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Heterogeneity in the Locomotory Behavior of Human Monocyte Subsets over Human Vascular Endothelium In Vitro

Joanna L. Collison,* Leo M. Carlin,† Martin Eichmann,* Frederic Geissmann,*† and Mark Peakman*

Monocytes constitute ~10% of all leukocytes in human peripheral blood, and play an important role in linking innate and adaptive immune responses (1). In disease states, monocytes are quickly recruited to the site of infection or inflammation, where they can migrate into the surrounding tissue and differentiate into dendritic cells, macrophages (1), and osteoclasts (2). Their dual role as both effector cells and effector cell precursors makes understanding their basic biology important for studying many infectious and inflammatory diseases.

Recent advances have enabled the distinction of two major subsets of monocytes in mice termed “inflammatory” and “resident” (3), on the basis of their expression of the Ly6C Ag and their migratory behaviors (1). In the human, monocytes have been assigned to three main subsets based on their CD14 and CD16 expression: CD14+CD16−, CD14+CD16+, and CD14−CD16−, otherwise termed “classical,” “intermediate,” and “nonclassical” (4). The different roles seen in murine monocyte subsets are conserved well in humans, although there are some important differences (5). Murine inflammatory monocytes are CD115+Ly6C+ and express CCR2, CD62L, and low levels of CX3CR1 (1). They are major producers of proinflammatory cytokines (6) and are thought to preferentially differentiate into dendritic cells in an inflammatory environment (7). This subset is usually equated to both CD14+CD16− and CD14+CD16 human monocyte subsets (8), as together they share many of the characteristics of the murine subset. Similar to murine inflammatory monocytes, human CD14+CD16− monocytes also express CCR2 (1) and CD62L (9), although they produce IL-10 in response to challenge with LPS (10). Human CD14+CD16+ monocytes, however, appear to be efficient phagocytes, expressing high levels of the Fc receptors CD64 and CD32 and are major producers of TNF-α and IL-1β (11).

Human CD14+CD16+ monocytes are slightly smaller than other monocytes (9) and do not express Fc receptors, so are poorly phagocytic and do not produce cytokines in response to LPS (12). They equate to murine resident monocytes, which are CD115+Ly6C− and express high levels of Cx3CR1 (1). Murine resident monocytes have recently been shown to belong to a functionally separate lineage, under the control of the transcription factor NR4A1 (13), and they are thought to be part of the early immune response to tissue damage (1) and to preferentially differentiate into macrophages in an inflammatory setting (7). Both human CD14+CD16+ and murine Ly6C− monocytes respond to TLR7 agonists, suggesting a role in the recognition and clearance of virally infected cells (9).

The first description of ‘patrolling’ behavior in monocytes emerged from studies into the behavior of murine Ly6C− monocytes expressing GFP-tagged Cx3CR1 in the microvasculature of the dermis (7). This crawling behavior is similar to that seen in

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The online version of this article contains supplemental material.

Abbreviations used in this article: EC, endothelial cell; HDBEC, human dermal blood EC; TEM, transendothelial migration.
NKT cells (14), in that the crawling is prolonged, independent of the direction of blood flow, and 100- to 1000-fold slower than rolling. Patrolling was also found to be LFA-1–dependent (7). These key locomotory functions have been less well studied in human monocytes. Human CD14⁺ monocytes were observed performing a short-range crawling and “pirouetting” motion under static conditions in vitro, which was LFA-1–dependent and was probably required to relocate to endothelial cell (EC) junctions for trans-endothelial migration (TEM) (15). More recently, human CD14⁺CD16⁻ and CD14dimCD16⁺ monocytes were adoptively transferred into mice and their locomotion was monitored by intravital microscopy (9). Human CD14dimCD16⁺ monocytes were found to be capable of performing a long-range crawling behavior similar to that seen in murine Ly6C⁺ monocytes, but no evidence for a similar behavior was found in human CD14⁺CD16⁺ monocytes.

As yet, no in-depth study of human monocyte locomotory behavior has been performed in which human monocyte subsets are studied independently during adhesion and migration over human endothelium under shear flow. The present study addresses this knowledge gap and begins to delineate the molecular requirements necessary for these interactions with the endothelium.

