Junctional Adhesion Molecule–C Mediates Leukocyte Infiltration in Response to Ischemia Reperfusion Injury

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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 adhesion and transmigration under conditions of I/R injury.

Conclusions—The findings demonstrate a role for EC JAM-C in mediating leukocyte adhesion and transmigration in response to I/R injury and indicate the existence of a novel regulatory mechanism for redistribution and hence function of EC JAM-C in vivo. (Arterioscler Thromb Vasc Biol. 2009;29:1509-1515.)

Key Words: JAM-C ▪ ischaemia reperfusion injury ▪ leukocyte transmigration ▪ inflammation ▪ adhesion molecules

Leukocyte migration into inflamed tissues is a characteristic feature of inflammatory disorders, including numerous cardiovascular conditions such as atherosclerosis, myocardial infarction, and stroke. This response involves a cascade of cellular and molecular events that have culminated in the paradigm of the leukocyte adhesion cascade.1 The final step in this process involves leukocyte migration through endothelial cells (ECs) which can occur via both para- or transcellular routes, and the subsequent breaching of the basement membrane underlying ECs and embedding pericytes.1 Leukocyte transendothelial cell migration involves a number of adhesion molecules, the expression of which is highly concentrated at junctions between adjacent ECs. These molecules include PECAM-1, ICAM-2, CD99, ESAM, and members of the junctional adhesion molecule (JAM) family.1−3 Our understanding of the roles, mechanisms of action, and potential interactions of these molecules has significantly enhanced in recent years, but many aspects of their functions in particular under in vivo pathological conditions remains unclear.

JAMs are members of an immunoglobulin subfamily, currently composed of JAM-A, -B, -C, JAM-4, ESAM (EC-selective adhesion molecule), and CAR (coxsackie virus and adenovirus receptor) that localize to cell–cell contacts and are specifically enriched at tight junctions with some being directly implicated in leukocyte transendothelial cell migration.4 Among these molecules, JAM-C is unique in terms of its broad expression and functional profile. Specifically, JAM-C expression has been reported on ECs, spermatids, intestinal epithelial cells, smooth muscle cells, fibroblasts, and has recently been detected on Schwann cells in the peripheral nervous system.4−11 Furthermore, in humans, JAM-C is expressed on platelets and lymphocytes, whereas murine hematopoietic cells only express JAM-C during early development.4,12−16 Because of this wide expression pattern, JAM-C has been implicated in numerous events such as leukocyte trafficking, regulation of cell polarity, vascular permeability, and angiogenesis and appears to be critical in maintaining the integrity of the myelin sheath and the function of peripheral nerves.3,5,6,10,17−19 A number of ligands
have been reported for JAM-C, namely JAM-C, JAM-B, and Mac-1, although their contributions in the diverse functions of JAM-C remains unclear.

The functional role of JAM-C has largely been investigated using in vitro models of cell–cell interactions, but more recently a growing body of in vivo studies have demonstrated a significant role for this molecule in inflammatory and vascular events. Despite these findings, however, many aspects of the role(s) of JAM-C remain unknown, in particular its role in different stages of the leukocyte adhesion cascade and regulation of expression under in vivo pathological conditions. In the present study we have investigated the functional role of JAM-C in leukocyte migration in two murine models of I/R injury, namely I/R injury in the kidney and the cremaster muscle, the latter being investigated by intravital microscopy (IVM). The role of JAM-C was investigated in these models using both a pharmacological blocker of JAM-C (soluble JAM-C; sJAM-C) and genetically modified mice deficient in JAM-C or selectively overexpressing JAM-C in their ECs. Collectively, the findings demonstrate a role for JAM-C in leukocyte infiltration as elicited by I/R injury and indicate that JAM-C can support this response by mediating both leukocyte adhesion and transmigration, two distinct phases of the leukocyte adhesion cascade. Furthermore, analysis of venules by immunoelectron microscopy (IEM) detected for the first time the expression of JAM-C in EC intracellular vesicles in vivo and indicated that I/R injury can lead to redistribution of JAM-C within ECs, most notably from EC junctions and intracellular vesicles to EC nonjunctional membrane sites. The findings provide novel insights into the role and mechanism of action of JAM-C and highlight a potentially novel mechanism through which regulated expression of JAM-C may mediate different phases of leukocyte–vessel wall interactions under pathological inflammatory conditions.

