Monocyte ADAM17 Promotes Diapedesis during Transendothelial Migration: Identification of Steps and Substrates Targeted by Metalloproteinases

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Monocyte ADAM17 Promotes Diapedesis during Transendothelial Migration: Identification of Steps and Substrates Targeted by Metalloproteinases

Yoshiaki Tsubota,* Jeremy M. Frey,* Phillip W. L. Tai†,1 Robert E. Welikson,†,2 and Elaine W. Raines*†

Despite expanded definition of the leukocyte adhesion cascade and mechanisms underlying individual steps, very little is known about regulatory mechanisms controlling sequential shifts between steps. We tested the hypothesis that metalloproteinases provide a mechanism to rapidly transition monocytes between different steps. Our study identifies diapedesis as a step targeted by metalloproteinase activity. Time-lapse video microscopy shows that the presence of a metalloproteinase inhibitor results in a doubling of the time required for human monocytes to complete diapedesis on unactivated or inflamed human endothelium, under both static and physiological-flow conditions. Thus, diapedesis is promoted by metalloproteinase activity. In contrast, neither adhesion of monocytes nor their locomotion over the endothelium is altered by metalloproteinase inhibition. We further demonstrate that metalloproteinase inhibition significantly elevates monocyte cell surface levels of integrins CD11b/CD18 (Mac-1), specifically during transendothelial migration. Interestingly, such alterations are not detected for other endothelial- and monocyte-adhesion molecules that are presumed metalloproteinase substrates. Two major transmembrane metalloproteinases, a disintegrin and metalloproteinase (ADAM)17 and ADAM10, are identified as enzymes that control constitutive cleavage of Mac-1. We further establish that knockdown of monocyte ADAM17, but not endothelial ADAM10 or ADAM17 or monocyte ADAM10, reproduces the diapedesis delay observed with metalloproteinase inhibition. Therefore, we conclude that monocyte ADAM17 facilitates the completion of transendothelial migration by accelerating the rate of diapedesis. We propose that the progression of diapedesis may be regulated by spatial and temporal cleavage of Mac-1, which is triggered upon interaction with endothelium. The Journal of Immunology, 2013, 190: 000–000.

Inflammation is a key process in disease pathogenesis, and leukocyte transendothelial migration into inflamed tissues controls both initiation and progression of acute and chronic inflammatory diseases. Transendothelial migration is a sequential, multistep process that consists of leukocyte rolling, capture, and firm adherence to endothelium, followed by locomotion across the endothelium and diapedesis through the intact vessel into the tissue, usually at endothelial junctions (1–3). Engagement of key ligands and their counterreceptors expressed on both leukocytes and endothelial cells have been shown to be responsible for each step of transendothelial migration (1–3). However, very little is known about regulatory mechanisms underlying the progression through each step and transitions between steps.

A molecular mechanism capable of rapidly reducing adhesion molecule interactions is their proteolytic cleavage or “ectodomain shedding” (4). In fact, soluble forms of multiple adhesion molecules involved in each step of leukocyte transendothelial migration are proteolytically shed, and elevated levels are detected in physiological fluids from various inflammatory diseases (4, 5). In addition, recent studies suggest roles for ectodomain shedding in leukocyte trafficking. For example, shedding of Mac-1 (CD11b/CD18), a major integrin dimer on leukocytes, has been reported to accelerate macrophage efflux from an inflammatory site (6) and regulate neutrophil detachment in vitro (7). However, specific steps in leukocyte transendothelial migration modulated by ectodomain shedding have not been determined.

Proteases responsible for shedding of a large number of cell-surface proteins involved in leukocyte transendothelial migration on both leukocytes and endothelial cells are zinc-dependent endopeptidases composed of a family of matrix metalloproteinases (MMPs), which include secreted and membrane types (MT)-MMPs, and the ADAM (a disintegrin and metalloproteinase) family of transmembrane metalloproteinases (4, 5). Analyses of specific enzymes have identified some key proteolytic cleavage events, but most studies have focused on regulation of neutrophil and lymphocyte transendothelial migration. For example, knockdown of ADAM10 on T cells, HUVECs, or both impairs T cell transendothelial migration in vitro (8). Although VE-cadherin was identified as a pos-

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Abbreviations used in this article: ADAM, a disintegrin and metalloproteinase; ALCAM, activated leukocyte cell adhesion molecule; CD62L, L-selectin; CM, conditioned medium; JAM, junctional adhesion molecule; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1 matrix metalloproteinase; siRNA, small interfering RNA.
possible endothelial target of ADAM10, the relative role of VE-cadherin cleavage was not determined (8). Neutrophil transendothelial migration is inhibited by soluble forms of junctional adhesion molecule (JAM)-A, which is shed from endothelium by ADAM10 and ADAM17 (9). Recently, ADAM17 shedding of L-selectin (CD62L) was shown to limit neutrophil recruitment, but monocyte emigration is independent of CD62L shedding (10).

A more limited number of studies have examined monocyte transendothelial migration. An Ab to MT1-MMP impairs transendothelial migration of human monocytes but only when HUVECs are preactivated and MCP-1 is present (11). Although ICAM-1 has been proposed as a possible substrate (12), the step(s) targeted by MT1-MMP is still unclear. Thus, there is a need to evaluate particular steps targeted by regulated proteolysis during transendothelial migration and to better define the enzymes and substrates involved.

In this report, we identify the metalloproteinase-regulated mechanisms underlying transendothelial migration used by monocytes, cells of central importance in the outcome of acute, and chronic inflammatory disease pathogenesis. In the presence of GM6001, which blocks metalloproteinases expressed on both monocytes and endothelial cells, we show that diapedesis is significantly delayed without any marked effect on adhesion or locomotion across the endothelial surface. To interrogate possible substrates whose impaired cleavage contributes to the delay of diapedesis, we separately screened monocytes and endothelial cells for adhesion molecules whose cell surface expression was elevated by GM6001 following their coincubation, which mimics transendothelial migration. Despite the presence of multiple substrates on both cells, only monocyte surface levels of Mac-1 increase upon coincubation with GM6001. We further show that ADAM10 and ADAM17 are responsible for metalloproteinase-dependent shedding of Mac-1 and that absence of monocyte ADAM17 leads to a significant prolongation of diapedesis. In contrast, depletion of monocyte ADAM10, or endothelial ADAM10 or ADAM17, does not affect diapedesis. Therefore, we demonstrate that monocyte ADAM17 promotes diapedesis and suggest that ADAM17 cleavage of Mac-1 may serve as a regulatory mechanism.