Materials and Methods

EC culture under shear flow

Primary HUVEC (macrovacular endothelium) were isolated from single donors by conventional methods and obtained after cryopreservation at passage 1 (PromoCell). Primary human dermal blood EC (HDBEC) were isolated from the dermis of juvenile foreskin from a single donor by conventional methods and obtained for study (PromoCell) after cryopreservation at passage 2. HUVEC and HDBEC were confirmed to have consistent EC characteristics (cell morphology, adherence rate), were positive for von Willebrand factor and CD31 by immunohistochemistry and for labeling with acetylated low-density lipoprotein, labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, and negative for podoplanin and smooth muscle–specific α-actin. Cells were thawed as per supplier’s directions and cultured in ECGM-MV2 (PromoCell) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% (v/v) Fungizone (all from Invitrogen, Paisley, U.K.) in T75 flasks at 37˚C, 5% CO₂. Cells were passaged upon reaching 90–100% confluence and used between passages four and eight for all experiments. Cells were...
Isolation of monocyte subsets from whole blood

Blood was obtained from healthy volunteers by venipuncture, following informed consent, in heparinized tubes, and RBCs were lysed to isolate peripheral blood leukocytes. CD2+ and CD15+ cells were depleted of CD2+ and CD15+ cells by magnetic-activated cell sorting using anti-CD2 and anti-CD15 MicroBeads and LD columns (all from Miltenyi Biotec, Bergisch Gladbach, Germany). Monocyte-enriched peripheral blood leukocytes were incubated in sorting buffer (PBS, 2% EDTA, 0.5% FCS) with FeR-blocking Ab at 37˚C, 5% CO2 for 2 h, before being gently washed twice with Accutase (Sigma-Aldrich) and washed in PBS to allow the cells to adhere. The µ-slide was then attached to a green/yellow perfusion set mounted in a fluids unit (both from ibidi) filled with 13.6 ml ECGM-MV2. Cells were cultured under shear flow using an air pressure pump controlled by ibidi’s pump control software for 10 min by replacing the media in the fluids unit with the monocyte mixture. Monocytes grown under shear flow for 48–72 h were incubated with the monocytes for 10 min by replacing the media in the fluids unit with the monocyte mixture. Monocytes were kept on ice for use in further experiments.

Classification of locomotory behaviors in human monocyte subsets

<table>
<thead>
<tr>
<th>Locomotory Behavior</th>
<th>Parameters</th>
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<tbody>
<tr>
<td>Rolling</td>
<td>Accumulative distance &gt; 50 µm in 30 min</td>
</tr>
<tr>
<td>Crawling</td>
<td>Accumulative distance &gt; 150 µm in 30 min</td>
</tr>
<tr>
<td>Arrested</td>
<td>Accumulative distance &lt; 50 µm in 30 min</td>
</tr>
<tr>
<td>TEM</td>
<td>Migrates through endothelium during 30-min recording period or is already beneath endothelium at start of recording</td>
</tr>
<tr>
<td>Detached</td>
<td>Detaches from endothelium and re-enters flow</td>
</tr>
</tbody>
</table>

Monocyte migration assay

Freshly sorted monocytes were incubated in ECGM-MV2 containing 1:200 Vybrant DiD, Vybrant DiO (both Invitrogen), or no dye at 37˚C, 5% CO2 for 15 min. Monocytes were washed and then added to ECGM-MV2 to give a final concentration of 10^6 cells from each subset per milliliter. EC grown under shear flow for 48–72 h were incubated with the monocytes for 10 min by replacing the media in the fluids unit with the monocyte suspension and operating the pump at 2 dynes/cm² shear flow. The entire pump system was then transferred to a Nikon Eclipse Ti microscope (Nikon U.K., Kingston upon Thames, U.K.) fitted with a humidified incubator, with flow maintained at 2 dynes/cm². Images were taken every 10 s during a 30-min period using a CoolSnap HQ2 CCD camera (Photometrics, Tucson, AZ) and NIS-Elements software (Nikon U.K.). Six fields of view were chosen at random, and images were taken using phase contrast, yellow fluorescent protein, and HcRed filters with an ×10 objective lens. Image sequences were analyzed using Image J’s manual tracking plug-in software (National Institutes of Health) and chemotaxis software (ibidi).

