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Elastase Release by Transmigrating Neutrophils Deactivates Endothelial–bound SDF-1α and Attenuates Subsequent T Lymphocyte Transendothelial Migration

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Abstract

Leukocyte trafficking to sites of inflammation follows a defined temporal pattern, and evidence suggests that initial neutrophil transendothelial migration modifies endothelial cell phenotype. We tested the hypothesis that preconditioning of human umbilical vein endothelial cells (HUVEC) by neutrophils would also modify the subsequent transendothelial migration of T lymphocytes across cytokine-stimulated HUVEC in an in vitro flow assay. Using fluorescence microscopy, preconditioning of HUVEC by neutrophils was observed to significantly reduce the extent of subsequent stromal cell–derived factor-1α (SDF-1α [CXCL12])-mediated T lymphocyte transendothelial migration, without reducing accumulation. In contrast, recruitment of a second wave of neutrophils was unaltered. Conditioned medium harvested after transendothelial migration of neutrophils or supernatants from stimulated neutrophils mediated a similar blocking effect, which was negated using a specific neutrophil elastase inhibitor. Furthermore, T lymphocyte transendothelial migration was inhibited by treatment of HUVEC with purified neutrophil elastase, which selectively cleaved the amino terminus of HUVEC-bound SDF-1α, which is required for its chemotactic activity. The reduction in T lymphocyte transendothelial migration was not observed using a different chemokine, ELC (CCL19), and was not reversed by replenishment of SDF-1α, indicating endothelial retention of the inactivated chemokine. In summary, transmigrating neutrophils secrete localized elastase that is protected from plasma inhibitors, and thereby modulate trafficking of other leukocyte subsets by altering the endothelial-associated chemotactic activities.

Key words: endothelium • chemokine • inflammation • lymphocyte • imaging • diapedesis

Introduction

The inflammatory response is an important component of innate immunity and a critical step is extravasation of leukocytes through the blood vessel wall. In recent years, the mechanisms and molecules involved in this process have been widely studied (1, 2). In many respects, the trafficking of T lymphocytes and neutrophils differs, despite the utilization of common molecular mechanisms. This may, in part, be due to the temporal expression of surface adhesion molecules or certain chemokines, leading to preferential recruitment of neutrophils or lymphocytes during the early and late parts of an inflammatory response, respectively. Additionally, in vitro studies have demonstrated that optimal T lymphocyte transendothelial migration requires the presence of laminar flow as well as an apical chemokine stimulus (3), which results in activation and polarization via a Rap-1–dependent mechanism (4). Furthermore, T lym-

Abbreviations used in this paper: DIC, differential interference contrast; EC, endothelial cell; EI, elastase inhibitor; HNE, human neutrophil elastase; HRP, horseradish peroxidase; HUVEC, human umbilical vein ECs; SDF-1α, stromal cell derived factor-1α.

phocyte subsets exhibit different adhesion and homing patterns to nonlymphoid tissues, according to their memory/naive phenotype (5, 6), their Th1/Th2 phenotype (7), and their chemokine receptor status (8, 9).

Recent evidence suggests that the interaction of neutrophils with endothelial cells (ECs) results in phenotypic changes in both ECs and neutrophils (for review see reference 10). Specifically, soluble products of neutrophil activation and transmigration can act in a pro- or antiinflammatory manner with respect to EC behavior (11–14). In particular, neutrophil proteases regulate other important inflammatory components such as chemokine activity (15–17) and expression of surface or junctional adhesion molecules (18, 19). Release of enzymes such as neutrophil elastase is itself augmented through adhesion to ECs (20) or to P-selectin (21).

Neutrophil trafficking has also been described to modulate the subsequent trafficking of other leukocyte subsets through a number of different mechanisms. For example, release of soluble IL-6 receptor from neutrophils has been implicated in mediating a switch from a neutrophilic to a mononuclear infiltrate (22), and neutrophil depletion reduces the capability of *Leishmania major*–infected mice to mount an effective Th2 response (23). In contrast, mononuclear cell infiltration into the peritoneum of LFA-1$^{-/-}$ mice (which have a relative defect in neutrophil trafficking) is normal and is mediated by MCP-1 release from resident macrophage and mesothelial cells (24). Most of these studies have examined the role of neutrophil trafficking on delayed leukocyte behavior. Few studies have examined the effects of transendothelial migration of neutrophils on the ability of ECs to support earlier T lymphocyte transendothelial migration.

In this work, we demonstrate that human umbilical vein ECs (HUVEC)–bound stromal cell–derived factor-1α (SDF-1α [CXCL12])-associated, but not ELC (CCL19), transendothelial migration of CD3$^+$ T lymphocytes is attenuated under flow conditions after transendothelial migration of an initial wave of neutrophils. This inhibition is mediated by neutrophil elastase and appears to affect both migration of T lymphocytes to the EC junction as well subsequent transendothelial migration. These findings suggest that locally secreted elastase from transmigrating neutrophils may be an important component in the regulation of the evolving inflammatory response by down-regulating early emigration of T lymphocytes and preventing the influx of cells of a more chronic inflammatory phenotype.

Materials and Methods

Cells and Cell Lines. HUVEC were isolated and cultured as described previously (25). HUVEC (at subculture 2) were seeded at 50% confluence on 25-mm glass coverslips (Assistent; Carolina Biological Supply Company), coated overnight with 5 μg/ml fibronectin (Sigma-Aldrich), and grown to confluence over 48 h. Routinely, HUVEC were stimulated with 10 U/ml of recombinant human IL-1β (a gift from Biogen) for 4 h and, where indicated, incubated with 20 ng/ml SDF-1α/CXCL12 or 200 ng/ml ELC/MIP-3β/CCL19 (both obtained from PeproTech) in growth medium (M199 + 20% FCS) at 37°C for 15 min before use (3). Human neutrophils (>95% pure) were isolated from sodium-citrated whole blood drawn from healthy volunteers using Histopaque 1077 (Sigma-Aldrich) centrifugation followed by 2% dextran (Sigma-Aldrich) sedimentation and hypotonic red cell lysis as described previously (26). Autologous human CD3$^+$ or CD4$^+$ T lymphocytes (>90% pure, of which 50–60% were CD45RO$^+$) were isolated from the original sample by negative selection using a RosetteSep T cell enrichment cocktail (StemCell Technologies Inc.) according to manufacturer's instructions. In experiments with perfusion of both neutrophils and lymphocytes, T lymphocytes were prelabeled with 1 μM of Cell Tracker orange or green (Molecular Probes) for 10 min at 37°C. Neutrophils were maintained at 8–10°C and T lymphocytes were maintained at 37°C before use. In experiments in which autologous donor plasma was also used, it was isolated by centrifugation of citrated whole blood (300 *g* for 20 min), followed by centrifugation to remove platelets (1,000 *g* for 20 min). Blood was drawn and handled according to appropriate institutionally approved Institutional Review Board protocols.