**Methods**

Mouse strains used were C57BL/6 (WT), JAM-C−/−, and mice overexpressing JAM-C in their ECs (EC JAM-C transgensics). Mice were purchased from Harlan-Olse, Bicester, UK or Charles River, Margate, UK. Analysis of JAM-C and PECAM-1 expression in murine tissues was performed by immunofluorescence staining and confocal microscopy. Mice pretreated with flag-tagged sJAM-C (3 mg/kg, i.v.) or a control molecule (flag-tag peptide or soluble fibronectin) were subjected to I/R injury. In the renal I/R injury model (30 minutes/24 hours), leukocyte infiltration into the kidneys was quantified by immunofluorescence and immunohistochemistry. Leukocyte adhesion and transmigration responses in mouse cremasteric venules as elicited by I/R injury (30 minutes/2 hours) was studied by IVM using WT mice (pretreated with a control molecule or sJAM-C), JAM-C−/−, and EC JAM-C transgenic mice, as compared with relevant controls. The expression level of different adhesion molecules was investigated in blood cells from JAM-C−/− and WT mice by flow cytometry. Cell transfer experiments were performed between WT and JAM-C−/−, and the response of fluorescently-labeled leukocytes in recipient mice was analyzed by fluorescent IVM in the cremasteric vasculature. Subcellular localization and redistribution of JAM-C by I/R injury was investigated by IEM. (Please see supplemental materials, available online at http://atvb.ahajournals.org).

**Results**

**JAM-C Is Expressed in the Vasculature of Multiple Organs in Mice**

As JAM-C protein expression has not been investigated in a systematic manner in murine tissues, initial studies aimed to address this point by immunofluorescence staining and confocal microscopy. In all tissues studied (heart, lung, liver, spleen, kidney, lymph nodes, small intestine, and cremaster muscle), JAM-C expression was closely associated with the EC marker PECAM-1, although the extent of colocalization varied between different organs (Figure 1). Please see the supplemental materials for more details. As strong vascular expression of JAM-C was noted in kidneys and the cremaster muscle, these organs were analyzed for the functional role of JAM-C under conditions of I/R injury.

**Soluble JAM-C Inhibits Leukocyte Infiltration in Models of Kidney and Cremaster Muscle I/R Injury**

The role of JAM-C in leukocyte migration in two models of I/R injury, kidney and cremaster muscle, was investigated.
using sJAM-C as a pharmacological blocker of JAM-C–mediated responses.\textsuperscript{12,23} In the kidney model, a significant leukocyte infiltration was noted in mice subjected to I/R injury as compared to sham-operated mice. In this model, the infiltrating leukocytes consisted of both neutrophils and monocytes/macrophages, though the latter appeared to form a minority population (1:4 ratio of CD68\textsuperscript{+} cells [monocyte/macrophage] to GR1\textsuperscript{+} cells [neutrophils and GR1\textsuperscript{+} inflammatory monocytes]; Figure 2A and supplemental Figure I). Pretreatment of mice with sJAM-C (3 mg/kg, i.v.), but not a flag control peptide, significantly suppressed the infiltration of both GR1\textsuperscript{+} and CD68\textsuperscript{+} cells into inflamed kidneys (37% and 55% inhibition, respectively), suggesting a role for JAM-C in both neutrophil and monocyte migration in this model. To investigate the stage in the leukocyte adhesion cascade mediated by JAM-C under conditions of I/R injury, the role of JAM-C in leukocyte migration was also investigated in real time by IVM\textsuperscript{24} in the mouse cremaster muscle. In the I/R injury of the cremaster muscle, after a 30-minute ischemia period, leukocyte–vessel wall interactions were observed by IVM during a 2-hour reperfusion period. In this model, leukocyte adhesion to and extravasation through venular walls increased in a time-dependent manner over the 2-hour reperfusion period, as compared to sham-operated animals. In mice pretreated with sJAM-C a marked reduction in leukocyte adhesion was noted (Figure 2B, left), which was associated with a significant suppression of leukocyte transmigration, as compared to mice receiving a flag-control peptide (51% inhibition at 120 minutes reperfusion time; Figure 2B, right). Fibronectin, used as a control soluble protein, had no significant effect on leukocyte adhesion or transmigration as compared to responses obtained in flag-control peptide-treated mice (not shown). These results demonstrate that sJAM-C suppresses leukocyte migration in the kidney and cremaster muscle models of I/R injury, indicating a role for JAM-C in this inflammatory scenario.

