

Vascular Biology, Atherosclerosis and Endothelium Biology

## Caveolin-1 Expression Determines the Route of Neutrophil Extravasation through Skin Microvasculature

Shana Marmon,\* Joseph Hinchey,<sup>†</sup> Philmo Oh,<sup>‡</sup>  
Michael Cammer,<sup>§</sup> Cecilia J. de Almeida,<sup>¶</sup>  
Leslie Gunther,<sup>§</sup> Cedric S. Raine,<sup>||</sup>  
and Michael P. Lisanti\*<sup>¶†††††</sup>

From the Departments of Molecular Pharmacology,\* Microbiology and Immunology,<sup>†</sup> Anatomy and Structural Biology,<sup>§</sup> and Pathology, Neurology and Neuroscience,<sup>||</sup> Albert Einstein College of Medicine, Bronx, New York; the Department of Molecular Pathogenesis,<sup>‡</sup> New York University School of Medicine, New York, New York; the Departments of Cancer Biology<sup>¶</sup> and Medical Oncology,<sup>††</sup> Kimmel Cancer Center, and the Stem Cell Biology and Regenerative Medicine Center,<sup>†††</sup> Thomas Jefferson University, Philadelphia, Pennsylvania

**Interleukin-8 plays a key role in the acute inflammatory response by mediating recruitment of neutrophils through vessel walls into affected tissues. During this process, molecular signals guide circulating blood neutrophils to target specific vessels for extravasation and to migrate through such vessels via particular routes. Our results show that levels of endothelial caveolin-1, the protein responsible for the induction of the membrane domains known as caveolae, are critical to each of these processes. We demonstrate that, in response to the intradermal injection of interleukin-8, neutrophils are preferentially recruited to a unique subset of venules that express high levels of intercellular adhesion molecule-1 and low levels of caveolin-1. Our results show that neutrophils traverse human dermal microvascular endothelial cells using one of two pathways: a transcellular route directly through the cell or a paracellular route through cellular junctions. Caveolin-1 expression appears to favor the transcellular path while down-regulation of caveolin-1 promotes the paracellular route. (*Am J Pathol* 2009, 174:684–692; DOI: 10.2353/ajpath.2009.080091)**

Wounding of the epithelium and entry of a foreign body elicit a series of responses from the innate immune system. One of the main hallmarks of acute inflammation is neutrophil infiltration at the affected site.<sup>1,2</sup> In response to

injury or infection, resident phagocytic cells become activated and release inflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-8. TNF- $\alpha$  activates the vascular endothelium causing vasodilation and cellular infiltration.<sup>3</sup> IL-8 functions as a critical chemotactic factor attracting neutrophils from the blood to the affected area.<sup>1,4</sup>

It is currently thought that leukocyte recruitment and migration through the vasculature is an active process not only for migrating blood cells but also for endothelial cells lining the vessels. Initially, inflammatory cytokines or bacterial endotoxins induce expression of P- and E-selectin on the surface of microvascular endothelial cells.<sup>5,6</sup> These molecules recognize carbohydrate counterligands on the surface of circulating leukocytes and mediate the tethering and rolling of these cells along vessel walls.<sup>5–7</sup> Firm adhesion is then initiated through the upregulation of endothelial adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and vascular adhesion molecule (VCAM)-1, which bind to integrins expressed on the leukocyte surface.<sup>5,7,8</sup> Finally, the leukocyte is induced to migrate through the vessel in a process known as diapedesis.<sup>5–7</sup>

Among the many proteins implicated in the process of diapedesis, the adhesion molecule ICAM-1, which is up-regulated on activated endothelium, and caveolin-1, which is expressed on most terminally differentiated cell types but is largely undetectable in white blood cells, have been most closely associated with the route of transendothelial migration in *in vitro* systems.<sup>7,9,10</sup> A recent study by Millan et al clearly demonstrates that ICAM-1 and caveolin-1 are involved in directing the path

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Address reprint requests to Dr. Michael P. Lisanti, Department of Cancer Biology, Kimmel Cancer Center, Thomas Jefferson University, 233 South 10<sup>th</sup> Street, Philadelphia, PA, 19107. E-mail: michael.lisanti@kimmeltcancercenter.org.

of T lymphoblast migration through human umbilical vein endothelial cells (HUVECs).<sup>7</sup>

Although both caveolin-1 and ICAM-1 have been associated with leukocyte transendothelial migration *in vitro*, the distribution of these proteins in vessels used by migrating leukocytes *in vivo* remain unclear. While all endothelial cells (ECs) share common features, the vascular tree is known to be extremely heterogeneous. As a result, the precise molecular profile of selectins and adhesion molecules defining vessels targeted for extravasation by circulating leukocytes is unknown. Furthermore, since the phenotype of vessel ECs is determined in large part by their unique *in vivo* microenvironment, site specific and regional differences in the expression of molecules contributing to the regulation of leukocyte transmigration have yet to be thoroughly characterized.<sup>11,12</sup> In this study, we have examined the *in vivo* molecular profile of vessels targeted by circulating neutrophils in response to IL-8 in the skin and have determined the effect of the expression of these factors on the route of neutrophil transmigration *in vitro*.

## Materials and Methods

### Mice

This study was conducted according to the guidelines of the National Institutes of Health (NIH) and the Albert Einstein College of Medicine Institute for Animal Studies. Caveolin-1 knockout mice have been generated as previously described.<sup>13</sup> Cav-1 transgenic mice ubiquitously overexpressing a C-terminally Myc-tagged form of the Cav-1 wild-type cDNA (canine; in the pCAGGS vector containing a  $\beta$ -actin/cytomegalovirus-based promoter) were generated using standard procedures, as previously described for other transgenes.<sup>14</sup> All mice used in these studies were on the C57BL/6 genetic background.

### Antibodies for Immunofluorescence

Rat anti-mouse antibodies to platelet endothelial cell adhesion molecule (PECAM)-1, MECA-32 (PV-1), PNA<sub>d</sub>, P-selectin, and hamster anti-mouse ICAM-1 were purchased from BD Pharmingen (San Jose, CA). Rabbit anti-caveolin-1 was purchased from Santa Cruz Biotechnology (SCBT, Santa Cruz, CA). Rat anti-mouse neutrophil (MCA771G) antibody was purchased from Serotec (Raleigh, NC) and rabbit anti-myeloperoxidase polyclonal antibody was purchased from LabVision Corporation (Fremont, CA). Mouse anti-human CD45 (clone MEM 28) was purchased from Chemicon International (Temecula, CA). Goat anti-human VE-Cadherin was purchased from R&D Systems (Minneapolis, MN). All CY3- and CY5-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Alexa 488-conjugated donkey anti-rabbit and anti-rat secondary antibodies were purchased from Invitrogen (Carlsbad, CA).

