al., 2007). Similar factors most likely activate trafficking SCs as they circulate through damaged tissue. We hypothesised that pre-treatment of HSCs with such factors would increase the likelihood of their adhesion. Indeed, we have previously demonstrated that H_2O_2 pre-treatment enhances adhesion of an immortalised HSC-like line (HPC7) within the ischaemia-reperfusion (IR) injured small intestine (SI) in vivo (Kavanagh et al., 2013a). This increase was mediated by clustering $\alpha 4$ and $\beta 2$ integrins on the HPC7 surface, increasing their affinity for endothelial counterligands such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and inducing HPC7 pro-migratory filopodia formation. Whether H_2O_2 pre-treated HSCs can adhere within the injured colon more efficiently than naïve HSCs is not known. An alternative strategy could involve HSC pre-treatment with the use of biological factors such as platelet derived microparticles (PMPs). On activation, platelets release these small (<1 μm) membranous vesicles that express many of the surface receptors found on platelets (Burnouf et al., 2014). PMPs are the biggest source of microparticles within humans and are known to be increased in various pathologies, including Crohn's disease (Chamouard et al., 2005). Initially, it was thought that PMPs were cellular debris without any distinct physiological or pathological function. However, it is increasingly evident that PMPs have many roles, including an ability to increase neutrophil adhesion by transfer of adhesion molecules to their surface (Jy et al., 1995). The use of PMPs as a pre-treatment option for HSCs is attractive as they have not only been shown to enhance HSC recruitment to bone marrow (Janowska-Wieczorek et al., 2001), but also increase cellular repair efficiency independently of affects on adhesion (Burnouf et al., 2014; Mause et al., 2010).

Determining whether systemic administration of transplanted HSCs can efficiently deliver cells to intestinal tissues, and whether this phenomenon can be enhanced, has received little attention, with no studies conducted in the injured colon in vivo. Therefore in this study, we used intravital microscopy, a methodology with single-cell sensitivity, to firstly detail the homing kinetics of an immortalised HSC-like line (HPC7) to the murine colon following two distinctly different injuries, namely an acute IR injury and a more chronic colitis injury. We further elucidated the molecular adhesive mechanisms governing HSC homing to injured colonic microvessels. Having already demonstrated an ability for H_2O_2 to improve HPC7 retention within IR injured SI, we tested this pre-treatment strategy within the two colon injury models. The efficacy of this chemical strategy was also compared with HPC7s pre-coated with PMPs. This is the first study to have tested HSC homing to two models of colonic injury and also utilised two distinctly different pre-treatment strategies in an attempt to enhance their local presence.

2. Materials and methods

2.1. Animals

Male C57Bl/6 mice (Harlan, UK) were used for procedures in accordance with the Animals (Scientific Procedures) Act of 1986 (Project Licence: 40/3336, 40/3226 or 30/2721). Anaesthetized animals (ketamine/xylazine administered intraperitoneally) underwent carotid artery cannulation to facilitate infusion of fluorescently labelled HPC7s and maintenance anaesthesia. The colon was exteriorised by gently moving the caecum out of the abdominal cavity and held in place with atraumatic hooks. A longitudinal cautery incision, made in the ascending colon along the anti-mesenteric border, was held open using atraumatic hooks. The exposed mucosa was cleaned gently with saline and a coverslip placed on it to improve image clarity when imaging microscopically. The mucosa was visualised using an upright fluorescent intravital microscope ($\times 10$ objective; BX61WI, Olympus). Images were captured and analysed using Slidebook V.5 (Intelligent Imaging Innovations, USA). Colonic blood flow was quantitated using the same preparation and monitored using a Moor FPLI laser speckle imager (Moor Instruments, UK).

2.2. Colon injury models and intravital microscopy

Intestinal IR injury was induced by occlusion of the superior mesenteric artery (SMA) with a non-traumatic clamp for 45 min and subsequent removal to initiate reperfusion. Control animals underwent sham surgery in which the SMA was isolated but not clamped. A single bolus dose of 2×10^6 CFSE labelled HPC7s was injected via the carotid artery cannula at 1 h reperfusion. Colitis injury was induced by allowing ad libitum access to 3% dextran sodium sulphate (DSS, MW36–50 kDa; MP Biomedicals, UK) in drinking water for 5 days. All colitis mice met a threshold injury score to ensure injury status as previously described (Cooper et al., 1993). For tracking HPC7s in the colitis colon, fluorescently labelled cells were infused via the carotid artery once the surgical preparation for intravital imaging was completed and the colon had been allowed to stabilise for 30 min. In both IR and colitis mice, a single pre-selected field of view was imaged intravital every 5 min for 1 h. Adherent HPC7s were defined as those that remained stationary for >30 s. Free flowing HPC7s were defined as those that passed through the field of view without slowing or becoming stationary. At no point were HPC7s seen to be rolling in the colonic microvasculature. For all experiments, the colon was excised at the end of intravital imaging and 5 additional fields of view were monitored ex vivo. This was to ensure the adhesive events in the pre-selected area for IR and colitis mice were representative of events taking place in the whole colon. The lungs were also analysed ex vivo to quantitate HPC7 presence in at least 5 random fields of view.

2.3. Platelet microparticle generation

Platelets were isolated from the descending aorta of donor C57BL/6 mice following CO_2 narcosis, into 100 μl acid citrate dextrose (120 mM sodium citrate, 110 mM glucose, 80 mM citric acid). Blood was diluted in Tyrode's-HEPES buffer (134 mM NaCl, 0.34 mM Na_2HPO_4 , 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, pH 7.3) and centrifuged ($200 \times g$, 5 min) to obtain a platelet rich plasma (PRP). The PRP was spiked with 10 $\mu g/ml$ of prostacyclin and centrifuged at $1000 \times g$ for 6 min, the platelet pellet washed and counted and finally resuspended in Stem Pro SFM 34 (Invitrogen, UK) at $3 \times 10^7/ml$. Platelets were subsequently activated with 2 U/ml of thrombin for 30 min and then centrifuged at $1000 \times g$ for 6 min. The pellet was discarded and the supernatant kept and classed as a platelet microparticle enriched supernatant (PES). This contained not only the platelet microparticles but also the soluble factors released by platelets following their activation. To generate purified platelet microparticles (PMP), the

PES was further microcentrifuged at $20,000 \times g$ for 20 min. The supernatant was then removed and the pellet resuspended in an equal volume of Stem Pro SFM 34. This PMP contained only the microparticles and not the releasate from the activated platelets. PES and PMP suspensions were used within 30 min of being generated.

2.4. Haematopoietic stem cells and pre-treatment protocols

Investigating the homing of individual HSCs at a cellular level using intravital microscopy has been hampered by the limited number of primary HSCs obtainable from individual mice (Kavanagh and Kalia, 2011). Therefore, an immortalised HSC line, HPC7, generated by transfecting embryonic SCs with the murine LIM-homeobox gene LH2 was used. HPC7s display many characteristics of primary HSCs including surface expression of common murine HSC markers ($c\text{-kit}^+$, CD34^- , Lin^-) (Pinto do et al., 1998) and similar behaviour at a molecular level (Pinto do et al., 2002). Importantly, these cells also reconstitute haematopoiesis when injected into lethally irradiated hosts (Pinto do et al., 2002). HPC7 cells also express adhesion molecules present on primary HSCs such as CD18 and CD49d, and we have used them extensively to model HSC recruitment to murine organs other than the gut, including the liver and kidney (Kavanagh et al., 2013a; White et al., 2013; Kavanagh et al., 2013b; Kavanagh et al., 2010). Cells were maintained at a density of 1×10^6 cells/ml in supplemented Stem Pro 34 serum free media (Life Technologies, UK), 100 ng/ml rmSCF (Life Technologies, UK), L-glutamine, penicillin and streptomycin.

