**Fundamentally different roles for LFA-1, Mac-1 and α4-integrin in neutrophil chemotaxis**

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**Summary**

Although the LFA-1, Mac-1 and α4 integrins are required for chemotaxis, it is unknown how they are regulated or what specific role they play. Previously we demonstrated that fMLP and IL-8 induce chemotaxis via the p38 MAPK and phosphoinositide 3-kinase (PI3K) pathways, respectively. Here we show that these chemoattractants also activate and use Mac-1 and LFA-1 in a differential manner during chemotaxis. Using integrin-specific substrata, we demonstrate that cell movement in response to IL-8 is mediated by Mac-1, whereas LFA-1 is required for directional migration. By contrast, chemotaxis to fMLP requires Mac-1 for cell movement, whereas LFA-1 and α4-integrin are required for directional migration. On serum protein, which contains ligands for LFA-1, Mac-1 and α4-integrin, chemotaxis to fMLP is dependent on Mac-1, whereas chemotaxis to IL-8 is dependent on LFA-1. These results suggest that Mac-1 is the dominant integrin involved in chemotaxis to fMLP, and LFA-1 is the dominant integrin involved in chemotaxis to IL-8. Consistent with these observations, higher quantities of high-affinity Mac-1 are found on cells chemotaxing to fMLP than on cells chemotaxing to IL-8. Moreover, a much larger quantity of clustered LFA-1 was found on cells migrating to IL-8 compared to cells moving towards fMLP. When cells are presented with competing gradients of fMLP and IL-8, they preferentially migrate towards fMLP and activate/utilize integrins in a manner identical to fMLP alone. Under the same conditions, p38 MAPK inhibition abolishes the preferential migration to fMLP; instead, the cells migrate preferentially towards IL-8. The activation and utilization of integrins under these conditions are consistent with patterns observed with IL-8 alone. Together, these data suggest that fMLP and IL-8 differentially activate integrins for use during chemotaxis, that p38 MAPK is a major mediator in the activation and utilization of integrins, and selective integrin activation occurs during chemotaxis between opposing gradients.

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**Introduction**

Neutrophil chemotaxis is a complex process involving close coordination of signaling pathways, the actin cytoskeleton, and effector adhesion molecules such as integrins. Two integrins have been shown to be central to neutrophil chemotaxis: LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) (Lindbom and Werr, 2002). The importance of these integrins for neutrophil chemotaxis was first identified in leukocyte adhesion deficiency type I (LAD I) patients. These patients have a genetic defect in CD18, the common subunit for both LFA-1 and Mac-1. Neutrophils from these patients cannot be recruited from the vasculature and are incapable of chemotaxis (Marshall and Haskard, 2002). The exact role of the LFA-1 and Mac-1 integrins is unclear, and at least one study suggests that these two integrins work in opposition. Ding et al. demonstrated that deletion of Mac-1 in mice increases leukocyte adhesion (Ding et al., 1999). A further complication arises when the α4-integrin is considered because this integrin can also mediate some aspects of neutrophil chemotaxis (Burns et al., 2001; Tasaka et al., 2002). Little is known about the regulation of LFA-1, Mac-1 and α4-integrin during chemotaxis, or if these integrins are regulated differentially by different chemoattractants. Finally, how these molecules respond in the presence of competing chemoattractant gradients remains to be elucidated.

Integrins are regulated at several levels including localization (Sims and Dustin, 2002), changes in affinity (Shimaoka et al., 2001; Shimaoka et al., 2002), and changes in avidity (Hogg and Leitinger, 2001; van Kooyk et al., 2004a; Kim et al., 2004b; Salas et al., 2004). Specific antibodies to high affinity Mac-1 and clustered LFA-1 have been generated [CBRM1/5 (Oxvig et al., 1999) and M24, respectively (van Kooyk et al., 1999)], allowing quantification of integrin activity on the surface of a cell. To date, this
quantification of integrin activation has been limited to cells in suspension; as such, it is not understood how integrin activity is mediated on chemotaxing cells.

Many signaling pathways have been identified that can activate Mac-1, LFA-1 and α4-integrins, but little work has been done to determine how these pathways influence chemotaxis. Recently, we demonstrated that neutrophils rely on different signaling pathways to move to end-target chemoattractants [i.e. endogenous and exogenous mediators produced predominantly at the site of the infection, such as C5a and fMLP (N-formyl methionyl-leucyl-phenylalanine)], versus intermediary chemoattractants [i.e. endogenous mediators found in the vicinity of an infection, such as interleukin 8 (IL-8) and leukotriene B4 (LTB4)] (Heit et al., 2002). Chemotaxis to end-target chemoattractants was found to be dependent on p38 MAPK and Mac-1, whereas chemotaxis to intermediary chemoattractants was dependent on phosphoinositide 3-kinase (PI3K) and LFA-1. Together these data suggest that Mac-1 and LFA-1 function and/or activation may lie downstream of different signaling pathways. In addition, we demonstrated the existence of a chemoattractant hierarchy in which neutrophils preferentially migrate towards end-target chemoattractants, even when intermediary chemoattractants are present. This hierarchy results from p38 MAPK activation by end-target chemoattractant signaling, which blocks the signaling of intermediary chemoattractants by inhibiting PI3K. It is likely that these pathways play a major role in mediating integrin function, as they are the central players in mediating neutrophil chemotaxis (Aikawa et al., 2002; Capodici et al., 1998). In this report, we address the role of integrin activation during chemotaxis and the role of p38 MAPK in mediating the activity of Mac-1, LFA-1 and α4-integrin.

