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GRO family chemokines are specialized for monocyte arrest from flow

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Smith, David F., Elena Galkina, Klaus Ley, and Yuqing Huo. GRO family chemokines are specialized for monocyte arrest from flow. *Am J Physiol Heart Circ Physiol* 289: H1976–H1984, 2005. First published June 3, 2005; doi:10.1152/ajpheart.00153.2005.—Chemokines participate in various processes of monocyte recruitment including monocyte arrest and migration. Our group and others have demonstrated that growth-related oncogene (GRO)-α (CXCL1) can support monocyte arrest in models of inflammation. Here we employed a parallel plate-flow chamber and Transwell reconstitution assay to test whether GRO family chemokines were sufficient for Mono Mac 6 (a human monocytic cell line) and isolated human monocyte recruitment. Our study shows that *1*) GRO-α, -β (CXCL2), and -γ (CXCL3) all act as arrest chemokines for monocyte adhesion on vascular cell adhesion molecule (VCAM)-1 under flow in the presence of P-selectin; *2*) CXCR2 is the functional receptor for GRO-family chemokines in monocyte arrest; however, CXCR2 is not an arrest chemokine receptor in general, since epithelial neutrophil-activating peptide ENA-78 failed to arrest monocytes; *3*) GRO-α, -β, and -γ all fail to increase intracellular free Ca^{2+} or mediate monocyte chemotaxis; and *4*) signaling through $G\alpha_i$ protein, phosphoinositide 3-kinase, and actin polymerization but not Ca^{2+} mobilization or the mitogen-activated kinases p38 and MAPK/extracellular signal-related kinase are necessary for GRO-α-mediated Mono Mac 6 cell arrest under flow. We conclude that the GRO-family chemokines are specialized monocyte-arrest chemokines. Their role in monocyte recruitment in inflammation can be inhibited by blocking CXCR2 function or downstream signaling events.

growth-related oncogene; P-selectin; vascular cell adhesion molecule-1; phosphoinositide 3-kinase; G protein; signaling

MONOCYTE RECRUITMENT IS A key step in the initiation and progression of various inflammatory disorders including atherosclerosis. Monocyte recruitment proceeds in a cascade that includes rolling, arrest, and migration, processes that are mediated by a variety of adhesion molecules and chemokines (14, 26). Chemokines are small chemoattractant peptides that share structural similarities (20). Distinguished by the number of amino acids between conserved cysteine residues, chemokines are divided into the four families CC, CXC, CX_3C, and C (16). Chemokines signal through heptahelical receptors linked to heterotrimeric G proteins to activate leukocytes (11). Human blood monocytes express an array of chemokine receptors including CXCR1, CXCR2, CXCR4, CCR1, CCR2, CCR4, and CCR7 (7). The interactions of these receptors with their chemokines induce monocyte arrest on endothelium, transmigration through the endothelium, and other functions (11).

The effects of chemokines on leukocyte arrest have received much interest over the past several years (16, 20). Growing evidence has shown that certain chemokines are able to mediate monocyte arrest, which is a transition from rolling to firm adhesion. For example, pretreatment of monocytes with most soluble chemokines including monocyte chemoattractant protein (MCP)-1 or interleukin (IL)-8 induced monocyte arrest on human umbilical vein endothelial cells (HUVECs) expressing E-selectin (9). Consistent with this observation, studies from several groups (27) including ours (15) revealed that immobilized growth-related oncogene (GRO)-α or KC on inflamed cultured endothelial cells or atherosclerotic endothelium was able to mediate monocyte arrest. However, conflicting data have also been reported. In an in vitro flow-chamber system that used a surface coated with adhesion molecules and chemokines, Cybulsky's group (4) has shown that only stromal cell-derived factor (SDF)-1α, but not other chemokines, is capable of arresting monocytes on a VCAM-1-coated surface. In contrast with the limitation to only VCAM-1 and chemokines in the reconstituted system, many more inflammatory molecules are presented to monocytes in the context of inflamed endothelial cells (27) or atherosclerotic endothelium (15). To determine the minimal molecular requirements for GRO chemokines to arrest monocytes, we investigated the role of GRO chemokines in monocyte arrest in a reconstituted system that contained VCAM-1, P-selectin, and chemokines. Previous data have shown that GRO-α does not induce significant migration of Mono Mac 6 cells (5), and the entire GRO family lacks the ability to induce migration of human monocytes (6). Here, we confirm these data and show that all GRO family members induce arrest of human monocytes under flow.

Signaling mechanisms involved in chemokine-mediated monocyte arrest are not well studied. Signals including pertussis toxin (PTX)-sensitive G proteins (1), phosphoinositide 3-kinase (PI3-kinase)-γ (12), and p38 mitogen-activated protein kinase (MAPK; Ref. 2) have been intensively studied in monocyte chemotaxis and/or migration and firm adhesion. Most models employed in these studies did not closely mimic physiological conditions. In the present study, using a parallel plate flow chamber with the surface coated with the minimal number of molecules required for monocyte recruitment in inflammation and/or atherosclerosis, we identified those signals important for GRO chemokine-mediated Mono Mac 6 cell arrest.

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MATERIALS AND METHODS

Reagents

OPI media supplement (that contains oxalacetic acid, sodium pyruvate, and insulin), cytochalasin D, and Histopaque density gradient 1077 were purchased from Sigma-Aldrich (St. Louis, MO). Human chemokines including RANTES and GRO-α, -β, and -γ were purchased from PeproTech (Rocky Hill, NJ). Recombinant human P-selectin, recombinant human VCAM-1, and PTX were purchased from R&D Systems (Minneapolis, MN). BAPTA and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR). Bovine serum albumin (BSA) was purchased from Boehringer Mannheim (Indianapolis, IN).