Generation of Conditioned Media from Neutrophil–EC Supernatants. HUVEC were grown to confluence on 100-mm plastic Petri dishes coated with 0.1% gelatin (Sigma-Aldrich) and stimulated with 10 U/ml of recombinant human IL-1β for 4 h or with growth medium alone. Neutrophils (20 × 10^6 in 3 ml flow buffer, approximating a ratio of 2 neutrophils for each HUVEC) were added to the apical surface and incubated under static conditions at 37°C for 30 min. This resulted in robust transmigration of neutrophils across activated, but not unactivated, HUVEC monolayers. The supernatant from this incubation was harvested and centrifuged at 200 *g* for 10 min at room temperature. This supernatant was further centrifuged at 1,000 *g* for 10 min at 4°C before use. Conditioned media generated in this way was added to IL-1β/SDF-1α–stimulated HUVEC at 37°C for 10 min.

Generation of Activated Neutrophil Supernatants. This protocol was modified from one described previously (11). In brief, 10^8 cells/ml neutrophils were incubated with 10^{-6} M FMLP in HBSS for 60 s at 37°C and centrifuged at 200 *g* at room temperature for 5 min. The supernatant was removed and further centrifuged at 1,000 *g* for 10 min at 4°C before use. Supernatants generated in this way were either used immediately or stored at −20°C. Alternatively, supernatants were produced by stimulation of neutrophils with 10^{-7} M C5a or 10^{-8} M IL-8 for 300 s and harvested using the same conditions as FMLP. Supernatants were added to IL-1β/SDF-1α–stimulated HUVEC at 37°C for 10 min. In certain experiments, these supernatants were pretreated with 10 μM MeOSuc-AAPV-CMK, a specific, irreversible elastase inhibitor (EI; Calbiochem) or 10 μM Z-GLF-CMK, a specific cathepsin G inhibitor (Enzyme Systems Products), both diluted in DMSO (itself at a final working concentration of 0.05%). In other experiments, FMLP-stimulated neutrophil supernatants were generated in the continued presence of 10 μM MeOSuc-AAPV-CMK, 25% autologous plasma, or both before their addition to IL-1β/SDF-1α–stimulated HUVEC at 37°C for 10 min. Alternatively, 33 nM of purified human neutrophil elastase (HNE; Calbiochem) in 50 mM NaOAc/200 mM NaCl was used in place of supernatants and, where indicated, this compound was pretreated with the EI. In one series of experiments, flow buffer (see next paragraph) was made up including either 0.05% DMSO or 10 μM MeO-Suc-AAPV-CMK.

Leukocyte Adhesion and Transmigration Assay. The in vitro flow model has been described previously in detail (27). Conflu-

ent HUVEC monolayers on coverslips were inserted into the flow chamber, and the entire chamber was mounted on an inverted differential interference contrast (DIC)– and fluorescence-equipped microscope (Nikon) connected to an Orca ER camera (Hamamatsu). Experiments were conducted using a 40 or 20× objective lens. Where indicated, to visualize EC junctional proteins, HUVEC monolayers were labeled with either anti–VE-cadherin, AlexaFluor-568 (Molecular Probes)–conjugated mAb, TEA 1/31 (Immunotech), or with AlexaFluor-488–conjugated anti–JAM-1 mAb, 1H2A9 (a gift from C. Parkos, Emory University, Atlanta, GA; reference 28). These mAb treatments have been shown not to alter HUVEC barrier function or the dynamics of neutrophil adhesion and transendothelial migration (unpublished data). The initial wave of neutrophils was resuspended in flow buffer (DPBS/0.1% HSA) at 0.5×10^6/ml and drawn across the HUVEC monolayer at an estimated shear stress of 1.0 dyne/cm^2, using a syringe pump (Harvard Apparatus) to minimize secondary capture of flowing neutrophils (29). Neutrophils were drawn into the chamber for 5 min to permit sufficient accumulation, followed by flow buffer alone for 10 min to allow maximum transmigration to occur. Fluorescent CD3$^+$ T lymphocytes (10^6/ml) were introduced at an estimated shear stress of 0.75 dynes/cm^2 for 6 min to maximize accumulation, followed by buffer alone for 10 min while transmigration of T lymphocytes occurred. Where indicated, SDF-1α (20 ng/ml in flow buffer) was introduced at an estimated shear stress of 0.5 dynes/cm^2 for 10 min, followed by 5 min of flow buffer alone, preceded T lymphocyte perfusion. In some experiments, flow buffer was supplemented with 10 or 25% autologous plasma. For a more detailed diagrammatic scheme of the experimental protocol, see Fig. 1 a.

Image Acquisition and Analysis. Fluorescence and DIC images were acquired using MetaMorph v4.6 software (Universal Imaging Corp.) controlling a Uniblitz VMM-D1 shutter (Vincent Associates) and a Lambda 10–2 optical filter changer (Sutter Instrument Co.). For transendothelial migration experiments, digital images were taken every 15 s for 10 min in one representative field of view from 3 min after T lymphocyte perfusion commenced. Where labeled T lymphocytes were used, both red fluorescence and DIC images were acquired sequentially. Where unlabeled T lymphocytes were used, DIC-only images were obtained at the same frequency. Acquired images were processed and analyzed using Confocal Assistant 4.02 shareware (Todd Clarke Brelje) and LaserSharp 2000 (Bio-Rad Laboratories). The number of sequentially accumulated cells (either rolling, arrested, or transmigrated) was counted for every minute of acquisition. The total number of accumulated leukocytes was tabulated and the number of transmigrated neutrophils or T cells (both in total and over time) was calculated as a percentage of this value. For analysis of junctional migration of T lymphocytes, sequential automated DIC-red-green images were taken from multiple fields of view using preprogrammed exposure times.

Generation of Biotinylated SDF-1α Compounds and ESI-MS Analysis of Elastase-mediated Proteolysis. SDF-1α-C-ter-bt was synthesized as described previously (30). This derivative retains the proadhesive and promigratory activities of native SDF-1α for T lymphocytes when overlaid on activated HUVEC (unpublished data). For SDF-1α-N-ter-bt, which does not bind its receptor CXCR4, the biotin was incorporated on the amino-terminal group before TFA cleavage of the protein resin. Chemokine purification and folding was identical as for SDF 1-67. Confirmation of elastase degradation of SDF-1α-N-ter-bt by reverse phase HPLC and subsequent electrospray ionization mass spectrometry (ESI-MS) was performed as described previously (17).

