

Shear-dependent Eosinophil Transmigration on Interleukin 4-stimulated Endothelial Cells: A Role for Endothelium-associated Eotaxin-3[⊙]

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Abstract

Leukocyte infiltration into inflammatory sites is regulated by the expression of adhesion and activation proteins, yet the role of these proteins in shear-dependent transmigration is poorly understood. We examined eosinophil recruitment on cytokine-stimulated human umbilical vein endothelial cells (HUVECs) under laminar flow conditions. Eosinophils rapidly transmigrated on interleukin (IL)-4-, but not TNF-stimulated HUVECs. Transmigration was shear dependent, with up to 90% of eosinophils transmigrating in the presence of shear and less than 25% of cells transmigrating under static conditions. Eosinophils express CC chemokine receptor CCR3 and are responsive to various CC chemokines. The effects of chemokines are mediated primarily through G_{αi}, which is pertussis toxin sensitive. Greater than 65% of shear-dependent eosinophil transmigration on IL-4-stimulated HUVECs was blocked by either pertussis toxin or by an anti-CCR3 monoclonal antibody. Using reverse transcription polymerase chain reaction (RT-PCR) and Western blots, we found that IL-4-stimulated HUVECs produce both mRNA and protein for eotaxin-3. Eotaxin-3 was both released by HUVECs and expressed on the endothelial cell surface. Pretreatment of HUVECs with an anti-eotaxin-3 antibody blocked eosinophil transmigration to the same extent as an anti-CCR3 antibody. These results indicate that IL-4-stimulated HUVECs support shear-dependent eosinophil transmigration by upregulating eotaxin-3, and that surface association is critical for the role of eotaxin-3 in transmigration.

Key words: chemokines • cell adhesion • cytokines • trafficking • leukocytes

Introduction

Inappropriate leukocyte recruitment is a hallmark of inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease and bronchial asthma. Selective recruitment of eosinophils and lymphocytes into the lung is thought to contribute to many of the features of asthma including increased cytokine production, epithelial cell shedding, mucous hyper-secretion, and airway hyper-responsiveness (1–3). The cytokines TNF, IL-1 β , IL-4, and IL-13 have all been used to stimulate human umbilical vein endothelial cells (HUVECs)* and these endothelial cells can sup-

port binding of several classes of leukocytes, including eosinophils (4–8). We have focused on IL-4 as a model for eosinophil recruitment, as this cytokine has been shown to participate in selective eosinophil recruitment both in vitro and in vivo. In vivo, IL-4 regulates several immune responses including IgE synthesis by B cells, promotion of the Th2 phenotype in T cells, and increased infiltration of eosinophils to inflammatory sites (9, 10). Recently we showed that IL-4-stimulated HUVECs support the selective recruitment of eosinophils from whole blood under flow conditions in vitro (11).

The generic cascade of events that leads to the movement of leukocytes from the bloodstream into the tissue includes tethering and rolling of leukocytes followed by activation and transmigration (12, 13). The first half of this cascade is well characterized, especially in regards to the roles of the selectins and vascular cell adhesion molecule-1 (VCAM-1) in the initial tethering and rolling of leukocytes along the inflamed endothelium (12, 13). In contrast, the

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*Abbreviations used in this paper: CCR, CC chemokine receptor; HRP, horseradish peroxidase; HUVEC, human umbilical vein endothelial cell; MCP, monocyte chemoattractant protein; RANTES, regulated upon activation, normal T cell expressed and secreted; RT, reverse transcription; TMB, tetramethylbenzidine.

subsequent events regulating activation and transmigration are less clear but are thought to involve activation by chemotactic factors and the use of adhesion molecules such as $\alpha 4$ - and $\beta 2$ -integrins and platelet-endothelial cell adhesion molecule-1 (PECAM-1). Together these proteins facilitate firm adhesion and subsequent transmigration through endothelial cell junctions (12, 14, 15). Although these interactions occur under the shear conditions found in the vasculature, *in vitro* laminar flow chamber systems have only recently been used to examine activation and transmigration of leukocytes (5, 16, 17).

Eosinophil transmigration across cytokine-activated endothelial cells has only been examined in static systems. These studies are limited by the fact that freshly isolated, normal eosinophils do not transmigrate across either IL-1- or IL-4-stimulated HUVECs (18–20). As a result, most eosinophil transmigration studies have focused on eosinophil migration across coated filters or unactivated HUVECs in response to chemoattractants such as chemokines. Chemokines are a family of small chemotactic proteins that are subdivided into two main groups, CC and CXC, based on the spacing of the first two conserved cysteine residues (14, 21, 22). Eosinophils express CC chemokine receptors CCR1 and CCR3 and respond to many chemokines including RANTES (regulated upon activation, normal T cell expressed and secreted), monocyte chemoattractant protein (MCP)-3, MCP-4, and the eotaxins (23, 24). Only the eotaxin family members (eotaxin, eotaxin-2, and eotaxin-3) act exclusively at CCR3 (23); however, these proteins have distinct patterns of expression. For example, mRNA for eotaxin-3, but not eotaxin, is specifically increased in IL-4- and IL-13-stimulated HUVECs but not in TNF- or IL-1-stimulated HUVECs (25). Thus these proteins will likely have distinct roles in eosinophil recruitment.

In this study we examined eosinophil transmigration on cytokine-stimulated endothelial cells using an *in vitro* laminar flow chamber to simulate the hydrodynamic shear conditions found *in vivo*. Eosinophils transmigrated on IL-4-stimulated HUVECs, but not on TNF-stimulated cells. Eosinophil transmigration on IL-4-stimulated HUVECs was rapid, with 50% of eosinophils transmigrating within 7 min. This rapid transmigration was absolutely dependent on shear conditions, as less than 15% of eosinophils transmigrated in the same time frame under static conditions. Even after 20 min, less than 25% of eosinophils transmigrated under static conditions, whereas transmigration under shear conditions climbed as high as 90%. Eosinophil activation was, in part, pertussis toxin sensitive and required CCR3. Using antibodies directed against eotaxin-3, we found that IL-4-stimulated HUVECs synthesized eotaxin-3 and both released the protein and expressed it on the cell surface. Furthermore, surface-associated eotaxin-3 was responsible for the CCR3-dependent component of eosinophil transmigration. This is the first time that a functional role for eotaxin-3 has been demonstrated. These results indicate that shear-dependent eosinophil transmigration is regulated by the expression of eotaxin-3. These data are

profoundly different from those obtained using static systems, suggesting that studying leukocyte transmigration under the flow conditions that exist in the vasculature may provide more accurate insight into the molecular mechanisms that govern this process.

