

The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils *in vivo*

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The migration of neutrophils into inflamed tissues is a fundamental component of innate immunity. A decisive step in this process is the polarized migration of blood neutrophils through endothelial cells (ECs) lining the venular lumen (transendothelial migration (TEM)) in a luminal-to-abluminal direction. By real-time confocal imaging, we found that neutrophils had disrupted polarized TEM ('hesitant' and 'reverse') *in vivo*. We noted these events in inflammation after ischemia-reperfusion injury, characterized by lower expression of junctional adhesion molecule C (JAM-C) at EC junctions, and they were enhanced by blockade or genetic deletion of JAM-C in ECs. Our results identify JAM-C as a key regulator of polarized neutrophil TEM *in vivo* and suggest that reverse TEM of neutrophils can contribute to the dissemination of systemic inflammation.

The migration of neutrophils from the vascular lumen to the extravascular tissue is a vital component of the host's defense 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 polarized manner^{1,2}. This process involves distinct cellular responses that begin with the capture of free-flowing leukocytes from the circulation and the formation of weak adhesive interactions with ECs, which results in the rolling of leukocytes along the venular wall. The 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 a complex series of overlapping molecular interactions involving selectins and integrins and their respective ligands¹.

The migration of neutrophils through ECs (transendothelial 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 studies of transcellular TEM have triggered many subsequent investigations into this phenomenon that have used mainly *in vitro* models; these have collectively provided insight into the characteristics and mechanisms of this mode of TEM⁷⁻¹². For example, invasive leukocyte protrusions

seeking permissive sites and EC structures such as caveolae and the membranous compartment that connects to the cell surface at cell borders (the lateral border recycling compartment), which acts 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 such studies, fundamental aspects of this response, including profile, frequency, dynamics and stimulus specificity in direct comparison with paracellular TEM, have not been investigated in real time *in vivo*.

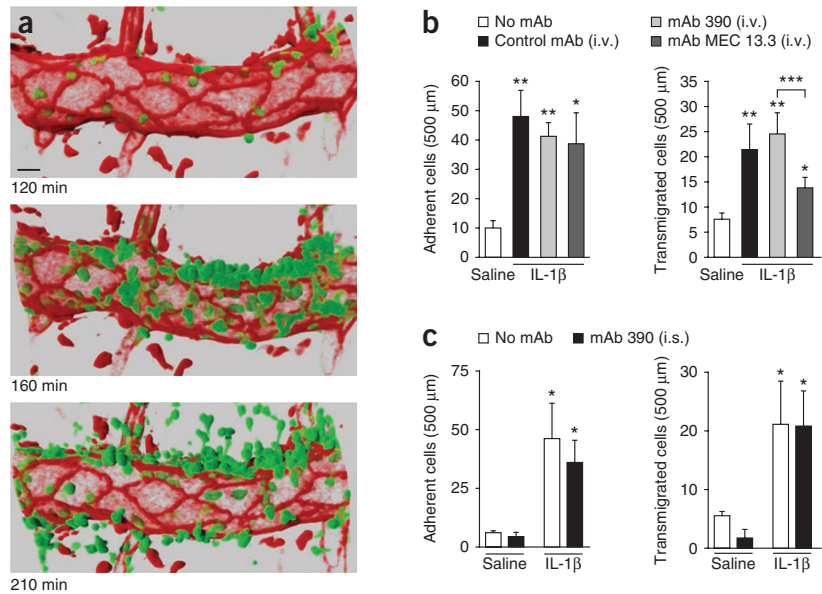
To further examine the mechanisms by which neutrophils breach venular walls, we have established a confocal intravital microscopy imaging platform for the three-dimensional observation of leukocyte transmigration in real time (four-dimensional imaging). The exceptional spatial and temporal resolution 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, because of the enhanced clarity with which leukocyte TEM can be tracked by this method, we observed neutrophils that unexpectedly migrated through EC junctions in an abluminal-to-luminal direction (for example, showed 'reverse TEM' (rTEM)). We observed such disrupted polarized neutrophil paracellular TEM responses relatively selectively under conditions of ischemia-reperfusion (I-R), an inflammatory insult that caused lower expression of JAM-C at EC junctions. The frequency of these responses was enhanced after pharmacological blockade or genetic deletion of EC JAM-C. The pathophysiological relevance of

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Figure 1 Development of a four-dimensional imaging platform for the analysis of leukocyte TEM *in vivo*. **(a)** Confocal intravital microscopy of cremasteric venules of lys-EGFP-ki mice (green leukocytes) immunostained *in vivo* for EC junctions by intrascrotal injection of Alexa Fluor 555-labeled mAb 390 to PECAM-1 (red) and stimulated for 120 min by intrascrotal injection of IL-1 β , followed by surgical exteriorization and capture of images *in vivo* at intervals of 1 min for a period of 90 min (from 120 to 210 min after IL-1 β injection), showing the development of an inflammatory response in a post-capillary venular segment (**Supplementary Video 1**). Original magnification, $\times 40$. Scale bar, 10 μ m. **(b,c)** Brightfield intravital microscopy of leukocyte adhesion and transmigration in wild-type mice given no pretreatment (No mAb) or pretreated intravenously **(b)** or intrascrotally **(c)** with mAb 390, mAb MEC 13.3 or IgG2b isotype-matched control mAb, then left untreated (saline) or given intrascrotal administration of IL-1 β , followed by exteriorization of tissues 4 h later. * $P < 0.05$ and ** $P < 0.01$, IL-1 β versus saline and *** $P < 0.001$ (analysis of variance (ANOVA)). Data are representative of six experiments **(a)** or are from three to eight **(b)** or three to five **(c)** experiments per group (one mouse per experiment; error bars **(b,c)**, s.e.m.).



our findings was demonstrated by evidence suggesting that rTEM of neutrophils stemming from a primary site of trauma (for example, after I-R injury) was associated with the development of injury in a second organ. Collectively our findings provide direct evidence that EC JAM-C has a key role in supporting the luminal-to-abluminal migration of neutrophils *in vivo* and suggest that rTEM of neutrophils can contribute to the dissemination of systemic inflammation.

RESULTS

Four-dimensional imaging for analysis of leukocyte TEM *in vivo*

To accurately investigate the profile and dynamics of leukocyte TEM *in vivo*, we established a four-dimensional imaging platform with advanced spatial and temporal resolution. A key component of the successful application of this imaging method was the reproducible and adequate labeling of EC junctions for *in vivo* fluorescence microscopy imaging. As preliminary studies indicated that intravenous injection of fluorescence-labeled monoclonal antibody (mAb) to PECAM-1 did not result in sufficiently uniform or strong labeling of EC contacts for accurate tracking of the route of leukocyte transmigration, it was necessary to develop an alternative protocol. Intrascrotal administration of directly labeled Alexa Fluor 555-conjugated mAb 390 to PECAM-1, a mAb that does not inhibit leukocyte transmigration¹⁴, resulted in strong and reliable labeling of EC borders in cremasteric venules (**Fig. 1a** and **Supplementary Fig. 1**). As well as showing junctional staining, labeled ECs also showed faint and diffuse cell-body expression of PECAM-1 on the luminal and abluminal surfaces, which did not seem 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 confirmed that both the junctional and nonjunctional endothelial labeling was specific (**Supplementary Fig. 1d**).