Blotting of Cleaved SDF-1α from EC Lysates. HUVEC were grown to confluence on 0.1% gelatin (Sigma–Aldrich)-coated
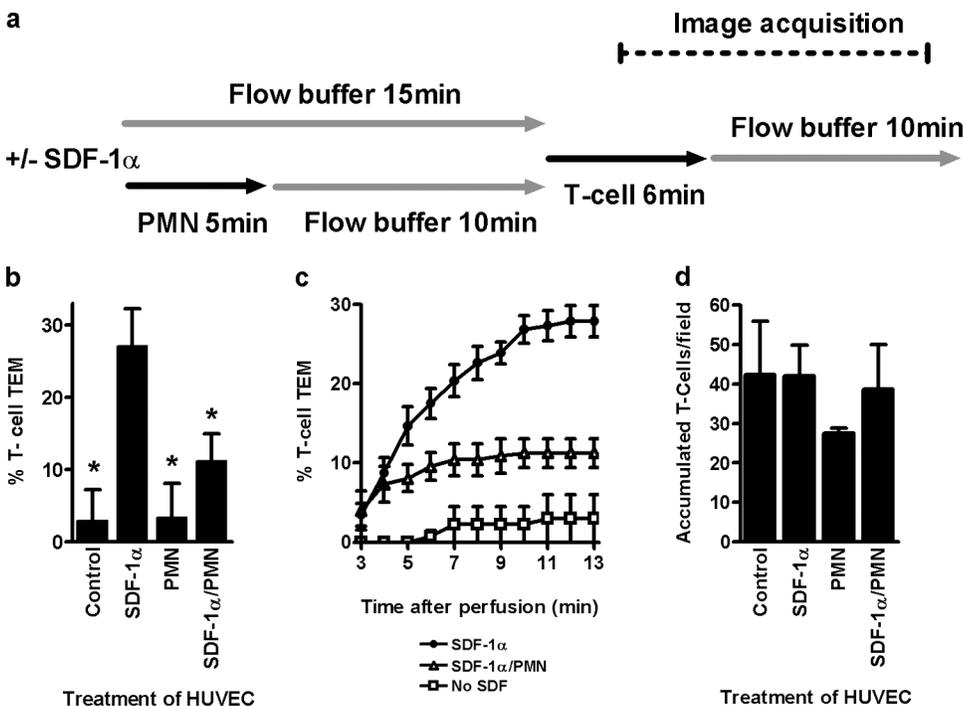

Figure 1. Pretreatment of IL-1β–stimulated HUVEC with neutrophils reduces CD3$^+$ T lymphocyte transmigration in response to SDF-1α. (a) Confluent HUVEC were stimulated with 10 μL/ml IL-1β for 4 h, treated with medium alone or 20 ng/ml SDF-1α before perfusion of PBS/0.1%HAS (flow buffer) for 15 min. Alternatively, neutrophils (0.5×10^6/ml) were drawn over these cells for 5 min at 1.0 dynes/cm^2, followed by flow buffer for 10 min, to allow robust transmigration. After each of the pretreatments, T lymphocytes (1.0×10^6/ml, labeled with Cell Tracker orange) were drawn over HUVEC at 0.75 dynes/cm^2 for 6 min, followed by flow buffer alone. Time–lapse DIC and fluorescent images were acquired after 3 min of T lymphocyte perfusion for 10 min every 15 s. Experiments were analyzed offline using image analysis software (see Materials and Methods). (b) After SDF-1α pretreatment of HUVEC monolayers, T lymphocyte transendothelial migration was 30%. In the absence of SDF-1α, transendothelial migration of T lymphocytes cells was <5%. Neutrophil preconditioning of HUVEC under flow did not affect subsequent baseline T lymphocyte transendothelial migration, but did significantly reduce that observed after SDF-1α pretreatment (*, P < 0.05). With all pretreatments, (c) there was no delay in transendothelial migration or (d) a reduction in the extent of subsequent T lymphocyte accumulation. ($n = 3$, separate experiments).

six-well plates (Costar; Corning Inc.). Confluent ECs were stimulated with IL-1β (for 4 h), incubated with either SDF-1α-N-ter-bt or SDF-1α-C-ter-bt (200 ng/ml; reference 30) (as indicated) for 30 min at 37°C, and washed three times with medium 199. HUVEC were treated with 33 μM purified HNE or control medium 199 at 37°C. ECs were lysed with boiling Tricine sample buffer (Bio-Rad Laboratories) under reducing conditions. Proteins were separated by SDS-PAGE on a 16.5% acrylamide Tris-Tricine gel (Bio-Rad Laboratories) at 4°C for 100 min at 100 V. Proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell) by wet transfer for 1 h at 4°C at 100 V. The blot was probed with 1:2,000 streptavidin–horseradish peroxidase (HRP; Amersham Biosciences) and developed using enhanced chemiluminescence (Pierce Biotechnology).

Data and Statistics. Data are expressed as mean ± SD for three separate experiments performed using different HUVEC isolates, with different leukocyte donors on separate days. Statistical significance was assumed to be P < 0.05 using one-way analysis of variance with Bonferroni post-test correction where appropriate.

Results

Perfusion of IL-1β–stimulated HUVEC with Neutrophils Reduces Subsequent CD3+ T Lymphocyte Transmigration in Response to SDF-1α. CD3+ T lymphocytes were labeled with Cell Tracker orange and drawn across IL-1β–stimulated HUVEC for 6 min, followed by buffer alone for 10 min. Lymphocyte adhesion and transendothelial migration were assessed by time-lapse videomicroscopy and were compared with that observed after pretreatment of endothelium with either SDF-1α, a bolus of neutrophils perfused for 5 min and followed by buffer, or both (Fig. 1 a).

As reported previously (3), in the absence of SDF-1α, <5% of adherent CD3+ T lymphocytes transmigrated across IL-1β–stimulated HUVEC despite robust adhesion to the HUVEC monolayer. After preincubation of HUVEC with SDF-1α, transendothelial migration increased significantly (Fig. 1 b), whereas adhesion remained constant (Fig. 1 d). In contrast, perfusion and robust transmigration of neutrophils in the absence of SDF-1α did not augment subsequent CD3+ T lymphocyte transendothelial migration. Surprisingly, in the presence of SDF-1α, prior perfusion of neutrophils reduced CD3+ T lymphocyte transendothelial migration by 59% (n = 4). Pretreatment of HUVEC with neutrophils and/or SDF-1α did not either cause a delay in CD3+ T lymphocyte transendothelial migration (Fig. 1 c) or a reduction in the number of cells accumulated on HUVEC (Fig. 1 d). Purified CD4+ T lymphocytes exhibited a similar reduction in transendothelial migration (unpublished data). The presence of SDF-1α did not alter the extent of neutrophil accumulation or rate of transmigration (unpublished data).