### Skin and Lymph Node Immunofluorescence

Mouse skin and inguinal lymph nodes were fixed for 2 hours in 4% paraformaldehyde in phosphate buffer then embedded in optimal cutting tissue compound and frozen. Tissue was sectioned to a thickness of 10  $\mu$ m, blocked with normal donkey serum (Caltag, Carlsbad, CA), permeabilized with 0.1% triton- $\times$ 100 and co-stained with anti-murine antibodies to PV-1, ICAM-1, and caveolin-1. To identify the phenotypic markers expressed by dermal vessels used by recruited neutrophils, shaved murine flank skin was injected with 2.5  $\mu$ g recombinant human IL-8 (Peprotech, Rocky Hill, NJ). The injection site was clearly marked and excised 1.5 hours postinjection. Biopsied skin was fixed and blocked as described and co-stained with antibodies to murine neutrophils and endothelial cell markers. Vessels containing aggregates of four or more neutrophils in the skin were analyzed for expression of PV-1, caveolin-1, PECAM-1, ICAM-1, and P-selectin. Images were captured using a BioRad Radiance 2000 Laser Scanning Confocal Microscope (Zeiss, Thornwood, NY).

### Electron Microscopy

Murine inguinal lymph nodes were excised and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixated with 1% osmium tetroxide followed by 1% uranyl acetate, dehydrated through a graded series of ethanol and embedded in LX112 resin (LADD Research Industries, Burlington VT). Ultrathin sections were cut on a Reichert Ultracut UCT, stained with uranyl acetate followed by lead citrate and viewed on a JEOL 1200EX transmission electron microscope at 80kv.

### Neutrophil Transmigration Assay

Primary dermal microvascular endothelial cells HDMVECs were purchased from Promocell (Heidelberg, Germany) and used at passage two or three. Cells were plated to confluence on fibronectin (10  $\mu$ g/ml) coated coverslips 24 hours before performing the transmigration assay. Cells were allowed to adhere and then stimulated with 100 ng/ml TNF- $\alpha$  (Peprotec, Rocky Hill, NJ) 12 hours before the assay. Activated dermal endothelial cells were washed twice with buffer (Hank's balanced salt solution, with 20 mmol/L HEPES, 1% human serum albumin)<sup>9</sup> and neutrophils were immediately added in the same buffer at a 2:1 ratio of neutrophils:endothelial cells and placed in a 37°C incubator for 10 minutes. Media was removed and cells were fixed for 20 minutes with 4% paraformaldehyde at room temperature. Coverslips were stained with anti-vein endothelial(VE)-cadherin, anti-caveolin-1 and anti-CD45. Active migration events were clearly identifiable by a unique pattern of intense CD45 staining localized in the caudal end of the migrating cell and scored as paracellular or transcellular based on whether or not they colocalized with VE-cadherin. A BioRad Radiance 2000 Laser Scanning Confocal Microscope (Zeiss, Thornwood, NY) was used to collect Z stacks at 0.45  $\mu$ m steps

with a  $\times 60$  N.A.1.40 Nikon infinity corrected objective. Typical Z stacks were 26 images deep (a range of 11.7  $\mu\text{m}$ ). Three-dimensional analysis was performed using both ImageJ (Rasband, W.S., ImageJ, NIH, Bethesda, Maryland, <http://rsb.info.nih.gov/ij/>, 1997–2007) and Volocity (Improvision, Lexington, MA) programs.

### Neutrophil Isolation

Human Neutrophils were isolated using a double gradient of Histopaque-1119 and Histopaque-1117 (Sigma-Aldrich, St Louis, MO) according to the manufacturer's protocol. After isolation neutrophils were resuspended in buffer (Hank's balanced salt solution with 20 mmol/L Hepes, 1% human serum albumin) and stimulated with 10 ng/ml TNF- $\alpha$  at room temperature for 20 minutes. An aliquot of the isolated cells were fixed with 3% acetone, counted by nuclear morphology and found to be >95% pure.

### Small Interfering RNA Knockdown

HDMVECs (Promocell, Heidelberg, Germany) were transfected at passage 2 using the HMVEC-L Nucleofector kit from Amaxa Biosystems (Gaithersburg, MD) according to the manufacturer's protocol. Briefly,  $5 \times 10^5$  cells were resuspended in nucleofection solution with 10  $\mu\text{l}$  (20  $\mu\text{mol/L}$  stock) of caveolin-1 small interfering (si)RNA (cat#1027110) or luciferase control siRNA (cat#1022070) purchased from Qiagen (Valencia, CA). 24 hours later, transfected cells were trypsinized, pooled, and plated to confluence onto fibronectin (10  $\mu\text{g/ml}$ )-coated coverslips in 24-well plates. An aliquot was removed from each of the pooled control and caveolin-1 siRNA transfected cells and plated into duplicate wells for Western blot analysis of knockdown efficiency. The primary antibodies used for the Western blot were as follows: mouse anti-human ICAM-1 (SCBT Santa Cruz, CA), mouse anti-caveolin-1 (Pharmingen, San Jose, CA), and goat anti- $\beta$ -actin (Sigma). Donkey anti-goat horseradish peroxidase was purchased from SCBT and goat anti-mouse horseradish peroxidase was purchased from Jackson Immunoresearch (West Grove, PA). Protein bands were analyzed using Scion Image (Scion Corporation, Fredrick, MD).

### Quantification of Endothelial Marker Expression Levels

Murine skin and inguinal lymph nodes were excised, fixed and stained as described above. Endothelial marker expression intensity was determined using ImageJ 1.38q running under Java 1.5 on WindowsXP (<http://rsb.info.nih.gov/ij/>). For each confocal image, background was subtracted and the image tagged to assure that the user could not measure an improperly subtracted image. The vessel was then traced in either channel and the histogram measurements were performed in the correct channel. Data were collated using the Histogram Data Analysis tool in Microsoft Excel 2003.