\textbf{JAM-C\textsuperscript{−/−} Mice Exhibit Reduced Leukocyte Adhesion and Transmigration in Cremasteric Venules as Induced by I/R Injury}

To further investigate the role of JAM-C in regulating leukocyte–vessel wall interaction, the response of JAM-C\textsuperscript{−/−} mice to I/R injury was studied by IVM in the cremaster muscle. Initially, some characterization of these mice was performed. Figure 3A shows the EC junctional colocalization of JAM-C with the EC marker PECAM-1 in WT cremasteric venules and its lack of expression in JAM-C\textsuperscript{−/−} mice. The expression of PECAM-1 and other EC adhesion molecules such as VE-Cadherin and ICAM-1 appeared normal in JAM-C\textsuperscript{−/−} mice (Figure 3A and not shown). In addition, flow cytometry analysis revealed that the expression of key leukocyte adhesion molecules (PECAM-1, ICAM-2, Mac-1, L-selectin, and \(\alpha_2\) integrins) on blood neutrophils, monocytes (inflammatory and noninflammatory), and lymphocytes was normal in JAM-C\textsuperscript{−/−} mice (data not shown). Finally, as this is the first reported IVM study on JAM-C\textsuperscript{−/−} mice, some hematologic parameters were also quantified (supplemental Table I), which showed elevated circulating leukocyte number in JAM-C\textsuperscript{−/−} mice in line with previous reports,\textsuperscript{7} whereas no differences in mean arterial blood pressure was noted between WT, JAM-C\textsuperscript{−/−}, JAM-C\textsuperscript{+/-}, and EC JAM-C transgenic mice.

In the cremaster muscle I/R injury model, JAM-C\textsuperscript{−/−} mice exhibited a significantly reduced leukocyte adhesion (88% inhibition at 40 minutes reperfusion) and extravasation (66% inhibition at 120 minutes reperfusion) response as compared to their littermate JAM-C\textsuperscript{+/-} control mice (Figure 3B), findings that are in line with the results obtained under conditions of pharmacological blockade of JAM-C with...
sJAM-C (Figure 2B). Of relevance, no differences in leukocyte responses were noted between JAM-C−/− mice and WT animals (not shown). Because the inhibitory effect noted in the JAM-C−/− may have been caused by defects in either leukocyte or EC functions, preliminary cell transfer experiments were performed to address this issue. Briefly, calcein-labeled JAM-C−/− leukocytes injected into WT recipients responded in the same manner as WT leukocytes, whereas fluorescently-labeled control leukocytes (from WT or JAM-C−/− mice) injected into JAM-C−/− recipients exhibited a reduced transmigration response (81% inhibition at 120 minutes reperfusion; not shown). Collectively these data provide the first indication that EC JAM-C plays a key role in mediating leukocyte adhesion and extravasation in vivo. To further address this point, I/R injury was assessed in mice overexpressing JAM-C in their ECs.

Transgenic Mice Overexpressing JAM-C in ECs Exhibit Enhanced Leukocyte Adhesion and Transmigration in Response to I/R Injury

To extend the findings above, the cremaster muscle model was used to study I/R injury in mice overexpressing JAM-C in their ECs (EC JAM-C transgenic).12 Confocal microscopy studies revealed that these mice exhibited an overall enhanced expression of JAM-C in ECs (not shown) as previously reported.12 Interestingly, in a small number of tissue samples (≈<1%), some ECs (in both venules and arterioles) exhibited a “patchy” expression profile of JAM-C where the distribution of the molecule was very different to that seen in WT mice (compare Figures 3A and 4A). Specifically, whereas in WT tissues JAM-C expression appeared to be largely localized at EC junctions, in some vessels of the transgenic mice JAM-C was detected both at EC junctions and also showing a strong apical/cytoplasmic expression. Of relevance the expression of other molecules under the control of this promoter has been reported to be unevenly distributed or patchy.25

The EC JAM-C transgenic mice exhibited a consistently higher level of leukocyte adhesion and extravasation in cremasteric venules (eg, 62% increase at 120 minutes reperfusion; Figure 4B) as compared to WT littermate controls in response to I/R injury. These results together with the findings of studies using sJAM-C and JAM-C−/− mice demonstrate that in this inflammatory scenario, EC JAM-C can support both leukocyte adhesion and transmigration. To gain a better understanding of the expression and regulation of expression of EC JAM-C under conditions of I/R injury, JAM-C subcellular localization was studied in cremaster muscle tissues by IEM.