Perfusion of Neutrophils Does Not Alter Subsequent Neutrophil Transmigration. Transendothelial migration of neutrophils across IL-1β–stimulated HUVEC is observed under flow conditions in the absence of SDF-1α. Therefore, we examined whether the neutrophil inhibition of transendothelial migration was still observed if the second cohort of leukocytes was a population of neutrophils, rather than T
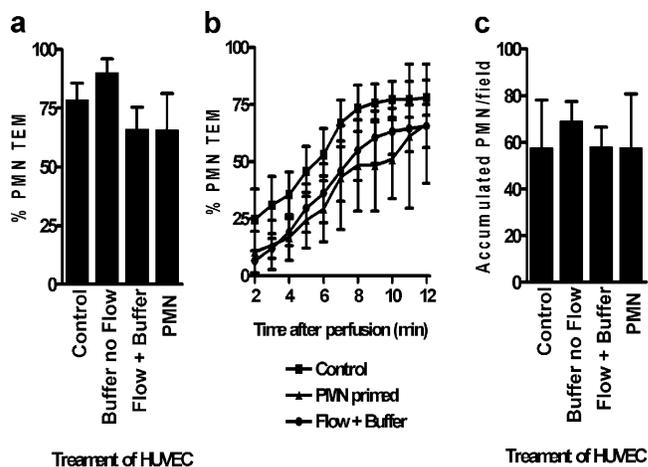
Figure 2. Neutrophil preconditioning does not affect the subsequent interaction of neutrophils across IL-1β–stimulated HUVEC. Confluent IL-1β–stimulated HUVEC monolayers were exposed to control, buffer with no flow, buffer at 1 dyne/cm² (each for 15 min), or neutrophils drawn across at 1 dyne/cm² for 5 min followed by buffer for 10 min, as before. Neutrophils were drawn across the HUVEC monolayer at 1 dyne/cm² for 5 min, followed by buffer alone for 10 min. The (a) extent and (b) rate of transendothelial migration and (c) accumulation of the second cohort of neutrophils was not affected by HUVEC preconditioning with either flow buffer or a first wave of neutrophils (n = 3, separate experiments).

lymphocytes. In this case, the second cohort of neutrophils was fluorescently labeled (to distinguish them from the first cohort) and perfused 10 min after the first. Transendothelial migration of the first cohort was compared with that of the second, as well as a cohort perfused after flow buffer alone (either under static or flow conditions for 15 min). No alteration in the extent or rate of transendothelial migration (Fig. 2, a and b) or in accumulation (Fig. 2 c) of the second wave of neutrophils was observed under any of these conditions. This suggests that the reduction in transmigration is specific for T lymphocytes and does not represent a global down-regulation of the capacity of HUVEC to support leukocyte transendothelial migration.

The Neutrophil-mediated Reduction in SDF-1α–associated CD3+ T Lymphocyte Transmigration Is Chemokine Specific and Is Not Reconstituted after Replenishment of SDF-1α. A second chemokine, ELC (CCL19, MIP-3β) is also known to augment T lymphocyte transendothelial migration in this system (3). Therefore, to test whether T lymphocyte transendothelial migration in response to a different chemokine was reduced by neutrophil perfusion, 200 ng/ml ELC was used in place of SDF-1α. As reported previously, T lymphocyte transendothelial migration was observed. Interestingly, after priming with neutrophils, no reduction of T lymphocyte transendothelial migration was observed (Fig. 3 a) demonstrating the specificity of this effect for SDF-1α–, but not ELC-associated transmigration.

One potential explanation for the observation that transmigration of T lymphocytes across HUVEC in response to SDF-1α is attenuated is that apically presented chemokine is dislodged or bound by the initial wave of neutrophils, although certain lines of evidence suggest that the SDF-1α
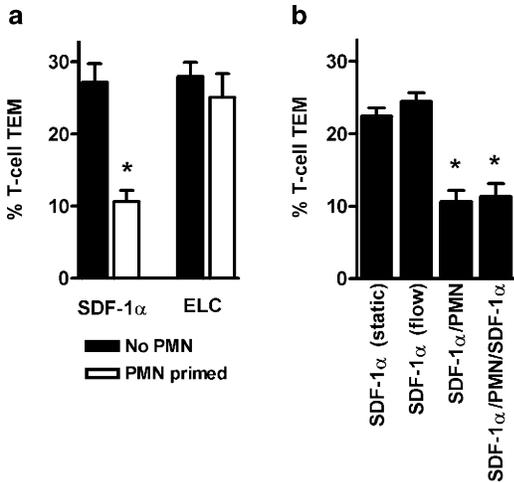
Figure 3. The neutrophil-mediated reduction in SDF-1α–associated CD3+ T lymphocyte transmigration is chemokine specific and is not reconstituted after replenishment of SDF-1α. IL-1β–stimulated HUVEC were incubated with either SDF-1α or ELC. Labeled T lymphocytes were perfused and transendothelial migration was assessed with DIC/fluorescence time-lapse microscopy with or without a prior bolus of neutrophils. (a) Preconditioning of EC with neutrophils reduced subsequent SDF-1α–associated T lymphocyte transendothelial migration, as shown previously; however, a similar reduction in ELC-associated T lymphocyte transendothelial migration was not observed. (b) 20 ng/ml SDF-1α was perfused into the flow chamber and was as effective at mediating T cell transendothelial migration as preincubation with SDF-1α. Replenishment with SDF-1α after transendothelial migration of neutrophils did not result in a rise in the percentage of T lymphocyte transendothelial migration back to baseline (*, P < 0.05). There was no delay in transendothelial migration, and the number of accumulated cells (not depicted) was not significantly different between all conditions (n = 3, separate experiments).

Figure 4. Supernatants generated after either transendothelial migration of neutrophils or activation of neutrophils modulate T lymphocyte transendothelial migration across IL-1β–stimulated HUVEC. Neutrophils were coincubated with unstimulated (control) or IL-1β–stimulated (conditioned) HUVEC, and the supernatants from these reactions were harvested after 30 min and used to pretreat IL-1β/SDF-1α–stimulated HUVEC (see Materials and Methods). (a) T lymphocyte transendothelial migration was significantly reduced after treatment of HUVEC with conditioned, but not control medium (*, P < 0.01, n = 3). (b) In separate experiments, neutrophils were activated with FMLP, and the supernatants were harvested (see Materials and Methods) and used to pretreat IL-1β/SDF-1α–stimulated HUVEC. Compared with supernatants from unactivated neutrophils or with FMLP alone, the extent of T lymphocyte transendothelial migration was significantly reduced (*, P < 0.05, n = 3) after pretreatment of HUVEC with FMLP-activated neutrophil supernatants. (c) A similar reduction in T lymphocyte transendothelial migration across IL-1β/SDF-1α–stimulated HUVEC after pretreatment of HUVEC with supernatants from neutrophils stimulated with either 10^{-6} M FMLP, 10^{-7} M C5a, or 10^{-8} M IL-8 (*, P < 0.05, n = 3).

receptor, CXCR4, is minimally expressed on freshly isolated peripheral blood neutrophils (31–33). To examine this possibility, the chemokine was replenished under flow after initial neutrophil perfusion. Delivery of SDF-1α in this manner was at least as effective as preincubation under static conditions because the extent of T lymphocyte transendothelial migration was similar for either condition (Fig. 3 b). After replenishment of SDF-1α and after transendothelial migration of neutrophils, labeled T lymphocytes were perfused and observed again. There was no restoration of T lymphocyte transendothelial migration (Fig. 3 b), although the number of accumulated cells was the same. Together, these data demonstrate that neutrophil transmigration specifically reduces T lymphocyte migration in response to SDF-1α, but not ELC, and furthermore that it is unlikely that SDF-1α is dislodged by neutrophils because its replenishment does not reconstitute T lymphocyte transendothelial migration.