### Statistics

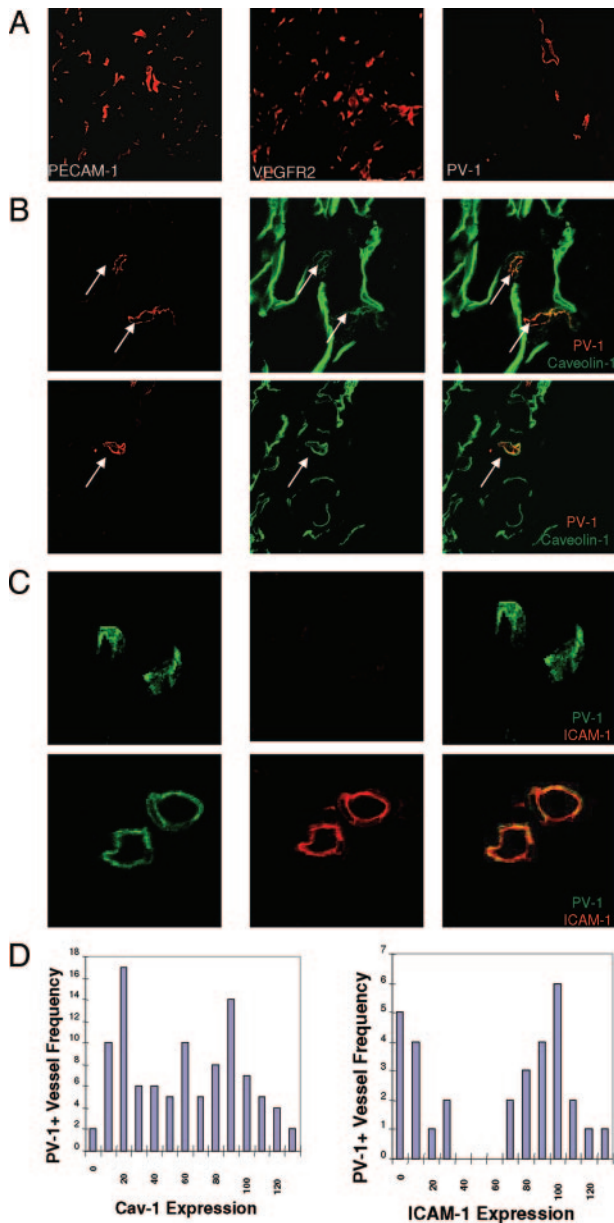
Numerical values in the figures are means  $\pm$  SD. The data for each group was analyzed as indicated in the respective figure legends. Statistical comparisons of the data were performed using the Student's *t*-test. A *P* value of less than 0.05 was considered to be significant.

### Results

#### Variable Levels of Caveolin-1 and ICAM-1 in the Microvasculature

Dermal vascular endothelium comprises a collection of vessels that form a barrier between circulating leukocytes and surrounding tissue.<sup>8,11</sup> The endothelium of microvasculature is especially critical to the inflammatory response because it forms the principle site of leukocyte transmigration.<sup>11,15,16</sup> To investigate molecular factors involved in neutrophil transmigration, we used the PV-1 protein as a marker to differentiate vessels of microvasculature from other cell types and larger vessels in the tissue. PV-1 (also known as MECA-32 or PAL-E), is uniquely expressed by endothelial cells of small to medium-sized vessels in human samples, can discriminate between blood vessel and lymphatic endothelium,<sup>17</sup> and in murine skin shows a more restricted expression pattern than other commonly used vascular endothelial markers such as PECAM-1 and vascular endothelial growth factor receptor-2 (Figure 1A).

The immunoglobulin superfamily member ICAM-1, has been shown to be critical to both leukocyte adhesion and transmigration.<sup>10</sup> Caveolin-1, responsible for the induction and maintenance of the plasmalemmal invaginations known as caveolae, has been demonstrated to work in concert with ICAM-1 to facilitate the transmigration of T cells through HUVECs *in vitro*.<sup>7</sup> However, the expression of these proteins in vessels responsible for recruitment and extravasation of leukocytes *in vivo* is not known. To determine the expression pattern of ICAM-1 and caveolin-1 in dermal microvasculature, untreated skin from wild-type mice was excised and stained with antibodies specific to these proteins, co-stained for the microvascular marker PV-1, and examined by immunofluorescence. Unexpectedly, we found variable levels of ICAM-1 and caveolin-1 in PV-1 positive vessels, with some vessels exhibiting high levels of staining and others, low to nearly undetectable levels of the proteins (Figure 1, B and C). To define more thoroughly the range of expression of ICAM-1 and caveolin-1 in the dermal microvascular, we systematically quantified the staining intensity of each marker (see Methods). As shown in Figure 1D, we observed that the microvasculature is comprised of a phenotypically heterogeneous population of vessels expressing a wide range of ICAM-1 and caveolin-1 intensities.



**Figure 1.** Variable levels of ICAM-1 and caveolin-1 in dermal microvasculature. **A:** PV-1 shows a restricted pattern of expression identifying small vessels in the murine dermis. Sections of wild-type mouse skin were stained with antibodies to PECAM-1, vascular endothelial growth factor receptor 2 and PV-1 (red). Images captured at original magnification  $\times 20$ . **B:** Representative confocal images of PV-1 (red) expressing vessels (arrows) colocalizing with vessels (arrows) alternately expressing low (top) or high levels (bottom) of caveolin-1 (green). **C:** Representative confocal images of PV-1 (green) expressing vessels colocalizing with vessels alternately expressing low (top) or high levels (bottom) of ICAM-1 (red). Images captured at original magnification  $\times 40$ . **D:** Graphs depicting the intensity of expression of the endothelial proteins caveolin-1 (left) and ICAM-1 (right) per PV-1 positive vessel counted (see Materials and Methods). Histograms depict pooled values of vessels counted from at least 24 skin sections from three experiments.

### *ICAM-1<sup>high</sup> Cav-1<sup>low</sup> Vessels Mediate the Majority of Neutrophil Recruitment in Response to Recombinant Human IL-8*

The entire process of inflammatory neutrophil recruitment can be recapitulated by the injection of IL-8 in the tis-

sue.<sup>1,4,18</sup> To identify more specifically the phenotype of microvessels used by extravasating leukocytes, we used intradermal injection of recombinant human IL-8 (rhIL-8) to elicit a chemotactic response from circulating murine neutrophils. The endogenous functional homologs of IL-8, Mip-2, and KC (GRO- $\alpha$ ) act as neutrophil chemoattractants in acute inflammation in the murine system. 1.5 hours postinjection, recruited neutrophils were clearly visible within the lumina of PV-1 expressing vessels in the dermis (Figure 2A). Vessels surrounding aggregates of neutrophils were then analyzed for expression of caveolin-1, the endothelial cell protein ICAM-1, the adhesion molecule PECAM-1, and the microvascular marker PV-1 (Figure 2A).