We systematically examined the role of LFA-1 and Mac-1 in neutrophil chemotaxis to single gradients of two prototype chemoattractants (fMLP and IL-8), as well as in competing gradients of these chemoattractants. We found that chemotaxis to fMLP required the cooperation of three integrins: Mac-1 providing 'traction' for the cell to move, while LFA-1 and α4-integrin provided directional signals to the cell. By contrast, migration to IL-8 was mediated by LFA-1. When neutrophils were presented with competing gradients of fMLP and IL-8 they preferentially migrated towards fMLP, and activated/utilized integrins in an fMLP-like manner. p38 MAPK inhibition reversed this hierarchy such that cells preferentially migrated towards IL-8. The reversal of this chemotactic hierarchy was associated with IL-8-like activation and utilization of integrins. Together our data suggest that different chemoattractants can selectively activate and utilize different integrins for chemotaxis. In addition, we demonstrate that p38 MAPK is the dominant signaling molecule involved in mediating how integrins are activated and utilized, and that outside-in signals through LFA-1 and α4-integrin are required for directional migration.

Materials and Methods
Reagents
The p38 MAPK inhibitor SB203580, anti-CD11a (clone m38), anti-CD11b (clone ICRF44), anti-β2, recombinant human ICAM-1, FBS, and the chemoattractants C5a and LTB4 were purchased from Calbiochem (San Diego, CA). CBMR1/5 antibody was purchased from e-Biosciences (San Diego, CA). M24 was a gift from Mike Gold (University of British Columbia, Vancouver, Canada). Ficoll-Histopaque 1077, human fibrinogen, mouse IgG, Triton X-100, BSA, HBSS, glycerol, paraformaldehyde, fMLP and IL-8 were purchased from Sigma-Aldrich (St Louis, MO). RPMI-1640 culture media, Zenon antibody labeling kits, DAPI and ultra pure agarose were purchased from Invitrogen (Carlsbad, CA). Dextran (M, 250,000) was purchased from Spectrum (Gardena, CA). 35×10 mm tissue culture dishes, 3 µm 24-well transwell inserts, 24-well tissue culture plates, #1.5 50×24mm glass coverslips, and FITC-conjugated goat anti–mouse IgG were purchased from Becton Dickinson (Franklin Lakes, NJ). Softworx software and the DeltaVision microscopy were purchased from Applied Precision (Issaquah, WA). ImageJ and the multitracker plugin (written by Jeffrey Kuhn, University of Texas) were provided by the National Institutes of Health (http://rsb.info.nih.gov/ij/). Openlab software was purchased from Improvision (Boston, MA). USAF 2′×2′ POS calibration slide was purchased from Edmund Industrial Optics (Barrington, NJ). Prism statistical software was purchased from Graphpad (San Diego, CA).

Neutrophil isolation and preparation
Blood was collected from healthy human donors. Erythrocytes were removed using dextran sedimentation (6% dextran in PBS) followed by two rounds of hypotonic lysis using ddH2O. Neutrophils were isolated from the resulting cell suspension using Ficol-Histopaque density centrifugation. The entire isolation was carried out at 4°C. Purified neutrophils were suspended in HBSS at a concentration of 1.0×10⁶ cells/ml and were kept on ice until needed. Neutrophils were >95% viable and >97.5% pure as determined by FACS. A single donor was used for one experiment; donors were picked randomly but had to be healthy, non-smokers and not taking any medications.

Under agarose assay procedure
The under agarose assay was used as described previously (Heit et al., 2002; Heit and Kubes, 2003). Briefly, Falcon 35×10 mm culture dishes were filled with 3 ml of a 1.2% agarose solution containing 50% HBSS and 50% RPMI-1640 culture medium with 20% heat-inactivated FCS. After the agarose solidified, three wells, 3.5 mm in diameter and 2.4 mm apart (edge-to-edge), were cut along a straight line in the gel. The gels were allowed to equilibrate for 1 hour in a 37°C/5% CO2 incubator, and were then loaded (see below). Once loaded, the gels were incubated for 2 hours in a 37°C/5% CO2 incubator, which allowed sufficient time for neutrophils to migrate the entire distance between the wells. Results were recorded using a video camera attached to a Zeiss Axiobserver 135 microscope. For experiments examining migration on specific substrata, the tissue culture dishes were coated with 500 µl of 10% serum, 25 µg/ml fibronectin, 2.5 mg/ml fibrinogen 4 µg/ml VCAM-1 or 0.5 µg/ml ICAM-1. The solution was spread evenly on the bottom of the 35 mm tissue culture dishes, and incubated for 1 hour at room temperature. Excess protein was then removed with a pipette, and the plate washed with PBS. Gels were cast as per usual, except the gel was cooled to 43°C before casting to prevent protein denaturation, and serum-free media was used to prevent serum from being incorporated into the substratum.

For most experiments, the central well of the gels was loaded with an optimal dose of chemoattractant, and neutrophils were placed in the outer wells. Optimal concentrations of each chemoattractant were determined previously (Heit et al., 2002) and used for all experiments: 10.0 pmol IL-8, LTB4 and C5a, and 1.0 pmol fMLP. For the hierarchy experiments, neutrophils were placed in the central well, fMLP was placed in the right well, and IL-8 was placed in the left well. For chemokinesis experiments 5 nM IL-8 or fMLP was directly incorporated into the gel, and the central well was omitted from the experimental design. To inhibit p38 MAPK, neutrophils were pretreated with 10 µM SB203580 for 30 minutes. This concentration of inhibitor has previously been shown to be the optimal inhibitory
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concentration without non-specific effects (Zu et al., 1998). In all experiments, 10 μl of a 1.0×10^5 cells/ml neutrophil suspension was loaded into each cell-containing well. To block the adhesion of specific integrins, neutrophils were treated with 10 μg/ml anti-LFA-1, 20 μg/ml anti-Mac-1, or 10 μg/ml anti-α,β-integrin.

