CD44-Mediated Monocyte Rolling in the Absence of Dominant α4-Integrin Ligands

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Article

CD44-Mediated Monocyte Rolling in the Absence of Dominant α4-Integrin Ligands

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Highlights

What are the main findings?

1. CD44–hyaluronan interactions can mediate monocyte rolling under low-shear conditions when α4-integrin engagement is lacking.
2. CD44-dependent rolling becomes functionally apparent on hyaluronan-presenting cellular monolayers that lack detectable α4-integrin–mediated adhesion.

What are the implications of the main findings?

1. Monocyte rolling reflects a hierarchical adhesion system in which CD44–hyaluronan interactions act as an alternative when dominant integrin pathways are unavailable.
2. CD44–hyaluronan-mediated interactions may contribute to monocyte adhesion in hyaluronan-rich or structurally altered vascular environments, including tumor-associated cellular interfaces.

Abstract

Leukocyte recruitment from blood into tissues usually involves sequential adhesive interactions, including selectin-mediated rolling followed by integrin-dependent arrest. Although CD44–hyaluronan interactions are known in leukocyte adhesion, their functional contribution to monocyte rolling under defined experimental conditions remains incompletely resolved. Here, we use controlled cell-based model systems differing in α4-integrin ligand availability to dissect the relative contributions of CD44 and integrins to monocyte rolling under physiologic shear. Using mouse monocytoid WEHI 78/24 cells and primary human monocytes, we show that CD44-hyaluronan interactions support rolling and adhesion on hyaluronan-presenting cellular monolayers lacking VCAM-1–mediated integrin engagement, whereas α4-integrin–dependent interactions dominate when VCAM-1 is available. Functional blockade of CD44, soluble hyaluronan competition, and enzymatic hyaluronan removal consistently reduced rolling and adhesion under these conditions. These findings demonstrate that CD44-mediated rolling represents a context-dependent adhesion pathway that becomes functionally apparent when dominant integrin-mediated interactions are limited, but is masked when VCAM-1 is present. By experimentally minimizing integrin engagement, this study provides a reductionist framework to resolve the hierarchical relationship between CD44 and integrin pathways in monocyte adhesion.

Keywords: CD44; hyaluronan (hyaluronate; HA); monocyte rolling; leukocyte trafficking; flow-dependent adhesion; leukocyte migration

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1. Introduction

Leukocyte recruitment to tissues proceeds through tethering, rolling, arrest, and transmigration under shear flow [1–4]. Rolling interactions are classically mediated by selectins, whereas firm adhesion and arrest are largely governed by integrins such as α4β1 (VLA-4) and αLβ2 (LFA-1) [5]. Chemokine-mediated integrin activation can trigger rapid arrest under flow [6]. In monocytes, integrin-dependent adhesion pathways are particularly important for inflammatory recruitment to tissues [2,7,8]. After extravasation, monocytes differentiate into macrophages or monocyte-derived dendritic cells and contribute to tissue homeostasis and immune responses [9,10].

CD44–hyaluronan (HA; also termed hyaluronate) interactions have been shown to support rolling of lymphocytes under flow, typically generating weaker and more transient interactions compared with integrin-mediated adhesion [11,12]. CD44 (HERMES) was originally identified as a lymphocyte homing-associated molecule [13,14], and subsequent studies have demonstrated its contribution to leukocyte trafficking in vivo. For example, interference with CD44-hyaluronan interactions reduces leukocyte rolling and infiltration in inflammatory settings such as experimental autoimmune uveoretinitis [15].

Despite this established role, the relative contribution of CD44 to monocyte rolling remains incompletely defined, particularly in relation to dominant integrin-dependent adhesion pathways. In most physiological and experimental settings, integrin ligands such as VCAM-1 and ICAM-1 are present and mediate strong adhesive interactions, which can obscure the contribution of alternative receptors.

Monocytes differ from lymphocytes in their integrin repertoire and adhesive behavior under flow, and their interactions are influenced by receptor organization and mechanical forces. This raises the question of whether CD44-hyaluronan interactions can function as an alternative rolling mechanism when integrin-mediated adhesion is limited.

To address this, we employed reductionist experimental conditions in which integrin-dependent adhesion is minimized. Using shaking and laminar flow adhesion assays, we analyzed CD44-dependent rolling of monocytoid cells on hyaluronidase-sensitive cellular monolayers that present hyaluronan but lack functional α4-integrin ligands. Using the mouse monocytoid cell line WEHI 78/24 and primary human monocytes, we dissected the relative contributions of CD44, hyaluronan, and α4 integrins under controlled conditions.

Over the past two decades, substantial work has established CD44-hyaluronan interactions as regulators of leukocyte recruitment and vascular inflammation [16–19]. In parallel, the endothelial glycocalyx has emerged as a dynamic regulator of leukocyte–surface interactions, with hyaluronan representing a key component whose organization influences adhesion [20]. However, the hierarchical relationship between CD44-mediated rolling and integrin-dependent adhesion remains difficult to resolve in complex in vivo settings.

Here, we use a controlled cell-based system to isolate these interactions and demonstrate that CD44-mediated rolling becomes functionally apparent when integrin engagement is limited. This approach defines the context-dependent contribution of CD44 within the hierarchy of adhesion mechanisms, rather than proposing a new adhesion pathway.

2. Materials and Methods

2.1. Cells and Reagents

bEnd.3 cells were obtained from the American Type Culture Collection (ATCC; CRL-2299) , a mouse brain endothelial cell line derived from primary brain endothelial cells transformed with polyomavirus middle T antigen [21], were maintained in complete DMEM (cDMEM; DMEM supplemented with 5% fetal bovine serum [endotoxin <10 pg/ml; Gemini Scientific] and 5% Fetal Clone [Hyclone Labs]). Cells were used between passages 22 and 30.

ECV304 cells were obtained from the American Type Culture Collection (ATCC; CRL-1998) and were originally described as spontaneously transformed human endothelial cells [22], but were later

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shown to be derived from the T24 bladder carcinoma line [23–26]. Cells were used as historically maintained laboratory stocks corresponding to the originally distributed ECV304 line. In the present study, ECV304 cells were therefore not used as a surrogate for authentic vascular endothelium, but as a T24-derived cellular monolayer providing a hyaluronidase-sensitive, hyaluronan-presenting surface for reductionist analysis of CD44-dependent interactions. ECV304 cells were maintained in M199 medium supplemented with 10% fetal bovine serum [endotoxin <10 pg/ml; Gemini Scientific].

HMEC-1 human dermal microvascular endothelial cells were obtained from the American Type Culture Collection (ATCC; CRL-3243) [27] were maintained in MCDB-131 medium supplemented with 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone and 10% fetal bovine serum [endotoxin <10 pg/ml; Gemini Scientific].

WEHI 78/24 murine monocytoid cells [28] were obtained as a gift from R. Coffman (DNAX Research Institute, Palo Alto, CA, USA), cultured in cDMEM and sub-cultured 36 h before use.