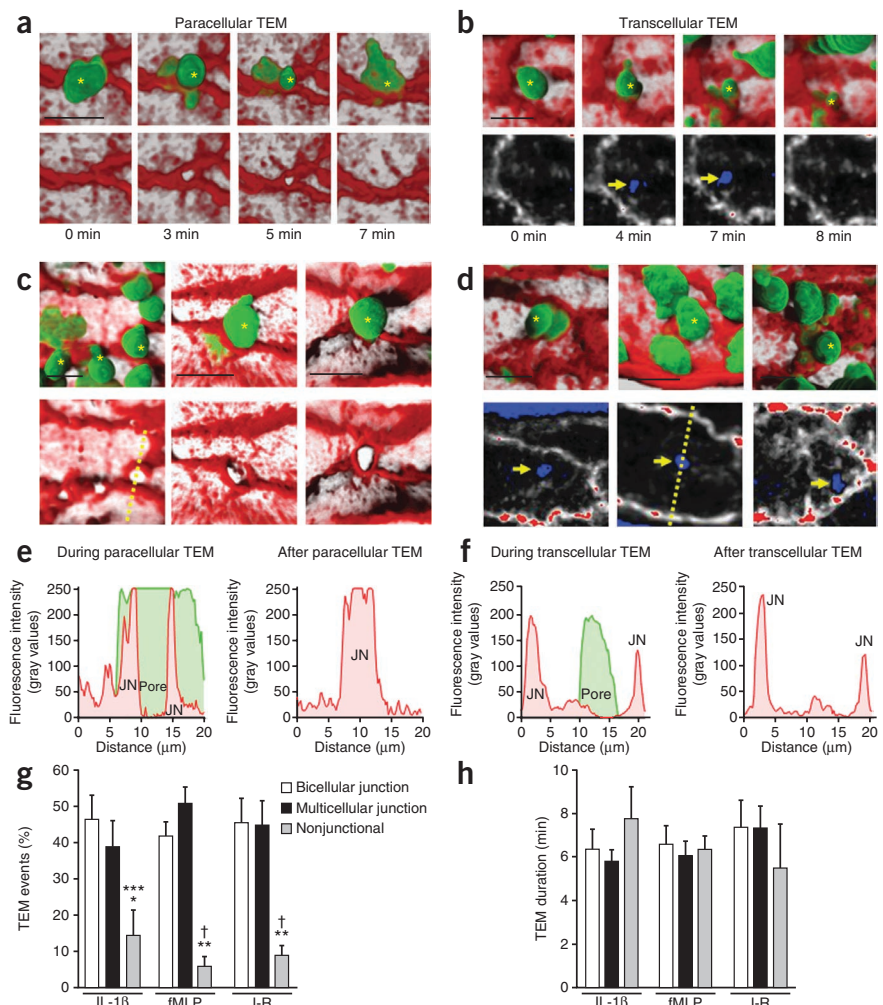
In addition to labeling ECs, we also labeled some extravascular cells with this approach, although these cells did not express PECAM-1, as demonstrated by analysis of tissues from PECAM-1-deficient mice and use of isotype-matched control mAbs (**Supplementary Fig. 1d**). An additional advantage of labeling EC junctions by intrascrotal injection of labeled mAb to PECAM (rather than the intravenous route) is

that this approach failed to label circulating leukocytes and thus provided a cleaner and hence more clear-cut mode of tracking the interactions of leukocytes with EC junctions (**Supplementary Fig. 1e,f**). Although we selected mAb 390 as the mAb to PECAM of choice because it has been shown not to suppress leukocyte TEM *in vivo*¹⁴, we did rigorous analysis to ensure that this new vascular labeling protocol had no effect 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 did not effect leukocyte transmigration as induced by interleukin 1 β (IL-1 β). As expected, the functional blocking mAb MEC 13.3 to PECAM (used as whole molecule) blocked TEM induced by IL-1 β (**Fig. 1b,c**). Intrascrotal injection of Alexa Fluor 555-labeled F(ab')₂ fragment of mAb 390 also effectively labeled EC junctions, but because the dynamics of leukocyte TEM as assessed by this approach were not substantially different from those noted when we used the whole mAb (data not shown), we used the latter in all subsequent experiments. Finally, fluorescence labeling of EC junctions by the approach described above, in conjunction with laser excitation of tissues at our standard confocal microscopy settings, did not elicit leukocyte TEM in unstimulated tissues, in contrast to the TEM observed in IL-1 β -treated tissues (data not shown). We applied the imaging approach and the EC junction-labeling method described above to lys-EGFP-ki mice, which express enhanced green fluorescent protein (GFP) driven by the promoter of the gene encoding lysozyme M and thus have endogenously labeled neutrophils and monocytes¹⁵. This allowed detailed spatiotemporal analysis of leukocyte TEM (**Fig. 1a** and **Supplementary Videos 1** and **2**).

Inflammation triggers mainly paracellular TEM *in vivo*

To mimic and analyze the effects of physiological and pathological insults on leukocyte TEM, we subjected cremaster muscles to the following three different types of stimuli: the proinflammatory cytokine IL-1 β ; the chemotactic formylated tripeptide fMLP; or I-R injury. Within the *in vivo* test periods (~2–4 h), the reactions induced by these stimuli were neutrophilic in nature, as indicated by published electron microscopy studies¹⁶ and analysis of infiltrates in stimulated tissues by immunofluorescence staining (data not shown).

Figure 2 Neutrophil paracellular and transcellular TEM *in vivo*. **(a)** Paracellular TEM of a leukocyte (*; top row) and its associated transient junctional pore formation (bottom row) in IL-1 β -stimulated, PECAM-1-labeled tissues (red) of lys-EGFP-ki mice (leukocytes, green; time (below images) relative to **Supplementary Video 3**). **(b)** Transcellular TEM through ECs with no disruption of PECAM-1-enriched junctions, in tissues as in **a** (top row); below, false-color images of the PECAM-1 channel (white, high intensity; blue, low intensity) for visualization of the transcellular pore (arrows); time (below images) relative to **Supplementary Video 4**. **(c,d)** Paracellular TEM **(c)** and transcellular TEM **(d)** in tissues as in **a**. Right, transcellular pores in close proximity to EC junctions without disruption of PECAM-1-labeled junctions. Dotted yellow lines indicate areas analyzed further in **e,f**. Scale bars **(a–d)**, 10 μ m. **(e,f)** Linear intensity profiles of the PECAM-1 channel (EC; red) and GFP (leukocyte; green) of TEM events along the dotted lines in **c,d**; intensity profiles after TEM illustrate pore closure. JN, junction. **(g,h)** Frequency **(g)** and duration **(h)** of TEM events induced by IL-1 β , fMLP or I-R. * $P < 0.01$ and ** $P < 0.001$, nonjunctional versus bicellular and *** $P < 0.05$ and $\dagger P < 0.001$, nonjunctional versus multicellular (ANOVA). Data are representative of four to seven experiments **(a)** or are from four to seven experiments with >103 TEM events per group **(e–h)**; one mouse per experiment; error bars **(g,h)**, s.e.m.).



In straight venular segments, the predominant mode of TEM was paracellular, where we noted the formation of a pore at EC contacts between two or multiple adjacent cells (**Fig. 2a** and **Supplementary Videos 2** and **3**).