Analysis of role of adhesion molecules on monocyte migration

The monocyte migration assay was adapted to allow for cell surface adhesion molecules on the endothelium to be blocked by uncoupling the µ-slide from the fluids unit and replacing the media with ECGM-MV2 containing anti-human blocking Abs directed against CD54 (BBIG-H1, 10 µg/ml), CD106 (BBIG-V1, 50 µg/ml), VEGFR2 (89106, 50 ng/ml), or CX3CL1 (81506, 5 µg/ml), or murine IgG1 isotype control (11711, 30 µg/ml). All Abs were supplied by R&D Systems. EC were incubated with the blocking Ab at 37˚C, 5% CO2 for 2 h, before being gently washed twice with ECGM-MV2 and reattached to the fluids unit. Flow was maintained at 2 dynes/cm² for 15 min prior to the addition of monocytes.

Statistical analysis

Individual monocyte tracks were analyzed using chemotaxis software (ibidi) to extract values for accumulative distance, Euclidean distance, confinement ratio, and average velocity. Data were evaluated for Gaussian distribution using the Kolmogorov–Smirnov test. Data found to be normally distributed were analyzed by Student t test, and remaining data were analyzed using the nonparametric Mann–Whitney U test to produce a Gaussian estimate of the p value.

Results

Locomotory behavior of human monocytes on resting microvascular endothelium

Sorted human monocyte subsets CD14^+CD16^−, CD14^+CD16^+, and CD14^dimCD16^+ were perfused over HDBEC at 2 dynes/cm² shear flow at a 1:1:1 ratio and distinguished using the membrane dyes DiD or DiO or no dye. Membrane dyes had no significant effect on the locomotory parameters of the different monocyte subsets (Supplemental Fig. 1). Individual adherent monocytes were tracked and assessed for average velocity, track length, and

![FIGURE 2. Classification of locomotory behaviors in human monocyte subsets. (A-C) Behavioral profiles of CD14^+CD16^− (A), CD14^+CD16^+ (B), and CD14^dimCD16^+ (C) human monocytes when interacting with HDBEC at 2 dynes/cm² shear stress. Bars represent the mean percentage of adherent monocytes performing each behavior from 18 fields of view and three donors.](http://www.jimmunol.org/)
confinement ratio, a measure of how directly the monocyte traveled from its start point to its end point. Under these conditions, monocytes of different subsets were observed to behave in different ways. Although the average confinement ratio was similar for all subsets (Fig. 1A), the range of track lengths and velocities observed differed between subsets (Fig. 1B, 1C). Fewer CD14+CD16+ monocytes were adherent (Supplemental Fig. 1), but those cells that anchored traveled significantly shorter distances at a significantly slower average velocity than CD14dimCD16+ monocytes and were significantly slower than CD14+CD16+ cells. These differences can clearly be seen when individual tracks are viewed (Fig. 1D–F). CD14+CD16− and CD14dimCD16+ monocytes were able to migrate up to ~250 μm during a 30-min period, with CD14dimCD16+ monocytes displaying the highest average velocity of the three subsets.

Upon close examination, the locomotory behavior of each monocyte subset could be assigned to one of four categories based on the velocity and direction of migration; rolling, crawling, arrested, and TEM (Table I). Cells moving quickly in the same direction as the flow and only loosely tethered to the endothelial surface were classified as rolling. Cells that performed TEM at any point during the 30-min observation period, or that had already undergone TEM, were classified as such. Most observed cells, regardless of subset, were either crawling or arrested (Fig. 2). Arrested cells were classified as those moving <50 μm in the 30-min observation period, a value chosen to compensate for the slight movement of the endothelium under shear flow. Crawling cells were firmly adherent, moved >50 μm during 30 min, traveled either with or against the flow, often changing direction, and could be seen to be performing an amoeboid-like movement (Supplemental Videos 1, 2). It was also noted when a cell detached from the endothelium and re-entered the flow.