Materials and Methods

Reagents. Hanks' balanced salt solution with Ca^{2+} and Mg^{2+} (HBSS), lymphoprep 1077, Media 199 (M199), Superscript II, TRIzol reagent, and tetramethylbenzidine (TMB) one-step ELISA substrate were from Invitrogen. PCR Master Mix was from QIAGEN. Human serum albumin (HSA) was from Immuno US. Enhanced chemiluminescence reagents were from Pierce Chemical Co. Pertussis toxin and histamine were from Sigma-Aldrich. MEK1 inhibitor PD 98059 was from Calbiochem-Novabiochem. Oligonucleotide probes were prepared by the DNA synthesis lab at the University of Calgary. All plasticware was from Becton Dickinson. All other chemicals were from BDH, Inc.

Proteins and Antibodies. Human recombinant IL-4, human recombinant TNF, anti-CCR3 mAb, anti-MCP-4 mAb, and ELISA kits for eotaxin and RANTES were from R&D Systems. Anti-eotaxin and anti-RANTES mAbs were purchased from Serotec. Anti-eotaxin-3 antibodies were from PeproTech. Goat anti-human isotype control antibody and donkey anti-goat horseradish peroxidase (HRP)-conjugated antibody were from Santa Cruz Biotechnology, Inc. Anti-CD16 and anti-CD3 magnetic beads were from Miltenyi Biotec. All antibodies were used according to manufacturers' instructions or as described.

Cell Isolation. Blood from healthy and mildly atopic adults was drawn into heparinized syringes and granulocytes were isolated by dextran sedimentation, hypotonic lysis, and density centrifugation on lymphoprep 1077. Eosinophils were then negatively selected by depleting neutrophils and any remaining lymphocytes using magnetic cell separation with anti-CD16 and anti-CD3 paramagnetic beads. The resulting eosinophils were routinely >95% pure by Kimura staining. Primary or first passage HUVECs were isolated as described (26) and maintained in M199 with 20% human serum. For all experiments, HUVECs were used 2 d postconfluence. The Ethics Committee at the University of Calgary approved all procedures.

Adhesion and Transmigration Under Flow Conditions. Eosinophil interactions under flow conditions were quantified as described (7). Briefly, confluent monolayers of endothelial cells were washed and stimulated with either M199 with 0.5% HSA (M199/A) alone or M199/A containing either 20 ng/ml IL-4 or 20 ng/ml TNF. After 6 or 24 h, the incubation buffer was removed, the flow cell was assembled and freshly isolated eosinophils (5×10^5 /ml) were perfused across endothelial monolayers. Accumulation was determined after 4 min at 2 dyn/cm² in all experiments. After 4 min of perfusion, the inlet line was transferred to HBSS to prevent the binding of new eosinophils and shear was maintained at 2 dyn/cm². Transmigration was then assessed between 6 and 7 min unless otherwise specified. Interacting cells were visualized using 400 \times magnification and recorded via a CCD camera for later analysis. We characterized transmigrated cells in a manner similar to previous studies (5, 16). Rolling and adherent cells on the surface of the endothelium appear phase-bright, whereas transmigrated cells are flattened and phase-dark (Fig. 1). Flattened, phase-dark cells were confirmed as being under the endothelial cell monolayer by observing the focal

plane of the eosinophils and the endothelial cells using 800× magnification. A cell was considered transmigrated if greater than 50% of the cell was under the monolayer at the point of quantification. Following cells for longer periods of time demonstrated that those cells that had become greater than 50% transmigrated continued to migrate and remain under the monolayer under shear conditions. Transmigration is expressed as the number of transmigrated cells divided by the total cells counted. In most experiments, 5–10 fields of view were used, and 100–150 cells were counted.

In some experiments eosinophils or HUVECs were treated with saturating concentrations of the specified antibodies for 10 min before assembly of the flow chamber. In other experiments, HUVECs were pretreated with 20 μ M PD 98059 or an equivalent amount of DMSO for 30 min or with 10 μ M histamine for 10 min before assembly of the flow chamber. Finally, eosinophils (5×10^6 /ml) were pretreated with 250 ng/ml pertussis toxin for 1 h at 37°C. A dose response curve was performed to ensure that the pertussis toxin was used at saturation. The cells were washed twice with HBSS, resuspended at 5×10^5 cells/ml, and used in transmigration experiments.

Transmigration Under Static Conditions. Eosinophils (5×10^5 /ml) were drawn into the flow chamber, the flow was stopped, and the cells were observed for up to 20 min. Percent transmigration was assessed at the specified times using video microscopy as described in the previous section. Unlike transmigration under shear conditions, cells that had partially transmigrated frequently retracted their processes (see online supplemental material). This resulted in an overestimate of transmigrated cells under static conditions.

Reverse Transcription PCR. Endothelial cells were stimulated with buffer alone or buffer containing 20 ng/ml IL-4 for 24 h as described above. Reverse transcription (RT)-PCR was performed to detect mRNA for RANTES, eotaxin, eotaxin-2, eotaxin-3, MCP-3, MCP-4, and β -actin. RNA was isolated using TRIzol according to manufacturer's instructions. Reverse transcription was performed using First strand synthesis kit from GIBCO BRL according to manufacturer's instructions. PCR was performed with QIAGEN Master Mix using 2 μ l of the RT reaction as template cDNA and the appropriate primer pairs. Primers pairs were designed based on published sequences. After 35 cycles, amplified PCR products were identified by electrophoresis as described previously (27).

ELISA for CCR3-active Chemokines. Endothelial cells were stimulated with buffer alone or buffer containing 20 ng/ml IL-4 for 24 h as described above. The supernatants from these cells were collected and ELISAs were performed to detect the presence of eotaxin, RANTES, or MCP-3 according to manufacturer's instructions. ELISA kits are not available for eotaxin-3; therefore we developed a sandwich ELISA for eotaxin-3. Immunosorp plates from NUNC were coated overnight with anti-eotaxin-3 antibody (0.5 μ g/ml) in carbonate coating buffer (0.1 M Na carbonate, pH 9.2). The plates were washed four times with HBSS and supernatants or eotaxin-3 standard diluted in M199/A were added to the plate. After 2 h at 37°C, the supernatants were discarded and the plates were washed four times before the addition of biotinylated anti-eotaxin-3 antibody (0.25 μ g/ml in HBSS/A). After an additional 2 h, the plates were washed four times and streptavidin-conjugated HRP at a 1:1,000 dilution in HBSS/A was added to the wells. Plates were again incubated for 2 h and washed four times before the addition of TMB one-step ELISA substrate. After 15 min the reaction was stopped by the addition of 1 M H_3PO_4 and the plates were read at 450 nm.

