Supplementary Online Material

Junctional adhesion molecule-C (JAM-C) regulates polarized neutrophil transendothelial cell migration *in vivo*

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Supporting Online Material

SUPPLEMENTARY MATERIALS AND METHODS

Animals

Male (20-25g) mice from several colonies were used. The principal strain employed was that of heterozygous *Lys-EGFP-ki* mice in which the gene for EGFP has been knocked into the lysozyme M (lys) locus, yielding mice that exhibit fluorescent myelomonocytic cells, with mature neutrophils comprising the highest percentage of $EGFP^{hi}$ cells [1]. These mice were used with the permission of Dr Thomas Graf (Center for Genomic Regulation and ICREA, Barcelona, Spain.) and were kindly provided for the study by Dr Markus Sperandio (Ludwig-Maximilians University, Munich, Germany). Other strains of mice used were wild type (WT) C57BL/6 (purchased from Harlan, Wyton, Uk), PECAM-1 deficient mice (*PECAM-1*$^{-/-}$) [2] (gift from Dr Tak Mak, Amgen Institute, Toronto, Canada), heterozygous *CX3CR1-EGFP-ki* that express EGFP in all monocytes (and also in natural killer cells and some T cells) but not in neutrophils [3] (obtained from the European Mutant Mouse Archive, Orleans, France) and EC specific JAM-C deficient mice crossed with *Lys-EGFP-ki* mice, exhibiting EC JAM-C knock-down and their littermate controls (see below for details). Animals were housed in open or individually ventilated cages and facilities were regularly monitored for health status and infections. All experiments were carried out under the UK legislation for the protection of animals and were approved by the institutional ethical policies of Queen Mary, University of London.

Endothelial cell specific JAM-C deficient mice (Tie2Cre;JAM-$C^{flox/flox}$) were obtained by Cre-mediated recombination of JAM-C exon 1 flanked by loxP sites under the control of the Tie2-promoter. The generation of JAM-$C^{flox/flox}$ mice was recently reported [4, 5]. To assess the effects of endothelial specific reduction of JAM-C, JAM-$C^{flox/flox}$ mutants were crossed with mice transgenic for the Cre-recombinase under the control of the Tie2-promoter enhancer [6]. As previously reported [7] the use of the Tie-2 promoter can lead to a patchy expression of JAM-C in ECs and so individual cells can be either WT or KO, resulting in an overall reduced (49%), rather than completely abolished, expression of JAM-C in the eJAM-$C^{-/-}$ KO mice. These animals were crossed with *Lys*-EGFP-*ki* mice to obtain a novel mouse colony exhibiting EC-specific

JAM-C reduction and GFP-tagged leukocytes (Tie-2Cre;JAM-C$^{flox/flox}$; *Lys-EGFP-ki*), providing a powerful tool for analysis of EC JAM-C in leukocyte responses *in vivo* as analyzed by real-time fluorescent imaging as detailed below. JAM-C$^{flox/flox}$*;Lys-EGFP-ki* littermate controls exhibiting GFP leukocytes but normal EC JAM-C expression were used as controls.

Reagents

Recombinant murine IL-1β and CCL2 were purchased from R&D Systems (Abingdon, Oxford, UK), Draq5 was purchased from Biostatus (Shepsted, Leicestershire, UK). Tyrode's salts DMEM, MEM, FCS, penicillin/streptomycin, L-glutamine, collagenase, DNAse, fibronectin, superoxide dismutase (SOD), catalase, Evans blue and fMLP were from Sigma-Aldrich (Poole, Dorset, UK). Dihydrorhodamine-123 (DHR) was from Invitrogen (Paisley UK). Murine endothelial growth factor was from Peprotech (New Jersey, USA). Anti-PECAM mAb clone 390 was generated as previously detailed [8], and conjugated to Alexa Fluor-555 using a commercially available kit (Invitrogen, Paisley, UK). F(ab')2 fragments of this antibody were generated using a commercially available kit (Thermo Scientific, Cramlington, UK) and were similarly conjugated to Alexa Fluor-555 in house. The blocking anti-JAM-A mAb BV-11 was a gift from Dr Elisabetta Dejana (IFOM, Milan) and was generated as previously detailed [9]. Anti-mouse PECAM-1 mAb (Mec13.3) and anti-mouse Gr-1 (RB6-8C5), Anti-Ly6G-Alexa Fluor 647, anti-ICAM-1-Alexa Fluor 488 and goat-anti-rat IgG-FITC were from BD Biosciences (Cowley, Oxford, UK). Control IgG2b and IgG2a antibodies were from Serotec (Oxford, UK). Anti-ICAM-1-PE was from eBiosciences, Anti JAM-C reagents (mAb H33 and H36, affinity purified polyclonal anti-JAM-C and soluble JAM-C (sJAM-C) were generated as previously detailed [10, 11]. Anti-mouse VE-Cadherin mAb (BV14, eBioscience) was directly conjugated to Alexa Fluor-488 using a commercial kit (Invitrogen, Paisley UK) and goat anti-rabbit Alexa Fluor 555 Ab was obtained from Invitrogen (Paisley, UK). Anti-CD115 (clone AFS98) was purchased APC-conjugated (for FACS studies) or un-conjugated (eBiosciences or BioLegend) and directly conjugated in-house to Alexa Fluor-555 (for *in vivo* labeling of monocytes) using a commercial kit (Invitrogen, Paisley, UK).

Induction of inflammation in the mouse cremaster

Mouse cremaster muscles were stimulated via intrascrotal injection (i.s.) of IL-1β (50 ng in 400 µl saline) or were injected with saline (400 µl). After 2-4 hr as governed by the experimental objective, the tissues were surgically exteriorized [12, 13] and analyzed by intravital microscopy or dissected out and fixed for immunofluorescent staining and subsequent analysis [14]. I-R injury was induced by placing an artery clamp at the base of the exteriorized cremaster muscle (30 min) followed by removal of the clamp to initiate reperfusion during which leukocyte-vessel wall interaction was analyzed by intravital microscopy and or after 2 hr the tissue was dissected out and fixed for immunofluorescent staining. Other stimuli employed were CCL2 (500 ng injected i.s. in 400 µl saline; 4 hr *in vivo* test period) and fMLP (5 µg/400 µl saline i.s.). The latter was injected for 2 hr prior to exteriorization of the tissue and was then applied topically for a further 2 hr during intravital microscopy image acquisition. Where necessary pre-treatments including anti-JAM-C mAb H33 or control mAb H36, soluble JAM-C (sJAM-C) (all at 3 mg/kg), or superoxide dismutase (SOD) and catalase (2,000 and 50,000 U/kg respectively) were given i.v via tail vein injection.

Transmitted light intravital microscopy

Intravital microscopy was used to directly observe leukocyte responses within mouse cremasteric venules as previously detailed [13]. Briefly, IL-1β or saline was injected intrascrotally and 4 hrs later mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The cremasters were surgically exteriorized and kept warm and moist by superfusion of warmed Tyrode's balanced salt solution. The vessels were visualized with transmitted light on an upright microscope (Zeiss Axioskop FS; Carl Zeiss Micro-Imaging). In some experimental groups, mice were pretreated with intravenous saline, an isotype control mAb, or mAbs directed against PECAM-1 (Mec13.3 or 390 both at 3 mg/kg). Leukocyte responses of rolling, firm adhesion, and extravasation in postcapillary venules of 20–40 µm diameter were quantified as described previously [13]. Leukocyte transmigration was defined as the number of leukocytes in the extravascular tissue across a 500 µm vessel segment and within 50 µm into the tissue of the vessel of interest (**Fig. 1b,c**). Of relevance, we have previously found that >90 % of transmigrated leukocytes in IL-1β–stimulated cremaster muscles (4 hr reaction), as analyzed by electron microscopy, are neutrophils [12].