Conditioned Medium from Neutrophil–HUVEC Transmigration Interactions Can Mediate a Reduction in T Lymphocyte Transendothelial Migration. Next, we considered whether the process of transmigration of neutrophils across IL-1β–stimulated HUVEC could release a soluble mediator capable of reducing subsequent T lymphocyte transendothelial migration. To assess this, conditioned medium was gener-
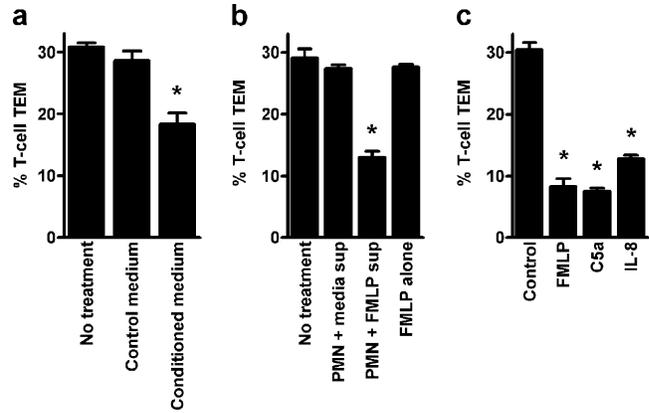
ated by incubating neutrophils (20×10^6 in 3 ml of flow buffer) with IL-1β–stimulated HUVEC under static conditions for 30 min as detailed in Materials and Methods. Significant transendothelial migration of neutrophils was observed across stimulated, but not unstimulated, HUVEC. The conditioned medium from each of these interactions was used to pretreat IL-1β/SDF-1α–stimulated HUVEC for 5 min at 37°C before perfusion of T lymphocytes. Significant reduction of T lymphocyte transendothelial migration was observed after pretreatment of HUVEC with conditioned medium from stimulated neutrophil–HUVEC interactions, but not with conditioned medium from unstimulated neutrophil–HUVEC interactions (control medium) (Fig. 4 a). There was no delay in transendothelial migration nor was there a reduction the number of accumulated cells (not depicted).

Supernatants from Neutrophils Stimulated with FMLP, C5a, or IL-8 Can Also Reduce T Lymphocyte Transendothelial Migration. Next, we examined whether a soluble factor released directly from interacting neutrophils might contribute to a reduction in T lymphocyte transendothelial migration. Preconditioning of IL-1β–stimulated/SDF-1α–pretreated HUVEC with FMLP-stimulated neutrophil supernatants for 5 min reduced T lymphocyte transendothelial migration, whereas preconditioning with supernatants from unstimulated neutrophils or with FMLP alone
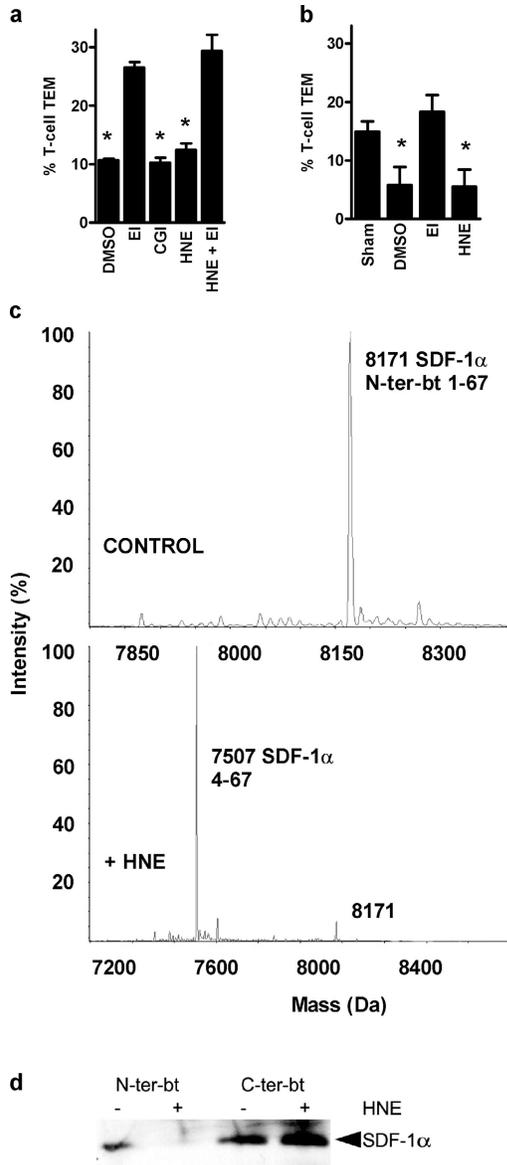
Figure 5. Neutrophil elastase reduces T lymphocyte transendothelial migration across IL-1β/SDF-1α–stimulated HUVEC and can directly inhibit the effect of SDF-1α. T lymphocyte transendothelial migration was observed across stimulated HUVEC after treatment of HUVEC with either FMLP-activated neutrophil supernatants preincubated with either DMSO, as vehicle, 10 μM MeOSucc-AAPV-CMK, a leukocyte elastase inhibitor (EI), 10 μM Z-GLF-CMK, a cathepsin G inhibitor (CGI), purified human neutrophil elastase (HNE), or HNE plus EI. (a) Elastase inhibition (EI) resulted in a reversal in the ability of neutrophil supernatants to reduce T lymphocyte transendothelial migration compared with vehicle alone (DMSO) or cathepsin G inhibitor (CGI). Similarly, treatment of HUVEC with purified HNE reproduced a reduction in T lymphocyte transendothelial migration that was reversed with pretreatment with EI (HNE + EI) (*, P < 0.05, n = 3). (b) In a similar series of experiments, SDF-1α was treated with either DMSO alone (sham) or with FMLP-neutrophil supernatants, themselves preincubated with either DMSO or EI or with HNE before addition to activated HUVEC monolayers. There was an overall reduction in baseline T lymphocyte transendothelial migration (by ∼50%), but elastase inhibition still resulted in a reversal in the ability of neutrophil supernatants to inhibit T lymphocyte transendothelial migration. Likewise, purified HNE treatment of SDF-1α resulted in a decrease in T lymphocyte transendothelial migration. (c) SDF-1α biotinylated at the NH₂ terminal (SDF-1α-N-ter-bt, 8171 D) was treated with HNE and analyzed by ESI-MS

had no effect on baseline T lymphocyte transendothelial migration (Fig. 4 b). There was no delay in transendothelial migration, and accumulation of T lymphocytes was unaffected in all cases (not depicted). Supernatants from neutrophils stimulated with either C5a or IL-8 could also mediate a significant reduction in T lymphocyte transendothelial migration (Fig. 4 c). These data demonstrate that a secreted neutrophil product can mediate a reduction in SDF-1α–associated T lymphocyte transendothelial migration.