Western Blotting and Cell Surface ELISA for Eotaxin-3. Endothelial cells were stimulated with buffer alone or buffer containing 20 ng/ml IL-4 for 24 h as described above. Total protein expression in control and IL-4-stimulated HUVECs was determined by Western blotting using an eotaxin-3 antibody to probe the membranes. Recombinant eotaxin-3 and recombinant MCP-4 were used as positive and negative controls, respectively. Surface expression of eotaxin-3 on control and IL-4-stimulated endothelial cells was determined using a modified ELISA as described (27). Briefly, stimulated cells were washed once with ice cold HBSS and 2 μ g/ml anti-eotaxin-3 or an isotype matched nonimmune antibody in HBSS/A was added and incubated for 60 min on ice. Cells were washed three times with HBSS and then incubated with a 1:1,000 dilution of HRP-conjugated donkey anti-goat IgG secondary antibody for 60 min on ice. Antibody binding was determined using TMB one-step ELISA substrate and quantified by measuring absorbance at 450 nm.

Statistics. All experiments were performed at least three times. The data were analyzed using either unpaired Student's *t* test or by using analysis of variance (ANOVA) followed by the Bonferroni test for intergroup comparisons. *P* values < 0.05 were considered significant.

Online Supplemental Material. Time-lapse images of eosinophils interacting with IL-4-stimulated HUVEC are shown in online supplemental Videos 1–3. All videos were captured for 4–5 min at 30 frames per second and compressed to 15–20 s using Adobe Premiere. Thus, the videos are presented at 15× time compression. Images were captured using a 40× objective on a ZEISS Axiovert 100 microscope coupled to a CCD camera as described in the previous section. Video 1 shows eosinophils interacting with IL-4-stimulated HUVECs under static conditions. Eosinophils move across the endothelial cell surface and remain phase bright, but do not migrate beneath the monolayer. Video 2 also shows eosinophils interacting under static conditions with IL-4-stimulated HUVECs. Here eosinophils attempt to transmigrate but are unable to do so and retract their pseudopods. Video 3 shows eosinophils interacting with IL-4-stimulated HUVECs under flow conditions. Eosinophils rapidly change shape and migrate beneath the monolayer, becoming phase-dark. Videos are available at <http://www.jem.org/cgi/content/full/194/12/1699/F2/DC1>.

Results

IL-4-, but Not TNF-stimulated Endothelial Cells Support Rapid Eosinophil Transmigration. HUVECs were stimulated with IL-4 or TNF, the flow chamber was assembled, and freshly isolated human eosinophils were perfused through the chamber at a wall shear stress of 2 dyn/cm². The inlet line was switched to buffer at 4 min to prevent the binding of additional eosinophils. After 6 min eosinophil transmigration was assessed by quantifying the percentage of phase dark, transmigrated cells. We found that both IL-4 and TNF supported equivalent levels of eosinophil accumulation (Fig. 1 A); however, only IL-4-stimulated HUVECs supported eosinophil transmigration (Fig. 1, B–D). The rapid transmigration of eosinophils in our system was a surprising finding, as only primed eosinophils have previously been reported to transmigrate across IL-4-stimulated HUVECs (20); however, these previous studies were performed under static conditions using transwell

