Junctional Adhesion Molecule-C Regulates the Early Influx of Leukocytes into Tissues during Inflammation

Michel Aurrand-Lions, Chrystelle Lamagna, John P. Dangerfield, Shijun Wang, Pedro Herrera, Sussan Nourshargh and Beat A. Imhof

J Immunol 2005; 174:6406-6415; doi: 10.4049/jimmunol.174.10.6406
http://www.jimmunol.org/content/174/10/6406

References
This article cites 43 articles, 27 of which you can access for free at:
http://www.jimmunol.org/content/174/10/6406.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Junctional Adhesion Molecule-C Regulates the Early Influx of Leukocytes into Tissues during Inflammation¹

Michel Aurrand-Lions,²*, Chrystelle Lamagna,*, John P. Dangerfield,‡, Shijun Wang,‡ Pedro Herrera,† Sussan Nourshargh,‡ and Beat A. Imhof*

Leukocyte recruitment from blood to inflammatory sites occurs in a multistep process that involves discrete molecular interactions between circulating and endothelial cells. Junctional adhesion molecule (JAM)-C is expressed at different levels on endothelial cells of lymphoid organs and peripheral tissues and has been proposed to regulate neutrophil migration by its interaction with the leukocyte integrin Mac-1. In the present study, we show that the accumulation of leukocytes in alveoli during acute pulmonary inflammation in mice is partially blocked using neutralizing Abs against JAM-C. To confirm the function of JAM-C in regulating leukocyte migration in vivo, we then generated a strain of transgenic mice overexpressing JAM-C under the control of the endothelial specific promoter Tie2. The transgenic animals accumulate more leukocytes to inflammatory sites compared with littermate control mice. Intravital microscopy shows that this is the result of increased leukocyte adhesion and transmigration, whereas rolling of leukocytes is not significantly affected in transgenic mice compared with littermates. Thus, JAM-C participates in the later steps of the leukoendothelial adhesion cascade. The Journal of Immunology, 2005, 174: 6406–6415.

Neutrophils and inflammatory monocytes are rapidly recruited into sites of infection. The initial influx of leukocytes into inflamed tissues is dominated by neutrophils, but a small number of inflammatory monocytes are also recruited at early time points (1). This dominance may reflect the higher concentration of neutrophils in the blood compared with monocytes. At later time points after tissue inflammation, the neutrophils progressively disappear and the predominant leukocyte population consists of monocyte/macrophages. One explanation for such a coordinated recruitment is that stimulated resident cells secrete CCL2/MCP-1 to attract inflammatory monocytes, which will themselves attract neutrophils and act as facilitators of neutrophil emigration (2, 3). The subsequent decrease in neutrophil numbers in inflamed tissues has been associated with their apoptosis and phagocytosis by the mononuclear phagocyte system. However, one could not exclude that differential adhesive mechanisms are involved in specific recruitment of monocytes and granulocytes as emphasized by the sustained recruitment of leukocyte subsets occurring in chronic inflammatory diseases.

Migration of leukocytes from blood to tissues occurs in a multistep process that involves cognate interactions between circulating cells and the vascular wall (4). The first interactive step mediated by selectins results in rolling of the leukocytes along the vessel. This is followed by chemokine activation of integrins on the leukocytes. Activated integrins engage ligands on vascular endothelial cells to mediate firm adhesion of leukocytes on the vessel wall and their movement toward interendothelial junctions (5). These steps precede the diapedesis of the leukocyte from the vascular lumen into the surrounding tissue, most likely by squeezing in the junctions between endothelial cells and in some cases by migrating through the endothelial cell body (6, 7).