Immunofluorescence staining and confocal microscopy analysis of whole-mounted cremaster muscles

To investigate the expression of EC junctional molecules under different inflammatory conditions, whole-mounted cremaster muscles were fixed, immunostained and analyzed by confocal microscopy as previously detailed [15, 16]. Briefly, cremaster muscles of WT or eJAM-C$^{-/-}$ mice were stimulated as detailed in Methods the animals then humanely sacrificed followed by a vascular wash-out step (10 ml PBS) and perfusion fixation (2 ml of 2% PFA). Tissues were then dissected away from the animals, pinned flat and further fixed in 4 % PFA for 10 minutes. Fixed whole mounted tissues were blocked and permeabilized in PBS containing 10 % normal goat serum, 10 % FCS, 5 % normal mouse serum and 0.5 % Triton X-100 for 3 hr at room temperature. The tissues were then incubated with rabbit anti-mouse JAM-C polyclonal antibody, rat anti-mouse VE-Cadherin (directly conjugated with Alexa Fluor-488) and rat anti-mouse PECAM-1 (directly conjugated with Alexa Fluor-633) in PBS containing 0.1 % Triton-X-100 and 0.01 % sodium azide for 3 days followed by 4 hr incubation with a goat anti-rabbit Ab (Alexa Fluor-555 labeled). Samples incubated with appropriate control primary Abs were run in parallel with test samples. The samples were imaged using a Leica TCS-SP5 confocal microscope at 400Hz employing sequential scanning of the 488 nm, 561 nm and 633 nm channels at a resolution of 512 x 1024 pixels, corresponding to a voxel size of approximately 0.25 x 0.25 x 0.3 µm in the *x*/*y*/*z* planes, respectively. 3D reconstructed images and the expression levels of junctional VE-Cadherin, JAM-C and PECAM-1 were analyzed using Imaris software (Bitplane, Switzerland). Briefly an isosurface rendering of the images was built with the VE-Cadherin labeled channel with equivalent parameters/thresholds for each image, and the intensity of VE-Cadherin, JAM-C and PECAM-1 immunofluorescence within that surface (expressed in terms of voxel intensities for each channel) was quantified (**Fig. 5a,b**). Similar results were obtained when PECAM-1 channel was used to create the isosurface. All scale bars are 10 µm.

Additionally the expression profile of PECAM-1 was investigated in fixed tissues, using the protocol employed for *in vivo* labeling of PECAM-1. Hence, following intrascrotal injection of the anti-PECAM-1 mAb 390 (3 µg; directly conjugated to Alexa Fluor-555), cremaster muscles

were dissected away from the animals, fixed and labeled with the nuclear dye Draq5 (1/1000 dilution). Samples were then viewed using a Zeiss LSM 5 Pascal laser-scanning confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) incorporating a 63X oil-dipping objective (NA 1.4).

PMN depletion

Neutrophil depletion of the *Lys-EGFP-ki* mice was induced by intraperitoneal injection of anti-GR1 (or an isotype-matched control antibody, both at 25 μg/day for 3 days) [14]. Blood neutrophil numbers were quantified in treated mice by flow cytometry ($Gr1^+$ cells) and found to be reduced by 99.5 % (n=5 mice/group). Of importance this pre-treatment had no effect on the proportion of blood $CD115^+/GR1^+$ monocytes (**Supplementary Fig. 4a**).

Flow cytometry

The efficiency and selectivity of the neutrophil depletion protocol detailed above (**Supplementary Fig. 4a**), and the relative GFP intensity of neutrophils and monocytes in *Lys-EGFP-ki* mice, was assessed by flow cytometry (**Supplementary Fig. 4b**). Briefly, whole blood was collected into 0.5M EDTA by cardiac puncture or tail vein sampling from *Lys-EGFP-ki* mice. Samples were stained with fluorescently conjugated antibodies against Gr-1 and CD115 (eBioscience), red blood cells were lysed with ACK lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA) and the surface expression of molecules of interest and GFP intensity was measured by flow cytometry on a FACSCalibur (BD Pharmingen) and analyzed using FlowJo software (TreeStar). The specificity of CD115 as a marker for murine monocytes was confirmed through labeling of blood samples obtained from *CX3CR1-EGFP-ki* mice for CD115 and GR1 (**Supplementary Fig. 4c**). Briefly, as *CX3CR1-EGFP-ki* mice express GFP-labeled monocytes, their use combined with immunofluorescent labeling of cells for Gr-1, enables unambiguous discrimination of neutrophils ($Gr\text{-}1^{hi}$ CX_3CR1^- and $CD115^-$) from GFP-labeled monocytes ($CD115^+$).

Immunoelectron microscopy

Small fragments of cremaster muscle were sampled from tissues subjected to I-R injury (n=4 tissues), stimulated with IL-1β (n=2 tissues) or control mice (n=5 tissues). For preparation, the

tissues were immediately immersed for 60 min in a mixture of 2% PFA and 0.002% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Thereafter, the tissues were washed three times in 0.1 M phosphate buffer, cut in small pieces, embedded in 12% gelatin and cooled on ice. Blocks of gelatin-embedded tissues were infused with 2.3 M sucrose, frozen in liquid nitrogen, and sectioned with a EMFCS ultracryomicrotome (Leica). Ultrathin sections were mounted on Parlodion-coated copper grids. The sections were processed for ultracryomicrotomy as previously described [7, 17], using a 15h exposure at 4°C to the affinity purified anti-JAM-C polyclonal Ab diluted 1:100, and a 20 min exposure at room temperature to a goat antibody against rabbit Igs, conjugated to 10 nm gold particles. Cryosections were screened and photographed in a CM10 electron microscope (Philips, Eindhoven, The Netherlands). The specificity of the staining was assessed by a strong labeling for JAM-C in the paranodal regions of non compacted myelin which closely apposed the axolemma of the nerves innervating the cremaster muscle, and its absence in the compacted myelin formed by the very same cells [17]. Further negative controls were run by exposing the sections to only the secondary antibody.

For evaluation of JAM-C distribution, over 200 ECs were screened per group. Out of these, 15-25 ECs were found in each group to feature junctional contacts, as identified by the close apposition of the cell membrane and the concentration of actin microfilaments on the cytosol. These cells were photographed at the original magnification of x 21,000 and the number and position of gold particles scored on prints at the final magnification of x 63,000. In agreement with our previous study [7], in venules JAM-C was detected at junctions between ECs, associated with non junctional membranes and also in small cytoplasmic vesicles (**Fig. 5c** and **Supplementary Fig. 5**). The distribution of gold particles between these different sites in I-R injured and IL-1β-stimulated tissues, as a % change over control samples was analyzed by the Chi-square test, as provided by the Statistical Package for Social Sciences program (SPSS Inc. Chicago, IL, USA).

In vitro generation and phenotypic analysis of rTEM neutrophils

Isolation and culture of murine ECs

Murine endothelial cells (mEC) were isolated from the hearts of wild type C57BL6 mice, as previously described [18]. Briefly, following removal of arteries or other large vessels, hearts were minced with a pair of fine forceps and treated with type I collagenase (0.2 mg/ml in HBSS) for 30 min at 37^{o}C. The cellular suspension was then filtered through a 70μm pore strainer, pelleted and resuspended in 1ml of base medium, to which approximately 6x10^{6} of anti-ICAM-2 coated magnetic beads were added for positive selection of the cells. The cells were cultured using standard protocols using fibronectin- and gelatin-coated flasks and were subjected to a second round of positive selection as detailed above prior to use. The endothelial phenotype of the cells was verified by FACS through analysis of expression of key endothelial markers such as CD31, ICAM-2 and VE-cadherin, as previously described [18].

