Published in final edited form as:
Nat Immunol. ; 12(8): 761–769. doi:10.1038/ni.2062.

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

Abigail Woodfin[1], **Mathieu-Benoit Voisin**[1], **Martina Beyrau**[1], **Bartomeu Colom**[1], **Dorothée Caille**[2], **Frantzeska-Maria Diapouli**[3], **Gerard B Nash**[3], **Triantafyllos Chavakis**[4,6], **Steven M. Albelda**[5,6], **G Ed Rainger**[3,6], **Paolo Meda**[2,6], **Beat A. Imhof**[2], and **Sussan Nourshargh**[1]

[1]William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK [2]Centre Médical Universitaire, CH-1211 Geneva, Switzerland [3]Centre for Cardiovascular Research, School of Clinical and Experimental Medicine, College of Medicine and Dentistry, University of Birmingham, B15 2TT [4]Dresden University of Technology, 01307 Dresden, Germany [5]University of Pennsylvania, Philadelphia, PA 19104-6160, USA.

Abstract

Neutrophil migration into inflamed tissues is a fundamental component of innate immunity. A decisive step in this process is the polarised migration of blood neutrophils through endothelial cells (ECs) lining the venular lumen (transendothelial cell migration; TEM) in a luminal to abluminal direction. Using real-time confocal imaging we report that neutrophils can exhibit disrupted polarised TEM ("hesitant" and "reverse") *in vivo*. These events were noted in inflammation following ischemia-reperfusion injury, characterised by reduced expression of junctional adhesion molecule C (JAM-C) from EC junctions, and were enhanced by EC JAM-C blockade or genetic deletion. The results identify JAM-C as a key regulator of polarised neutrophil TEM *in vivo* and suggest that reverse TEM neutrophils can contribute to dissemination of systemic inflammation.

Migration of neutrophils from the vascular lumen to the extravascular tissue is a vital component of the host's defence reaction to injury and infection. To exit the blood circulatory system neutrophils establish a cascade of adhesive interactions with endothelial cells (ECs) lining the lumen of venular walls and ultimately breach the endothelium in a polarised manner1, 2. This process involves distinct cellular responses beginning with the capture of free-flowing leukocytes from the circulation and the formation of weak adhesive interactions with ECs resulting in the rolling of leukocytes along the venular wall. Activation of rolling neutrophils by surface bound stimulating factors such as chemokines promotes their firm attachment to venular walls and intravascular crawling to sites where the endothelium is eventually breached. These responses are mediated by complex series of

Corresponding author: Professor Sussan Nourshargh (s.nourshargh@qmul.ac.uk).
AUTHOR CONTRIBUTIONS A.W. designed and performed most experiments, analyzed data and contributed to the writing of the manuscript, M-B.V. designed and performed the immunofluorescent staining experiments and contributed to method development data analysis and interpretation. M.B. designed and performed flow cytometry assays, and contributed to data analysis and interpretation, B.C., D.C., and F.R.D. designed and performed selected assays. G.N., T.C., S.M.A., G.E.R. and P.M. provided key reagents and/or contributed to the design of experiments, B.A.I. provided key reagents and made intellectual contributions to the study. S.N. provided overall project supervision, contributed to the design of the experiments and the writing of the manuscript.
[6]These authors contributed equally to this work.

COMPETEING FINANCIAL INTERESTS The authors declare no competing financial interests.

Europe PMC Funders Author Manuscripts

overlapping molecular interactions involving selectins, integrins and their respective ligands 1.

Neutrophil migration through endothelial cells (transendothelial cell migration; TEM) can occur via junctions between adjacent ECs (paracellular route) 3, 4, a response that is supported by the active involvement of numerous EC junctional molecules such as PECAM-1, CD99, ICAM-2, ESAM and members of the junctional adhesion molecule (JAM) family 1, 2, 5. In addition, neutrophils can migrate through the body of endothelial cells (transcellular route) 6. Electron microscopy observations of transcellular TEM triggered many subsequent investigations into this phenomenon largely employing *in vitro* models that have collectively provided valuable insights into the characteristics and mechanisms of this mode of TEM 7-12. For example, invasive leukocyte protrusions seeking permissive sites and EC structures such as caveolae and a membranous compartment connected to the cell surface at cell borders (termed lateral border recycling compartment; LBRC), acting as a source of unligated PECAM-1, CD99 and JAM-A, have all been associated with mechanisms of transcellular leukocyte TEM 2, 7, 9, 13. Despite these studies, fundamental aspects of this response such as profile, frequency, dynamics and stimulus-specificity in direct comparison to paracellular TEM have not been investigated in real-time *in vivo*.

To further examine the mechanisms through which neutrophils breach venular walls we have established a confocal intravital microscopy imaging platform for the 3 dimensional observation of leukocyte transmigration in real-time (4D imaging). The exceptional spatial and temporal resolution of the technique and its application to the study of neutrophil TEM in inflamed microvessels has enabled rigorous analysis of key characteristics of both paracellular and transcellular TEM. Furthermore, due to the advanced clarity with which leukocyte TEM can be tracked we observed neutrophils that unexpectedly exhibited migration through EC junctions in an abluminal to luminal direction such as ‘reverse TEM’ (rTEM). Disrupted polarized neutrophil paracellular TEM responses were relatively selectively observed under conditions of ischemia-reperfusion (I-R), an inflammatory insult that caused reduced expression of JAM-C from EC junctions and their frequency was enhanced following pharmacological blockade or genetic deletion of EC JAM-C. The pathophysiological relevance of these findings is demonstrated by evidence suggesting that rTEM neutrophils stemming from a primary site of trauma (e.g. following I-R injury) are associated with the development of second organ injury. Collectively our findings provide direct evidence to show that EC JAM-C plays a key role in supporting luminal to abluminal neutrophil migration *in vivo* and suggest that rTEM neutrophils can contribute to dissemination of systemic inflammation.

RESULTS

Use of 4D imaging for analysis of leukocyte TEM *in vivo*

To enable us to accurately investigate the profile and dynamics of leukocyte TEM *in vivo* a 4D imaging platform with advanced spatial and temporal resolution was established. A key component to the successful application of this imaging method was the need for reproducible and adequate labelling of EC junctions for *in vivo* fluorescent microscopy imaging. As preliminary studies indicated that intravenous (i.v.) injection of fluorescently-labelled anti-PECAM-1 mAbs does not result in sufficiently uniform or strong labelling of EC contacts for accurate tracking of the route of leukocyte transmigration, it was necessary to develop an alternative protocol. Intrascrotal (i.s.) administration of directly-labelled Alexa Fluor-555 PECAM-1 mAb 390, a mAb that does not inhibit leukocyte transmigration 14, resulted in strong and reliable labelling of EC borders in cremasteric venules (Fig. 1a and Supplementary Fig. 1). As well as junctional staining, labelled ECs also exhibited a faint and

diffuse cell-body expression of PECAM-1 on the luminal and abluminal surfaces, which did not appear to be cytoplasmic as indicated by its lack of exclusion from nuclear regions when ECs were viewed *en face* (Supplementary Fig. 1a-c). Analysis of PECAM-1-deficient tissues (*Pecam1*$^{-/-}$) confirmed that both the junctional and non-junctional endothelial labeling was specific (Supplementary Fig. 1d, two left panels).

In addition to ECs, some extravascular cells were also labelled with this approach though these cells were not PECAM-1 expressing as demonstrated through analysis of tissues from *Pecam1*$^{-/-}$ mice and use of isotype control mAbs (Supplementary Fig. 1d). An additional advantage of labelling EC junctions through i.s. injection of labelled-anti-PECAM mAb (as opposed to the i.v. route) is that this approach failed to label the circulating leukocytes and so provided a cleaner and hence more clear-cut mode of tracking leukocyte interactions with EC junctions (Supplementary Fig. 1e,f). Although mAb 390 was selected as the anti-PECAM-1 mAb of choice as it has previously been shown not to suppress leukocyte TEM *in vivo* 14, we conducted rigorous analysis of our own to ensure that this novel vascular labelling protocol had no effects on leukocyte TEM in our model. We confirmed that intravenous or local administration of mAb 390 did not elicit an inflammatory response by itself and/or does not effect leukocyte transmigration as induced by interleukin-1β (IL-1β). As expected the functional blocking anti-PECAM-1 mAb Mec 13.3 (used as whole molecule) blocked TEM as induced by IL-1β (Fig. 1b,c). Intrascrotal injection of Alexa Fluor-555-labelled F(ab')$_2$ fragment of mAb 390 also effectively labeled EC junctions but as the dynamics of leukocyte TEM with this approach were not significantly different to when the whole mAb was employed (data not shown), the latter was used in all subsequent experiments. Finally, fluorescent labelling of EC junctions with the above approach in conjunction with laser excitation of tissues at our standard confocal microscopy settings did not result in stimulation of leukocyte TEM in unstimulated tissues as compared to tissues injected with IL-1β (data not shown).