Cell tracking experiments

The under agarose assay was used to track neutrophil migration. The assay was prepared as described above, and the gels incubated for 1.5 hours to allow the neutrophils to migrate into the target region (Fig. 1A). At this point the gel was transferred to an Olympus IX70 inverted microscope equipped with a heated enclosure. The enclosure was maintained at 37°C, and the gel perfused with humidified air plus 5% CO2. The migration of the cells was recorded at 10×/0.30 NA magnification, using Openlab software, with images taken every 20 seconds over a period of 20 minutes (Fig. 1B, supplementary material Movie 1). Cells would move approximately a half cell length between frames, thus allowing for accurate tracking of the cells. Analysis was carried out using ImageJ version 1.33, with the 2001/7/14 version of the multi-tracker plugin. First, the background was removed using the ImageJ standard background correction (rolling-ball value of 10). Next, the image was binarized using the ImageJ threshold feature, and noise removed using the despeckle command. The neutrophil trajectories were then determined using the multi-tracker plugin, with a minimal tracking size of 50 pixels (approximately ¼ of a cell diameter) to prevent the tracking of artefacts. This procedure generated x and y coordinates for the center of each cell at each time point. These data were exported to Excel for further analysis. An image of a USAF calibration slide was taken after each experiment in order to convert pixels to μm.

In this article we present two values, chemotactic index and speed (Fig. 1B). To quantify the directionality of migration the chemotactic index (C.I.) was calculated. This was done by dividing the displacement along the x-axis (i.e. the distance the cell moved towards the chemoattractant) (Δx) by the total distance the cell moved (Σdist). Using this measure, randomly moving cells have a C.I. of ~0.20 to 0.20, whereas directionally moving cells have a C.I. of 0.20 to 1.0 (Fig. 2). The speed of cell migration was also calculated by dividing Σdist by the time of the experiment in minutes.

Formulas

\[
\Sigma_{\text{dist}} = \Delta_{\text{dist}(n=0)} + \Delta_{\text{dist}(n=1)} + \ldots + \Delta_{\text{dist}(n=t)}
\]

C.I. = \frac{\Delta_x}{\Sigma_{\text{dist}}}

V_{\text{net}} = \frac{\Sigma_{\text{dist}}}{\text{time}}

Transwell migration

Transwell inserts were coated with either 10% serum diluted in PBS, or with 25 μg/ml fibronectin for 4 hours. Inserts were then rinsed in PBS and loaded into clean 24-well tissue culture plates. Five nM FMLP or 5 nM IL-8 was loaded into the lower chamber, and 200 μl of a 1×10^6 cells/ml of neutrophils loaded into the top chamber. To ensure that we were observing directional migration, rather than random migration, duplicate wells were set up, but 5 nM FMLP or IL-8 was also added to the upper chamber. This generated a uniform concentration of chemoattractant, which allowed random migration to be assayed. After the incubation was complete, the upper well was rinsed well with PBS and gently scraped with a cell scraper to remove any non-migrated neutrophils. The membrane was then removed from the insert, fixed in methanol and stained with a Wright-Giemsa stain using the manufacturer’s directions.

The stained membranes were then mounted on slides and three randomly selected regions of each membrane visualized on an Olympus IX70 inverted microscope at 40× magnification, using Openlab software. The number of completely transmigrated cells was counted. Note that all migrating neutrophils remained adherent to the bottom of the membrane, and no cells were observed in the fluid of the lower well. To measure the directionality of migration, the number of cells crossing the membranes in response to a chemotactic gradient was divided by the number of cells crossing the membrane in the uniform concentration of chemoattractant. This generated a chemotactic ratio, where ratios >1 indicate chemotaxis, and ratios <1 indicate random migration.

Fluorescent microscopy

These experiments were conducted as previously described (Heit and Kubes, 2003). Briefly, the under agarose assay
was performed as described above, except the gels were cast on 10% FCS (10% FCS in PBS) coated #1.5 glass coverslips. After a 2 hour migration period the gel was carefully removed, and the coverslips fixed in 2% ice-cold paraformaldehyde for 15 minutes. Antibodies were pre-labeled with fluorescent labels using a Zenon labeling kit according to the manufacturer’s instructions. Anti-Mac-1, anti-LFA-1 and an isotype antibody were labeled using Alexa-488, Alexa-555 and Alexa-647 Zenon dyes. The fluorescent label used to label each antibody was changed for every experiment to ensure that any observed changes were not due to the dye characteristics, but rather due to changes in integrin concentration. After labeling, antibodies were pooled and diluted to 1.0 μg/ml in PBS + 0.5% BSA, and added to the coverslips for 1 hour. 300 nM DAPI was added for the last 10 minutes of the antibody incubation to stain the nuclei of the cells. The coverslips were then washed twice in PBS and mounted in 100% glycerol.

Fluorescently labeled neutrophils were then imaged using an Olympus IX70 microscope equipped with a digital deconvolution apparatus (DeltaVision, API, Issaquah, WA). Cells were imaged in both fluorescence and differential interference contrast (DIC) using a 100×/1.35 NA objective. Cells were selected randomly for imaging, but had to meet three criteria: (1) the cells had to be located in between the neutrophil containing well and the chemoattractant containing well, (2) the cell must show no signs of visible damage via DIC imaging, and (3) the nucleus of the cell must be intact and multi-lobed, as determined by DAPI staining. Cell morphology and the intensity of integrin staining were not used as criteria for selecting cells. Z-sections were recorded at successive 0.2 μm intervals through the entire thickness of the cell. The same excitation times and intensities were used during a single experiment used to allow for semi-quantitative comparisons between samples. Activation was always measured after 2 hours of migration.

**Image analysis**

A full-iterative deconvolution based on an experimental point-spread function (Softworx software) was used to reassign out-of-focus photons within the z-stacks. Photobleaching was less than 2% over the duration of the experiment, and so no bleaching corrections were applied. To limit analysis to one cell, the outer edge of the cell was identified using a DIC image and the corresponding fluorescent signal from one image plane quantified by summing the total fluorescence within the cell body. To ensure that only those integrins in direct contact with the substratum (i.e. coverslip) were quantified, only the last in-focus z-section was used for analysis. All images shown in this paper are a single z-slice corresponding to the region of the cell in contact with the coverslip. To account for non-specific binding, any sample in which the isotype staining of the cell was greater than the isotype staining on the coverslip was eliminated from analysis. Total fluorescent intensity was measured by summing the fluorescence intensities across the cell. Relative comparisons of fluorescence levels were only carried out on samples on the same slide or between slides from the same experiment. For comparisons between experiments, and for statistics, the fluorescent intensities were standardized to Mac-1 fluorescence on an uninhibited cell.