Primary human monocytes were isolated from peripheral blood samples from healthy adult donors using standard previously published procedures. Quality controls included collection of samples from each major step, followed by FACS analysis. All samples were obtained with written informed consent; no identifying information was associated with the samples.

FITC-labelled hyaluronan (FITC-HA) was prepared fresh, tested (Figure S1) and stored in aliquots as described previously [29].

Antibodies used in this study were as follows: HERMES-3 (mouse IgG2a, anti-human CD44) [30]; 84H10 (anti-human ICAM-1); L133 (anti-human CD31); TY1138 (anti-human VCAM-1); WAPS1.2 (anti-human P-selectin) [31]; DREG56 (mouse IgG1, anti-human L-selectin) [31]; 9B5 (anti-human CD44); IM7.8.1 or TJB1.7 (anti-mouse CD44, depending on experiment); Mel-14 (anti-mouse L-selectin) [32]; PS/2 (rat IgG2b, anti-mouse α4 integrin) [33]; MI/70 (anti-mouse αM/Mac-1) [34]; TIB213 (anti-mouse αL); and 30G12 (rat IgG2a, anti-mouse CD45) [35]. Additional antibodies used in specific experiments are indicated in the corresponding figure legends.

2.2. Monolayer Activation

Where indicated, HMEC-1, bEnd.3 or ECV304 monolayers were grown to confluence and treated with recombinant human TNF-α (1 ng/ml; R&D Systems) for the indicated times before use in adhesion assays.

2.3. Hyaluronidase Treatment

Monolayers were washed extensively in DMEM and incubated with hyaluronidase (10 or 100 μg/ml) in DMEM containing 10 mM HEPES for 1 h at 37 °C. Monolayers were then washed twice to remove enzyme and used immediately in binding or flow assays to prevent synthesis of new hyaluronate.

2.4. Flow Cytometry

Binding of soluble FITC-labelled hyaluronan to WEHI 78/24 cells was analyzed by flow cytometry. Cells were incubated with FITC-hyaluronan in the presence or absence of blocking antibodies under standard conditions and analyzed on a FACScan (BD) using CellQuest software. Experiments were repeated at least three times with comparable results.

2.5. Static and Shaking Adhesion Assays

Monolayer-forming cell lines were seeded into 1 cm^2 wells of 8-well Lab-Tek chamber slides (Nunc Inc., Naperville, IL) and grown to confluence for 2–3 days. Where indicated, monolayers were treated with TNF-α (1 ng/ml) for 18 h, washed twice with assay buffer and left in 100 μl/well before addition of WEHI 78/24 cells or primary human monocytes.

Before the assay, WEHI 78/24 cells were resuspended at 2×10^6 cells/ml and preincubated with saturating concentrations of antibody (10 μg/ml) for 15 min at room temperature or with soluble

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hyaluronan (400 µg/ml) for 30 min at room temperature. A total of 2 × 10^5 cells was added in 100 µl/well for a final volume of 200 µl. Assays were performed at room temperature with continuous rocking. Chambers were rotated by 90° after 10 min to facilitate even binding. After 20 min, the chamber top and gasket were removed, slides were dipped twice in PBS to remove non-adherent cells and fixed in 1.5% glutaraldehyde in PBS. Bound cells were quantified by manual counting under light microscopy. The mean number of bound cells per field or well was determined as indicated in the corresponding figures.

Primary human monocyte adhesion assays were performed analogously under static or shaking conditions as specified in the figure legends.

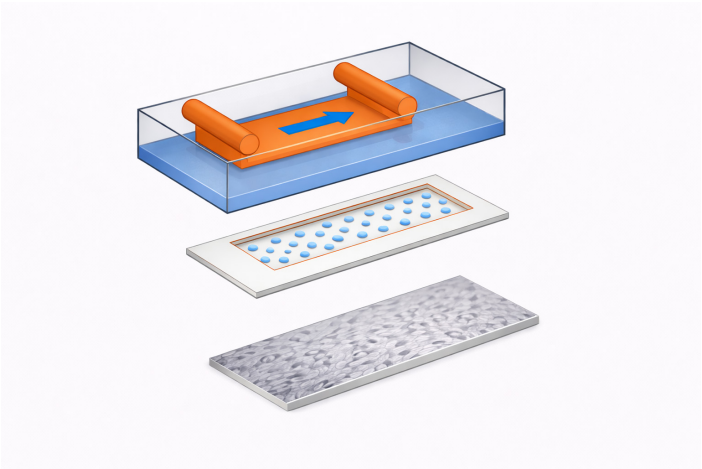
2.6. Monocyte–Hyaluronate Binding Assay

Hyaluronan (3.3 mg/ml in PBS, 0.1% BSA) was diluted in PBS to 400 µg/ml. A volume of 200 µl was added to 1 cm^2 wells of 8-well Lab-Tek chamber slides (Nunc Inc., Naperville, IL) and allowed to bind overnight at 4 °C. Remaining binding sites were blocked for 30 min at room temperature with PBS containing 1% BSA. Slides were washed twice with assay buffer and left in 100 µl/well before addition of cells. During the blocking step, WEHI 78/24 cells were preincubated with antibodies (10 µg/ml) for 15 min at room temperature. After incubation, slides were examined by light microscopy and the mean number of bound cells was determined.

2.7. Laminar Flow Assays

Parallel-plate laminar flow assays were performed using a custom-made flow chamber (Figure 1a) based on the design described by Lawrence et al. [36–38]. Monolayer cells were grown to confluence on glass slides (Superfrost Microscope Slides, Erie Scientific, Portsmouth, NH) and assembled in a parallel-plate chamber with a 250 µm gap thickness, generating uniform wall shear stress across the monolayer.

(**a**)



(**b**)

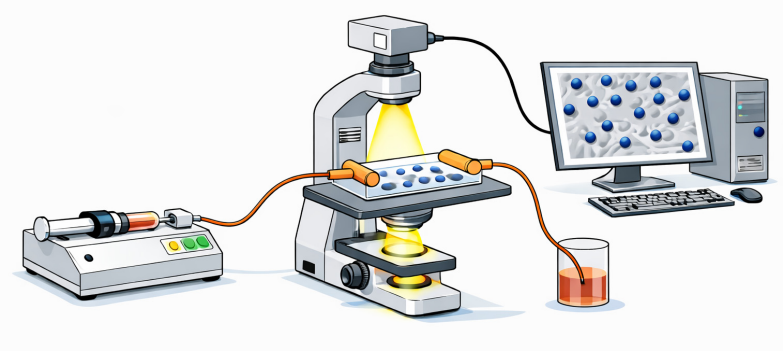


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Figure 1. Parallel-plate flow chamber assay for analysis of CD44–hyaluronan-dependent monocyte interactions. (a) Schematic representation of the custom-made transparent parallel-plate flow chamber (gap height 250 μm; not drawn to scale). Confluent cellular monolayers are assembled in the chamber and exposed to defined laminar shear flow. (b) Monocytes are perfused through the chamber using a syringe pump and analyzed under controlled shear conditions. Rolling and transient adhesion are visualized on hyaluronan-presenting cellular monolayers (e.g., ECV304) under low shear stress using an inverted microscope equipped with video acquisition. This setup allows quantitative analysis of CD44–hyaluronan-mediated interactions under conditions of limited α4-integrin engagement.