Over the last decade, a great deal of efforts has led to the characterization of several endothelial adhesion molecules that act as leukocyte integrin ligands and are involved in leukocyte migration. Some of them belong to the Ig superfamily molecules and can be subdivided into two classes. Proteins, which are constitutively expressed by endothelial cells such as ICAM-2 or PECAM-1, and molecules, which are up-regulated by inflammation such as VCAM-1 or ICAM-1 (8, 9). The members of the junctional adhesion molecular (JAM) family and nectins expressed by endothelial cells have been proposed to constitute additional leukocyte ligands (10, 11). The JAM family protein consists of three members called JAM-A, JAM-B, and JAM-C, which are Ig superfamily molecules with two extracellular Ig domains and a short cytoplasmic tail ending with a PDZ binding motif (12). The prototypical member of the family, JAM-A, has been initially described as a tight junctional molecule expressed by endothelial and epithelial cells and involved in monocyte migration in vivo (13). The protein JAM-A has been found to bind the leukocyte integrin LFA-1 (αLβ2) and to regulate the adhesion and transmigration of lymphocytes in vitro (14). The two other members of the family, JAM-B and JAM-C, were described, respectively, as endothelial adhesion molecules highly expressed by high endothelial venules and lymphatic endothelial cells in lymphoid organs (15, 16). Notably, JAM-C appeared to be expressed at lower levels on endothelial cells of peripheral tissues than lymphoid organs except the kidney (17). In addition, JAM-C has also been shown to be expressed on human leukocytes and to interact with JAM-B (18, 19).

This led to postulating that JAM-C expressed by leukocytes could act as a ligand for JAM-B expressed by endothelial cells to support...
leukocyte adhesion and migration in vivo. However, a recent study has shown that JAM-C expressed on human platelets counterinteracts with the leukocyte integrins αmβ2 and αxβ2 on monocytes (20, 21). The relevance of JAM-C interaction with leukocyte integrins was confirmed by the remarkable findings that JAM-C expressed by epithelial cells is involved in neutrophil transmigration in vitro (22). More recently, Chavakis et al. (23) used recombinant soluble JAM-C to demonstrate that Mac-1-dependent neutrophil transendothelial migration involves JAM-C as counterreceptor on endothelial cells in vitro and in vivo. However, the use of soluble JAM-C in this study does not allow excluding that additional ligands of JAM-C, such as JAM-B, were also affected (24).

The present study extends these findings by investigating the role of JAM-C in several inflammatory models in vivo using both neutralizing Abs and a novel strain of mice overexpressing JAM-C on their endothelial cells. Mice overexpressing JAM-C on endothelial cells show that JAM-C participates in the recruitment of neutrophils into tissues and that increased expression of JAM-C on peripheral endothelial cells correlates with increased neutrophil migration into tissues. Using intravital video microscopy, we show that JAM-C expressed on endothelial cells does not play a significant role in the rolling step of leukocytes but rather participates in their adhesion and transmigration.

Materials and Methods

Mice

Eight- to 10-wk-old C57BL/6 mice weighing 25–30 g were provided by Iffa-Credo. The colony of pHNNS-JAM-C transgenic animals outbred on C57BL/6 for more than six generations was handled and maintained in the animal facility according to the Veterinary Swiss National Law.

Materials

Anti-VE-Cadherin, CD45, and secondary probes were purchased from BD Pharmingen and Jackson ImmunoResearch (Milan Analytica, La Roche, Switzerland). Monoclonal anti-JAM-C Abs were generated in the laboratory and have been previously described (15, 25). Dr. B. Engelhardt (Theodor Kocher Institute, Bern University, Bern, Switzerland) kindly provided the hybridoma secreting isotype control Ab 9B5 (rat IgG2a) against human CD44. LPS (Escherichia coli 0111:B4) was a gift from Difco Laboratories. Neutrophils were recovered from mouse peripheral blood at the 72/62% interface using a three-layer Percoll gradient of 72/62/45%. Purity was assessed by flow cytometry and was routinely >95%. The chemokine fMLP was added to the lower chamber at a final concentration of 10^{-8} M at the start of the transmigration assay, and soluble recombinant JAM-C (20 μg/ml) or control FLAG peptide (0.6 μg/ml) were left throughout the duration of the transmigration assay. In some experimental conditions, endothelial cells were stimulated with 10 U/ml TNF-α 4 h prior to the transmigration assay.