Isolation of mouse neutrophils

Mouse bone-marrow (BM) neutrophils were isolated from the femur and tibia of wild type C57BL6 mice by flushing the bone marrow with buffered medium. For generation of rTEM neutrophils, the cells were then subjected to red blood cell hypotonic lysis and mature neutrophils were purified using Percoll density centrifugation. Collected cells were ~95% pure neutrophils, with ~70-80% being mature cells.

Mouse blood neutrophils were assessed for their phenotype using a whole blood flow cytometry assay and peritoneal neutrophils were assessed using exudates from an IL-1β (10ng in 1ml for 4 hrs) elicited peritonitis model as previously detailed [19].

Generation of rTEM neutrophils in mEC cultures

Reverse transmigrated (rTEM) murine neutrophils were generated using a modified version of a previously published protocol [20]. Briefly, confluent mEC monolayer were stimulated with IL-1β (2.5 ng/ml) for 4 hours and washed before the addition of 5x10^{6} murine BM neutrophils. After 1 hour incubation at 37^{o}C (5% CO_2) non-adherent neutrophils were removed through washing and the cells were cultured for a further 24 hrs. Random fields were captured on video at 2, 4 and 24 hours. All recordings were analysed off-line and reverse transmigration was quantified as a

percentage of the total number of neutrophils that had transmigrated during the initial 60 minutes post introduction. At 24h > 60% of initially transmigrated neutrophils had reverse transmigrated, and were collected from the apical surface of the endothelial cells by washing the surface of the endothelium with DMEM/BSA (0.15 %) several times. As control, neutrophils in complete medium were added in a well without mECs and cultured at 37^{o}C (5% CO_2) for 24 hours.

Characterization of murine rTEM neutrophils

Fresh, rTEM neutrophils and 24 hour cultured control neutrophils were collected and analysed for expression of ICAM-1 by flow cytometry. Briefly, Fc-receptor mediated antibody binding was blocked by incubation with anti-mouse CD16-CD32 (10 µg/ml) followed by incubation with anti-ICAM-1-PE or IgG-PE (0.4 µg/ml). Following washing, samples were analysed using the Cyan flow cytometer (DAKO) and Summit version 4.3 software package (DAKO). The binding of the primary mAb, relative to the binding of control mAb is expressed in terms of relative fluorescence intensity (RFI).

Induction of I-R injury models and analysis of pulmonary lung vascular washouts for rTEM neutrophil phenotype

Models of I-R injury

Two I-R models were employed, namely cremaster muscle and lower limb I-R injury. The former was induced as detailed in the main text and as previously described [7]. The latter lower limb model was established as follows. A 1 cm mid-line incision was made in the abdomen to allow access to the interior vena cava and aorta. A clamp was placed on the aorta below the mesenteric, renal and hepatic vessels, and the abdominal incision covered with saline dampened gauze during a 20 min ischemia period. The clamp was then removed and reperfusion allowed for 60 min. Sham surgeries were performed in the same way, bar the placing of a clamp on the cremaster or aorta. During reperfusion periods anaesthesia was maintained, and animals kept on a heated stage.

Measurement of lung oedema

Effect of lower limb I-R injury on lung oedema was quantified by measuring accumulation of i.v. injected Evans blue into lung tissue. Briefly, following induction of I-R animals were given an

i.v. injection of 100µl of 5 % Evans blue solution. The dye was allowed to circulate for 5 min before the animals were killed by cervical dislocation. The chest was opened immediately and the pulmonary vasculature of the lungs was perfused with 10 ml of warm PBS by direct injection into the right ventricle. Following vascular wash-out, the lung tissue was excised, weighed, and the accumulated Evans blue was eluted in formamide (500 µl for 24 h at 55˚C) and optical density (OD) readings were taken at 620 nm. Plasma extravasation was presented as Evans blue OD readings after subtraction of the OD of formamide alone, and normalization to lung weight.

Collection of lung vascular wash-outs and lung tissue

Following the above models, the left carotid artery was canulated, a bolus injection of heparin (50 U) administered and the mice fully exsanguinated with the blood being collected into heparin. The carotid artery was then ligated, the canula removed and mice killed by cervical dislocation. Immediately after, the chest was opened and a clamp was placed on the thoracic vena cava and aorta just above the diaphragm. The pulmonary vasculature of the lungs was then perfused with 10 ml of warm PBS (containing heparin and EDTA) by direct injection into the right ventricle, and collection via a canula inserted into the left ventricle. Following this vascular wash-out, the lung tissue was excised and digested for 30 min in 5 ml DMEM with collagenase and DNAse (500 U each), and passed through a 40 µm cell sieve, enable quantitative analysis of the number of neutrophils in the lung tissue.

Analysis of leukocyte counts and neutrophil phenotype by flow cytometry

Flow cytometric analysis of cells was performed largely as detailed above. Specifically, after Fc-receptor mediated antibody binding was blocked by incubation with anti-mouse CD16-CD32 (5 µg/ml), samples of whole blood, pulmonary vascular wash out, BM leukocytes and lung tissue digests in suspension were stained with fluorescently conjugated antibodies against Ly-6G (Alexa Fluor-647, 0.1 µg/ml) and ICAM-1 (Alexa Fluor-488, 0.1 µg/ml) or the respective isotype control antibodies. Red blood cells were lysed with ACK lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA) and the surface expression of molecules of interest was measured by flow cytometry as detailed above. Leukocytes were identified by FSC and SSC characteristics, dead cells were excluded by 7-Aminoactinomycin D staining and neutrophils were identified based on Ly-6G positivity. ICAM-1-positive neutrophils were gated after determining the level

of background fluorescence in the isotype control stained samples and data is expressed as RFI. In some experiments the oxidative phenotype of neutrophils was investigated using DHR as previously described [21]. Cells were loaded with 1 μM DHR for 45 minutes at 37˚C and median DHR fluorescence was then quantified by flow cytometry at 488-530 nm excitation-emission.

Statistics

Data analysis was performed using Graph-pad Prism 4 (La Jolla, CA, USA) or the Statistical Package for Social Sciences program (SPSS Inc. Chicago, IL, USA). Results are mean ± standard error of mean (S.E.M). Statistical significance was assessed by Chi-square test (**Fig. 5c** and **Supplementary Fig. 5c**), Multinomial logistic regression analysis (**Fig. 6a**), one way ANOVA with Student-Newman-Keuls multiple comparison test, or unpaired t-test as appropriate. $P < 0.05$ was considered significant. Correlations were quantified by Pearson's correlation coefficient.