Monocytes

Human blood-derived monocytes were isolated from whole blood drawn from healthy individuals. This protocol has been approved by the Institutional Review Board at the University of Virginia. A 1:3 blood-PBS dilution was placed over Histopaque 1077 density gradient before centrifugation at 400 *g* for 30 min. The interface was collected and washed twice with PBS. Monocytes were isolated using the Human Monocyte Isolation Kit II from Milteny Biotec (Auburn, CA). Approximately 100 million cells obtained from the Histopaque separation were resuspended in 300 μl of degassed buffer (PBS without Ca^{2+} and Mg^{2+} at pH 7.2 with 0.5% BSA and 2 mM EDTA) before addition of 100 μl of Fc receptor-blocking reagent and 100 μl of the biotin-antibody cocktail. The cell suspension was incubated at 4°C for 10 min. Buffer (300 μl) and anti-biotin microbeads (200 μl) were added to the cell suspension. After the cells were incubated for 15 min at 4°C, 6 ml of the buffer was added to the cell suspension, and the cell suspension was centrifuged at 300 *g* for 10 min. The cell pellet was resuspended in 500 μl of the buffer and run through a magnetic separation LS column (Milteny Biotec). Buffer (9 ml) was added to the column, and the effluent (monocytes) was collected. The monocyte suspension was centrifuged at 300 *g* for 10 min, and the cell pellet was resuspended in RPMI 1640 with 10% FCS. Monocyte purity was determined by flow cytometry based on the percentage of CD14-positive cells. The purity was >90%. Isolated monocytes showed minimal loss of L-selectin or increase in Mac-1, which suggests that minimal activation occurred during isolation by negative selection.

In some experiments, the monocytic cell line Mono Mac 6 (28) was used. Mono Mac 6 cells were cultured in 75-cm^2 flasks (Corning; Fisher Scientific; Hampton, NH) in RPMI 1640 medium (Invitrogen; Life Technologies; Carlsbad, CA) supplemented with 10% FBS; OPI supplement containing oxalacetic acid, sodium pyruvate, and insulin; MEM nonessential amino acids; 100 U/ml of penicillin; 100 μg/ml of streptomycin; and 250 ng/ml of amphotericin B.

Ca^{2+} Flux

Isolated monocytes were centrifuged at 1,000 *g* for 5 min, and the cell pellet was resuspended in culture medium to a final concentration of 5×10^6 cells/ml. Indo-1 AM (Molecular Probes) dissolved in DMSO was added to a final concentration of 1 μM, and the cells were incubated at 37°C for 1 h. After three washes with five volumes of Hanks' balanced salt solution (Cambrex Bio Science) that contained 1% FBS, 1 mM Ca^{2+}, and 1 mM Mg^{2+}, the cell pellet was resuspended to a final concentration of 1×10^6 cells/ml. Cells were prewarmed to 37°C, and a 30-s baseline reading was taken before addition of the activators. Immediately before analysis with the SLM spectrophotometer (SLM Instruments; Rochester, NY), the activators were added to 2 ml of the cell suspension in a quartz cuvette. Changes in intracellular Ca^{2+} were monitored with excitation at 340 nm and emission as the ratio of fluorescence at 398/480 nm. The data were converted to intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$, in nM) by adding 50 mM ionomycin and 10 mM EGTA and using the formula $[Ca^{2+}]_i = [(y - R_{min})/(R_{max} - y)] \times K_d \times \beta'$, where R_{min} is 398-nm fluorescence with EGTA/480-nm fluorescence with EGTA, R_{max} is 398-nm fluorescence with ionomycin/480-nm fluorescence with ionomycin, K_d is 125, and β' is 480-nm fluorescence with EGTA/480-nm fluorescence with ionomycin.

Chemokine-Mediated Monocyte Arrest

Immobilization of P-selectin, VCAM-1, and chemokines. A microparallel plate flow chamber was employed to conduct experiments using primary blood monocytes. The chamber was made from 2 × 0.2-mm rectangular glass capillaries (VitroCom; Mountain Lakes, NJ) connected to polyethylene-90 tubing (Becton-Dickinson; San Diego, CA; Ref. 23). P-selectin (3 μg/ml) and VCAM-1 (100 ng/ml) were added with or without 5 μg/ml chemokines to PBS. These concentrations were determined in preliminary experiments. A 15-μl aliquot of solution that contained both VCAM-1 and P-selectin with and/or without chemokine was added to each capillary tube and incubated for 2 h at room temperature. Capillaries were then blocked with 1% BSA in PBS for 1 h. After blocking, capillaries were washed with RPMI 1640.

A GlycoTech parallel plate flow chamber (GlycoTech; Gaithersburg, MD) was employed to conduct flow-chamber experiments on Mono Mac 6 cells. P-selectin (10 μg/ml) and VCAM-1 (150 ng/ml) were added with or without the tested chemokine (5 μg/ml) to PBS. A 100-μl aliquot of solution that contained both VCAM-1 and P-selectin with and/or without the tested chemokine was added to the center of each 35-mm petri dish (Falcon; Fisher Scientific) and incubated at 4°C overnight. The plates were blocked with 1% BSA in PBS for 1 h and washed with RPMI 1640, and the parallel plate flow chamber was attached.

Parallel plate flow chamber. Human monocytes or Mono Mac 6 cells were placed in RPMI 1640 with 1% FBS, 1 mM Ca^{2+}, 1 mM Mg^{2+}, and 10 mM HEPES at a concentration of 1×10^6 cells/ml. The cell suspension was pulled through the parallel plate flow chambers with a Harvard Apparatus 22 pump (Instech Laboratories; Plymouth Meeting, PA) and a 10-ml syringe (BD Biosciences; San Jose, CA) at 1 dyn/cm^2. Cells were visualized using an Axioskop 100 inverted light microscope (Carl Zeiss Optical; Thornwood, NY) with a ×10 objective, and adherent and rolling cells were counted by videomicroscopy (Olympus; Melville, NY) from 10-min recordings.

Analysis of arrested cells. For flow-chamber experiments involving human monocytes, four fields of view were taken from each capillary chamber down the length of the glass tubing. Each field of view was taken from approximately the same area in all of the capillary tubes. Each recording lasted 30 s. For Mono Mac 6 cell experiments, five fields of view were recorded, which also lasted 30 s. The first field of view was in the center of the flow chamber midline from the inlet and outlet ports. Two other fields of view were taken between the center field of view and the walls of the flow chamber. The last two fields of view were from the right and left sides of the center field of view, halfway between the field of view and the inlet/outlet port. Arrested cells were defined as those cells that were adherent for 20 s. The average of four or five fields of view was considered one measurement.

Tracking data. Video recordings were digitized into a Macintosh computer (Apple Computers; Cupertino, CA) with Adobe Premiere software (Adobe Systems; San Jose, CA) using a MicroMotion DC30 video compression card (Pinnacle Systems; Mountain View, CA). The public domain NIH Image program (http://rsb.info.nih.gov/nih-image) was used to analyze the video clips. Cells were tracked every three frames per second. Sliding averages of the velocity were determined by taking the average of every 10 velocity measurements and plotted vs. time.