A minority of TEM events analyzed (~10%) occurred via nonjunctional routes; during these, we noted leukocytes breaching the body of the endothelium, which resulted 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 (additional examples of such TEM events with linear intensity profiles for each pore type, **Fig. 2c–f**). The linear intensity profiles showed a paracellular pore immediately flanked by junctions with abundant PECAM-1 labeling (**Fig. 2e**), whereas a transcellular pore formed in the EC body with PECAM-1 labeling of lower intensity (**Fig. 2f**). We analyzed the frequency and dynamics of neutrophil TEM via junctional and nonjunctional routes for all three stimuli and found that ~90% of the observed TEM events were via the paracellular route, with no significant difference between bicellular or multicellular EC junctions or between different stimuli (**Fig. 2g**). The mean duration of each type of TEM response (bicellular, multicellular or transcellular) was ~6 min, with no significant difference between routes or stimuli (**Fig. 2h**). Collectively these results show that in our model, TEM induced by a variety of distinct proinflammatory stimuli occurred predominantly via the paracellular route, with no significant difference in the profile or dynamics of the observed responses.

Inflammation triggers several forms of paracellular TEM

Although paracellular was the predominant mode of leukocyte TEM, we observed various forms of this response. Most paracellular

TEM events involved the normal passage of leukocytes through EC junctions in a luminal-to-abluminal direction with no pause, but a smaller proportion of transmigrating leukocytes showed either rTEM or what seemed to be a multidirectional or 'hesitant' mode of paracellular TEM (**Fig. 3**). In 'hesitant' TEM, leukocytes moved back and forth in the EC junction several times (about two to three oscillations) before finally completing migration into the sub-EC space (**Fig. 3a** and **Supplementary Video 5**; additional examples of hesitant TEM, **Supplementary Fig. 2** and **Supplementary Videos 6** and **7**). In rTEM, leukocytes migrated through EC junctions in the abluminal-to-luminal direction, disengaged from the junction and crawled away from the junction across the luminal surface of the endothelium (**Fig. 3b** and **Supplementary Video 8**). We analyzed the frequency of these various forms of paracellular TEM in tissues stimulated with IL-1 β , fMLP and I-R injury and noted different profiles with different stimuli (**Fig. 3c**). IL-1 β -stimulated tissues showed relatively little rTEM or hesitant TEM (~3% of all paracellular events quantified), whereas in tissues subjected to I-R injury, ~15% of paracellular TEM events were hesitant TEM or rTEM. The difference in the frequency of these responses under different inflammatory conditions was not governed by the overall magnitude of the inflammatory responses, as there was no substantial difference in the absolute number of leukocytes that transmigrated in response to IL-1 β or I-R injury (**Supplementary Fig. 3**). Quantification of the duration of the various forms of paracellular TEM in tissues stimulated by I-R

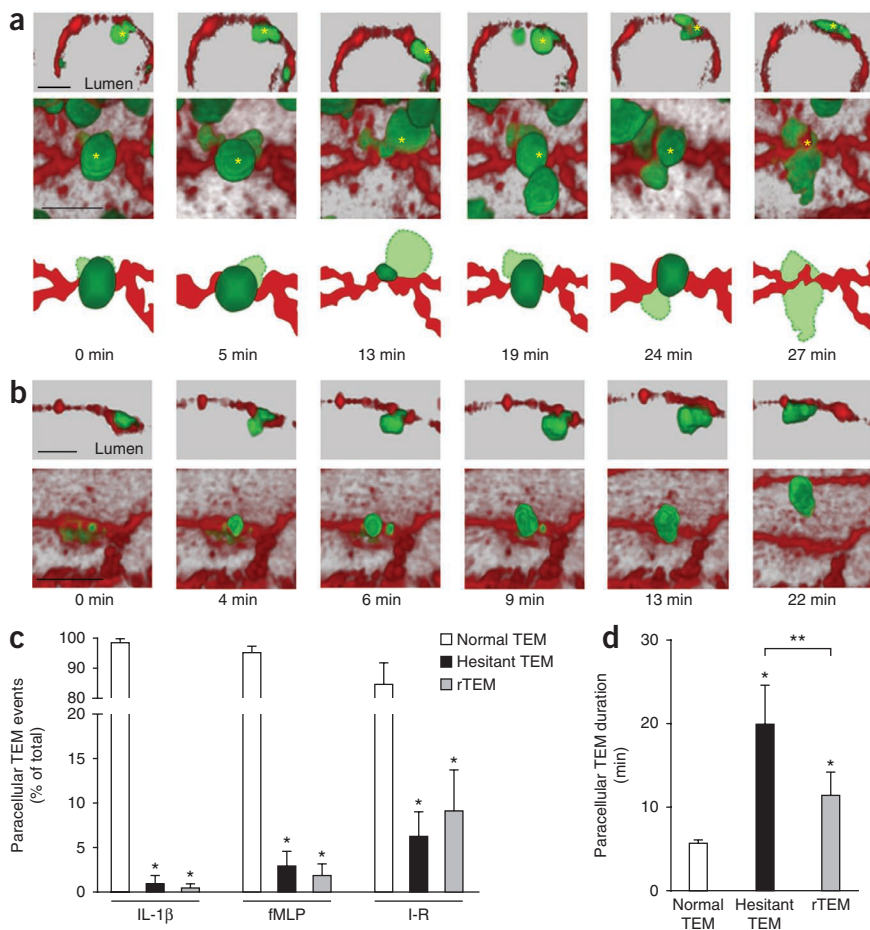


Figure 3 Disrupted forms of polarized paracellular TEM. **(a)** Time-lapse images of a GFP-expressing leukocyte (*) undergoing hesitant paracellular TEM: top, transverse section of venule; middle, luminal view; bottom, sub-EC segments of the migrating leukocyte in light green with dashed outline (time (below images) relative to **Supplementary Video 5**; additional examples, **Supplementary Fig. 2** and **Supplementary Videos 6** and **7**). **(b)** Time-lapse images of a leukocyte undergoing rTEM as it migrates through a bicellular junction in an abluminal-to-luminal direction, disengages from the junction and crawls away on the luminal surface: top, transverse section of venule; bottom, luminal view (time (below images) relative to **Supplementary Video 8**). **(c)** Frequency of normal, hesitant and reverse paracellular TEM events induced by IL-1 β , fMLP or I-R, presented as frequency among total paracellular TEM events. **(d)** Duration of normal, hesitant and rTEM events in tissues injured by I-R. Scale bars **(a,b)**, 10 μ m. * P < 0.001, normal versus disrupted (hesitant TEM and rTEM) and ** P < 0.001, hesitant TEM versus rTEM (ANOVA). Data are representative of seven experiments **(a,b)** or are from four to seven experiments with >103 TEM events per group **(c,d)**; one mouse per experiment; error bars **(c,d)**, s.e.m.).

indicated that hesitant TEM and rTEM were significantly slower than normal paracellular responses (**Fig. 3d**).

Neutrophils show disrupted polarized TEM

We next sought to further investigate the profile of hesitant TEM and rTEM, collectively called 'disrupted TEM' here, under conditions of I-R injury. Specifically, as we observed these events in I-R-injured tissues from lys-EGFP-ki mice, which express GFP in both neutrophils and monocytes¹⁵, it was important to elucidate which leukocyte subtype had disrupted TEM responses. We investigated the contribution of neutrophils and monocytes to the observed TEM events by various approaches, analyzing neutrophil-depleted lys-EGFP-ki mice (**Fig. 4a** and **Supplementary Fig. 4a**) and the differentiation of neutrophils from monocytes on the basis of significant differences in their GFP intensities (**Fig. 4b,c**, **Supplementary Fig. 4b** and **Supplementary Results**) and, finally, through the use of CX3CR1-GFP-ki mice, which express enhanced GFP in all monocytes but not in neutrophils¹⁷ (**Supplementary Fig. 4c**). In these last mice, I-R injury elicited very little normal paracellular TEM of GFP⁺ cells, and we detected no hesitant TEM or rTEM in >20 vessels analyzed. Collectively, these findings indicated that neutrophils were the sole participants in hesitant TEM and rTEM in our I-R injury model.