The application of the above imaging approach and EC junctional labelling method to *lys-EGFP-ki* mice that exhibit endogenously-labelled neutrophils and monocytes 15 allowed detailed spatiotemporal analysis of leukocyte TEM (Fig. 1a and Supplementary Videos 1 and 2).

Inflammation primarily triggers paracellular TEM *in vivo*

In order to mimic and analyze the effects of physiological and pathological insults on leukocyte TEM, cremaster muscles were subjected to three different types of stimuli, namely the pro-inflammatory cytokine IL-1β, the chemotactic formylated tri-peptide fMLP or I-R injury. Within the *in vivo* test periods employed (~2-4 hrs) the reactions induced by these stimuli were neutrophilic in nature, as indicated by previous EM studies 16 and analysis of infiltrates in stimulated tissues by immunofluorescent staining (results not shown). Within straight venular segments, the predominant mode of TEM was paracellular, where the formation of a pore at EC contacts could be seen between two or multiple adjacent cells (Fig. 2a and Supplementary Videos 2 and 3). A minority of analyzed TEM events occurred via non-junctional routes during which leukocytes could be observed breaching the body of the endothelium resulting in the transient formation of pores in the cell-body (Fig. 2b and Supplementary Video 4). The location and size of both the paracellular and transcellular pores were diverse and additional examples of such TEM events are shown alongside linear intensity profiles of an example of each pore type (Fig. 2c-f). The frequency and dynamics of neutrophil TEM via junctional and non-junctional routes for all three stimuli was analyzed and ~90% of the observed TEM events were via the paracellular route, with no significant difference being seen between bi-cellular or multi-cellular EC junctions or between different stimuli (Fig. 2g). The mean duration of different types of TEM responses (i.e. bi-cellular, multi-cellular and transcellular) was ~6 min with

no significant differences being noted between the different routes or stimuli (Fig. 2h). Collectively the results show that within the present model, TEM as induced by a range of distinct proinflammatory stimuli occurs predominantly via the paracellular route, with no significant difference being detected in the profile and dynamics of the observed responses.

Inflammation triggers multiple forms of paracellular TEM

Although paracellular was the predominant mode of leukocyte TEM, multiple forms of this response were observed. While the majority of the paracellular TEM events involved the normal passage of leukocytes through EC junctions in a luminal to abluminal direction with no pause, a smaller proportion of transmigrating leukocytes exhibited either reverse TEM, or what appeared to be a multi-directional or 'hesitant' mode of paracellular TEM (Fig. 3). In 'hesitant' TEM leukocytes were observed to move back and forth within the EC junction a number of times (~2-3 oscillations), before finally completing migration into the sub-EC space (Fig. 3a and Supplementary Video 5). Additional examples of hesitant TEM are shown in Supplementary Figure 2 and Supplementary Videos 6 and 7. In instances of reverse TEM, leukocytes were seen to migrate through EC junctions in the abluminal to luminal direction, disengage from the junction and crawl away from the junction across the luminal surface of the endothelium (Fig. 3b and Supplementary Video 8). The frequency of these multiple forms of paracellular TEM were analyzed in tissues stimulated with IL-1β, fMLP and I-R injury and different profiles were noted with different stimuli (Fig. 3c). IL-1β-stimulated tissues exhibited a relatively low level of reverse and hesitant forms of TEM (~3 % of all quantified paracellular events), whereas in tissues subjected to I-R injury in total ~15 % of paracellular TEM events were hesitant or reverse. The differential frequency of these responses under different inflammatory conditions was not governed by the overall magnitude of the inflammatory responses in that there was no significant difference in the absolute number of transmigrated leukocytes in response to IL-1β and I-R injury (Supplementary Fig. 3). Quantification of the duration of the different forms of paracellular TEM in I-R stimulated tissues indicated that hesitant and reverse TEM were significantly slower events than normal paracellular responses (Fig. 3d).

Neutrophils exhibit disrupted polarised TEM

We next sought to further investigate the profile of hesitant and reverse TEM, collectively termed 'disrupted' TEM, under conditions of I-R injury. Specifically, as these events were observed in I-R injured tissues in *Lys-EGFP-ki* mice that express GFP in both neutrophils and monocytes 15, it was important to elucidate which leukocyte sub-type was exhibiting disrupted TEM responses. The contribution of neutrophils and monocytes to the observed TEM events was investigated using multiple approaches namely, the analysis of neutrophil depleted *Lys-EGFP-ki* mice (Fig. 4a and Supplementary Fig. 4a), the differentiation of neutrophils from monocytes based on the significant differences in their GFP intensities (Fig. 4b,c, Supplementary Fig. 4b and Supplementary Results text) and finally through the use of *Cx3cr1-EGFP-ki* mice that express enhanced GFP in all monocytes but not in neutrophils 17 (Supplementary Fig. 4c). With respect to the latter, in *Cx3cr1-EGFP-ki* mice I-R injury elicited very low level of normal paracellular TEM of GFP^+ cells, and no hesitant or reverse modes of TEM were detected in >20 vessels analyzed. Collectively the findings strongly indicate that neutrophils are the sole participants of hesitant and reverse modes of TEM in the present I-R injury model.

I-R injury lowers expression of JAM-C from EC junctions

Having characterised the responses of disrupted TEM in terms of frequency, stimulus-specificity, dynamics and leukocyte sub-type, we next addressed the mechanism associated with these events. We hypothesised that "hesitant "and "reverse" forms of TEM were associated with disrupted expression of EC junctional molecules. To address this possibility

we used immunofluorescent staining and confocal microscopy to investigate the expression of VE-Cadherin, and PECAM-1, key EC junctional molecules implicated in maintaining the integrity of EC junctional contacts 18 and in mediating leukocyte TEM, respectively 1, 2, 19, in control, IL-1β-stimulated and I-R injured tissues. Furthermore, as JAM-C supports polarized monocyte migration through cultured ECs *in vitro* 20, the expression of this adhesion molecule was also analyzed. Under conditions of I-R injury, but not in response to IL-1β, cell surface expression of JAM-C was selectively reduced at EC junctions whereas there was no change in VE-Cadherin or PECAM-1 (Fig. 5a,b). This loss of EC junctional JAM-C was partially prevented in mice pretreated with superoxide dismutase (SOD) and catalase, indicating that reactive oxygen intermediates contributed to the reduced junctional expression of JAM-C in response to I-R injury (Fig. 5b).

The expression of JAM-C in inflamed tissues was also investigated by immunoelectron microscopy (Fig. 5c and Supplementary Fig. 5). This approach confirmed previous findings that JAM-C can be detected on ECs at three locations, junctional membranes, non-junctional membrane and cytosolic vesicles 21. In control (sham-operated and saline-injected) tissues, JAM-C was almost equally distributed between these three localizations. In tissues subjected to I-R injury, the junctional labeling of JAM-C was decreased, while that of the non-junctional membrane increased, and that of cytoplasmic vesicles dropped. In contrast and in agreement with our immunofluorescent staining studies, no change in expression of JAM-C was noted in IL-1β-stimulated tissues as analyzed by immunoelectron microscopy (Fig. 5c). Together, the findings show the ability of I-R injury but not IL-1β to reduce EC junctional expression of JAM-C, findings that are in line with the almost undetectable level of disrupted paracellular TEM induced by IL-1β (Fig. 3c).

EC JAM-C expression mediates polarized neutrophil TEM

Since hesitant and reverse neutrophil TEM occurred in tissues where JAM-C expression at EC junctions was disrupted, we sought to investigate the functional consequence of blocking EC junctional JAM-C. As JAM-C is maintained at EC junctions through an interaction with JAM-B and blocking JAM-C-JAM-B interaction has been shown to result in reduced EC junctional and enhanced luminal expression of JAM-C *in vivo* 21, 22, the effect of a mAb blocking JAM-C-JAM-B interaction (mAb H33) was investigated. Mice pre-treated with mAb H33 (but not a non-blocking anti-JAM-C mAb, H36) exhibited reduced percentage of normal paracellular TEM and enhanced levels of neutrophil hesitant and reverse TEM in response to I-R injury (Fig. 6a), i.e. ~50% disrupted neutrophil TEM (Fig. 6b). In mice pretreated with soluble JAM-C (sJAM-C), a reagent previously shown to suppress total leukocyte infiltration as induced by I-R injury 21, the duration of normal paracellular TEM was significantly enhanced (5.7±3.5 and 11.6±9.5 min in control and sJAM-C-treated mice, respectively; n=47-79 TEM events in n=4 mice analyzed) but there was no effect on frequency of disrupted polarized TEM (Fig. 6b). This is in contrast to mAb H33 treated mice in which the frequency of disrupted TEM was increased, but the duration of normal TEM was not affected (5.7±3.5 and 7.0±4.5 min in control and mAb H33 treated mice, respectively). Antibody blockade of a related junctional adhesion molecule, JAM-A23, did not cause increased frequency of disrupted polarized TEM (Fig. 6b).