Chemotaxis Assay

Human blood-derived monocytes or Mono Mac 6 cells were placed in RPMI 1640 with 10% FBS at a final concentration of 2×10^6

cells/ml. Appropriate chemokine solution (600 μl) was placed in the bottom of a Corning Costar 24-well plate (Fisher Scientific). Corning Transwell inserts (Fisher Scientific), 8 μm for Mono Mac 6 and 5 μm for blood monocytes, were placed over each well, and 200 μl of cell suspension was then placed in the insert. Cells were allowed to migrate for 2.5 h in an incubator at 37°C with 5% CO_2. After incubation, the inserts were removed, and cells were counted with a hemacytometer.

Statistical Analysis

Data are represented as the means ± SE of 3–12 independent experiments. A two-tailed Student's *t*-test was used with the null hypothesis rejected at $P < 0.05$.

RESULTS

Role of GRO Chemokines in Monocyte Arrest and Chemotaxis

To determine the minimal molecular requirements for GRO-α to arrest monocytes under flow, we tested surfaces coated with GRO-α and either P-selectin, VCAM-1, or both. A human monocytic cell line that closely mimics monocytes was used to identify the substrate for human monocyte arrest assays. Neither GRO-α alone nor a combination of GRO-α coimmobilized with either P-selectin or VCAM-1 was able to effectively mediate Mono Mac 6 cell arrest (Fig. 1*A*). There

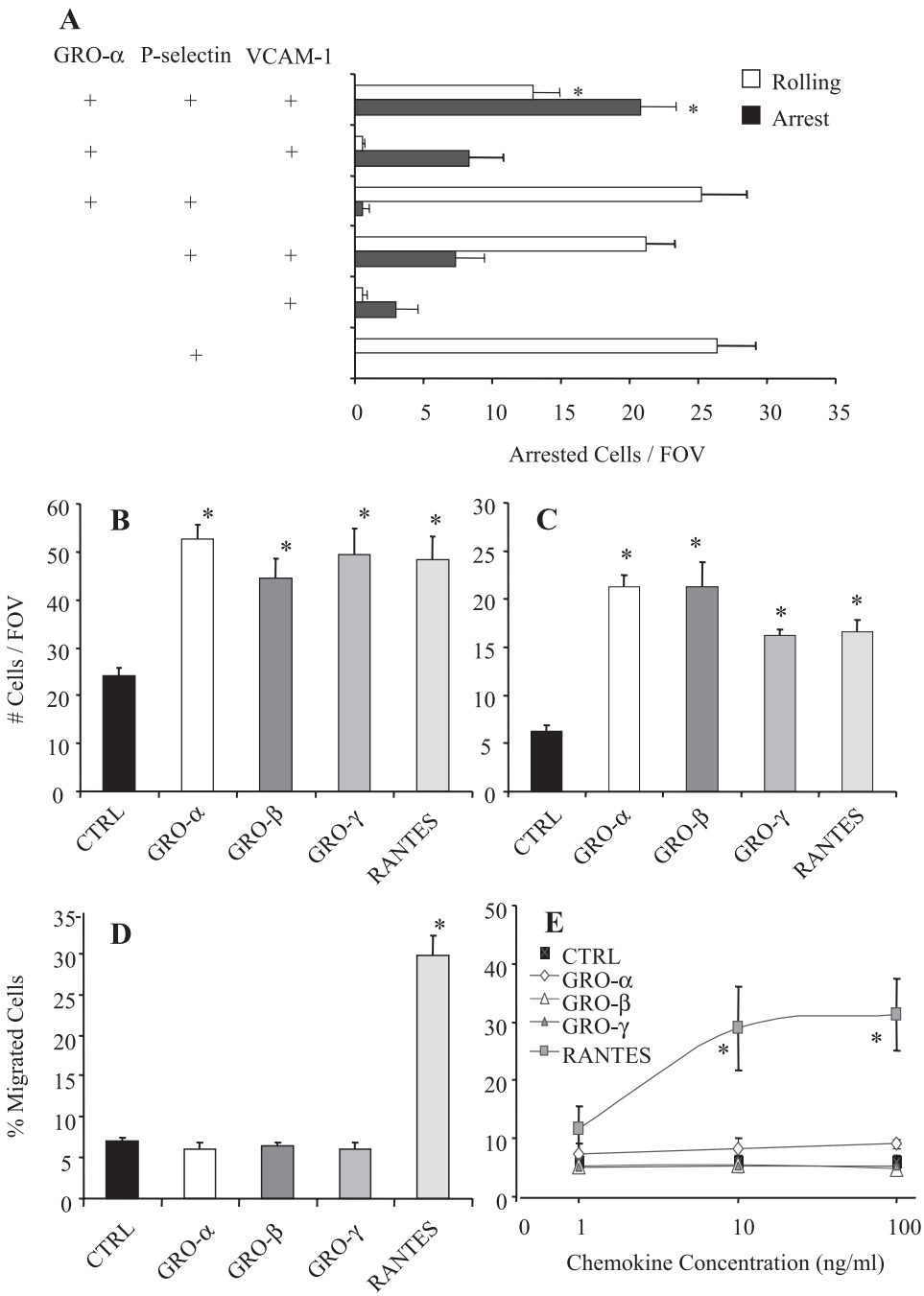

Fig. 1. Arrest and chemotaxis of Mono Mac 6 cells and human blood-derived monocytes in response to growth-related oncogene (GRO) chemokines. *A*: in the flow chamber at a shear stress of 1 dyn/cm^2 on P-selectin (10 μg/ml) alone, vascular cell adhesion molecule (VCAM)-1 (150 ng/ml) alone, P-selectin and VCAM-1, or in combination with the chemokine GRO-α (5 μg/ml), the number of rolling and arrested Mono Mac 6 cells on P-selectin/VCAM-1/GRO-α are significantly different from the number of rolling and arrested Mono Mac 6 cells on both VCAM-1/GRO-α and P-selectin/VCAM-1 (substrate considered a negative control). *B* and *C*: coimmobilizing the chemokines GRO-α, -β, and -γ or RANTES (5 μg/ml) with P-selectin (10 μg/ml for Mono Mac 6 cells and 3 μg/ml for monocytes) and VCAM-1 (150 ng/ml for Mono Mac 6 cells and 100 ng/ml for monocytes) significantly increased arrest of isolated blood monocytes (*B*) and Mono Mac 6 cells (*C*) compared with a control (CTRL) of P-selectin and VCAM-1 only. Data are presented as the number of cells arrested in a 0.55-mm^2 field of view (FOV). *D*: RANTES (100 ng/ml) significantly induced migration of human blood-derived monocytes, whereas GRO-family chemokines (also 100 ng/ml) did not. *E*: RANTES (10 and 100 ng/ml) induced significant migration above background of Mono Mac 6 cells. Similar to the results seen with human monocytes, the GRO chemokines failed to induce chemotaxis of Mono Mac 6 cells at 1, 10, or 100 ng/ml. Data are shown as percents of total cells added to the top of the insert before incubation. *$P <$ 0.05 compared with control.

were significantly more arrested Mono Mac 6 cells on the surface cocoated with GRO-α, P-selectin, and VCAM-1, which indicates that selectin-mediated monocyte rolling facilitates GRO-α-mediated monocyte arrest (Fig. 1*A*).