I-R injury lowers JAM-C expression at EC junctions

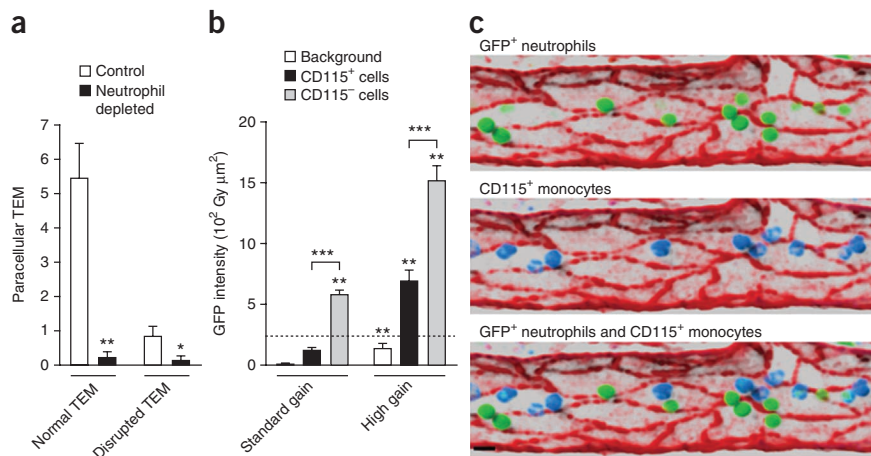
Having characterized the responses of disrupted TEM in terms of frequency, stimulus specificity, dynamics and leukocyte subtype, we next addressed the mechanism associated with these events. We hypothesized that hesitant TEM and rTEM were associated with

disrupted expression of EC junctional molecules. To address this possibility, we used immunofluorescence staining and confocal microscopy to investigate the expression of vascular endothelial cadherin (VE-cadherin) and PECAM-1, key EC junctional molecules

linked to maintenance of the integrity of EC junctional contacts¹⁸ and the mediation of leukocyte TEM, respectively^{1,2,19}, in control, IL-1 β -stimulated and I-R-injured tissues. Furthermore, as JAM-C supports the polarized migration of monocytes through cultured ECs *in vitro*²⁰, we also analyzed the expression of this adhesion molecule. Under conditions of I-R injury, but not in response to IL-1 β , cell surface expression of JAM-C was lower at EC junctions. We noted no change in VE-cadherin or PECAM-1 with I-R injury or in response to IL-1 β (**Fig. 5a,b**). The loss of JAM-C at EC junctions after I-R injury was partially prevented by pretreatment of mice with superoxide dismutase and catalase (**Fig. 5b**), which indicated that reactive oxygen intermediates contributed to the lower junctional expression of JAM-C in response to I-R injury.

We also investigated the expression of JAM-C in inflamed tissues by immunoelectron microscopy (**Fig. 5c** and **Supplementary Fig. 5**). This approach confirmed published findings that JAM-C can be detected on ECs at the following three locations: junctional membranes, nonjunctional membranes and cytosolic vesicles²¹. In control (sham-operated and saline-injected) tissues, JAM-C was distributed almost equally among those three locations. In tissues subjected to I-R injury, there was less labeling of JAM-C at junctions and cytosolic vesicles, whereas labeling of the nonjunctional membrane was greater. In contrast and in agreement with our immunofluorescence staining studies, we noted no change in JAM-C expression in IL-1 β -stimulated tissues, as analyzed by immunoelectron microscopy (**Fig. 5c**). Together these findings demonstrated the ability of I-R injury, but not of IL-1 β , to induce lower expression of JAM-C at EC junctions, findings that were in line with the almost undetectable disrupted paracellular TEM induced by IL-1 β (**Fig. 3c**).

Figure 4 Disrupted forms of polarized paracellular neutrophil TEM. (a) Frequency of normal and disrupted TEM events (rTEM and hesitant TEM) induced by I-R in control and neutrophil-depleted lys-EGFP-ki mice, assessed over 30 min (standard image sequence capture time). (b) GFP intensity of monocytes (CD115⁺; $n = 81$) and neutrophils (CD115⁻; $n = 158$) in CCL2-stimulated cremaster muscles from lys-EGFP-ki mice ($n = 4$), quantified from two-dimensional projections at standard settings (routinely used and optimized for analysis of GFP⁺ neutrophils) and high-GFP-gain image-capture settings, compared with the threshold intensity for visibility in three-dimensional reconstructions (dashed line; ~ 200 Gy/ μm^2). (c) Three-dimensional reconstruction image of a CCL2-stimulated lys-EGFP-ki mouse cremasteric venule, with EC junctions labeled with antibody to PECAM-1 (red), acquired by standard GFP gain settings: green, GFP⁺ neutrophils; blue, monocytes immunostained with intravenous mAb to CD115. Scale bar, 10 μm . * $P < 0.05$ and ** $P < 0.01$, control versus neutrophil depletion (ANOVA) or *** $P < 0.01$ and **** $P < 0.001$, background versus leukocyte associated (ANOVA). Data are from seven to nine (a) or four experiments (b) or are representative of four experiments (c; one mouse per experiment; error bars (a,b), s.e.m.).



EC JAM-C expression mediates polarized neutrophil TEM

As hesitant TEM and rTEM of neutrophils occurred in tissues in which JAM-C expression at EC junctions was disrupted, we sought to investigate the functional consequence of blocking JAM-C at EC junctions. Because JAM-C is maintained at EC junctions through an interaction with JAM-B, and blocking the JAM-C–JAM-B interaction has been shown to result in lower JAM-C expression at EC junctions and enhanced luminal JAM-C expression *in vivo*^{21,22}, we investigated the effect of a mAb (H33) that blocks the JAM-C–JAM-B interaction. Mice pretreated with mAb H33 had a lower frequency of normal paracellular TEM of neutrophils and enhanced hesitant and rTEM of neutrophils in response to I-R injury ($\sim 50\%$), but in mice pretreated with the nonblocking mAb H36 to JAM-C there was no such effect (Fig. 6a). In mice pretreated with soluble JAM-C, a reagent shown

to suppress total leukocyte infiltration induced by I-R injury²¹, the duration of normal paracellular TEM was significantly greater (5.7 ± 3.5 min (control mice) and 11.6 ± 9.5 min (mice treated with soluble JAM-C); $n = 47\text{--}79$ TEM events in four mice), but there was no effect on frequency of disrupted polarized TEM (Fig. 6b). This was in contrast to TEM in mice treated with mAb H33, in which the frequency of disrupted TEM was greater but the duration of normal TEM was not affected (5.7 ± 3.5 min (control mice) and 7.0 ± 4.5 min (mice treated with mAb H33)). Antibody blockade of the related junctional adhesion molecule JAM-A²³ did not cause a greater frequency of disrupted polarized TEM (Fig. 6b).

