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# GRO Family Chemokines are Specialized for Monocyte Arrest from Flow

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**Abstract**

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) could 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: (a) 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. (b) CXCR2 is the functional receptor for GRO family chemokines in monocyte arrest. However, CXCR2 is not an arrest chemokine receptor in general, since ENA-78 failed to arrest monocytes. (c) GRO-α, β and γ all fail to raise intracellular free calcium or mediate monocyte chemotaxis. (d) Signaling through $G\alpha_i$ protein, phosphoinositide 3-kinase (PI3K), and actin polymerization, but not calcium mobilization or the mitogen-activated kinases p-38 and MEK, 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.

## Introduction

Monocyte recruitment is a key step in the initiation and progression of various inflammatory disorders including atherosclerosis. Monocyte recruitment proceeds in a cascade including rolling, arrest, and migration, processes mediated by a variety of adhesion molecules and chemokines(14; 26). Chemokines are small chemoattractant peptides which share structural similarities(20). Distinguished by the number of amino acids between conserved cysteine residues, chemokines are divided into 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 effect of chemokines on leukocyte arrest has received much interest over the past several years(16; 20). Growing evidence has shown that certain chemokines are able to mediate monocyte arrest, 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 (HUVEC) expressing E-selectin(9). Consistent with this observation, studies from several groups(27), including ours(15), revealed that immobilized GRO-α or KC on inflamed cultured endothelial cells or atherosclerotic endothelium were able to mediate monocyte arrest. However, conflicting data has also been reported. In an *in vitro* flow chamber system using a surface coated with adhesion

molecules and chemokines, Cybulsky's group 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(4). In contrast to 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 containing VCAM-1, P-selectin, and chemokines. Previous data has 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 (PI3K) γ(12), and p38 mitogen-activated protein kinase (MAPK)(2) have been intensively studied in monocyte chemotaxis/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/atherosclerosis, we identified those signals important for GRO chemokine-mediated Mono Mac 6 cell arrest.

## Materials and Methods

***Reagents*.** OPI Media Supplement (containing 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, GRO-α, GRO-β, and GRO-γ, were purchased from PeproTech (Rocky Hill, NJ). Recombinant human P-selectin, recombinant human VCAM-1 and pertussis toxin 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. A 1:3 blood/PBS dilution was placed over Histopaque 1077 Density Gradient (Sigma-Aldrich), followed by centrifugation at 400 g for 30 minutes. 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 w/o $Ca^{++}$ and $Mg^{++}$ at a pH of 7.2 + 0.5% BSA + 2 mM EDTA), followed by the addition of 100 μl of FcR Blocking Reagent and 100 μl of the Biotin-Antibody Cocktail. The cell suspension was incubated at 4°C for 10 minutes. 300 μl of the buffer and 200 μl Anti-Biotin Microbeads were added to the cell suspension. After the cells were incubated for 15 minutes at 4°C, 6 ml of the buffer was added to the cell suspension, and the cell suspension was centrifuged at 300g for 10 minutes. The cell pellet was resuspended in 500 μl of the buffer and run through a Magnetic Separation LS column (Milteny Biotec). 9 ml of the buffer was added to the column, and the effluent

(monocytes) was collected. The monocyte suspension was centrifuged at 300 g for 10 minutes, and the cell pellet was resuspended in RPMI + 10% FCS. Monocyte purity was determined by flow cytometry based on the percentage of CD14 positive cells. The purity was greater than 90%. Isolated monocytes showed minimal loss of L-selectin or increase in Mac-1 suggesting 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 Corning 75 $cm^2$ flasks (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 non-essential amino acids, 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 250 ng/ml of amphotericin B.

***Calcium Flux.*** Isolated monocytes were centrifuged at 1000g for 5 minutes, and the cell pellet was resuspended in culture medium to a final concentration of $5x10^6$ 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 hour. Following three washes with 5 volumes of Hank's Balanced Salt Solution (Cambrex Bio Science) containing 1% fetal bovine serum, 1 mM $Ca^{2+}$ and 1 mM $Mg^{2+}$, the cell pellet was resuspended to a final concentration of $1x10^6$ cells/ml. Cells were pre-warmed to 37°C, and a 30 second baseline reading was taken prior to 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 calcium were monitored with excitation at 340 nm and emission as the ratio

of fluorescence at 398/480. The data was converted to intracellular calcium concentration (nM) by adding 50 mM ionomycin and 10 mM EGTA and using the formula: $[Ca^{2+}]_i=((y-R_{min})/(R_{max}-y))*k_d*\beta'$, where $R_{min}$ is 398 fluorescence with EGTA/480 fluorescence with EGTA, $R_{max}$ is 398 fluorescence with ionomycin/480 fluorescence with ionomycin, $k_d$ is 125, and $\beta'$ is fluorescence with EGTA/480 fluorescence with ionomycin.

***Chemokine-mediated monocyte arrest.***

*a. Immobilization of P-selectin, VCAM-1 and Chemokines*. A micro-parallel plate flow chamber was employed to conduct experiments using primary blood monocytes. The chamber was made from 2x0.2 mm rectangular glass capillaries (VitroCom, Mountain Lakes, NJ) connected to Polyethylene 90 tubing (Becton-Dickinson, San Diego, CA)(23; 23). P-selectin (at 3 μg/ml) and VCAM-1 (at 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 containing both VCAM-1 and P-selectin with/without chemokine was added to each capillary tube and incubated for 2 hours at room temperature. Capillaries were then blocked with 1% BSA in PBS for 1 hour. After blocking, capillaries were washed with RPMI.

A GlycoTech parallel plate flow chamber was employed to conduct flow chamber experiments on Mono Mac 6 cells. P-selectin (at 10 μg/ml) and VCAM-1 (at 150 ng/ml) were added with or without the tested chemokine (at 5 μg/ml) to PBS. A 100 μl aliquot of solution containing both VCAM-1 and P-selectin with/without the tested chemokine was added to the center of each Falcon 35 mm petri dish (Fisher Scientific, Hampton, NH) and incubated at 4$^{o}$C overnight. The plates were blocked with 1% BSA in PBS for 1

hour, washed with RPMI, and the parallel plate flow chamber (GlycoTech, Gaithersburg, MD) was attached.

