Junctional adhesion molecule-C mediates leukocyte infiltration in response to ischemia reperfusion injury

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Abstract

OBJECTIVE: Junctional adhesion molecule-C (JAM-C) is an adhesion molecule that has multiple roles in inflammation and vascular biology, but many aspects of its functions under pathological conditions are unknown. Here we investigated the role of JAM-C in leukocyte migration in response to ischemia reperfusion (I/R) injury. METHODS AND RESULTS: Pretreatment of mice with soluble JAM-C (sJAM-C), used as a pharmacological blocker of JAM-C-mediated reactions, significantly suppressed leukocyte migration in models of kidney and cremaster muscle I/R injury (39 and 51% inhibition, respectively). Furthermore, in the cremaster muscle model (studied by intravital microscopy), both leukocyte adhesion and transmigration were suppressed in JAM-C-deficient mice (JAM-C(-/-)) and enhanced in mice overexpressing JAM-C in their endothelial cells (ECs). Analysis of JAM-C subcellular expression by immunoelectron microscopy indicated that in I/R-injured tissues, EC JAM-C was redistributed from cytoplasmic vesicles and EC junctional sites to nonjunctional plasma membranes, a response that may account for the role of JAM-C in both leukocyte [...]
Supplement Material

Online Supplementary Methods

Animals
Male mice of 25-30g were used for all studies. Commercially obtained wild-type C57BL/6 mice (Harlan-Olac, Bicester, UK or Charles River, Margate, UK) were used for studies where JAM-C function was pharmacologically blocked. JAM-C deficient mice (JAM-C<sup>−/−</sup>, on a 129Sv x C57BL/6 background) and mice over-expressing JAM-C in their ECs under the control of the promoter Tie2 (EC JAM-C transgenics, on a C57BL/6 background) were generated as previously detailed. For studies involving these mice, control animals were male littermates (WT or JAM-C<sup>+/−</sup>). All animals were housed at Imperial College London or Barts and the London School of Medicine and Dentistry animal house facilities. Experiments were conducted in accordance with the United Kingdom legislation.

Expression of JAM-C in murine tissues by immunofluorescence and confocal microscopy
Freshly isolated tissues (heart, lung, liver, spleen, kidney, lymph node, small intestine and cremaster muscle) from WT mice were embedded in OCT and frozen in liquid nitrogen. Tissue sections of 30µm-thickness were prepared using a cryostat (Bright, UK), collected on polylysine-coated slides and allowed to dry at room temperature for 2 hours. Sections were post-fixed in 100% ice-cold methanol for 20 min, blocked/permeabilised for 60 min in PBS supplemented with 10-20% FCS/NGS and 0.1-0.5% Triton X-100, and incubated with primary and appropriate secondary Abs
coupled to fluorescent Alexa Fluor dyes (Molecular Probes, Invitrogen, Paisley, UK). Primary antibodies used were rabbit anti-mouse JAM-C polyclonal antibody, and rat anti-mouse PECAM-1 mAb (clone Mec13.3; BD-Pharmingen, Oxford, UK). Samples were mounted with coverslips and observed at room temperature using a Zeiss LSM 5 PASCAL confocal laser-scanning microscope (Zeiss Ltd, Welwyn Garden City, U.K.) equipped with Argon (excitation wavelength: 488nm) and Helium/Neon (He/Ne) (excitation wavelength: 543nm) lasers. Multiple optical sections of tissue samples, running through the whole depth of the tissue, were captured with the software’s automatic scanning mode. Z-stack images were obtained for 3D-reconstruction using the LSM 5 Pascal software (version 3.2).

**Murine model of kidney ischemia/reperfusion injury**

Mice were subjected to renal I/R injury as previously detailed. Mice were anaesthetised with ketamine (100 mg/ml) and xylazine (20 mg/ml) (2:1; 1.5 ml/kg, i.p.) and their abdominal hair was shaved and the skin cleaned with 70% alcohol (v/v). The mice were then placed on a heated blanket set to 37 °C and a mid-line laparotomy was performed and their renal pedicles (consisting of the renal artery, vein and nerve) were isolated and clamped using non-traumatic microvascular clamps at time 0. After 30 minutes of acute bi-lateral ischemia, the clamps were removed, the skin was then sutured following administration of buprenorphine (0.1 mg/kg, s.c.) for pain control, and mice were allowed to recover from anaesthesia and returned to cages. Following the 24h reperfusion period, mice were re-anaesthetised and killed by excising the heart. Mice subjected to I/R injury were pre-treated with flag-tagged sJAM-C (3mg/kg, i.v.) or the control flag-tag peptide, 15 min prior to induction of ischemia. Sham operated mice underwent the same surgical procedures and were
performed in parallel. Kidneys from the mice were analysed for leukocyte infiltration by immunofluorescence and immunohistochemistry as detailed below.