The results reported above suggested that JAM-C can mediate neutrophil motility through EC junctions (suppressed by soluble JAM-C, presumably via blockade of the interaction of the integrin Mac-1 with JAM-C^{24,25}) and regulate the directionality of the migration of neutrophils through EC junctions in a luminal-to-abluminal direction

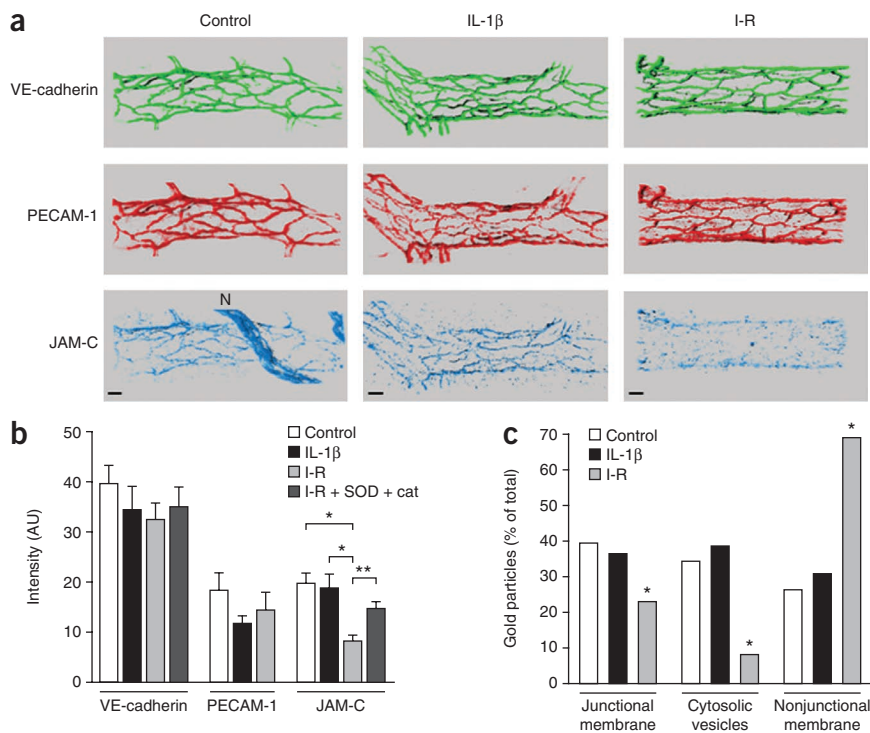
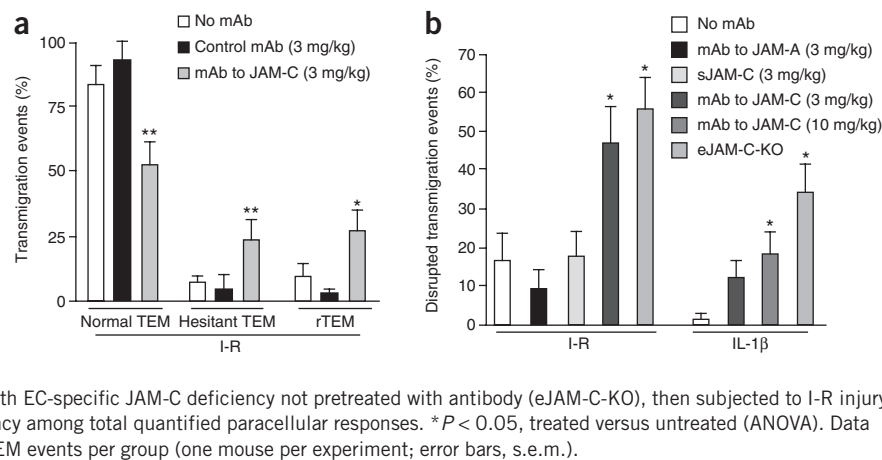


Figure 5 Disruption of JAM-C at EC junctions in response to I-R injury but not in response to IL-1 β . (a) Microscopy of cremasteric venules in wild-type mouse tissues left unstimulated (control), stimulated with IL-1 β or subjected to I-R injury and then immunostained *ex vivo* for VE-cadherin (green), PECAM-1 (red) or JAM-C (blue); N, JAM-C+ nerve. Scale bars, 10 μm . (b) Fluorescence intensity at EC junctions in mice treated as in a or pretreated with superoxide dismutase and catalase (SOD + cat) before the induction of I-R injury (Supplementary Methods). AU, arbitrary units. * $P < 0.05$ and ** $P < 0.01$, stimulus versus treatment (ANOVA). (c) Immunoelectron microscopy analysis of the distribution of JAM-C in ECs in control (saline-injected or sham-operated) tissues and cremaster muscles stimulated with IL-1 β or subjected to I-R injury (image examples and raw data, Supplementary Fig. 5a,b). * $P < 0.001$, I-R versus IL-1 β (chi-squared test). Data are representative of (a) or are from (b) seven to fifteen experiments with four to ten vessels per mouse or two to five experiments with 17–33 total ECs per group (c; one experiment per mouse; error bars (b,c), s.e.m.).

Figure 6 Critical role for EC JAM-C in mediating polarized neutrophil paracellular TEM. (a) Normal, hesitant and rTEM responses in lys-EGFP-ki mice pretreated with intravenous saline (no mAb), control nonblocking mAb H36 to JAM-C or blocking H33 mAb to JAM-C (each at a dose of 3 mg per kg body weight (3 mg/kg)), then subjected to I-R injury; results are presented as frequency among total observed paracellular responses. * $P < 0.05$ and ** $P < 0.01$ (multinomial logistic regression analysis). (b) All disrupted TEM events (hesitant TEM and rTEM) in lys-EGFP-ki mice given no pretreatment (no mAb) or pretreated with mAb to JAM-A, mAb to JAM-C or soluble JAM-C (all administered intravenously at a dose of 3 mg or 10 mg per kg body weight) and in lys-EGFP-ki mice with EC-specific JAM-C deficiency not pretreated with antibody (eJAM-C-KO), then subjected to I-R injury or treated with IL-1 β ; results are presented as frequency among total quantified paracellular responses. * $P < 0.05$, treated versus untreated (ANOVA). Data are from four to ten experiments with 52–109 total TEM events per group (one mouse per experiment; error bars, s.e.m.).



(suppressed by interventions that diminish expression of JAM-C at EC junctions, such as treatment with mAb H33). In agreement with that, offspring of lys-EGFP-ki mice crossed with mice with EC-specific JAM-C deficiency (we used Lys-EGFP-ki progeny with EC-specific JAM-C deficiency, which had on average 49% lower expression of EC JAM-C than did their wild-type littermates; data not shown) had >50% disrupted paracellular TEM in response to I-R injury (Fig. 6b), compared with ~8% in wild-type littermate control mice with normal EC JAM-C expression (data not shown). We also observed one incident of transcellular rTEM in these mice after I-R injury, which indicated that such a response can occur. However, its rarity (1 of >700

TEM events analyzed) suggested that this mode of TEM may not be of physiological or pathological importance.