Transendothelial migration assays

Static transmigration assays were performed as previously described except that 10-mm diameter polycarbonate membranes with 3-μm diameter pores (Nunc) and b.end.5 murine endothelioma cell line were used (25). Neutrophils were recovered from mouse peripheral blood at the 72/62% interface using a three-layer Percoll gradient of 72/62/45%. Purity was assessed by flow cytometry and was routinely >95%. The chemokine fMLP was added to the lower chamber at a final concentration of 10^{-8} M at the start of the transmigration assay, and soluble recombinant JAM-C (20 μg/ml) or control FLAG peptide (0.6 μg/ml) were left throughout the duration of the transmigration assay. In some experimental conditions, endothelial cells were stimulated with 10 U/ml TNF-α 4 h prior to the transmigration assay.
FIGURE 2. JAM-C is involved in the recruitment of monocytes and granulocytes to site of inflammation. A, The phenotype of resident and recruited inflammatory cells to the lungs is shown at various time points after intranasal instillation of LPS. On the left panel, the phenotype of resident alveolar macrophages purified from BALs in the absence of LPS challenge is shown. Alveolar macrophages are Mac-1⁺, F4/80⁺, and Gr1⁻. On the right panel, the changes in the phenotype of the cells recovered from the BALs of mice challenged with LPS are shown at various time points. The granulocytes (Mac-1⁺, Gr1⁺, F4/80⁻) represent the dominant population found at 48 h after challenge, whereas inflammatory monocytes (Mac-1⁺, Gr1⁻, F4/80⁻) are found at early and later time points (6, 16, and 96 h). B, The D33 Ab against JAM-C (plain line) inhibits the recruitment of total leukocytes to the lungs of mice treated with LPS compared with the 9B5 isotype matched Ab (dashed line). The D33 mAb reduces by 40% the number of granulocytes and slightly decreases the number of inflammatory monocytes migrated into the alveoli 16 h after challenge. Data represent means ± SEM obtained 1 h (n = 4), 3 h (n = 4), 6 h (n = 4), 16 h (n = 15), and 120 h (n = 12) after LPS challenge. *, p < 0.05, as calculated by Mann-Whitney method using StatView software.
**IL-1β-induced peritonitis**

Quantification of leukocyte migration into the peritoneal cavity of age-matched mice injected i.p. with saline (1 ml; control group) or IL-1β (10 ng/cavity), was performed as previously described (27). Briefly, 4 h post-injection, the animals were killed by asphyxiation with CO₂ and the peritoneal cavity was opened via midline incision and lavaged with 3 ml of modified saline solution (0.9% saline containing 0.25% BSA and 2 mM EDTA). Differential cell analysis was determined in exudate smears prepared in a cytocentrifuge (Cytospin-3; Shandon) and stained with May-Grünwald/Giemsa stains. For quantification, 500 cells per slide were counted, and the results were expressed as the number of total leukocytes and percentage of neutrophils recovered from each cavity.

**Immunohistochemistry**

Cryosections of lungs used for immunofluorescence staining were obtained from lungs filled and embedded with OCT. Immunofluorescence staining was then performed as previously described (17). Immunostaining of cremasteric venules was performed as follows. Cremaster muscles were fixed in 4% paraformaldehyde and washed in PBS before blocking 3 h at room temperature in PBS containing 20% horse serum, 2% BSA, and 0.5% Triton X-100. Samples were then incubated overnight with primary Abs (rabbit anti-JAM-C polyclonal Ab at 1/100 dilution and rat anti-CD31 monoclonal Ab MEC-13.3 at 1/50 dilution) diluted in PBS/BSA (2%). After washing, samples were sequentially reacted with Alexa 488-conjugated goat anti-rabbit secondary Ab diluted 1/200 and Alexa 555-conjugated goat anti-rat secondary Ab diluted 1/200 (Molecular Probes). Pictures were acquired using a laser-scanning confocal microscope (Zeiss LSM510).

**Intravital microscopy**

Intravital microscopy on the mouse cremaster muscle was performed as described previously (28). Briefly, mice received intrascrotal administration of 400 μl of saline (control mice) or IL-1β (30 ng/animal). Four hours later, the mice were anesthetized by i.p. administration of ketamine (100 mg/kg) and xylazine (10 mg/kg) and were placed on a custom-built, heated (37°C) microscope stage where the surgical procedure was performed. Following incision of the scrotum, one testis was gently withdrawn to allow the cremaster muscle to be incised and was pinned out flat over the window in the microscope stage. The cremaster muscle was kept warm and moist by continuous application of warmed Tyrodes balanced salt solution.

Postcapillary cremasteric venules (20–40 μM in diameter) were viewed on an upright fixed-stage microscope (Axioskop FS; Zeiss), fitted with water-immersion objectives. Images were then captured using a color video camera (JVC; model KY-F55BE) and stored by videocassette recorder (model MD830E; Panasonic).