**Analysis of renal leukocyte infiltration**

Leukocyte infiltration into the kidney was investigated by two different techniques i.e immunofluorescence and immunohistochemistry. Kidneys were removed from mice at the end of the *in vivo* test period after tying the renal pedicle, and were cut into two halves. Tissue samples were snap-frozen in liquid nitrogen, embedded in OCT compound and transferred to -80°C storage until further use.

Immunofluorescence protocol: 10µm cryosections were cut and dried overnight, fixed with ice-cold methanol for 10min, treated with 50µg/ml of proteinase K (Roche Diagnostics, Indianapolis, USA) in dionized water at room temperature for 30min and blocked with 10% FCS, 10% NGS, 5% mouse serum in TBS for 30min. Sections were incubated overnight at 4°C with primary antibodies and for 2 hours with appropriate secondary Abs coupled to fluorescent Alexa Fluor dyes (Molecular Probes, Invitrogen, Paisley, UK). Primary antibodies used were rabbit anti–mouse collagen IV polyclonal Ab (Abcam, Cambridge, UK), rat anti-mouse Gr-1 mAb (clone RB6-8C5; BD Pharmigen, Oxford, UK) for immunostaining of inflammatory leukocytes and rat anti-mouse CD68 (clone FA-11; AbD serotec, Oxford, UK) for specific immunostaining of monocytes/macrophages. Finally sections were analyzed by confocal microscopy as detailed above.

Immunohistochemistry protocol: Serial cryosections (5µm) were cut, mounted and air dried overnight. For analysis, sections were fixed in acetone for 10 mins at 4°C and blocked at different steps using biotin blocking system, peroxidase-blocking, and protein block solutions (Dako, Cambridge, UK). Sections were then immunostained
for with anti-Gr-1 mAb (clone RB6-8C5; BD Pharmigen, Oxford, UK) and an appropriate biotinylated polyclonal secondary antibody (Dako, Cambridge, UK). For the detection of positive cells, sections were incubated with avidin-biotin-HRP complex (Vectastain Elite ABC Kit, Vector Laboratories, Peterborough, UK) and 3,3-diaminobenzidine (Dako, Cambridge, UK) was used as chromogen. Finally, the sections were counterstained with hematoxylin before mounting. To assess the number of infiltrating leukocytes, positive (brown) cells in the renal cortex region of the kidney were quantified by image analysis. Results are expressed as the mean number of cells/mm², where 10 fields per section were quantified from at least 3 sections per animal (with at least 100µm between sections) and using samples obtained from at least 4 separate animals for analysis of each group.

**Intravital microscopy in a cremaster muscle model of I/R injury**

Leukocyte/vessel wall interactions in murine cremasteric venules as elicited by I/R injury was studied as previously detailed. Briefly, male mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and maintained at 37°C on a custom-built heated Perspex microscope stage. Testes were gently withdrawn by making an incision in the scrotum. The cremaster muscle around one testis was incised and laterally pinned out over the optical window of a microscopic stage. The tissue was kept warm and moist by superfusion of warmed Tyrode’s balanced salt solution. To induce I/R injury, the blood flow to the muscle was stopped by placing a clamp at the base of the tissue for 30 min to induce ischemia, after which the clamp was removed to allow reperfusion and leukocyte responses were quantified by intravital microscopy over a 2 h reperfusion period. Sham operated mice underwent surgical procedures identical to those of I/R mice.
except that clamps were not applied. Leukocyte/endothelial cell interactions were observed on an upright fixed stage microscope (Axioskop FS, Carl Zeiss, Welwyn Garden City, UK) fitted with water immersion objectives. Post-capillary venules, ranging from 20-40 µm in diameter were identified and leukocyte firm adhesion and transmigration was quantified. Firmly adherent leukocytes were considered as those remaining stationary for at least 30 s within a given 100 µm vessel segment. Extravasated leukocytes were quantified as those in a perivascular area along 500 µm vessel segments and within 50 µm in the tissue. Several vessel segments (3-5) from multiple vessels (3-5) were studied for each animal. In selected experiments, blood pressure was measured by cannulation of the carotid artery with fine polyethylene tubing and recording of the mean arterial blood pressure in mmHg with a blood pressure transducer (Harvard Apparatus, Kent, UK). Total and differential leukocyte counts were determined as previously detailed. WT mice (some treated with soluble JAM-C or control molecules, the control flag-tag peptide detailed above or soluble fibronectin [Biopur AG, Bubendorf, Switzerland]), or genetically modified mice were employed in these studies as detailed in the text.