Under the same conditions, we perfused isolated human blood monocytes through the flow chamber. Immobilized GRO-α effectively arrested human monocytes on the surface coated with P-selectin and VCAM-1. To determine whether the role of GRO-α in monocyte arrest can be generalized to other members of the GRO family, we investigated monocyte arrest on surfaces coated with GRO-β or -γ with P-selectin and VCAM-1. GRO-β and -γ induced monocyte arrest similar to GRO-α (Fig. 1*B*). The role of GRO-β and -γ as arrest chemokines was also confirmed with Mono Mac 6 cells (Fig. 1*C*). In contrast with the ability of the GRO family to induce monocyte arrest, GRO chemokines were not able to mediate isolated human monocyte chemotaxis (Fig. 1*D*). The inability of GRO chemokines to mediate monocyte chemotaxis was further confirmed at multiple concentrations using Mono Mac 6 cells (Fig. 1*E*). RANTES was used as a positive control for chemokine-mediated monocyte chemotaxis.

Characterization of Monocyte Arrest Induced by GRO-α in Reconstituted System

By tracking isolated human monocytes interacting with the adhesive surface under flow conditions, we obtained rolling and arrest profiles of GRO-α-mediated monocyte arrest. On a P-selectin/VCAM-1 surface (Fig. 2*A*), rolling monocytes showed random velocity fluctuations for the duration of tracking. Adding GRO-α to the surface did not change the pattern of rolling velocity (Fig. 2*C*). On both P-selectin/VCAM-1 (Fig. 2*A*) and P-selectin/VCAM-1/GRO-α (Fig. 2*C*) surfaces, those monocytes that arrested to either substrate did so within a few seconds in a similar fashion. GRO-α-induced conversion of monocyte rolling to arrest does not appear to result from a gradual activation. The total number of visible monocytes that transiently tethered, rolled, and arrested (all interacting cells) to the P-selectin/VCAM-1-coated surface (Fig. 2*B*) was identical to that on the P-selectin/VCAM-1/GRO-α-coated surface (Fig. 2*D*). However, of the ~80 cells traveling near the substrate, only ~12 monocytes arrested on P-selectin/VCAM-1 within 1 min (Fig. 2*B*). This number significantly increased to ~36 in the presence of coimmobilized GRO-α (Fig. 2*D*), which suggests that GRO-α increased monocyte arrest by activating monocytes without recruiting more cells to interact with the surface.

To further investigate mechanisms by which GRO-α mediates monocyte arrest, we tested whether GRO-α could induce Ca^{2+} flux in isolated monocytes. In contrast with a large increase in intracellular free Ca^{2+} in monocytes induced by RANTES (Fig. 3*A*), GRO-α did not cause a change in intracellular free Ca^{2+} (Fig. 3*A*). Because P-selectin is required for effective GRO-α-mediated monocyte arrest, we also tested whether P-selectin could induce an increase in intracellular free Ca^{2+}. Similar to GRO-α, the Ca^{2+} flux induced by P-selectin was not significant (Fig. 3*A*). The addition of P-selectin with GRO-α also failed to induce intracellular free Ca^{2+} mobilization compared with the results seen with the addition of either GRO-α or P-selectin alone (Fig. 3*C*). These experiments were repeated with Mono Mac 6 cells, and RANTES induced a large Ca^{2+} response (Fig. 3*B*), whereas P-selectin, GRO-α, or P-selectin with GRO-α did not (Fig. 3*D*). Also, whole blood labeled with fluo-4 AM was tested for Ca^{2+} response in monocytes using flow cytometry. Similar to the isolated monocytes, monocytes in whole blood did not show a Ca^{2+} response to GRO-α (data not shown). This suggests that the ability of