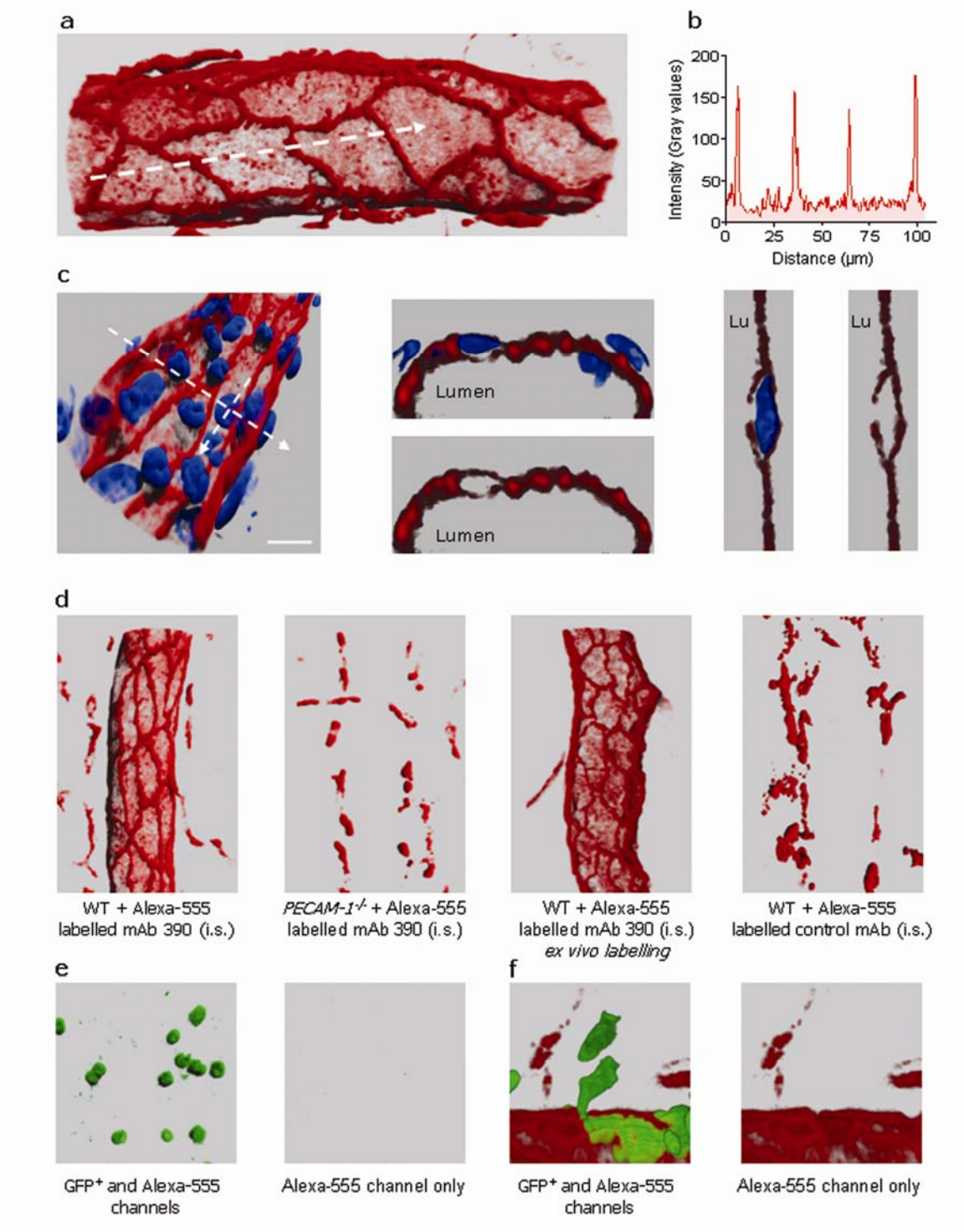
SUPPLEMENTARY RESULTS

Differentiation of neutrophil and monocyte TEM responses

As disrupted leukocyte TEM was observed in I-R injured tissues in *Lys-EGFP-ki* mice, that express GFP in both neutrophils and monocytes [1], it was important for us to elucidate which leukocyte sub-type was exhibiting the quantified disrupted TEM events. This was addressed by several means. Firstly, TEM was analyzed in *Lys-EGFP-ki* mice under conditions of neutrophil depletion (**Fig. 4a**). This intervention that resulted in >99% depletion of circulating neutrophils without effecting monocyte numbers (**Supplementary Fig. 4a**), almost totally inhibited the occurrence of all normal and disrupted forms of paracellular TEM events (**Fig. 4a**). Secondly, we took advantage of the significant difference in GFP levels of neutrophils and monocytes in the *Lys-EGFP-ki* mice to distinguish between these cell types in our *in vivo* imaging platform. Specifically, *Lys-EGFP-ki* mice express a higher level of GFP in neutrophils than monocytes [1], a phenomenon confirmed to be ~6 fold intensity difference by FACS (**Supplementary Fig. 4b**). To establish that this intensity variation between neutrophils and monocytes could be used to accurately discriminate between these cell types within our real-time *in vivo* confocal imaging model, CD115 labeling was used to positively identify the circulating monocyte population. Initially the specificity of CD115 as a marker for monocytes was confirmed using *CX3CR1-EGFP-ki* mice that express GFP-labeled monocytes [3]. Briefly, immunofluorescent labeling of blood leukocyte samples from these mice for Gr-1 enables unambiguous discrimination of neutrophils (Gr-1^{hi} CX_3CR1^- and shown to be $CD115^-$) from monocytes (Gr-$1^{med/lo}$ CX_3CR1^+ and confirmed to be $CD115^+$) (**Supplementary Fig. 4c**). These *in vitro* studies were extended to the *in vivo* setting as follows. The cremaster muscles of *Lys-EGFP-ki* mice were stimulated with i.s. injections of CCL2, vessels were identified by i.s. PECAM-1 labeling, and monocytes were labeled *in vivo* by i.v. injection of a directly-conjugated anti-CD115 mAb. Following *in vivo* image acquisition, using two settings in the GFP channel, a "high gain" or a "standard gain" setting (routinely used in our studies), $CD115^+$ and $CD115^-$ cells were identified visually, and their GFP intensity values determined to be above or bellow 200 Grays/μm^2 in analysis of 2D projections of the image using the LAS-AF software. This value was found to be the approximate limit for visibility post 3D reconstruction of images using Imaris. At lower gain settings

(standard gain), optimized for GFP^{hi} neutrophil visualization and routinely used in this work, $CD115^+$ cells (monocytes) had a mean GFP intensity of 112±24, while $CD115^-$ (neutrophils) were 571±37 and the background intensity was 8.1±2 Grays/ μm^2 (**Fig. 4b**). With this imaging methodology, the GFP^{lo} $CD115^+$ monocyte population is barely visible, exhibiting a fluorescence signal close to that of background, and below the ~ 200 Grays/ μm^2 limit for visibility in 3D models, shown by a dotted line on **Figure 4b**. **Figure 4c** shows a 3D reconstruction of a vessel containing both GFP^{hi} neutrophils and $CD115^+$ monocytes. With this approach of distinguishing neutrophils from monocytes based on their GFP intensity levels, both hesitant and reverse TEM could still be observed as induced by I-R injury. Together with the use of *CX3CR1-EGFP-ki* mice that express enhanced GFP in all monocytes but not in neutrophils (see main text), through three different techniques, the findings indicate that disrupted TEM event is only exhibited by migrating neutrophils in the present I-R injury model.