Rolling cells were defined as those cells moving slower than the flowing erythrocytes, and rolling flux was then quantified as the number of rolling cells moving past a fixed point on the venular wall per minute for 5 min. Firmly adherent leukocytes were defined as those cells that remained stationary for at least 30 s within a 100-μm segment of a venule. Transmigrating leukocytes were defined as those cells in the perivascular tissue adjacent to but remaining within a distance of 50 μm of a 100-μm vessel segment under study. In each animal, three to five vessel segments and three to four vessels were quantified, and averages were taken.

**Results**

**Mouse monocytes and granulocytes do not express JAM-C**

Because expression of JAM-C on circulating human leukocytes has been reported (18, 19), we first analyzed the expression of JAM-C on murine leukocytes. For this purpose, mouse peripheral circulating cells were stained for Mac-1, Gr1, and F4/80 to discriminate between granulocytes (Mac-1+, Gr1+, F4/80−), inflammatory monocytes (Mac-1−, Gr1+, F4/80+), and resident monocytes (Mac-1−, Gr1−, F4/80−) (29, 30). As shown in Fig. 1, none of these subpopulations expressed JAM-C. This was demonstrated by the lack of detectable JAM-C signals on the cells gated with regions R2, R3, and R4, which corresponded, respectively, to granulocytes (mainly neutrophils), inflammatory monocytes, and resident monocytes.

**JAM-C is involved in early recruitment of granulocytes during inflammation**

Having shown that leukocytes did not express JAM-C, we tested whether their migration to inflammatory sites was regulated by JAM-C expressed on endothelial cells. For this purpose, Abs against JAM-C were screened for their ability to inhibit leukocyte migration from blood to inflammatory sites. Localized inflammation of the lungs was used as an inflammatory model, allowing the recruitment of leukocytes in an α4β1-dependent manner (31). For this purpose, LPS was instilled by intranasal challenge to induce recruitment of inflammatory cells into the alveolar space of the lungs. Such a stimulus induced the sequential recruitment of granulocytes and monocytes. Quantitative and qualitative analysis of emigrated cells was performed on BALs. In this model, granulocytes represented the dominant leukocyte populations accumulating within the first 48 h, whereas monocytes were prominent at later time points (Fig. 2A). When the mice received a single i.v. injection of D33 Ab against JAM-C simultaneously to the LPS challenge, the recruitment of total leukocytes, especially granulocytes, was reduced by 40% 16 h after challenge (Fig. 2B). We did not observe a significant reduction in the number of emigrated leukocytes at other time points. Interestingly, a slight significant decrease in the number of emigrated inflammatory monocytes was observed in anti-JAM-C-treated mice 16 h after intranasal instillation, whereas the peak of monocyte accumulation occurred at later time points. These results suggest that JAM-C preferentially acts on the recruitment of leukocytes occurring during early phases of inflammatory response.

To confirm that JAM-C may control early neutrophil migration, we then performed static transendothelial migration assays using unstimulated and TNF-α-stimulated endothelial cells, and recombinant soluble JAM-C to inhibit JAM-C-dependent migration of neutrophils. TNF-α stimulation of endothelial cells was used to induce an inflammatory phenotype of endothelial cells mimicking the conditions encountered by neutrophils in vivo after inflammatory stimulation. As shown in Fig. 3, recombinant soluble JAM-C was able to reduce neutrophil transendothelial migration by 50% when endothelial cells were not stimulated with TNF-α. In contrast, we did not observe such a blocking effect when endothelial cells were stimulated with TNF-α, indicating that alternative JAM-C-independent transmigration occurs under these conditions.

![FIGURE 3. JAM-C is involved in neutrophil transendothelial migration through nonactivated murine endothelial cells. The transendothelial migration of neutrophils purified from peripheral blood of mice toward 10−7 M fMLP is shown. Results obtained on monolayers untreated or treated with 100 U/ml TNF-α for 4 h in the presence of control FLAG peptide (□) or recombinant soluble JAM-C (■) are shown. Data represent means (n = 6) ± SEM from one typical experiment; similar results were obtained in three independent experiments.](http://www.jimmunol.org/content/journals/bundles/6428982/6429609/6409)
Increased expression of JAM-C on endothelial cells using a transgenic approach