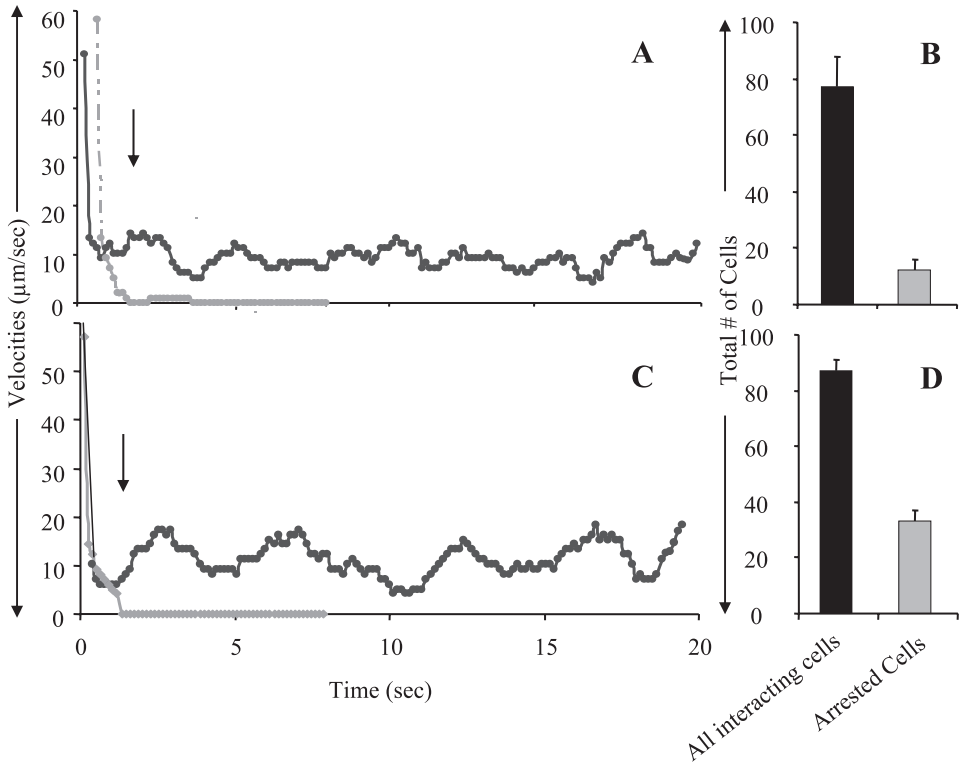

Fig. 2. Profiles of monocyte rolling and arresting in the presence of GRO-α. Cells were tracked for ~20 s and a sliding average of 10 was determined. *A* and *C*: instantaneous velocities of arrested monocytes are shown (gray lines) on P-selectin (10 μg/ml)/VCAM-1 (150 ng/ml; *A*) and P-selectin/VCAM-1/GRO-α (5 μg/ml; *C*) substrates. Arrows indicate where monocytes arrested. Instantaneous velocities of human blood-derived monocytes (black lines) on either a P-selectin (10 μg/ml)/VCAM-1 (150 ng/ml; *A*) or a P-selectin/VCAM-1/GRO-α (5 μg/ml; *C*) substrate are shown. *B* and *D*: fraction of arrested cells in the total number of cells near the substrate. Number of cells that transiently tethered, rolled, and arrested to the substrate during the first minute of cell suspension perfusion through the flow chamber (solid bars) and fraction of arrested cells on a substrate (gray bars) consisting of P-selectin and VCAM-1 (*B*) are much lower than that on a substrate consisting of P-selectin, VCAM-1, and GRO-α (*D*).

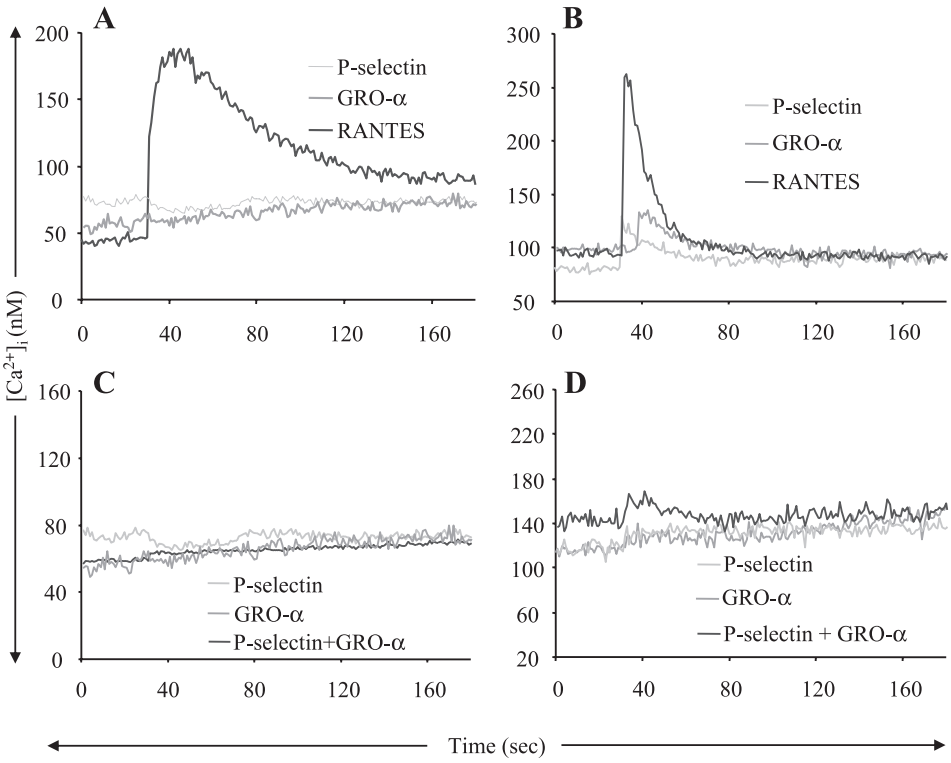

Fig. 3. Ca^{2+} flux of human blood-derived monocytes and Mono Mac 6 cells in the presence of varying activators. *A* and *B*: RANTES (100 ng/ml)-induced intracellular Ca^{2+} flux in monocytes (*A*) and Mono Mac 6 cells (*B*) was large compared with the response induced by P-selectin (1 μg/ml) or GRO-α (100 ng/ml). *C* and *D*: addition of GRO-α (100 ng/ml) and P-selectin (1 μg/ml) together failed to induce a substantial Ca^{2+} flux in either monocytes (*C*) or Mono Mac 6 cells (*D*), similar to the results seen with the addition of GRO-α or P-selectin alone. All activators tested were added at 30 s. These graphs are one of two or three independent experiments performed. Data are presented as free intracellular Ca^{2+} concentrations.

GRO-α or its combination with P-selectin to mediate monocyte arrest is not related to its ability to stimulate a change in intracellular free Ca^{2+} in monocytes.

For chemotaxis assays, it is known that the functional receptor for the chemokines of the GRO family is CXCR2 (20). Therefore, we investigated the role of CXCR2 in GRO-α-mediated monocyte arrest. Blockade of CXCR2 using a neutralizing antibody or desensitization of CXCR2 by soluble GRO-α reduced GRO-α-mediated monocyte arrest to below baseline levels (Fig. 4*A*). As a control, it was shown that treatment of monocytes with CXCR2 antibody did not reduce arrest to P-selectin/VCAM-1/RANTES thereby verifying the specificity of the antibody (Fig. 4*A*). We then tested the other known CXCR2 ligands to determine whether CXCR2 acts as a unique monocyte-arrest chemokine receptor. Immobilized IL-8 induced significant monocyte arrest to a surface coimmobilized with P-selectin and VCAM-1 (Fig. 4*B*), although its effect was lower than that of GRO-α on arrest. Under the same conditions, epithelial neutrophil activating peptide (ENA)-78 (CXCL5), another ligand for CXCR2, did not induce significant arrest of monocytes compared with the control (Fig. 4*B*). However, ENA-78 was found to arrest neutrophils under flow (data not shown). This suggests that the potent role of GRO chemokines in monocyte arrest may be mainly attributed to the nature of these chemokines and cannot be generalized to all chemokines that bind CXCR2.