Supplementary Figures



Supplementary Figure 1

Supplementary Figure 1

***In vivo* labeling of PECAM-1 in cremasteric venules.**

Intrascrotal injection of Alexa Fluor-555-anti-PECAM-1 mAb (PECAM-555) (clone 390, 3μg in 300 μl saline) yielded strong and specific labeling of venular EC PECAM-1 in the mouse cremaster muscle. The images acquired by confocal microscopy as detailed show some of the characteristics of this labeling protocol. **(a)** Image of the abluminal side of a PECAM-555 labeled post capillary venule. The junctions between ECs are strongly labeled while the EC cell bodies have a more diffuse low intensity labeling pattern. **(b)** Intensity profile of PECAM-1 labeling along the arrow in panel (**a**). High intensity junctional peaks are labeled 'Jn'. **(c)** PECAM-555 labeled tissues were fixed and labeled with Draq5 to visualize nuclei. The lobed nuclei of neutrophils can be seen in the lumen, and the flattened endothelial nuclei in the vessel wall. Transverse and longitudinal sections through the image along the arrows in the left hand panel show that PECAM-555 labeling is present on the luminal and abluminal side of the endothelial nucleus. The absence of a visible nuclear exclusion region in the *en face* images of ECs suggests that the PECAM-555 labeling is not cytoplasmic. It has been reported that at EC borders, PECAM-1 enters an intracellular membranous compartment connected to the lateral borders of cells (termed LBRC) and recycles to the junctions during TEM [22]. As these structures have largely been detected in permeabilized cultured ECs by immunofluorescent labelling or immunoelectron microscopy, it is possible that they were not detected within our studies due to poor access of the Alexa Fluor-555 PECAM-1 mAb 390 to internal EC structures in live tissues. **(d)** Intrascrotal PECAM-555 labeling of WT mice shows labeling of perivascular structures as well as the EC junctions and cell body (left). Using the same labeling protocol on a tissue from *PECAM-1*$^{-/-}$ mice shows no EC labeling whilst the perivascular structures were still labeled (left middle). The same PECAM-555 mAb used on fixed and permeabilized WT tissues labels EC PECAM-1 but not the perivascular structures right middle). An Alexa Fluor-555 conjugated IgG2a isotype control Ab given intrascrotally to a WT animal also labels these structures, but not the endothelial expressed PECAM-1 (right). As the same labeled anti-PECAM-1 mAb selectively labeled ECs in fixed tissues (ie no labeling of mural cells was noted) and an Alexa Fluor-555

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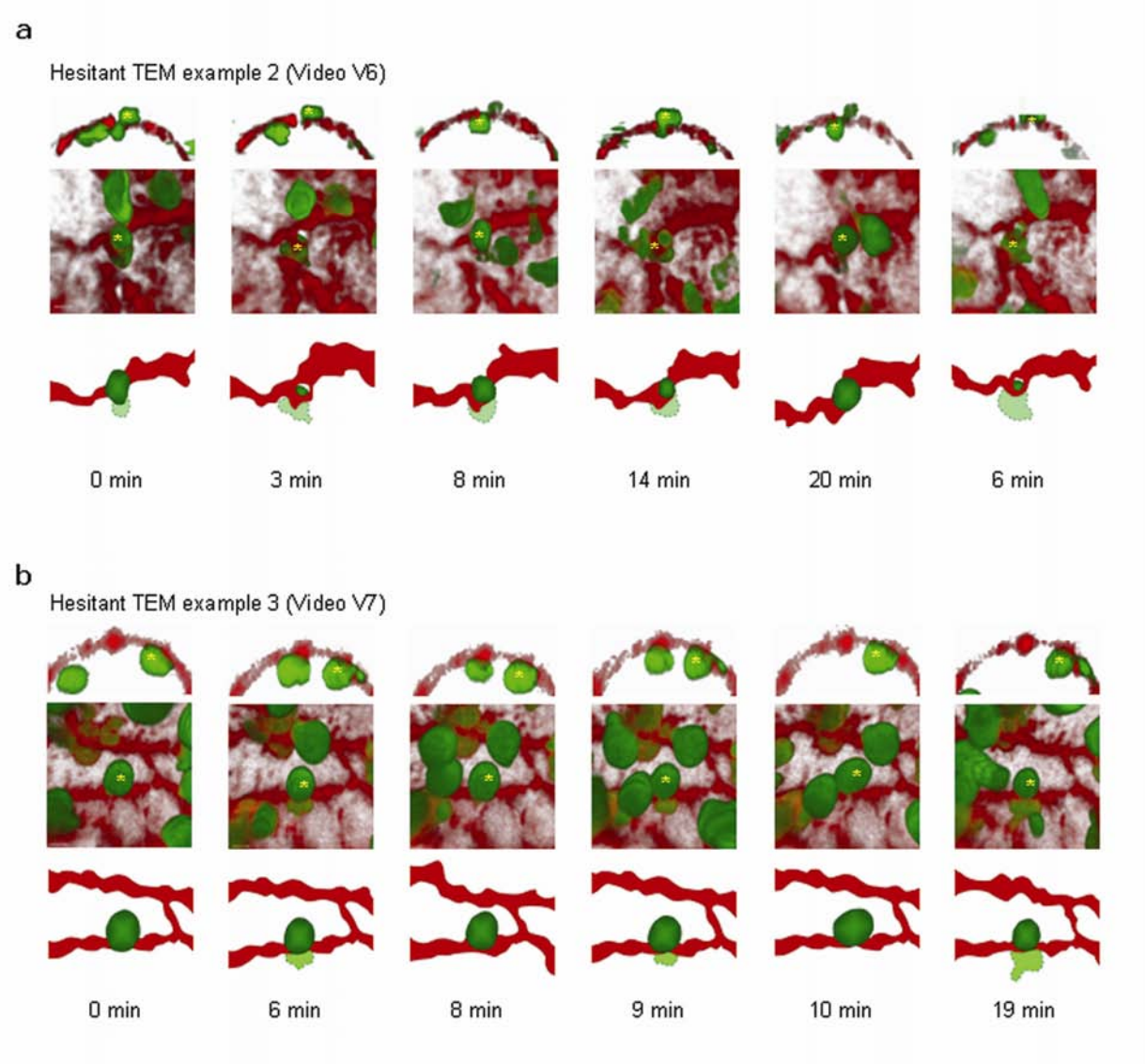
conjugated isotype control IgG2a mAb when administered via the intrascrotal route labeled the extravascular cells but not ECs (**Supplementary Fig. 1d**), collectively the data suggest that the perivascular cell labeling noted with the local administration of mAb occurs as a result of non-specific active uptake of fluorescently tagged antibody. **(e-f)** A *Lys-EGFP-ki* mouse was injected with intrascrotal injection of Alexa Fluor-555-anti-PECAM-1 390 mAb and leukocyte labeling with Alexa Fluor-555 was analyzed using both a blood sample **(e)** and *in vivo* imaging of cremaster muscles **(f)**. Scale bars = 10 μm.

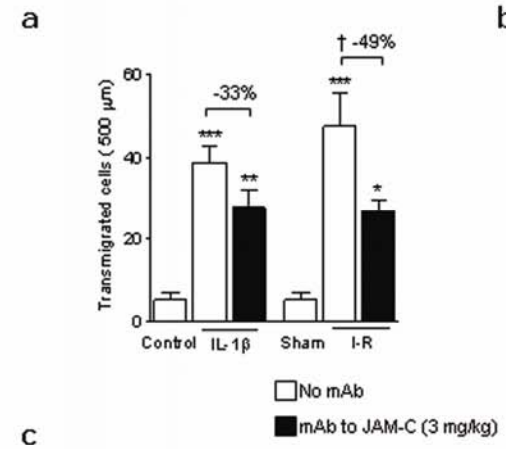


Supplementary Figure 2

Supplementary Figure 2

Examples of hesitant TEM.

TEM events in cremasteric venules of *Lys-EGFP-ki* mice immunostained *in vivo* for EC junctions with an anti-PECAM-1 mAb (red). **(a** and **b)** The two panels (Example 2 and 3) track the behavior of green GFP-leukocytes (*) exhibiting 'hesitant' paracellular TEM as induced by I-R injury. In each example, the top panels show the transverse section of the venule, the middle panel shows the event as imaged from the luminal side as supported by illustrative drawings showing the sub-EC segments of the migrating leukocyte in light green. **Supplementary Videos V6** and **V7** show these TEM events.