Finally, in tissues stimulated with IL-1 β in which we did not observe disrupted TEM or lower JAM-C expression at EC junctions, pharmacological blockade of the JAM-C–JAM-B interaction or lowering EC JAM-C by genetic means also resulted in a greater frequency of disrupted TEM (~12–30%; Fig. 6b). A higher percentage of disrupted TEM events in mAb H33-treated mice was associated with a greater frequency and absolute number of disrupted TEM events (Supplementary Fig. 3), which demonstrated that blockade of JAM-C led to true enhancement of disrupted neutrophil TEM. Together these results indicate that JAM-C

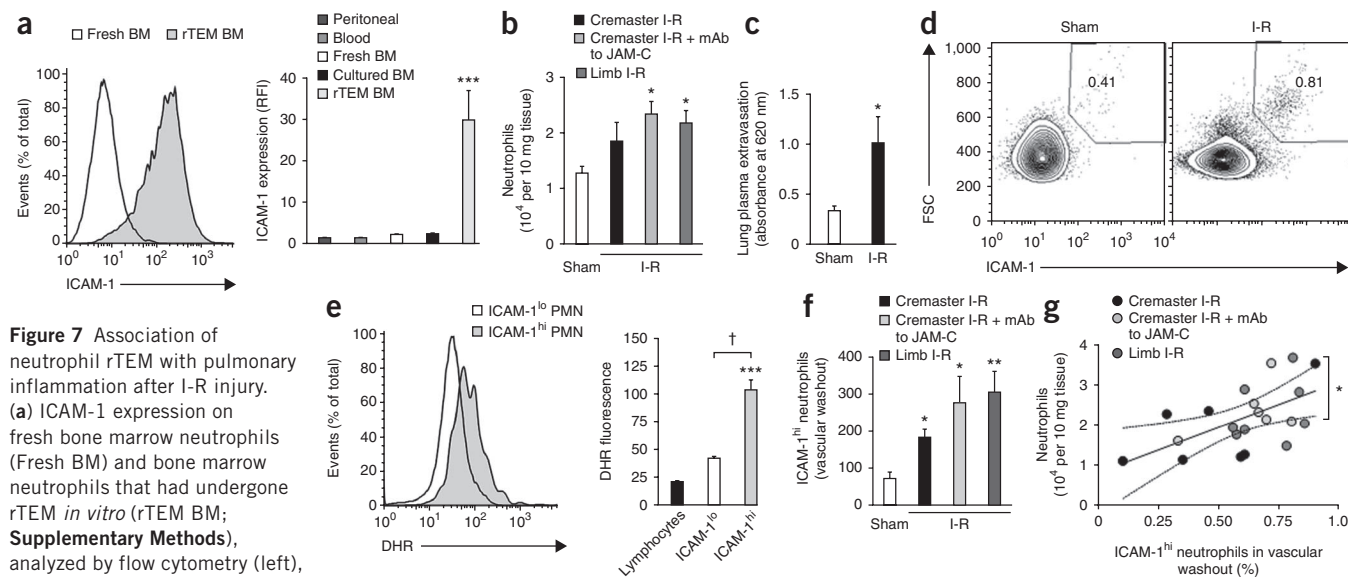


Figure 7 Association of neutrophil rTEM with pulmonary inflammation after I-R injury. (a) ICAM-1 expression on fresh bone marrow neutrophils (Fresh BM) and bone marrow neutrophils that had undergone rTEM *in vitro* (rTEM BM; Supplementary Methods), analyzed by flow cytometry (left), and quantification of ICAM-1

expression on peritoneal and blood neutrophils, fresh bone marrow neutrophils, bone marrow neutrophils cultured for 24 h (cultured BM) and bone marrow neutrophils that had undergone rTEM *in vitro*, presented as relative fluorescence intensity relative to (the binding of isotype-matched control mAb RFI; right). (b) Infiltration of lung tissue by neutrophils after sham operation or I-R injury of the cremaster muscle (cremaster I-R) or lower limb (limb I-R), with (light gray) or without (black and dark gray) treatment with mAb to JAM-C (3 mg/kg), quantified by flow cytometry of cells from digested and homogenized tissues. (c) Edema formation after sham operation or lower-limb I-R injury, assessed as local accumulation of intravenously injected Evans blue. (d) Flow cytometry analysis of ICAM-1 expression by neutrophils in pulmonary vascular washouts from mice subjected to sham operation or lower-limb I-R. FSC, forward scatter. Numbers in outlined areas indicate percent FSC^{hi}ICAM-1^{hi} cells. (e) Generation of ROS (quantified as the fluorescence intensity of dihydrorhodamine 123 (DHR) by ICAM-1^{lo} and ICAM-1^{hi} neutrophils in pulmonary vascular washouts of mice subjected to lower-limb I-R; lymphocytes serve as a negative control (right). (f) Total ICAM-1^{hi} neutrophils in pulmonary vascular washouts of mice subjected to sham operation or I-R injury with or without treatment with mAb to JAM-C (as in b). (g) Correlation of the frequency of ICAM-1^{hi} neutrophils in pulmonary vascular washouts and number of neutrophils infiltrating lung tissue in all mice subjected to I-R injury. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, control versus experimental and † $P < 0.001$ (ANOVA (a–f) or Spearman's rank correlation (g)). Data are representative of three experiments (a, left) or six experiments (e, left) or are from five to nine (a (right), b, f, g) or six (c, d, e) experiments per group (one mouse per experiment; error bars, s.e.m.).

expression has a key role in maintaining polarized neutrophil paracellular TEM and that under conditions in which JAM-C expression at EC junctions is lower, TEM occurs in a disrupted form, as shown by the hesitant TEM and rTEM of neutrophils.

Pathophysiological role of neutrophil rTEM

Finally, we sought to investigate the potential pathophysiological role of disrupted neutrophil TEM, the most extreme form of this being rTEM of neutrophils. As we observed the latter mainly 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 (for example, lung inflammation) and is believed to be mediated by multiple factors, including the generation of systemic inflammatory mediators and circulation of activated forms of neutrophils²⁶. As human neutrophils that undergo rTEM have been shown to have a distinct phenotype *in vitro* (high expression of ICAM-1 (ICAM-1^{hi})) and to have a greater ability to generate reactive oxygen species (ROS)²⁷, we investigated the potential association of neutrophils undergoing rTEM with lung inflammation after I-R injury. In an *in vitro* model, mouse bone marrow neutrophils that had transmigrated through cultured mouse ECs in the reverse direction were ICAM-1^{hi}, whereas bone marrow neutrophils that had not transmigrated and control bone marrow neutrophils cultured for 24 h and blood neutrophils were ICAM-1^{lo} (Fig. 7a). Of note, peritoneal neutrophils derived from an IL-1 β -driven peritonitis model that represented a pool of 'normal' transmigrated neutrophils were also ICAM-1^{lo} and had expression of ICAM-1 similar to that detected on blood and bone marrow neutrophils (Fig. 7a). Using ICAM-1 expression as a means of detecting neutrophils that had undergone rTEM *in vivo*, we next sought to investigate the presence of such neutrophils in the circulation of mice subjected to I-R injury. For these studies, we used the cremaster muscle model of I-R injury and a more severe model of lower-limb I-R injury. Both of these models showed lung inflammation, manifested by tissue infiltration of neutrophils and/or edema formation, as measured by enhanced vascular permeability to Evans blue dye injected intravenously (Fig. 7b,c). In these inflammatory reactions, we detected a small percentage of ICAM-1^{hi} neutrophils (with expression similar to that quantified for neutrophils generated *in vitro* that underwent rTEM) in washouts of the lung pulmonary circulation after I-R injury (Fig. 7d). ICAM-1^{hi} cells had significantly more ROS than did ICAM-1^{lo} neutrophils from pulmonary vascular washout (and lymphocytes, which we used as a negative control; Fig. 7e); this indicated that ICAM-1^{hi} neutrophils form a distinct subset of activated neutrophils. Both the percentage and total number of these ICAM^{hi} lung vascular washout neutrophils were significantly higher in I-R-injured groups than in sham-operated control mice (Fig. 7f,g and data not shown). These data demonstrate that the presence of more ICAM-1^{hi} neutrophils in the pulmonary vascular washouts was not governed simply by an overall increase in the total number of neutrophils in the lung vasculature after I-R injury. Pretreatment of mice with mAb H33 to JAM-C to enhance the incidence of neutrophil rTEM (Fig. 6) led to 83% more 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 frequency of ICAM-1^{hi} neutrophils in the pulmonary vasculature of I-R-stimulated mice and the extent of lung inflammation, as indicated by the presence of neutrophils that had infiltrated the lung tissue (Fig. 7g). Together these findings show that neutrophils that had undergone rTEM were more responsive in terms of ROS generation, re-entered the circulation and were detected in

a distant organ after local I-R injury, and that the presence of these cells was associated with tissue inflammation in a second organ (Supplementary Fig. 6).