The chamber was mounted on the stage of an inverted phase-contrast microscope. WEHI 78/24 cells or primary human monocytes were resuspended at 2×10^6 cells/ml in assay buffer and perfused through the chamber with a Harvard syringe pump at defined flow rates (Figure 1b). Wall shear stress was calculated from chamber geometry and volumetric flow rate, assuming a viscosity of 1.0 cP. Experiments were performed over a shear-stress range of 0.7–2 dyn/cm^2, as indicated in the Results and figure legends. For low-shear rolling assays, a wall shear stress of 1 dyn/cm^2 was used.

The flow rate was stepped down, where indicated, to allow measurement of rolling and firm adhesion at different shear stresses. Two minutes were allowed for equilibration after each change in flow. Interacting cells were counted every 30 s for approximately 2–4 min. Cell behavior, including rolling and arrest, was recorded by video microscopy and quantified by analysis of recorded images.

2.8. Statistics

Unless otherwise indicated, quantitative data are presented as mean ± standard deviation (SD) from at least three independent experiments. Statistical significance for comparisons between two groups was assessed using two-tailed unpaired Student's *t*-tests. A $p < 0.05$ was considered statistically significant. Representative images and flow cytometry plots are shown from at least three independent experiments repeated with comparable results.

2.9. Study Approval and Human Samples

The study was conducted in accordance with institutional guidelines and ethical standards applicable at the time the human samples were obtained. Written informed consent was obtained from all healthy adult donors. No identifying information was associated with the samples.

2.10. AI Tool Disclosure

AI-assisted tools (ChatGPT, GPT-5.3 model, OpenAI) were used exclusively for language editing, improvement of text clarity, and refinement of author-generated schematic illustrations (Figure 1a and 1b). No AI tools were used for data generation, analysis, or interpretation. All scientific content and conclusions were generated and verified by the authors.

3. Results

To determine whether monocytes can engage CD44–hyaluronan as a rolling mechanism when α4-integrin engagement is limited, we compared monocyte interactions with cellular monolayers that differ in adhesive properties and in their support of α4-integrin–dependent interactions. Throughout this study, authenticated endothelial monolayers (HMEC-1 and bEnd.3) were used as reference systems for α4-integrin–dependent adhesion, whereas ECV304 cells were used as a T24-derived, hyaluronan-presenting cellular substrate to analyze CD44-dependent interactions under reductionist conditions.

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3.1. Distinct α4-Integrin– and CD44-Dependent Adhesion Pathways Are Revealed on Different Cellular Monolayers

WEHI 78/24 monocytoid cells express major adhesion receptors including L-selectin and α4 integrins [39]. To assess whether these cells can engage CD44-hyaluronan independently of α4-integrin ligands, we compared their adhesion to HMEC-1, bEnd.3 and ECV304 monolayers in shaking adhesion assays (Figure 2). Adhesion to HMEC-1 and bEnd.3 monolayers was inhibited by function-blocking antibodies against α4 integrin, whereas the same treatment had no detectable effect on adhesion to ECV304 cells. Conversely, function-blocking anti-CD44 antibodies selectively inhibited adhesion to ECV304 monolayers, but not to HMEC-1 or bEnd.3. Control antibodies against L-selectin, CD45 and Mac-1 had no detectable effect.

These findings indicate that WEHI 78/24 cells can use two experimentally separable adhesion pathways: an α4-integrin–dependent pathway on HMEC-1 and bEnd.3 monolayers, and a CD44-dependent pathway on ECV304 cells. Thus, under the conditions used here, ECV304 serves not as a surrogate for intact vascular endothelium, but as a cell-based hyaluronan-presenting substrate that allows CD44-mediated adhesion to be examined under conditions of minimal α4-integrin engagement.

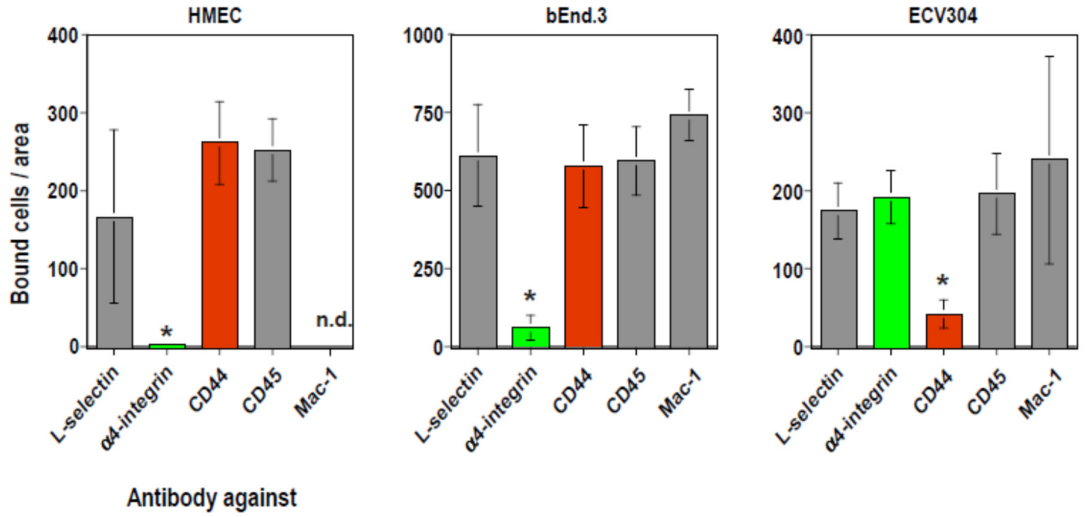

Figure 2. Distinct α4-integrin– and CD44-dependent adhesion pathways in monocytoid cells. Shaking adhesion assays were performed to quantify binding of WEHI 78/24 cells to HMEC-1, bEnd.3 and ECV304 monolayers in the presence of function-blocking antibodies. Adhesion to HMEC-1 and bEnd.3 was inhibited by blockade of α4 integrins (PS/2), whereas adhesion to ECV304 was selectively inhibited by anti-CD44. Control antibodies against L-selectin, CD45 and Mac-1 had no detectable effect. Data are shown as mean ± SD from n = 3. *$p < 0.05$ versus control.