**Statistics**

All data are expressed as the arithmetic mean±s.e.m. Data were compared using a Student’s t-test unless otherwise noted. Values of $P<0.05$ were considered to be statistically significant. All analysis was carried out using Prism Statistical software.

**Results**

**Characterization of random migration**

Although the under agarose assay is a robust assay that maintains long-lasting physiological gradients (Campbell et al., 1997), and can be adapted to allow for cell tracking over long periods of time (Heit and Kubes, 2003), it has one drawback: all cells start at the same location (i.e. the cell-containing well).

**Fig. 2.** Random migration (chemokinesis) of neutrophils in uniform concentrations of fMLP and IL-8. Cells are migrating on substrata consisting of serum protein (serum), fibrinogen (Fbg), fibrinogen and ICAM-1 (Fbg+ICAM), or fibronectin (Fn). (A) Chemotactic index of cells migrating in uniform concentrations of fMLP and IL-8. (B) Speed of migration in uniform concentrations of fMLP and IL-8. (C) Tracks plotting the migration of ten representative cells in uniform concentrations of fMLP (left) and IL-8 (right). Scale is in μm. Results are shown as mean±s.e.m., n=5. No statistical differences were observed (Student’s t-test).
As such, the assay has a potential mass-action effect; that is, since all the cells start at one location, random migration (chemokinesis) can yield a chemotactic index of greater than zero. In this case, chemokinesis could be mistaken for chemotaxis. Chemotactic index is a measure of the distance a cell moves towards a chemoattractant compared with the total distance the cell moves which, in essence, is a measure of the directionality of migration. To account for mass action we set up uniform concentrations of chemoattractants in the gel, and then measured the chemotactic index (C.I.) of the migrating cells to determine what range of C.I. accounts for random migration in the under agarose assay. As there is no overall gradient of chemoattractant, all cells moving in these conditions are migrating randomly. Under these conditions cells migrating

![Diagram](image)

**Fig. 3.** Chemotaxis of neutrophils to fMLP, IL-8, and in the hierarchy on substrata of serum protein (serum) or fibrinogen (Fbgn). (A) Effect of different substrata on the number of cells migrating towards (white bars) and away (black bars) from fMLP and IL-8. (B) Chemotactic index (C.I.) of cells migrating to fMLP and IL-8. (C) Speed of migration to fMLP and IL-8. (D) Tracks plotting the migration of ten representative cells to fMLP on serum and fibrinogen. (E) Tracks plotting the migration of ten representative cells to IL-8 on serum and fibrinogen. Scale is in μm. (F) Patterns of cell migration in competing gradients of fMLP and IL-8. The number of cells migrating to fMLP and IL-8 (x-axis), as well as the number of cells migrating perpendicular to the chemoattractants (y-axis) are shown. Results are shown as means ± s.e.m., n=6 for panels A,D,E,F; n=4 for panels B,C. *P<0.05 compared with migration on serum protein.
in uniform concentrations of fMLP or IL-8 migrate with a chemotactic index of 0.12 to 0.20 (Fig. 2A). Based on this data, we have defined chemotaxis in this assay as cells migrating with a chemotactic index of >0.20, and chemokinesis (random migration) as a chemotactic index of <0.20.

Although cells did move faster in response to uniform gradients of IL-8 than in response to uniform gradients of fMLP (Fig. 2B), there was no significant difference in the chemotactic index or the speed of migration of cells migrating on different substrata to the same chemoattractant (Fig. 2A,B).
Interestingly, most cells in these uniform concentrations of chemoattractants moved very little, but 10-15% of cells moved rapidly, in a non-directional fashion (Fig. 2C).

Cells migrate randomly on fibrinogen
Cells migrated directionally towards both fMLP and IL-8 on serum protein, which contains ligands for LFA-1, Mac-1 and \( \alpha_4 \)-integrins (Foxman et al., 1997; Heit et al., 2002). On fibrinogen, a Mac-1-specific ligand (Flick et al., 2004; Lishko et al., 2004), cells migrated randomly in response to fMLP or IL-8 (Fig. 3A), with nearly 40% of cells migrating away from the chemoattractant. This trend was reinforced when the chemotactic index (C.I.) of individual cells was measured. Neutrophils moved directionally on serum protein (C.I. of 0.4 to 0.5). When migrating on a fibrinogen substratum the

![Fig. 5. Chemotaxis of neutrophils to fMLP and IL-8 on VCAM-1. (A) Effect of different substrata on the number of cells migrating towards (white bars) and away (black bars) from fMLP and IL-8. (B) Chemotactic index (C.I.) of cells migrating to fMLP and IL-8. (C) Speed of migration to fMLP and IL-8. (D) Paths of ten representative cells migrating to fMLP (left) and IL-8 (right) on VCAM-1. Scale is in \( \mu \text{m} \). Results are shown as mean±s.e.m., \( n=5 \) for panels A,D; \( n=3 \) for panels B,C. *\( P<0.05 \) compared to migration on serum protein.](image-url)
chemotactic index dropped by 60%, to the levels seen during chemokinesis (Fig. 2A), demonstrating that fibrinogen lacks the ligands required for directional migration (Fig. 3B). Interestingly, this drop in chemotactic index had no discernible impact on the speed at which cells migrate (Fig. 3C), indicating a loss in directional movement, rather than a loss in the cell’s
motility. This loss of motility is clearly illustrated by examining the tracks of the individual cells (Fig. 3D,E), where the cells move directionally towards the chemoattractant on serum protein (left panels), but move randomly on fibrinogen (right panels).

We and others previously described a chemotactic hierarchy (Campbell et al., 1997; Heit et al., 2002), in which neutrophils are placed between competing gradients of an intermediary (IL-8) and end-target chemoattractant (fMLP). Under these conditions, on serum protein, neutrophils preferentially moved towards the end-target chemoattractant (Fig. 3F, top panel). However, when migrating on fibrinogen (i.e. in the absence of LFA-1 and α4-integrin ligands) this preferential migration to fMLP was lost and the cells moved in all directions (Fig. 3F, bottom panel), which demonstrates that the lack of appropriate integrin ligands can have a significant impact on intracellular signaling cascades.