Neutrophil Elastase Mediates a Reduction in T Lymphocyte Transendothelial Migration. Of interest, both neutrophil-derived MMP-2 (16) and elastase (17) have been reported to reduce the activity of SDF-1α by cleavage of the amino-terminus, although both previous papers used prolonged incubation of purified protein. Mononuclear cell–derived cathepsin G has also been reported to cleave SDF-1α (34). Thus, we postulated that neutrophil-derived elastase might account for the inactivation of some or all of the apically presented chemokines on HUVEC that are mandatory for T lymphocyte transendothelial migration. Elastase activity in FMLP-activated neutrophil supernatants was quantified and was found to be equivalent to 33 nM purified HNE (unpublished data). The FMLP-activated neutrophil supernatants were pretreated for 20 min with either 10 μM MeO-Suc-AAPV-CMK (a specific EI), 10 μM Z-GLF-CMK (a specific cathepsin G inhibitor, CGI), or 0.05% DMSO (as vehicle control) before addition for 10 min to IL-1β/SDF-1α–stimulated HUVEC at 37°C. Treatment with the EI, but not cathepsin G inhibitor or DMSO, abrogated the blocking effect of FMLP-neutrophil supernatant (Fig. 5 a). Similarly, addition of 33 nM HNE to IL-1β/SDF-1α–stimulated HUVEC at 37°C for 10 min reproduced the inhibitory effect of FMLP-neutrophil supernatants in reducing T lymphocyte transendothelial migration by ∼60%. This effect of HNE was prevented by treatment with MeO-Suc-AAPV-CMK (EI). Next, we incubated SDF-1α with the same inhibitor or compound before addition to IL-1β–stimulated HUVEC to demonstrate a direct, elastase-dependent effect of FMLP-neutrophil supernatants on chemokine function. Sham treatment (0.05% DMSO) of SDF-1α resulted in a reduction in transendothelial migration of T lymphocytes by 50% to that observed in the absence of DMSO (Fig. 1 b), perhaps reflecting an effect due to manipulation or dilution of SDF-1α. However, incubation with either FMLP-neutrophil supernatant or 33 nM HNE still resulted in further reduction in T lymphocyte transendothelial migration to <5% (Fig. 5 b). As expected, addition of EI to FMLP-neutrophil supernatant before incubation with SDF-1α reversed its ability to inactivate the chemokine.

To further examine this phenomenon, we used SDF-1α, biotinylated either at the carboxy (SDF-1α-C-ter-bt) or amino (SDF-1α-N-ter-bt) terminus. SDF-1α-C-ter-bt is rapidly cleaved by purified HNE when in solution as described previously (17), and the same property was confirmed by mass spectrometry for SDF-1α–N-ter-bt. After incubation with HNE, >90% of the synthetic chemokine was cleaved to a 7,507 D species and <10% was the original uncleaved 8,171 D parent species (Fig. 5 c). To confirm that purified HNE could directly cleave the SDF-1α bound to HUVEC monolayers, we incubated IL-1β–stimulated HUVEC with each synthetic SDF-1α compound. After treatment with purified HNE, HUVEC lysates were resolved on a 16.5% Tris-Tricine gel and SDF-1α–biotin species were detected with HRP-streptavidin. Although both compounds are susceptible to elastase cleavage of the amino terminal, the biotin conjugate was lost only from SDF-1α-N-ter-bt, whereas little loss of biotin signal from SDF-1α-C-ter-bt was observed. This series of experiments confirmed the ability of HNE to cleave the functional NH_2 terminus of HUVEC-bound SDF-1α, resulting in a significant reduction in the ability of truncated SDF-1α to trigger T lymphocyte transendothelial migration.

Figure 7. Pretreatment with neutrophils reduces the capacity of T lymphocytes to migrate to EC junctions associated with a reduction in polarization in response to SDF-1α. T lymphocytes, labeled with Cell Tracker green, were perfused over stimulated HUVEC that had been stained with an anti–VE cadherin, AlexaFluor 568–conjugated antibody to clearly visualize HUVEC junctions. Data are expressed as percentages of all T lymphocytes interacting during the time course of observation. The behavior of T lymphocytes was observed under high power (40×) to determine whether cells remained attached to the monolayer (Non-junctional), migrated to and remained at HUVEC junctions (junctional), or underwent transendothelial migration. In the presence of SDF-1α, the majority of cells (∼90%) either underwent transendothelial migration or remained at HUVEC junctions. In contrast, in the absence of SDF-1α, or in the presence of SDF-1α and neutrophil preperfusion, <50% of T lymphocytes either underwent transendothelial migration (reference 3) or remained at HUVEC junctions. We observed the same behavior when these experiments were performed in 25% autologous plasma. No alteration in localization or intensity of VE-cadherin staining or in HUVEC confluence was observed with any of these treatments (not depicted).
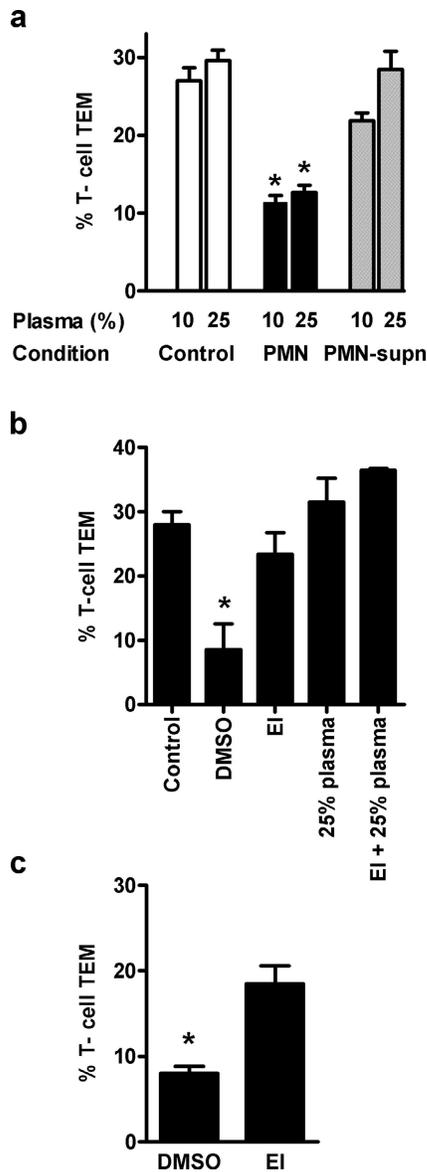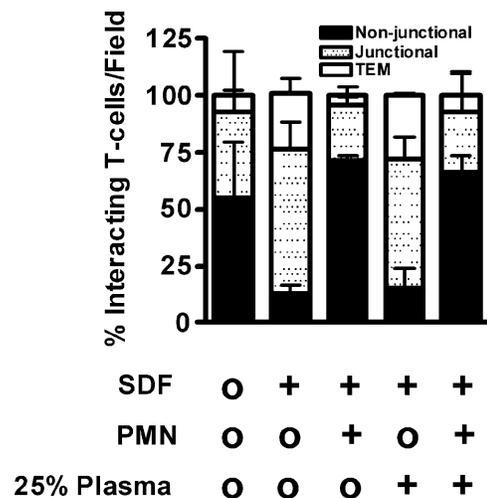
Figure 6. Whole neutrophils, but not treatment with stimulated neutrophil supernatants, reduces T lymphocyte transendothelial migration in the presence of autologous plasma. (a) T lymphocytes were drawn across stimulated HUVEC in normal flow buffer or flow buffer supplemented with 10 or 25% autologous plasma (white bars). Inclusion of either 10 or 25% autologous plasma did not affect the ability of whole neutrophils to reduce subsequent T lymphocyte transendothelial migration (black bars; *, P < 0.05, n = 3). In contrast, inclusion of 10 or 25% autologous plasma did reverse the ability of FMLP-neutrophil supernatants to mediate a similar reduction (diagonally striped bars). (b) When FMLP-neutrophil supernatants were generated in the presence of DMSO, a significant reduction in T lymphocyte transendothelial migration compared with control (no supernatant added) was observed (* P < 0.05, n = 2). However, if these supernatants were generated in the presence of either EI, 25% plasma, or EI + 25% plasma, this reduction was not observed. (c) In a separate series of experiments, the effect of neutrophil preconditioning on T lymphocyte transendothelial migration across IL-1β/SDF-1α–stimulated HUVEC was observed in the continued presence of 10 μM MeO-Succ-AAPV-CMK (EI) or vehicle control (DMSO). In this case, elastase inhibition partially reversed the capacity of neutrophil preconditioning to mediate a reduction in T lymphocyte transendothelial migration, whereas DMSO had no effect (*, P < 0.05, n = 3).