Materials and Methods

Human endothelial cell culture and monocyte isolation

HUVECs (Cascade Biologies—Invitrogen) were cultured in gelatin-coated flasks using M199 supplemented with 20% FCS, EGM-2 SingleQuots (Lonza), and antibiotics and used up to passage 3. Human PBMCs were freshly isolated from citrate anticoagulated whole blood of healthy donors by Ficoll–Paque Plus (Amersham Biosciences) separation and subjected to enrichment for monocytes by negative selection using the Monocyte Isolation Kit II (Miltenyi Biotec) plus biotinylated Abs against CD42b (GenexTex) in some experiments to remove platelets (13). Enriched monocytes (>90% estimated by FACS analysis) were resuspended in assay medium (phenol red-free M199 with 20% FCS and 20 mM HEPES [pH 8]) and kept on ice unless specified. Metalloproteinase activity was blocked by a 30-min preincubation with 50 μM GM6001 (Elastin Products) and 0.1% DMSO as a control, unless specified otherwise. Because GM6001 blockade is reversible, all subsequent experiments also included inhibitor or control. The University of Washington Human Subjects Review Committee has approved all protocols.

Transwell assays

HUVECs were seeded at 3 × 10^5 cells/well onto Costar Transwell filters (3-μm pore, 6.5-mm diameter) previously coated with 5 μg/ml human fibronectin and grown to confluence. PBMCs (3.0 × 10^6 cells/100 μl) were labeled with calcine-AM, and migrated leukocytes in the lower well and bottom filter were counted using ImageJ software (National Institutes of Health).

Static assay of monocyte transendothelial migration

The in vitro system previously reported to model monocyte transendothelial migration under static conditions using confluent HUVEC monolayers grown on type I collagen gels (14) was modified to enable simultaneous recording of multiple conditions. Briefly, flat collagen gels (~0.12-mm thickness) were prepared in 4-well silicon chamber (9 mm diameter, ~3-mm depth), which is made in a 40-mm glass-bottom dish (Willco Wells) in combination with Secure-Seal Adhesive Spacer and Press-to-Silicon Seal Silicon Isolator (Molecular Probes). Detailed methodology is available upon request. The collagen gels were coated with human plasma fibronectin, and HUVECs were plated and grown to confluence. After 30 min of equilibration at 37°C and 30 min of pretreatment with DMSO or GM6001, both in assay medium, experiments were started by addition of a monocyte suspension (50 μl droplet/well, 3–4 × 10^6 cells) to the HUVECs (100 μl/ well). Multiple fields were randomly chosen so that at least 25 diapedesis events/condition could be monitored. The recording was started after 20 min for unactivated HUVECs and 10 min for TNF-activated HUVECs.

Flow assay of monocyte transendothelial migration

Analysis of transendothelial migration under shear flow was performed as described previously (15). Briefly, HUVECs were grown to confluence on 25-mm diameter fibronectin-coated glass coverslips and preactivated for 4 h with 10 ng/ml TNF-α, followed by preincubation with DMSO (0.1%) or 50 μM GM6001 for 30 min at 37°C. The prepared coverslip was inserted into the in vitro flow device as described previously. Platelet-free monocytes were kept at 8°C and used within 6 h of preparation. Preincubation of monocytes with DMSO or GM6001 was at 37°C for 5 min to minimize the loss of CD62L, which is essential for adhesion under flow conditions. The monocytes were then introduced into the flow chamber as a bolus (10^6 cells/100 μl), followed by the culture medium, which was allowed to equilibrate at 37°C and 30 min of pretreatment with DMSO or GM6001, respectively. At 37°C for 5 min to minimize the loss of CD62L, which is essential for adhesion under flow conditions. The monocytes were then introduced into the flow chamber as a bolus (10^6 cells/100 μl), followed by the culture medium, which was allowed to equilibrate at 37°C and 30 min of pretreatment with DMSO or GM6001, respectively. The monocytes were then allowed to attach to the HUVECs for 5 min and then introduced into the flow chamber. The recording was started after 20 min for unactivated HUVECs and 10 min for TNF-activated HUVECs.

Optimal diapedesis depends on monocyte ADAM17

Monocyte migration on endothelium was analyzed in a blinded manner by two separate investigators using ImageJ with MTrackJ plug-in software (E. Meijering, Biomedical Imaging Group Rotterdam of the Erasmus MC–University Medical Center, Rotterdam, The Netherlands). Monocyte cell bodies were traced on the time-lapse images by moving the MTrackJ pointer when they displaced more than their radii. Analyses were done only on monocytes that ultimately underwent diapedesis, and monocytes undergoing transmigration at impaired endothelial junctions were excluded.

Flow cytometry-based screening of metalloproteinase substrates on coincubated endothelial cells and monocytes

HUVECs were grown to confluence in human plasma fibronectin (5 μg/ml)-coated 10-cm dishes and stained for 10 min with 1 μM CellTracker Green CMFDA (Molecular Probes) 16 h prior to use. Monolayers were stimulated for 4 h with TNF-α at 10 ng/ml and subjected to preincubation with DMSO or GM6001 for 20 min. Freshly prepared PBMCs (10^6 per condition) were similarly preincubated and then coincubated with the HUVEC monolayers in the presence of DMSO or GM6001 for 2 h. The cocultures were washed three times to remove nonadherent cells and collected with ice-cold PBS supplemented with 0.1% BSA and 10 μM EDTA (1 ml/dish) using cell lifters. Following incubation with FcR blocking reagent (Miltenyi Biotec), cell suspensions were stained at 4°C for 30 min with anti-CD14 and saturating amounts of PE-labeled Abs for candidate molecules (Supplemental Table I). Simultaneously, cells incubated separately for 2 h were collected and stained as described above and cell suspensions with the same ratio of endothelial cells and monocytes. All stained cells were analyzed on FACScan (BD Biosciences), and flow data were analyzed using FlowJo 8.4 software (Tree Star).