3.2. Rolling Interactions on ECV304 Monolayers Are Weaker than on bEnd.3 Monolayers

To compare rolling supported by α4-integrin–dependent versus CD44-dependent interactions, monolayers of bEnd.3 and ECV304 cells were analyzed in a parallel-plate flow chamber over a range of wall shear stresses from 0.7 to 2 dyn/cm^2 (Figure 3). As shear stress decreased, WEHI 78/24 cells exhibited increased interactions on both substrates. However, across the full range of shear stresses tested, bEnd.3 monolayers supported more interacting cells than ECV304 monolayers. Because bEnd.3 cells provide α4-integrin ligands whereas ECV304 cells do not support detectable α4-integrin–dependent adhesion in this assay system, these findings indicate that α4-integrin–mediated interactions are more efficient than CD44-dependent interactions under otherwise comparable flow conditions. At the same time, the presence of measurable rolling on ECV304 monolayers

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demonstrates that CD44-hyaluronan interactions are sufficient to support rolling when dominant integrin ligands are unavailable.

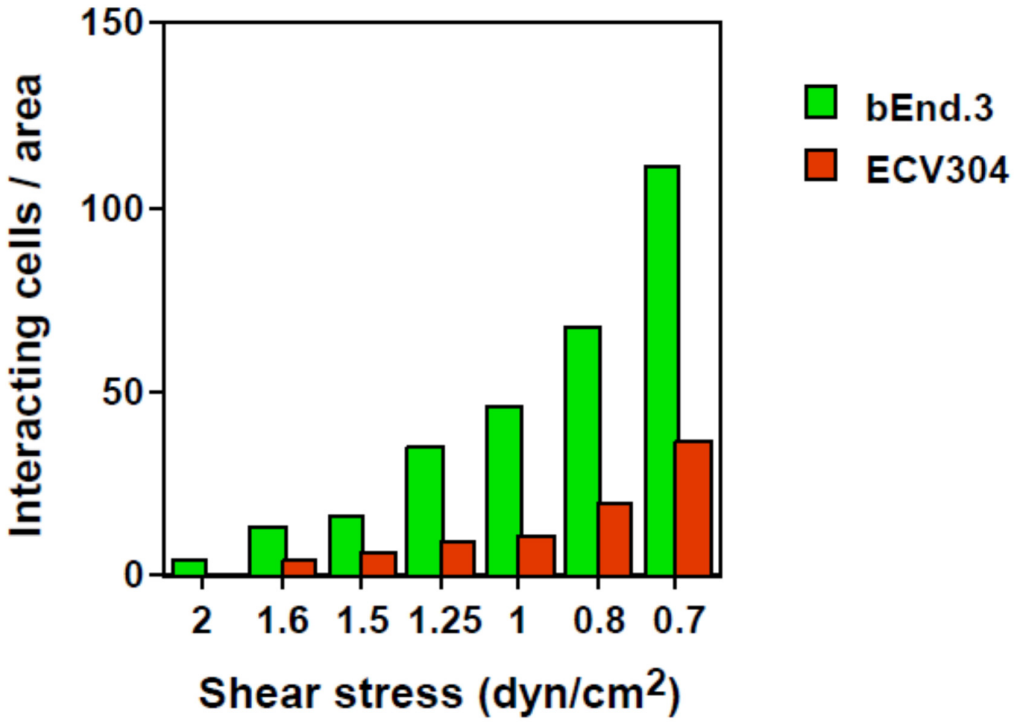

Figure 3. Rolling interactions of WEHI 78/24 cells under defined flow conditions. Rolling and adhesion of WEHI 78/24 cells were analyzed in a parallel-plate flow chamber on bEnd.3 and ECV304 monolayers at wall shear stresses between 0.7 and 2 dyn/cm². Under identical flow conditions, bEnd.3 monolayers supported more interacting cells across the full range tested, whereas interactions on ECV304 monolayers were consistently lower. Data shown are representative of three independent experiments.

3.3. CD44 Mediates Rolling and Arrest of WEHI 78/24 Cells on ECV304 Monolayers via Hyaluronan

To define the mechanism of rolling on ECV304 monolayers, WEHI 78/24 cells were analyzed under low shear stress of 1 dyn/cm² (Figure 4). Under these conditions, cells progressively accumulated on the ECV304 surface over time through a combination of rolling and firm arrest. Pretreatment with a function-blocking anti-CD44 antibody markedly reduced both rolling and arrest, whereas antibodies against L-selectin, α4 integrin, Mac-1 or CD45 had no detectable effect (Figure 4A). Pretreatment with soluble hyaluronan likewise reduced rolling and arrest, although inhibition was less complete than that observed with direct CD44 blockade (Figure 4B).

Together, these results show that CD44 is the principal receptor mediating WEHI 78/24 rolling on ECV304 monolayers under these low-shear conditions, and that hyaluronan is the relevant ligand in this reductionist assay system.

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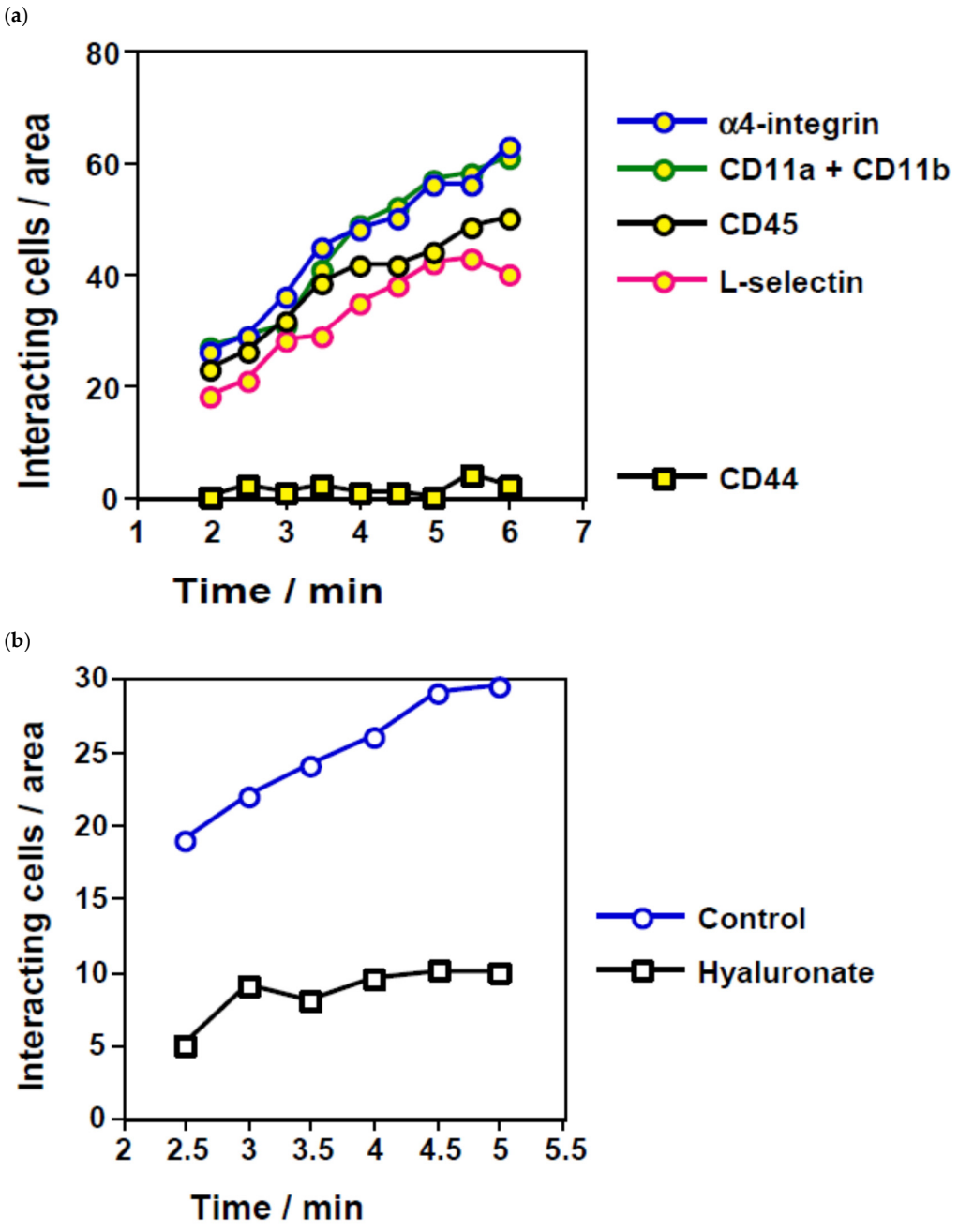