*b. Parallel Plate Flow Chamber*. Human monocytes or Mono Mac 6 cells were placed in RPMI with 1% FBS, 1 mM $Ca^{++}$, 1 mM $Mg^{++}$, and 10 mM HEPES at a concentration of $1x10^6$ cells/ml. The cell suspension was pulled through the parallel plate flow chambers with a Harvard Apparatus (Instech Laboratories, Plymouth Meeting, PA) 22 pump and a 10 ml syringe (BD Biosciences, San Jose, CA) at 1 dyne/$cm^2$. Cells were visualized using an Axioskop 100 inverted light microscope (Carl Zeiss Optical, Thornwood, NY) with 10X objective, and adherent and rolling cells were counted by videomicroscopy (Olympus, Melville, NY) from 10-minute recordings.

*c. 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 seconds. For Mono Mac 6 cell experiments, five fields of view were recorded, also lasting 30 seconds. 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 seconds. The average of four or five fields of view was considered one measurement.

*d. 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 ten velocity measurements and plotted versus time.

***Chemotaxis Assay.*** Human blood-derived monocytes or Mono Mac 6 cells were placed in RPMI+10% FBS at final concentration of $2x10^6$ cells/ml. 600 µl of the appropriate chemokine solution were 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 were then placed in the insert. Cells were allowed to migrate for 2.5 hours in an incubator at $37^oC$, 5% $CO_2$. After incubation, the inserts were removed, and cells were counted with a hemacytometer.

***Statistical Analysis.*** Data are represented as the mean ± SE of 3-12 independent experiments. A two-tailed Student 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 co-immobilized with either P-selectin or VCAM-1 was able to effectively mediate Mono Mac 6 cell arrest (Figure 1A). There were significantly more arrested Mono Mac 6 cells on the surface co-coated with GRO-α, P-selectin and VCAM-1, indicating that selectin-mediated monocyte rolling facilitates GRO-α-mediated monocyte arrest (Figure 1A).

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 GRO-γ with P-selectin and VCAM-1. GRO-β and GRO-γ induced monocyte arrest similar to GRO-α (Figure 1B). The role of GRO-β and GRO-γ as arrest chemokines was also confirmed with Mono Mac 6 cells (Figure 1C). In contrast to the ability of the GRO family to induce monocyte arrest, GRO chemokines were not able to mediate isolated human monocyte chemotaxis (Figure 1D). The inability of GRO chemokines to mediate monocyte chemotaxis was further confirmed at multiple concentrations using Mono Mac 6 cells (Figure 1E). RANTES was used as a positive control for chemokine-mediated monocyte chemotaxis.

***Characterization of monocyte arrest induced by GRO-α in the 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 (Figure 2A), 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 (Figure 2C). On both P-selectin/VCAM-1 (Figure 2A) and P-selectin/VCAM-1/GRO-α (Figure 2C) 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 (B) was identical to that on the P-selectin/VCAM-1/GRO-α coated surface (D). However, of the approximately 80 cells traveling near the substrate, only ~12 monocytes arrested on P-selectin/VCAM-1 within one minute (B). This number significantly increased to ~36 in the presence of co-immobilized GRO-α (D), suggesting 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 calcium flux in isolated monocytes. In contrast to a large rise in intracellular free calcium in monocytes induced by regulated upon activation, normal T cell expressed and secreted (RANTES) (Figure 3A), GRO-α did not cause a change in intracellular free calcium (Figure 3A). Since P-selectin is required for effective GRO-α-mediated monocyte arrest, we also tested whether P-selectin could induce a rise in intracellular free calcium. Similar to GRO-α, the calcium flux induced

by P-selectin was not significant (Figure 3A). The addition of P-selectin with GRO-α also failed to induce intracellular free calcium mobilization as compared to the results seen with the addition of either GRO-α or P-selectin alone (Figure 3C). These experiments were repeated with Mono Mac 6 cells, and RANTES induced a large calcium response (B) while P-selectin, GRO-α, or P-selectin with GRO-α did not (D). Also, whole blood labeled with fluo-4-AM was tested for calcium response in monocytes using flow cytometry. Similar to the isolated monocytes, monocytes in whole blood did not show a calcium response to GRO-α (data not shown). This suggests that the ability of 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 calcium 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 (Figure 4A), As a control, it was shown that treatment of monocytes with CXCR2 antibody did not reduce arrest to P-selectin/VCAM-1/RANTES, verifying the specificity of the antibody (Figure 4A). 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 co-immobilized with P-selectin and VCAM-1 (Figure 4B), though 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 as compared to the control (Figure 4B). ENA-78, though, 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 binding CXCR2.

***Signal transduction pathways involved in GRO-α-mediated monocyte arrest.*** Signaling in monocyte arrest is poorly understood. In contrast, signal transduction in chemotaxis/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. Then, we 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, PI3K, and p-38 MAPK, but not calcium flux, actin polymerization or MEK signaling. A dramatic suppression of Mono Mac 6 cell chemotaxis to RANTES was found in experiments using Mono Mac 6 cells pretreated with pertussis toxin (PTx), the PI3K inhibitors wortmannin or LY294002, or the p-38 MAPK inhibitor SB203580 (Figure 5F, H, 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 PI3K inhibitors wortmannin or LY294002 (Figure $5A_1$, $C_1$), 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), suggesting the PI3K is involved in the signaling initiated once the Mono Mac

6 cells bind chemokine on the substrate. The blockade of calcium flux did not affect Mono Mac 6 cell arrest (Figure $5B_1$). Inhibition of MEK and p-38 MAPK, two kinases downstream of PI3K, did not have any influence in GRO-α-mediated Mono Mac 6 cell arrest (Figure $5D_1$). Actin polymerization, though 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 (Figure $5E_1$).