Knockdown of ADAM10 and ADAM17 in primary monocytes

Two reported small interfering RNA (siRNA) sequences for knockdown of ADAM10 and ADAM17 were synthesized by Ambion, and those with the
best downregulation of target ADAMs were used for the experiments shown: ADAM10-construct, 5'-AGA CAI UAU GAA GGA UUA U-3' (16), and ADAM17-construct, 5'-GCU UGA UUC UU GCU CUC A-3' (17). A nontargeting siRNA (Silencer Negative Control number 1; Ambion) was used as control.

Platelet-free primary monocytes (5 × 10⁶) were transfected with siRNA (300 nM siRNA/reaction) using a Nucleofector II Kit and a Human Monocyte Nucleofector Kit (Ammaxa). To achieve maximal knockdown, monocytes were allowed to recover at 37°C for 16 h in IMDM (Lonza) containing 20% human plasma–derived serum (or FCS to evaluate soluble CD18 dimers). Monocytes to be used for transmigration were incubated in a 50-ml conical tube on an orbital shaker to give an estimated shear of 7.5 dyn/cm² to maintain monocyte migratory properties as determined in pilot experiments. Monocytes were subjected to an Annexin V–based Dead Cell Removal Kit (Miltenyi Biotec) to enrich for healthy cells (>95%) and then applied to the static assay of transendothelial migration.

Statistical analysis

For analysis of two groups within a single experiment of time-lapse microscopy using monocytes from a single donor, unpaired t tests were performed after data were confirmed to fulfill the criteria. Otherwise, Mann–Whitney U tests were applied. In comparison of means or medians obtained from three or four experiments, paired t tests were performed. To validate significance for fold differences, paired t test was performed after log transformation. All statistical analyses were performed two-sided using InStat (GraphPad), and p < 0.05 was considered significant.

Results

A broad-spectrum metalloproteinase inhibitor impairs transendothelial migration of monocytes

We put forward the hypothesis that proteolytic shedding of cell surface proteins provides a mechanism to aid in the rapid transition of cells between different steps and to coordinate the complex, multistep process of leukocyte recruitment to inflammatory sites (4). To test this hypothesis, we examined the impact of a broad-spectrum, zinc-dependent metalloproteinase inhibitor, GM6001, on net migration of human PBMCs across HUVEC monolayers using a Transwell assay. As shown in Fig. 1A, GM6001 impairs net migration of leukocytes across unactivated endothelium to 40% of vehicle control. When endothelial cells are preactivated by the inflammatory stimulant TNF-α (0.1 ng/ml for 4–5 h), GM6001 reduces net leukocyte migration to a similar extent (Fig. 1B). We confirmed that monocytes are the major cell type that migrates across endothelial monolayers under all conditions tested (>85% as estimated by FACS analysis; data not shown), and quantification of net monocyte migration shows that GM6001 impairs migration by 70% (Fig. 1C).

Metalloproteinase blockade delays completion of monocyte transendothelial migration under both static and flow conditions

Time-lapse video microscopy was then used to monitor the interactions of purified monocytes with endothelial cells to dissect at which step(s) GM6001 is acting in transendothelial migration. To assess migration under static conditions, unactivated endothelial monolayers on collagen gels were coincubated with primary monocytes (18), an in vitro assay in which key mediators of transmigration have subsequently been confirmed in vivo (19–23), and almost all leukocytes cross the endothelial cells at the cell borders, undergoing paracellular migration (24). As shown in Fig. 2A, GM6001 postpones the frequency of monocyte completion of transendothelial migration, the point at which remnants of monocyte cell body disappear from the apical surface of endothelial monolayers in the process of diapedesis. Because the total frequency of transendothelial migration in the presence of GM6001 is comparable to vehicle control (Fig. 2A), GM6001 does not inhibit transendothelial migration. Rather, GM6001 delays monocyte transendothelial migration under static conditions.

The effects of GM6001 on monocyte transendothelial migration were also tested under flow conditions that more closely model physiological conditions. Monocytes were drawn across a TNF-α–activated endothelial layer for 30 min in medium including vehicle or GM6001 at an estimated flow rate of 0.5 dyn/cm², conditions that induce maximal initial adhesion to allow evaluation of post-adhesion events (15). In vehicle control (Supplemental Video 1), monocytes rapidly adhere, spreading on the activated endothelium followed by locomotion for a relatively short distance to reach endothelial junctions. Monocytes then undergo diapedesis, thereby finishing their paracellular transendothelial migration, which is particularly easy to distinguish under flow conditions. It takes monocytes ~10 min to complete all of these steps (data not shown). However, in the presence of GM6001 (Supplemental Video 2), monocyte tails remain on the apical surface of endothelial monolayers for longer durations until they complete transendothelial migration (Fig. 2B), and GM6001 delays the completion of transendothelial migration under flow conditions as observed under static conditions (Fig. 2A). Taken together, our data indicate that metalloproteinase activity is rate limiting and is required for optimal monocyte transendothelial migration.

Metalloproteinase activity facilitates progression of diapedesis but not other steps of monocyte transendothelial migration