Figure 4. CD44-mediated rolling and arrest of WEHI 78/24 cells at low shear stress. (A) WEHI 78/24 cells were analyzed on ECV304 monolayers in parallel-plate flow chamber assays at 1 dyn/cm^2 following pretreatment with function-blocking antibodies. Anti-CD44 markedly reduced rolling and firm arrest, whereas antibodies against L-selectin, α4 integrin, Mac-1 and CD45 had no detectable effect. (B) Pretreatment with soluble hyaluronan similarly inhibited rolling and arrest, confirming ligand specificity. Data shown are representative of three independent experiments.

3.4. WEHI 78/24 Cells Bind Soluble Hyaluronan via CD44

To determine whether WEHI 78/24 cells directly bind hyaluronan via CD44, FITC-labelled hyaluronan was analyzed by flow cytometry (Figure 5). Untreated WEHI 78/24 cells showed strong hyaluronan binding, whereas pretreatment with a function-blocking anti-CD44 antibody abolished binding and reduced fluorescence to baseline levels. Control antibodies had no detectable effect. These data confirm that CD44 on WEHI 78/24 cells functions as the principal hyaluronan receptor in

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this system and support the interpretation that rolling on ECV304 monolayers is mediated through CD44-hyaluronan interactions.

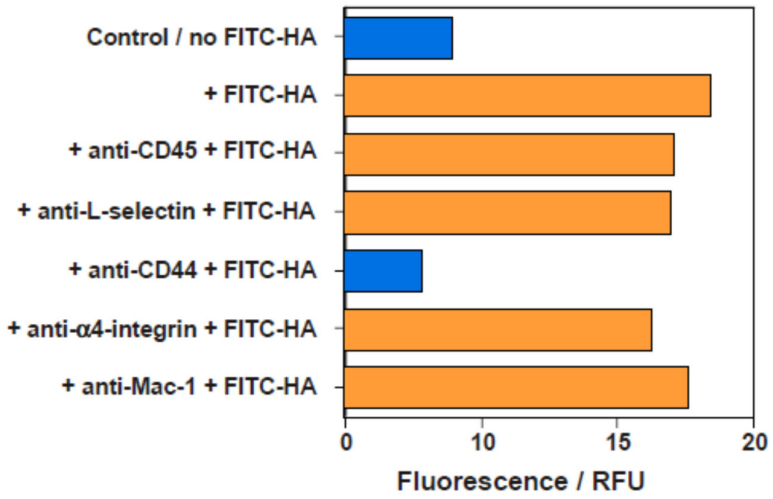

Figure 5. CD44 mediates soluble hyaluronan binding by WEHI 78/24 cells. Flow cytometric analysis demonstrated robust binding of FITC-labelled hyaluronan (FITC-HA) to WEHI 78/24 cells. Binding was abolished by a function-blocking anti-CD44 antibody, whereas control antibodies had no detectable effect. Representative histograms from three independent experiments are shown.

3.5. Surface-Associated Hyaluronan on ECV304 Monolayers Supports Adhesion of WEHI 78/24 Cells

To test whether hyaluronan presented by ECV304 cells contributes to monocyte adhesion, static adhesion assays were performed in the presence of blocking antibodies, soluble hyaluronan, or hyaluronidase treatment (Figure 6). Binding of WEHI 78/24 cells to ECV304 monolayers was inhibited by anti-CD44, but not by antibodies against CD45 or L-selectin (Figure 6, left). Soluble hyaluronan partially reduced binding (Figure 6, middle), consistent with competition for CD44. Enzymatic treatment of ECV304 monolayers with increasing concentrations of hyaluronidase led to a dose-dependent reduction in adhesion (Figure 6, right).

These results demonstrate that adhesion of WEHI 78/24 cells to ECV304 monolayers depends on CD44 and on surface-associated hyaluronan. Within the limits of this assay, they support the interpretation that hyaluronan presented by the cellular substrate is required for the observed CD44-dependent adhesion.

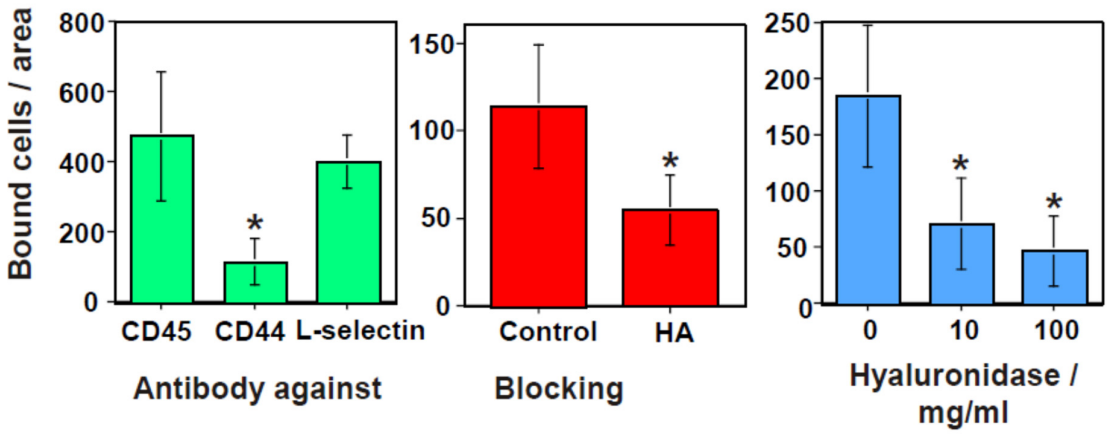

Figure 6. CD44-hyaluronan-dependent adhesion of WEHI 78/24 cells to ECV304 monolayers. Adhesion of WEHI 78/24 cells to ECV304 monolayers was assessed in static assays following pretreatment with function-blocking

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anti-CD44 antibodies (left), soluble hyaluronan (middle), or hyaluronidase treatment of the monolayer (right). Anti-CD44 and soluble hyaluronan inhibited adhesion, and hyaluronidase reduced adhesion in a dose-dependent manner. Control antibodies, including anti-L-selectin, had no detectable inhibitory effect. Data are shown as mean ± SD from n = 3. *p < 0.05 versus control.