These results suggest that JAM-C can mediate both neutrophil motility through EC junctions (suppressed by sJAM-C, presumably via blockade of Mac-1-JAM-C interaction 24, 25) and regulate the directionality of neutrophil migration through EC junctions in a luminal to abluminal direction (suppressed by interventions that reduce EC junctional expression of JAM-C, eg mAb H33). In agreement with this, *Lys-EGFP-ki* mice crossed with EC-specific JAM-C deficient mice (exhibiting on average 49% reduction in EC JAM-C) showed >50% disrupted form of paracellular TEM in response to I-R injury (Fig. 6b). In these animals one incidence of reverse transcellular TEM was also observed post I-R injury, indicating that

such a response can occur. However, its rarity (1 out of >700 TEM events analyzed), suggests that this mode of TEM may not be of much physiological or pathological importance.

Finally, in tissues stimulated with IL-1β where disrupted forms of TEM and reduced EC junctional expression of JAM-C was not observed, pharmacological blockade of JAM-C-JAM-B interaction or genetic reduction of EC JAM-C also resulted in an increased frequency of disrupted TEM (~12-30%) (Fig. 6b). An increased percentage of disrupted TEM events in mAb H33-treated mice was associated with significantly increased frequency and absolute numbers of disrupted TEM events (Supplementary Fig. 3), demonstrating that JAM-C blockade leads to a true enhancement of disrupted neutrophil TEM responses. Together, these results indicate that JAM-C expression plays a key role in maintaining a polarized neutrophil paracellular TEM and under conditions where its EC junctional expression is reduced TEM occurs in a disrupted format as illustrated through the responses of hesitant and reverse neutrophil TEM.

Pathophysiological role of reverse TEM neutrophils?

Finally we sought to investigate the potential pathophysiological role of disrupted neutrophil TEM, the most extreme form of this being neutrophil reverse TEM (rTEM). As the latter was largely observed in response to I-R injury, we hypothesized that this phenomenon might be associated with systemic consequences characteristic of I-R-induced tissue damage. This response is most notably manifested in the form of second organ tissue injury (e.g. lung inflammation), and is believed to be mediated by multiple factors including generation of systemic inflammatory mediators and circulation of activated forms of neutrophils 26. As rTEM human neutrophils have previously been shown to exhibit a distinct phenotype *in vitro* (ICAM-1^{hi}) and to have a greater ability to generate reactive oxygen species (ROS) 27, we investigated the potential association of rTEM neutrophils with lung inflammation post I-R injury. Using an *in vitro* model, mouse neutrophils that had reverse transmigrated through cultured mouse ECs had elevated levels of ICAM-1 (ICAM-1^{hi}) whereas non-transmigrated and control cultured bone-marrow neutrophils (for 24hrs) and blood neutrophils were ICAM-1^{lo} (Fig. 7a). Of note, peritoneal neutrophils derived from an IL-1β-driven peritonitis model and representing a pool of “normal” transmigrated neutrophils, were also ICAM-1^{lo} and expressed comparable levels of ICAM-1 to those detected on blood and bone-marrow neutrophils (Fig. 7a). Using ICAM-1 expression as a means of detecting rTEM neutrophils *in vivo*, we next sought to investigate the presence of rTEM neutrophils in the circulation of mice subjected to I-R injury. For these studies the cremaster muscle model of I-R injury and a more severe model of lower limb I-R injury were employed. Both of these models exhibited lung inflammation as manifested by tissue infiltration of neutrophils and or edema formation as measured through enhanced vascular permeability to i.v. injected Evans blue dye (Fig. 7b and c). Within these inflammatory reactions a small percentage of ICAM-1^{hi} neutrophils (with expression comparable to that quantified for rTEM neutrophils generated *in vitro*) were detected in washouts of the lung pulmonary circulation post I-R injury (Fig. 7d). Compared to ICAM-1^{lo} pulmonary vascular washout neutrophils (and lymphocytes which were used as a negative control), ICAM-1^{hi} cells exhibited significantly higher levels of ROS, indicating that the latter form a distinct subset of activated neutrophils (Fig. 7e). Both the percentage and total number of these ICAM^{hi} lung vascular washout neutrophils was significantly higher in I-R injured groups as compared to sham operated control mice (Fig. 7f,g and data not shown). These data demonstrate that the elevated levels of ICAM-1^{hi} neutrophils in the pulmonary vascular washouts was not simply governed by an overall increase in the total number of neutrophils in the lung vasculature post I-R injury. Pre-treatment of mice with the anti-JAM-C mAb H33 to enhance the incidence of neutrophil rTEM (Fig. 6), led to 83 % increase in the number of ICAM-1^{hi} neutrophils in the lung

vasculature of mice subjected to cremaster muscle I-R injury (Fig. 7f). Finally, there was a significant association between the percentage of ICAM-1^{hi} neutrophils within the pulmonary vasculature of I-R stimulated animals and the extent of lung inflammation as indicated by the presence of tissue infiltrated neutrophils (Fig. 7g). Taken together, these findings show that rTEM neutrophils are more responsive with respect to ROS generation, re-enter the circulation and can be detected in a distant organ post local I-R injury and that the presence of these cells is associated with second organ tissue inflammation (Supplementary Fig. 6).

DISCUSSION

Through development and application of an advanced 4D imaging method with high spatiotemporal resolution we have analyzed the mode and dynamics of neutrophil TEM in inflamed tissues *in vivo*. In response to several diverse inflammatory stimuli neutrophil TEM predominantly occurs via the paracellular route (~90%) but multiple forms of this response were observed. Specifically, as well as polarized neutrophil TEM, neutrophils were observed to exhibit abluminal to luminal EC junctional motility. In investigating the mechanism associated with the latter, this study identifies EC JAM-C as a critical molecule in supporting polarized neutrophil TEM *in vivo*. Furthermore, the study provides evidence to suggest that neutrophils undergoing reverse TEM contribute to dissemination of systemic inflammation and secondary organ tissue damage.

Despite tremendous interest and recent progress in our understanding of the mechanisms that mediate leukocyte transmigration through venular walls, many aspects of this response within *in vivo* settings remain unknown. A key limiting factor here has been the lack of sufficiently advanced imaging modalities for clear tracking of leukocytes through different components of blood vessel walls. To aid our on-going investigations in this area we have developed an imaging platform rigorously optimized for unambiguous tracking and analysis of leukocyte TEM through cremasteric venules of *Lys-EGFP-ki* mice (which express GFP-labelled neutrophils and monocytes). This method enabled us to investigate the frequency and dynamics of paracellular and transcellular neutrophil TEM in straight venular segments as induced by a range of diverse inflammatory stimuli, IL-1β, fMLP and in response to I-R injury. All three stimuli elicited a largely paracelluar TEM reaction (~90%) with similar frequency of these events occurring via bi-cellular and multi-cellular junctions for all stimuli. Previous studies have indicated that tri-cellular junctions act as the preferred sites of leukocyte TEM through cultured ECs because of the discontinuity of key junctional molecules such as occludin, ZO-1, cadherin, and β-catenin 28. There is also evidence for this phenomenon *in vivo* especially in convergence venular sites where due to differences in EC morphology (less ordered alignment of ECs), there is a greater number of tri-cellular EC junctions as compared to straight venular regions 29.

The chemotactic formylated peptide fMLP has previously been reported to induce neutrophil TEM in guinea-pig skin and mouse lip via the transcellular route 6, 30, but neither of these studies involved quantitative comparison of transcellular vs. paracellular TEM responses. Although it is potentially possible that the relative modes of neutrophil TEM in these tissues is in line with our findings in the mouse cremaster muscle, it is also likely that the heterogeneous nature of ECs in different tissues may govern the mode and mechanism of leukocyte TEM in different vascular beds. For example the association of caveolin-1 expression with transcellular leukocyte TEM 10, 31 suggests that tissues with high levels of EC caveolin-1 (e.g. skin) may support a greater level of transcellular TEM 31. However, analysis of the duration of TEM responses within mouse cremasteric venules showed that irrespective of the stimulus or the route, all TEM events occurred with similar dynamics

(completing within ~6 minutes), implying the involvement of common molecular pathways in paracellular and transcellular TEM, in line with recent evidence 7, 9.