Addition of an LFA-1 ligand supports directional migration

The addition of ICAM-1, a ligand for LFA-1 and Mac-1 (Diamond et al., 1990; Stautton et al., 1990), to the fibrinogen substratum is sufficient to restore directional migration to both fMLP and IL-8 (Fig. 4A). The addition of ICAM-1 restored the chemotactic index (C.I.) to levels seen on serum protein (Fig. 4B,D), although the speed at which the neutrophils migrated was decreased (Fig. 4C). This suggested that either the composition of the substratum and/or the density of ligands can affect the motility of a cell. Finally, the introduction of ICAM-1 also caused the neutrophils to regain their ability to preferentially migrate to fMLP in our opposing gradient model (Fig. 4E). These results indicated that the addition of ICAM-1 is sufficient to restore both directional migration to chemoattractants, as well as hierarchal responses when competing gradients of chemoattractants are present.

α4-integrin ligands can replace LFA-1 ligands for migration to fMLP, but not IL-8

The presence of α4-integrin on neutrophils suggests that this integrin is involved in neutrophil chemotaxis. To test this hypothesis, we analyzed neutrophil migration on a substratum consisting of VCAM-1, a ligand for the α4-integrin (Elies et al., 1990). Neutrophils were incapable of significant migration on VCAM-1 (Fig. 5A). The small amount of migration that occurred was non-directional (Fig. 5B) and very slow (Fig. 5C). This is reflected in the tracks of these cells, where all but one or two cells moved less than one cell-length during the experiment (Fig. 5D).

Since VCAM-1 was unable to support any significant migration on its own, we coated plates with fibronectin, a ligand for both Mac-1 and the α4-integrin (Burns et al., 2001; Lishko et al., 2003). When placed on a fibronectin substratum, neutrophils migrated directionally towards fMLP, but moved randomly in response to IL-8 (Fig. 6A,D). Indeed, a low chemotactic index of only 0.15 was observed during chemotaxis to IL-8 on fibronectin (Fig. 6B). Fibronectin had no impact on the speed of migration in response to either chemoattractant (Fig. 6C).Although fibronectin supported chemotaxis to fMLP in the hierarchy system, there was a small degree of random migration towards IL-8 (Fig. 6E). However, the amount of random migration was less than half of that seen on fibronogen (Fig. 3F), which suggests that the α4-integrin can provide some of the hierarchal directionality normally provided by LFA-1.

The ability of neutrophils to chemotax on fibronectin is controversial. Some studies have observed chemotaxis on fibronectin (Loike et al., 2001), whereas other studies have only observed random migration on fibronectin (Harler et al., 1999). To determine if our observation was an assay-specific phenomenon, we tested migration to IL-8 on fibronectin using the transwell migration assay. In this assay, neutrophils migrate between two wells through a porous membrane. Since cells can only move in one direction in this assay (i.e. into the membrane), the assay must be set up to discriminate between directional and random migration. To do this, we tested migration across membranes where a chemotactic gradient existed across the membrane (i.e. chemoattractant only in the lower well), as well as across membranes where a uniform concentration of chemoattractant was present (i.e. chemoattractant in both wells). If the ratio between the numbers of cells migrating in the gradient vs the number moving in the uniform concentration (termed the chemotactic ratio) is ≈1.0, the cells are moving randomly. Chemotactic ratios of >1 suggest that the cells are undergoing chemotaxis. In this assay cells migrating to fMLP chemotaxed equally well on membranes coated in serum protein or fibronectin (Fig. 6F). However, cells chemotaxed strongly to IL-8 on serum protein, but chemotaxed weakly to IL-8 on fibronectin. Although the neutrophils did move directionally to IL-8 on fibronectin in this system (compared with cells moving in uniform gradients), the directionality of this migration was significantly lower then the directionality of migration on serum protein. This is in agreement with our observations in the under agarose assay (Fig. 6B), and suggests that the conflict in the literature over the ability of neutrophils to chemotax on fibronectin is due to different groups using different cut-off levels for discriminating between chemotaxis and chemokinesis.

LFA-1 and Mac-1 are the dominant integrins for IL-8- and fMLP-induced chemotaxis

The effects of blocking anti-LFA-1, anti-Mac-1 and anti-α4-integrin antibodies on chemotaxis in response to fMLP and IL-8 was tested (Fig. 7). Isotype antibodies had no effect on
chemotaxis to any of the chemoattractants, whereas antibodies against LFA-1 were able to block chemotaxis to IL-8, but not to fMLP (Fig. 7). By contrast, antibodies against Mac-1 were able to block chemotaxis to fMLP, but not IL-8 (Fig. 7). To ensure this observation held true for other chemoattractants, a second intermediary chemoattractant (LTB₄) and a second end-target chemoattractant (C5a) were tested. Indeed, identical responses were observed, with intermediary chemoattractants using LFA-1 for chemotaxis and end-target chemoattractants using Mac-1 for chemotaxis.

Antibodies against α₄β₁-integrin had no impact on migration to any of the four chemoattractants unless they were combined with antibodies against LFA-1. When anti-LFA-1 and anti-α₄β₁-integrin were used together, migration to both end-target chemoattractants was blocked (Fig. 7). Combining anti-LFA-1 and α₄β₁-integrin antibodies did not further affect migration to intermediary chemoattractants compared with anti-LFA-1 alone. These data suggest that α₄β₁-integrin and LFA-1 can function interchangeably for chemotaxis to end-target chemoattractants, but not for chemotaxis to intermediary chemoattractants.