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 co-immobilized with P-selectin and VCAM-1. Those inhibitors that blocked GRO-α-mediated arrest also inhibited RANTES-mediated arrest (Figure 5). Inhibition of $G\alpha_i$, PI3K and actin polymerization blocked RANTES-mediated Mono Mac 6 cell arrest to P-selectin/VCAM-1/GRO-α (Figure $5A_2$,$C_2$, $E_2$), while inhibition of calcium mobilization, MEK or p38 MAPK did not block arrest (Figure $5B_2$,$D_2$). This data suggests 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 apoE$^{-/-}$ mice, while 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 human umbilical vein endothelial cells (HUVEC) under flow conditions, but, in that study, the chemokine was added to the monocyte reservoir prior to perfusion, not immobilized to the endothelial cells(9). GRO chemokines were previously implicated in monocyte arrest to MM-LDL-activated endothelial cells, but arrest was studied under static conditions(21). Under flow conditions, SDF-1α co-immobilized with VCAM-1 arrested monocytes, while MCP-1, RANTES, and GRO-α did not(4). Certainly, the method of chemokine presentation and the nature of the substrate could be the cause 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 to 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 calcium 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 as well as human monocytes. As members of the CXC family, it has long been demonstrated that GRO chemokines induce neutrophil chemotaxis(6). KC, a mouse homolog of GRO-α, is also able to trigger neutrophil chemotaxis/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. Since 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 versus arrest. It is known that PI3Kγ can be directly activated by the βγ subunits of G proteins *in vitro*(24). Neutrophils isolated from PI3Kγ$^{-/-}$ mice had reduced chemotactic activity towards IL-8, fMLP, and C5a, but chemokine-mediated neutrophil adhesion in a static system was not affected by the lack of PI3Kγ(12). In this same study, peritoneal macrophages from PI3Kγ deficient mice showed reduced chemotaxis towards SDF-1α, RANTES and macrophage derived chemokine (MDC)(12). Another study found that MCP-1-mediated monocyte adhesion signaling could involve the PI3Kα

isoform, and both PI3K inhibitors wortmannin and LY294002 reduced monocyte arrest to E-selectin-transduced HUVEC(8). In our experiments, wortmannin and LY294002 significantly reduced Mono Mac 6 chemotaxis to RANTES (Figure 5), as well as arrest on P-selectin/VCAM-1 in response to GRO-α and RANTES (Figure 5). BAPTA-AM, a chelator of intracellular free calcium, did not inhibit chemotaxis or arrest of Mono Mac 6 cells in our study. This was similar to previous data which showed that blood lymphocytes pretreated with BAPTA did not have reduced arrest to an SDF-1α/VCAM-1 substrate as compared to untreated cells(10). However, in a calcium flux assay, the concentration of BAPTA used in the arrest assay blocked a calcium response of Mono Mac 6 cells to RANTES (data not shown), confirming that this concentration did block calcium influx in the arrest study. This data suggests that an increase in intracellular free calcium secondary to activation of $G\alpha_i$ protein-coupled receptors is not necessary for either monocyte chemotaxis or arrest.

In a study using a static adhesion assay, Ashida et al. reported a significant contribution of MEK signaling in monocyte adhesion on a VCAM-1-coated surface(2). Using the same static adhesion assay, we confirmed that PD98059, 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: the state of integrin activation could vary between monocyte adhesion in a static system and monocyte arrest under shear flow. Notably, in the past 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, suggesting 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, suggesting 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 prototypic 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, one of the earliest steps in monocyte recruitment, thereby efficiently regulating inflammation.

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Reference List


1. **Arai H and Charo IF**. Differential regulation of G-protein-mediated signaling by chemokine receptors. *J Biol Chem* 271: 21814-21819, 1996.

2. **Ashida N, Arai H, Yamasaki M and Kita T**. Distinct signaling pathways for MCP-1-dependent integrin activation and chemotaxis. *J Biol Chem* 276: 16555-16560, 2001.

3. **Bozic CR, Kolakowski LF, Jr., Gerard NP, Garcia-Rodriguez C, Uexkull-Guldenband C, Conklyn MJ, Breslow R, Showell HJ and Gerard C**. Expression and biologic characterization of the murine chemokine KC. *J Immunol* 154: 6048-6057, 1995.

4. **Chan JR, Hyduk SJ and Cybulsky MI**. Chemoattractants induce a rapid and transient upregulation of monocyte alpha4 integrin affinity for vascular cell adhesion molecule 1 which mediates arrest: an early step in the process of emigration. *J Exp Med* 193: 1149-1158, 2001.

5. **Cross AK, Richardson V, Ali SA, Palmer I, Taub DD and Rees RC**. Migration responses of human monocytic cell lines to alpha- and beta-chemokines. *Cytokine* 9: 521-528, 1997.

6. **Geiser T, Dewald B, Ehrengruber MU, Clark-Lewis I and Baggiolini M**. The interleukin-8-related chemotactic cytokines GRO alpha, GRO beta, and GRO gamma activate human neutrophil and basophil leukocytes. *J Biol Chem* 268: 15419-15424, 1993.

7. **Geissmann F, Jung S and Littman DR**. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19: 71-82, 2003.

8. **Gerszten RE, Friedrich EB, Matsui T, Hung RR, Li L, Force T and Rosenzweig A**. Role of phosphoinositide 3-kinase in monocyte recruitment under flow conditions. *J Biol Chem* 276: 26846-26851, 2001.

9. **Gerszten RE, Garcia-Zepeda EA, Lim YC, Yoshida M, Ding HA, Gimbrone MA, Jr., Luster AD, Luscinskas FW and Rosenzweig A**. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature* 398: 718-723, 1999.