**Flow cytometry**

For analysis of leukocyte adhesion molecules, cell samples were stained with the following primary antibodies directed against molecules of interest: anti-PECAM-1 mAb (clone Mec13.3), anti-CD11b (clone M1/70), anti-integrin α4 (clone R1-2), anti-L-selectin (clone MEL-14), anti-ICAM-2 (clone 3C4) and Ly6G/C (Gr-1) (clone RB6-8C5), all from BD Pharmingen, Oxford, UK. After washing, samples were incubated with appropriate fluorescently-labelled secondary Abs and analyzed using a Beckman Coulter flow cytometer Epics XL. The ratio of fluorescence intensities
associated with the binding of primary mAbs and isotype-matched control mAbs was used to express specific binding of test mAbs in terms of relative fluorescence intensity (RFI).

**Cell transfer experiments**

Bone marrow-derived leukocytes were obtained from WT or JAM-C⁻/⁻ mice by flushing femurs and tibias and labelled with the fluorescent dye calcein-AM as previously detailed.⁶ Labelled cells (12x10⁶ cells/recipient) were injected via the tail vein into recipient mice (WT or JAM-C⁻/⁻) and left to circulate for 2 h, after which I/R injury was performed as described above. Fluorescent adherent and extravasated leukocytes were quantified by IVM as previously detailed.⁶

**Immunoelectron microscopy**

Cremaster muscles dissected from WT sham operated mice or animals subjected to I/R injury were fixed for 5 min at room temperature in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde, followed by 60 min in 4% paraformaldehyde (all fixatives diluted in 0.1 M phosphate buffer, pH 7.4). Cremasters were washed three times in 0.1M phosphate buffer, cut into small segments, embedded in 12% gelatin and cooled on ice. Blocks were infused with 2.3M sucrose, frozen in liquid nitrogen, and sectioned with an EMFC ultracryomicrotome (Leica). Ultrathin sections were mounted on Parlodion-coated copper grids. The sections were processed as per previously described protocols⁸,⁹ which, in these experiments, included a 15h exposure at 4°C to affinity purified rabbit anti-JAM-C antibody³ diluted 1:100, and a 20 min exposure at room temperature to either Protein A-coated gold particles of 15 nm diameter, diluted 1:150 or a goat antibody against rabbit Igs and conjugated to 10 nm gold particles. Sections were
screened and photographed in a CM10 electron microscope (Philips, Eindhoven, The Netherlands). The specificity of the staining was assessed by a strong labelling 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 (not shown). Negative controls were run by exposing the sections to only the Protein A-coated particles (not shown). For evaluation of JAM-C distribution, at least 10 endothelial cells per cremaster 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. For evaluation of vesicle size and labelling, 40-50 vesicles per group were measured at a 441,000 x magnification, using a graduated lens. Sizes were calculated relative to a calibrated reference grid (2160 lines/mm; E.F. Fullam Inc., Schenectady, NY, USA).

Online Supplementary Figure legends

Figure 1. JAM-C expression profile in different murine organs. The expression profile of JAM-C (green) as compared to that of PECAM-1 (red) was investigated in multiple murine organs by immunofluorescence staining and confocal microscopy. Each image is representative of sections analysed from n=3 WT mice. Scale bars: 20µm.