We have previously demonstrated that pre-treatment of HPC7s with $100 \mu\text{M}$ H_2O_2 enhanced their adhesion to endothelial cells in vitro and IR injured SI in vivo (Kavanagh et al., 2013a). Therefore, the same concentration was utilised for the current experiments in the colon. HPC7s were incubated at 37°C with $100 \mu\text{M}$ H_2O_2 for 1 h at a density of 2×10^6 cells/ml. Some cells were incubated at 37°C with either PES, PMP or thrombin for 1 h at a density of 4×10^6 cells/ml. HPC7s were then washed by centrifugation and resuspended in un-supplemented Stem Pro at the required density immediately prior to use. To visualise HPC7s, they were fluorescently labelled with $5 \mu\text{M}$ (5/6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Life Technologies, UK) prior to in vitro and in vivo imaging. For intravital studies, 2×10^6 CFSE labelled cells were injected as a bolus dose via the carotid artery. To determine whether HPC7 surface integrin subunits CD18 ($\beta 2$) and CD49d ($\alpha 4$) mediated their recruitment in vivo, function blocking antibodies to these integrins were used. 2×10^6 HPC7s were incubated in $80 \mu\text{g/ml}$ of either LEAFTM (low endotoxin, azide free) rat anti-mouse CD18 (Game 46; BD Pharmingen, UK), rat anti-mouse CD49d (R1-2; Cambridge Bioscience, UK) or rat IgG (RTK2758; Cambridge Bioscience, UK) for 15 min, washed by centrifugation and used immediately following wash.

2.5. Flow cytometry

We have previously documented the effects of H_2O_2 on HPC7 surface integrin expression (Kavanagh et al., 2013a). In the current study, flow cytometry was also used to determine changes in integrin expression following PES and PMP incubation. HPC7s were resuspended in PBS containing 5% FBS and were then incubated with a primary fluorescent antibody at a concentration of 1:250 for 15 min. Antibodies used were fluorescein isothiocyanate (FITC) conjugated rat anti-mouse CD49d (R1-2, Cambridge Bioscience, UK), FITC rat anti-mouse CD18 (Game-46; Santa Cruz Biotechnology, USA) or FITC rat anti-mouse IgG (poly4060; Biolegend, UK). Cells were then washed in cold PBS and expression analysed using a BD FACSCalibur (Beckton Dickinson, USA) flow cytometer and analysed using Summit v.4.3 (Dako Cytomation). Flow cytometry was also used to determine whether PMPs coated the surface of HPC7s following incubation by measuring increases in the expression of platelet surface markers, namely GPIIb/CD41 (GPIIb

component of GPIIb/IIIa integrin) and GPIIb α /CD42 (GPIIb α component of GPIIb-V-IX complex) on the HPC7 surface. This was assessed using FITC conjugated rat anti-mouse GPIIb (MWReg30; BD Biosciences, UK), FITC conjugated rat anti-mouse GPIIb (Clone: Xia.B2, Emfret, Germany) or FITC rat anti-mouse IgG (poly4060; Biolegend, UK).

2.6. Confocal and electron microscopy

The physical interaction of PMPs with HPC7s was also further confirmed confocally in two different ways. Firstly, in order to visualise PMPs, a platelet rich plasma was incubated with DiOC₆ at a final concentration of $4 \mu\text{g/ml}$ and mixed gently in the dark for 30 min. DiOC₆ (3,3'-dihexyloxycarbocyanine iodide) is a cell permeant, green fluorescent dye used for staining the membrane of vesicles. Platelets were washed as described above and were then activated with thrombin and stained microparticles were isolated. Attachment of DiOC₆ labelled PMPs to HPC7s was visualised confocally. Interactions were also visualised confocally by labelling HPC7s with CellTracker Orange (CTO; Invitrogen, UK; red) and incubating with platelet microparticles labelled with an anti-GPIIb monoclonal antibody, counterstained with Alexa Fluor-488 antibody (green).

We have previously determined the effects of H_2O_2 on HPC7 surface integrin clustering (Kavanagh et al., 2013a). In the current study, confocal microscopy was also used to monitor integrin clustering following PES and PMP pre-treatment. CTO labelled HPC7s were incubated with PES, PMP or thrombin, formalin fixed and then incubated with either LEAF rat anti-mouse CD18 or LEAF rat anti-mouse CD49d for 60 min on ice. Cells were washed and incubated with Alexa488 goat anti-rat IgG (Invitrogen, UK) for 30 min on ice. Cells were washed thoroughly and allowed to settle onto poly-L-lysine coated coverslips overnight and then mounted using Hydromount (Fisher Scientific, UK) ready for confocal imaging (Leica TCS-SP2; $\times 63$ oil immersion lens). Off-line image analysis of clustering was conducted using ImageJ software (NIH, US).

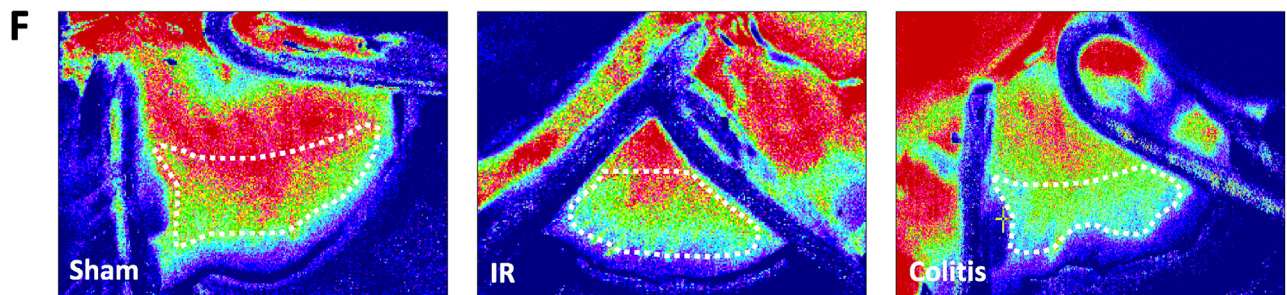
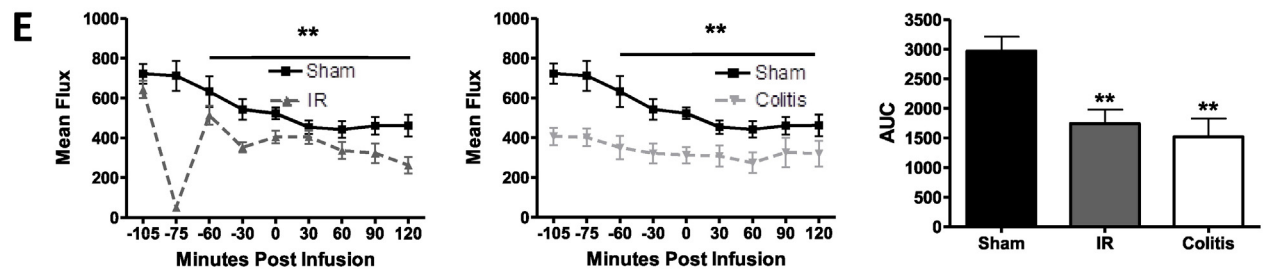
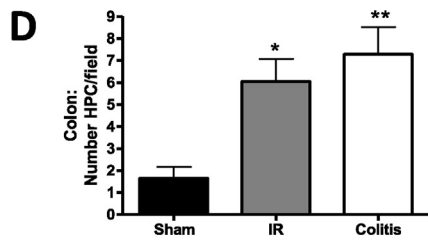
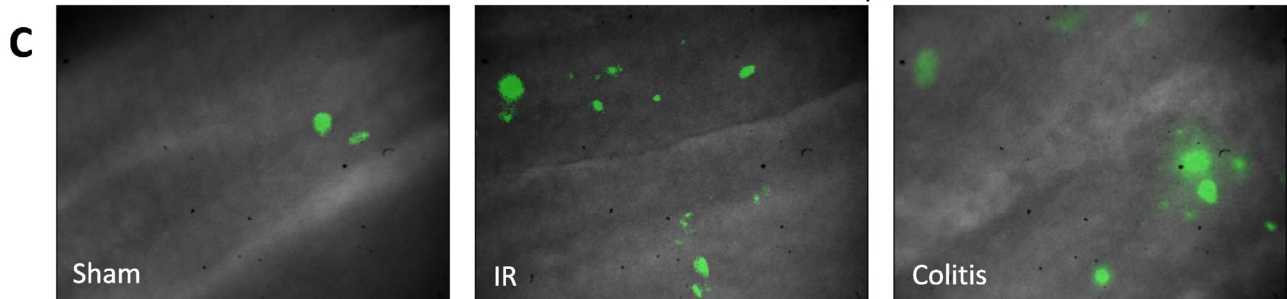
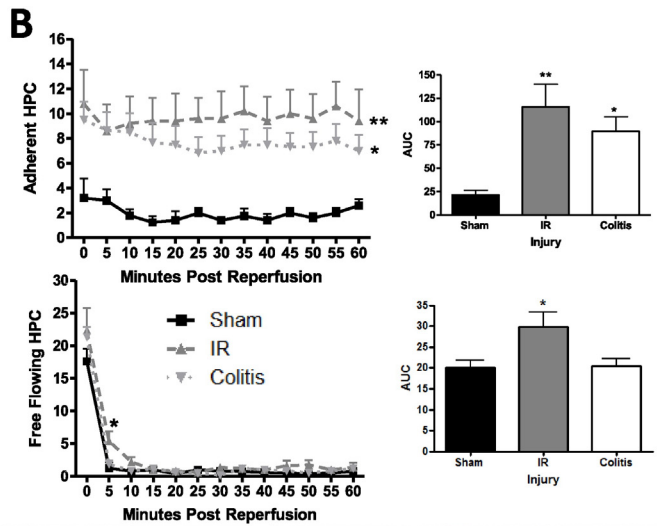
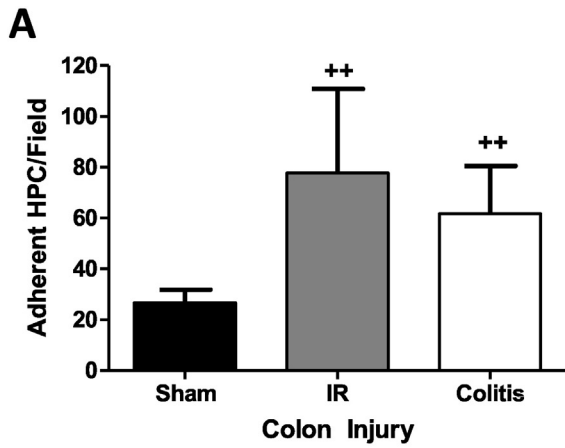
Scanning electron microscopy (SEM) was utilised to assess phenotypic changes in HPC7s following PES/PMP pre-treatment and was performed on 2% glutaraldehyde fixed coated HPC7s. Briefly, samples were settled onto poly-L-lysine coverslips overnight, dehydrated through a graded ethanol series and critical point dried and mounted onto SEM stubs. Samples were coated with evaporated carbon and examined using a Jeol 7000F SEM.