Although our findings indicated paracellular TEM to be the principal mode of leukocyte transmigration, rigorous analysis of our imaging files identified unexpected modes of junctional motility characterized by abluminal to luminal migration. Specifically three forms of paracellular TEM were identified: 'normal', in which leukocytes migrated through EC junctions in a luminal to abluminal direction without pause, 'hesitant', in which leukocytes exhibited bi-directional movement within junctions (~2-3 oscillations in a luminal to abluminal direction) before entering the sub-EC space, and 'reverse', in which leukocytes migrated in an abluminal to luminal direction before disengaging from the junction and crawling on the luminal surface. Reverse TEM may be a more severe form of hesitant TEM and these responses have been collectively termed here as disrupted polarized paracelluar events. The occurrence of these responses was governed by the inflammatory reaction in that their frequency was low in tissues stimulated with IL-1β and fMLP (<5%) but was markedly elevated in tissues subjected to I-R injury (~15%). Using a number of approaches we demonstrated that the leukocytes exhibiting disrupted forms of polarized paracellular TEM were neutrophils. Although reported for other leukocyte sub-types, most notably for monocytes 20, 32, neutrophil reverse TEM is a contentious subject but there is evidence for its occurrence *in vivo* using a zebra fish model 33, 34 and through cultured human ECs 27.

In addressing the mechanism associated with disrupted polarized paracellular responses we observed that these events were most pronounced under conditions were there was reduced functional expression of the adhesion molecule JAM-C from EC junctions. Specifically, significant levels of disrupted polarized neutrophil TEM were noted following I-R injury, an inflammatory reaction that led to reduced EC junctional expression of JAM-C. The latter appeared to be mediated at least in part by generation of reactive oxygen species. Furthermore, interference with JAM-C expression and or function led to a dramatic enhancement of the frequency of disrupted polarized paracellular TEM events in response to I-R injury. These interventions also led to significant levels of disrupted polarized paracellular TEM in response to IL-1β (~20%), an inflammatory reaction which in control mice did not induce disruption of EC JAM-C expression. Together, these results provide direct and conclusive evidence that EC JAM-C plays a key role in maintaining a luminal to abluminal neutrophil paracellular TEM response *in vivo*. Blockade of JAM-A did not result in enhanced frequency of disrupted neutrophil TEM, indicating the specificity of JAM-C in regulating this process. Furthermore, the present results are in agreement with previous findings showing that blockade of EC JAM-C leads to enhanced reverse TEM of monocytes through cultured ECs 20. Although there is ample evidence supporting a role for JAM-C in leukocyte TEM 35, details of how this response is mediated remains unknown. Taken together with previous findings it appears that JAM-C can mediate neutrophil TEM via at least three modes: mediating migration of cells through EC junctions by providing an adhesive ligand for neutrophil Mac-1 24, 25, regulation of endothelial adherens junctions and barrier integrity 36, and finally by regulating the directionality of neutrophil migration through EC junctions in a luminal to abluminal direction. Exactly how EC JAM-C supports polarized neutrophil TEM is at present unclear but may involve stimulating migrating leukocytes to exhibit shape-change, extend protrusions into EC junctions and/or respond to chemotactic gradients, key components of polarized cell migration. JAM-C may also mediate neutrophil migration in a luminal to abluminal direction through regulation of expression and/or localization of EC chemokines.

Due to the relative prevalence of neutrophil rTEM under conditions of I-R injury, we sought to investigate the association of this process with systemic consequences characteristic of an I-R insult such as lung inflammation, a common and adverse outcome of major surgeries

and trauma 26. In line with previous studies using human neutrophils 27, we now report that murine neutrophils that have undergone rTEM *in vitro* have a distinctive ICAM-1^{hi} phenotype in contrast to blood, bone-marrow and normal transmigrated neutrophils that are ICAM-1^{lo}. Using this characteristic phenotype, rTEM neutrophils were detected in the lung vasculature of mice subjected to cremaster muscle or lower limb I-R injury. The retrieved ICAM-1^{hi} neutrophils were primed for enhanced ROS generation and their presence in the pulmonary vasculature was associated with lung inflammation. These results suggest that rTEM neutrophils may contribute to turning a local inflammatory response into a systemic multi-organ response. Although we cannot exclude the possibility that ICAM-1^{hi} neutrophils detected in the pulmonary vasculature may have originated from the lung tissue itself, such an event would also require rTEM of neutrophils, yet again supporting our hypothesis that the response of neutrophil rTEM is associated with second organ inflammation. Hence, by providing a direct link between the response of neutrophil rTEM and the pathogenesis of I-R injury, the data presented supports the hypothesis that as well as other factors such as circulating inflammatory mediators, neutrophils undergoing reverse TEM that are primed for enhanced ROS generation contribute to dissemination of systemic inflammation and secondary organ tissue damage. These results are in line with findings of a recent study performed in zebrafish embryos showing that rTEM neutrophils can be detected in multiple organs post local inflammation 34. It is also potentially possible that rTEM may provide a physiological means of dampening down an inflammatory reaction 33, 37 and/or may be associated with chronic inflammatory conditions 27, 37.

Collectively we have reported on the occurrence of disrupted polarized paracellular neutrophil TEM under conditions of reduced availability of functional JAM-C at EC junctions. These results suggest that previously reported anti-inflammatory effects of JAM-C blockade/deletion *in vivo* 21, 25, 38 may be partly accounted for via enhanced neutrophil rTEM, highlighting the need for more detailed analysis of TEM dynamics in different inflammatory scenarios and vascular beds. Through associating rTEM with dissemination of systemic inflammatory responses, the study also highlights the need for further investigations into the occurrence, mechanisms and implications of neutrophil reverse transmigration in other tissues and inflammatory reactions.

METHODS

The principal *in vivo* imaging technique employed is described below, for other details see Supplementary Methods. All experiments were carried out under UK legislation for the protection of animals and approved by the Ethical Review Process of Queen Mary, University of London.

Confocal intravital microscopy of mouse cremaster muscles

Mice expressing endogenously labeled GFP-leukocytes (*Lys-EGFP-ki, CX3CR1-EGFP-ki*, Tie-2Cre;JAM-C$^{flox/flox}$;*Lys*-EGFP-*ki* and JAM-C$^{flox/flox}$;*Lys-EGFP-ki* littermate controls) were used. Cremaster muscles were inflamed as detailed in Supplementary Information and EC junctions were labeled through co-administration of Alexa Fluor-555-labeled anti-PECAM-1 mAb (clone 390; 3μg i.s.). Where necessary, anti-JAM-C or anti-JAM-A mAbs or sJAM-C (3mg/kg), or a SOD and catalase cocktail (2,000 and 50,000 U/kg) were administered i.v. immediately prior to induction of inflammation.

Straight post-capillary venules of 20-40 μm in diameter were selected for analysis of leukocyte-vessel wall interactions. Z-stacks of images were captured by confocal microscopy using a single beam Leica TCS-SP5 confocal laser-scanning microscope equipped with argon and helium-neon lasers and incorporating a 20X water dipping objective (NA 1.0; Leica, Milton Keynes, UK). The microscope incorporates an optical

zoom function and most image sequences were captured at ~40-60X final magnification. Images were acquired with sequential scanning of the 488 nm (to capture GFP^+ leukocytes) and 561 nm (to capture Alexa Fluor-555-anti-PECAM-1 labeled EC junctions) channels at a resolution of 512×024 pixels, corresponding to a voxel size of approximately 0.25×0.25×0.7 μm in the *x/y/z* planes, respectively. Stacks of images of ~1μm optical sections were routinely acquired at 1 min intervals, and using the incorporated 8,000 Hz resonance scanner, acquiring a single Z stack of ~60 images would routinely take ~40 seconds. This approach enabled acquisition of full 3D confocal images of venules, yielding the generation of high resolution 4D (real-time in 3D) video clips of dynamic events. In the majority of preparations tissues were stable and movement from the heartbeat did not interfere with image acquisition. Slower movements resulting from muscular contraction-relaxation occasionally resulted in movement of the vessel of interest in *x/y/z* directions, and a drift correction algorithm was applied during the 3D reconstruction stage using the 4D modeling software Imaris (Bitplane, Zurich Switzerland).

Following acquisition, the sequences of Z-stack images were analyzed using the Leica software (LAS-AF Lite, Leica) or more commonly the image stacks were linked and subsequently analyzed off-line using Imaris which renders the optical sections into 3D models enabling analysis of the dynamics of leukocyte-vessel wall interactions. All images and resultant video clips show half vessels to allow the luminal surface of the endothelium to be seen clearly. Confocal microscopy images are traditionally shown as 'maximum intensity projections' in which each channel is 'transparent' and as such a labeled object with one colour 'behind' a second object of another colour will still be visible in the projection. As the present work is concerned with the movement of objects in relation to each other the images were displayed as opaque 3D objects which enable the observer to determine where the objects are in relation to each other. With this approach, the development of an inflammatory reaction and leukocyte TEM into tissues could be observed with high clarity, exhibiting remarkable temporal and spatial resolution, as exemplified in Supplementary Video 1. All scale bars are 10 μm.