10. **Grabovsky V, Feigelson S, Chen C, Bleijs DA, Peled A, Cinamon G, Baleux F, Arenzana-Seisdedos F, Lapidot T, van Kooyk Y, Lobb RR and Alon R**. Subsecond induction of alpha4 integrin clustering by immobilized chemokines stimulates leukocyte tethering and rolling on endothelial vascular cell adhesion molecule 1 under flow conditions. *J Exp Med* 192: 495-506, 2000.

11. **Haribabu B, Zhelev DV, Pridgen BC, Richardson RM, Ali H and Snyderman R**. Chemoattractant receptors activate distinct pathways for chemotaxis and secretion. Role of G-protein usage. *J Biol Chem* 274: 37087-37092, 1999.

12. **Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, Sozzani S, Mantovani A, Altruda F and Wymann MP**. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science* 287: 1049-1053, 2000.

13. **Huo Y, Hafezi-Moghadam A and Ley K**. Role of vascular cell adhesion molecule-1 and fibronectin connecting segment-1 in monocyte rolling and adhesion on early atherosclerotic lesions. *Circ Res* 87: 153-159, 2000.

14. **Huo Y and Ley K**. Adhesion molecules and atherogenesis. *Acta Physiol Scand* 173: 35-43, 2001.

15. **Huo Y, Weber C, Forlow SB, Sperandio M, Thatte J, Mack M, Jung S, Littman DR and Ley K**. The chemokine KC, but not monocyte chemoattractant protein-1, triggers monocyte arrest on early atherosclerotic endothelium. *J Clin Invest* 108: 1307-1314, 2001.

16. **Johnston B and Butcher EC**. Chemokines in rapid leukocyte adhesion triggering and migration. *Semin Immunol* 14: 83-92, 2002.

17. **Kucik DF, Dustin ML, Miller JM and Brown EJ**. Adhesion-activating phorbol ester increases the mobility of leukocyte integrin LFA-1 in cultured lymphocytes. *J Clin Invest* 97: 2139-2144, 1996.

18. **Laudanna C, Kim JY, Constantin G and Butcher E**. Rapid leukocyte integrin activation by chemokines. *Immunol Rev* 186: 37-46, 2002.

19. **Lira SA, Zalamea P, Heinrich JN, Fuentes ME, Carrasco D, Lewin AC, Barton DS, Durham S and Bravo R**. Expression of the chemokine N51/KC in the thymus and epidermis of transgenic mice results in marked infiltration of a single class of inflammatory cells. *J Exp Med* 180: 2039-2048, 1994.

20. **Olson TS and Ley K**. Chemokines and chemokine receptors in leukocyte trafficking. *Am J Physiol Regul Integr Comp Physiol* 283: R7-28, 2002.

21. **Schwartz D, Andalibi A, Chaverri-Almada L, Berliner JA, Kirchgessner T, Fang ZT, Tekamp-Olson P, Lusis AJ, Gallegos C, Fogelman AM and .** Role of the GRO family of chemokines in monocyte adhesion to MM-LDL-stimulated endothelium. *J Clin Invest* 94: 1968-1973, 1994.

22. **Singer II, Scott S, Kawka DW, Chin J, Daugherty BL, DeMartino JA, DiSalvo J, Gould SL, Lineberger JE, Malkowitz L, Miller MD, Mitnaul L, Siciliano SJ, Staruch MJ, Williams HR, Zweerink HJ and Springer MS**.

CCR5, CXCR4, and CD4 are clustered and closely apposed on microvilli of human macrophages and T cells. *J Virol* 75: 3779-3790, 2001.

23. **Smith ML, Sperandio M, Galkina EV and Ley K**. Autoperfused mouse flow chamber reveals synergistic neutrophil accumulation through P-selectin and E-selectin. *J Leukoc Biol* 76: 985-993, 2004.

24. **Stephens LR, Jackson TR and Hawkins PT**. Agonist-stimulated synthesis of phosphatidylinositol(3,4,5)-trisphosphate: a new intracellular signalling system? *Biochim Biophys Acta* 1179: 27-75, 1993.

25. **van Kooyk Y and Figdor CG**. Avidity regulation of integrins: the driving force in leukocyte adhesion. *Curr Opin Cell Biol* 12: 542-547, 2000.

26. **Weber C, Schober A and Zernecke A**. Chemokines: key regulators of mononuclear cell recruitment in atherosclerotic vascular disease. *Arterioscler Thromb Vasc Biol* 24: 1997-2008, 2004.

27. **Weber KS, von Hundelshausen P, Clark-Lewis I, Weber PC and Weber C**. Differential immobilization and hierarchical involvement of chemokines in monocyte arrest and transmigration on inflamed endothelium in shear flow. *Eur J Immunol* 29: 700-712, 1999.

28. **Ziegler-Heitbrock HW, Thiel E, Futterer A, Herzog V, Wirtz A and Riethmuller G**. Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. *Int J Cancer* 41: 456-461, 1988.

**Figure Legends**

Figure 1. **Arrest and chemotaxis of Mono Mac 6 cells and human blood derived monocytes in response to GRO chemokines.** A, in the flow chamber at a shear stress of 1 dyne/cm$^2$ on P-selectin (10 μg/ml) alone, 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 negative control). B and C, co-immobilizing the chemokines GRO-α, β, γ or RANTES at 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) as compared to a control of P-selectin and VCAM-1 only. Data presented as the number of cells arrested in a 0.55 mm$^2$ field of view (FOV). D, RANTES (at 100 ng/ml) significantly induced migration of human blood-derived monocytes, while GRO family chemokines, also at 100 ng/ml, did not. E, RANTES, at 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 is shown as percent of total cells added to the top of the insert before incubation. *, $p<0.05$, compared to control.