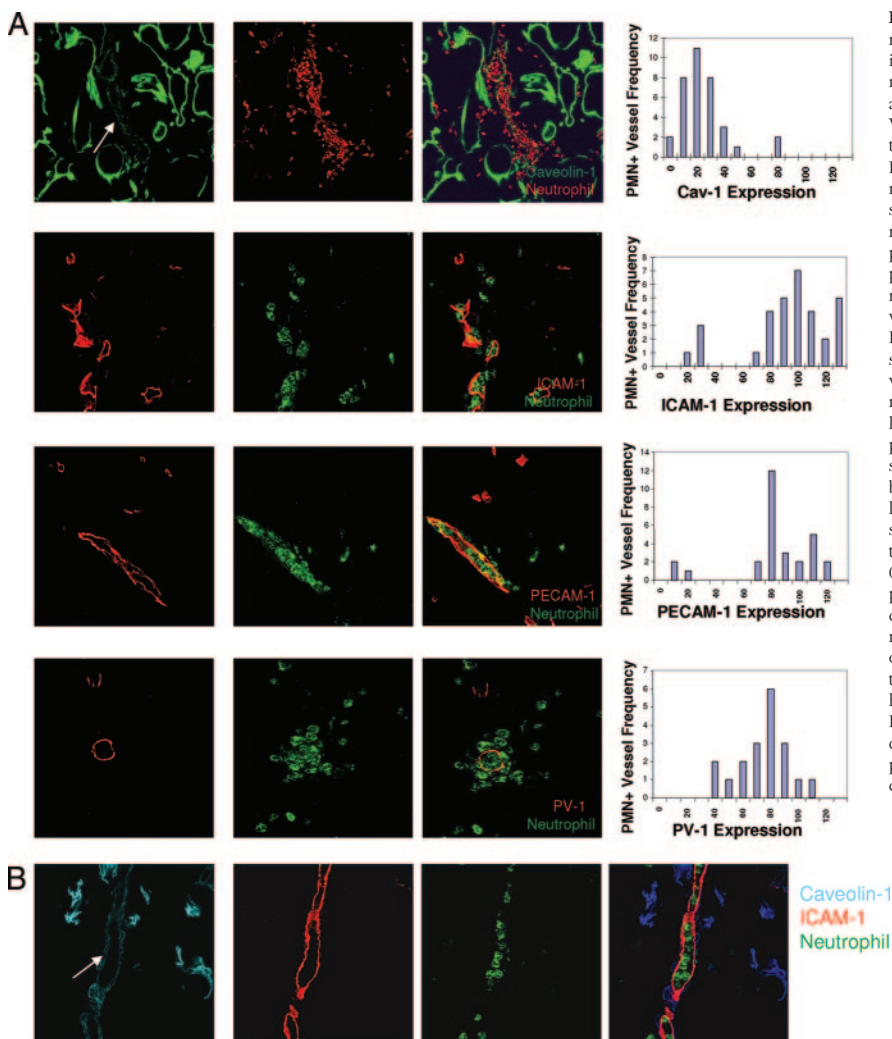
The majority of vessels enclosing recruited neutrophils expressed relatively high levels of ICAM-1 (*ICAM-1<sup>high</sup>*), PECAM-1, and PV-1 (Figure 2A, lower panels). Surprisingly, however, most of these vessels were found to express low levels of caveolin-1 (*cav-1<sup>low</sup>*), as compared with other PV-1 positive vessels, which expressed high levels of the protein (Figure 2A, upper panels). These results suggested that IL-8 preferentially recruited neutrophils to *ICAM-1<sup>high</sup> cav-1<sup>low</sup>* vessels (Figure 2B).

### *Variable Levels of Caveolin-1 in P-Selectin-Positive Venules*

Within the microvasculature, postcapillary venules have been shown to mediate the majority of neutrophil recruitment during an inflammatory response. On injection of rhIL-8 in rabbit skin, IL-8 immunoreactivity was detected solely in the walls of postcapillary venules and small veins and not other blood vessel subtypes.<sup>15</sup> Thus, it remained a possibility that *cav-1<sup>low</sup>* vessels mediating neutrophil recruitment were venules while *cav-1<sup>high</sup>* vessels were other subtypes of microvessels such as arterioles and capillaries. To identify specifically levels of caveolin-1 expression in small veins and venules in the dermis, wild-type skin was excised and co-stained with the venule-specific markers P-selectin and caveolin-1. P-selectin, stored in Weibel-Palade bodies, is constitutively expressed in venular ECs but is absent from capillaries and arterioles.<sup>19</sup> As demonstrated in Figure 3A, variable expression of caveolin-1 was identified in P-selectin-expressing venules in wild-type murine skin. In contrast, all P-selectin-expressing vessels were observed to express high levels of ICAM-1 (Figure 3B and data not shown). Finally as expected, all vessels surrounding collections of recruited neutrophils in response to IL-8 expressed P-selectin (Figure 3C). These results demonstrate that a subset of *ICAM-1<sup>high</sup>, cav-1<sup>low</sup>*, P-selectin-positive venules mediated neutrophil recruitment in response to IL-8 in the skin.