Analysis of GFP- or Alexa Fluor-555 anti-CD115-labeled (following i.v. mAb) leukocytes in terms of intensity profiles, as illustrated in Figure 6b, was carried out using the LAS-AF Leica software to quantify the intensity in Gray values per μm^2 of 2D projections of cells.

Analysis of leukocyte behaviors

Images acquired from the confocal intravital microscopy studies were analyzed for profile and dynamics of leukocyte TEM. In every video clip, only TEM events that were imaged in full in terms of their duration through the EC barrier and were clearly visible in terms of their location and dynamics were analyzed. Occurrence of quantified responses was thus presented as the mean % of total TEM events observed/vessel under observation. Routinely, ~ 50-100 TEM events from up to 4 venules/animal and from a minimum of 4 mice were analyzed per treatment.

As the focus of the present study was on TEM, the imaging settings employed (detailed above) were optimized for acquisition of TEM events from which the following parameters were quantified: (a) ***Paracellular TEM***: A paracellular TEM was identified through the occurrence of EC junctional disruption (labeled using anti-PECAM-1 mAb), i.e. occurrence of transient pores within the PECAM-1 staining during a TEM event. The association of such events with bi-cellular EC contacts or at junctions between three or more ECs was noted and quantified. (b) ***Transcellular TEM***: A transcellular TEM was identified as an event associated with the transient occurrence of pores within the EC body (stained faintly and diffusely with anti-PECAM-1) but not associated with disruption of PECAM-1-labeled EC junctions. Such responses could be observed either clearly separate from EC junctions or

adjacent to a junction but with no visible junctional disruption occurring during TEM. (c) ***Duration of TEM:*** The duration of TEM events was calculated as the time between the first frame in which disruption of EC or cell body PECAM-1 labeling could be seen to the frame in which the leukocyte being tracked had fully traversed the EC barrier. (d) ***'Reverse' TEM***: This response was defined as an event where the movement of a leukocyte from an abluminal to luminal direction followed by disengagement from the junction into the lumen could be observed. (e) ***'Hesitant' TEM***: The definition of this previously un-observed phenomenon was established following the careful observation of many such events under conditions of I-R injury. The event was defined as the occurrence of a bi-directional movement of a leukocyte within a junction (~2-3 oscillations), with an abluminal end point at which time the leukocyte disengaged from the EC junction, apparently continuing its passage through the venular wall although the possibility that these cells subsequently exhibited a reverse TEM beyond the field of view cannot be excluded.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Professor Stephen J. Weiss for critical reading of the manuscript, Dr Rashmi Yadav for advice on surgical procedures and Dr Helen M. McGetterick for contributions to analysis of *in vitro* generated rTEM neutrophils. This work was supported by funds from the Wellcome Trust (Ref: 081172/Z/06/Z to S.N.). S.N.'s work contributes to the translational research portfolio of Barts and the London Cardiovascular Biomedical Research Unit, supported and funded by the NIHR. The study is also supported by the Swiss National Science Foundation (310000-122423, 310000-109402, CR32I3_129987 for P.M. and 310030-120184 for B.A.I.), the Juvenile Diabetes Research Foundation (40-2011-11 for P.M.), the European Union (BETAIMAGE 222980; IMIDIA, C2008-T7 for P.M.) and the NIH Intramural Research Program, NCI, and the Deutsche Forschungsgemeinschaft (for T.C.).

References

1. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat. Rev. Immunol. 2007; 7:678–689. [PubMed: 17717539]
2. Nourshargh S, Hordijk PL, Sixt M. Breaching multiple barriers: leukocyte motility through venular walls and the interstitium. Nat. Rev. Mol. Cell Biol. 2010; 11:366–378. [PubMed: 20414258]
3. Hurley JV. An electron microscopic study of leucocytic emigration and vascular permeability in rat skin. Aust. J. Exp. Biol. Med. Sci. 1963; 41:171–186. [PubMed: 13955841]
4. Marchesi VT, Florey VT. Electron micrographic observations on the emigration of leucocytes. Q. J. Exp. Physiol Cogn Med. Sci. 1960; 45:343–348. [PubMed: 13766495]
5. Muller WA. Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. Trends Immunol. 2003; 24:327–334. [PubMed: 12810109]
6. Feng D, Nagy JA, Pyne K, Dvorak HF, Dvorak AM. Neutrophils emigrate from venules by a transendothelial cell pathway in response to FMLP. J. Exp. Med. 1998; 187:903–915. [PubMed: 9500793]
7. Carman CV, et al. Transcellular diapedesis is initiated by invasive podosomes. Immunity. 2007; 26:784–797. [PubMed: 17570692]
8. Cinamon G, Shinder V, Shamri R, Alon R. Chemoattractant signals and β2 integrin occupancy at apical endothelial contacts combine with shear stress signals to promote transendothelial neutrophil migration. J. Immunol. 2004; 173:7282–7291. [PubMed: 15585851]
9. Mamdouh Z, Mikhailov A, Muller WA. Transcellular migration of leukocytes is mediated by the endothelial lateral border recycling compartment. J. Exp. Med. 2009; 206:2795–2808. [PubMed: 19887395]
10. Millan J, et al. Lymphocyte transcellular migration occurs through recruitment of endothelial ICAM-1 to caveola- and F-actin-rich domains. Nat. Cell Biol. 2006; 8:113–123. [PubMed: 16429128]

11. Nieminen M, et al. Vimentin function in lymphocyte adhesion and transcellular migration. Nat. Cell Biol. 2006; 8:156–162. [PubMed: 16429129]
12. Yang L, et al. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-α-activated vascular endothelium under flow. Blood. 2005; 106:584–592. [PubMed: 15811956]
13. Marmon S, Cammer M, Raine CS, Lisanti MP. Transcellular migration of neutrophils is a quantitatively significant pathway across dermal microvascular endothelial cells. Exp. Dermatol. 2009; 18:88–90. [PubMed: 19040450]
14. Christofidou-Solomidou M, Nakada MT, Williams J, Muller WA, DeLisser HM. Neutrophil platelet endothelial cell adhesion molecule-1 participates in neutrophil recruitment at inflammatory sites and is down-regulated after leukocyte extravasation. J. Immunol. 1997; 158:4872–4878. [PubMed: 9144503]
15. Faust N, Varas F, Kelly LM, Heck S, Graf T. Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages. Blood. 2000; 96:719–726. [PubMed: 10887140]
16. Thompson RD, et al. Platelet-endothelial cell adhesion molecule-1 (PECAM-1)-deficient mice demonstrate a transient and cytokine-specific role for PECAM-1 in leukocyte migration through the perivascular basement membrane. Blood. 2001; 97:1854–1860. [PubMed: 11238129]
17. Jung S, et al. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. Mol. Cell Biol. 2000; 20:4106–4114. [PubMed: 10805752]
18. Dejana E. Endothelial cell-cell junctions: happy together. Nat. Rev. Mol. Cell Biol. 2004; 5:261–270. [PubMed: 15071551]
19. Muller WA. Mechanisms of transendothelial migration of leukocytes. Circ. Res. 2009; 105:223–230. [PubMed: 19644057]
20. Bradfield PF, et al. JAM-C regulates unidirectional monocyte transendothelial migration in inflammation. Blood. 2007; 110:2545–2555. [PubMed: 17625065]
21. Scheiermann C, et al. Junctional adhesion molecule-C mediates leukocyte infiltration in response to ischemia reperfusion injury. Arterioscler. Thromb. Vasc. Biol. 2009; 29:1509. [PubMed: 19574560]
22. Lamagna C, et al. Dual interaction of JAM-C with JAM-B and αMβ2 integrin: function in junctional complexes and leukocyte adhesion. Mol. Biol. Cell. 2005; 16:4992–5003. [PubMed: 16093349]
23. Nourshargh S, Krombach F, Dejana E. The role of JAM-A and PECAM-1 in modulating leukocyte infiltration in inflamed and ischemic tissues. J. Leukoc. Biol. 2006; 80:714–718. [PubMed: 16857733]
24. Santoso S, et al. The junctional adhesion molecule 3 (JAM-3) on human platelets is a counterreceptor for the leukocyte integrin Mac-1. J. Exp. Med. 2002; 196:679–691. [PubMed: 12208882]
25. Chavakis T, et al. The junctional adhesion molecule-C promotes neutrophil transendothelial migration in vitro and in vivo. J. Biol. Chem. 2004; 279:55602–55608. [PubMed: 15485832]
26. Tsukamoto T, Chanthaphavong RS, Pape HC. Current theories on the pathophysiology of multiple organ failure after trauma. Injury. 2010; 41:21–26. [PubMed: 19729158]
27. Buckley CD, et al. Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. J. Leukoc. Biol. 2006; 79:303–311. [PubMed: 16330528]
28. Burns AR, et al. Neutrophil transendothelial migration is independent of tight junctions and occurs preferentially at tricellular corners. J. Immunol. 1997; 159:2893–2903. [PubMed: 9300713]
29. Sumagin R, Sarelius IH. Intercellular adhesion molecule-1 enrichment near tricellular endothelial junctions is preferentially associated with leukocyte transmigration and signals for reorganization of these junctions to accommodate leukocyte passage. J. Immunol. 2010; 184:5242–5252. [PubMed: 20363969]
30. Hoshi O, Ushiki T. Scanning electron microscopic studies on the route of neutrophil extravasation in the mouse after exposure to the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP). Arch. Histol. Cytol. 1999; 62:253–260. [PubMed: 10495880]