I/R Injury Promotes JAM-C Redistribution in ECs In Vivo

Our immunofluorescent and confocal microscopy studies in WT animals indicated the EC JAM-C expression to be predominantly junctional (Figures 1 and 3A), in line with previous reports.5 More recent studies have also reported on the intracellular expression of JAM-C in cultured microvascular (HDMECs) and macrovascular (HUVECs) ECs.19,26 To investigate the expression profile of EC JAM-C in vivo and more importantly to assess how this may be regulated under conditions of I/R injury, JAM-C subcellular localization was studied in cremasteric venules by IEM.
JAM-C at the subcellular level. Using this technique, in control sham operated mice, JAM-C expression was noted both in ECs and in the myelin sheath of nearby nerves (not shown) as previously reported.10 In ECs, the expression of JAM-C was mostly at junctions between adjacent cells (3.6±0.4 particles per junction, n=28 ECs; Figure 5Ai) with a significantly lower level of immunolabeled JAM-C being found along the nonjunctional plasma membrane of ECs (1.4±0.1 particles/field, n=49 ECs; Figure 5Aii). Interestingly, JAM-C was also localized in small single membrane-bound cytoplasmic vesicles (56±1.8 nm diameter vesicles expressing 2.2±0.2 particles/vesicle, n=41; Figure 5Aiii). These vesicles represented <1% of all EC vesicles and were found throughout the cytoplasm.

In cremaster muscles subjected to I/R injury, JAM-C was detected in the same cellular compartments but there was a clear shift in the distribution of JAM-C (P<0.0001 by the χ² test). Specifically, the number of particles per field of nonjunctional membrane was higher and the number of cytosolic vesicles and of junctional membrane domains immunolabeled for JAM-C was lower as compared to sham-operated tissue samples (Figure 5B). Hence, under conditions of I/R injury JAM-C expression in cytoplasmic vesicles was reduced by ≈7-fold while increasing in the nonjunctional membrane by ≈2.5-fold. This in vivo redistribution of JAM-C was associated with an unusually frequent presence of leukocytes in the lumen of the vessels (not shown).

Collectively, the present results provide the first in vivo indication for redistribution of JAM-C from EC junctional sites and intracellular compartments toward the plasma membrane under inflammatory conditions, demonstrating the existence of a novel regulatory mechanism for relocalization and hence function of EC JAM-C in vivo.

**Discussion**

JAM-C is a relatively new addition to the growing number of EC junctional adhesion molecules implicated in leukocyte trafficking,3,12,17,20,23 and as such many aspects of its mechanism of action, in particular within in vivo pathological scenarios, remain unknown. Here we provide evidence for the involvement of JAM-C in leukocyte infiltration as elicited by I/R injury and demonstrate that in mediating this response, JAM-C can support both leukocyte adhesion and transmigration, two distinct phases of the leukocyte adhesion cascade. Furthermore, the study reports on the in vivo existence of EC vesicular stores of JAM-C that are redistributed to the plasma membrane in response to I/R, providing a novel regulatory mechanism for EC JAM-C function in vivo.
ter muscle in close association with that of PECAM-1. The broad cellular and tissue distribution of JAM-C suggests a role for this molecule in multiple essential biological functions, as illustrated by the diverse and severe defects (e.g., immune-deficiency, growth retardation, and neurological and reproductive defects) exhibited by JAM-C–deficient mice.5,7,10 JAM-C has also been implicated in the pathogenesis of numerous inflammatory and cardiovascular conditions such as arthritis, acute pancreatitis, peritonitis, pulmonary inflammation, and atherosclerosis as largely investigated using murine disease models.7,8,21,23,29 The aim of the present studies was to further investigate the role of JAM-C under pathological cardiovascular conditions by investigating its involvement in leukocyte infiltration as induced by I/R injury. For this purpose, two murine models were used, namely I/R injury in the kidney and in the cremaster muscle, the latter being studied in real-time by IVM. The functional role of JAM-C was investigated here using both a pharmacological blocker of JAM-C, sJAM-C, and genetically modified mice lacking JAM-C (JAM-C−/−) or overexpressing JAM-C in their ECs (EC JAM-C transgenic).5,6,12,27 Treatment of mice with i.v. sJAM-C resulted in a significant suppression of leukocyte infiltration (both neutrophils and monocytes) into kidneys subjected to I/R injury. Analysis of leukocyte–vessel wall interactions in the cremaster muscle model by IVM showed that sJAM-C could suppress leukocyte adhesion and extravasation in response to I/R injury. These results were in line with findings in JAM-C−/− mice where a significant suppression of leukocyte adhesion and extravasation was observed under conditions of I/R injury. Overall, the findings suggest that the noted suppression of leukocyte extravasation under conditions of pharmacological or genetic deletion of JAM-C may be at least partly attributable to reduced leukocyte adhesion. As murine circulating leukocytes are reported not to express JAM-C,12,16 the results are likely to be caused by loss of function of endothelial JAM-C. In agreement with this hypothesis, studies performed using a cell transfer technique demonstrated that vascular (but not leukocyte) JAM-C deficiency lead to suppression of leukocyte–vessel wall interactions. In addition, EC JAM-C transgenic mice exhibit consistently higher levels of leukocyte adhesion and a significantly enhanced leukocyte extravasation response as induced by I/R injury.