### *Lymph Node High Endothelial Venules Contain Populations of Cav-1<sup>high</sup> and Cav-1<sup>low</sup> vessels*

As discussed, EC differentiation is determined in large part by the unique *in vivo* microenvironment. To evaluate



**Figure 2.** *ICAM-1<sup>high</sup> Cav-1<sup>low</sup>* vessels mediate the majority of neutrophil recruitment in response to injection of rhIL-8 in murine dermis. Wild-type murine skin injected with 2.5  $\mu$ g of rhIL-8 was removed and processed for immunofluorescence at 1.5 hours. Vessels containing aggregates of four or more neutrophils were analyzed for surface expression of PV-1, caveolin-1, ICAM-1, and PECAM-1 (see Materials and Methods). **A:** Sections of injected skin co-stained with anti-caveolin-1 (green) and a rat anti-mouse neutrophil antibody (Serotec) recognizing a polymorphic 40-kDa antigen expressed by polymorphonuclear cells, but absent on resident tissue macrophages (red) (**top left**). **Arrow** depicts a *cav-1<sup>low</sup>* vessel enclosing neutrophils recruited in response to IL-8. Graph depicting intensity of caveolin-1 expression per vessel counted (**top right**). Sections stained with hamster anti-mouse ICAM-1 (red) and rat anti-mouse neutrophil antibody (Serotec; green, middle left). Graph depicting intensity of ICAM-1 expression per vessel counted (**upper middle right**). Sections stained with rat anti-mouse PECAM-1 (red) and rabbit anti-neutrophil myeloperoxidase (green, middle left). Graph depicting intensity of PECAM-1 expression per vessel counted (**lower middle right**). Sections stained with anti-neutrophil myeloperoxidase (green) and anti-PV-1 (red, **bottom left**). Graph depicting intensity of PV-1 expression per vessel counted (**bottom right**). Images captured at original magnification  $\times 40$ . Histograms depict pooled values of vessels counted from at least 24 skin sections from three experiments. **B:** IL-8 injected murine skin at 1.5 hours co-stained with anti-caveolin-1 (blue), anti-ICAM-1 (red) and anti-neutrophil (Serotec, green) depicting an *ICAM-1<sup>high</sup> cav-1<sup>low</sup>* vessel. **Arrow** depicts a *cav-1<sup>low</sup>* vessel enclosing neutrophils recruited in response to IL-8. Image captured at 60X.

if similar molecular heterogeneity extends to vessels used by extravasating leukocytes in other vascular beds, caveolin-1 expression was examined in high endothelial venules (HEVs) in the murine lymph node. HEVs are specialized postcapillary venules critical for homing of monocytes and T cells to the lymph node during development and in chronic inflammatory conditions.<sup>12,20</sup> Identified by the HEV specific marker PNAd, the majority of the HEVs were identified as *cav-1<sup>low</sup>* with the remainder expressing high levels of the protein (Figure 4A–D). Accordingly, we detected substantial variability in the number of caveolae among individual HEVs from the same lymph node (Figure 4E). Within the same sample, some HEVs displayed many caveolae lining the luminal surface while others had relatively very few.

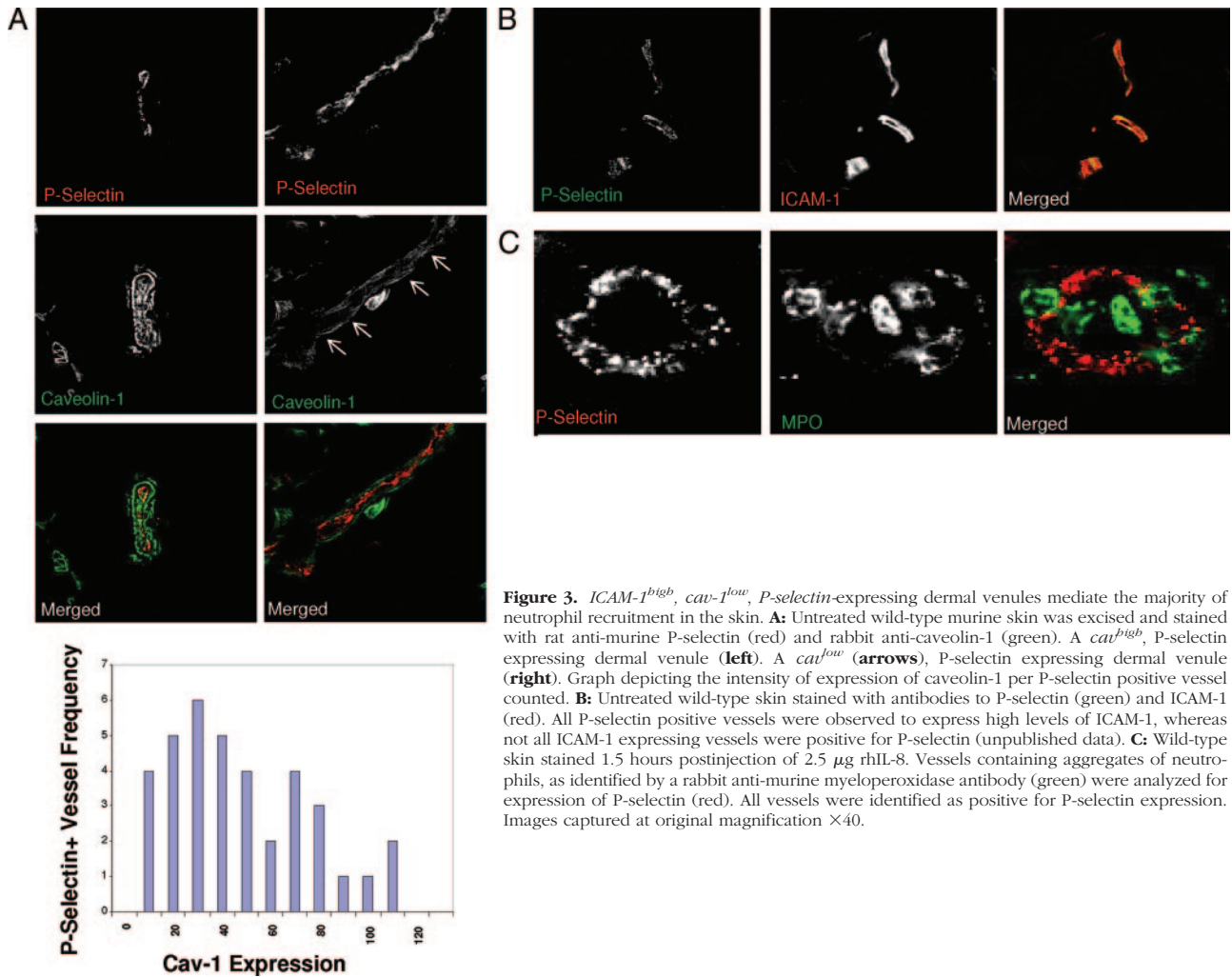
### Normal Neutrophil Recruitment and Extravasation in the Absence of Caveolin-1

In light of these observations, we investigated whether the presence of caveolin-1 in vessels of the microvasculature was inhibitory to neutrophil extravasation. In such a case, *cav-1<sup>-/-</sup>* animals would be expected to have greater

numbers of extravasated neutrophils in the dermis in response to IL-8. To test this, we injected shaved skin of wild-type and *cav-1<sup>-/-</sup>* animals with 1, 2.5, and 5.0  $\mu$ g of rhIL-8 and analyzed the total number of neutrophils in the dermis after three hours. As depicted in Figure 5, on injection of rhIL-8, no appreciable difference between wild-type and *cav-1<sup>-/-</sup>* animals was detected in the total number of extravasated neutrophils in the skin.