31. Marmon S, et al. Caveolin-1 expression determines the route of neutrophil extravasation through skin microvasculature. Am. J. Pathol. 2009; 174:684–692. [PubMed: 19164603]
32. Randolph GJ, Furie MB. Mononuclear phagocytes egress from an in vitro model of the vascular wall by migrating across endothelium in the basal to apical direction: role of intercellular adhesion molecule 1 and the CD11/CD18 integrins. J. Exp. Med. 1996; 183:451–462. [PubMed: 8627158]
33. Mathias JR, et al. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish. J. Leukoc. Biol. 2006; 80:1281–1288. [PubMed: 16963624]
34. Yoo SK, Huttenlocher A. Spatiotemporal photolabeling of neutrophil trafficking during inflammation in live zebrafish. J. Leukoc. Biol. 2011
35. Bradfield PF, Nourshargh S, Aurrand-Lions M, Imhof BA. JAM family and related proteins in leukocyte migration. Arterioscler. Thromb. Vasc. Biol. 2007; 27:2104–2112. [PubMed: 17615384]
36. Orlova VV, Economopoulou M, Lupu F, Santoso S, Chavakis T. Junctional adhesion molecule-C regulates vascular endothelial permeability by modulating VE-cadherin-mediated cell-cell contacts. J. Exp. Med. 2006; 203:2703–2714. [PubMed: 17116731]
37. Mulero V, Sepulcre MP, Rainger GE, Buckley C. Neutrophils live on a two-way street. Journal of Leukocyte Biology. 2011; 89
38. Aurrand-Lions M, et al. Junctional adhesion molecule-C regulates the early influx of leukocytes into tissues during inflammation. J. Immunol. 2005; 174:6406–6415. [PubMed: 15879142]

Europe PMC Funders Author Manuscripts

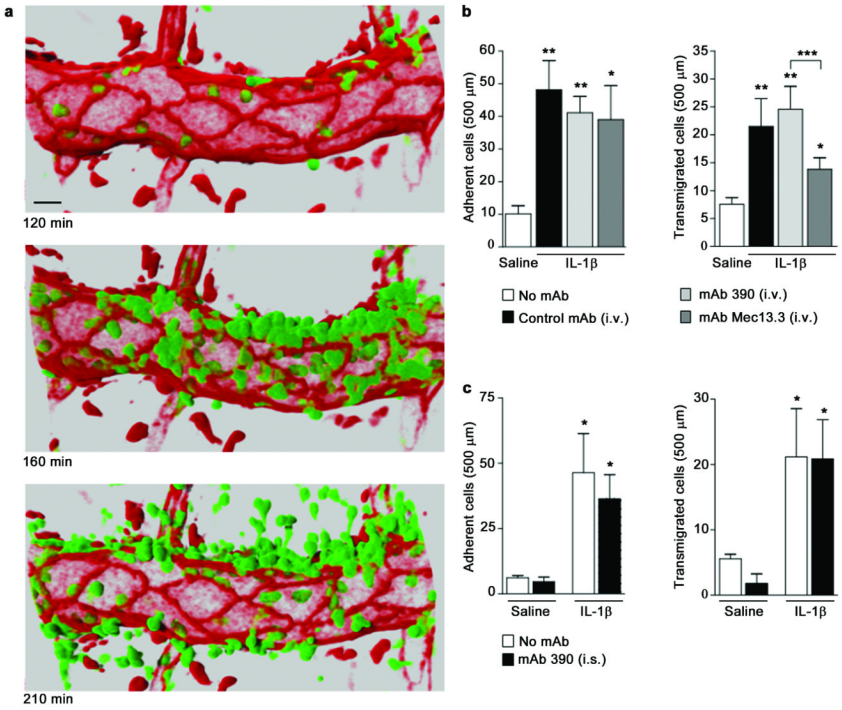

Figure 1. Development of a 4D imaging platform for analysis of leukocyte TEM *in vivo*

Cremasteric venules of *Lys-EGFP-ki* mice (exhibiting green leukocytes) were immunostained *in vivo* for EC junctions with intrascrotal (i.s.) injection of Alexa Fluor-555-labeled anti-PECAM-1 mAb 390 (PECAM-555; 3μg/mouse; shown in red), and were stimulated with i.s. IL-1β (50 ng/mouse). 2 h later tissues were surgically exteriorised and images were captured at 40x magnification by *in vivo* confocal intravital microscopy (IVM) at 1min intervals for a period of 90min. **(a)** Panels show images of a post-capillary venular segment immediately after tissue exteriorisation (at t=120, 160, and 210 min post IL-1β application), showing the development of an inflammatory response (Supplementary Video 1). **(b)** and **(c)** show that intravenous (i.v.) or i.s. mAb 390 has no effect on leukocyte adhesion or transmigration induced by IL-1β as analyzed by brightfield IVM. **(b)** WT mice were pretreated with i.v. mAb 390, Mec13.3 (a blocking anti-PECAM-1 mAb), an IgG2b isotype control mAb (all at 3mg/kg) prior to i.s. administration of IL-1β, or were untreated. 4 h later, tissues were exteriorized and leukocyte responses of adhesion and transmigration were quantified by IVM. **(c)** WT mice were injected with i.s. saline or IL-1β, with or without i.s. Alexa Fluor-555-labeled mAb 390 (3μg). After 4 h leukocyte adhesion and transmigration responses were quantified by IVM (n = 3-8 animals, 3-5 vessels per animal. Error bars show S.E.M). Statistically significant responses in IL-1β-stimulated tissues as compared to saline-injected cremaster muscles are indicated by asterisks, *P<0.05 and **P<0.01. Other statistical comparisons are shown by ***P<0.001.