Because JAM-C is not expressed at the same level on all endothelial cells of peripheral tissues (17), we generated transgenic mice strains overexpressing JAM-C under the control of the endothelial specific promotor Tie-2 (Fig. 4A). When the full promotor including 5’ and 3’ enhancer regions (pHHNS) was used (26), increased JAM-C expression was detected on endothelial cells of peripheral tissues as illustrated by Western blotting using total lung extracts of pHHNS-JAM-C transgenic mice compared with littermate animals (Fig. 4B). This was confirmed by immunoprecipitation and Western blotting experiments, showing increased quantities of immunoprecipitated JAM-C in lung lysates from transgenic animals compared with control mice (Fig. 4C). The increased level of expression was due to the specific overexpression of JAM-C on endothelial cells, because a 3-fold increase in mean fluorescence intensity was observed on VE-cadherin-positive endothelial cells and not on CD45-positive hemopoietic cells (Fig. 4, D and E). No difference in the numbers of circulating white blood cells and leukocytes were observed at the steady state (Table I), making this animal model suitable to explore the role of JAM-C in leukocyte recruitment independently of its endogenous variable level of expression on endothelial cells of peripheral tissues.

Increased recruitment of monocytes and granulocytes in pHHNS-JAM-C transgenic mice

The profile of leukocyte recruitment in pHHNS-JAM-C transgenic mice was then investigated in three inflammatory models. Following i.p. injection of LPS, a massive drop in the number of circulating leukocytes was observed in both strains as early as 30 min after challenge (Table I), indicating that systemic leukocyte trapping occurred in both strains. The histological analysis of the lungs

**FIGURE 4.** Generation and characterization of pHHNS-JAM-C transgenic mice. A, Strategy for transgene generation. The coding region for murine JAM-C was inserted in place of *lacZ*-SV40pA into pBSIIISK-HHNS construct using Sse8783I and MluI. The transgene comprising the promotor, the first intron of Tie2 (gray box), the JAM-C coding region, and the 3’ enhancer (gray and black boxes) was excised using ApaI and injected into oocytes. The use of the entire 3’ enhancer region is required for uniform expression of the transgene in endothelial cells (26). B, JAM-C protein overexpression is shown by Western blot on total tissue lysates obtained from mesenteric lymph nodes (MLN) or lungs. The sample loading is shown on the lower panel depicting the results obtained using anti-actin Ab for Western blot. C, Immunoprecipitation and Western blot for JAM-C showing quantitative differences of immunoprecipitated material between lungs of littermate animals as compared with pHHNS-JAM-C transgenic mice. Because JAM-C is highly expressed in MLN of littermate animals, the increased expression in MLN of pHHNS-JAM-C mice is not observed by immunoprecipitation due to saturating signals obtained with lysates of littermate animals. D, Flow cytometry analysis of JAM-C expression on pulmonary cells obtained from collagenase-dissociated lung tissue. Staining for the endothelial marker VE-cadherin and the hemopoietic marker CD45 show that JAM-C overexpression is specifically detected on endothelial cells. E, Quantification of the mean fluorescence intensity shown in D. A 3-fold increase in fluorescence signals for JAM-C on endothelial cells is observed in pHHNS transgenic mice compared with littermate animals (*n* = 5).
after i.p. LPS challenge revealed that leukocytes accumulated around vessels and airways in the lungs of transgenic mice 30 min after challenge, whereas this accumulation was not detectable in littermate animals (Fig. 5A). This suggested that, in this model, JAM-C overexpression on endothelial cells redirects or increases the early leukocyte recruitment of inflammatory cells into the lungs of transgenic mice.

To extend our analysis to other tissues, we then used the pHHNS-JAM-C transgenic mice in a peritonitis model where local injection of IL-1β/H9252 was used to elicit leukocyte migration into the peritoneal cavity (Fig. 5B). In this model, we observed a slight increase in total leukocyte count and a 3-fold increase in the percentage of neutrophils migrated into the peritoneal cavity in pHHNS-JAM-C mice compared with control animals (Fig. 5C). These changes resulted in a 6-fold increase in the numbers of neutrophils recruited in transgenic mice compared with control mice (2.44 × 10^6 ± 1 × 10^6 vs 0.124 × 10^6; p < 0.05). This indicates that increased expression of JAM-C on endothelial cells enhances the migration of neutrophils into the peritoneal cavity.