Each subset was seen to preferentially perform different types of locomotory behavior. CD14+CD16+ monocytes were typically arrested (Fig. 2B), CD14+CD16− were predominantly crawling or arrested (Fig. 2A), and CD14dimCD16+ monocytes showed mainly crawling behavior (Fig. 2C). Transendothelial migration was observed at low levels for all subsets except CD14 dimCD16+ monocytes. The propensity for arrest on the endothelium noted for

### Table II. Characteristics of short- and long-range crawling

<table>
<thead>
<tr>
<th>Crawling Behavior</th>
<th>Defining Parameters</th>
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<tbody>
<tr>
<td>Short-range</td>
<td>Accumulative distance 50–100 μm in 30 min</td>
</tr>
<tr>
<td></td>
<td>Average velocity &lt; 3 μm/min</td>
</tr>
<tr>
<td>Long-range</td>
<td>Accumulative distance &gt; 100 μm in 30 min</td>
</tr>
<tr>
<td></td>
<td>Average velocity 3–12 μm/min</td>
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**FIGURE 3.** Crawling behavior in human monocyte subsets. (A and B) Tracks of total monocytes (all subsets combined) performing short-range (A) or long-range (B) crawling from 12 separate fields of view and two donors, plotted after aligning their starting positions. (C) Ratio of short-range (open bars) to long-range (filled bars) crawling performed by each monocyte subset from 18 separate fields of view and three donors. (D and E) Comparison of average velocity (D) and track length (E) for short-range (○) and long-range (●) crawling between human monocyte subsets interacting with HDBEC at 2 dynes/cm² shear stress. Dot plots show median and range, with data from 18 fields of view and three donors. *p < 0.05 calculated by Mann–Whitney U test. (F) Graph shows numbers of monocytes observed in each subset to generate analyses in (D) and (E). Left panel is short-range and right panel long-range crawling.
CD14+CD16+ monocytes explains the reduced velocity and track length observed for this subset as a whole (Fig. 1B, 1C). The same is true for the propensity for crawling behavior in the CD14dimCD16+ subset and its associated higher average velocity (Fig. 1C). In general there was little rolling behavior in any subset. This was not clearly attributable to reduced or altered expression of CD62L. Analysis of CD62L expression during monocyte preparation (Supplemental Fig. 2) showed increasing expression on the CD14+CD16+ subset, sustained but low-level expression on CD14+CD16+ cells, and absence of expression on CD14dimCD16+ subset, as previously reported.

During the performance of these studies, secondary adhesion events (i.e., incoming monocytes binding to EC-adherent monocytes) were not observed.

Crawling behavior of human monocyte subsets

During analysis of the image sequences, we noted two major forms of crawling behavior, a short-range crawling and a long-range crawling. These two behaviors were easily identifiable, with short-range crawling appearing slower and covering a shorter distance, with the cells often pausing. Cells performing long-range crawling appeared to travel more quickly and at a constant speed, often performing loops and hairpins as they migrated. The long-range crawling appeared very similar to that previously described for Ly6C+ murine monocytes (7). The two behaviors were distinguished using a track length cut-off of 100 μm in 30 min, and an average velocity cut-off of 3 μm/min (Table II). Long-range and short-range crawling behaviors appeared similar across all monocyte subsets, and they showed archetypal patterns when crawling tracks were analyzed for all subsets (Fig. 3A, 3B). When looking at total crawling cells for each subset, it was interesting that long-range crawling was never observed in the CD14+CD16+ subset, but it occurred in approximately a third of CD14dimCD16+ and CD14+CD16+ crawling cells (Fig. 3C). Short-range crawling was similar between subsets, as were the median track lengths and average velocities (Fig. 3D, DE). However, differences in track length were observed for long-range crawling, with CD14dimCD16+ monocytes traveling farther on average than CD14+CD16+ monocytes (Fig. 3E). Note again that far fewer CD14+CD16+ monocytes were adherent and available for these analyses (Fig. 3F).