α₄β₁-integrin can provide directional signals during chemotaxis to fMLP but not IL-8

fMLP migration on a Mac-1 ligand (fibrinogen) was random unless an LFA-1 or α₄β₁-integrin ligand was provided (Figs 4 and 6). Therefore we hypothesised that both LFA-1 and α₄β₁ ligands could provide directional signals for chemotaxis to fMLP on a multiprotein substratum. To test this hypothesis, we treated cells with blocking antibodies against both LFA-1 and α₄β₁-integrin, and then assayed their ability to migrate towards fMLP on serum protein. Simultaneous treatment of neutrophils with antibodies against LFA-1 and α₄β₁-integrin resulted in a significant number of cells migrating away from fMLP (Fig. 8A). This was clearly reflected by a dramatic drop in the C.I. (Fig. 8B), which indicates that directional migration was lost, and that the cells were moving randomly. There was no impact on the speed of migration (Fig. 8C). Individually, α₄β₁-integrin antibodies and LFA-1 antibodies only had a minor effect, demonstrating that both LFA-1 and α₄β₁-integrin can support directional migration to fMLP.

Mac-1 is preferentially activated by fMLP

Since integrins can exist in at least two states – an inactive (weak ligand binding) and active (strong ligand binding) state, we determined the activation patterns of LFA-1 and Mac-1 on chemotaxing neutrophils. This was done using activation-specific antibodies against LFA-1 (M24), which recognizes an affinity-independent LFA-1 activation epitope (van Kooyk et al., 1999), and Mac-1 (CBRM1/5), which recognizes an epitope that is exposed upon conformational change (Lu et al., 2001). We quantified only those integrins in contact with the substratum, thus limiting our analysis to those integrins that were in contact with their ligands and, as such, actively involved in moving the cells. Mac-1 activation was highest on cells migrating to fMLP, and was minimally activated on cells migrating to IL-8 (Fig. 9A, black bars).

Previously, we demonstrated that chemotaxis to fMLP could be inhibited by p38 MAPK inhibitors (Heit et al., 2002). In uninhibited cells fMLP preferentially activated Mac-1, whereas IL-8 induced a weak activation of Mac-1 (Fig. 9A, black bars). Using the p38 MAPK inhibitor SB203580, we determined that the activation of Mac-1 was p38 MAPK dependent (Fig. 9A, open bars). These changes in Mac-1 activation were not due to a change in integrin numbers, as measurements of total cell-surface Mac-1 with an activation-independent antibody showed no difference between fMLP and IL-8, or between uninhibited and p38 MAPK-inhibited cells (Fig. 9B). Most of the active Mac-1 is found in the uropod of
the cell, although small amounts can be seen on the leading edge (Fig. 9C). Not only did p38 MAPK inhibition block Mac-1 activation, but it also blocked chemotaxis to fMLP (Fig. 9D). Inhibition of p38 MAPK had no impact of IL-8-induced chemotaxis, consistent with minimal activation of p38 MAPK and Mac-1 with IL-8 (Fig. 9A,D).

LFA-1 is preferentially activated by IL-8
fMLP minimally activated LFA-1, whereas IL-8 potently activated LFA-1 (Fig. 10A). These changes in LFA-1 activation were independent of the number of integrins on the cell, as measured using an activation-independent antibody (Fig. 10B). Unlike active Mac-1 (Fig. 9C), active LFA-1 was found throughout the region of the cell in contact with the substratum, with clearly enhanced LFA-1 activation on cells migrating to IL-8 versus fMLP (Fig. 10C). Interestingly, the localization of Mac-1 and LFA-1 was similar on cells moving to IL-8 and fMLP. Mac-1 was located predominantly in the uropod of the migrating cells, whereas LFA-1 was found throughout the cell body. These observations are in direct agreement with studies by Seo et al., who demonstrated an identical pattern of Mac-1 and LFA-1 localization using ICAM-1- and albumin-coated beads (Seo et al., 2001). Interestingly, the uropod pool of active Mac-1 appears to be the major site that anchors cells migrating to fMLP (Hughes et al., 1992).

Integrin activation in the hierarchy is identical to integrin activation on cells moving to fMLP
When faced with opposing gradients of fMLP and IL-8, neutrophils always migrate towards fMLP, regardless of the concentrations of the two chemoattractants (Heit et al., 2002). This is due to a hierarchal response in which the fMLP signaling pathway inhibits the IL-8 signaling pathway. One would predict that this would result in an activation and utilization of integrins similar to fMLP alone. Indeed, neutrophils preferentially activated Mac-1 when placed in the hierarchy system (Fig. 11A, black bar), and minimally activated LFA-1 (Fig. 11B, black bar). When treated with a p38 MAPK inhibitor, Mac-1 activation was suppressed (Fig. 11A, open bar), whereas LFA-1 activation was enhanced (Fig. 11B, open bar). These changes in activation occurred independently of the total number of integrins on the cell surface (Fig. 11C). Chemotaxis in the hierarchy was independent of LFA-1 and dependent on Mac-1 (Fig. 11D). Overall, chemotaxis in the hierarchy was identical to chemotaxis to fMLP alone (Fig. 7), which suggests that there is a complete dominance of the fMLP/p38 MAPK signaling pathway over the IL-8/Pi3K signaling pathway.

As we described previously (Heit et al., 2002), p38 MAPK inhibition removes the inhibition of Pi3K by p38 MAPK, thus allowing cells to respond to IL-8 within the hierarchy system. p38 MAPK inhibition resulted in a reversal of the direction of cell migration, without inducing any significant random migration (Fig. 11E). When p38 MAPK was inhibited, there was a drop in the concentration of active Mac-1 on the cells and a large increase in the amount of active LFA-1 (Fig. 11A). This is identical to what we observed when cells migrating to fMLP were treated with p38 MAPK inhibitors (Fig. 9A). However, in this case we have an opposing gradient of IL-8 and, as such, the cells moved effectively to IL-8. In fact, nearly double the number of cells moved to IL-8 in this system then to IL-8 alone (>150 vs. 80, Fig. 9D and Fig. 11E). This migration to IL-8 could be blocked with antibodies against both Mac-1 and LFA-1, demonstrating that both integrins are required for chemotaxis in the p38 MAPK inhibited hierarchy.
(Fig. 11E, bottom panels). Together these data suggest that the high levels of active LFA-1 on these cells may enhance migration to intermediary chemoattractants such as IL-8.