Figure 2. Soluble JAM-C (sJAM-C) decreases leukocyte migration in kidney and cremaster muscle models of I/R injury. (A) WT mice were subjected to kidney ischemia (30min) and reperfusion (24h), or were sham-operated, and the leukocyte
infiltration response in the renal cortex region was quantified by immunofluorescence as described in Methods. Inflammatory leukocytes infiltration was immunodetected using anti-Gr1 Ab or anti-CD68 Ab. The I/R injured mice were pre-treated with sJAM-C (3mg/kg, iv) or a control peptide and leukocyte infiltration quantified by confocal microscopy. Results are expressed as mean ± SEM of cells/mm² (x40 magnification) and a minimum of 4 animals per group were quantified. (B) Surgically exteriorized mouse cremaster muscles were subjected to I/R injury (or were sham-operated) as detailed in Methods. Mice under-going I/R injury were pre-treated with sJAM-C (3mg/kg, iv) or a control peptide. The number of adherent and extravasated leukocytes was quantified per 500 µm vessel segment and 500x50 µm² extravascular area, respectively, during a 120 min reperfusion period. Results are presented as means±SEM for n = 4-5 mice/group with ≥3 vessels/cremaster quantified. Statistically significant differences as assessed by one-way ANOVA between sham operated and I/R groups are indicated by # symbols, #P < 0.05, ##P < 0.01 and ###P < 0.001. Additional statistical comparisons between sJAM-C and control I/R injured groups are indicated by asterisks, *P < 0.05, **P < 0.01 and ***P < 0.01.

Figure 3. JAM-C deficient mice (JAM-C−/−) exhibit reduced leukocyte adhesion and transmigration in cremasteric venules in response to I/R injury. (A)
Cremaster muscle tissues from WT or JAM-C−/− mice were immunostained for JAM-C (green) and PECAM-1 (red) and analysed by confocal microscopy, demonstrating the lack of JAM-C expression and normal PECAM-1 expression in the knock-out mice. Scale bar:20µm (B) Surgically exteriorized cremaster muscles of JAM-C−/− or JAM-C+/− (used as controls) were subjected to I/R injury (or were sham-operated) as detailed in Methods. The number of adherent and extravasated leukocytes was
quantified per 500 µm vessel segment and 500x50 µm² extravascular area, respectively, during a 120 min reperfusion period. Results are presented as means± SEM for n = 4-7 mice/group with ≥ 3 vessels/cremaster muscle quantified.

Statistically significant differences as assessed by one-way ANOVA between sham operated and I/R groups are indicated by # symbols, # P < 0.05 and ## P < 0.01.

Additional statistical comparisons between JAM-C⁻/⁻ or JAM-C⁺/⁺ I/R injured groups are indicated by asterisks, * P < 0.05 and ** P < 0.01.

Figure 4. Transgenic mice over-expressing JAM-C in their ECs exhibit enhanced leukocyte adhesion and transmigration in cremasteric venules in response to I/R injury. (A) Cremaster muscle tissues from EC JAM-C transgenic mice were immunostained for JAM-C (green) and PECAM-1 (red) and analysed by confocal microscopy, demonstrating an enhanced and patchy expression profile of JAM-C in the transgenic animals. Scale bar:20 µm (B) Surgically exteriorized cremaster muscles of EC JAM-C transgenic mice or WT littermates were subjected to I/R injury (or were sham-operated) as detailed in Methods. The number of adherent and extravasated leukocytes was quantified per 500 µm vessel segment and 500x50 µm² extravascular area, respectively, during a 120 min reperfusion period. Results are presented as means± SEM for n = 4-5 mice/group with ≥ 3 vessels/cremaster muscle quantified. Statistically significant differences as assessed by one-way ANOVA between sham operated and I/R groups are indicated by # symbols, # P < 0.05, ## P < 0.01 and ### P<0.001. Additional statistical comparisons between EC JAM-C transgenic mice and WT I/R injured groups are indicated by asterisks, * P < 0.05.
Figure 5. JAM-C localizes in different compartments within ECs and redistributes after I/R injury. A) Localization of JAM-C in different cellular regions in ECs of cremasteric venules, (i) at EC junctional sites (black arrowhead), (ii) at non-junctional domains of the EC membrane (arrow), and (iii) within cytoplasmic vesicles (white arrowhead). Scale bar: 100nm. B) The graph shows redistribution of immunodetected JAM-C from junctional and vesicular regions towards non-junctional compartments following I/R injury. Statistically significant comparisons of immunodetected JAM-C expression in different EC cellular compartments under sham and I/R injury conditions is indicated by asterisks, *P<0.0001 (n=40-42 ECs from 4 cremasters/group) as compared to the distribution of gold particles in the sham-operated mice by the chi square test ($\chi^2 = 127.9; df = 3$).