Locomotory behavior of human monocytes on resting macrovascular endothelium

To establish whether the locomotory behaviors observed for each monocyte subset were affected by the type of endothelium used as a substrate, the migration experiments were repeated using HUVEC as a model macrovascular endothelium to compare with those carried out with HDBEC, a model microvascular endothelium. To simplify the analysis, only the two major forms of locomotion, arrested and crawling, were compared, because together these account for >90% of all observed cells. CD14+CD16+ monocytes showed crawling behavior predominantly on HDBEC, compared with most cells being arrested on HUVEC (Fig. 4A).
CD14<sup>+</sup>CD16<sup>-</sup> monocytes favored arrest on both substrates and CD14<sup>dim</sup>CD16<sup>-</sup> monocytes favored crawling on both (Fig. 4A). Both CD14<sup>+</sup>CD16<sup>-</sup> and CD14<sup>dim</sup>CD16<sup>-</sup> monocytes showed a greater propensity to detach from HUVEC, suggesting a reduced ability to adhere to macrovascular endothelium (Fig. 4B). In contrast, CD14<sup>-</sup>CD16<sup>+</sup> monocytes showed a reduced tendency to detach from HUVEC compared with HDBEC (Fig. 4B). Coupled with the switch to a higher proportion of arrested cells, this suggested that CD14<sup>-</sup>CD16<sup>+</sup> monocytes adhere more strongly to macrovascular endothelium than to microvascular endothelium. Of note, crawling CD14<sup>-</sup>CD16<sup>-</sup> monocytes also exhibited different behavior on different substrates (less long-range crawling on HUVEC compared with HDBEC) whereas CD14<sup>dim</sup>CD16<sup>-</sup> monocytes retained similar function (Fig. 4C), suggesting that long-range crawling may be facilitated by different adhesion molecules for each monocyte subset.

**Immunophenotype of micro- and macrovascular endothelium and effects of activation**

HUVEC and HDBEC cultured under static conditions were immunophenotyped for nine different cell surface adhesion molecules and receptors by flow cytometry to generate potential candidate molecules for blocking studies. Static culture conditions were deemed appropriate, as they allowed for a greater number of candidate molecules to be screened. Both EC types were found to stably express all nine markers up to passage 12, with ICAM1 (CD54), VCAM1 (CD106), and VEGFR2 being reproducibly induced by a combination of IFN-γ and TNF-α on both types of endothelium (Supplemental Fig. 3). In terms of differences between the two EC types, HUVEC expressed higher levels of MCAM (CD146), neuropilin-1 (NRP1), and VCAM1 (CD106) in a resting state than HDBEC, whereas HDBEC expressed higher levels of PECAM1 (CD31), E-selectin (CD62E), ICAM1 (CD54), and VEGFR2 (Fig. 5A). Both types of endothelium express comparable levels of endoglin (CD105) and P-selectin (CD62P). Under inflammatory conditions (10 ng/ml TNF-α and 10 ng/ml IFN-γ for 8 h), HUVEC maintained higher expression levels of CD146, NRP1, and CD106, and HDBEC expressed significantly higher levels of CD31 and CD62E (Supplemental Fig. 3). Higher expression of CD146 on HUVEC indicates a strong intercellular junction (16), which would be required to withstand the larger shear to which macrovascular cells are subject. VEGFR2 signaling is enhanced by the action of NRP1 as a coreceptor (17); higher HUVEC expression could be due to lower VEGFR2 expression, as greater costimulation would be required to achieve the same level of signaling. Higher expression of the adhesion molecules CD54 and CD62E on resting HDBEC fits with the barrier function provided by the skin, as well as the need for rapid leukocyte access in the dermal microenvironment.

We next examined the impact of such EC activation on monocyte behavior. Using HDBEC as the exemplar, monocyte locomotion profiles showed small differences when comparing resting and activated endothelium, with the most notable being the increased number of CD14<sup>+</sup>CD16<sup>-</sup> cells performing TEM and the emergence of similar behavior in CD14<sup>dim</sup>CD16<sup>-</sup> cells (Fig. 5B–D), both of which facilitate involvement of these cells in tissue inflammation.

We also analyzed expression of the chemokines CCL2 and CX3CL1 on both ECs under static conditions. Expression of CX3CL1 was not detectable under resting conditions on HUVEC or HDBEC, but both showed induced expression following activation. CCL2 was expressed at low levels on HUVEC and HDBEC and levels were boosted following activation (Supplemental Fig. 4).