Because the process of monocyte transendothelial migration involves multiple steps, each was quantified to identify the metalloproteinase-targeted step(s). As summarized in Table I, GM6001 does not affect the initial adhesion or locomotion across the endothelium. We also verified that GM6001 does not alter endothelial junctions during the period of the migration assay as compared with vehicle control (data not shown). To analyze diapedesis, a frame-by-frame analysis of vehicle control and GM6001 conditions was performed on unactivated endothelial monolayers under static conditions (Fig. 3A). In vehicle control (Fig. 3A, top panel; Supplemental Video 3) monocytes first squeeze a membrane protrusion, and then their main cell body, between the endothelial junctions, and within a short time their tails retract.
Diapedesis is dependent upon both endothelial and monocyte adhesion molecules, several of which have been shown to be shed by metalloproteinases, including leukocyte-specific integrin CD18 (6, 25) and its partner α integrins (CD11a, CD11b (6), CD11c and CD11d), ICAM-1 (12, 26), activated leukocyte cell adhesion molecule (ALCAM) (27), platelet/endothelial cell adhesion molecule (PECAM)-1 (28), and JAM-A (9). To identify specific molecules involved in metalloproteinase-mediated regulation of diapedesis, we initially screened these adhesion molecules by flow cytometry to determine whether monocyte surface levels were increased in the presence of GM6001 under the same conditions used for evaluation of transendothelial migration (Fig. 4A, 4B, Supplemental Table II). CD14+ monocytes were evaluated following PBMC incubation with or without TNF-activated HUVEC monolayers in the presence of vehicle and GM6001 for 2 h, a time period during which the majority of monocytes complete transendothelial migration (Fig. 2A). As highlighted in Fig. 4A and 4B, only surface levels of CD11b are upregulated (~1.4-fold) following coincubation with HUVECs in the presence of GM6001 (Fig. 4B). ICAM-1 levels were also increased by GM6001 treatment but both with and without HUVEC coinoculation (Supplemental Table II). Monocyte ICAM-1 does not seem relevant to transendothelial migration because monocyte-targeted functional blockade of ICAM-1 did not significantly affect net Transwell migration of mononuclear cells (data not shown). CD11a, ALCAM-1, PECAM-1, and JAM-A levels were not altered by GM6001 (Fig. 4A, Supplemental Table II). Taken together, these data demonstrate a metalloproteinase-dependent manner upon coincubation with endothelial cells and CD11b/CD18 is cleaved by transmembrane proteases ADAM17 and ADAM10.

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To confirm that CD18 is shed from primary monocytes in a metalloproteinase-dependent manner, we analyzed cell lysates and

Table I. GM6001 does not alter initial adhesion or locomotion of monocytes under static conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Index of Initial Adhesion</th>
<th>Locomotion Speed (µm/min²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated HUVECs</td>
<td></td>
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</tr>
<tr>
<td>DMSO</td>
<td>0.139 ± 0.012</td>
<td>3.01 ± 1.42</td>
</tr>
<tr>
<td>GM6001</td>
<td>0.147 ± 0.011</td>
<td>3.82 ± 3.35</td>
</tr>
<tr>
<td>TNF-activated HUVECs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0.455 ± 0.021</td>
<td>3.63 ± 1.91</td>
</tr>
<tr>
<td>GM6001</td>
<td>0.646 ± 0.047</td>
<td>3.22 ± 2.03</td>
</tr>
</tbody>
</table>

Adhesion and locomotion were measured as described in footnotes. Values are means ± SD of representative data from at least three experiments.

Footnotes:

(a) Calcein-AM-labeled PBMCs (3 × 10⁷/well) were coincubated for 20 min with unstimulated or TNF-activated HUVEC monolayers in a 96-well plate. The index of adhesion is the ratio of fluorescent intensities of adhering cells after washing over the input intensity.

(b) The locomotion step of individual monocytes was tracked on time-lapse movies until the frame where the membrane protrusion of the cells reaches the site of diapedesis. To estimate speeds of locomotion, time-course plots of inclusive migration distance for individual monocytes were subjected to the linear least-square method. The slopes of resultant approximated lines were referred to as locomotion speeds.
conditioned media (CM) prepared from monocytes treated with GM6001 for 16 h. Soluble integrin CD18 is decreased in 16-h CM with GM6001 treatment as determined by Western blot analysis (Fig. 4C), and this is associated with increased integrin CD18 in cell lysates. The modest difference in m.w. of soluble and cellular integrin CD18 is similar to previous reports (6, 7, 29), indicating that metalloproteinase cleavage within the ectodomain close to the cell membrane results in loss of the transmembrane and the small cytoplasmic domains. The relatively small increase in cellular CD18 with GM6001 is consistent with significant intracellular stores of CD18 (30). Further analysis of monocyte CMs demonstrates that both CD11b/CD18 (Mac-1) and CD11a/CD18 (LFA-1) are shed by metalloproteinases, but the amount of shed Mac-1 was ∼7.8 times higher than that of LFA-1, suggesting a difference in the extent of constitutive shedding (Fig. 4D).

Candidate endothelial substrates are not altered by metalloproteinase blockade and neither endothelial ADAM10 nor ADAM17 promotes monocyte diapedesis

Because endothelial junctions may serve as gatekeepers for leukocyte transmigration (31), we also assessed changes in surface levels of endothelial candidate substrates during monocyte transendothelial migration in the presence of GM6001 (Supplemental Table III) as shown in the analysis of monocyte surface adhesion molecules (Supplemental Table II). Four candidate substrates were screened, including VCAM-1 (32), ICAM-1 (12, 26), ALCAM (27), and JAM-A (9). However, GM6001 treatment has no effect on surface levels of any of the four candidate substrates, with or without coincubation (Supplemental Table III).

FIGURE 3. Monocyte diapedesis is prolonged by metalloproteinase inhibition under both static and flow conditions. (A) Sequential time-lapse images from the beginning to the end of diapedesis in the presence of DMSO (Supplemental Video 3, top panel) and GM6001 (Supplemental Video 4, bottom panels) are shown every 75.6 s. The beginning of diapedesis (start) was defined as the image frame where the cell body or monocyte membrane protrusion reached the site of diapedesis (asterisk). Completion of diapedesis (end) was denoted as the image frame where the remnant of the monocyte cell body disappeared from the apical surface of HUVEC monolayer. Arrows indicate the direction of monocyte migration. Original magnification ×40. (B) Histograms of the diapedesis duration in DMSO and GM6001 are shown for unactivated HUVEC monolayers. Diapedesis duration is defined as the interval between the start and the end frames of diapedesis, as defined in (A). A representative data set from three different experiments is shown. Medians for this data sets are 8.8 min, n = 47 monocytes for DMSO; 21.7 min, n = 26 monocytes for GM6001; p < 0.0001 (Mann–Whitney U test). (C) Means ± SD of diapedesis duration from different experiments on unactivated (n = 3) and TNF-activated HUVEC (0.1 ng/ml for 4 h, n = 4) are shown for monocytes prepared from different donors. (D) Duration of diapedesis under flow conditions in the presence of DMSO or GM6001 was evaluated as described above. Means ± SD of medians obtained from three different experiments are shown. In a single experiment, an average of 45 monocytes were evaluated for DMSO, and an average of 37 were evaluated for GM6001. *p < 0.05, **p < 0.02, ***p < 0.005 (paired t test).
Supplemental Table I for Abs used. *Abs for CD11a and CD11b for capture and CD18 for detection. See complexes shed in 16-h CM were quantitated by sandwich ELISA using test).