2.7. Static adhesion assays

Adhesion of HPC7s to endothelium, immobilised endothelial counterligands and frozen colon tissue sections were also assessed in vitro. Immortalised colon endothelial cells (CECs) were a kind gift from Prof. Steven Alexander (Louisiana State University, USA). CECs were maintained in culture on gelatin coated flasks in D-Valine Minimum Essential Media (Promocell, UK), 10% foetal bovine serum (FBS, Invitrogen, UK), $50 \mu\text{g/ml}$ streptomycin, 50 U/ml penicillin, 2 mM L-glutamine, $1/100$ non-essential amino acids (Sigma, UK), $1/100$ vitamin mix (Gibco, UK) and 10 U/ml mIFN γ (Peprotech, UK) at 33°C , 5% CO_2 . For experiments, CECs were cultured on the base of 24 well tissue culture dishes at 37°C , 5% CO_2 in Dulbecco's Modified Eagles Medium (4.5 g/l glucose; Invitrogen, UK), 10% FBS, 50 U/ml penicillin, $50 \mu\text{g/ml}$ streptomycin until confluency. 4 h prior to experiment wells were stimulated with 100 U/ml TNF α (Peprotech, UK), then washed thoroughly prior to addition of HPC7s. CECs were grown to confluency and HPC7s were added for 20 min at 37°C , washed with PBS and then fixed with 2% glutaraldehyde (Sigma, UK) before imaging. We have previously compared the adhesion of HPC7s and primary lineage negative (Lin^-) cells to endothelium with and without an array of different pre-treatment strategies (White et al., 2013). This particular subset of cells was selected as it provides a sufficient number of cells from three donor

mice to perform the endothelial adhesion assays in vitro. The data obtained showed that the HPC7 line responded to all the tested pre-treatment strategies in a similar way to primary cells.

To determine adhesion to immobilised counterligands, flat bottomed 96-well plates were coated with 500 ng of murine VCAM-1, ICAM-1 (R&D Systems, UK) or 1% bovine serum albumin in phosphate



buffered saline (PBSA) for 60 min at room temperature. Wells were washed twice and subsequently blocked with 1% PBSA for 60 min at room temperature. 10^5 treated HPCs in 50 μ l were incubated on the well surface for 20 min at 37 °C. Wells were washed twice and fixed in 2% glutaraldehyde for 15 min at 37 °C. Wells were then washed twice and 100 μ l PBS added prior to imaging using an inverted microscope (Olympus IX81). Frozen colon tissue sections were prepared by taking 20 mm lengths of excised ascending colon, opened to expose the mucosa and snap frozen in liquid nitrogen. Tissue sections were then cryostat sectioned to remove 20 μ m from the mucosal surface in order to expose the mucosal vasculature. Tissues were subsequently stored at –80 °C until ready for use.

2.8. Hydrogen peroxide in tissue

H₂O₂ content in colon tissues was assessed as previously described (Kavanagh et al., 2013a) using PeroXOquant Quantitative Peroxide Assay Kit (Thermo Scientific, UK) as per manufacturer's instructions. Similarly, 8-oxo-2'-deoxyguanosine staining was performed as previously described (Kavanagh et al., 2013a) on 10 μ m transverse cryosectioned colon tissue.

3. Statistics

All statistical analyses and graph plotting were performed using Prism GraphPad v4 (GraphPad Software Inc., USA). The area under the curve (AUC) values were calculated using the same software for each intravital and laser speckle experiment from the serial data plots and presented alongside the serial plots as bar charts. This is the most appropriate way of statistically interpreting serial measurements such as these. The AUC mean + SEM values were calculated and ANOVA with Dunnett's post-test used to test for significant differences. Some experiments were analysed using Student's t-test as appropriate. Details of statistical tests are contained within figure legends. Data is presented as mean + standard error or mean (SEM) in all cases.