Finally, the model of pulmonary leukocyte recruitment induced by intranasal LPS challenge was performed. Migrated cells in transgenic and littermate animals were analyzed 96 h after intranasal challenge. This time point was chosen, because granulocytes and inflammatory monocytes were almost equally represented in the BAL at that time and accounted for 15% of the total cell count in C57BL/6 animals (Fig. 2A). A 4-fold increase in the number of granulocytes and a 2-fold increase in inflammatory monocytes recovered from the BALs of transgenic mice were observed compared with the littermate animals (Fig. 6). These results confirm that JAM-C expressed on the vascular tree regulates the migration of granulocytes to sites of inflammation, whereas monocyte

Table I. Numbers of circulating cells in the blood of the indicated mouse strains

<table>
<thead>
<tr>
<th></th>
<th>WBC* (cells/ml)</th>
<th>Neutrophils (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Littermates (n = 11)</td>
<td>10.22 × 10^6 ± 1.1</td>
<td>3.56 × 10^6 ± 0.52</td>
</tr>
<tr>
<td>pHHNS-JAM-C (n = 14)</td>
<td>7.96 × 10^6 ± 0.63</td>
<td>3.61 × 10^6 ± 0.44</td>
</tr>
<tr>
<td>Littermates (n = 10); 1 h LPS</td>
<td>4.84 × 10^6 ± 0.9</td>
<td>0.62 × 10^6 ± 0.08</td>
</tr>
<tr>
<td>pHHNS-JAM-C (n = 9); 1 h LPS</td>
<td>4.71 × 10^6 ± 1</td>
<td>0.56 × 10^6 ± 0.94</td>
</tr>
</tbody>
</table>

*a Abbreviation: WBC, White blood cell.

FIGURE 5. Leukocyte recruitment to inflammatory sites is increased in pHHNS-JAM-C transgenic mice. A, H&E staining of lung sections 30 min after systemic i.p. challenge with 4 mg/kg LPS. As indicated by arrowheads, pHHNS-JAM-C transgenic mice (left panel) show increased accumulation of leukocytes around vessels (V) and airways (Aw) compared with littermates animals. At this early time point after inflammatory challenge, leukocytes are not found in the alveolar space (Al). B, Total leukocyte counts in the peritoneal cavity of wild-type and pHHNS-JAM-C transgenic mice injected with saline or IL-1β (10 ng/cavity) are shown. C, Percentages of neutrophils emigrated in the peritoneal cavity 4 h after saline or IL-1β injection in the peritoneal cavity. Results are from n = 3–9 mice/group. A significant difference from responses obtained from saline-injected animals is shown by asterisks: *, p < 0.05; **, p < 0.01 (Mann-Whitney method; using StatView software). Additional statistical comparisons are indicated by lines.
migration appears to be less dependent on JAM-C. Although the results from the three inflammatory models detailed above collectively demonstrate a functional role for endothelial JAM-C in regulation of leukocyte migration, the findings do not indicate at what stage of leukocyte migration JAM-C plays a role. This was addressed by the intravital observation of leukocyte migration across cremasteric venules of transgenic and control animals.

**JAM-C acts at the adhesion and transmigration steps of neutrophil emigration**

Intravital video microscopy was performed on IL-1β-stimulated cremasteric venules, which express low levels of JAM-C in nontransgenic animals and high levels in pHHNS-JAM-C mice (Fig. 7A). Although basal leukocyte rolling flux in saline-injected mice appeared to be slightly higher in pHHNS-JAM-C mice compared with control, this difference was not statistically significant. Furthermore, no difference in rolling flux was observed between the two mouse strains in response to IL-1β (Fig. 7B, upper panel). By contrast, firm adhesion and transmigration of leukocytes induced by IL-1β were significantly enhanced in JAM-C transgenic mice compared with control animals (Fig. 7B, middle and lower panels). This 2-fold increase in adhesion and emigration of leukocytes observed in transgenic mice upon inflammatory stimulation indicates that JAM-C is involved in the adhesion and the subsequent trans-migration steps of neutrophils recruited by IL-1β. Moreover, the lack of difference between control and transgenic animals in the absence of stimulation confirms that the function of JAM-C expressed on endothelial cells is revealed only when inflammatory cell recruitment is induced. This supports the hypothesis that JAM-C acts as a protein specifically involved in migration of leukocyte subpopulations under inflammatory conditions.