### Knockdown of Caveolin-1 in HDMVECs Decreases Transcellular Migration

Our results indicate that loss of caveolin-1 in the microvasculature does not affect total neutrophil extravasation in response to IL-8. We therefore designed an *in vitro* model to examine more closely the behavior of migrating neutrophils in the presence of alternately high or low levels of caveolin-1. To approximate the exact type of cells encountered by extravasating leukocytes *in vivo*, we used caveolin-1 specific siRNA to reduce protein expression in primary human dermal microvascular endothelial cells (HDMVECs). Transfection with caveolin-1 siRNA in HDMVECs resulted in a greater than 90% decrease in the



**Figure 3.** *ICAM-1<sup>high</sup>, cav-1<sup>low</sup>, P-selectin*-expressing dermal venules mediate the majority of neutrophil recruitment in the skin. **A:** Untreated wild-type murine skin was excised and stained with rat anti-murine P-selectin (red) and rabbit anti-caveolin-1 (green). A *cav<sup>high</sup>*, P-selectin expressing dermal venule (left). A *cav<sup>low</sup>* (arrows), P-selectin expressing dermal venule (right). Graph depicting the intensity of expression of caveolin-1 per P-selectin positive vessel counted. **B:** Untreated wild-type skin stained with antibodies to P-selectin (green) and ICAM-1 (red). All P-selectin positive vessels were observed to express high levels of ICAM-1, whereas not all ICAM-1 expressing vessels were positive for P-selectin (unpublished data). **C:** Wild-type skin stained 1.5 hours postinjection of 2.5  $\mu$ g rhIL-8. Vessels containing aggregates of neutrophils, as identified by a rabbit anti-murine myeloperoxidase antibody (green) were analyzed for expression of P-selectin (red). All vessels were identified as positive for P-selectin expression. Images captured at original magnification  $\times 40$ .

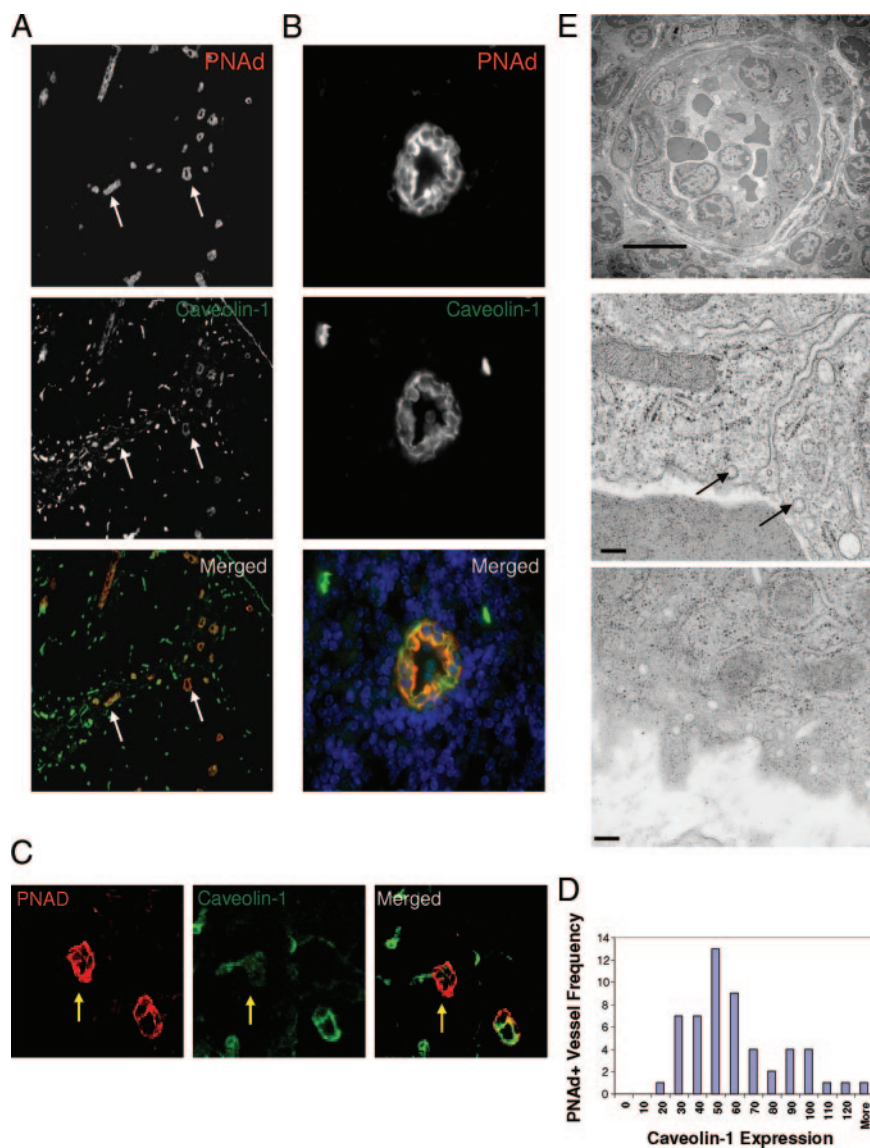
level of caveolin-1, with no appreciable reduction in the level of ICAM-1 (Figure 6, A and B). To assess the effect of caveolin-1 levels on neutrophil diapedesis, purified human neutrophils were added to TNF- $\alpha$ -stimulated HDMVECs transfected with either caveolin-1 siRNA or control siRNA. Migrating neutrophils were easily distinguished from resting or adherent cells by their perpendicular orientation relative to the endothelial cell monolayer, causing a distinct staining pattern of CD45 that distributed with F-actin in the trailing end of the neutrophil during diapedesis.<sup>21</sup> Active migration events were scored as paracellular or transcellular based on the presence or absence of colocalization with the junctional marker VE-cadherin, respectively. In non-transfected cells, a significant fraction of migrating neutrophils (nearly 30%) were found to traverse HDMVECs in a purely transcellular manner unrelated to VE-cadherin-expressing cellular junctions (data not shown). A similar number of neutrophils were found to use a transcellular route of diapedesis after transfection with the control siRNA oligos (Figure 6, C, D, left panels) as in the untransfected cells. However, knockdown of caveolin-1 in HDMVECs resulted in about a 50% loss in the percentage of cells undergoing transcellular migration and an in-

crease in cells using the paracellular route (Figure 6, C, D, right panels).