3.6. Primary Human Monocytes use CD44 to Bind Hyaluronan-Presenting ECV304 Monolayers

To determine whether the CD44-dependent interaction observed in WEHI 78/24 cells is conserved in primary cells, human peripheral blood monocytes were analyzed on ECV304 monolayers under static and shaking conditions (Figure 7). Only function-blocking anti-human CD44 antibodies inhibited monocyte adhesion, whereas non-blocking anti-CD44 antibodies such as HERMES-3 and additional control antibodies had no detectable effect. A blocking antibody cocktail directed against P-selectin, β1 integrins and β2 integrins did not reduce adhesion compared with untreated controls.

These findings indicate that primary human monocytes, like WEHI 78/24 cells, can engage CD44-dependent adhesion on hyaluronan-presenting ECV304 monolayers under conditions in which alternative adhesion pathways make no detectable contribution.

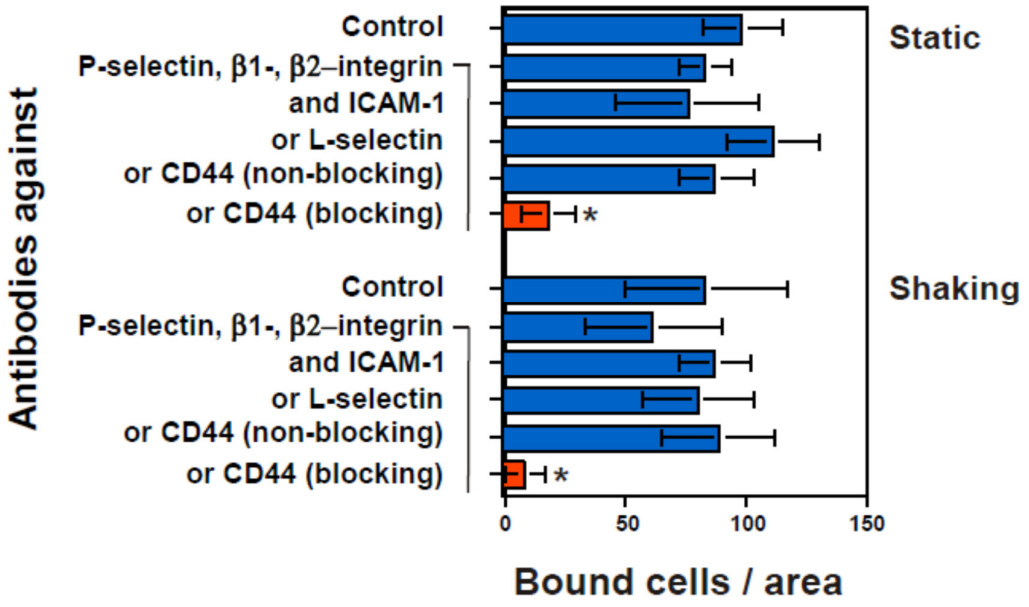

Figure 7. CD44-dependent adhesion of primary human monocytes to ECV304 monolayers. Human monocyte adhesion to ECV304 monolayers was assessed under static and shaking conditions. A function-blocking anti-CD44 antibody (9B5) produced near-complete inhibition of adhesion, whereas the non-blocking anti-human CD44 antibody (HERMES-3) and other control antibodies had no detectable effect. A blocking cocktail against P-selectin, β1 integrins and β2 integrins did not reduce adhesion relative to untreated controls. Data are shown as mean ± SD from n = 3. *p < 0.05 versus control.

3.7. Primary Human Monocytes also Roll on ECV304 Monolayers via CD44 Under Low Shear

Because primary human monocytes represent the most relevant non-transformed cell system in this study, we next tested whether they also exhibit CD44-dependent rolling under flow (Figure 8). At low shear stress of 1 dyn/cm², human monocytes displayed robust rolling interactions on ECV304 monolayers. Pretreatment with a function-blocking anti-CD44 antibody markedly reduced rolling, whereas control antibodies had no detectable effect.

Thus, the CD44-dependent rolling behavior identified in WEHI 78/24 cells is conserved in primary human monocytes. These experiments extend the reductionist findings obtained with the

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murine monocytoid line to human cells and establish that CD44-mediated rolling on hyaluronan-presenting cellular monolayers is not restricted to the transformed model system.

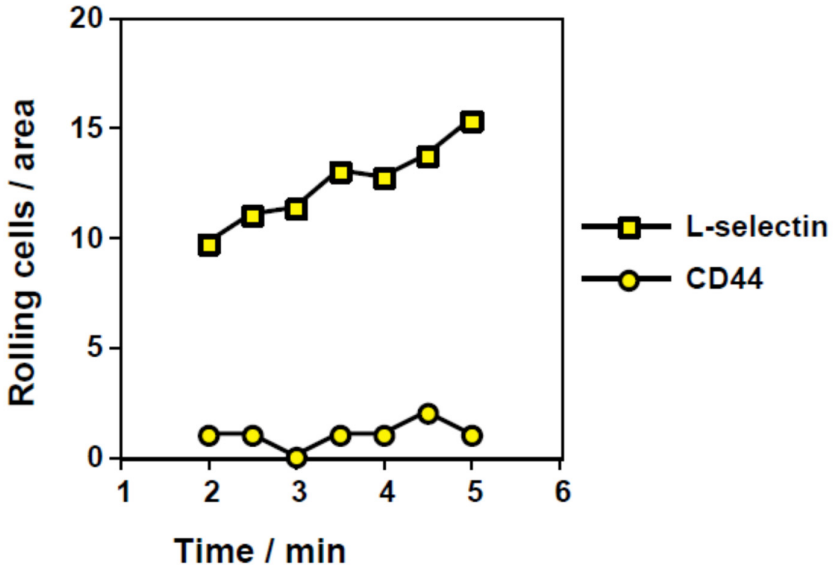

Figure 8. CD44 mediates rolling of primary human monocytes under low shear. Primary human monocytes were analyzed on ECV304 monolayers in parallel-plate flow chamber assays at 1 dyn/cm^2. A function-blocking anti-CD44 antibody markedly reduced rolling interactions, whereas control antibodies had no detectable effect. These findings demonstrate that CD44-dependent rolling on hyaluronan-presenting cellular monolayers is conserved in human monocytes. Data shown are representative of three independent experiments.

4. Discussion

CD44–hyaluronan interactions have been studied before as contributors to leukocyte adhesion and rolling under inflammatory and tissue-specific conditions [12,17–19]. The present study does not identify CD44 as a new rolling receptor, nor does it seek to redefine the established importance of integrins in monocyte recruitment. Rather, our data address a narrower mechanistic question: whether CD44-hyaluronan interactions are sufficient to support monocyte rolling under defined low-shear conditions when dominant α4-integrin–ligand interactions are absent or functionally minimized. Under these experimental conditions, CD44-dependent rolling becomes clearly detectable and can be separated from the stronger α4-integrin–dependent pathway.