**Discussion**

In the present study, we addressed the question whether JAM-C is involved in leukocyte migration. For this purpose, we used in vivo inflammatory models inducing the recruitment of leukocytes expressing the integrin αMβ2. Although we cannot detect JAM-C expression on these cells, we show that they migrate in a JAM-C-dependent manner to sites of inflammation. Using transgenic mice overexpressing JAM-C on endothelial cells, we further demonstrate that increased JAM-C expression on endothelial cells correlates with enhanced recruitment of leukocytes at inflammatory sites. In addition, the function of JAM-C in leukocyte trafficking independently of its endogenous variable level of expression on endothelial cells of peripheral tissues was studied using this animal model and intravital real-time video microscopy.

Leukocyte migration from blood into tissues involves a series of molecular interactions between leukocyte and endothelial cells.
This allows leukocyte rolling, firm arrest, and transendothelial migration to occur. Recently, adhesion molecules such as PECAM, CD99, poliovirus receptor, vascular adhesion protein-1, JAM-A, or JAM-C have been shown to be involved in leukocyte transendothelial migration in vitro (10, 25, 32). Although the ligands for CD99, poliovirus receptor, and JAM-A expressed by endothelial cells have been shown to be, respectively, CD99, DNAX accessory molecule 1, and LFA-1 on leukocytes (11, 14, 33), the situation is by far less clear concerning the JAM-B and JAM-C family members. Indeed, homo- and heterophilic interactions between these molecules have been shown to contribute to cell-cell interactions (18, 19, 22). In addition, human JAM-C interacts with α4β2 or α6β2, and JAM-B binds to α4β1 if JAM-C is coexpressed with this integrin on leukocytes (21, 24). This led to the proposal that JAM-C might be involved in leukocyte migration in vivo, even though this question has never been addressed in these studies and several scenarios are possible. A first possibility is that JAM-C expressed on leukocyte subset may counterinteract with its ligand JAM-B expressed by endothelial cells and as such plays a role in the specific migration of those leukocytes. Alternatively, JAM-C expressed by endothelial cells regulates the structure of interendothelial junctions to facilitate emigration of adherent leukocytes from the vascular lumen into tissues. A third possibility is that JAM-C expressed by endothelial cells may interact with the leukocyte integrins αMβ2 or α5β2 to allow leukocyte adhesion and transmigration. This latter scenario was confirmed in vitro and in vivo by a recent study by Chavakis et al. (23) showing that the interaction between Mac-1 and JAM-C is involved in leukocyte transendothelial migration but not in leukoendothelial adhesion.

Murine granulocytes can be distinguished from either inflammatory monocytes or resident monocytes by means of Mac-1 (αMβ2), Gr-1, and F4/80 expression (29). Using flow cytometry, we show that none of these leukocyte populations express detectable levels of JAM-C, making it unlikely that these cells would use JAM-C as an adhesive ligand for JAM-B expressed on endothelial cells. One Ab against JAM-C reduced by 40% the recruitment of granulocytes into inflamed lungs 16 h after challenge, whereas no significant effects were observed at other time points. In agreement with this result, soluble JAM-C blocked transendothelial migration of neutrophils through unstimulated murine endothelial cells.

**FIGURE 7.** Enhances neutrophil migration through IL-1β-stimulated cremasteric venules of pHHNS-JAM-C transgenic mice. A, Cremasteric venules from littermate (a, a’, a”) and transgenic animals (b, b’, b”) were immunostained for PECAM (a, b) and JAM-C (a’, b’, b”) and observed by laser-scanning confocal microscopy as detailed in Materials and Methods. Note that, in transgenic animals, interendothelial junctions are stained with anti-JAM-C, whereas the junctional staining is barely detectable in littermate mice. The merged pictures are shown on the right panel (a”, b”). B, Wild-type or pHHNS-JAM-C transgenic were treated with intrascrotal saline or IL-1β (30 ng/animal), and 4 h later, the cremaster muscle was surgically exteriorized, and leukocyte responses of rolling, firm adhesion, and transmigration were quantified by intravital microscopy. The data represent mean ± SEM from n = 4–8 mice/group. A significant difference from responses obtained from saline-injected animals is shown by asterisks as follows: *, p < 0.05; ***, p < 0.001. Additional statistical comparisons are indicated by lines.
whereas the effect is lost upon TNF-α treatment. This could be explained by JAM-C acting as leukocyte α4β2 integrin ligand during the early phase of the inflammatory process, whereas JAM-C-independent mechanisms occur later on. Different mechanisms have been proposed recently to orchestrate the regulation of endothelial adhesion molecules during transmigration: 1) targeted recycling of PECAM at the site of monocyte emigration (34), 2) formation of ICAM-1 ringlike structures at the site of neutrophil emigration through TNF-α-activated endothelial cells (35), and 3) relocalization of JAM-A from interendothelial junctions to apical surface by proinflammatory treatment of endothelial cells (36, 37). Although further studies are required to understand the molecular mechanisms by which JAM-C regulates leukocyte transmigration, it seems unlikely that JAM-C is involved in one of the above-mentioned mechanisms, because additive blocking effects of JAM-C and PECAM have been observed on neutrophil transmigration (23) and because we do not detect a blocking effect of JAM-C on neutrophil transmigration through TNF-α-stimulated endothelial cells.