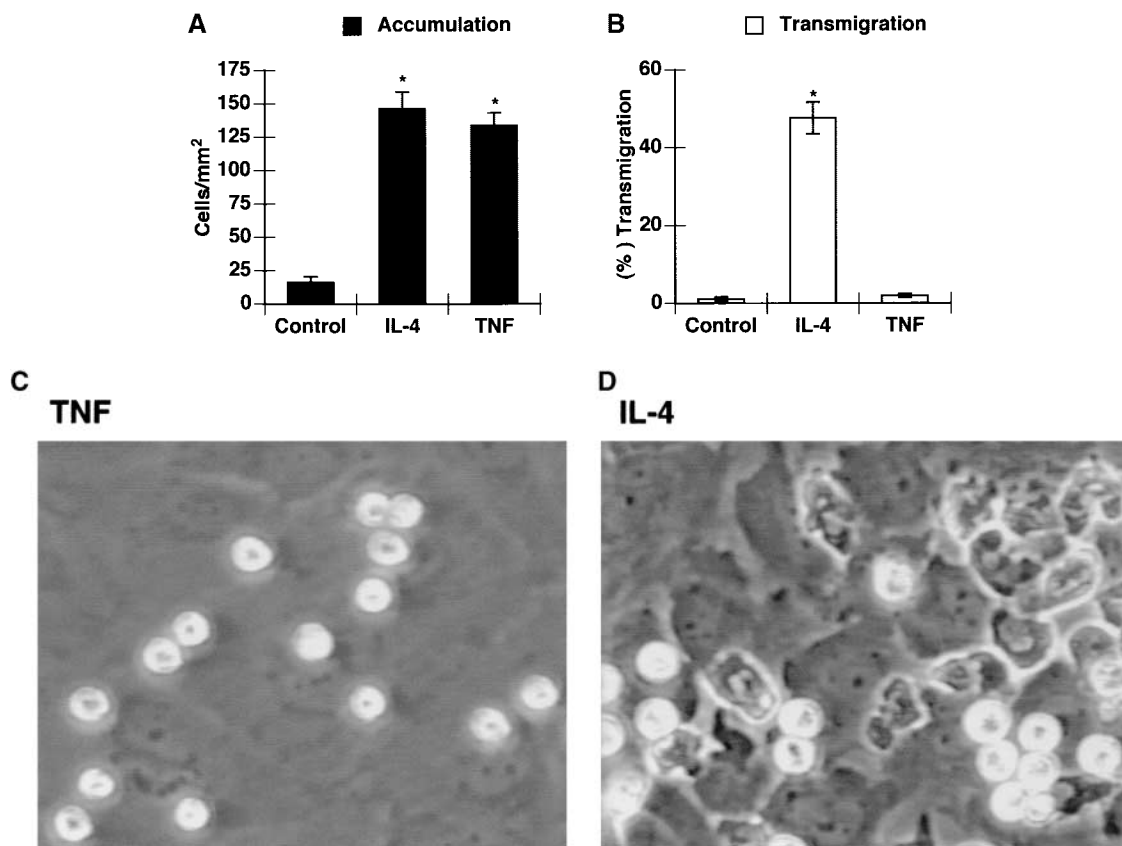


Figure 1. Eosinophils transmigrate across IL-4-stimulated HUVECs. Tightly confluent monolayers of HUVECs were stimulated for 24 h with M199/A alone (Control) or M199/A containing 20 ng/ml IL-4 (IL-4). Alternatively, cells were stimulated for 6 h with M199/A containing 20 ng/ml TNF (TNF). After the incubation, the flow chamber was assembled and isolated eosinophils (5×10^5 /ml) were perfused over the HUVECs for 4 min at a wall shear stress of 2 dyn/cm². (A) Accumulation and (B) transmigration were quantified as described in Materials and Methods. Data are mean \pm SEM of between 3 and 15 experiments. * $P < 0.001$. Representative images of interactions between eosinophils and (C) TNF-stimulated HUVECs (TNF) or eosinophils and (D) IL-4-stimulated HUVECs (IL-4) were digitally captured at 400 \times magnification. Eosinophils bound to TNF-stimulated HUVECs remained phase-bright, whereas eosinophils bound to IL-4-stimulated HUVECs changed shape and transmigrated, becoming phase-dark.

plates. Shear stress has recently been shown to be critical for lymphocyte transmigration across cytokine-stimulated HUVECs (28); thus, we next examined the role of shear stress in eosinophil transmigration.

Eosinophil Transmigration on IL-4-stimulated HUVECs Is Shear Dependent. To assess the role of shear stress in eosinophil transmigration, eosinophils were drawn into the flow chamber and the inlet line was closed to allow the eosinophils to settle onto the monolayer in the absence of shear. The percentage of transmigrated cells was measured every 2 min for 20 min. Few eosinophils transmigrated under the monolayer under these conditions (Fig. 2 A), although the cells were clearly being activated as demonstrated by increased motility on the surface of the endothelial cells (Fig. 2 B, and online supplemental videos). In addition, under static conditions eosinophils were frequently observed to put out processes in an attempt to transmigrate, but, after failing to transmigrate, they retracted these processes and moved to another site on the monolayer (online supplemental videos). These partially transmigrated cells were occasionally observed on the monolayer and account for some of the cells quantified as

transmigrated (Fig. 2 A). These interactions were recorded and time-lapse video microscopy clearly showed that under static conditions eosinophils move on the surface of the endothelium but generally do not migrate beneath the surface (Fig. 2 B, and online supplemental videos), whereas under flow conditions, a majority of eosinophils migrate completely beneath the monolayer (Fig. 2, and online supplemental videos). IL-4 stimulation does lead to some endothelial cell retraction; however, without shear stress, eosinophils do not migrate under the monolayer even at sites of exposed matrix. These data show that eosinophil transmigration is shear dependent.

Endothelial Cell Activation in Shear-dependent Eosinophil Transmigration. Shear stress alone has been shown to activate extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein (MAP) kinases within minutes in HUVECs (29). To determine if MAP kinase activation in endothelial cells was associated with shear-dependent transmigration, we pretreated endothelial cells with PD 98059, an inhibitor of the upstream kinase, MEK1. We found that PD 98059 significantly attenuated shear-dependent eosinophil transmigration (Fig. 3 A). The incubation time and