4. Results

4.1. HPC7 adhesion to IR and colitis injured colon is increased despite poor blood flow

HPC7 adhesion to frozen sections of IR and colitis colon was significantly ($p < 0.01$) increased in vitro by ~2 fold when compared with healthy sham control tissue (Fig. 1A). Similar increases in adhesion were also observed in vivo in IR and colitis injured colonic mucosa when assessed intravitaly (Fig. 1B–C). Indeed, AUC analysis demonstrated significant increases in HPC7 adhesion in IR ($p < 0.01$) and colitis mice ($p < 0.05$) (Fig. 1B: right side). There was no difference in the number of free flowing cells observed trafficking through the colitis colon microcirculation and, after first pass, HPC7 were generally not observed again. However, there was a small but significant ($p < 0.05$) increase in free flowing cells within IR injured colon immediately after their injection as determined when analysing AUC data for free flowing cells (Fig. 1B). Since intravital microscopy determined adhesion within a single, randomly pre-selected region of the colonic mucosa, ex vivo analysis of multiple sites in excised tissues, removed at the end of in vivo imaging, was used to confirm the intravital data. Again, HPC7 adhesion was increased in IR and colitis colon (Fig. 1D). A very high

number of HPC7s were present within the lungs of all mice with no differences between groups (Fig. 1D). It was possible that the increased HPC7 adhesion observed in vivo occurred as a result of greater blood perfusion (hyperemic response to injury), leading to a greater delivery of cells to the injured colon. However, laser speckle microscopy demonstrated that in both IR and colitis injury, the mucosa had significantly lower blood flow compared with the sham tissue (Figs. 1E–F). Indeed, AUC analysis demonstrated a significantly lower blood flow in both IR ($p < 0.01$) and colitis ($p < 0.01$) injured colons (Fig. 1E: right side).

4.2. HPC7 integrins govern adhesion within injured colon but in an injury specific manner

Functionally blocking CD18 on HPC7s resulted in a significant ($p < 0.001$) reduction in their adhesion to IR injured colon (Fig. 2A), but was not observed when CD49d was blocked. There was no effect observed on the number of free flowing cells trafficking through the colon (data not presented). Ex vivo analysis of multiple additional sites in the colon confirmed these results but it also demonstrated that pulmonary retention was not integrin dependent (Fig. 2B: right side). Blocking either CD18 or CD49d significantly ($p < 0.05$) reduced HPC7 recruitment within colitis injured colon (Fig. 2C). Again, there was no effect on the number of free flowing cells observed (data not presented). It is noteworthy that when CD18 was blocked on HPC7s infused into IR injured mice, HPC7 adhesion was reduced to the baseline values seen in sham colons. However, blocking either HPC7 CD18 or CD49d did not lower adhesion to baseline values in the colitis colon, suggesting a possible redundancy in the adhesion molecules used in this injury. Again ex vivo analysis of tissues reflected what was observed in vivo and pulmonary recruitment was again not affected (Figs. 2D: right side).

4.3. Pre-treating HPC7s with H₂O₂ enhances adhesion but only in the colitis colon in vivo

H₂O₂ was significantly ($p < 0.05$) increased within homogenised IR injured colonic tissue. Although raised in the colitis colon, it did not attain statistical significance (Fig. 3A). Similarly, 8-oxo-dG labelling in colon tissue sections revealed that both IR and colitis injury resulted in greater amounts of oxidative damage within the colon when compared with sham tissue (Fig. 3B). As shown earlier in Fig. 1A, HPC7 adhesion to frozen sections of IR and colitis colon was significantly ($p < 0.01$) increased in vitro (Fig. 1A). However, this adhesion was enhanced on all colon tissue sections when HPC7s were pre-treated with H₂O₂ including sham ($p < 0.001$), IR ($p < 0.01$) and colitis tissue sections ($p < 0.05$) (Fig. 3C). Interestingly, H₂O₂ pre-treatment did not increase adhesion to the IR injured colon when imaging intravitaly in vivo (Fig. 3D). Ex vivo analysis supported these intravital observations but it also demonstrated that the number of HPC7s within the lungs was not altered either (Fig. 3E: right side). However, pre-treatment did significantly ($p < 0.05$) increase adhesion within the colitis colon in vivo (Fig. 3F), which was confirmed by AUC analysis of the data (Fig. 3F: right side). Again, ex vivo analysis supported these observations and showed no changes in pulmonary recruitment (Fig. 3G: right side). H₂O₂ pre-treatment did not affect the number of free flowing cells in either IR or colitis colon (data not presented). The inability of H₂O₂ to enhance HPC7 adhesion to IR injured colon was speculated to be due to the systemically delivered cells becoming recruited to the simultaneously injured small intestine, thus limiting the pool of circulating HPC7s

Fig. 1. Inflammatory injury to the colon results in increased recruitment of HSCs. (A) HSC adhesion is increased on IR and colitis injured frozen colon tissue compared with vehicle treated shams. (B) Intravital microscopy also demonstrated increased HSC adhesion within both IR and colitis injured colons (upper left) but no major changes in the number of free flowing HSCs (lower left). The right panels display the area under the curve (AUC) data for these intravital graphs. (C) Representative intravital images of the mucosa from sham, IR injured and colitis injured colons. (D) Ex vivo analysis of excised tissues confirmed increases in HSC adhesion to IR and colitis injured colon and also demonstrated high numbers of HSCs within the lungs. (E) IR and colitis injury resulted in reduced blood flow (measured as mean flux) to colon mucosa. Black line represents data used for statistical comparison. Right panel displays AUC data for region delineated by black line. (F) Images from in vivo laser speckle microscopy are shown in which red indicates areas of high blood flow and blue, areas of least blood flow. Data presented as mean \pm SEM. Statistical analysis performed with (A) 2 way ANOVA and (B–D) 1 way ANOVA with Bonferroni post-test comparisons. $N \geq 5$ –6 mice for each group; * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$.