Discussion

During an infection many chemoattractants are released from various locations including the vascular endothelium, interstitial cells (macrophages and mast cells) and the infectious agent itself (Murdoch and Finn, 2000). As a result, neutrophils must find their way through an environment of multiple, and often conflicting, chemotactic cues. In this study, we use the under agarose migration assay (Campbell et al., 1997; Heit and Kubes, 2003; Nelson et al., 1975) to study the integrins used by neutrophils to navigate through this complex environment. This system allows for the establishment of multiple chemotactic gradients, and is adaptable to both high-resolution fluorescent microscopy and cell tracking. By adapting this system for fluorescence microscopy and cell tracking we can measure the chemotaxis of neutrophils in complex chemotactic environments, quantify the localization and activation of integrins, as well as monitor individual neutrophils while they chemotax. This offers many advantages over traditional methods of measuring integrin activation, such as FACS, as it allows us to measure the activation of integrins on a relevant substratum, rather than in suspension. In addition, it allows us to quantify integrin localization and activation at the cell-substratum interface. We report profound differential responses for an end-target chemoattractant (fMLP) and an intermediary chemoattractant (IL-8) in the activation and utilization of individual integrin subunits and the directionality of movement.

Although the β2 integrins have been shown to be important for chemotaxis, it is unclear what specific roles LFA-1 and Mac-1 have in mediating chemotaxis. Some evidence has suggested that LFA-1 and Mac-1 play different roles during recruitment – indeed a study comparing CD11a (LFA-1)- and CD11b (Mac-1)-knockout mice showed that these two integrins play opposing roles in mediating neutrophil recruitment to TNF-α. Ding et al. demonstrated that the LFA-1-knockout neutrophils had decreased recruitment, whereas the Mac-1-knockout neutrophils had greatly enhanced recruitment, suggesting that Mac-1 may act as a ‘brake’ during this process (Ding et al., 1999). In contrast to the study described above, Mac-1 was found to be the predominant integrin involved in chemotaxis through synovial and dermal fibroblast barriers (Gao and Issekutz, 1996). Our data resolve the apparent conflict in these studies by demonstrating that the role of Mac-1 and LFA-1 in mediating chemotaxis is dependent on the nature of the chemoattractant involved, and on the substratum upon which the cell is moving.

Our data suggest that non-β2 integrins, notably α4-integrin, also play a role in chemotaxis. Unlike the β2 integrins, the α4-integrin is expressed at low levels on human neutrophils, although its expression is increased during chronic inflammatory diseases (Issekutz et al., 2003) and during acute systemic inflammation (Ibbotson et al., 2001). In this study we demonstrate that α4-integrin plays a central role in neutrophil chemotaxis, but only during chemotaxis to fMLP and in the fMLP/IL-8 hierarchy. Under these conditions, α4-integrin and
LFA-1 appear to function together in order to provide directional signals to the cells (Figs 5-8). This is in agreement with Henderson et al., who demonstrated that the $\alpha_4$-integrin and LFA-1 had overlapping roles during tethering and rolling in post-capillary venules (Henderson et al., 2001). Interestingly, $\alpha_4$-integrin appears to have no role in mediating chemotaxis to IL-8 or LTB4 (Fig. 7), which suggests that it is preferentially used for chemotaxis to end-target chemoattractants, but not to intermediary chemoattractants.

LFA-1 mediates chemotaxis through 'outside-in' signals generated when LFA-1 binds to its ligands (Menegazzi et al., 1999; Takagi et al., 2002). To determine whether LFA-1 signaling was playing a role in mediating chemotaxis we coated tissue culture dishes with fibrinogen – an extracellular matrix component that acts as a ligand for Mac-1, but not for LFA-1 or $\alpha_4$-integrin (Harler et al., 1999; Lishko et al., 2003; Miettinen et al., 1998; Wang et al., 2002). Interestingly, chemotaxis of neutrophils was random in response to fMLP and IL-8, which suggests that Mac-1 alone was not sufficient to establish directional migration (Fig. 3A,B). It is important to note that there was no significant difference in the speed of migration compared with migration on serum protein (which contain ligands for Mac-1, LFA-1 and $\alpha_4$-integrin), demonstrating that the defect in chemotaxis was due to a loss of directional migration rather then a loss in a cell’s mobility (Fig. 3C). When placed between competing gradients of fMLP and IL-8, cells lost their ability to preferentially migrate towards fMLP and began to move randomly on the fibrinogen substratum (Fig. 3D), demonstrating that substratal proteins may contribute to the intracellular signaling hierarchy. Indeed, addition of either an LFA-1 ligand (Fig. 4) or an $\alpha_4$-integrin ligand (Fig. 6) restored directional chemotaxis in the hierarchy.

Outside-in signaling through integrins has been shown to play an important role in neutrophil biology, including activation of the NF-\(\kappa\)B pathway (Kim et al., 2004a), cell spreading and oxidative burst (Menegazzi et al., 1999), transmigration (Cinamon et al., 2001), and activation of pathways that can modulate the cytoskeleton (Zheng et al., 1996). Several signaling pathways have been implicated, including the ILK/PI3K pathway (Attwell et al., 2000; Attwell et al., 2003; Dedhar et al., 1999), Rap1 (Shimonaka et al., 2003), RanBPM (Denti et al., 2004) and Vav-1 (Gakidis et al., 2004; Riteau et al., 2003). Clearly, outside-in signaling has the potential to affect chemotaxis, and our data suggest that this signaling is required for directional neutrophil chemotaxis. Specifically, the integrins that can provide these directional signals appear to differ depending on the chemoattractant towards which a cell is migrating.