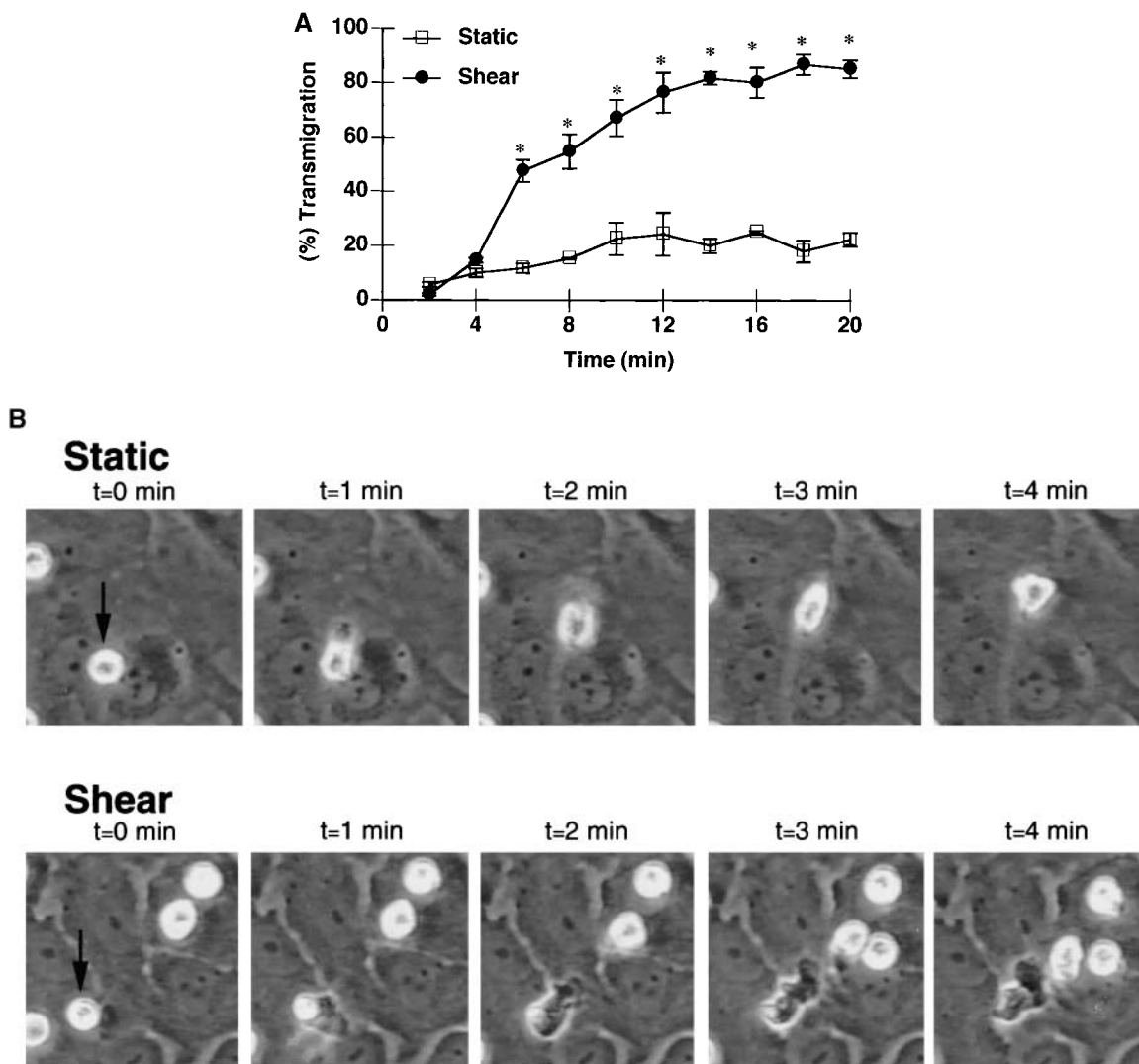


Figure 2. Eosinophil transmigration across IL-4-stimulated HUVECs is shear dependent. HUVECs were stimulated with M199/A containing 20 ng/ml IL-4 for 24 h. Eosinophils (5×10^5 /ml) were allowed to interact with IL-4-stimulated HUVECs under static (0 dyn/cm²) or flow (2 dyn/cm²) conditions in the flow chamber. (A) Transmigration was measured every 2 min for 20 min as described in Materials and Methods. Data are mean \pm SEM of at least four experiments. * $P < 0.05$. (B) Digitally captured images are presented at 1-min intervals to illustrate the interactions between eosinophils (black arrow) and IL-4-stimulated HUVECs under static or shear conditions. Data are representative of at least four experiments.

concentration of PD 98059 used on HUVECs completely blocked phosphorylation of ERK1/2 in response to histamine (data not shown). These data suggest that activation of ERK 1/2 MAP kinases may promote shear-dependent eosinophil transmigration; however, other mechanisms are clearly required to induce maximal transmigration.

We next determined if shear stress alone was sufficient to induce eosinophil transmigration. IL-4-stimulated HUVECs were preexposed to shear conditions for 5 min before perfusion of eosinophils. Transmigration was then examined in the absence of shear stress. We found that preexposure of HUVECs to shear stress had no effect on eosinophil transmigration (Fig. 3 B). We also examined the role of endothelial cell permeability in shear-dependent transmigration. Histamine acts through a G protein-coupled receptor to increase tyrosine phosphorylation of

adherens junction proteins and induce cellular permeability (30). We pretreated IL-4-stimulated endothelial cells with histamine to determine if changes in endothelial cell permeability would alter eosinophil transmigration under static or shear conditions. We found that pretreating endothelial cells with histamine did not affect the magnitude or kinetics of the shear-dependent eosinophil transmigration (Fig. 3 C). Histamine also had no effect on static transmigration at early time points (Fig. 3 C), but did modestly enhance transmigration at later time points. Despite the negative effects in this assay, we found that histamine treatment of endothelial cells did lead to activation as measured by prostaglandin synthesis (data not shown). Furthermore, histamine treatment of endothelial cells did not affect shear-dependent transmigration of lymphocytes in a similar system (28). Thus changes in permeability are not

sufficient to induce eosinophil transmigration in the absence of shear stress.

Eosinophil Activation through CCR3 Is Required for Shear-dependent Transendothelial Migration. The rapid shape change and transmigration of eosinophils on IL-4-stimulated HUVECs clearly demonstrated that these eosinophils were being activated. Furthermore, we previously showed that greater than 95% of eosinophils bound to IL-4-stimu-

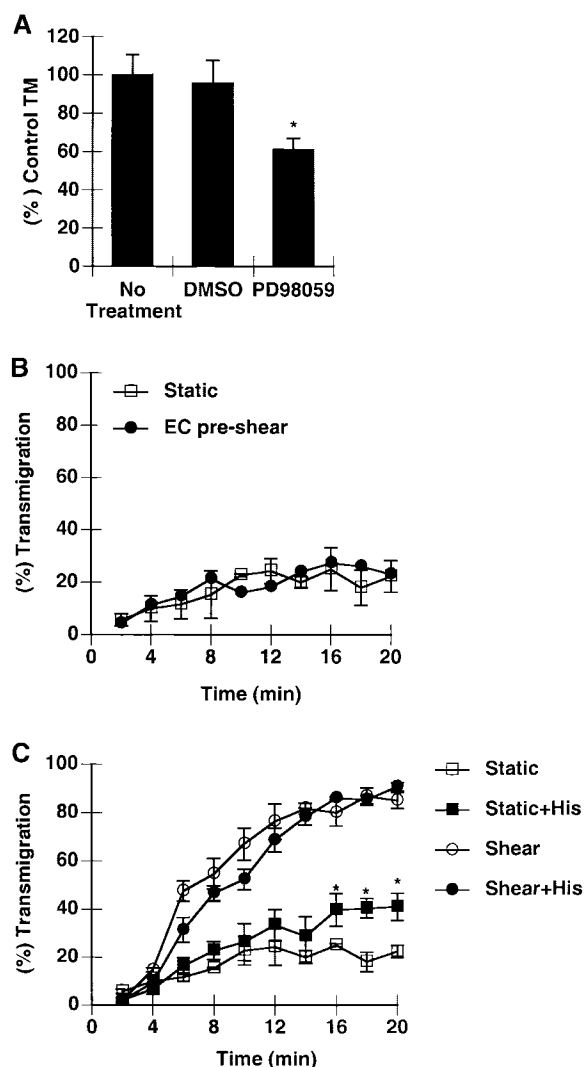


Figure 3. Role of ERK1/2 MAP kinase and endothelial cell permeability in shear-dependent eosinophil transmigration. HUVECs were stimulated with M199/A containing 20 ng/ml IL-4 for 24 h. (A) HUVECs were pretreated with 20 μ M PD 98059 or an equivalent amount of DMSO for 30 min before the assembly of the flow chamber. Transmigration was assessed as described in Fig. 1. (B) The flow chamber was assembled and buffer was perfused across the surface at 2 dyn/cm^2 for 5 min. Eosinophils were then drawn into the chamber, the flow was stopped, and transmigration was determined every 2 min for 20 min as described in Fig. 2. (C) IL-4-stimulated HUVECs were treated with 10 μ M histamine for 5 min before the assembly of the flow chamber. Eosinophils were allowed to interact with IL-4-stimulated HUVECs under static (0 dyn/cm^2) or flow (2 dyn/cm^2) conditions in the flow chamber and transmigration was assessed as described in Fig. 2. Data are mean \pm SEM of at least three experiments. * $P < 0.05$.