### Discussion

Neutrophil recruitment and extravasation through vessels of the microvasculature is critical to the acute inflammatory response. Using injection of rhIL-8 in the skin, we established a model for this condition in the mouse. We observed that in response to injection of chemokine, neutrophils largely extravasated from *ICAM-1<sup>high</sup> cav-1<sup>low</sup>* venules. Additionally, we found that specialized vessels in other vascular beds, lymph node HEVs also expressed alternately high and low levels of caveolin-1.

It is as yet unclear as to why *cav-1<sup>low</sup>* venules are targeted by circulating neutrophils in response to IL-8. We found no difference in the number of extravasated neutrophils in response to an intradermal injection of IL-8 in transgenic mice expressing high levels of caveolin-1 (data not shown), or in *cav-1<sup>-/-</sup>* animals, suggesting that caveolin-1 neither facilitated nor inhibited total neutrophil transmigration. *In vitro*, we observed that decreased caveolin-1 in HDMVECs favors an increase in the per-



**Figure 4.** *Cav-1<sup>bigb</sup>* and *cav-1<sup>low</sup>* expressing HEVs in the murine lymph node. Untreated wild-type murine inguinal lymph nodes were excised and co-stained with an HEV-specific antibody to peripheral node addressin-PNAd (red) and caveolin-1 (green). **A:** Section from a wild-type inguinal lymph node co-stained with anti-PNAd and anti-caveolin-1 depicting a mixed population of *cav<sup>bigb</sup>* and *cav<sup>low</sup>* expressing HEVs (**arrows**) Images captured at original magnification  $\times 10$ . **B:** Section from a wild-type lymph node stained with anti-PNAd (red), anti-caveolin-1 (green) and Dapi (blue) depicting an HEV with relatively low levels of caveolin-1 as compared with the level of expression in the surrounding vessels. Images captured at original magnification  $\times 40$ . **C:** A *cav-1<sup>low</sup>* HEV (**arrow**) next to a *cav-1<sup>bigb</sup>* HEV. Images captured at original magnification  $\times 20$ . **D:** Graph depicting the distribution of *cav-1<sup>bigb</sup>* and *cav-1<sup>low</sup>* in PNAAd positive HEVs in the murine inguinal lymph node. **E:** Inguinal lymph nodes were excised and processed for electron microscopy and HEVs were identified by their distinct cuboidal cellular morphology and rounded appearance. An HEV in the murine inguinal lymph node (scale bar = 2  $\mu\text{m}$ ) (**top**). An endothelial cell from an HEV with few visible caveolae. **Arrows** indicate coated pits (**middle**) (scale bar = 200 nm). An endothelial cell from another HEV taken from the same lymph node sample, a large number of caveolae can be detected (**bottom**) (scale bar = 200 nm).

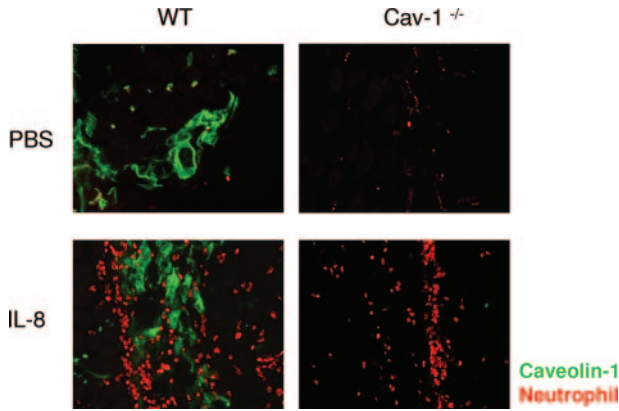
centage of neutrophils migrating along a paracellular path. Taken together, these results suggest that in response to IL-8, neutrophils are recruited to venules expressing relatively low levels of caveolin-1 and migrate across such vessels in a largely paracellular manner.

More research is needed to determine whether recruitment to *cav-1<sup>low</sup>* vessels is characteristic of acute inflammation in general or represents a specific response to the injection of IL-8. We have found that microvasculature is composed of a phenotypically diverse collection of vessels expressing varying levels of both ICAM-1 and caveolin-1. It remains possible that in response to different inflammatory stimuli or different concentrations of the same chemokine, neutrophils may be preferentially recruited to *cav<sup>high</sup>* vessels.

There is conflicting evidence as to the predominant route of neutrophil diapedesis *in vivo*. Early studies involving leukocyte trafficking through inflamed rat mesenteric vessels show that neutrophils follow a predominantly paracellular path of diapedesis.<sup>22,23</sup> A later study dem-

onstrated that neutrophils migrated through microvessels of the pig dermis in a transcellular manner in response to the chemotactic agent, FMLP.<sup>24,25</sup> Largely as a consequence of the technical difficulty of *in vivo* analysis, no consensus has been reached as to the predominant route of migration *in vivo*.

Prior studies conducted *in vitro* have suggested that the route of neutrophil diapedesis is mainly paracellular, with cells preferentially crossing endothelial monolayers at tricellular corners devoid of tight junctions.<sup>26,27</sup> However, nearly all such studies examining neutrophil migration *in vitro* were performed in large-vessel derived HUVECs, while leukocyte extravasation *in vivo* is known to be mediated by microvascular endothelium. There is substantial evidence that macro- and microvascular endothelium differ in their inflammatory response. Microarray comparison of gene expression profiles in HUVECs and human microvascular endothelial cells after TNF- $\alpha$  stimulation shows that few of the genes examined were regulated in a similar manner in both cell types.<sup>28</sup> In



**Figure 5.** Normal neutrophil recruitment and extravasation in the absence of caveolin-1 and caveolae. Wild-type and *Cav-1*<sup>-/-</sup> mice were shaved on their flanks and injected with PBS on one side (**top**), and 2.5  $\mu$ g of rhIL-8 on the other (**bottom**). Three hours after injection, skin was removed, fixed and stained with rat-anti-mouse neutrophil antibody (Serotec) (red) and rabbit anti-caveolin-1 (green). Figure shows a similar number of extravasated neutrophils in the dermis of both wild-type and *Cav-1*<sup>-/-</sup> mice. Images captured at original magnification  $\times 20$ .