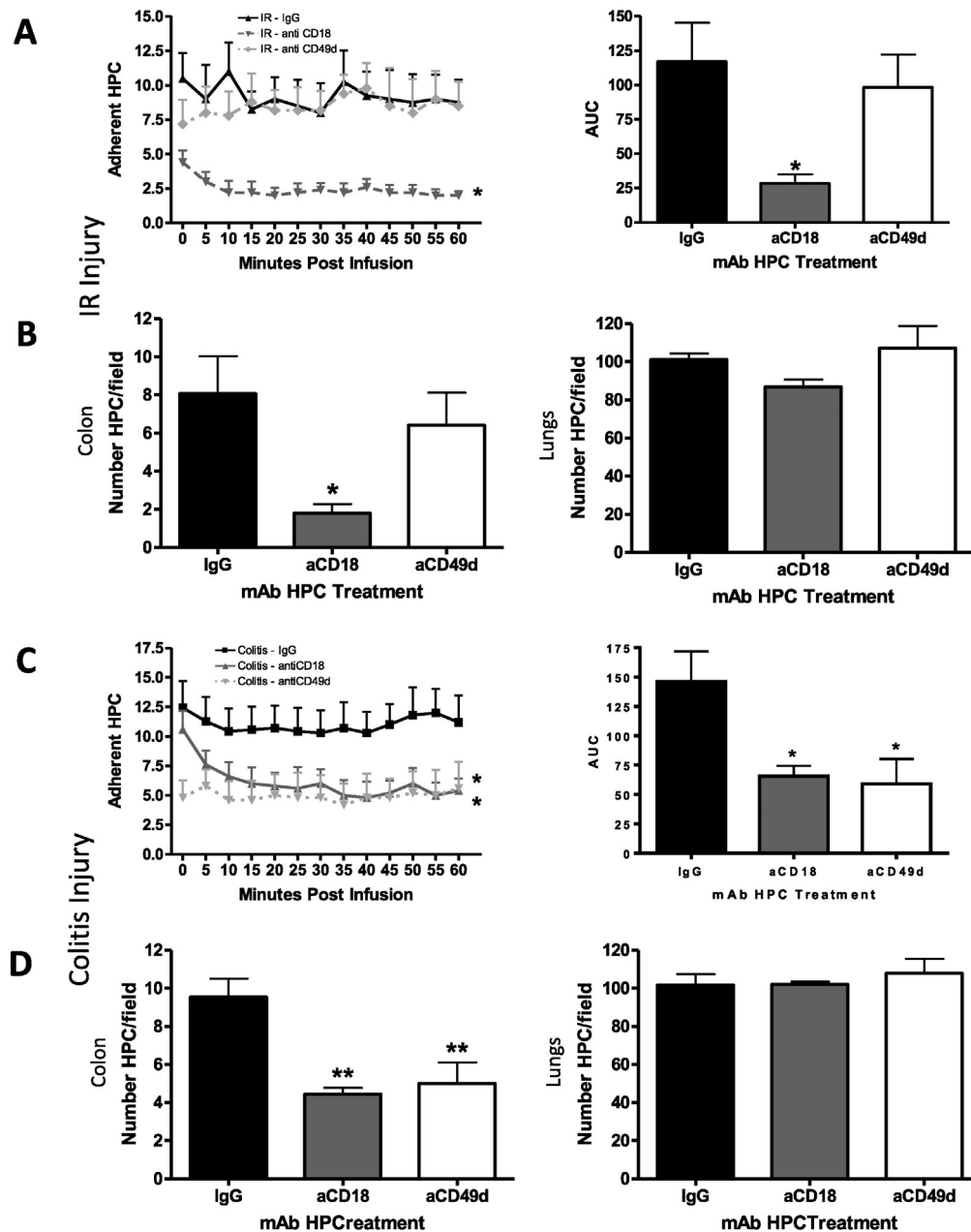


Fig. 2. HSC adhesion within injured colon is integrin mediated, but differs depending on injury. (A) Blocking HSC surface CD18, but not CD49d, reduced adhesion in IR injured colon. The right panel displays the AUC data for this intravital graph. (B) Ex vivo analysis of the colon and lungs from IR injured mice confirmed that recruitment is lowered in the colon with anti-CD18ab, but unaltered in the lungs. (C) Blocking either CD18 or CD49d reduced HSC recruitment in the colitis colon. The right panel displays the AUC data for this intravital graph. (D) Ex vivo analysis of the colon and lungs from colitis injured mice confirmed these results and that recruitment remained unaltered in the lungs. For all experiments $N \geq 5$ mice. Data presented as mean \pm SEM. Statistical analysis performed using 1 way ANOVA with Bonferroni post-test comparisons.

available for the colon. Indeed, ex vivo analysis revealed that H_2O_2 pre-treatment significantly ($p < 0.05$) increased HPC7 adhesion within IR injured small intestinal ileum (Fig. 3H). To further test this, we intravitaly imaged the small intestinal ileum where H_2O_2 pre-treated HPC7s were present in abundance (Fig. 3H: right side).

4.4. Pre-treating HPC7s with PES, but not PMPs, enhances adhesion within colitis colon in vivo

Expression levels of the platelet surface markers GPIIb and GPIIb were increased on HPC7s following incubation with either PES or PMP (Fig. 4A). Interestingly, GPIIb was found to be expressed on naïve HPC7s, but this was not surprising as it is found on early haematopoietic progenitor cells (Fig. 4A). DiOC₆, used to label PMPs, was observed on

HPC7s incubated with PES and PMP (Fig. 4B). HPC7s incubated with PES or PMP also expressed more platelet GPIIb when imaged confocally (Fig. 4B). HPC7s incubated with PES demonstrated a significantly ($p < 0.001$) increased adhesion on murine colonic endothelial cells when compared with vehicle controls (Fig. 4C). However, PMP pre-treatment failed to modulate adhesion in vitro (Fig. 4C). Intravitaly it was observed that PES, but not PMPs, marginally but again not significantly, increased adhesion within the IR injured colon (Fig. 4D), which was confirmed by AUC analysis of the data (Fig. 4D: right side). Ex vivo analysis of IR injured colon did not show any changes although, interestingly, PES pre-treatment did significantly ($p < 0.01$) increase recruitment to the lungs of intestinal IR injured mice (Fig. 4E). PES treatment did significantly ($p < 0.05$) increase adhesion within the colitis colon (Fig. 4F), which was confirmed by AUC analysis of the data (Fig. 4F:

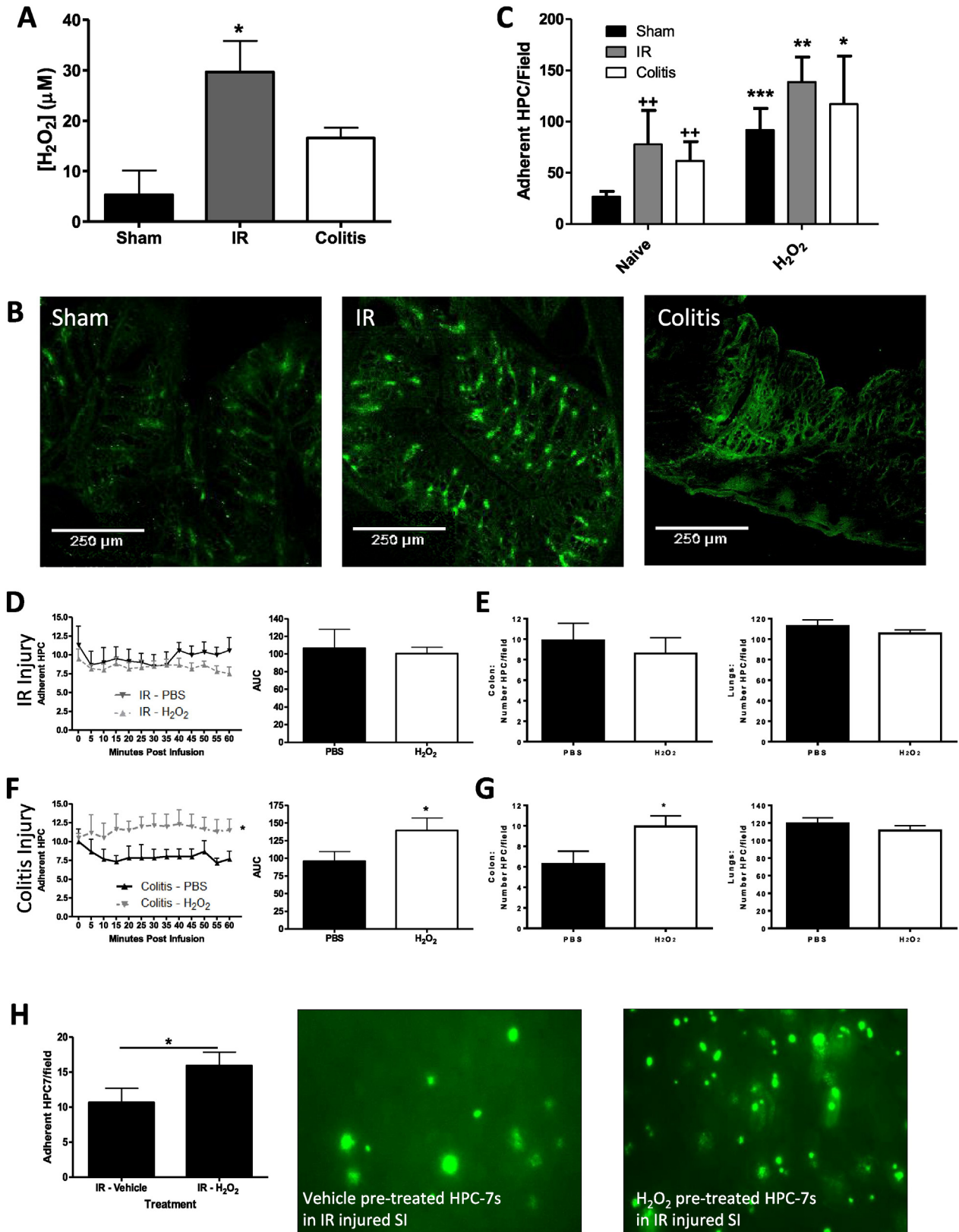


Fig. 3. Pre-treatment of HSCs with H₂O₂ increases adhesion in colitis colon. (A) H₂O₂ is significantly increased in IR and somewhat raised in colitis colon as determined by measuring H₂O₂ content in homogenised tissue. (B) 8-Oxo-dG was detected in both IR and colitis colon and reflects oxidative damage following inflammatory injury. (C) HSC adhesion was enhanced on all colon tissue sections when pre-treated with H₂O₂. (D) H₂O₂ did not increase HSC within IR injured colon. The right panel displays the AUC data for this intravital graph. (E) Ex vivo analysis also demonstrated no enhanced HSC adhesion to colon or pulmonary tissue. (F) H₂O₂ did increase HSC recruitment to colitis colon. The right panel displays the AUC data for this intravital graph (G) This increase was confirmed by ex vivo analysis but pulmonary recruitment again remained unchanged. Ex vivo analysis revealed that H₂O₂ pre-treatment significantly (*p* < 0.05) increased HPC7 adhesion within IR injured small intestinal ileum (H). Intravital images of the small intestinal ileum also confirmed that H₂O₂ pre-treated HPC7s were present in abundance in this region (H). For IVM experiments, N = 6 for all groups. Data presented as mean + SEM. Statistical analysis performed using 1 way ANOVA with Bonferroni post-test comparisons. **p* < 0.05.