In vivo, migration of neutrophils is known to occur in an α4β2-dependent or -independent manner according to the inflammatory model used (31, 38). In the latter case, it seems possible that α4β2-dependent/ICAM-1-independent migration exists, as illustrated by the unaffected migration of neutrophils into the alveoli of ICAM-1-deficient mice (39, 40). Our results indicate that JAM-C is a good candidate as alternative ligand for α4β2 in this model, because an Ab to JAM-C is able to reduce the early onset of granulocytes and inflammatory monocytes migration to the lungs. Whether JAM-C acts indirectly on granulocytes by decreasing the early influx of monocytes, which are then required to get neutrophil accumulation, remains to be determined. However, the increased accumulation of monocytes and granulocytes found in transgenic mice 96 h after LPS challenge together with the reduced numbers of inflammatory monocytes found in the lungs of Ab-treated mice strongly suggest that JAM-C directly regulates migration of both leukocyte populations in vivo. This will occur through an α4β2-dependent pathway and will be in agreement with the finding that JAM-C acts at the integrin-dependent steps of leukocyte emigration (i.e., adhesion and transmigration).

An important conclusion of our study is that JAM-C specifically acts on adhesion and the subsequent step of transendothelial migration of neutrophils recruited by IL-1β in vivo. Intravital microscopy shows that, although rolling flux of leukocytes is not significantly affected by increased expression of JAM-C on c germany vessels, their adhesion and transmigration is greatly enhanced. This demonstrates that JAM-C expression might directly influence the levels of inflammatory reactions and its duration. To date, there is no evidence of JAM-C expression levels regulated by inflammatory stimulus. However, we previously reported that expression of JAM-C on vascular endothelial cells is heterogeneous (17), suggesting that JAM-C, which participates in leukocyte recruitment, can contribute to the tropism and development of local inflammatory responses. In the present study, we use for the first time a transgenic mouse model consisting of overexpressing an adhesion molecule under the control of the endothelial specific promoter Tie2. This model allows us to draw important conclusions on the function of JAM-C expressed by endothelial cells using intravital video microscopy on cremasteric venules, which normally express rather low levels of JAM-C. Such a transgenic approach would be easily transposable to other adhesion molecules to dissect the molecular mechanisms of rolling, adhesion, and transmigration by intravital microscopy independently of the timing and the type of inflammatory stimulus used to activate endothelial cells (41–43).

Together, our results set the basis for the function of JAM-C as regulator of neutrophil recruitment in vivo. Such recruitment occurs mainly during bacterial infections, and is not taking place in absence of inflammatory stimulus. The lack of an obvious phenotype in the transgenic mice in absence of inflammatory stimulus allows excluding that JAM-C acts nonspecifically on leukocyte transmigration by regulating the paracellular space and strongly favors the hypothesis that the interaction of JAM-C with its cognate ligand(s) on leukocytes is dependent on local inflammatory activation.

Acknowledgments

We thank D. Ducrest-Gay for her invaluable technical expertise in handling the animals and C. Magnin and P. Ropraz for technical help. We are grateful to Dr. Urban Deutsch in providing the pBSIJKS-HHNS plasmid.

Disclosures

The authors have no financial conflict of interest.

References


Downloaded from http://www.jimmunol.org/ by guest on April 13, 2017