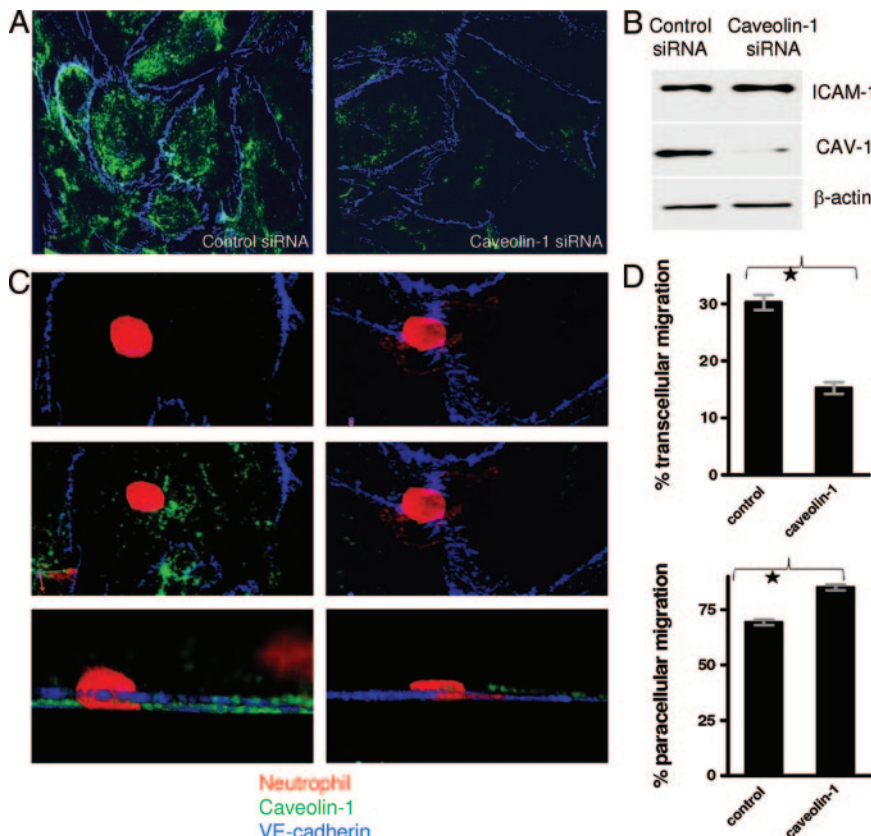
addition, TNF- $\alpha$ -stimulated HUVECs and microvascular endothelial cells were found to have clear differences in the regulation of a number of genes critical to leukocyte transmigration, including ICAM-1 and IL-8.<sup>28</sup>

We have found that a significant percentage of neutrophils migrated across the endothelial monolayer along a purely transcellular path unrelated to cell-to-cell junction. Using siRNA specific for caveolin-1 we achieved a greater than 90% reduction in protein expression in pri-

mary HDMVECs with no diminishment in caveolin-1 levels on transfection with control oligonucleotides. Although we cannot completely exclude the possibility of off-target effects, as in the *cav-1* knockout mouse *in vivo*, we observed no defect in total neutrophil transmigration in our *in vitro* knockdown system. We determined that the percentage of neutrophils capable of migrating along the transcellular path was dependent on the level of expression of caveolin-1 in HDMVECs.

The mechanism by which caveolin-1 facilitates migration along a transcellular route is unclear. Millan et al demonstrated that ICAM-1, a critical factor in leukocyte adhesion and transmigration, is transcytosed by caveolae during T cell diapedesis through HUVECs. Activated T lymphoblasts were found to extend pseudopodia into regions enriched with caveolin-1, ICAM-1 and F-actin, and migrated through those regions.<sup>7</sup> This finding led to speculation that during T cell transmigration, caveolae may fuse forming a transcellular pore whereby the leukocyte can traverse endothelium without substantially disturbing the structural integrity of the cell.<sup>7</sup>

Using siRNA specific for caveolin-1 they showed that reduction of levels of caveolin-1 in HUVECs led to a concurrent decrease in the expression of ICAM-1. In our model, knockdown of caveolin-1 in HDMVECs had no effect on the level of ICAM-1 but resulted in a comparable decrease in the percentage of leukocyte transcellular migration. Further research is needed to determine whether the role of ICAM-1 in transcellular migration differs according to EC subtype.



**Figure 6.** Knockdown of caveolin-1 in HDMVECs decreases transcellular neutrophil migration. Neutrophils were added to primary HDMVECs transfected with caveolin-1 siRNA and luciferase control siRNA and allowed to migrate (see Materials and Methods). Fixed coverslips containing neutrophils and endothelial cells were stained with antibodies to caveolin-1 (green), VE-cadherin (blue), and CD45 (red). Neutrophils in the process of diapedesis could be distinguished by their perpendicular orientation to the monolayer and ring-like cluster of intense CD45 expression, which marked the point of penetration through the endothelium. Active migration events were scored as paracellular or transcellular based on the presence or absence of colocalization with the junctional marker VE-cadherin, respectively. **A:** Representative confocal images of HDMVECs transfected with luciferase control siRNA (**left**) and caveolin-1 siRNA (**right**) stained with caveolin-1 (green) and VE-cadherin (blue). **B:** Immunoblot analysis of protein levels of caveolin-1 and ICAM-1 in control and siRNA transfected HDMVECs.  $\beta$ -actin was used to normalize protein loading. **C:** Top (**top**) and side (**bottom**) views of representative confocal images depicting a single neutrophil migrating in a transcellular manner in control siRNA transfected HDMVEC (**left**) and another neutrophil migrating along a paracellular route (**right**) through caveolin-1 siRNA transfected HDMVECs. **D:** Graphs quantifying the percentage of neutrophils migrating in a transcellular and paracellular manner in control and caveolin-1 siRNA transfected HDMVECs. Values represent the mean  $\pm$  SD from multiple replicate coverslips from two independent experiments. \* $P < 0.001$ ; Student's *t*-test.

Our studies have demonstrated that in response to IL-8, venules identified as *ICAM-1<sup>high</sup> cav-1<sup>low</sup>* mediated the majority of neutrophil extravasation in the skin and that differential levels of caveolin-1 influenced the route of migration across the microvasculature. These results present a functional link between the phenotype of microvessels used by extravasating neutrophils and their migratory behavior across the endothelium.

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