**FIGURE 5.** Cell surface adhesion molecule expression on human endothelium and effects of EC activation on locomotion. (A) Bar chart of fold change in mean fluorescence intensity over FMO control for nine cell surface adhesion molecules and receptors as measured by flow cytometry on HDBEC (open bars) and HUVEC (filled bars). Data represent triplicate repeats of passages 5–12, showing mean and SEM. Statistical significance was determined using unpaired t tests. (B–D) To study the effects of EC activation on locomotion, HDBEC were used in a resting state or pre-treated with 10 ng/ml TNF-α and 10 ng/ml IFN-γ for 8 h. Sorted human monocyte subsets migrating over HDBEC at 2 dynes/cm² were tracked manually and their movement described as rolling, crawling, TEM, or arrested, with detachment from the endothelial surface noted. Locomotion profiles are shown for CD14<sup>+</sup>CD16<sup>-</sup> (B), CD14<sup>-</sup>CD16<sup>-</sup> (C), and CD14<sup>dim</sup>CD16<sup>-</sup> (D) subsets, comparing monocytes perfused over resting HDBEC (plain bars) with HDBEC stimulated with 10 ng/ml TNF-α and 10 ng/ml IFN-γ for 8 h (striped bars). For each condition, n = 2 donors, with data collected from six randomly chosen fields of view in each case and the mean percentages plotted. *p < 0.05, **p < 0.01, ***p < 0.001.

**Effects of adhesion molecule blockade on human monocyte locomotion**

Migration experiments were carried out on HDBEC that had been preincubated with blocking Abs against ICAM1, CX3CL1, VEGFR2, or VCAM1 to examine the roles of these adhesion molecules and receptors in the locomotory behavior of human monocyte subsets. ICAM1 blockade induced a shift toward more arrested behavior in the CD14<sup>-</sup>CD16<sup>-</sup> subset, but it had no effect on the ratio of arrested to crawling monocytes in the other subsets (Fig. 6A). The range of track lengths was altered (Fig. 6B), but it was only significantly reduced compared with the isotype control in the CD14<sup>-</sup>CD16<sup>-</sup> subset (Fig. 6C). Blocking VCAM1 had no...
effect on the ratio of arrested to crawling monocytes in the CD14+ CD16− and CD14dimCD16+ subsets, but it increased the proportion of arrested monocytes in the CD14+CD16+ subset (Fig. 6A). This increase in arrested CD14+CD16+ monocytes can be seen as a significant difference in track length when compared with other subsets under the same conditions (Fig. 6B), but not when compared with the isotype control (Fig. 6D). Blocking CX3CL1 shifted the ratio of arrested to crawling monocytes toward the former in CD14+CD16+ and CD14+CD16− monocytes, and toward crawling for CD14dimCD16+ monocytes (Fig. 6A). The increase in crawling CD14dimCD16+ monocytes and arrested CD14+CD16+ monocytes can be seen as a significant difference in the track lengths (Fig. 6B). Blocking VEGFR2 induced a greater tendency toward arrested behavior for CD14+CD16+ monocytes, and toward crawling for both the CD14+CD16− and CD14dimCD16+ subsets, with only crawling being observed for the CD14dimCD16+ monocytes (Fig. 6A). These changes are reflected in the track lengths of these subsets in comparison with each other (Fig. 6B), but not in comparison with the isotype control (Fig. 6C–E).

As stated, long-range crawling was never observed in the CD14+ CD16 subset (Fig. 6D). However, CD14+CD16− monocyte long-range crawling was markedly reduced by blockade of ICAM1 and VCAM1 (Fig. 7A), with crawling cells traveling significantly shorter distances (Fig. 7C). Within the CD14dimCD16− subset, there was a complete absence of long-range crawling upon blockade of ICAM1, VCAM1, or CX3CL1 (Fig. 7B), although the average track length of crawling cells is unperturbed (Fig. 7D).

Taken together, these data suggest that long-range and short-range crawling behaviors require different adhesion molecules, and that each monocyte subset has a different requirement for adhesion molecule ligands to perform long-range crawling.