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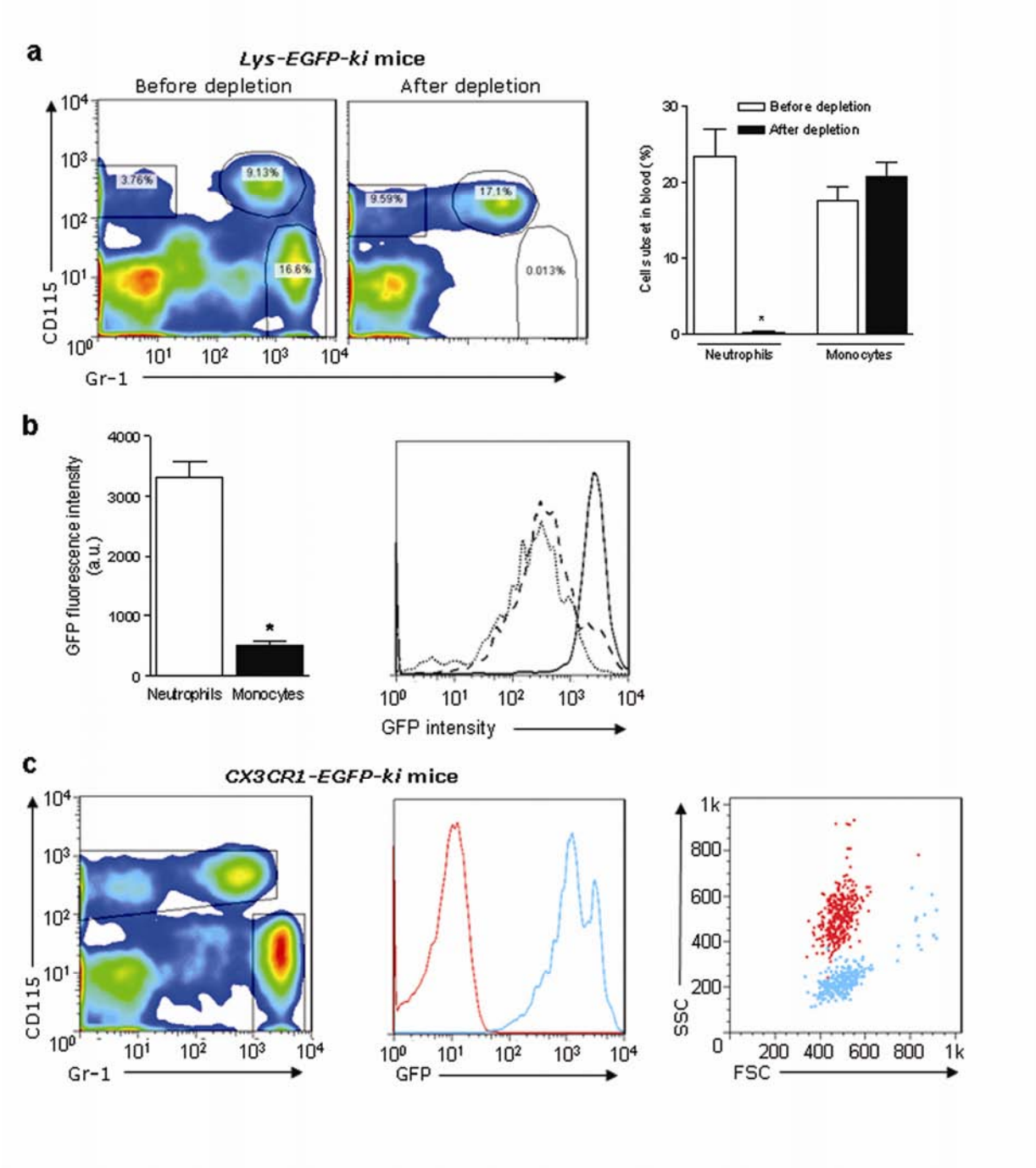
Group	Animals (n)	Total TEM	Normal TEM	Disrupted TEM
IR	10	109	93	16
IR + H33	10	103	65	38

Supplementary Figure 3

Supplementary Figure 3

The effect of JAM-C blockade on total extravasation and frequency of neutrophil TEM.

The cremasteric venules of *Lys-EGFP-ki* mice (exhibiting green leukocytes) were immunostained *in vivo* for EC junctions with an intrascrotal (i.s.) injection of Alexa Fluor-555-labeled anti-PECAM-1 mAb 390 (PECAM-555; 3μg/mouse) and were stimulated with i.s. IL-1β (50ng/mouse) or saline control. 4 hrs later tissues were surgically exteriorised and single images of post capillary venules were captured at ~40x magnification by *in vivo* confocal intravital microscopy (see Methods). Alternatively the cremaster muscle was subjected to I-R injury and after 2 h reperfusion, single confocal images of post capillary venules were captured at ~40x magnification. **(a)** The number of extravasated GFP^+ leukocytes within 50 μm of each venule (per 500 μm lengths) was quantified in mice pretreated with control mAb or anti-JAM-C mAb H33 (3mg/kg for both; n = 4 animals, and at least 25 vessels per group). **(b)** 30 minute image sequences of the development of an inflammatory response in I-R stimulated cremasters were generated and the dynamics of observed TEM events quantified as described (see Methods). The graphs show the frequency of observed normal and disrupted TEM events per 30 minutes in control mAb and H33-treated mice (n = 10-15 animals, and at least 103 TEM events per group in total). **(c)** Table shows the total absolute numbers of normal and disrupted TEM events observed and analysed in all animals within the indicated treatment groups. Statistical differences between sham and I-R groups (* $P<0.05$, ** $P<0.01$, *** $P<0.001$), and between mAb treated and untreated groups († $P<0.001$) were analysed by unpaired t-test

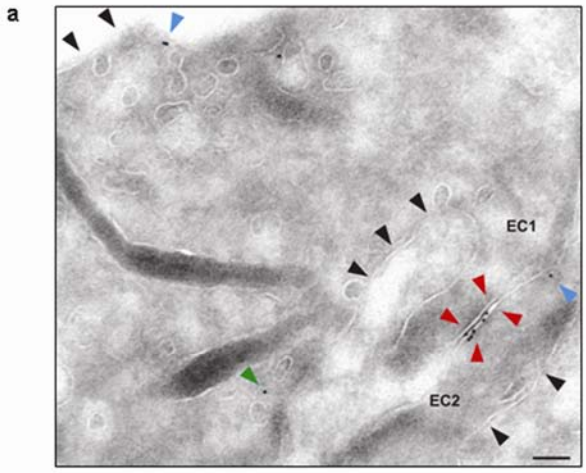


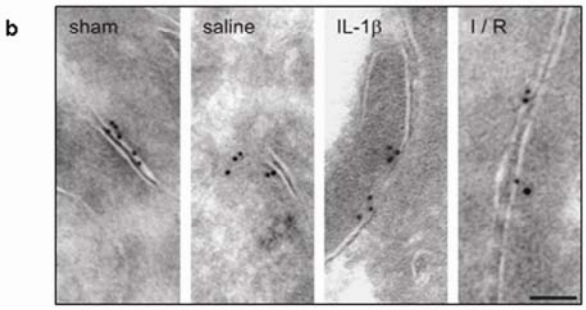
Supplementary Figure 4

Supplementary Figure 4

Distinguishing neutrophils from monocytes.