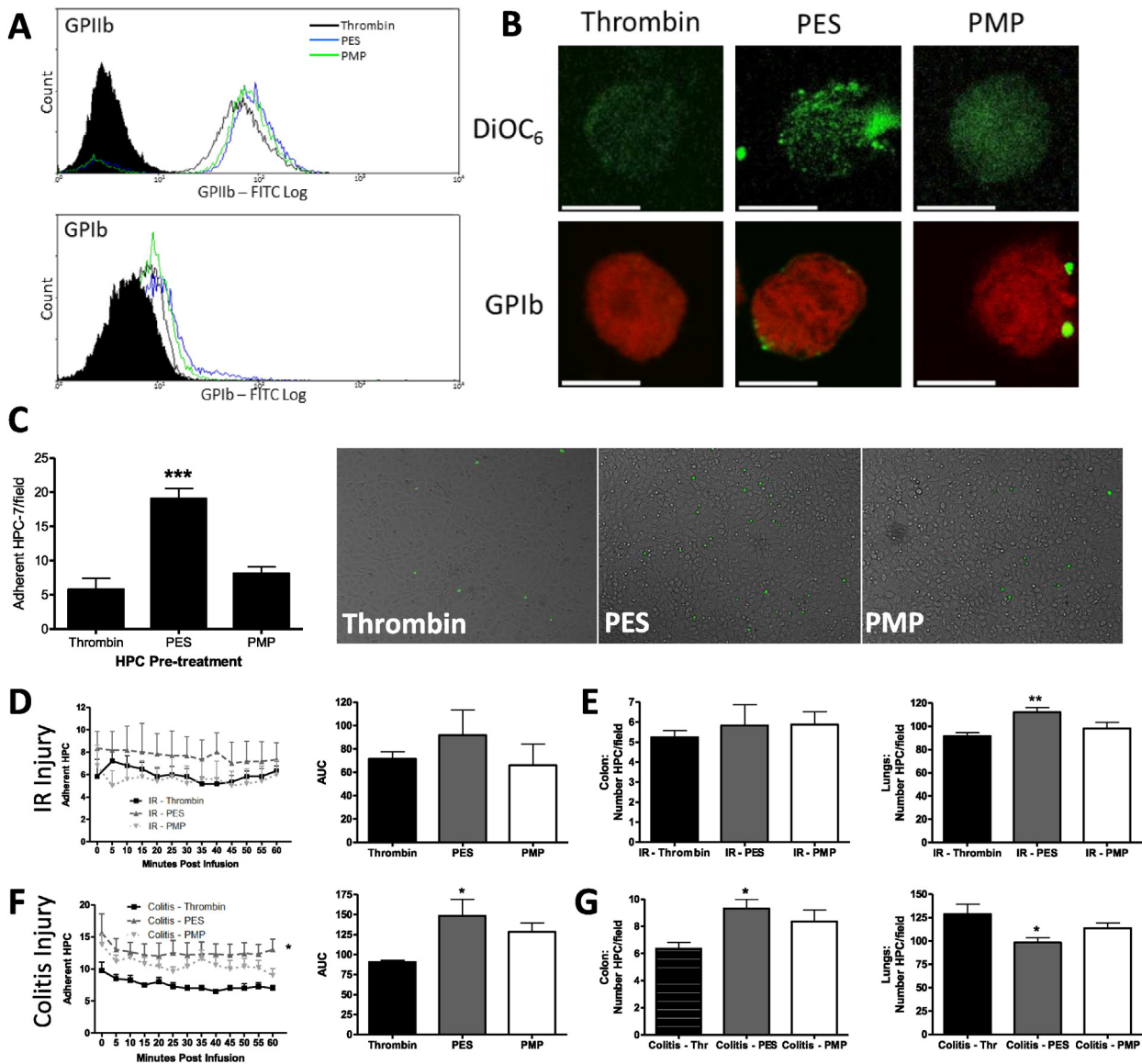


Fig. 4. Pre-treatment of HSCs with PES/PMPs increases adhesion in colitis colon. (A) HSCs incubated with PES (blue line) or PMPs (green line) are coated with microparticles as indicated by the increase in GPIIb (top) and GPIb (bottom) expression, compared with thrombin (black line). Isotype control is shown as black fill. (B) Upper panels are confocal images of green microparticles labelled with DiOC₆ attached to HSCs. In the lower panels CTO labelled HSCs (red) are interacting with microparticles labelled with an anti-GPIb monoclonal antibody and counterstained with Alexa Fluor-488 antibody (green). Scale bar = 5 μ m. (C) PES pre-treatment increased HSC adhesion to colon endothelial cells in vitro. Representative images are shown. (D) Neither PES or PMP pre-treatment enhanced HSC adhesion within IR injured colon. The right panel displays the AUC data for this intravital graph. (E) Ex vivo analysis demonstrated increased adhesion within the lungs following PES pre-treatment. (F) PES pre-treatment enhanced adhesion within colitis colon in vivo and lowered pulmonary (G) retention. For IVM $N \geq 5$ across all groups. Data presented as mean + SEM. Scale bars are 5 μ m. Statistical analysis performed using 1 way ANOVA with Bonferroni post-test comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

right side). PMPs also induced a marginal and sustained increase in the colitis injured colon, but the difference was not significant (Fig. 4F). Again, these observations were confirmed when the colon was investigated ex vivo and reduced ($p < 0.05$) retention was also observed in the lungs of colitic mice (Fig. 4G). There was no effect observed on the number of free flowing HPC7s within the IR or colitic injured colon (data not presented).

4.5. PES/PMPs modulate HPC7 adhesion by increasing surface integrin clustering and their affinity for endothelial counterligands

Electron micrographs demonstrated that PES and PMP incubation triggered an activated HPC7 phenotype in which they exhibited membrane ruffling and extension of pseudopodia (Fig. 5A). This HPC7 phenotype has also been demonstrated previously by us following H₂O₂ pre-treatment (Kavanagh et al., 2013a). PES pre-treatment

significantly increased adhesion to immobilise endothelial counterligands, ICAM-1 ($p < 0.01$) and VCAM-1 ($p < 0.001$; Fig. 5B). However, PMP pre-treatment only resulted in a significant ($p < 0.01$) increase to VCAM-1 coated surfaces (Fig. 5B). PES and PMP had no effect on CD18 or CD49d surface expression (Fig. 5C). The number of CD18 clusters increased with both PES and PMP ($p < 0.05$) (Fig. 5D), whereas PES only triggered an increase in CD49d clustering ($p < 0.05$; Fig. 5D). There were no significant differences in the size of the integrin clusters (Fig. 5D).