Signal Transduction Pathways Involved in GRO-α-Mediated Monocyte Arrest

Signaling in monocyte arrest is poorly understood. In contrast, signal transduction in chemotaxis and/or migration is relatively well studied. To test signaling in GRO-α-mediated monocyte arrest, we first tested signaling in Mono Mac 6 cell chemotaxis to RANTES. We then examined whether the same pathways involved in chemotaxis also participated in GRO-α-mediated monocyte arrest. Mono Mac 6 cell chemotaxis to RANTES requires G protein-coupled receptors, PI3-kinase, and p38 MAPK, but not Ca^{2+} flux, actin polymerization, or MAPK/ERK kinase (MEK) signaling. A dramatic suppression of Mono Mac 6 cell chemotaxis to RANTES was found in experiments using Mono Mac 6 cells pretreated with PTX, the PI3-kinase inhibitors wortmannin or LY-294002, or the p38 MAPK inhibitor SB-203580 (Fig. 5, *F, H*, and *I*). To investigate whether Mono Mac 6 cells employ these pathways in their arrest response to GRO-α, we pretreated Mono Mac 6 cells with the same inhibitors and perfused them through the surface coated with P-selectin, VCAM-1, and GRO-α. Similar to the chemotaxis assay, Mono Mac 6 cell arrest was significantly inhibited when cells were pretreated with PTX or the PI3-kinase inhibitors wortmannin or LY-294002 (Fig. 5, A_1 and C_1), which was even more pronounced than its effect on chemotaxis. As a control, wortmannin-treated Mono Mac 6 cells were perfused over a substrate consisting of P-selectin and VCAM-1. There was no significant difference in arrest between treated and untreated cells to the control flow chambers (data not shown), which suggests that PI3-kinase is involved in the signaling initiated once the Mono Mac 6 cells bind chemokine on the substrate. The blockade of Ca^{2+} flux did not affect Mono Mac 6 cell arrest (Fig. 5B_1). Inhibition of MEK and p38 MAPK, two kinases downstream of PI3-kinase, did not have any influence in GRO-α-mediated Mono Mac 6 cell arrest (Fig. 5D_1). Actin polymerization, although not involved in chemotaxis, is required for GRO-α-mediated Mono Mac 6 cell arrest as demonstrated by the inhibitory effect of low-dose cytochalasin D (Fig. 5E_1).

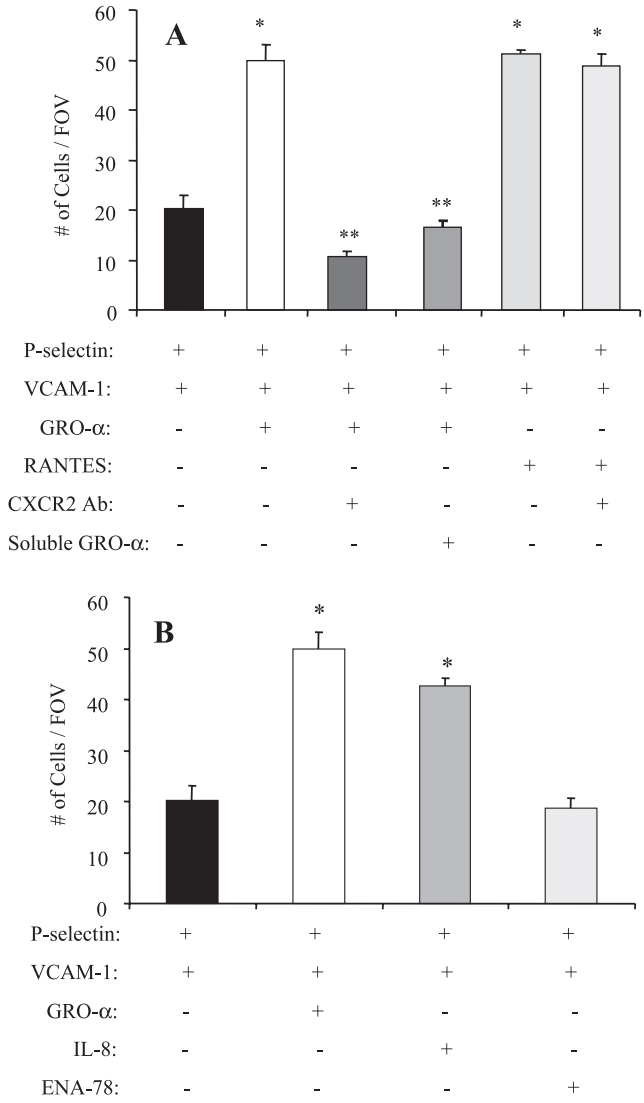

Fig. 4. Arrest of human blood-derived monocytes to varying substrates. *A*: when monocytes are either treated with CXCR2 antibody (Ab; 20 μg/ml) for 20 min or desensitized with soluble GRO-α (100 ng/ml) for 30 min, the number of arrested cells on the substrate including GRO-α (5 μg/ml), P-selectin (10 μg/ml), and VCAM-1 (150 ng/ml) is significantly reduced compared with untreated monocytes arresting to the same substrate. Monocytes treated with CXCR2 Ab still arrested to P-selectin/VCAM-1 and RANTES. *B*: IL-8 (5 μg/ml), when coimmobilized with P-selectin and VCAM-1, was found to induce significant arrest of monocytes compared with a P-selectin/VCAM-1 substrate, whereas coimmobilized epithelial neutrophil-activating peptide (ENA)-78 (5 μg/ml) failed to induce arrest of the cells. Data are shown as the number of arrested cells per 0.55-mm^2 field of view. *$P < 0.05$ compared with control; **$P < 0.05$ compared with untreated Mono Mac 6 cells.

To determine whether GRO-α induces arrest via the same signaling pathways used by other chemokines, signaling pathways tested in GRO-α-mediated arrest were compared with those important for RANTES-induced arrest of Mono Mac 6 cells when coimmobilized with P-selectin and VCAM-1. Those inhibitors that blocked GRO-α-mediated arrest also inhibited RANTES-mediated arrest (Fig. 5). Inhibition of $G\alpha_i$, PI3-kinase, and actin polymerization blocked RANTES-mediated Mono Mac 6 cell arrest to P-selectin/VCAM-1/GRO-α (Fig. 5, A_2, C_2, and E_2), whereas inhibition of Ca^{2+} mobilization, MEK, or p38 MAPK did not block arrest (Fig. 5, B_2 and D_2). These data suggest that RANTES and GRO-α use similar signaling pathways to arrest Mono Mac 6 cells.