Nonidet P-40 buffer supplemented with proteinase inhibitors, and CM were concentrated using YM-30 membrane (Millipore) following centrifugation. CM (40 times concentrated, 40 μg/ml) were collected. The expression levels are presented as ratios relative to separate experiments for CD11a and five for CD11b. (A and B) Cell surface expression of CD11a (A) and CD11b (B) on monocytes was determined by FACS analysis after coincubation with TNF-activated HUVEC monolayers (endothelial cell [EC] + PBMC) or separate incubation (PBMC alone) for 2 h in the presence of DMSO or GM6001. For analysis of cell surface expression on monocytes, first, green fluorescent ECs were gated out, and then, CD14+ cells were analyzed further. At least 5000 events were collected. The expression levels are presented as ratios relative to separate incubation with DMSO. Each column represents mean ± SD from three different experiments for CD11a and five for CD11b. (C) Platelet-free monocytes were incubated in Opti-MEM at 10^6/ml in the presence of DMSO (–) or GM6001 (+) for 16 h at 37˚C. Cells were lysed on ice with Nonidet P-40 buffer supplemented with proteinase inhibitors, and CM were concentrated using YM-30 membrane (Millipore) following centrifugation. CM (40 times concentrated, 40 μg/ml) and cell lysates (4 μg/ml) were resolved by 7.5% SDS-PAGE and evaluated by Western blotting with Ab to the ectodomain of human CD18. Arrows indicate full-length CD18 in the lysates and shed forms (soluble) of CD18 in CM. (D) CD18 integrin complexes shed in 16-h CM were quantitated by sandwich ELISA using Abs for CD11a and CD11b for capture and CD18 for detection. See Supplemental Table I for Abs used. *p < 0.05, **p < 0.01 (paired t test).

![CD18 integrins are shed from human monocytes in a metalloproteinase-dependent manner both constitutively and inducibly upon interaction with endothelial cells.](Image)

**FIGURE 4.**

Diapedesis duration was also assessed following separate knockdown of endothelial ADAM10 and ADAM17 from HUVECs using siRNA (Supplemental Fig. 1). Depletion of ADAM10 consistently leads to a significant decrease in endothelial–junctional permeability (Supplemental Fig. 1C), which agrees with a previous report (8). However, neither ADAM10 nor ADAM17 depletion from HUVECs reproduces GM6001-mediated prolongation of monocyte diapedesis (Supplemental Fig. 1A, 1B). Taken together, our data demonstrate that cleavage of metalloproteinase substrates on endothelial cells and endothelial ADAM10 and ADAM17 proteolytic activity are not major contributors to metalloproteinase-mediated facilitation of monocyte diapedesis.

ADAM17 expressed on monocytes, but not ADAM10, promotes their diapedesis

To determine the monocyte metalloproteinases that contribute to the regulation of diapedesis, we first analyzed MT1-MMP previously reported to promote monocyte transmigration on activated endothelium based on partial inhibition by an MT1-MMP Ab, but only if MCP-1 was present as a chemottractant (11). Using the same Ab, we observed no effect on monocyte transmigration under our conditions lacking MCP-1 (data not shown). Thus, MT1-MMP does not appear to be a major regulatory mechanism for diapedesis. We then used siRNA knockdown to evaluate whether monocyte ADAM17 and/or ADAM10 are involved in metalloproteinase-mediated regulation of diapedesis. GM6001-dependent prolongation of diapedesis was verified for control-transfected monocytes (Fig. 6A), and a similar extent of inhibition (1.5-fold delay) to that with freshly prepared monocytes (compare with Fig. 2C) was observed. In contrast, depletion of ADAM17 (Supplemental Video 6), but not ADAM10 (Fig. 6B), extends the duration of monocyte diapedesis compared with control-transfected monocytes (Supplemental Video 5), although the extent of inhibition (1.3-fold) was less than that observed with GM6001 (Fig. 6A). Taken together, these data demonstrate that monocyte ADAM17, but not monocyte ADAM10, makes a significant contribution to metalloproteinase-dependent promotion of diapedesis.

Discussion

Although metalloproteinase-mediated “ectodomain shedding” has been implicated in the regulation of leukocyte trafficking (4–9), the steps targeted, enzymes responsible, and key substrates are still unclear, particularly for monocytes. The present study shifts the paradigms regarding metalloproteinase regulation of monocyte
Duration of monocyte diapedesis was determined for monocytes transfected with control siRNA were generated as described in Materials and Methods and subjected to time-lapse video analysis on TNF-activated endothelium in the presence of DMSO or GM6001. Representative data from two independent experiments are expressed as means ± SEM. (B) Depletion of ADAM17, but not ADAM10, from monocytes prolongs the diapedesis step. (A) Untransfected monocytes and monocytes transfected with control siRNA were generated as described in Materials and Methods and subjected to time-lapse video analysis on TNF-activated endothelium in the presence of DMSO or GM6001. Representative data from two independent experiments are expressed as means ± SEM. (B) Duration of monocyte diapedesis was determined for monocytes transfected with control, ADAM10, or ADAM17 siRNA. Means ± SD from three different experiments are shown. All monocyte preparations show compatible viability (>95% as determined by trypan blue exclusion). *p < 0.01 (paired t test), **p < 0.0005 (unpaired t test with Welch correction; n = 19, 37, and 27 monocytes for untransfected, DMSO, and GM6001, respectively).