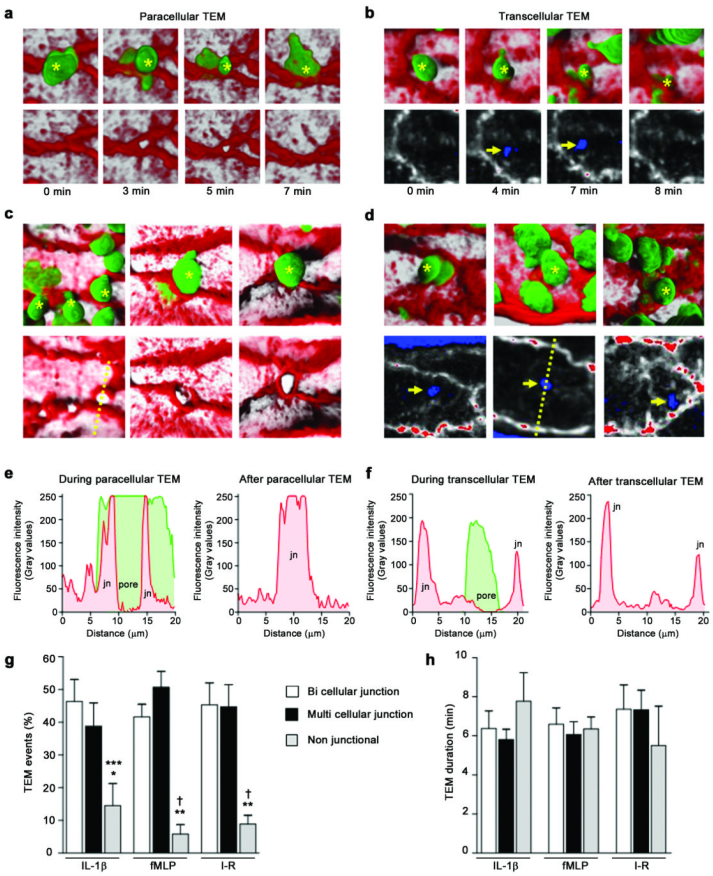

Figure 2. Analysis of neutrophil paracellular and transcellular TEM *in vivo*

TEM responses were imaged in IL-1β-stimulated PECAM-1-labelled (red) tissues of *Lys-EGFP-ki* mice (leukocytes shown in green). **(a)** Paracellular TEM response of a leukocyte (*, upper panels) and its associated transient junctional pore formation (lower panels) (Supplementary Video 3). **(b)** Transcellular TEM event illustrating migration through ECs with no disruption of PECAM-1 enriched junctions. False color images of the PECAM-1 channel (high and low intensity sites shown in white and blue, respectively) enable visualization of the transcellular pore (arrow) (Supplementary Video 4). **(c)** Additional examples of paracellular TEM and **(d)** transcellular TEM events. Some transcellular pores occurred in close proximity to EC junctions without disrupting PECAM-1-labelled junctions (right panel). **(e-f)** Linear intensity profiles of PECAM-555 (EC; red) and GFP (leukocyte; green) of TEM events along the indicated dotted lines shown in **(c)** and **(d)**. **(e)** The paracellular pore is immediately flanked by high PECAM-1-labelled junctions (Jn), while in **(f)** the transcellular pore is formed in the EC body where lower PECAM-1 labeling is observed. Intensity profiles post TEM illustrates pore closures. Scale bars = 10μm. The frequency **(g)** and duration **(h)** of TEM responses as induced by multiple stimuli was quantified (n > 103 TEM events observed in 4-7 mice; mean ± S.E.M). Statistical significance between frequency of non-junctional and bi-cellular TEM events (*P < 0.01 and ** P < 0.001) and between non junctional and multi cellular locations (***P < 0.05 and †P < 0.001) are indicated.

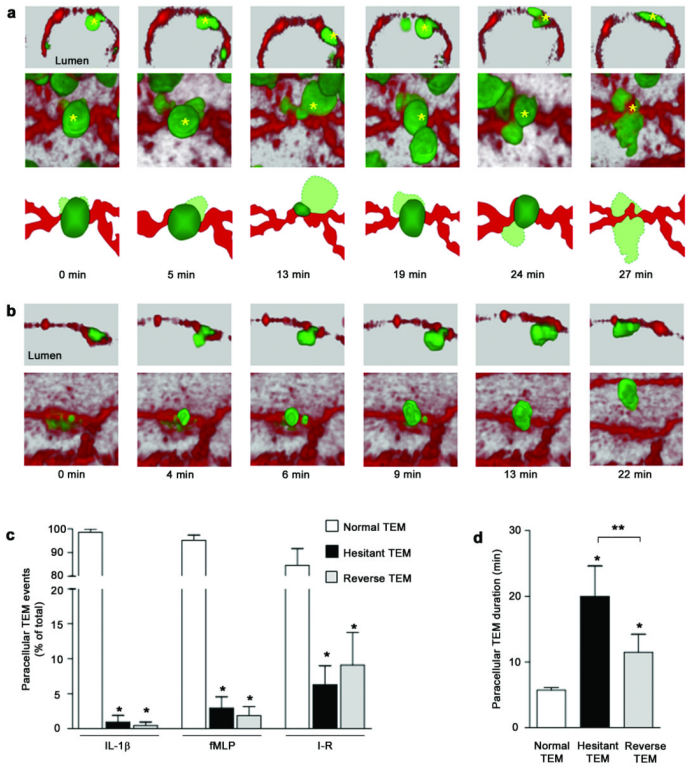

Figure 3. Observation and quantification of disrupted forms of polarized paracellular TEM

Disrupted polarised paracellular TEM behaviours, termed "hesitant" and "reverse" TEM were observed. **(a)** Time-lapse images of a GFP-leukocyte (*) exhibiting hesitant paracellular TEM. Top panels show a transverse section of the venule, middle panels show the event from the luminal side as supported by illustrative drawings (lower panels), showing the sub-EC segments of the migrating leukocyte in light green with dashed outline (Supplementary Video 5). Additional examples are shown in Supplementary Figure 2 and **Supplementary Videos** 6 and 7. **(b)** Time-lapse images of a leukocyte exhibiting reverse TEM. The leukocyte migrates through a bi-cellular junction in an abluminal to luminal direction, disengages from the junction and crawls away on the luminal surface. Top panels show transverse sections of the venule and bottom panels show the event from the luminal side (Supplementary Video 8). **(c)** Frequency of normal, hesitant and reverse paracellular TEM as induced by multiple stimuli expressed as a % of total paracellular TEM events ($n \leq 103$ TEM events as observed in 4-7 mice per inflammatory reaction). **(d)** The duration of normal, hesitant and reverse TEM events in I-R injured tissues were analyzed. Statistical differences between normal and disrupted (hesitant and reverse) TEM events (*$P < 0.001$), and differences between hesitant and reverse TEM events (**$P < 0.001$) are indicated. Data are shown as mean ± S.E.M.

Europe PMC Funders Author Manuscripts

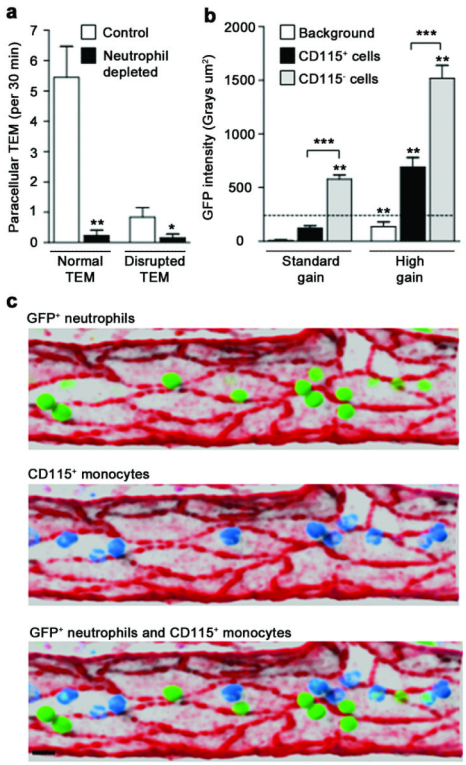

Figure 4. Neutrophils exhibit disrupted forms of polarized paracellular TEM

A number of approaches demonstrated that within the employed models the leukocyte sub-type that exhibited disrupted forms of polarized TEM were neutrophils. **(a)** I-R-induced TEM was analyzed in control and neutrophil depleted *Lys-EGFP-ki* mice (Supplementary Figure 4a). The frequency of normal and disrupted TEM events (both reverse and hesitant) per 30 min (standard image sequence capture time) is shown (results are from n=7-9 animals). **(b)** The GFP intensity of monocytes ($CD115^+$; n=81) and neutrophils ($CD115^-$; n=158) (Supplementary Figure 4b) in CCL2-stimulated *Lys-EGFP-ki* mouse cremaster muscles (n = 4 animals) was quantified from 2D projections at standard (routinely employed and optimized for analysis of GFP^+ neutrophils) and high GFP gain image capture settings, and compared to the threshold intensity for visibility in 3D reconstructions (indicated by a dotted line; intensity of ~200 Grays/μm^2). **(c)** A 3D reconstruction image of a CCL2-stimulated *Lys-EGFP-ki* mouse cremasteric venule, with anti-PECAM-1-labeled EC junctions (red), acquired using standard GFP gain settings. The image shows the visibility of GFP^+ neutrophils (green) but not monocytes, immunostained with i.v. anti-CD115 mAb (blue), using the standard GFP settings. Statistically significant differences between control and neutrophil depleted mice (*P<0.05 and **P<0.01), or between background and leukocyte-associated fluorescence intensity (**P<0.01 and ***P<0.001) are shown. Data are shown as mean ± S.E.M. Scale bars = 10 μm.