Because JAM-C is largely expressed at EC junctions and has to date been heavily implicated in leukocyte transendothelial cell migration, identifying a role for JAM-C in leukocyte adhesion in vivo was of interest. We hypothesized that JAM-C may mediate leukocyte adhesion, an earlier step in the leukocyte adhesion cascade that is prerequisite to leukocyte transendothelial cell migration, under conditions where JAM-C is enhanced on the luminal surface of the endothelium. In this context, a number of in vitro studies have demonstrated enhanced expression of EC junctional molecules on nonjunctional regions. Specifically, nonjunctional expression of PECAM-1 and JAM-A on cultured ECs has been shown to be enhanced by certain cytokine combinations.30,31 More recently, Keiper et al found that cultured ECs stimulated with oxidized LDL can support enhanced monocyte adhesion in a manner that is partly JAM-C-dependent and appears to be associated with enhanced expression of JAM-C on ECs at nonjunctional sites.8 A similar phenomenon was also observed under conditions of blocking JAM-B–JAM-C interactions.27 To explore the potential mechanisms that may account for JAM-C–mediated leukocyte adhesion, the subcellular expression and localization of EC JAM-C was studied in cremaster muscles by IEM. The findings identified JAM-C in three distinct EC regions, junctional membrane, nonjunctional membrane, and an intracellular vesicular store of JAM-C. Although representing a small proportion of EC vesicles, the latter novel finding may provide mechanistic insights to regulation of expression and function of JAM-C under different inflammatory conditions. Indeed, comparison of tissues from control and I/R injured mice showed a clear redistribution of JAM-C from junctional and vesicular domains to nonjunctional regions, a finding that could well account for JAM-C–mediated leukocyte adhesion under conditions of I/R injury. Such a redistribution may provide a means of enhancing leukocyte–vessel wall interaction within the vascular lumen, possibly via JAM-C–Mac-1 interactions.27 In addition, increased luminal expression of JAM-C may promote migration of leukocytes to EC junctions through increased intravascular crawling, a response that appears to support efficient leukocyte transmigration and is reportedly Mac-1–dependent,32 as well as by creating an adhesive haptotactic gradient that guides luminal leukocytes to EC junctions. Of relevance, in humans, vascular expression of JAM-C appears to be enhanced under certain inflammatory disease conditions such as atherosclerosis and rheumatoid arthritis,8,20 though the associated mechanisms are unknown, highlighting the need for a better understanding of the molecular events that regulate JAM-C expression under different disease conditions.

Collectively, the present results show a role for JAM-C in leukocyte adhesion and transmigration in response to I/R injury and demonstrate the redistribution of EC JAM-C during this vascular insult in a manner that could potentially determine the functional role of JAM-C.

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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\(^{-/-}\), 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.\(^1\)\(^,\)\(^2\) For studies involving these mice, control animals were male littermates (WT or JAM-C\(^{+/+}\)). 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 30\(\mu\)m-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
with an 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/mm$^2$, 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.$^6$ 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 (12x10\(^6\) 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 EMFCS ultracryomicrotome (Leica). Ultrathin sections were mounted on Parlodion-coated copper grids. The sections were processed as per previously described protocols\(^8,\)\(^9\) which, in these experiments, included a 15h exposure at 4°C to affinity purified rabbit anti-JAM-C antibody\(^3\) 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:20µm (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 (χ² = 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.\textsuperscript{3, 10} Sections from lumbar lymph nodes showed strong expression of JAM-C on high endothelial venules in line with previous findings.\textsuperscript{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


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