Autologous Plasma Inhibits the Proteolytic Activity of Neutrophil Supernatants, But Not That of Intact Neutrophils. Endogenous plasma-born protease inhibitors could potentially neutralize the capability of HNE to mediate SDF-1α proteolysis. Therefore, the ability of either adhesive/transmigrating neutrophils or neutrophil-derived supernatants to mediate reduction in T lymphocyte transendothelial migration was examined in the presence of autologous plasma. Perfusion of neutrophils in the presence of either 10 or 25% autologous plasma could still mediate a reduction in subsequent T lymphocyte transendothelial migration; in contrast, the ability of FMLP-stimulated neutrophil supernatants to mediate a parallel reduction was now lost (Fig. 6 a). Similarly, if these supernatants were generated in buffer supplemented with either 10 μM Meo-Suc-AAPV-CMK, 25% autologous plasma, or both, their ability to mediate a reduction in T lymphocyte transendothelial migration was also lost (Fig. 6 b).

In addition, neutrophils were perfused across IL-1β/SDF-1α–stimulated HUVEC before T lymphocytes again in the continued presence of the EI MeO-Suc-AAPV-CMK (or 0.05% DMSO as vehicle control). In this case, T lymphocyte transendothelial migration was reduced in the presence of 0.05% DMSO, but only partially when 10 μM MeO-Suc-AAPV-CMK was included in the assay (Fig. 6 c), suggesting that a small molecule inhibitor of elastase could mediate a partial reversal in the neutrophil-mediated effects on T lymphocyte transendothelial migration.

Neutrophil Transmigration Reduces Migration of T Lymphocytes to HUVEC Junctions before Transendothelial Migration. T cell transmigration is predominantly paracellular and, therefore, arrested lymphocytes must first undergo chemokine-driven migration on the endothelial surface toward intercellular junctions (35). In this system, addition of SDF-1α potentiates junctional migration and transmigration, but not initial accumulation. To examine whether neutrophil preconditioning of IL-1β/SDF-1α–treated HUVEC monolayers affected surface migration of T lymphocytes, HUVEC were labeled with a nonblocking fluorescent antibody to the adherens junction protein VE-cadherin. The ability of adherent T lymphocytes to migrate to junctions and undergo subsequent transendothelial migration was examined using a 40× objective. Consistent with previous reports (35), in the absence of SDF–1α, adherent T lymphocytes did not consistently migrate to HUVEC junctions (Fig. 7). In contrast, in the presence of SDF-1α, the majority of cells migrated to HUVEC junctions with a fraction subsequently undergoing transendothelial migration. After neutrophil transmigration across IL-1β/SDF-1α HUVEC monolayers, the percentage of T lymphocytes that failed to position at HUVEC junctions or subsequently transmigrate was significantly higher despite comparable accumulation of T lymphocytes (Fig. 7). We observed the same behavior when 25% autologous plasma was included in the flow buffer. This suggests that transmigrating neutrophils impair the ability of HUVEC-associated SDF-1α to support the migration of T lymphocytes toward cell junctions, a key step in subsequent transendothelial migration (35).

Discussion

Our data have demonstrated a significant reduction in SDF-1α–associated T lymphocyte transendothelial migration across cytokine-stimulated HUVEC under in vitro flow conditions immediately after transendothelial migration of neutrophils. This effect can be reproduced by conditioned medium from neutrophil–HUVEC interactions or supernatants from neutrophils stimulated with FMLP, C5a, or IL-8. This effect is mediated by the release of neutrophil elastase, which causes inactivation of apically presented SDF-1α by cleavage of its NH_2 terminal, a process protected from endogenous plasma-born EIs. Of considerable interest is that this novel mechanism appears to be important for attenuation of endothelial-bound SDF-1α function but does not occur when the promigratory chemokine is ELC.

We did not witness a reduction in the number of accumulated T lymphocytes after neutrophil transmigration. This is noteworthy as neutrophil-derived elastase and cathepsin G have also been reported to rapidly (<15 min) cleave PSGL-1 (18), an important mediator of initial neutrophils and T lymphocyte attachment, as well as VCAM-1 (albeit in bone marrow stroma; reference 36). EC-derived nitric oxide also possesses antiadhesive properties (37), and other neutrophil-derived products have been reported to possess antiinflammatory activity, notably adenosine/5′AMP (11, 38).