Our study highlights the role of metalloproteinases, specifically monocyte ADAM17, in regulating the rate of diapedesis. The delay in diapedesis by metalloproteinase blockade is a form of regulation distinct from total blockade of the process as previously shown with blocking Abs to PECAM-1 (18) and CD99 (21). As our time-lapse imaging shows, the main cell body of the monocyte migrates into the endothelial junction, even in the presence of GM6001. Thus, blocking metalloproteinase-mediated cleavage does not impair initiation of diapedesis and functions related to movement of the cell body into the junction between endothelial cells. The marked effect of GM6001 treatment is that monocyte tails are persistent on the apical surface of endothelium, even after the main cell body is spreading in the subendothelial space. Therefore, we suggest that metalloproteinase inhibition impairs organized retraction of the monocyte cell body from the endothelial apical surface and thus diapedesis. The greater inhibitory efficacy of GM6001 shown in the Transwell assay as compared with apical video microscopy (Figs. 1, 3) further suggests that postdiapedesis mechanisms may additionally be targeted by metalloproteinases. Interesting possibilities for future studies include new steps that have been recently defined beneath the endothelial for leukocyte emigration into tissues in vivo in which uropod release from the endothelial basal surface (33) is followed by cell crawling and penetration of vascular basement membranes and pericyte sheaths (34).

A surprising finding in our study is the failure to observe any increase in endothelial surface expression of previously demonstrated substrates of metalloproteinases, including those of ADAM10 and ADAM17, upon coincubation of monocytes and endothelial cells in the presence of GM6001. Coincubation was limited to 2 h to focus on cleavage events during transendothelial migration. Using a similar detection system, Koenen et al. (9) showed that enhanced shedding of endothelial JAM-A upon 2-h coincubation with neutrophils is associated with significant decrease in their endothelial surface levels. Consistent with our inability to detect surface changes in endothelial ADAM substrates, which could contribute to the regulation of diapedesis, endothelial knockdown of ADAM10 or ADAM17 had no effect on the rate of diapedesis. These data are in contrast to previous reports that endothelial ADAM10 regulates T cell transendothelial migration, although the specific step targeted by ADAM10 was not determined (8). Endothelial ADAM10 and ADAM17 also control neutrophil transendothelial migration through generation of soluble JAM-A (9). These data strongly suggest that transendothelial migration of different leukocytes may be regulated by distinct mechanisms.

In contrast, analysis of expression levels of candidate monocyte substrates under conditions comparable to transendothelial migration identified a selective increase in surface levels of the integrin dimer Mac-1 (CD11b/CD18). Mac-1 has been shown to be involved in diapedesis (35) as well as other steps of leukocyte transendothelial migration (1). We were able to confirm that the broad inhibitor GM6001, as well as knockdown of ADAM17 and ADAM10, decreased shedding of soluble Mac-1. To our knowledge, this is the first report of ADAM-mediated cleavage of leukocyte integrins.

Our data further suggest that ADAM17 cleavage of Mac-1 may play a role in facilitation of monocyte diapedesis because knockdown of monocyte ADAM17 impairs diapedesis (Fig. 6B). Under normal conditions, soluble Mac-1 may also contribute to the facilitation of diapedesis because it retains its ability to bind to its endothelial ligand, ICAM-1 (6, 7). Interaction of leukocytes and endothelial cells triggers multiple signaling cascades and activation of leukocyte integrins (36). Because we have shown that integrin activation is not sufficient to induce cleavage of Mac-1 (6), we speculate that monocyte–endothelial contact may activate monocyte ADAM17 or promote its binding to Mac-1 and thus enhance temporally and spatially regulated cleavage. The dissociation of Mac-1/ICAM-1 interactions could then facilitate monocyte retraction from the apical side of endothelium, thereby achieving rapid progression of diapedesis (22). However, because Mac-1 functional blocking Abs impair locomotion and thus prevent paracellular diapedesis (22), it is not possible to use this approach to define the contribution of Mac-1 cleavage to progression of monocyte diapedesis. Alternate strategies, such as uncleavable mutants, will be needed to eliminate the possible contribution of other effects of ADAM17 knockdown and to provide a direct link between ADAM17 cleavage of Mac-1 and facilitation of diapedesis. It also remains possible that additional metalloproteinases may be involved because GM6001 resulted in a greater suppression of diapedesis and soluble integrin CD18 dimer release than those following siRNA knockdown of ADAM17. Shedding of additional substrates not included in our screen may also contribute to the regulation of monocyte diapedesis.

Interestingly, depletion of monocyte ADAM10 did not alter diapedesis, suggesting that ADAM10-mediated cleavage of Mac-1 is not enhanced by interaction with endothelial cells. Indeed, it is

![Figure 6](http://www.jimmunol.org/DownloadedFrom)
known that constitutive shedding by ADAM17 and ADAM10 can be enhanced through distinct stimuli and signaling pathways (37, 38). Previous experiments that defined the monocyte locomotion step also demonstrated a requirement for both monocyte CD11a/CD18 and CD11b/CD18 interaction with endothelial ICAM-1 and ICAM-2 (22). Because endothelial ICAM-1 has been identified as a substrate of ADAM17 (26) and MT1-MMP (12), inhibition of ICAM-1 cleavage has been suggested as a mechanism to regulate its interaction with CD18 integrins capable of altering the locomotion step. However, our inability to detect any effect of metalloproteinase blockade on the locomotion step, despite demonstrating metalloproteinase-dependent cleavage of both CD11a/CD18 and CD11b/CD18, suggests that other mechanisms to modulate their ICAM interactions, such as alteration of integrin affinity (39), may be more important for transitioning between locomotion and diapedesis.

Potential in vivo relevance for our prolongation of in vitro monocyte diapedesis is suggested by intriguing similarities with data from a recent study of in vivo neutrophil diapedesis. Wooddin et al. (40) described two modes of abnormal neutrophil diapedesis in ischemia–reperfusion stimulated cremaster venules: hesitant diapedesis and reverse diapedesis. In hesitant diapedesis, neutrophils move back and forth at endothelial junctions before finally completing migration into the subendothelial space as we observed with monocyte diapedesis in the presence of GM6001, most clearly seen under flow conditions (Supplemental Video 2). In vivo, neutrophils show delayed diapedesis similar to our studies. The delayed diapedesis of neutrophils is caused by relocalization of JAM-C to the venular surface from endothelial junctions (40), which is triggered by ischemia–reperfusion. JAM-C has been reported to mediate monocyte diapedesis in kidney models of ischemia–reperfusion (41) and to maintain monocyte unidirectional diapedesis in vitro (42), suggesting that monocytes may show delayed diapedesis in specific organs and conditions. Interestingly, JAM-C controls diapedesis thorough interaction with Mac-1 (35, 43, 44). Thus, it is possible that metalloproteinase cleavage of Mac-1 may be involved in JAM-C-regulated unidirectional diapedesis. Although we observed no delay in vivo in monocyte accumulation in the peritoneal cavity 24 h after injection of the sterile irritant thioglycollate in mice lacking ADAM17 in circulating cells (10), the potential role of Mac-1 and JAM-C has not been investigated in a 30% decrease in monocyte accumulation 48 h after thioglycollate seen in the same mice (J. Tang, C.L. Wilson, and E.W. Raines, unpublished observations). However, as discussed above, alternate approaches such as generation of uncleavable mutants are needed to address this question.