DISCUSSION

The ability of certain chemokines to arrest monocytes is controversial under different conditions. Previous research from our laboratory has shown that blocking KC (murine GRO-α) or its receptor reduced monocyte adhesion to the endothelium of atherosclerotic carotid arteries of apolipoprotein $E^{-/-}$ mice, whereas blocking JE (murine MCP-1) had no effect (15). However, in another study, MCP-1 along with IL-8 was found to arrest human monocytes to E-selectin-transduced HUVECs under flow conditions, but in that study, the chemokine was added to the monocyte reservoir before perfusion and was not immobilized to the endothelial cells (9). GRO chemokines were previously implicated in monocyte arrest to minimally modified LDL-activated endothelial cells, but arrest was studied under static conditions (21). Under flow conditions, SDF-1α coimmobilized with VCAM-1-arrested monocytes, whereas MCP-1, RANTES, and GRO-α did not (4). Certainly, the method of chemokine presentation and the nature of the substrate could be causes of some of these apparent discrepancies.

In this study, we developed a reconstituted system to test the role of GRO chemokines in monocyte arrest under flow. In this system, P-selectin and VCAM-1, molecules known to be involved in monocyte recruitment (13), formed the minimal molecular condition for determination of chemokine-mediated monocyte arrest. In contrast with a previous study (4), the present study shows that certain chemokines, especially chemokines in the GRO family, are able to mediate robust monocyte arrest when reconstituted with P-selectin and VCAM-1. P-selectin could enhance signaling induced by chemokines and/or contribute to chemokine-mediated monocyte arrest. However, P-selectin did not induce Ca^{2+} flux in monocytes in our study, whether alone or in combination with GRO-α. Alternatively, the role of P-selectin in chemokine-mediated monocyte arrest may be simply to cause monocyte rolling and facilitate the interactions of the chemokine receptor with the immobilized chemokine.

GRO family members were incapable of inducing monocyte chemotaxis in our study. This is consistent with a previous study (6). We confirmed this finding with both Mono Mac 6 cells and human monocytes. It has long been demonstrated that as members of the CXC family, GRO chemokines induce neutrophil chemotaxis (6). KC, a mouse homolog of GRO-α, is also able to trigger neutrophil chemotaxis and/or infiltration both in vitro (3) and in vivo (19). The reasons for this difference between neutrophils and monocytes are not clear. It is certainly possible that the response to GRO-α is cell-type specific. Considering the significant contrast in the role of GRO chemokines in monocyte arrest and chemotaxis, it is likely that different cell activation and signaling events are required for monocyte arrest and chemotaxis. Because GRO chemokines only participate in monocyte arrest, monocyte activation and signaling induced by these chemokines may be the prototype of cell activation and signaling in monocyte arrest.

Different signals downstream of the G protein-coupled receptors are required for cell chemotaxis vs. arrest. It is known