This distinction is important because, in most physiological and experimental settings, adhesion pathways are not examined in isolation. Integrin ligands such as VCAM-1 and ICAM-1 typically dominate rolling, arrest and firm adhesion, making it difficult to define the contribution of weaker alternative interactions. By comparing monocyte interactions across cellular substrates with different adhesive properties, our data reveal a hierarchical organization in which α4-integrin–dependent interactions dominate when available, whereas CD44–hyaluronan interactions become functionally apparent when integrin engagement is limited. These pathways should not be viewed as mutually exclusive but rather as context-dependent modules that can be differentially engaged depending on ligand availability, shear conditions and cellular activation states. In this sense, the conceptual advance of the present study is not the identification of CD44 as a hyaluronan receptor or as a leukocyte adhesion molecule—both of which are well established—but the demonstration of conditional sufficiency and hierarchy in a monocyte context. An important limitation of the present study is the use of ECV304 cells. ECV304 cells were originally described as endothelial-like, but were later shown to be derived from the T24 bladder carcinoma line. We therefore do not interpret ECV304 as a surrogate for authentic vascular endothelium. In the present study, ECV304 serves as a T24-

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derived, hyaluronan-presenting cellular monolayer that lacks detectable α4-integrin–dependent adhesion in our assay system and thereby provides a useful reductionist substrate for dissecting CD44-hyaluronan interactions. The findings obtained on ECV304 should therefore be interpreted as a mechanistic demonstration in a controlled cell-based model, not as a comprehensive representation of endothelial adhesion biology in vivo.

A further aspect supporting biological relevance is the existence of abnormal vessel-like structures in tumors, including tumor cell-lined channels or vasculogenic mimicry, which can create non-canonical cellular interfaces under low-shear conditions. In such pathological environments, circulating monocytes may encounter hyaluronan-presenting cellular surfaces that are more similar to tumor-derived monolayers than to intact vascular endothelium. Thus, although ECV304 cells are not interpreted here as bona fide endothelial cells, their use as a T24-derived hyaluronan-presenting cellular substrate may still capture aspects of tumor-associated cellular interfaces that could support direct CD44-hyaluronan-dependent monocyte adhesion under low-shear conditions.

This distinction also clarifies why our conclusions are intentionally limited. We do not claim that monocyte rolling in vivo is generally mediated by CD44 on vascular endothelium in the absence of selectins or integrins. Instead, we show that monocytes are capable of rolling through CD44-hyaluronan interactions when stronger α4-integrin–dependent interactions are unavailable. This reductionist approach complements, rather than replaces, more complex in vivo studies. It helps explain why CD44-dependent rolling can be difficult to detect in systems in which integrin pathways are fully active and dominant.

The observation that rolling on ECV304 monolayers is weaker than on bEnd.3 monolayers across a defined range of shear stresses further supports this interpretation. bEnd.3 cells, which support α4-integrin–dependent interactions, consistently promoted more rolling and transient arrest than ECV304 cells. Thus, when both pathways are functionally available, integrin-dependent rolling is more efficient. CD44-mediated rolling therefore appears not to compete with α4-integrin pathways on equal terms, but rather to represent a weaker adhesion mechanism that becomes functionally relevant in a distinct adhesive context. This interpretation is consistent with the broader leukocyte adhesion literature, in which the relative contribution of adhesion pathways depends on receptor–ligand affinity, ligand density, force loading and wall shear stress [7,40].

One plausible explanation for the low-shear dependence of CD44-mediated rolling relates to receptor topography. Efficient tethering and rolling under flow depend on the spatial presentation of adhesion receptors on the leukocyte surface, particularly their localization on microvilli [41]. Molecules excluded from microvillous tips are less efficient in initiating contacts under higher shear. CD44 was shown to be largely excluded from microvilli in the leukocyte subsets analyzed in that study, in contrast to selectins, which are enriched at microvillous tips. Although monocytes were not specifically analyzed there, this provides a structural framework for why CD44-hyaluronan interactions in our study become apparent primarily under low-shear conditions. In this view, CD44-mediated rolling is expected to be weaker and more context-dependent than classical selectin- or integrin-based rolling.

The present data also help to place previous in vivo observations into a clearer mechanistic framework. CD44-hyaluronan interactions have been implicated in leukocyte rolling and tissue recruitment in several settings, including uveoretinitis, inflamed liver sinusoids and vascular inflammatory models [15,17,18]. Those studies established in vivo relevance, but they did not resolve how CD44 function relates hierarchically to stronger adhesion pathways such as α4-integrin–VCAM-1. Our results suggest that CD44-dependent rolling may be underappreciated in many systems precisely because it is masked by dominant integrin engagement. Once this dominant pathway is experimentally removed, CD44-hyaluronan interactions are sufficient to sustain rolling and transient arrest.

The issue of alternative CD44 ligands also deserves comment. CD44 has been reported to interact with non-hyaluronan ligands under some conditions, including selectins, and cholesterol-dependent redistribution of CD44 has been shown to modulate monocyte rolling in the context of E-selectin [42].

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However, in our system, rolling was inhibited by soluble hyaluronan and by hyaluronidase treatment of the cellular monolayer, strongly supporting a hyaluronan-dependent mechanism. Moreover, blockade of classical selectin pathways did not measurably inhibit rolling in the murine monocytoid assays. Together, these observations indicate that hyaluronan-dependent, rather than selectin-dependent, CD44 interactions dominate under the present conditions. This does not exclude a role for CD44–selectin cooperation in other settings, but it argues against it as the major explanation for the rolling observed here.

An additional point to consider is that activated ECV304 cells have been reported to express VCAM-1 under certain conditions [43]. Under the experimental conditions used here, we did not detect a functional contribution of α4-integrin–dependent adhesion. Our conclusions therefore do not rest on the assumption that ECV304 cells are inherently devoid of all endothelial adhesion molecules under all conditions. Rather, they rest on the functional observation that α4-integrin blockade did not reduce adhesion or rolling in our assays on ECV304 monolayers, whereas CD44 blockade, soluble hyaluronan and hyaluronidase treatment did. Thus, within the conditions used here, α4-integrin–dependent adhesion was not detectably contributing to the observed phenotype. This functional readout is central to our argument and is more relevant for interpretation of the present experiments than the possibility of inducible VCAM-1 expression under different conditions.