lated HUVECs are firmly attached but that blocking both $\alpha 4$ - and $\beta 2$ -integrins could decrease eosinophil firm adhesion and increase the percentage of rolling cells (7). In this study we found that blocking both $\alpha 4$ - and $\beta 2$ -integrins could also block most eosinophils transmigration (Fig. 4). This was expected, as few cells were firmly adherent and firm adhesion is a prerequisite for transmigration. To address the mechanisms by which eosinophils were being activated, we used pertussis toxin to prevent activation by chemoattractants acting via $G_{\alpha i}$ -coupled receptors. We found that pertussis toxin blocked $\sim 65\%$ of eosinophil transmigration indicating that activation was primarily pertussis toxin-sensitive (Fig. 4). CCR3 is the major chemokine receptor on eosinophils and CCR3 mediates its effects via $G_{\alpha i}$ -coupled receptors, thus we next examined the ability of an anti-CCR3 antibody to affect eosinophil transmigration. Anti-CCR3 mAb blocked eosinophil transmigration to the same extent as pertussis toxin (Fig. 4). Neither pertussis toxin nor anti-CCR 3 mAb prevented the firm adhesion of eosinophils to IL-4-stimulated HUVECs (data not shown). This was not unexpected since we have previously shown that interactions with high concentrations of VCAM-1 alone can mediate both rolling and firm adhesion of eosinophils through interactions with $\alpha 4$ -integrins (7). These data suggest that a majority of eosinophil transmigration across IL-4-stimulated HUVECs is CCR3-dependent, but CCR3-independent pathways also participate in eosinophil firm adhesion and transendothelial migration.

IL-4-stimulated HUVECs Synthesize mRNA and Protein for Eotaxin-3. Many CC chemokines act at CCR3 (23). We used RT-PCR, and in some cases ELISA, to screen IL-4-stimulated HUVECs for the expression of mRNA and protein for several of these CCR3-specific chemokines. IL-4-stimulated HUVECs did not express mRNA or protein for eotaxin, RANTES, or MCP-3 (Fig. 5 A, and

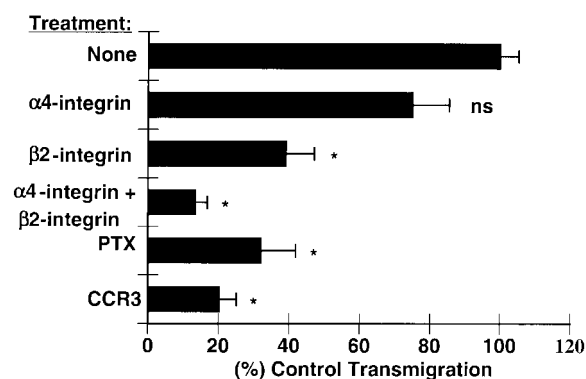


Figure 4. Eosinophil transmigration across IL-4-stimulated HUVECs is pertussis toxin sensitive and CCR3 dependent. HUVECs were stimulated with IL-4 as described in Fig. 1. Eosinophils were pretreated with 250 ng/ml pertussis toxin for 1 h (PTX). Alternatively, eosinophils were pretreated for 10 min with the following antibodies: 5 μ g/ml anti- $\alpha 4$ -integrin mAb, 5 μ g/ml anti- $\beta 2$ -integrin mAb, both anti- $\alpha 4$ -integrin mAb and anti- $\beta 2$ -integrin mAb, or 10 μ g/ml anti-CCR3 mAb (CCR3). The flow chamber was assembled and eosinophil transmigration was assessed as described in Fig. 1. Data are mean \pm SEM of at least three experiments. * $P < 0.001$; ns, not significant.

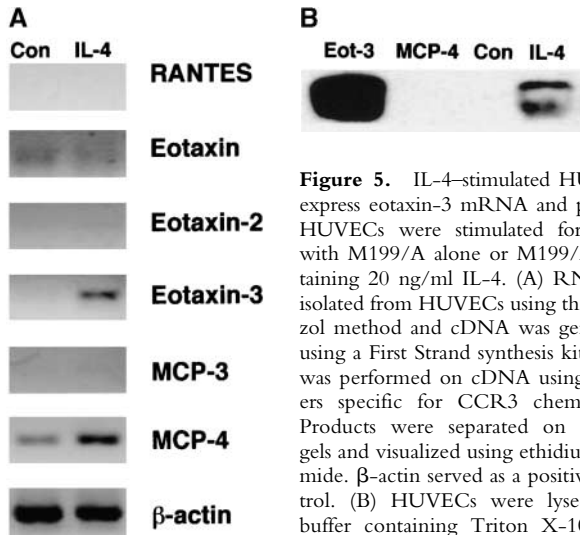


Figure 5. IL-4-stimulated HUVECs express eotaxin-3 mRNA and protein. HUVECs were stimulated for 24 h with M199/A alone or M199/A containing 20 ng/ml IL-4. (A) RNA was isolated from HUVECs using the TRIzol method and cDNA was generated using a First Strand synthesis kit. PCR was performed on cDNA using primers specific for CCR3 chemokines. Products were separated on agarose gels and visualized using ethidium bromide. β -actin served as a positive control. (B) HUVECs were lysed in a buffer containing Triton X-100 and lysates were separated by SDS-PAGE.

Proteins were transferred to PVDF and eotaxin-3 protein was detected using an eotaxin-3-specific antibody. Figures are representative of at least three experiments.

data not shown). In contrast, we did observe mRNA expression for both MCP-4 and eotaxin-3. MCP-4 showed basal mRNA expression in control endothelial cells that was slightly enhanced after stimulation with IL-4 (Fig. 5 A); however, we were unable to detect MCP-4 protein by Western blotting (data not shown). mRNA for eotaxin-3 was not detected in control HUVECs but was clearly expressed by IL-4-stimulated HUVECs (Fig. 5 A). Eotaxin-3 is a recently described chemokine that acts at CCR3 (25, 31). Western blotting showed that eotaxin-3 protein was expressed in IL-4-stimulated HUVECs (Fig. 5 B). The antibody we used had been tested for cross-reactivity with the eotaxin family, and the data in Fig. 5 B confirmed that there was no binding to recombinant MCP-4, the closest known homologue to eotaxin-3 (Fig. 5 B).

Eotaxin-3 Is Both Secreted by IL-4-stimulated HUVECs and Associated with the Surface of IL-4-stimulated HUVECs. Newly synthesized chemokines are released into the medium; however, chemokines can also remain cell-associated through interactions with proteoglycans on the cell surface (32, 33). We developed a sandwich ELISA to quantify eotaxin-3 secretion by IL-4-stimulated HUVECs and used a cell surface ELISA to evaluate the association of eotaxin-3 with the surface of HUVEC. Eotaxin-3 was not detected in the conditioned supernatant from control HUVECs, but considerable amounts of eotaxin-3 were detected in supernatants from IL-4-stimulated HUVECs (Fig. 6 A). The amount of eotaxin-3 detected in the conditioned supernatants from IL-4-stimulated HUVECs was 7.97 ± 1.79 ng/ml. Using a cell surface ELISA, eotaxin-3 was also found associated with the cell surface of IL-4-stimulated HUVECs (Fig. 6 B).