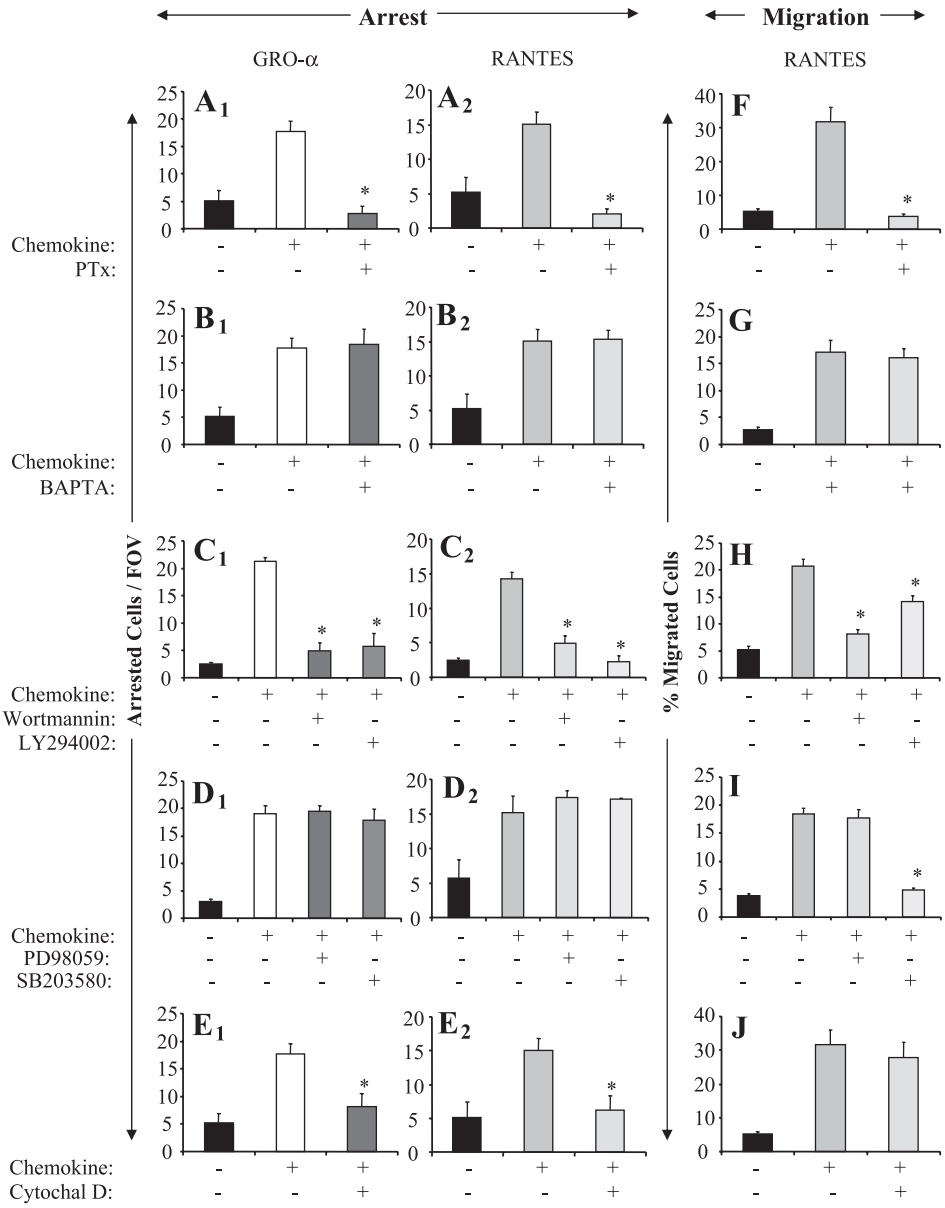

Fig. 5. Effects of inhibitors of signaling pathways on arrest and migration of Mono Mac 6 cells. *A, C,* and *E*: pretreatment with pertussis toxin (PTx; 250 ng/ml) for 3 h (A_1 and A_2), wortmannin (100 ng/ml) for 30 min or LY-294002 (50 μmol/l) for 30 min (C_1 and C_2), or cytochalasin D (Cytochal D; 1 μmol/l) for 1 h (E_1 and E_2) all significantly reduced cell arrest to GRO-α (5 μg/ml) or RANTES coimmobilized with P-selectin and VCAM-1, compared with untreated, arrested Mono Mac 6 cells. *B* and *D*: pretreatment with BAPTA (10 μmol/l) for 30 min (B_1 and B_2), PD-98059 (100 μmol/l) for 1 h, or SB-203580 (50 μmol/l) for 1 h (D_1 and D_2) did not inhibit cell arrest on GRO-α or RANTES coimmobilized with P-selectin and VCAM-1. Arrested cells were defined as cells that did not roll for at least 20 s. Data are presented as the number of arrested cells per 0.55-mm^2 field of view. For chemotaxis, RANTES was used at a concentration of 100 ng/ml. *F, H, and I*: pretreatment with pertussis toxin (250 ng/ml) for 3 h (*F*) or wortmannin (100 ng/ml) for 30 min or LY-294002 (50 μM) for 30 min (*H*) or SB-203580 (50 μM) for 1 h (*I*) all significantly inhibited migration of Mono Mac 6 cells compared with migration without the inhibitor. G, *I,* and *J*: pretreatment with BAPTA (10 μM) for 30 min (*G*), PD-98059 (100 μM) for 1 h (*I*), or cytochalasin D (1 μM) for 1 h (*J*) did not significantly block migration of Mono Mac 6 cells. Data are presented as the percents of total cells added to the top of the insert. All controls were treated with 0.1% DMSO. *$P < 0.05$ compared with untreated cells.

that PI3-kinase-γ can be directly activated by the β,γ-subunits of G proteins in vitro (24). Neutrophils isolated from PI3-kinase-$\gamma^{-/-}$ mice had reduced chemotactic activity toward IL-8, formyl-Met-Leu-Phe-OH, and C5a, but chemokine-mediated neutrophil adhesion in a static system was not affected by the lack of PI3-kinase-γ (12). In this same study, peritoneal macrophages from PI3-kinase-γ-deficient mice showed reduced chemotaxis toward SDF-1α, RANTES, and macrophage-derived chemokine (12). Another study found that MCP-1-mediated monocyte-adhesion signaling could involve the PI3-kinase-α isoform, and both PI3-kinase inhibitors wortmannin and LY-294002 reduced monocyte arrest to E-selectin-transduced HUVECs (8). In our experiments, wortmannin and LY-294002 significantly reduced Mono Mac 6 chemotaxis to RANTES (Fig. 5) as well as arrest on P-selectin/VCAM-1 in response to GRO-α and RANTES (Fig. 5). BAPTA-AM, a chelator of intracellular free Ca^{2+}, did not inhibit chemotaxis or arrest of Mono Mac 6 cells in our study. This was similar to previous data that showed that blood lymphocytes pretreated with BAPTA did not have reduced arrest to an SDF-1α/VCAM-1 substrate compared with untreated cells (10). However, in a Ca^{2+}-flux assay, the concentration of BAPTA used in the arrest assay blocked Ca^{2+} response of Mono Mac 6 cells to RANTES (data not shown), thereby confirming that this concentration did block Ca^{2+} influx in the arrest study. These data suggest that an increase in intracellular free Ca^{2+} secondary to activation of $G\alpha_i$ protein-coupled receptors is not necessary for either monocyte chemotaxis or arrest.

In a study that included a static adhesion assay, Ashida et al. (2) reported a significant contribution of MEK signaling in monocyte adhesion on a VCAM-1-coated surface. Using the same static adhesion assay, we confirmed that PD-98059, a MEK inhibitor, dramatically suppressed GRO-α-mediated Mono Mac 6 cell adhesion on a VCAM-1-coated surface (data not shown). However, inhibition of MEK signaling did not influence GRO-α-mediated Mono Mac 6 cell arrest under shear

flow. This difference highlights an interesting issue, namely, the state of integrin activation could vary between monocyte adhesion in a static system and monocyte arrest under shear flow. Notably, in the last few years, the different roles of integrin affinity and avidity in leukocyte recruitment have been investigated (18, 25).

Cytochalasin D treatment prevents actin polymerization and can enable integrin rearrangement in the membrane (17). It has been previously shown that cytochalasin D does not inhibit the binding of soluble VCAM-1 to VLA-4 under basal conditions or after chemokine treatment, which suggests that cytochalasin D does not affect VLA-4 affinity (4). The present study showed that cytochalasin D dramatically inhibited GRO-mediated monocyte arrest on a P-selectin/VCAM-1/GRO-α-coated surface under shear flow, which suggests a role for actin polymerization in monocyte arrest. Several possibilities are consistent with this result. First, suppression of basal actin polymerization may affect the maintenance of microvilli, which are required for proper presentation of integrins and other molecules (22). Consequently, the interactions of VLA-4 integrin with VCAM-1 may be altered in cytochalasin D-treated cells. Second, conformational changes of monocyte integrins regulated by actin polymerization might be important in monocyte arrest under flow. Finally, cytochalasin D-treated monocytes may not be able to properly undergo the shape change required for stable adhesion under flow. Residual actin polymers may be sufficient for cellular activities involved in monocyte chemotaxis. Therefore, monocyte chemotaxis is not affected by treatment with cytochalasin D at the concentration used in this study.

In conclusion, the GRO family is the first and perhaps a prototypical example of selective arrest chemokines for monocytes with no ability to induce significant chemotaxis. The signaling pathways involved in monocyte chemotaxis and arrest are similar but not identical. Blockade of the GRO-family chemokines, their receptors, or inhibition of involved downstream signals may suppress monocyte arrest, which is one of the earliest steps in monocyte recruitment, and thereby efficiently regulate inflammation.

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