(a) Blood samples from *Lys-EGFP-ki* mice (before and after being subjected to neutrophil depletion) were analyzed by flow cytometry. Neutrophil and monocyte populations were identified by gating on Gr-1 and CD115 (neutrophils are Gr-1^{hi} and $CD115^{-}$; inflammatory monocytes are Gr-1^{med} and $CD115^{+}$; resident monocytes are Gr-1^{lo} and $CD115^{+}$). The neutrophil population is totally absent (>99 % depletion) following treatment of mice with i.p. anti-Gr-1 mAb. The right hand graph shows the efficiency and specificity of the neutrophil depletion protocol. Statistical difference between samples analyzed before and after Gr-1 treatment of mice is indicated by asterisks *P<0.001 (n=6 animals). **(b)** Using the same gating protocol the GFP intensity of neutrophils and monocytes in blood samples of *Lys-EGFP-ki* mice was analyzed. Neutrophils were found to be 6.4 fold brighter than monocytes. N = 3 mice (*P<0.001). A representative histogram is shown on the right in which signals from neutrophils (solid line), resident monocytes (dotted line) and inflammatory monocytes (dashed line) are depicted. **(c)** Blood from *CX3CR1-EGFP-ki* mice, with GFP^{+} monocytes, was analyzed by flow cytometry. The different myeloid cell subsets were distinguished based on Gr-1 and CD115 staining (Left panel). GFP fluorescence of cell subsets (neutrophils: red and monocytes: blue, middle panel) and the FSC/SSC properties (right panel) are analyzed, showing that the CD115 expressing monocyte population does not contain neutrophils.





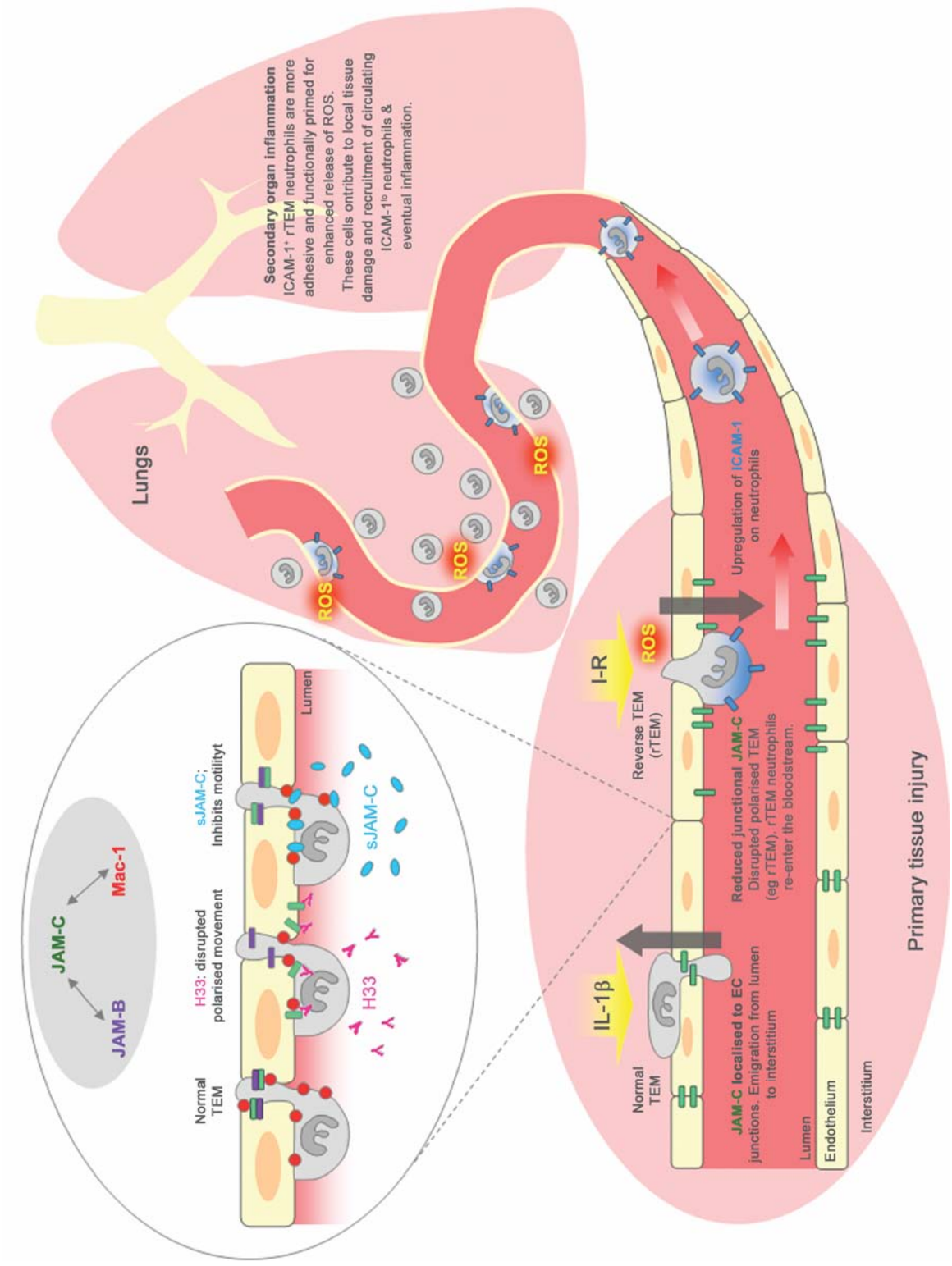
c

Group	N (mice)	N (ECs)	Total number of gold particles		
			Cytoplasmic vesicles	Non-junctional membrane	Junctional membrane
Saline	3	18	29	22	33
IL-1β	2	25	34**	32**	38**
Sham	2	15	27	21	31
I-R	4	17	6*	51*	17*

Supplementary Figure 5

Supplementary Figure 5

Immunogold labelling of JAM-C on endothelial cells. (a) Low power view of endothelial cells in a sham treated tissue, illustrating labeling for JAM-C at EC junctions (red arrow heads) and less abundantly in the non junctional membrane (blue arrow heads) and cytoplasmic vesicles (green arrow head). Black arrow heads point to non labeled portions of the non junctional membrane . Scale bar = 240 nm. **(b)** Images show the junctional region of apposed endothelial cells from saline or IL-1β treated, and sham or I-R treated cremasters, with apposed membranes showing as pale lines. Immunogold particles (black spots) bound to JAM-C were significantly less frequent at the junctions from I-R treated tissues. JAM-C was also less highly expressed in cytoplasmic vesicles, and more highly expressed on non junctional membranes from these tissues, while no difference was seen between the other groups (images not shown). Scale bar = 100 nm. **(c)** The Table shows the frequency of anti-JAM-C immunogold particles quantified at different sub-cellular locations in sham, saline, IL-1β and I-R treated cremaster muscles. Data was analysed by CHi square and statistical differences compared to the labelling of sham-operated mice (*P< 0.0001; 2 = 29.8; df = 2) and compared to the labelling of I-R mice (**P< 0.0001; 2 = 27.7; df = 2) is indicated.



Supplementary Figure 6

Supplementary Figure 6

Schematic presentation of JAM-C-mediated polarized neutrophil TEM and potential contribution of rTEM neutrophils to remote organ inflammation post I-R injury

The diagram illustrates a model depicting the role of rTEM neutrophils in second organ injury. Physiological inflammation mediated by locally generated inflammatory mediators such as cytokines (eg IL-1β) and chemoattractant molecules (eg fMLP) stimulates a polarised neutrophil TEM response that occurs in a luminal to abluminal direction. However the pathological insult ischemia-reperfusion (I-R) injury induces disrupted polarized neutrophil TEM, with the cells exhibiting abluminal to luminal EC junctional motility. This phenomenon is caused by disrupted expression of the adhesion molecule JAM-C from EC junctions, a response at least partly mediated by generation of reactive oxygen intermediates/species (ROS). The most severe form of disrupted polarized TEM is manifested into reverse TEM (rTEM) with rTEM neutrophils having a distinct phenotype (ICAM-1^{hi}) and being functionally primed for enhanced generation of ROS both *in vitro* [20] and *in vivo* (present study). rTEM neutrophils are also pro-adhesive, resistant to apoptosis and exhibit increased rigidity [20], properties that can promote their sequestration in capillaries and small venules of the pulmonary vasculature. In support of this the present study provides evidence to suggest that rTEM neutrophils stemming from a primary site of I-R injury can re-enter the blood stream and contribute to lung inflammation. Collectively it is proposed that rTEM neutrophils can contribute to the dissemination of systemic inflammation post I-R injury.