Figure 2. **The profiles of monocyte rolling and arresting in the presence of GRO-α.** Cells were tracked for approximately 20 seconds, and then a sliding average of 10 was determined. Grey dotted lines show the instantaneous velocities of arrested monocytes

on either P-selectin (10 μg/ml)/VCAM-1 (150 ng/ml) (A) or P-selectin/VCAM-1/GRO-α (5 μg/ml) (C). Bold arrows indicate where monocytes arrested. Black dotted lines show the instantaneous velocities of human blood-derived monocytes 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) substrate (C). B and D show the fraction of arrested cells in the total number of cells near the substrate. Black bars indicate the number of cells that transiently tethered, rolled, and arrested to the substrate during the first minute of cell suspension perfusion through the flow chamber. The fraction of arrested cells on a substrate consisting of P-selectin and VCAM-1 (B) is much lower than that on a substrate consisting of P-selectin, VCAM-1, and GRO-α (D).

Figure 3. **Calcium flux of human blood-derived monocytes and Mono Mac 6 cells in the presence of varying activators.** RANTES (100 ng/ml)-induced intracellular calcium flux in monocytes (A) and Mono Mac 6 cells (B) was large compared to the response induced by P-selectin (1 μg/ml) or GRO-α (100 ng/ml). The addition of GRO-α (100 ng/ml) and P-selectin (1 μg/ml) together failed to induce a substantial calcium 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 seconds. These graphs are one of 2-3 independent experiments performed. Data is presented as the free intracellular calcium concentration (nM).

Figure 4. **Arrest of human blood-derived monocytes to varying substrates.** When monocytes are either treated with 20 μg/ml CXCR2 antibody for 20 minutes or desensitized with 100 ng/ml soluble GRO-α for 30 minutes, 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 as compared to untreated monocytes arresting to the same substrate (A). Monocytes treated with CXCR2 mAb still arrested to P-selectin/VCAM-1 and RANTES (A). IL-8 at 5 μg/ml, when co-immobilized with P-selectin and VCAM-1, was found to induce significant arrest of monocytes when compared to a P-selectin/VCAM-1 substrate (B), while co-immobilized ENA-78 at 5 μg/ml failed to induce arrest of the cells (B). Data is shown as the number of arrested cells per 0.55 $mm^2$ field of view. *, $p<0.05$ when compared to control. **, $p<0.05$ when compared to untreated Mono Mac 6 cells.

Figure 5. **Effects of inhibitors of signaling pathways on arrest (A-E) and migration (F-J) of Mono Mac 6 cells.** Pretreatment with pertussis toxin at 250 ng/ml for 3 hours ($A_1$,$A_2$), wortmannin at 100 ng/ml for 30 minutes ($C_1$,$C_2$), LY 294002 at 50 μmol/L for 30 minutes ($C_1$,$C_2$), or cytochalasin D at 1 μmol/L for 1 hour ($E_1$,$E_2$) all significantly reduced cell arrest to 5 μg/ml GRO-α or RANTES, co-immobilized with P-selectin and VCAM-1, when compared to those untreated, arrested Mono Mac 6 cells. Pretreatment with BAPTA at 10 μmol/L for 30 minutes ($B_1$,$B_2$), PD98059 at 100 μmol/L for 1 hour ($D_1$,$D_2$) or SB203580 at 50 μmol/L for 1 hour ($D_1$,$D_2$) did not inhibit cell arrest on GRO-α or RANTES co-immobilized with P-selectin and VCAM-1. Arrested cells were defined as cells that did not roll for at least 20 seconds. Data is 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. Pretreatment with pertussis toxin at 250 ng/ml for 3 hours (F), wortmannin at 100 ng/ml for 30 minutes(H), LY 294002 at 50 μM for 30 minutes(H) or SB203580 at 50 μM for 1 hour all significantly inhibited migration of Mono Mac 6 cells when compared to migration without the inhibitor. Pretreatment with BAPTA at 10

μM for 30 minutes (G), PD98059 at 100 μM for 1 hour (I) or cytochalasin D at 1 μM for 1 hour (J) did not significantly block migration of Mono Mac 6 cells. Data is presented as the percent of total cells added to the top of the insert. All controls were treated with 0.1% DMSO. *, $p<0.05$, when compared to the untreated cells.