Discussion
In the present study, we show, to our knowledge for the first time, heterogeneity in the locomotory behavior of human monocytes of different subsets when interacting with human micro- or macrovascular endothelium under shear flow. Using human endothelium cultured under physiologically relevant shear flow (18) and purified populations of human monocyte subsets in a novel closed-loop, multicellular migration assay, we were able to closely monitor the movement and migration of monocytes by live-cell time-lapse microscopy, and to classify the various behaviors observed as rolling, crawling, arrested, or TEM, with arrested and crawling being the most common. Each monocyte subset was shown to preferentially perform different types of locomotory behavior in a resting state, with CD14+CD16+ monocytes displaying the highest degree of arrested behavior, and CD14dim CD16+ monocytes the highest degree of crawling behavior. A long-range crawling behavior, similar to the patrolling behavior of murine Ly6C2 monocytes (7), was observed in CD14+CD16− and CD14dimCD16+ monocytes, but not in CD14+CD16+ monocytes, and TEM was only observed in the CD14+CD16− and CD14+CD16+ subsets. CD14dimCD16+ and CD14+CD16+ monocytes showed a preference for adhering to microvascular over macro-
or human CD14dimCD16+ monocytes adoptively transferred into in vivo CD14+CD16+ has previously been examined only in murine Ly6C

crawling behavior within the confines of an in vitro assay, which and 3 donors. (C and D) Track lengths of total crawling CD14+CD16− (C) and CD14dimCD16+ (D) monocytes interacting with HDBEC with isotype control Ab or Abs against endothelial-expressed adhesion molecules at 2 dynes/cm² shear stress from 18 fields of view and three donors. *p < 0.05 calculated by Mann–Whitney U test.

FIGURE 7. Effects of endothelial-expressed adhesion molecule blockade on human monocyte crawling behavior. (A and B) Ratio of short-range (open bars) to long-range (filled bars) crawling performed by human CD14+CD16+ (A) and CD14dimCD16+ (B) monocyte subsets when interacting with HDBEC with isotype control Ab or Abs against endothelial-expressed adhesion molecules at 2 dynes/cm² shear stress from 18 fields of view and three donors. (C and D) Track lengths of total crawling CD14+CD16− (C) and CD14dimCD16+ (D) monocytes interacting with HDBEC with isotype control Ab or Abs against endothelial-expressed adhesion molecules at 2 dynes/cm². Dot plots show median and range, with data from 18 fields of view and three donors. *p < 0.05 calculated by Mann–Whitney U test.

vascular endothelium. We used Ab-mediated blockade of candidate receptor-ligand pairs to investigate the molecular basis of these different behaviors and showed a complex series of effects that suggests that each form of locomotion has a different requirement for adhesion molecules, and that each subset may use different adhesion molecules to perform each type of locomotion. In particular, long-range crawling behavior in CD14dimCD16+ monocytes was abrogated by blockade of ICAM1, VCAM1, or CX3CL1, whereas only blockade of ICAM1 stopped this behavior in CD14+CD16+ monocytes.

The ability to observe human monocytes performing long-range crawling behavior within the confines of an in vitro assay, which has previously been examined only in murine Ly6C− monocytes (7), or human CD14dimCD16+ monocytes adoptively transferred into mice may represent a useful new technology for studying human monocyte function. Although crawling behavior was noted for all three subsets, long-range crawling was observed only in CD14+ CD16− cells and CD14dimCD16+ cells. Notably, long-range crawling by CD14+CD16− monocytes was a highly reproducible observation on both human macro- and microvascular endothelium. At this stage we have only examined these phenomena in relationship to two relatively distantly related vascular beds (large vessel and skin capillary), and it is conceivable that there are important monocyte locomotion differences across different types of microvascular vessels (e.g., lung versus skin capillaries). The new methodology we outline could be used to address specific questions such as these about vascular biology and monocyte locomotory behavior. There are additional limitations in the scope of our study, namely the effect that shear stress, as well as its duration, has on the phenotype of endothelium and, importantly, its expression of adhesion molecules, which could impact differently on each monocyte subset. In our studies, endothelial cells were exposed to shear stress for >24 h, which is generally considered long-term. Future studies using varying durations will be required to establish to what extent endothelial phenotype changes and what impact this has on observed monocyte behavior. A further limitation is that the study of locomotory behaviors is dependent on monocyte adhesion to the endothelium, the frequency of which differs for the different subsets. In our studies we consider the extent of sampling of monocytes with low adhesion properties (CD14+CD16+) to be reasonable to draw preliminary conclusions, but our findings will need to be confirmed using systems that enable greater depth of subset analysis, for example by using larger cell beds.