In conclusion, this investigation demonstrates that metalloproteinase activity promotes the diapedesis step of monocyte transendothelial migration under both static and physiological flow conditions in vitro by increasing the rate of diapedesis. During transendothelial migration, metalloproteinase activity leads to cleavage of monocyte Mac-1 but not other reported adhesion substrates expressed on monocytes and endothelial cells. Knockdown of monocyte ADAM17 impairs Mac-1 shedding and diapedesis, suggesting that the metalloproteinase-mediated promotion of diapedesis may involve monocyte ADAM17 cleavage of Mac-1. Because diapedesis is a central component of leukocyte recruitment, our study establishes cell-type specific regulatory mechanisms of diapedesis, which are critical for the development of cell-targeted therapy in chronic inflammatory diseases such as atherosclerosis.

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Disclosures

The authors have no financial conflicts of interest.

References


### Supplemental Tables

**Table SI**: Antibodies used for flow cytometric analyses, Westerns and ELISAs

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Source</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies for flow cytometry (directly conjugated with PE, unless specified)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11b</td>
<td>M1/70</td>
<td>BD Pharmingen</td>
<td></td>
</tr>
<tr>
<td>CD11a</td>
<td>38</td>
<td>AbD Serotec</td>
<td></td>
</tr>
<tr>
<td>VCAM-1</td>
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<td>BD Pharmingen</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
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<td>eBioscience</td>
<td></td>
</tr>
<tr>
<td>PECAM-1</td>
<td>WM59</td>
<td>BD Pharmingen</td>
<td>Does not react with endothelial PECAM-1</td>
</tr>
<tr>
<td>ALCAM-1</td>
<td>3A6</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>JAM-A</td>
<td>M.Ab.F11</td>
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<td>Labeled with Zenon antibody-labeling Kit (Molecular Probes).</td>
</tr>
<tr>
<td>Isotype control</td>
<td>MOPC-21</td>
<td>BD Pharmingen</td>
<td>Labeled with Zenon antibody-labeling Kit (Molecular Probes).</td>
</tr>
<tr>
<td>CD14</td>
<td>61D3</td>
<td>eBioscience</td>
<td>PerCP-Cy5.5</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>CD18, ectodomain</td>
<td>MEM48</td>
<td>Chemicon</td>
<td></td>
</tr>
<tr>
<td>CD18, cytoplasmic domain</td>
<td>Goat IgG</td>
<td>Santa Cruz; C-20</td>
<td></td>
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<tr>
<td>ADAM10</td>
<td>Rabbit IgG</td>
<td>AbD Serotec</td>
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<td>Rabbit IgG</td>
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<td>Rabbit IgG</td>
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<td></td>
</tr>
<tr>
<td>Antibodies for ELISAs</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CD18</td>
<td>TS1/18</td>
<td>Biolegend</td>
<td>For capture</td>
</tr>
<tr>
<td>CD18</td>
<td>Goat IgG</td>
<td>R&amp;D Systems</td>
<td>For detection; biotinylated</td>
</tr>
<tr>
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<td>TS2/4</td>
<td>Biolegend</td>
<td>For capture</td>
</tr>
<tr>
<td>CD11b</td>
<td>LM2/1</td>
<td>eBioscience</td>
<td>For capture</td>
</tr>
</tbody>
</table>
**Table SII. Metalloproteinase inhibition differentially alters monocyte cell-surface adhesion molecules implicated in regulation of adhesion and diapedesis steps.**

<table>
<thead>
<tr>
<th>2-h incubation</th>
<th>Expression levels* of adhesion molecules on the monocyte surface</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD11a</td>
</tr>
<tr>
<td>without HUVECs:</td>
<td></td>
</tr>
<tr>
<td>n=</td>
<td>3</td>
</tr>
<tr>
<td>DMSO</td>
<td>27.01 ± 14.6</td>
</tr>
<tr>
<td>GM6001</td>
<td>25.63 ±15.56</td>
</tr>
<tr>
<td>p value**</td>
<td>ns</td>
</tr>
<tr>
<td>with activated HUVECs:</td>
<td></td>
</tr>
<tr>
<td>n=</td>
<td>3</td>
</tr>
<tr>
<td>DMSO</td>
<td>34.13 ± 18.14***</td>
</tr>
<tr>
<td>GM6001</td>
<td>32.29 ± 14.43</td>
</tr>
<tr>
<td>p value**</td>
<td>ns</td>
</tr>
</tbody>
</table>

* Expression levels in each condition are presented as a ratio of mean geometric fluorescent intensity of samples stained with PE-conjugated specific antibody/background intensity of an identical unstained sample. Means ± SD of at least 3 different experiments are shown.

** The expression levels described above were converted to the relative expression levels/2-h incubation with DMSO without HUVEC, and then two-tailed p values were calculated by paired t test. ns, not significant.

*** For comparison with DMSO without HUVECs, p<0.05 (calculated as described above).
Table SIII. Metalloproteinase inhibition does not alter endothelial cell-surface adhesion and junctional molecules implicated in regulation of locomotion and diapedesis steps.