DISCUSSION

Through the development and application of an advanced four-dimensional 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 occurred mainly via the paracellular route (~90%), but we observed many forms of this response. Specifically, as well as undergoing polarized TEM, neutrophils showed abluminal-to-luminal motility through the EC junction. In investigating the mechanism associated with the latter, we identified EC JAM-C as a molecule critical to the support of polarized neutrophil TEM *in vivo*. Furthermore, our study has provided evidence to suggest that neutrophils undergoing rTEM contribute to the dissemination of systemic inflammation and tissue damage in secondary organs.

Despite tremendous interest and progress in the understanding of the mechanisms that mediate the transmigration of leukocytes through venular walls, many aspects of this response in *in vivo* settings remain unknown. A key limiting factor has been the lack of sufficiently advanced imaging modalities for distinct tracking of leukocytes through various components of blood vessel walls. To aid our ongoing investigations in this area, we have developed an imaging platform rigorously optimized for unambiguous tracking and analysis of the TEM of leukocytes through cremasteric venules of lys-EGFP-ki mice (which express GFP-labeled neutrophils and monocytes). This method enabled us to investigate the frequency and dynamics of paracellular and transcellular neutrophil TEM in straight venular segments induced by a range of diverse inflammatory stimuli, including IL-1 β , fMLP and I-R injury. All three stimuli elicited mainly paracellular TEM (~90%), with similar frequency of these events occurring via bicellular and multicellular junctions for all stimuli. Published studies have indicated that tricellular junctions are the 'preferred' sites for the TEM of leukocytes through cultured ECs because of the discontinuity of key junctional molecules, such as occludin, ZO-1, cadherin and β -catenin²⁸. There is also evidence for this phenomenon *in vivo*, especially in convergence venular sites where, because of differences in EC morphology (less ordered alignment of ECs), there are more tricellular EC junctions than straight venular regions²⁹.

The chemotactic formylated peptide fMLP has been reported to induce neutrophil TEM via the transcellular route in guinea pig skin and mouse lips^{6,30}, but neither of those studies involved quantitative comparison of transcellular and paracellular TEM. Although it is potentially possible that the relative modes of neutrophil TEM in those tissues were in line with our findings obtained with 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 expression of caveolin-1 in ECs (for example, skin) may support more transcellular TEM³¹. However, analysis of the duration of TEM in mouse cremasteric venules showed that regardless of the stimulus or the route, all TEM events occurred with similar dynamics (were complete within ~6 min), which suggests the involvement of common molecular pathways in paracellular and transcellular TEM, in line with published evidence^{7,9}.

Although our findings indicated paracellular TEM was 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, we identified the following three forms of paracellular TEM: normal TEM, in which leukocytes migrated through EC junctions in a luminal-to-abluminal direction without pause; hesitant TEM, in which leukocytes showed bidirectional movement in junctions (about two to three oscillations in a luminal-to-abluminal direction) before entering the sub-EC space; and rTEM, in which leukocytes migrated in an abluminal-to-luminal direction before disengaging from the junction and crawling on the luminal surface. This rTEM may have been a more severe form of hesitant TEM, and we have collectively called these responses 'disrupted polarized paracellular events' here. The occurrence of these responses was governed by the inflammatory reaction, as their frequency was low in tissues stimulated with IL-1 β and fMLP (<5%) but was much greater in tissues subjected to I-R injury (~15%). Through the use of various approaches, we have demonstrated that the leukocytes with disrupted forms of polarized paracellular TEM were neutrophils. Although rTEM has been reported for other leukocyte subtypes, most notably for monocytes^{20,32}, rTEM of neutrophils is a contentious subject, but there is evidence for its occurrence *in vivo* in a zebrafish model^{33,34} and through cultured human ECs²⁷.

In addressing the mechanism associated with disrupted polarized paracellular responses, we observed that these events were most pronounced under conditions in which there was lower functional expression of the adhesion molecule JAM-C at EC junctions. Specifically, we noted substantial disrupted polarized neutrophil TEM after I-R injury, an inflammatory reaction that led to less junctional expression of JAM-C by ECs. The latter seemed to be mediated at least in part by the generation of ROS. Furthermore, interference with the expression and/or function of JAM-C led to a much greater frequency of disrupted polarized paracellular TEM events in response to I-R injury. Such interventions also led to considerable disrupted polarized paracellular TEM in response to IL-1 β (~20%), an inflammatory reaction that in control mice did not induce disruption of EC JAM-C expression. Together these results provide direct and conclusive evidence that EC JAM-C has a key role in maintaining luminal-to-abluminal neutrophil paracellular TEM *in vivo*. Blockade of JAM-A did not result in enhanced frequency of disrupted neutrophil TEM, which indicated the specificity of JAM-C in regulating this process. Furthermore, our results are in agreement with published findings showing that blockade of EC JAM-C leads to enhanced rTEM of monocytes through cultured ECs²⁰. Although there is ample evidence to support the idea that JAM-C is involved in leukocyte TEM³⁵, details of how this response is mediated remains unknown. Our findings here, together with other published studies, indicate that JAM-C can mediate neutrophil TEM via at least three modes: by mediating the migration of cells through EC junctions by providing an adhesive ligand for neutrophil Mac-1 (refs. 24,25); by regulating endothelial adherens junctions and barrier integrity³⁶; and by regulating the directionality of the migration of neutrophils through EC junctions in a luminal-to-abluminal direction. Exactly how EC JAM-C supports polarized neutrophil TEM is unclear at present, but this may involve stimulating migrating leukocytes to change their shape, extend protrusions into EC junctions and/or respond to chemotactic gradients, which are key components of polarized cell migration. JAM-C may also mediate the migration of neutrophils in a luminal-to-abluminal direction by regulating the expression and/or localization of EC chemokines.

Because of the greater prevalence of neutrophil rTEM under conditions of I-R injury than after other inflammatory reactions, 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 trauma²⁶. In line with published studies of human neutrophils²⁷, we found that mouse neutrophils that had undergone rTEM *in vitro* had a distinctive ICAM-1^{hi} phenotype, in contrast to blood, bone marrow and normal transmigrated neutrophils, which were ICAM-1^{lo}. Through the use of this characteristic phenotype, we detected rTEM neutrophils in the lung vasculature of mice subjected to I-R injury of the cremaster muscle or lower limb. The ICAM-1^{hi} neutrophils retrieved were primed for enhanced generation of ROS, and their presence in the pulmonary vasculature was associated with lung inflammation. These results suggest that neutrophils that have undergone rTEM may contribute to turning a local inflammatory response into a systemic multiorgan response. Although we cannot exclude the possibility that the 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, which yet again supports our hypothesis that neutrophil rTEM is associated with inflammation in a second organ. Hence, by providing a direct link between neutrophil rTEM and the pathogenesis of I-R injury, our data support the hypothesis that as well as other factors, such as circulating inflammatory mediators, neutrophils undergoing rTEM that are primed for enhanced ROS generation contribute to the dissemination of systemic inflammation and tissue damage in secondary organs. Our results are in line with findings of a study of zebrafish embryos showing that neutrophils undergoing rTEM can be detected in many organs after local inflammation³⁴. It is also potentially possible that rTEM may provide a physiological means of dampening an inflammatory reaction^{33,37} and/or may be associated with chronic inflammatory conditions^{27,37}.