Online supplementary Results

JAM-C is expressed in the vasculature of multiple organs in mice

In the heart, JAM-C was present in the myocardial microcirculation where it located to capillaries and larger calibre vessels. The staining here showed a strong overlap with that of PECAM-1 in capillaries, whereas in larger vessels (exemplified with arrow in Figure 1), JAM-C expression appeared to be distributed in a more basal manner, possibly indicating an abluminal expression of JAM-C. In lungs, JAM-C expression was detected in vessels surrounding the airways, indicating an expression in pulmonary endothelial cells and possibly pulmonary epithelial cells (as suggested by a small level of JAM-C positive but PECAM-1 negative regions). In contrast, there was almost no JAM-C expression in the bronchial microvasculature where strong
PECAM-1 staining was detected. In the hepatic circulation, low JAM-C expression was detected surrounding larger calibre vessels whereas neither JAM-C nor PECAM-1 could be detected in the liver sinusoids. In the spleen and kidney JAM-C was expressed in the vasculature and also showed extravascular distribution as indicated by PECAM-1 positive and PECAM-1 negative regions, respectively. In kidneys, strong JAM-C expression was noted in the glomeruli and within the tubular vasculature, in agreement with previous reports.3,10 Sections from lumbar lymph nodes showed strong expression of JAM-C on high endothelial venules in line with previous findings.3,10 JAM-C was also closely associated with PECAM-1 in the microvasculature of the small intestine and in cremasteric venules, in the latter JAM-C expression being clearly co-localized with PECAM-1 at endothelial cell junctions. As control, tissue samples were also stained with isotype control antibodies where no binding was noted (data not shown). Furthermore, the specific binding of the anti-JAM-C Ab was demonstrated by its lack of binding to tissues obtained from JAM-C deficient mice (data not shown and Figure 3A).

Leukocyte infiltration into the inflamed kidney

Supplemental Figure IA shows the infiltration of leukocytes into the inflamed kidney measured by immunohistochemistry. Sections were processed as detailed in supplementary methods and immunostained with anti-GR1 Ab. Results showed an increase in leukocyte infiltration in kidneys subjected to ischemia/reperfusion. Mice pre-treated with sJAM-C showed a decreased leukocyte infiltration as compared to animals injected with the control peptide (39% inhibition). Figure IB shows the infiltrated monocytes/macrophages in kidney tissues as detected by
immunofluorescence using anti-Gr1 Ab or anti-CD68 Ab. Confocal microscopy quantification of this series of experiments is shown in Figure 2A.
Supplemental Figure I. WT mice were subjected to kidney ischemia (30min) and reperfusion (24h), or were sham-operated. The I/R injured mice were pre-treated with sJAM-C (3mg/kg, iv) or a control peptide. A) The leukocyte infiltration response in the renal cortex region was quantified by immunohistochemistry as described in supplemental Methods. Inflammatory leukocyte infiltration (neutrophils and monocytes) were immunodetected using an anti-Gr1 Ab and positive cells are stained brown. Results are expressed as mean ± SEM of leukocytes/field of view (x40 magnification), where 10 fields/tissue sections was quantified from at least 3 kidney sections/animal (with 100µm between sections) and a minimum of 4 animals per group. Scale bar represents 50 µm. Statistically significant differences were assessed by one-way ANOVA between sham operated and I/R groups (###P<0.001) or between sJAM-C and control I/R groups (**P<0.01). B) The infiltration of inflammatory leukocytes (Gr1+ cells) or monocytes/macrophages (CD68+ cells) into inflamed kidneys was analyzed by immunofluorescence as described in Methods. Confocal microscopy quantification of this series of experiments is shown in Figure 2A. Scale bar represents 50 µm.

Table I. Haematological parameters from the different mouse strains used in the study. Results are presented as mean±SEM of cells/ml of blood (n=5-11 mice/group) or as mmHg (n=4-5 mice/group). Statistically significant differences between genetically modified mice as compared to WTs is shown by asterisks, * P<0.05 and **P<0.01.
References for online supplemental data


10. Aurrand-Lions M, Johnson-Leger C, Wong C, Du Pasquier L, Imhof BA. Heterogeneity of endothelial junctions is reflected by differential expression