5. Discussion

The clinical success of haematopoietic stem/progenitor cell (HSPC) therapy for large bowel disorders is poor, partly due to their limited recruitment following systemic administration. However, cellular therapy may be made effective if systemically transplanted cells are targeted

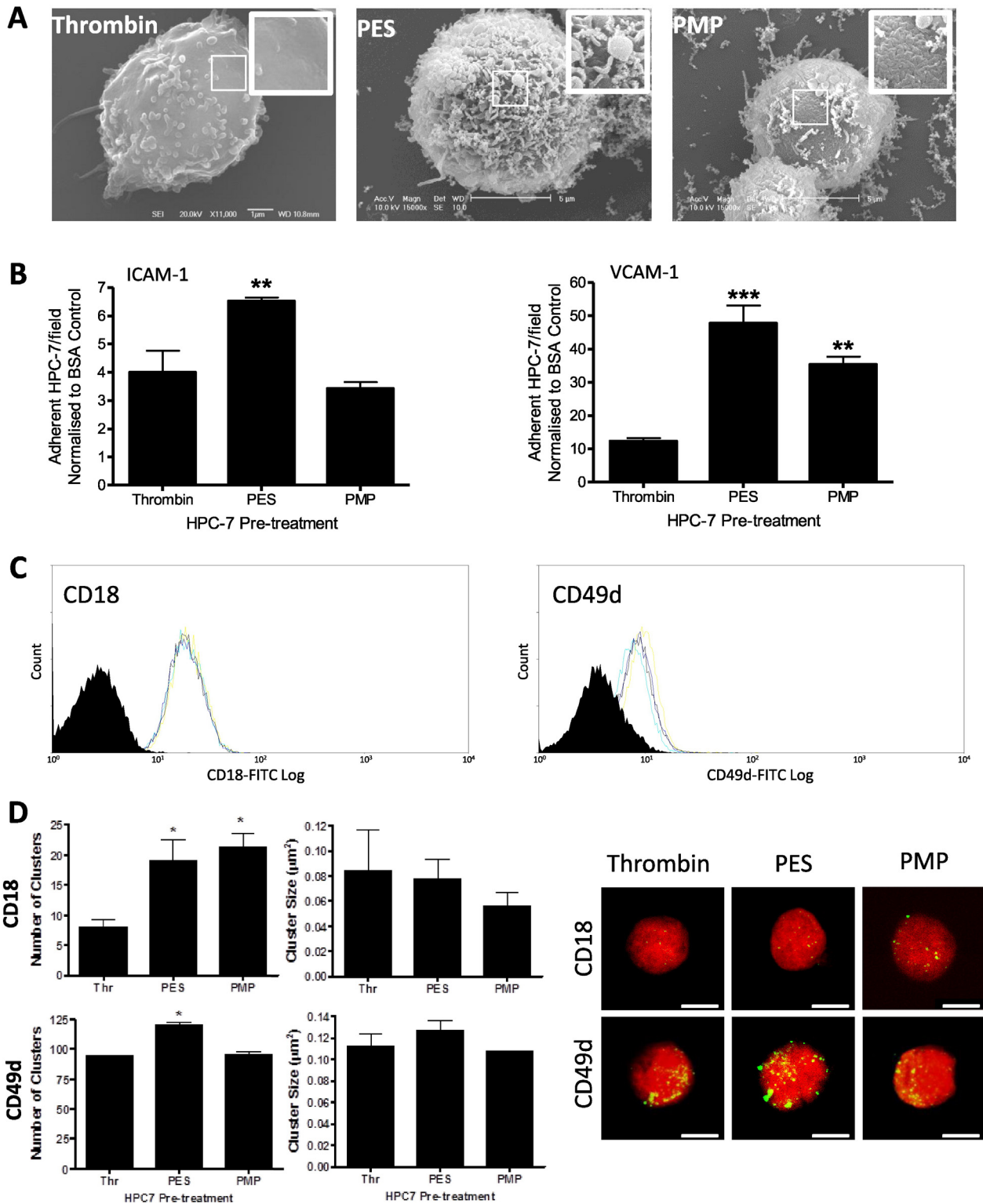


Fig. 5. PES/PMPs modulate HSC recruitment by increasing their integrin clustering and affinity for endothelial counterligands. (A) Thrombin treated HSCs demonstrated pseudopodia, but this was increased following PES and PMP pre-treatment. PES and PMP pre-treatments also increased membrane ruffling (insert). (B) PES pre-treatment significantly enhanced HSC adhesion to ICAM-1, but both increased adhesion to VCAM-1. (C) Pre-treatment of HSCs with thrombin (yellow), PES (light blue) or PMP (dark blue), failed to increase surface expression of CD18 or CD49d compared with vehicle (black line) controls. Isotype control represented as black fill. N = 3 (D) PES and PMP significantly increased CD18 cluster number but not size, whereas, PES increased only CD49d cluster number. Data presented as mean + SEM. Scale bars are 5 μm. Statistical analysis performed using 1 way ANOVA with Bonferroni post-test comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.

directly to the colon. This is highly desirable for local treatment of a variety of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease. The colon is highly susceptible in humans (and mice) to inflammatory bowel disorders, with approximately 60% of patients

with Crohn's disease suffering from colonic involvement (Mills and Stamos, 2007). Although the colon is a site affected by many inflammatory disorders, the dynamics of HSPC recruitment within the colonic microcirculation has not previously been imaged experimentally.

This study therefore determined whether pre-treating a purified immortalised HSC-like line with factors released during or at injury sites could enhance their adhesion within the murine colon. These pre-treatments were tested in two different inflammatory injuries. Our novel data provides the first evidence that immortalised HSC-like HPC-7 adhesion within the injured colon microvessels can occur almost immediately after their systemic infusion. Within the colon, we show that recruitment is not a passive event but is actively regulated by injury specific surface receptors, namely CD18 and/or CD49d. More importantly, recruitment achieved by injury alone was not maximal as pre-treatment strategies could enhance the local presence of HPC7s. Indeed, both chemical (H₂O₂) and biological (PES) pre-treatment strategies enhanced HPC7 adhesion in vitro, but only to the colitis colon in vivo. These approaches were utilised as both H₂O₂ generation and PMP formation are known to be elevated following intestinal IR and colitis injury (Fraticegli et al., 1996; Kavanagh et al., 2013a; Chamouard et al., 2005). However, while PMPs are elevated in both these injuries, only PES was effective in increasing HPC7 adhesion whereas PMPs were not. The development of strategies that enhance colon homing through modulation of HPC7 integrin affinity (or avidity/expression) may be a worthwhile clinical pursuit if associated with improved therapeutic benefit.

Increased HPC7 adhesion within IR and colitis injured colon was observed on both frozen tissue sections and in vivo when compared with un-injured colon. Increased adhesion observed in vivo was not due to a reactive hyperaemia, an increased blood perfusion, often associated with tissue injury. Interestingly, blood flow within the exposed colonic mucosa was significantly reduced to a similar degree following both injuries as determined by laser speckle microscopy. 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 the colon also undergoes a significant reduction in blood flow post-injury. Since preservation of the gut luminal lining is essential to ensure nutrient absorption and prevent bacterial translocation, it was reassuring to observe that systemically infused HPC7s could 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 ulcerative colitis patients, a reduction in flow is often detected in the chronically inflamed and remodelled colon (Hulten et al., 1977). It is possible that this reduced perfusion resulted in increased HPC7 adhesion due to lower shear rates. Reduced blood flow in injury sites may be more conducive to adhesive stem-endothelial cell interactions taking place. Therefore, systemically delivered SCs may have advantages over pharmacological drugs for the targeted treatment of inflammatory diseases of the colon.