Importantly, the human monocyte experiments strengthen the biological relevance of the study and should not be viewed merely as confirmatory add-ons. Primary human monocytes showed the same qualitative dependence on CD44 when interacting with ECV304 monolayers under both static/shaking conditions and low-shear flow. Inhibitory anti-CD44 antibodies markedly reduced adhesion and rolling, whereas non-blocking anti-CD44 antibodies and other control antibodies had no detectable effect. This establishes that the adhesion hierarchy observed in WEHI 78/24 cells is conserved beyond a single murine monocytoid cell line and can also be detected in human monocytes. While additional quantitative analyses of rolling velocity and selectin comparison would certainly be valuable in future work, the present human data are sufficient to support the central mechanistic conclusion that CD44 can mediate monocyte rolling under defined low-shear, hyaluronan-dependent conditions.

The physiological significance of this mechanism is likely to be restricted to specific vascular contexts rather than representing a general pathway of monocyte recruitment. CD44-hyaluronan interactions are most plausibly relevant in low-shear, hyaluronan-rich microenvironments, such as specialized vascular beds or pathologically remodeled tissues, where integrin ligand availability is low or where local architecture reduces the efficiency of classical adhesion pathways. In that sense, CD44-dependent rolling may act as a low-affinity, context-dependent adhesion module that facilitates transient interactions that enable vascular scanning and tissue surveillance, allowing circulating cells to sample local endothelial or cellular environments before committing to firm adhesion or transmigration.

This framework may also be relevant when dominant pathways are therapeutically blocked. α4-integrin blockade is an established treatment strategy in multiple sclerosis, highlighting the importance of α4–VCAM-1 interactions in leukocyte recruitment to the central nervous system. Our findings do not challenge that paradigm. Rather, they suggest that under conditions in which α4-integrin engagement is limited, residual adhesive interactions mediated by CD44-hyaluronan may become more apparent, especially in hyaluronan-rich or low-shear environments. Consistent with this possibility, anti-CD44 treatment reduced disease in experimental autoimmune encephalomyelitis [44]. Extrapolation to human disease clearly requires caution, but the present results provide a mechanistic basis for considering integrin-independent rolling modules in settings of therapeutic α4 blockade.

A similar logic may apply in vascular inflammatory disease. CD44 has been implicated in atherosclerosis and inflammatory cell recruitment, and hyaluronan is a dynamic component of the luminal glycocalyx whose organization influences leukocyte interactions [16,19,20]. In addition, endothelial changes that promote monocyte adhesion have been described in experimental models

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of atherogenesis. Pathological vascular environments may therefore provide conditions under which CD44–hyaluronan-mediated rolling becomes functionally relevant. Inflamed tissues and tumor-associated vasculature are characterized by pronounced structural and hemodynamic abnormalities, including irregular vessel architecture, heterogeneous vessel diameters, sluggish or intermittent blood flow, and regions of markedly reduced shear stress [45–47]. Such low-shear environments are predicted to favor adhesion mechanisms based on weaker, non-integrin interactions, in contrast to canonical integrin–ligand pathways that sustain rolling and arrest under higher shear conditions. In this context, CD44–hyaluronan interactions may contribute to transient rolling or retention phenomena when integrin ligand availability is limited or when local flow conditions fall below the threshold required for efficient integrin-mediated adhesion.

A further point that may increase the biological relevance of this reductionist system is the existence of abnormal vessel-like structures in tumors, including tumor cell-lined channels described as vasculogenic mimicry [48–50]. Although vasculogenic mimicry remains a specialized and debated phenomenon, it illustrates how non-canonical cellular interfaces can generate atypical flow patterns and shear conditions distinct from those of normal microvessels. In such pathological environments, circulating monocytes may encounter hyaluronan-presenting cellular surfaces that are more similar to tumor-derived monolayers than to intact vascular endothelium. Thus, although ECV304 cells are not interpreted here as bona fide endothelial cells, their use as a T24-derived hyaluronan-presenting cellular substrate may still capture aspects of tumor-associated cellular interfaces that could support direct CD44–hyaluronan-dependent monocyte adhesion under low-shear conditions. Under these circumstances, altered receptor topography and reduced shear forces could permit CD44–hyaluronan-mediated contacts that would be inefficient in intact, high-shear vascular endothelium.

Importantly, hyaluronan is a dynamically regulated component of the luminal glycocalyx and can be organized into adhesive structures on vascular surfaces that support leukocyte interactions under flow [19,20,51,52]. Moreover, dysregulation of hyaluronan synthesis and accumulation of hyaluronan-rich microenvironments are common features of many tumors [53]. Because hematogenous dissemination requires circulating cells to engage vascular surfaces under flow, adhesion mechanisms characterized in monocytes may reveal principles that are also exploited by rare metastasis-initiating tumor cells [54–57]. In this sense, tumor cells may co-opt physiological leukocyte adhesion mechanisms, including CD44–hyaluronan interactions, to facilitate transient vascular contacts under permissive flow conditions. While the present study does not address tumor cell behavior directly, these observations provide a plausible biological setting in which CD44–hyaluronan-mediated rolling could contribute to transient interactions of monocytes—or other CD44-expressing cells—with cellular monolayers under low-shear pathological conditions. This interpretation is necessarily speculative but consistent with established principles of leukocyte adhesion biology.

Beyond immune cell trafficking, CD44–hyaluronan interactions are also discussed in the context of tumor cell dissemination, where transient adhesive contacts may contribute to multistep homing and vascular interactions of circulating tumor cells [58–61]. Experimental modulation of adhesion molecules on vascular surfaces can alter metastatic patterns in vivo, illustrating the principle that vascular ligands can shape dissemination routes [62]. CD44 variant isoforms have been linked to metastatic phenotypes in experimental models and are detected in human tumors [59,60,63]. More broadly, these concepts intersect with cancer stem cell frameworks that aim to explain how rare tumor subpopulations can drive progression [64]. Modern liquid biopsy approaches quantify tumor-derived material in blood, including circulating tumor cells and cell-free DNA [57,65–70].

In summary, this study does not claim discovery of CD44-mediated rolling or seek to overturn the established role of selectins and integrins in leukocyte recruitment. Instead, it provides mechanistic clarification of how CD44-hyaluronan interactions can independently support monocyte rolling under defined low-shear conditions and demonstrates that this pathway becomes functionally apparent when dominant α4-integrin–ligand interactions are limited. By confirming this behavior in both murine monocytoid cells and primary human monocytes, the study defines CD44-mediated

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rolling as a context-dependent, hierarchically unmasked adhesion mechanism whose contribution is likely to be restricted to specific adhesive environments.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki. Under U.S. federal regulations in effect at the time of the research (1997–1998), specifically 45 CFR 46.101(b)(2), the collection of peripheral blood from healthy adult volunteers for non-interventional, minimal-risk immunological research was exempt from full Institutional Review Board review. Accordingly, no individual ethics approval number or waiver certificate was issued.

Informed Consent Statement: Written informed consent was obtained from all healthy adult volunteers prior to blood donation. No identifying information was associated with the samples.

Data Availability Statement: The original contributions presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author(s).

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Conflicts of Interest: Marcus Hubbe is currently employed by Pfizer Pharma GmbH. This employment is unrelated to the subject matter of the present manuscript. Pfizer Pharma GmbH had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results. Robert H. Eibl declares no conflicts of interest.

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