**Figure 1**

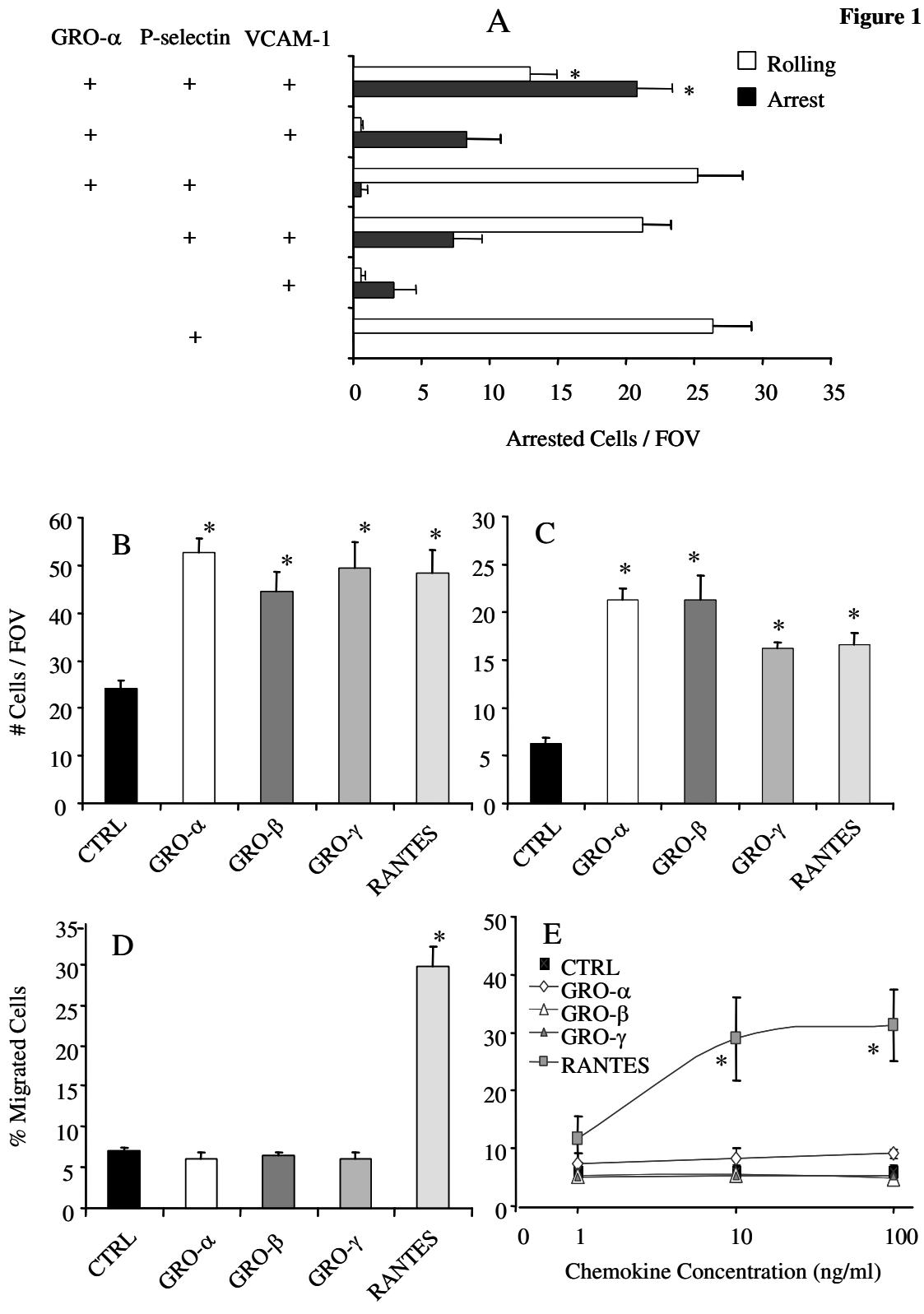

A
GRO-α
P-selectin
VCAM-1
Rolling
Arrest
Arrested Cells / FOV
B
# Cells / FOV
CTRL
GRO-α
GRO-β
GRO-γ
RANTES
C
D
% Migrated Cells
E
Chemokine Concentration (ng/ml)

**Figure 2**

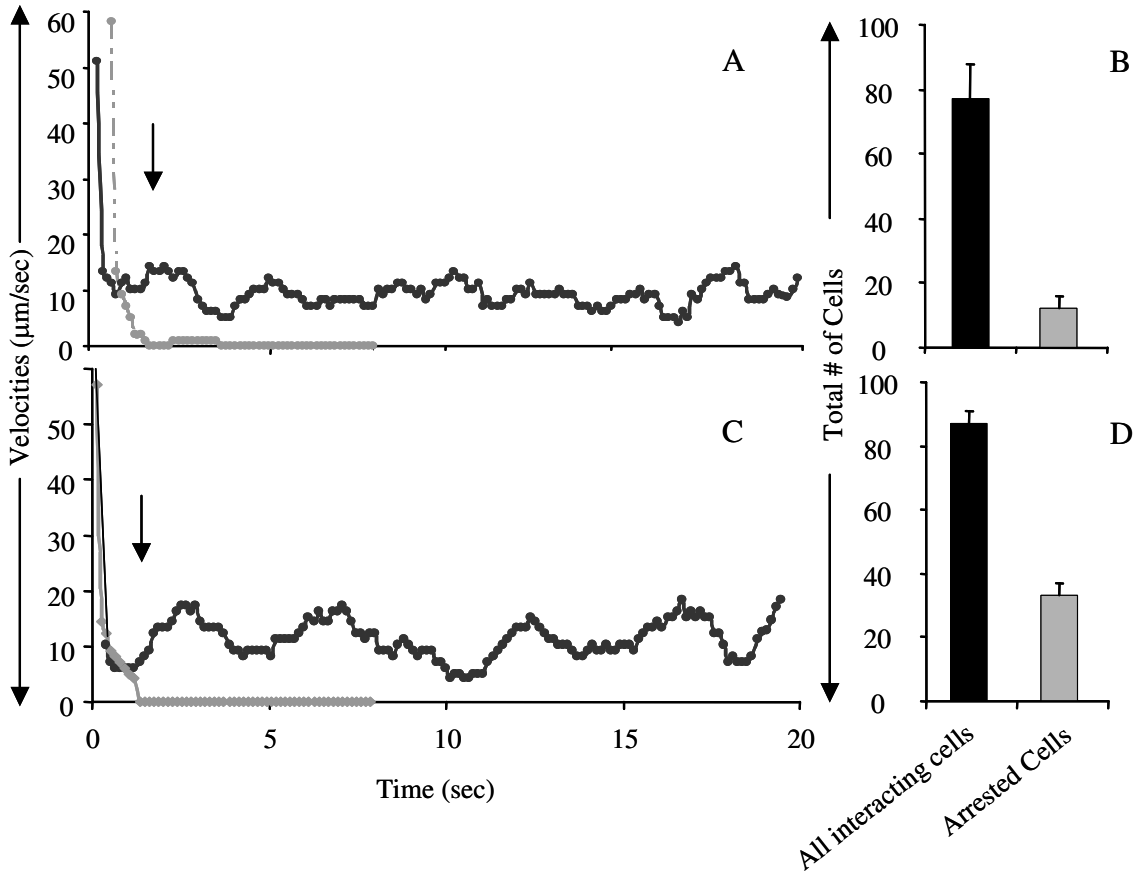

A
B
C
D
Velocities (µm/sec)
Total # of Cells
Time (sec)
All interacting cells
Arrested Cells