<table>
<thead>
<tr>
<th>2-h incubation</th>
<th>Expression levels* of candidate substrate molecules on the activated endothelial surface</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VCAM-1</td>
</tr>
<tr>
<td>without PBMCs (HUVEC alone)</td>
<td></td>
</tr>
<tr>
<td>n=</td>
<td>4</td>
</tr>
<tr>
<td>DMSO</td>
<td>17.44 ± 2.83</td>
</tr>
<tr>
<td>GM6001</td>
<td>18.05 ± 2.96</td>
</tr>
<tr>
<td>p value**</td>
<td>ns</td>
</tr>
<tr>
<td>with PBMCs:</td>
<td></td>
</tr>
<tr>
<td>n=</td>
<td>4</td>
</tr>
<tr>
<td>DMSO</td>
<td>17.40 ± 4.20</td>
</tr>
<tr>
<td>GM6001</td>
<td>16.95 ± 3.40</td>
</tr>
<tr>
<td>p value**</td>
<td>ns</td>
</tr>
</tbody>
</table>

* Surface expression on endothelial cells were determined by gating on green-fluorescence positive cells, and expression levels under each condition are presented as a ratio of mean geometric fluorescent intensity of samples stained with PE-conjugated specific antibody/background intensity of an identical unstained sample. Means ± SD of at least 3 different experiments are shown.

** The expression levels described above were converted to the relative expression levels/2-h incubation with DMSO without PBMCs, and then two-tailed p values are calculated by paired t test. ns, not significant.
Supplemental Figure

Figure S1. Endothelial-specific deletion of ADAM10 and ADAM17 does not alter monocyte diapedesis on TNF-activated endothelium in spite of enhanced barrier function with knockdown of ADAM10. HUVECs were transfected with siRNAs for ADAM10, ADAM17 and a non-specific control sequence with DharmaFECT-1 (Dharmacon) according to the manufacturer’s instructions. For functional assays, transfection was done in the vessels for experiments. The duration of monocyte diapedesis on these monolayers was analyzed by time-lapse microscopy.  

A. Protein levels of ADAM10 and ADAM17 were evaluated by immunoblotting of whole cell lysates 72 h and 96 h post siRNA transfection, respectively, with antibodies against their cytoplasmic domains. Arrowheads indicate specific ADAM protein bands.

B. Duration of monocyte diapedesis on control or ADAM-knockdown (KD) TNF-activated endothelial monolayers is shown for representative data from 3 independent experiments for ADAM10 and 2 for ADAM17. Means ± SEM were expressed based on evaluation of 26 (control) and 22 (KD) monocytes for ADAM10, and, 20 and 18, respectively, for ADAM17.

C. HUVECs were cultured in Transwell inserts and treated with the indicated reagents or siRNAs for ~70 h. Endothelial resistance was tracked daily, and maximal plateau levels are shown. Resistance is expressed as mean ± SD from measurement of triplicate wells per condition. Note that GM6001 does not alter endothelial resistance during the period of the migration assay (< 4 h) as compared with vehicle control (see “Results”), but does during long-term culture (~70 h; this figure). Representative data are shown for each knockdown experiments from at least 2 independent experiments, *p <0.01, **p <0.0005 (unpaired t test).
Tsubota et al., Figure S1
Legends for supplemental videos

Video 1. Under flow conditions, monocyte transendothelial migration shows rapid completion of diapedesis in the vehicle control. This video is part of the data summarized in Figure 2B. Monocytes were drawn at 0.5 dyn/cm² (toward the left) across TNF-activated HUVEC monolayers, and monitored every 10 s for 30 min. The 10-frames/s compressed movie shows two monocytes in the first frame and an additional two appearing in the third frame that demonstrate rapid monocyte diapedesis under flow conditions. The cell on the far left stays at the site of adhesion for a relatively long time (~800 s), but once it reaches the endothelial junction, it completes diapedesis rapidly as observed with the other three cells in this video. Note that endothelial borders can be distinguished by their ruffling membranes, and all diapedesis occurs at endothelial junctions (paracellular).

Video 2. GM6001 prolongs diapedesis under flow conditions and is associated with a delay in monocyte tail retraction. Time-lapse imaging was performed as shown in Video 1 using the same monocytes. Monocytes appearing before the 9th frame represent transendothelial migration with GM6001 under flow conditions. Note that monocyte tails remain on the endothelial surface for a longer duration.

Video 3. Monocytes rapidly undergo diapedesis under static conditions. This video corresponds to an image sequence from data in Figure 3A with DMSO. Time-lapse images (40x DIC) of monocyte interactions with unactivated endothelial monolayers were acquired at 37.8-s intervals and compressed to 10 frames/s. A monocyte at the left top corner is locomoting over the endothelial surface and undergoes diapedesis at the right bottom corner. During the
diapedesis step, the monocyte squeezes its membrane protrusion into the endothelial junction; subsequently the main cell body and the tail are retracted into the subendothelial space within a short time (duration of the step, 11.3 min).

**Video 4. Metalloproteinase inhibition prolongs monocyte diapedesis under static conditions.** Video corresponding to GM6001 treatment in data from Figure 3A and was acquired simultaneously with Video 3. A monocyte at the right bottom corner approaching the center of the video is an example of diapedesis in the presence of GM6001 under static conditions. Note that the monocyte tail remains on the endothelial surface before it is completely retracted into the subendothelial space, resulting in the longer duration for the diapedesis step (23.9 min for this monocyte) as compared to vehicle control (Video 3).

**Video 5. Control-siRNA-transfected monocytes can complete diapedesis.** Figure 6B video supplement shows transendothelial migration of a control siRNA-transfected monocyte on a TNF-activated HUVEC monolayer under static conditions (control for Video 6. Phase-contrast Images (20x) were taken every 97 s and compressed to a 4-frames/s movie. An arrow will appear to denote a monocyte that locomotes on the endothelium, and then undergoes diapedesis. The diapedesis step is indicated in the movie during its progression through the endothelium. The duration of diapedesis for the monocyte shown is 37 min.

**Video 6. siRNA-mediated suppression of monocyte ADAM17 prolongs the diapedesis step.** Video supplement to Figure 6B that shows an example of transendothelial migration of monocytes, which were transfected with ADAM17 siRNA. HUVECs are TNF-activated but not
treated with siRNAs. An arrow will appear to denote a monocyte that undergoes diapedesis after spreading on the endothelium without locomotion. Duration of the diapedesis for the monocyte that is shown in the movie is 63 min.