Interestingly, Mac-1 was required for chemotaxis to end-target chemoattractants (fMLP and C5a), but not required for chemotaxis to intermediary chemoattractants (IL-8 and LTB4, Fig. 7). One explanation for this is that intermediary chemoattractants, but not end-target chemoattractants, can potently activate LFA-1 (Fig. 10). It is possible that in the presence of LFA-1 ligands, active LFA-1 is capable of providing both directional signals as well as traction for cell movement – in other words, LFA-1 may act as both the motor and steering wheel for a cell. By contrast, Mac-1 was unable to provide directional signals for chemotaxis to any of our tested chemoattractants (Fig. 3), which suggests that the sole role of Mac-1 is to act as a cell's motor. Together, these data suggest that the two $\beta_2$ integrin subunits are utilized differentially by different chemoattractants. Notably, Mac-1 acts as a motor for cell movement whereas LFA-1 can act to both steer and move a cell. The ability of LFA-1 to steer a cell, versus its ability to move a cell, may be dependant on its activation state, with increased LFA-1 activation resulting in LFA-1-dependent motility (Fig. 10A).

The lack of a role for LFA-1 in mediating chemotaxis to fMLP (Fig. 7) would appear to contradict our observations in Figs 3 and 4, where it is clear that LFA-1 signaling is required for chemotaxis to fMLP. However, our data shows that $\alpha_4$-integrin can also provide directional signals for chemotaxis to fMLP, and as such both LFA-1 and $\alpha_4$-integrin

![Fig. 10. LFA-1 activation on cells moving on serum towards single gradients of fMLP or IL-8. (A) Relative activation of LFA-1. Values are expressed as fold-activation, compared with fMLP. (B) Total LFA-1 on cells, as measured using an activation-independent antibody. (C) DIC and fluorescent microscopy showing the localization of active LFA-1 on cells moving to fMLP (top) or IL-8 (bottom). Integrin data represent only the integrins in contact with the substratum, and is expressed as fluorescent intensity relative to fMLP. Results are shown as mean±s.e.m., n=minimum of 5. *P<0.05 compared with fMLP.](image-url)
must be blocked to reduce chemotaxis to fMLP (Fig. 7). The preferential utilization of Mac-1 and LFA-1 for chemotaxis to fMLP and IL-8, respectively, is reflected in the activation of these integrins. Indeed, fMLP preferentially activates Mac-1 (Fig. 9A), whereas IL-8 preferentially activates LFA-1 (Fig. 10A).

This preferential utilization of Mac-1 and LFA-1 by end-target and intermediary chemoattractants may explain the apparent conflict in many chemotaxis studies. For example, Ding et al. found that LFA-1 was required for recruitment to TNF-α, a molecule that induces the expression of intermediary chemoattractants such as IL-8 and other chemokines (Ding et al., 1999; Hashimoto et al., 1999). By contrast, the Mac-1-dependent migration across the synovial and dermal fibroblast layers observed by Gao et al. (Gao and Issekutz, 1996) was driven by C5a, which we have identified as an end-target chemoattractant (Heit et al., 2002). This apparent conflict in the role of LFA-1 and Mac-1 could be explained by the preferential use of LFA-1 by intermediary chemoattractants (LTB4, IL-8) and the preferential use of Mac-1 by end-target chemoattractants (fMLP, C5a). Our results contribute to the resolution of these conflicting data, but they also reveal that outside-in signaling through the integrins plays a central role in determining both the magnitude and direction of chemotaxis (Figs 4 and 5) and, as such, the composition of the substratum may also play a significant role in the usage of Mac-1 and LFA-1 during chemotaxis.

The affinity of Mac-1 for its ligands is dynamically regulated through a conformational change (Lee et al., 1995; Oxvig et al., 1999). This conformational change is mediated by ‘inside-out’ signaling in which signals generated by another receptor, such as a chemoattractant receptor, induce a conformational change in Mac-1 (Takagi et al., 2002). This conformational change involves a separation of the cytoplasmic tails of the integrins α and β subunits (Kim et al., 2003), which then leads to an opening of the integrin that resembles the motion of a switchblade (Shimaoka et al., 2001; Shimaoka et al., 2002). These conformational changes produce a high-affinity form of Mac-1, which binds to its substrate with an affinity many times greater than the low affinity form of Mac-1. In this study we used the CBRM1/5 monoclonal antibody, which specifically binds to the high-affinity form of Mac-1 (Weber et al., 1996) to quantify the activation of Mac-1 in response to end-target and intermediary chemoattractants (Fig. 9A).

Mac-1 activation was greatest when cells were chemotaxing to fMLP, and much lower on cells migrating towards IL-8. Interestingly, the activation of Mac-1 could be inhibited with SB203580, demonstrating that Mac-1 activation lies directly downstream of p38 MAPK (Fig. 9A). These data correlate well with observations made in this study, where we found that chemotaxis to end-target chemoattractants, but not intermediary chemoattractants, was Mac-1 dependent (Fig. 7) and p38 MAPK dependent (Fig. 9D). These data provide strong evidence that the high affinity form of Mac-1 is required for chemotaxis to end-

Fig. 11. Integrin activation on cells migrating on serum in competing gradients of fMLP and IL-8. Cells are either uninhibited (UT) or treated with the p38 MAPK inhibitor SB203580. (A) Relative Mac-1 activation. Values are expressed as fold-activation, compared with UT. (B) Relative LFA-1 activation. Values are expressed as fold-activation, compared with UT. (C) Total Mac-1 and LFA-1 on cells, as measured using activation-independent antibodies. (D) Effect of blocking antibodies against Mac-1 and LFA-1 on chemotaxis in the hierarchy. (E) Effect of p38 MAPK inhibition on chemotaxis in the hierarchy, and the roles of LFA-1 and Mac-1 in mediating this chemotaxis. Integrin data represents only the integrins in contact with the substratum, and are expressed as fluorescent intensity relative to fMLP, UT. Results are shown as mean±s.e.m., n=minimum of 5. *P<0.05 compared to untreated.
Fig. 12. Proposed signaling model. Chemotaxis involves three signals that, together, direct the cell towards a site of infection. The first of these signals is generated by p38 MAPK, and allows a cell to ‘ignore’ intermediary chemoattractants (a). The second signal is an inside-out signal, generated by the chemoattractants, which results in the activation of the integrins (b). Lastly, a signal is generated by integrin-ligand interactions, and provides outside-in signals that are required to ‘steer’ the cell (c). Lines with arrow heads at the ends represent stimulatory pathways. Lines with bars at the end represent inhibitory pathways.

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