**Figure 3**

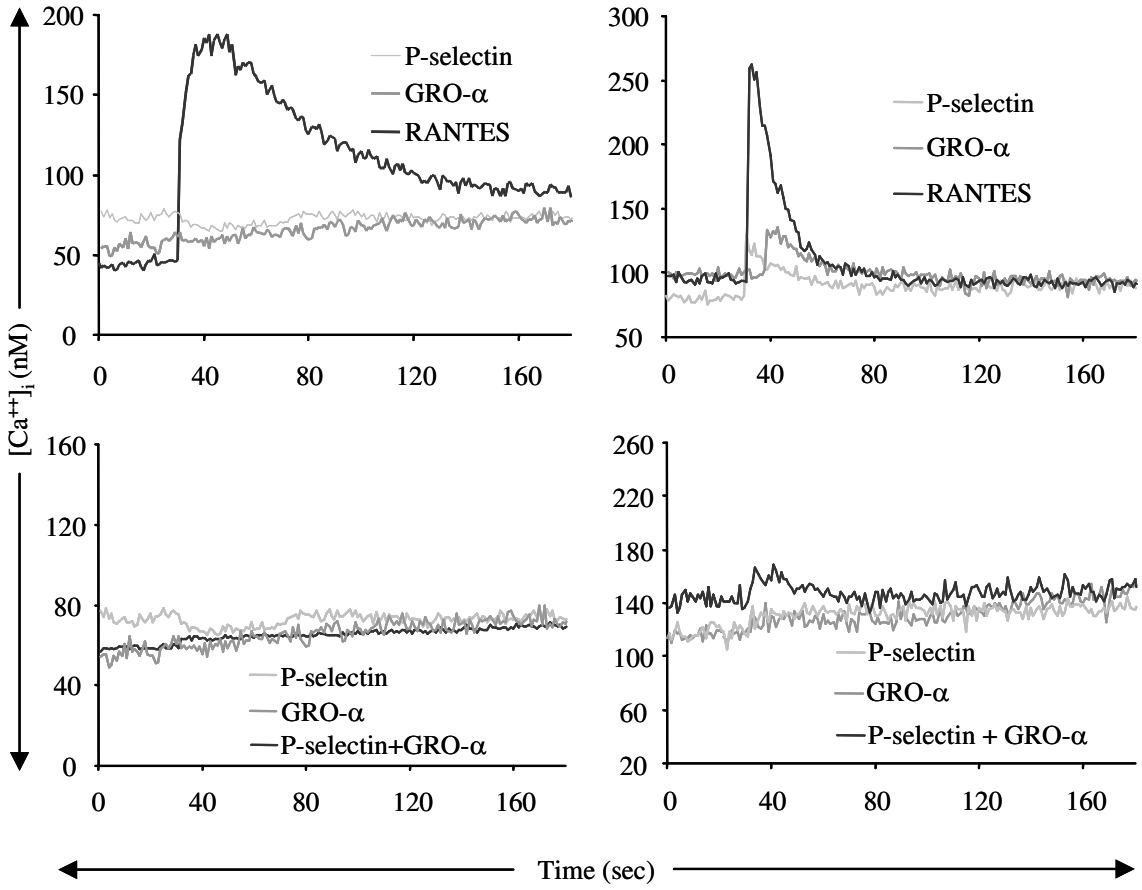

[Ca++]i (nM)
P-selectin
GRO-α
RANTES
P-selectin+GRO-α
P-selectin + GRO-α
Time (sec)