Cell-associated Eotaxin-3 Participates in Eosinophil Transmigration on IL-4-stimulated HUVECs. Our eotaxin-3 antibody blocks functional activity of this chemokine, thus we

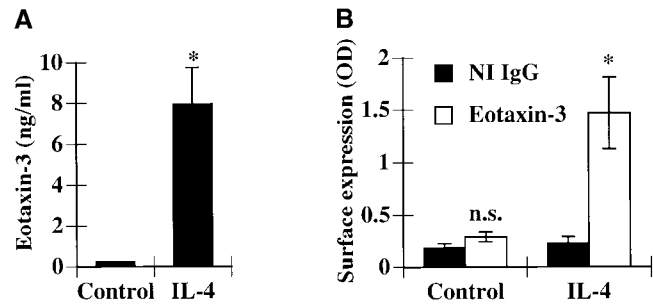


Figure 6. Eotaxin-3 protein is secreted by and associated with the surface of IL-4-stimulated HUVECs. HUVECs were stimulated for 24 h with M199/A alone or M199/A containing 20 ng/ml IL-4. (A) Supernatants were collected, and soluble eotaxin-3 was measured using a sandwich ELISA as described in Materials and Methods. (B) Surface-associated eotaxin-3 was detected using a cell-surface ELISA with nonimmune goat antibody (NI IgG) and anti-eotaxin-3 antibody as described in Materials and Methods. Data are mean \pm SEM of at least five experiments. * $P < 0.05$; ns, not significant.

used this tool to determine if eotaxin-3 was responsible for the CCR3-dependent eosinophil activation and subsequent transmigration on IL-4-stimulated HUVECs. As our studies are performed under flow conditions, eotaxin-3 released into the supernatant is unlikely to participate in eosinophil activation. We therefore treated HUVECs with anti-eotaxin-3 antibody for 10 min before assembly of the flow chamber. Pretreatment of IL-4-stimulated HUVECs with an anti-eotaxin-3 antibody blocked eosinophil transmigration (Fig. 7). This was specific for eotaxin-3, as neither an isotype control antibody nor antibodies against eotaxin or RANTES had any effect on transmigration (data

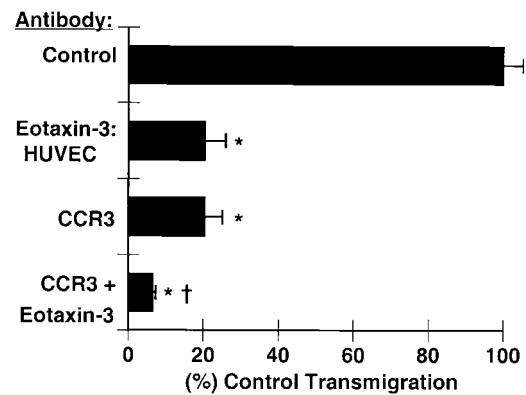


Figure 7. Surface-associated eotaxin-3 is responsible for the CCR3-dependent transmigration of eosinophils across IL-4-stimulated HUVECs. HUVECs were stimulated with IL-4 as described in Fig. 1. Freshly isolated eosinophils (5×10^5 /ml) were preincubated with 10 μ g/ml of antibody against CCR3. In some experiments, HUVECs were preincubated for 10 min with 20 μ g/ml of antibody against eotaxin-3 and then 10 μ g/ml of eotaxin-3 was maintained in the perfusate. Isotype-matched control antibodies were used in all cases. The flow chamber was assembled and eosinophils were perfused over the HUVECs as described in Fig. 1. Transmigration was measured as described in Materials and Methods. Data are mean \pm SEM of at least three experiments. * $P < 0.001$ compared with control and † not significant compared with Eotaxin-3 or CCR3 alone.

not shown). Both eotaxin-3 and CCR3 antibodies inhibited transmigration to the same extent (Fig. 7), and the effect of the antibodies together was not statistically different from the effect of either antibody alone (Fig. 7). This indicates that eotaxin-3 is responsible for all of the CCR3-dependent eosinophil activation and transmigration. In addition, cell-associated eotaxin-3 was critical in eosinophil transmigration, as the addition of eotaxin-3 antibody to the perfusate alone without pretreating the HUVECs resulted in only a small degree of inhibition (data not shown). Furthermore, preexposure of HUVECs to shear for 5 min to remove any soluble eotaxin-3 before perfusion of eosinophils had no effect on shear-dependent transmigration (data not shown).

Discussion

In this study, we present the first *in vitro* examination of eosinophil transmigration under shear conditions. We observed that eosinophils rapidly transmigrated across IL-4-stimulated HUVECs under flow conditions. After only 6 min of interaction with the endothelial cell monolayer, ~50% of eosinophils had transmigrated. This number increased even further, with over 80% of all recruited cells transmigrating in less than 20 min. This rapid transmigration under flow conditions *in vitro* is consistent with *in vivo* findings (34), but differs significantly from previous reports of eosinophil transmigration measured using static assays. Using these systems, Moser et al. showed that eosinophils will selectively bind to IL-4-stimulated HUVECs (35), but will not transmigrate unless they have been primed (20, 35). Priming is also required for eosinophil transmigration across IL-1 β -stimulated HUVECs (36). Priming can occur *in vivo*, as in the case of cells isolated from allergic asthmatics, or *in vitro* by treatment with IL-5, or GM-CSF (20, 36). Even using eosinophils from allergic asthmatics, transmigration still requires hours to occur and only 35–40% of the cells transmigrate (20, 36). Consistent with these reports, we also found that few cells completely transmigrated in the absence of shear. These data suggest that shear stress strongly promotes eosinophil transmigration across IL-4-stimulated HUVECs.

The transmigration of leukocytes including neutrophils, monocytes, and lymphocytes has been examined under flow conditions (5, 16, 17, 37). Only recently, however, have groups such as Cinamon et al. (28) and Kitayama et al. (17) systematically examined the role of shear stress in facilitating leukocyte transmigration. Cinamon et al. showed that shear stress is absolutely required for T lymphocyte transmigration across cytokine-stimulated HUVECs, as no transmigration occurs under static conditions (28). In contrast, Kitayama et al. showed that neutrophil transmigration across cytokine-stimulated HUVECs is accelerated by shear stress, but is not dependent on shear stress, as transmigration eventually plateaus at the same level under either static or shear conditions (17). Our study shows that eosinophils more closely resemble lymphocytes

than neutrophils in that eosinophils require shear stress to reach maximal levels of transmigration. Unlike lymphocytes, however, eosinophils showed a basal level of transmigration under static conditions. These data indicate that the effect of shear stress on transmigration is dependent upon the subclass of leukocyte examined.

The specific mechanisms that govern shear-dependent eosinophil transmigration may be related to signaling events within the endothelial cells. Endothelial cell signaling events induced by fluid shear stress include increases in intracellular calcium (38), activation of Src-family kinases and focal adhesion kinase (39, 40), and activation of MAP kinases (40, 41). Activation of these kinases occurs within minutes and has been shown to result in dynamic changes in the actin cytoskeleton (42). Thus fluid shear stress rapidly activates multiple signaling cascades in endothelial cells. However, shear stress alone is not capable of promoting eosinophil transmigration. Preexposure of the endothelium to shear stress did not increase eosinophil transmigration under static conditions (Fig. 3), consistent with studies of shear-dependent transmigration of neutrophils and lymphocytes (17, 43). This may reflect an inability of shear stresses of 2 dyne/cm² alone to induce signaling in the endothelium. These relatively low shear stresses may instead serve to prime endothelial cells for activation of signaling pathways, and a second stimulus, such as leukocyte adhesion, may be required for a full response.

Neutrophil adhesion to endothelial cells results in calcium mobilization (44), myosin light chain kinase phosphorylation (45, 46), and MAP kinase activation (47, 48) in endothelial cells. Many of these events are critical for subsequent neutrophil transmigration (46, 49). Unlike neutrophils, unprimed eosinophils will not transmigrate across stimulated HUVECs under static conditions. This may reflect an inability for unprimed eosinophils to elicit these responses in endothelial cells. Eosinophil adhesion combined with shear stress could synergistically activate signaling cascades within endothelial cells, thereby inducing changes in the cytoskeleton and cellular junctions that promote transmigration. Our data suggests that endothelial ERK1/2 is being activated after eosinophil adhesion under shear conditions, as an inhibitor of ERK1/2 activation blocked ~35% of eosinophil transmigration. We are currently investigating both the specific targets of ERK1/2 as well as the upstream activators of this MAP kinase.

Eosinophils accumulated on both TNF- and IL-4-stimulated HUVECs under shear conditions, but transmigration was selective for IL-4-stimulated HUVECs (Fig. 1). This suggests that although fluid shear stress is required for eosinophil transmigration, shear stress alone is not sufficient to induce transmigration. Both *in vivo* and *in vitro* investigations into the mechanisms by which IL-4 supports increased eosinophil recruitment have focused primarily on adhesion molecule expression. In this study we instead focused on the role of chemokines in eosinophil transmigration across activated endothelial cells, however, unlike other studies, we did not use exogenously added chemokines to promote transmigration (43). We found that eo-

sinophil transmigration was, in part, pertussis toxin-sensitive and inhibited by an antibody directed against CCR3. Endogenously synthesized eotaxin-3 was responsible for the CCR3-dependent eosinophil activation and transmigration observed in this system. Eotaxin-3 is a newly described CC chemokine that was cloned from IL-4-stimulated HUVECs (25, 31). Eotaxin-3 mRNA is upregulated in dermal fibroblasts after stimulation with IL-4 and in the airways after antigen challenge (50, 51); however, this is the first example of a role for endogenous eotaxin-3 in eosinophil recruitment.

Endothelial cell-associated eotaxin-3, and not secreted eotaxin-3, was involved in eosinophil transmigration. Several chemokines are cell-associated due to interactions with proteoglycans expressed by endothelial cells (33, 52–54). It has been proposed that surface association of endothelial chemokines leads to the establishment of haptotactic gradients at sites of inflammation. These gradients could be maintained even in the presence of shear, where soluble gradients would be rapidly dispersed. Although we did not examine the distribution of eotaxin-3 on the surface of HUVECs, increased expression near the cellular junctions and deposition within the subendothelial cell matrix may account for the ability of this chemokine to promote transmigration. Alternatively, a haptotactic gradient may not be required for eotaxin-3 to stimulate transmigration in the presence of shear. Soluble monocyte chemoattractant protein-1 (MCP-1) has been shown to promote monocyte transmigration across TNF-stimulated HUVECs under flow conditions, suggesting that immobilized chemokine gradients are not required (37). Additionally, lymphocytes transmigrate from high to low concentration across an immobilized gradient of stromal cell-derived factor-1 α (SDF-1 α) or EBI-1 molecular ligand chemokine (ELC) in the presence of shear (28). These studies challenge the assumption that chemokine gradients are required for leukocyte transmigration, and suggest that endothelial cell-derived chemokines simply serve to initiate transmigration, while other signals give directionality to the leukocyte migration. Our laboratory is currently examining both the mechanisms that govern eotaxin-3 cell-association and the localization of eotaxin-3 on HUVECs.

Several groups have begun to focus on the role of chemokine and cytokine networks in selective leukocyte recruitment (37, 55, 56). Chemokines associated with endothelial cells promote leukocyte activation, firm adhesion, and transmigration. These activated cells must now ignore this chemokine and follow a hierarchy of alternating chemotactic gradients. By using multiple chemotactic signals, each of which is active toward a particular leukocyte subclass, specific subclasses of leukocytes can be attracted while others are left behind. Surface-associated eotaxin-3 may serve as the first chemokine in a network of signals that leads to eosinophil infiltration into sites of allergic inflammation. Both groups that cloned and initially characterized eotaxin-3 showed that eotaxin-3 is ~ 10 -fold less active than eotaxin. When these two chemokines are used at equal concentrations, eotaxin-3 does not completely de-

sensitize eosinophils to subsequent stimulation by eotaxin (25, 31). Thus, once cells have transmigrated in response to eotaxin-3, they may still be able to respond in a CCR3-dependent manner to an eotaxin gradient established in the tissue. In addition, CCR3-independent mechanisms may also modulate responsiveness to secondary chemotactic gradients.

This study demonstrates that shear stress is critical in the rapid transendothelial migration of eosinophils and suggests that examining leukocyte transmigration under the flow conditions found in the vasculature may better recapitulate the mechanisms of leukocyte trafficking in vivo. Furthermore, we show that a novel eosinophil chemokine, eotaxin-3, is both surface associated and critical in supporting eosinophil transmigration across IL-4-stimulated HUVECs under flow conditions. Eotaxin-3 may also serve as a selective signal for eosinophil infiltration into sites of allergic inflammation. Future studies will focus on the signaling pathways activated during adhesion under shear forces and how these pathways influence the transmigration process.

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