Junctional Adhesion Molecule Distribution Is Not Altered by Transendothelial Migration of Neutrophils. Another potential explanation for our findings could be that transendo-
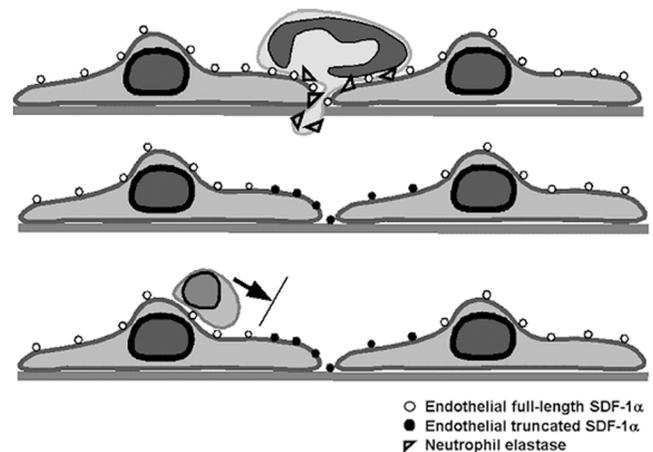

Figure 8. Schematic representation of HNE cleavage of apically presented SDF-1α. (first row) Neutrophil transendothelial migration with localized elastase secretion (gray triangles) at the leading edge of the cell. This elastase release is protected from endogenous protease inhibitors (see Fig. 6 a and Autologous Plasma Inhibits the Proteolytic Activity of Neutrophil Supernatants, But Not That of Intact Neutrophils). (second row) Truncation of NH_2 terminus of junctional SDF-1α. Apically presented SDF-1α (white dots), located at or near EC junctions, is cleaved at the amino terminus, producing inactive SDF-1α (black dots), primarily at or near HUVEC junctions. (third row) Normal T cell accumulation but failure of SDF-1a to trigger T cell locomotion and subsequent transendothelial migration. Although primary accumulation is unimpaired, truncated SDF-1α is unable to promote T lymphocyte migration and subsequent transendothelial migration.

Legend:
○ Endothelial full-length SDF-1α
● Endothelial truncated SDF-1α
▽ Neutrophil elastase

thelial migration of neutrophils affects the function or distribution of junctional adhesion molecules. We and others have previously demonstrated reversible changes during leukocyte transendothelial migration in the distribution of junctional proteins such as VE-cadherin, PECAM-1, and JAM-A (25, 26, 39–41). Our previous observations suggest that such changes were transient and fully reversible. In addition, our observation that a defect occurs not only in transendothelial migration but also in the preceding step of migration to the HUVEC junction would reiterate the view that the distribution and function of junctional adhesion molecules is unaffected at this level. Furthermore, it would be expected that any major redistribution of these proteins would similarly affect transendothelial migration of neutrophils as well as that of T lymphocytes in response to other chemokines (e.g., ELC).

The Selective Effect of Neutrophil-derived Elastase on SDF-1α–induced Transendothelial Migration of T Lymphocytes. We have demonstrated a role for HNE, but not cathepsin G, by both inhibiting the effects of FMLP-neutrophil supernatant on T lymphocyte transendothelial migration with a specific inhibitor and by confirming these observations with purified neutrophil elastase. Although neutrophil-derived serine proteases are secreted during transendothelial migration (42) and may induce disorganization of HUVEC junctions (19), it remains unclear whether HNE has a direct role in the migration of neutrophils through HUVEC junctions (43, 44). From our data, it would appear that any effect of HNE on junctional proteins may be transient as no reduction in transendothelial migration of a subsequent population of neutrophils was observed. However, neutrophil–derived enzymes selectively truncate multiple chemokines resulting in alteration in their function (16, 17, 34, 45, 46). These papers have used prolonged incubation of these enzymes with the respective chemokines in solution, and pertain to chemokine processing in the extravascular space. To our knowledge, our work is the first demonstration of in situ modulation of chemokine activity at the blood vessel wall by transmigrating blood-born cells.

The physiological impact of our results is further substantiated by the observation that, even in the presence of autologous plasma, transmigration of HUVEC by neutrophils can still impair SDF-1α–associated T lymphocyte transendothelial migration and migration to the junction. Neutrophil elastase has been reported to circumvent inactivation by its endogenous inhibitors using a variety of mechanisms. These include compartmentalization away from serum protease inhibitors (47), by remaining bound to the neutrophil surface (48) or its substrate (49), or by secretion of mediators that inactivate protease inhibitors (50, 51). Elastase has been reported to be released at the leading edge of the transmigrating (rather than adherent) neutrophils (42). The current paradigm for T lymphocyte transendothelial migration proposes that cells sequentially attach, arrest, polarize, and migrate to EC junctions before transendothelial migration (3). Our observation in this work is that without SDF-1α, or after neutrophil priming in the presence of SDF-1α, T lymphocytes exhibit a defect

in migration and positioning at the HUVEC junction as well as in subsequent transendothelial migration but not initial, nonjunctional accumulation. Therefore, it would seem reasonable to speculate that selective elastase-mediated cleavage of SDF-1α occurs preferentially at the HUVEC junction and is protected from plasma inhibitors (as outlined in Fig. 8). At the apical surface, on the other hand, HNE may not be released or its activity could be inhibited by plasma inhibitors.

Notably, replenishment of SDF-1α (or of a second chemokine, TARC; unpublished data) immediately after perfusion of neutrophils did not reconstitute T lymphocyte transendothelial migration and we could also still detect SDF-1α-C-ter-bt after HNE treatment. Therefore, this would suggest that NH$_2$ terminally cleaved inactive SDF-1α occupies structures on inflamed HUVEC that might otherwise serve to present native SDF-1α, or other chemokines, such as TARC. Thus, generation of truncated chemokine products by neutrophils could result in distinct outcomes depending on the type and distribution of the proteolysed chemokine.

Both SDF-1α and its receptor CXCR4 are widely distributed and have well-described roles in the physiology of hematopoetic (52), angiogenic (53), and neurological (54) systems as well as in activation and/or recruitment of leukocytes and stem cells (55). They are implicated in the pathogenesis of a number of disease processes including rheumatoid arthritis (56), metastatis (57), and HIV encephalitis and dementia (58). Hence, the observation that transendothelial migration of neutrophils specifically down-regulates SDF-1α function, while preserving the potency of other chemokines (e.g., ELC), might represent an important control mechanism in evolving inflammation. This mechanism is of potential significance with respect to regulating early lymphocyte recruitment, which may have potentially deleterious effects, as well as to other SDF-1α–dependent processes characteristic of chronic disease, such as stem cell recruitment and angiogenesis. It is of interest that a similar mechanism has been described in vivo after GM-CSF stimulation, whereby neutrophil elastase, but not cathepsin G, cleaves SDF-1α expressed on bone marrow stroma, inhibiting retention and facilitating the subsequent mobilization of stem cells from bone marrow into the peripheral circulation (59, 60), a process which may augment the CXCL1-dependent release of cells from the bone marrow (33). In addition, CXCR4 appears to be up-regulated on senescent neutrophils and, thus, mediates their SDF-1α–dependent return to the bone marrow (33). It would be of interest to examine whether senescent neutrophils can cleave bone marrow EC SDF-1α and thereby influence haematopoetic progenitor cell recirculation through bone marrow compartments (55).

In summary, we have described a novel regulatory level in T lymphocyte recruitment to inflammatory sites, in which transmigration of inflamed HUVEC by neutrophils results in a reduction in chemokine triggered T lymphocyte diapedesis. The observation that the recruitment of one type of immune cell modulates the migratory behavior of another type indicates that trafficking of leukocytes is

not only dictated by endothelial-specific signals, but also by the leukocyte response to these signals.

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