Insert: The EC junctional localization of JAM-C is stabilized through the formation of heterodimers with JAM-B [11]. JAM-C also interacts with the leukocyte integrin Mac-1 [23], and these interactions collectively support neutrophil polarized migration (luminal to abluminal) and motility through EC junctions, respectively. Anti-JAM-C mAb H33 disrupts the interaction of JAM-C-JAM-B, leading to reduced junctional expression of JAM-C and so enhances the frequency of disrupted polarized neutrophil TEM. Conversely soluble JAM-C (sJAM-C) has no affect on EC junctional expression of JAM-C (as JAM-C has greater affinity for JAM-B than it does for JAM-C), and so does not affect the polarity of neutrophil TEM. sJAM-C does however, through its ability to block JAM-C-Mac-1, inhibit neutrophil adhesive interactions with EC JAM-C and so suppress neutrophil motility through EC junctions. This results in significantly enhanced duration of neutrophil paracellular TEM. Hence, under physiological inflammatory conditions the combined interaction of JAM-C with JAM-B (at EC junctions) and Mac-1 (on

neutrophils) ensures directionally correct and efficient movement of neutrophils through the endothelium.

Supplementary Video Legends

Supplementary video V1: Development of an inflammatory response in an IL-1β-stimulated tissue.

The video shows a cremasteric venule (~50μm diameter) of a *Lys-EGFP-ki* mouse (exhibiting GFP-leukocytes), immunostained *in vivo* for EC junctions with Alexa Fluor-555-labeled anti-PECAM-1 mAb 390 (red) and stimulated with i.s. IL-1β (50ng/mouse). The video (captured at ~40X) shows the development of an inflammatory response (starting approximately 2 hrs post injection of the cytokine), with leukocyte adhesion and crawling preceding migration of leukocytes through venular walls. Images were captured at one frame per 2 minutes and the sequence shows a 90 min period. Still images are shown in **Figure 1a**.

Supplementary video V2: Migration of leukocytes in a paracellular mode.

The video shows a higher magnification (~40X) capture of an inflammatory response in a cremasteric venule of a *Lys-EGFP-ki* mouse (exhibiting GFP-leukocytes), immunostained *in vivo* for EC junctions with Alexa Fluor-555-labeled anti-PECAM-1 mAb 390 (red) and stimulated with i.s. IL-1β (50ng/mouse). The video then zooms in to show two leukocytes crawling across the luminal surface and migrating through EC junctions in a paracellular manner. Images were captured at one frame per minute and the sequence shows a 19 min period.

Supplementary video V3: Paracellular transmigration and pore formation. The video captures an inflammatory response in a cremasteric venule of a *Lys-EGFP-ki* mouse (exhibiting GFP-leukocytes), immunostained *in vivo* for EC junctions with Alexa Fluor-555-labeled anti-PECAM-1 mAb 390 (red) and stimulated with i.s. IL-1β (50ng/mouse) in which high optical zoom shows a single leukocyte migrating through a multi-cellular junction (viewed from the luminal side). The sequence is repeated showing the PECAM-555 channel (red) alone in order to

illustrate the formation of a paracellular pore during TEM. Images were captured at 1 frame per minute and show a 7 min period. Still images of this sequence are shown in **Figure 2a**.

Supplementary video V4: Transcellular TEM and pore formation. The video captures an inflammatory response in a cremasteric venule of a *Lys-EGFP-ki* mouse (exhibiting GFP-leukocytes), immunostained *in vivo* for EC junctions with Alexa Fluor-555-labeled anti-PECAM-1 mAb 390 (PECAM-555, red) and stimulated with i.s. IL-1β (50ng/mouse) in which high optical zoom shows a single leukocyte migrating by the transcellular route (viewed from the luminal side). The sequence is repeated showing the PECAM-555 channel (red) alone in order to illustrate the formation of a transcellular pore during TEM. Images were captured at 1 frame per minute and show a 7 min period. Still images of this sequence are shown in **Figure 2b**.

Supplementary video V5: Hesitant TEM as induced by I-R injury (example 1). The video captures an inflammatory response in a cremasteric venule of a *Lys-EGFP-ki* mouse (exhibiting GFP-leukocytes), immunostained *in vivo* for EC junctions with Alexa Fluor-555-labeled anti-PECAM-1 mAb 390 (red) and stimulated with I-R injury. High optical zoom shows a single leukocyte migrating through a multi-cellular junction as viewed from the luminal side. The leukocyte enters the junctional pore and partially extends into the sub-endothelial space before returning most of the cell body to the luminal side of the junction, and finally returning again to the sub-endothelial side and fully passing through the junction. Images were captured at 1 frame per minute and show a 27 min period. Still images, transverse sections and line drawings are shown in **Figure 3a**.

Supplementary video V6: Hesitant TEM as induced by I-R injury (example 2). The video captures an inflammatory response in a cremasteric venule of a *Lys-EGFP-ki* mouse (exhibiting GFP-leukocytes), immunostained *in vivo* for EC junctions with Alexa Fluor-555-labeled anti-PECAM-1 mAb 390 (red) and stimulated with I-R injury. The clip shows a leukocyte within a bi-cellular junction viewed from the luminal side. The cell partially returns to the lumen several times without disengaging from the junction before completing its migration into the sub-endothelial space. Images were captured at 1 frame per minute and show a 26 min period. Still images, transverse sections and line drawings are shown in **Supplementary Figure 2a**.

Supplementary video V7: Hesitant TEM as induced by I-R injury (example 3). The video captures an inflammatory response in a cremasteric venule of a *Lys-EGFP-ki* mouse (exhibiting GFP-leukocytes), immunostained *in vivo* for EC junctions with Alexa Fluor-555-labeled anti-PECAM-1 mAb 390 (red) and stimulated with I-R injury. The clip shows a leukocyte engaging with a bi-cellular junction viewed from the luminal side. The leukocyte extends and retracts a protrusion through the junctional pore several times before a more substantial part of the cell body enters the pore. Images were captured at 1 frame per minute and show a 19 min period. Still images, transverse sections and line drawings are shown in **Supplementary Figure 2b**.

Supplementary video V8: Reverse TEM as induced by I-R injury. The video captures an inflammatory response in a cremasteric venule of a *Lys-EGFP-ki* mouse (exhibiting GFP-leukocytes), immunostained *in vivo* for EC junctions with Alexa Fluor-555-labeled anti-PECAM-1 mAb 390 (red) and stimulated with I-R injury. The clip shows high optical zoom of a single leukocyte migrating through a multi-cellular junction viewed from the luminal side. The leukocyte is initially on the abluminal side of the endothelial junction and subsequently migrates through the junction in an abluminal to luminal or 'reverse' direction. On the luminal side the leukocyte disengages with the junction and crawls across the luminal surface. Images were captured at 1 frame per minute and show a 22 min period. Still images of this sequence are shown in **Figure 3b**.

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