Europe PMC Funders Author Manuscripts

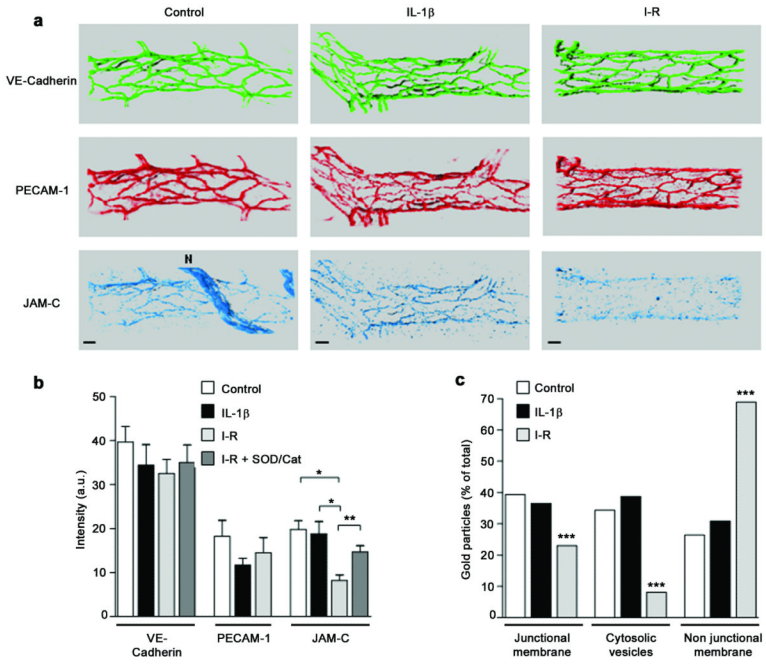

Figure 5. JAM-C is disrupted from EC junctions in response to I-R injury but not IL-1β

The expression and localisation of JAM-C was investigated on post capillary cremasteric venules in un-stimulated tissues, IL-1β-stimulated tissues and tissues subjected to I-R injury by immunofluorescence staining and immunoelectron microscopy. One group of mice was also pretreated with superoxide dismutase (SOD) and catalase (2,000 and 50,000 U/kg respectively, i.v.) prior to induction of I-R injury. **(a)** Cremasteric venules of WT mice were immunostained *ex-vivo* for VE-Cadherin (green), PECAM-1 (red) and JAM-C (blue; a JAM-C^{+} nerve [N] is also shown). Images are representative from n = 7-15 mice/treatment group, 4-10 vessels per mouse. Scale bars=10 μm. The fluorescence intensity at EC junctions was quantified (see Supplementary Methods) and presented in (**b**). Statistically significant differences in protein intensity between stimulus and treatment groups were analysed by ANOVA and are indicated by asterisks *P<0.05 and **P<0.01. Data are shown as mean ± S.E.M. **(c)** The distribution of JAM-C on ECs was also analyzed by immunoelectron microscopy in control tissues (saline-injected or sham-operated) and cremaster muscles stimulated with IL-1β or I-R injury. Examples of images acquired are shown in Supplementary Fig. 5a and b. Quantification revealed that JAM-C was significantly displaced from junctional regions in I-R, but not IL-1β-stimulated tissues (17-33 ECs from n=2-5 mice/group were quantified and analyzed by Chi-square test, ***P<0.001).

Europe PMC Funders Author Manuscripts

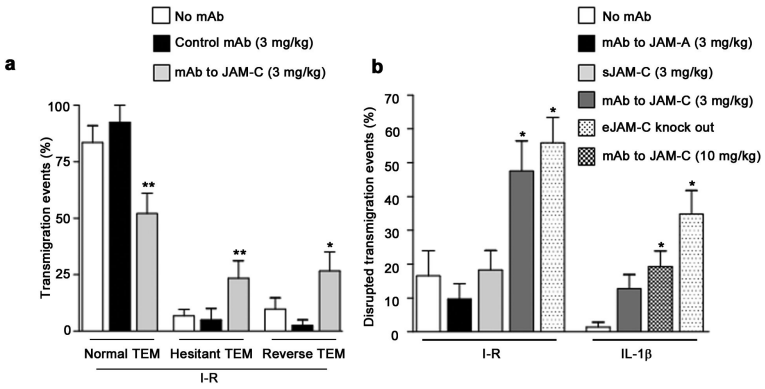

Figure 6. EC JAM-C plays a critical role in mediating polarized neutrophil paracellular TEM

The functional role of EC JAM-C in mediating disrupted polarized forms of paracellular TEM was investigated through pharmacological blockade and genetic reduction of EC JAM-C. **(a)** *Lys-EGFP-ki* mice were subjected to I-R injury and different groups of mice were pretreated with i.v. saline, a control non-blocking anti-JAM-C mAb (H36) or with a blocking anti-JAM-C mAb (H33) (mAbs given at 3mg/kg). The occurrence of normal, hesitant and reverse TEM responses was quantified and presented as a % of total paracellular TEM events. Statistically significant differences in data sets, as analyzed by multinomial logistic regression analysis, are shown by asterisks (*P<0.05 and **P<0.01). **(b)** The occurrence of all disrupted TEM events (hesitant and reverse) expressed as a % of total paracellular responses as induced by I-R injury in control *Lys-EGFP-ki* mice and *Lys-EGFP-ki* mice pretreated with anti-JAM-A mAb, anti-JAM-C mAb or soluble JAM-C (all given i.v. at 3 mg/kg) and *Lys-EGFP-ki* mice crossed with Tie2Cre:JAM-C$^{flox/flox}$ mice (exhibiting EC JAM-C reduction and statistically compared with littermate controls exhibiting normal EC JAM-C) are shown. These results are compared with % of disrupted TEM events in IL-1β-stimulated tissues in un-treated and anti-JAM-C mAb pretreated *Lys-EGFP-ki* mice and in *Lys-EGFP-ki* mice crossed with Tie2Cre:JAM-C$^{flox/flox}$ mice. Results are from 43-109 TEM events as quantified in n = 4-10 mice. Data are shown as mean ± S.E.M. Statistically significant differences as analysed by ANOVA, between control and experimental groups are indicated by asterisks (*P<0.05).

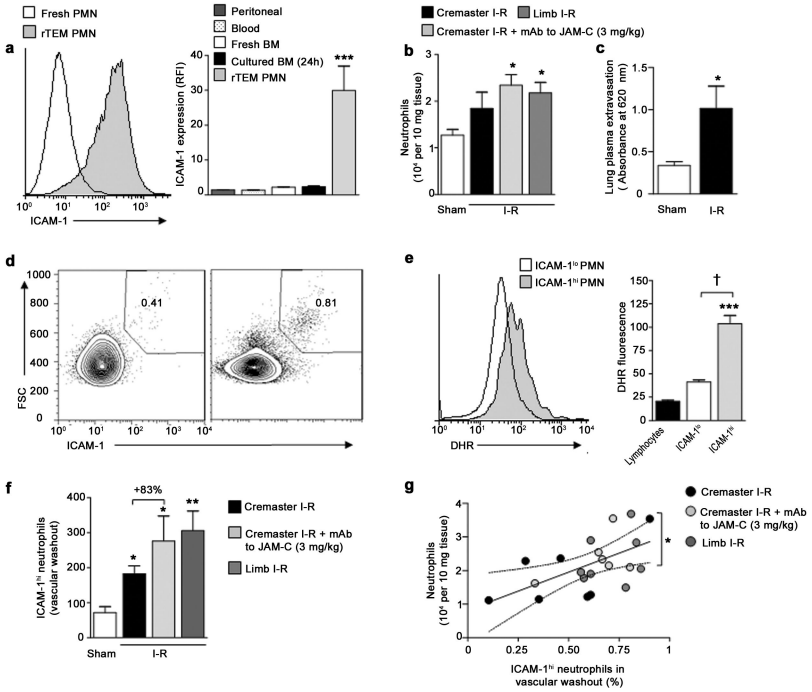

Figure 7. Reverse transmigrated neutrophils are associated with pulmonary inflammation following I-R injury

(a) Reverse transmigrated (rTEM) bone-marrow (BM) murine neutrophils (PMN) were generated *in vitro* (Supplementary Methods) and ICAM-1 expression on rTEM and fresh PMN as well as multiple other PMN preparations was analysed by flow cytometry (right panel; n≥3 preparations). **(b-g)** Cremasteric and lower limb I-R procedures were performed. Post I-R, mice were exsanguinated and pulmonary vascular washout leukocytes collected. **(b)** Lung tissue neutrophil infiltrate was quantified by flow cytometry of digested and homogenised tissues. **(c)** Oedema formation was quantified by local accumulation of i.v. injected Evans blue. **(d)** Pulmonary vascular washout neutrophils from sham (left) and limb I-R (right) animals were analysed for ICAM-1 expression by flow cytometry and examples of scatter plots of ICAM-1 and FSC profiles are shown. The numbers in the gates demonstrate the greater % of ICAM-1^{hi}/FSC^{hi} population in the I-R group. **(e)** Pulmonary vascular washout ICAM-1^{lo} and ICAM-1^{hi} neutrophils were analysed for ROS generation (both panels) with lymphocytes acting as a negative control (right). **(f)** The total number of ICAM-1^{hi} neutrophils in the pulmonary vascular washout of mice subjected to sham and I-R protocols was analysed. **(g)** Collation of all I-R stimulated groups shows a positive correlation between the % of ICAM-1^{hi} pulmonary vascular and the number of tissue infiltrated neutrophils. Data are shown as mean ± S.E.M (n= 6–9 animals per group). Statistical differences between control and experimental groups, and correlations, are shown with asterisks (*P<0.05, **P<0.01, ***P<0.001). Additional comparisons are indicated by lines (†P<0.001).