In conclusion, we have reported the occurrence of disrupted polarized paracellular neutrophil TEM under conditions of less availability of functional JAM-C at EC junctions. Our results suggest that published reports of anti-inflammatory effects of JAM-C blockade and/or deletion *in vivo*^{21,25,38} may be partly accounted for by enhanced neutrophil rTEM, which emphasizes the need for more detailed analysis of TEM dynamics in various inflammatory scenarios and vascular beds. By associating rTEM with the dissemination of systemic inflammatory responses, we have also shown the need for further investigation into the occurrence, mechanisms and implications of the reverse transmigration of neutrophils in other tissues and inflammatory reactions.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

A.W. designed and did most experiments, analyzed data and contributed to the writing of the manuscript; M.-B.V. designed and did the immunofluorescence staining experiments and contributed to method development and data analysis

and interpretation; M.B. designed and did flow cytometry assays and contributed to data analysis and interpretation; B.C., D.C. and F.-M.D. designed and did some assays; G.B. T.C., S.M.A., G.E.R. and P.M. provided reagents and/or contributed to the design of experiments; B.A.I. provided reagents and made intellectual contributions to the study; and S.N. provided overall project supervision and contributed to the design of the experiments and the writing of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Confocal intravital microscopy of mouse cremaster muscles. Mice expressing endogenously labeled GFP in leukocytes (*lys-EGFP-ki*, CX3CR1-GFP-*ki*, Tie-2Cre;JAM-C^{flox/flox}; *lys-EGFP-ki* and JAM-C^{flox/flox}; *lys-EGFP-ki* littermate controls) were used. EC junctions were labeled with Alexa Fluor 555-labeled mAb to PECAM-1 (3 µg injected intrascrotally; 390; prepared 'in-house'), along with an inflammatory mediator. Alternatively, inflammation was induced by I-R injury after exteriorization. (**Supplementary Methods**). Where necessary, mAb to JAM-C (H33; prepared 'in-house'), mAb to JAM-A (BV11; a gift from E. Dejana) or soluble JAM-C (each at a dose of 3 mg or 10 mg per kg body weight), or a 'cocktail' of superoxide dismutase and catalase (2,000 and 50,000 U/kg) was administered intravenously immediately before induction of inflammation. All experiments followed UK legislation for the protection of animals and were approved by the Ethical Review Process of Queen Mary, University of London.

Straight post-capillary venules 20–40 µm in diameter were selected for analysis of leukocyte–vessel wall interactions. Z-stacks of images were captured by confocal microscopy with a single-beam Leica TCS-SP5 confocal laser-scanning microscope equipped with argon and helium-neon lasers and incorporating a 20× water-dipping objective (numerical aperture, 1.0; Leica). This microscope incorporates an optical zoom function, and most image sequences were captured at a final magnification of ~×40–×60. Images were acquired by sequential scanning of the 488-nm channels (for GFP⁺ leukocytes) and 561-nm channels (for EC junctions labeled with Alexa Fluor 555-antibody to PECAM-1 (390)) at a resolution of 512 × 1024 pixels, which corresponds to a voxel size of approximately 0.25 × 0.25 × 0.7 µm in the *x-y-z* planes. Stacks of images of optical sections ~1 µm in thickness were routinely acquired at intervals of 1 min, and with the incorporated resonance scanner of 8,000 Hz, acquisition of a single *z* stack of ~60 images routinely took ~40 s. This approach enabled acquisition of full three-dimensional confocal images of venules, which yielded high-resolution four-dimensional videos (real time in three dimensions) of dynamic events. For most 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 three-dimensional reconstruction stage with Imaris four-dimensional modeling software (Bitplane).

After acquisition, sequences of *z*-stack images were analyzed with LAS-AF Lite software (Leica application suite advanced fluorescence; Leica) or, more commonly, linked image stacks were subsequently analyzed off-line with Imaris, which renders stacks of optical sections into three-dimensional models, enabling analysis of the dynamics of leukocyte–vessel wall interactions. All images and resultant videos show half vessels to allow clear viewing of the luminal surface of the endothelium. Confocal microscopy images are traditionally shown as 'maximum-intensity projections' in which each channel is 'transparent' and thus a labeled object with one color 'behind' a second object of another color will still be visible in the projection. As the work here was aimed at visualizing the movement of objects in relation to each other, images

are presented as opaque three-dimensional objects, which allows the observer to determine where the objects are in relation to each other. By this approach, the development of an inflammatory reaction and TEM of leukocytes into tissues were detectable with high clarity and considerable temporal and spatial resolution (**Supplementary Video 1**).

LAS-AF software (Leica) was used for analysis of the intensity profiles of leukocytes labeled with GFP- or Alexa Fluor 555-labeled mAb to CD115 (AFS98; eBioscience) after intravenous injection of mAb to CD115 (**Fig. 6b**) for quantification of the intensity in Gray values per µm² of two-dimensional projections of cells.

Analysis of leukocyte activity. Images acquired by confocal intravital microscopy were analyzed for profile and dynamics of leukocyte TEM. In every video, only TEM events imaged in full in terms of their duration as the cell traversed the EC barrier and clearly visible in terms of their location and dynamics were analyzed. The occurrence of quantified responses is thus presented as the mean percent of total TEM events observed per vessel under observation. Routinely, ~50–100 TEM events from up to four venules per mouse and from a minimum of four mice were analyzed per treatment.

As the focus of the study here was on TEM, imaging settings (described above) were optimized for acquisition of TEM events from which the following parameters were quantified. First, paracellular TEM events were identified by the occurrence of EC junctional disruption (labeled with mAb to PECAM-1); that is, the occurrence of transient pores in junctional PECAM-1 staining during a TEM event. The association of such events with bicellular EC contacts or at junctions between three or more ECs was noted and quantified. Second, transcellular TEM events were identified as those associated with the transient occurrence of pores within the EC body (stained faintly and diffusely with antibody to PECAM-1) but not associated with disruption of EC junctions labeled with antibody to PECAM-1. Such responses were observed either distinctly separate from EC junctions or adjacent to a junction but with no visible junctional disruption occurring during TEM. Third, the duration of TEM events was calculated as the time between the first frame in which disruption of EC or labeling of the cell body with antibody to PECAM-1 could be seen to the frame in which the leukocyte being tracked had fully traversed the EC barrier. Fourth, rTEM was defined as an event in which a leukocyte moved in an abluminal-to-luminal direction, followed by disengagement from the junction into the lumen. Fifth, the definition of hesitant TEM was established by careful observation of many such events under conditions of I-R injury; it was defined as the occurrence of a bidirectional movement of a leukocyte within a junction (about two to three 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 moved by rTEM beyond the field of view cannot be excluded.

Additional methods. The principal *in vivo* imaging techniques used are described above; additional details are in the **Supplementary Methods**.