The propensity of CD14+CD16+ monocytes to adhere to the endothelium and remain relatively static, coupled with the complete absence of long-range crawling behavior, is worthy of note. The lack of long-range crawling by CD14+CD16+ monocytes on either macro- or microvascular endothelium suggests that this subset performs a unique role within monocytes. Cells of this subset adhere tightly to the endothelium, and they were observed to travel against the flow of media. This behavior may be linked to the proposed function of these cells as producers of large amounts of proinflammatory cytokines (19, 20), as this kind of "anchoring" behavior would allow the precise delivery of cytokines into either the bloodstream or the tissues, as required. However, note again that the number of CD14+CD16+ monocytes adhering in order for these observations to be made is small and therefore the data are inadequate for a robust comparison across subsets at this stage.

Transendothelial migration, including movement of monocytes beneath the endothelium, and reverse transendothelial migration were observed to take place across both micro- and macrovascular endothelium in vitro, although never by the CD14dimCD16+ subset. This is consistent with the behavior observed in murine Ly6C− monocytes in a steady-state (7), in which these cells rarely migrated out of the vasculature. The preference for adhering to microvascular over macrovascular endothelium observed in this subset is also consistent with the observation of murine Ly6C− monocytes patrolling postcapillary venules (7). This preference was also noted for CD14+CD16+ monocytes, but not for CD14+ CD16− monocytes, suggesting that the CD14+CD16+ monocytes may preferentially use a different range of adhesion molecules for firm adhesion to the endothelium. This would include making greater use of VCAM1, which is expressed at higher levels on resting HUVEC than HDBEC, and the blocking of which reduces the distance traveled by crawling CD14+CD16+ monocytes.

The adhesion molecule blocking studies confirmed already known roles for adhesion molecules in leukocyte migration. ICAM1 was shown to play an important role in crawling behavior, corroborating...
previous results (7, 15). The role of CX3CL1 in mediating adhesion of CD16+ monocytes, but not CD16– monocytes, was also confirmed (21), as blockade of CX3CL1 had no effect on the long-range crawling of CD14+CD16+ monocytes. Previous murine studies found no role for VLA-4–VCAM1 interactions in monocyte crawling in noninflamed vessels, and a reduction in adherence, but not crawling, in inflamed vessels (22). In contrast to this, VCAM1 blockade was seen to affect the distance traveled by CD14+CD16+ crawling monocytes and to completely abrogate long-range crawling in CD14dimCD16+ monocytes. This highlights the importance of performing migration assays with human monocytes and human endothelium, and in separating human monocytes into their separate subsets to gain a clear picture of monocyte–endothelial interactions.

Interestingly, blockade of VEGFR2 caused every observed adherent CD14dimCD16+ monocyte to exhibit crawling, suggesting that blockade of VEGFR2 promotes crawling behavior. The Ab used in these studies is known to block interactions between VEGFR2 and VEGF-A, but could potentially be acting to cross-link VEGF2 molecules, thus bypassing the need for a ligand and increasing signaling via VEGFR2. This would cause an upregulation in ICAM1 and VCAM1 expression at the endothelial cell surface, as both adhesion molecules are known to be VEGF-A inducible (23) via the canonical NF-kB pathway. Although not yet corroborated, upregulation of ICAM1 and VCAM1 as a result of incubation with the VEGFR2 blocking Ab would be likely to increase crawling behavior, as blockade of either ICAM1 or VCAM1 disrupted the crawling of CD14dimCD16+ monocytes.

These studies have shown clear differences in the locomotory behavior of human monocytes of different subsets and highlighted the importance of studying each subset separately. We describe a method for analyzing interactions between human leukocytes and human endothelium under physiological levels of shear flow in vitro, which could be used to investigate physiological locomotion, inflammatory states, and therapeutic strategies.

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References