Although it is possible that reduced shear stresses in the injured colon may have favoured HPC7 adhesion, this study suggests that active adhesive mechanisms, similar to those utilised by inflammatory neutrophils, were actually involved in mediating their recruitment. There has been much recent discussion surrounding the best and most effective route for HSPC delivery. This study demonstrates that for the colon, these cells can be delivered systemically and they can home to and be actively captured by inflamed colonic vasculature. Interestingly, the adhesive mechanisms governing recruitment varied in the same tissue depending on the type of injury incurred with either CD18 only or both CD18 and CD49d utilised. This differential use of integrins is likely due to the timeframe of the injury investigated. VCAM-1, the counterligand for CD49d, is known to be expressed as a result of injury but its expression requires time. This is unlike ICAM-1, the CD18 endothelial counterligand which is expressed constitutively. The time window in which the IR injury was investigated was 1–2 h post-reperfusion, which was likely too short a period for expression of VCAM-1. However, the colitis injury, established over 1 week, is known to exhibit VCAM-1 in the colitis colon vasculature (Soriano

et al., 2000). As well as the adhesive mechanisms varying depending on the injury to the organ, we have previously demonstrated that site-specific mechanisms also occur for similar injuries. Within IR injured SI, adhesion of the same immortalised HSC-like line was solely CD18 dependent, whereas in IR injured kidney additional and multiple adhesion molecules, including CD49d and CD44 were also involved (White et al., 2013; Kavanagh et al., 2013b; Kavanagh et al., 2010). Collectively, this data provides important information on the complexity surrounding the adhesive mechanisms involved, which is important knowledge to gain if effective targeted delivery strategies are to be designed.

Having investigated the kinetics of HPC7 homing to two different colon injuries, we further investigated whether injury mediated recruitment could be enhanced. H₂O₂ has been known to increase leukocyte rolling and chemotaxis (Fraticegli et al., 1996). Furthermore, we previously showed that H₂O₂ pre-treatment, at physiologically relevant concentrations, effectively 'primed' the same HPC7 cells for maximal adhesion prior to their systemic delivery (Kavanagh et al., 2013a). This subsequently improved their retention within injured SI. Increased H₂O₂ was also detected in homogenates primarily of IR injured, but also colitis injured, colon along with increased levels of 8-oxo-dG, a marker used to indicate signs of tissue oxidative stress. However, H₂O₂ only enhanced HPC7 adhesion within colitis injured colon in vivo. This may be due to the fact that occlusion of the SMA, which supplies blood to the SI as well as the colon, would also have led to SI injury. Indeed the SI is highly susceptible to ischaemic insults and comprises a much larger length of bowel than the colon. Therefore, many of the pre-treated HPC7s would have been recruited to the simultaneously injured SI, limiting the pool of circulating HPC7s available for the colon. This has important clinical implications as the targeting of systemic HSPCs to sites of injury may be compounded by their retention in other areas where known or unknown injury also exists.

Enhanced HPC7 adhesion was also observed in the colitis colon following platelet releasate pre-treatment. Approximately 6–7 control HPC7s were identified in the colitis microcirculation intravitaly 60 min post-infusion, ~11–12 were identified after H₂O₂ pre-treatment and ~13–14 after PES pre-treatment. Confocal and flow cytometric analyses confirmed physical interactions between microparticles, from PES and PMP supernatants, with HPC7s in a manner similar to that described by others for HSPCs (Janowska-Wieczorek et al., 2001; Mause et al., 2010). It is worth noting that others have also shown that PMPs significantly augment the arrest of endothelial progenitor cells on blood vessels and moreover, accelerated and enhanced their vasoregenerative potential (Mause et al., 2010). Interestingly, the PES pre-treatment was more effective than the PMPs in enhancing adhesion both in vitro to endothelial cells and in vivo. This suggests that activated platelets release important soluble factors that, in addition to the membrane fragments, modulate HSPC adhesion. Indeed, it has previously been demonstrated that platelets, often found adherent in abundance in ischaemic and inflammatory environments, may guide the homing of HSPCs to sites of injury (de Boer et al., 2006). Further characterisation of the chemical and cellular factors involved in this process may contribute to the development of other innovative strategies to augment HSPC mediated tissue repair for colon pathophysiological conditions. It is not clear whether the transfer of platelet adhesion molecules such as GPIIb/IIIa, an integrin that allows platelets to tether to immobilised von Willebrand factor, aided in modifying HPC7 recruitment. This would need to be determined using an anti-GPIIb antibody in our animal models. If transfer of GPIIb/IIIa is useful, improving the protocol to generate a greater number of PMPs or improving the coating of HSPCs with PMPs could be a worthwhile pursuit.

In addition to PES pre-treatment increasing HPC7 adhesion within the colon, it also concomitantly reduced pulmonary presence, thereby reducing non-specific retention in healthy tissues. Pulmonary entrapment is a major obstacle for systemic HSPC delivery for regenerative purposes both experimentally and clinically (Fischer et al., 2009). Indeed, patients receiving exogenous HSCs for haematologic disorders

experience post-transplant pulmonary injury due to cells becoming trapped in lung capillaries. In addition to promoting lung damage, pulmonary entrapment significantly reduces the available pool of circulating transplanted cells available for recruitment to injured tissues. This study is the first to demonstrate that microparticle coating not only effectively enhanced recruitment into specific sites of tissue injury, but also concomitantly decreased their entrapment within lungs. This observation may simply be related to cell availability, i.e. enhanced intestinal recruitment means less cells are available for pulmonary entrapment and vice-versa.

The mechanisms by which microparticles and activated platelet releasate may modify HPC7 adhesion appear to be similar to those we have previously seen with H₂O₂ (Kavanagh et al., 2013a). Although no change in the actual expression of HPC7 surface integrins was induced, the number of CD18 and/or CD49d clusters did increase. Evidence is emerging that the dynamic reorganisation of surface integrins into microclusters, through F-actin polymerisation, is the major mechanism regulating integrin binding strength and is a prerequisite for their activation and ligand binding (van Kooyk and Figdor, 2000). Hence, these observed changes in integrin clustering may play a major role in microparticle modification of HPC7 adhesion. Electron microscopic examination of the HPC7 surface also revealed that PMP/PES induced ruffling of the cell membrane and the extension of cellular protrusions, reminiscent of pseudopodia and filopodia. These phenotypic changes 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). 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 PES/PMP pre-treatment.

In conclusion, although evidence suggests that HSPCs are beneficial following tissue injury, the efficacy of such therapies is likely to be proportional to the degree of cell recruitment which can be achieved. Indeed, we have recently demonstrated that increasing the local presence of the same immortalised HSC-like line (HPC7) in the mouse small intestine was associated with significantly reduced neutrophil infiltration at 4 h post-reperfusion injury (Kavanagh et al., 2013b). Hence a greater local presence of HPC7s was required for an anti-inflammatory benefit to be realised. Enhancing the effectiveness of regenerative processes, particularly in diseases where leukocytes are key players in mediating injury, may therefore depend on identifying and then modulating the adhesive mechanisms that underpin HSPC trafficking. This study demonstrated that HPC7 recruitment to different injuries of the colon is increased, but that this recruitment can only be increased further in colitis mice. This can be achieved by chemical and biological pre-treatment strategies in the form of H₂O₂, PMPs or platelet releasate. Observed enhanced adhesion was obtained by a short pre-treatment duration that may be translated into clinical settings as an adjuvant to cellular therapy. Our observations that individual pre-treatments are effective only in certain organs or diseases are novel as it highlights how a single universal approach may be difficult to identify. Previous studies have enhanced SC recruitment using genetically modified cells including the local introduction of genes which encode for the potent SC chemoattractant SDF-1 α . However, the clinical applicability of such techniques is debatable and may be associated with aggravated tissue injury due to side effects such as SDF-1 α -dependent lymphocyte recruitment. The current study offers the benefit of not requiring genetic manipulation and, crucially, does not require manipulation of host tissues to achieve enhanced recruitment. The data in this study may help in the design of future cellular therapies using haematopoietic

progenitors. It is anticipated that enhancing their recruitment to injured organs may expedite the recovery process and encourage therapeutic success clinically in the field of regenerative medicine.

Author contributions

AIY designed, performed, analysed and interpreted the experiments and data and also wrote the manuscript. DA assisted in interpreting the data and in designing the experiments. NK designed the experiments, interpreted the data and edited the manuscript.

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Disclosures

The authors confirm that they have no conflicts of interest to disclose.

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