**Figure 4**

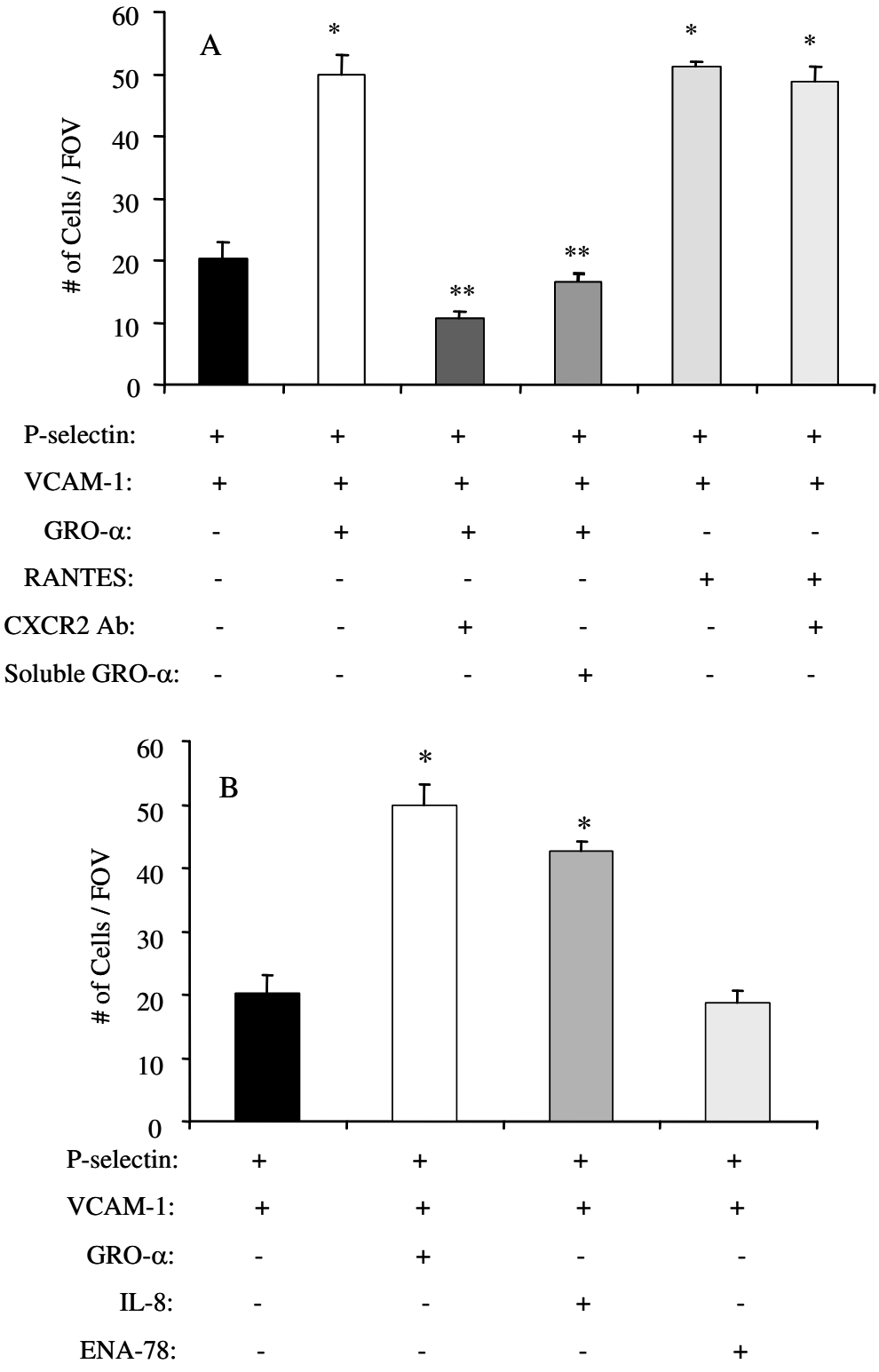
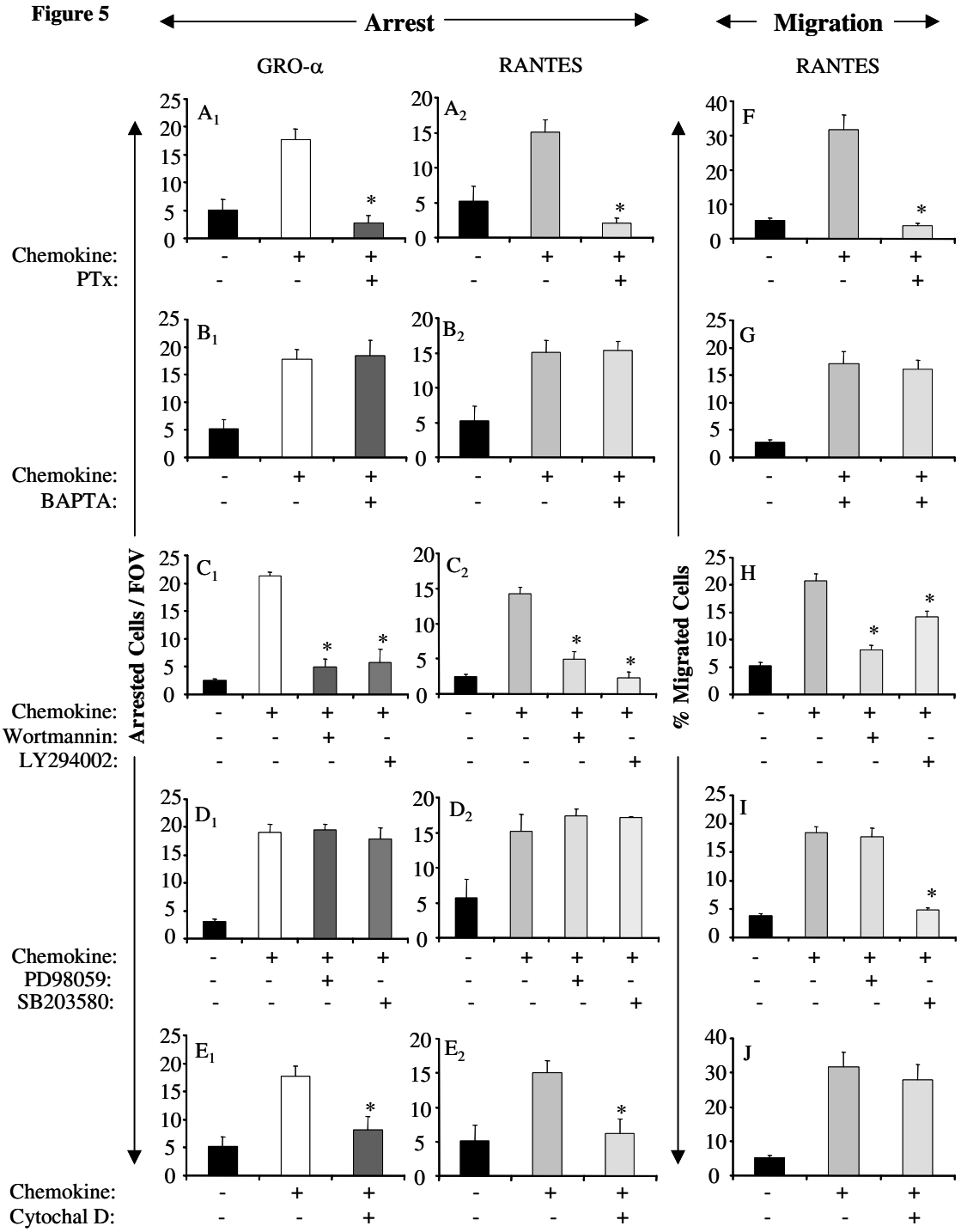

A
# of Cells / FOV
60
50
40
30
20
10
0
*
**
**
*
*
P-selectin: + + + + + +
VCAM-1: + + + + + +
GRO-α: - + + + - -
RANTES: - - - - + +
CXCR2 Ab: - - + - - +
Soluble GRO-α: - - - + - -
B
# of Cells / FOV
60
50
40
30
20
10
0
*
*
P-selectin: + + + +
VCAM-1: + + + +
GRO-α: - + - -
IL-8: - - + -
ENA-78: - - - +

**Figure 5**


Arrest
Migration
GRO-α
RANTES
RANTES
A1
A2
F
B1
B2
G
C1
C2
H
D1
D2
I
E1
E2
J
Arrested Cells / FOV
% Migrated Cells
Chemokine:
PTx:
BAPTA:
Wortmannin:
LY294002:
PD98059:
SB203580:
Cytochal D: