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Article

Separating CD44-Mediated Monocyte Rolling from Dominant VLA-4 Adhesion

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Abstract

Leukocyte recruitment from blood into tissues involves sequential adhesive steps, including rolling and integrin-dependent arrest. The integrin VLA-4 is known to mediate firm adhesion, but can also support rolling. CD44–hyaluronan interactions have also been implicated in leukocyte rolling. Here, we used parallel-plate flow chamber assays to compare the contributions of CD44 and VLA-4 to monocyte rolling on different cellular monolayers. Monocytoid WEHI 78/24 cells rolled and adhered through CD44 on hyaluronan-presenting ECV304 monolayers, whereas VLA-4 dominated adhesion on endothelial monolayers expressing functional VCAM-1. Primary human monocytes showed similar CD44-dependent rolling on ECV304 monolayers. Blocking CD44, adding soluble hyaluronan, or removing surface hyaluronan with hyaluronidase reduced rolling and adhesion. These results show that CD44 can support monocyte rolling when VLA-4/VCAM-1 adhesion is not the dominant interaction. This cell-based flow model distinguishes CD44/hyaluronan-mediated rolling from VLA-4/VCAM-1-rolling and may help analyze monocyte rolling on hyaluronan, including tumor-derived monolayers.

Keywords: CD44; hyaluronan (hyaluronate; HA); VLA-4; VCAM-1; monocyte; rolling; leukocyte trafficking; parallel-plate flow chamber; cell adhesion

1. Introduction

Leukocyte recruitment to tissues proceeds through tethering, rolling, arrest, and transmigration under shear flow [1–3]. Rolling interactions are classically mediated by selectins, whereas firm adhesion and arrest largely depend on integrins such as α4β1 (VLA-4) and αLβ2 (LFA-1) [4,5]. In addition to classical selectin ligands and integrins, other glycosylated leukocyte surface molecules can contribute to adhesive interactions under flow. These include heat-stable antigen/CD24, which has been implicated in myeloid cell interactions with endothelial or platelet P-selectin under shear conditions [6,7], and CD44, a major hyaluronan receptor involved in leukocyte adhesion and trafficking [8–11]. Chemokine-mediated integrin activation can trigger rapid arrest under flow [12]. In monocytes, integrin-dependent adhesion pathways are particularly important for inflammatory recruitment to tissues [13–15]. After extravasation, monocytes differentiate into macrophages or monocyte-derived dendritic cells and contribute to tissue homeostasis and immune responses [16,17]. CD44–hyaluronan (HA; also termed hyaluronate) interactions can support rolling of lymphocytes under flow and usually generate weaker, more transient interactions than integrin-mediated adhesion [8,9]. CD44, originally described as the Hermes lymphocyte homing-associated antigen [10,11], has since been linked to leukocyte trafficking in several inflammatory models. Blocking CD44–hyaluronan interactions reduces leukocyte rolling and infiltration in experimental

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autoimmune uveoretinitis [18]. In lipopolysaccharide (LPS)-induced airway inflammation, CD44-deficient mice show impaired macrophage recruitment and reduced endothelial adhesion of cells, supporting a role for CD44 in monocyte/macrophage trafficking [19]. However, under physiological flow conditions, CD44-dependent rolling is frequently superimposed by dominant integrin-mediated interactions, particularly involving VLA-4/VCAM-1. This makes it difficult to experimentally dissect the relative contribution of CD44-mediated rolling in cell-cell interaction systems. 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.

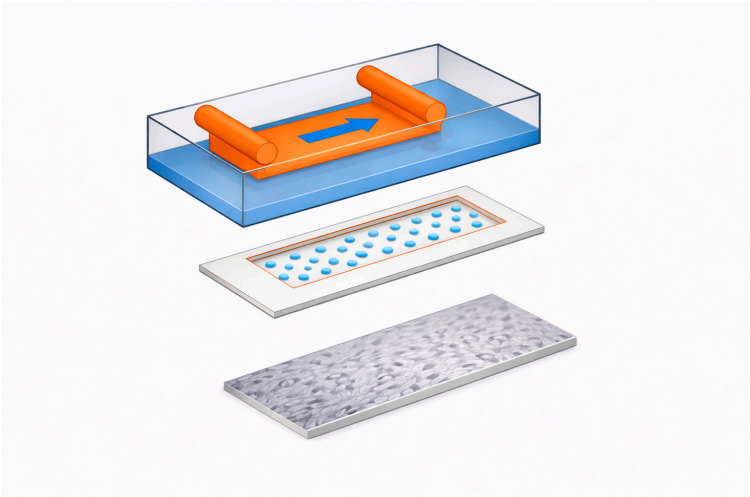
To experimentally distinguish CD44-mediated rolling from dominant α4-integrin-dependent adhesion, we compared monocyte interactions with different cellular monolayers under shear flow conditions. Using endothelial and tumor-derived cellular substrates with distinct adhesive properties, we analyzed the relative contributions of CD44, hyaluronan, and α4 integrins to monocyte rolling and transient adhesion. Over the past two decades, substantial work has established CD44-hyaluronan interactions as regulators of leukocyte recruitment and vascular inflammation [20–23]. 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 [24]. 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 cell-based flow system to separate these interactions and show that CD44-mediated rolling can be detected when integrin engagement is reduced. Thus, the study analyzes the contribution of CD44 within established adhesion mechanisms rather than proposing a new adhesion pathway.

2. 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. The flow chamber setup used for rolling measurements is summarized in Figure 1.

(**a**)



(b)

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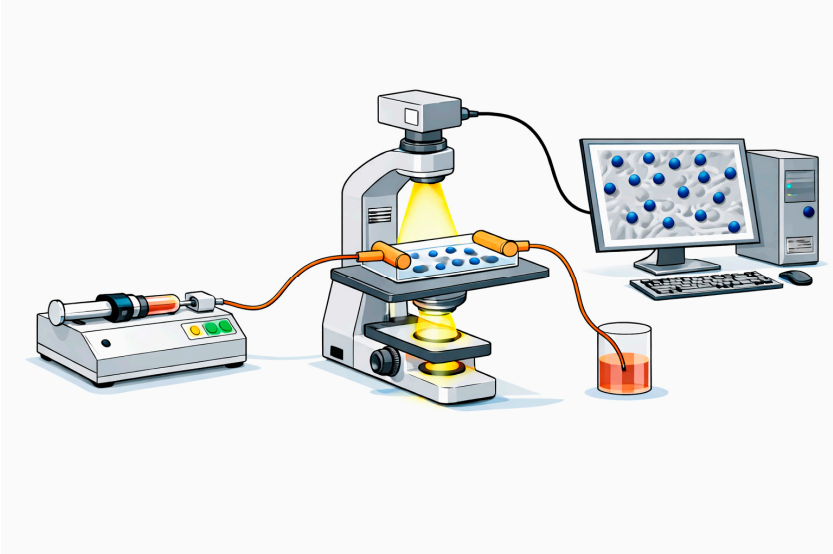

Figure 1. Parallel-plate flow chamber assay for analysis of CD44–hyaluronan-dependent monocyte interactions. **(a)** Schematic representation of the custom-made transparent parallel-plate flow chamber (gap height 250 μm; not drawn to scale). Confluent cellular monolayers are assembled in the chamber and exposed to defined laminar shear flow. The arrow indicates the direction of 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.

2.1. 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 [25]. 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. ECV304 cells were originally described as endothelial cells but were later identified as derivatives of the T24 bladder carcinoma line. In the present study, they were intentionally used as a hyaluronan-presenting cellular monolayer lacking dominant α4-integrin-dependent adhesion. This experimental setting enabled analysis of CD44-mediated rolling interactions under controlled flow conditions.

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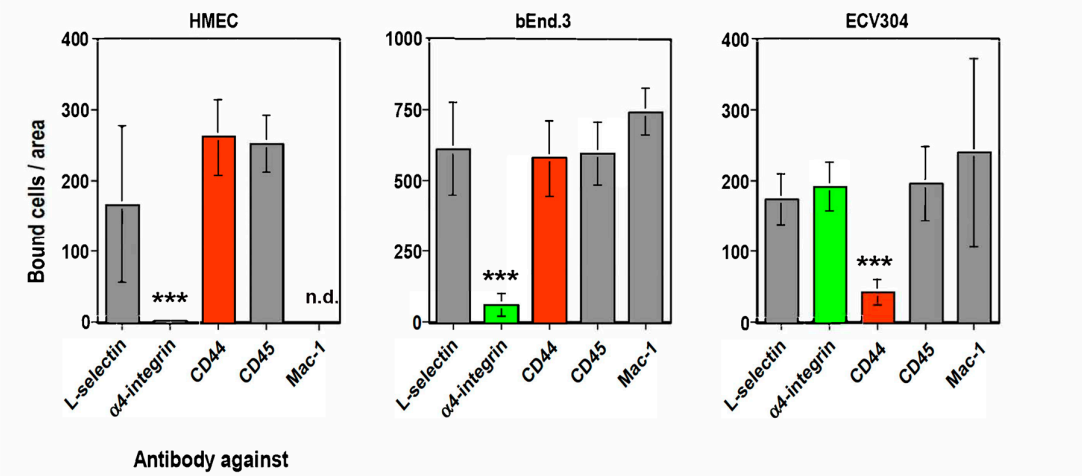

Figure 2. Distinct α4-integrin– and CD44-dependent adhesion pathways in monocytoid cells. Shaking adhesion assays were performed to quantify binding of WEHI 78/24 cells to HMEC-1, bEnd.3 and ECV304 monolayers in the presence of function-blocking antibodies. Adhesion to HMEC-1 and bEnd.3 was inhibited 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 independent experiments. ***p < 0.001 versus CD45 control.

2.2. 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/cm² (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 demonstrates that CD44-hyaluronan interactions are sufficient to support rolling when dominant integrin ligands are unavailable.

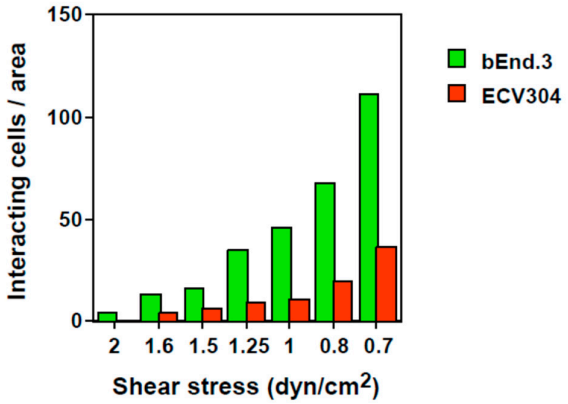

Figure 3. Rolling interactions of WEHI 78/24 cells under defined flow conditions. Rolling and adhesion of WEHI 78/24 cells were analyzed in a parallel-plate flow chamber on bEnd.3 and ECV304 monolayers at wall shear

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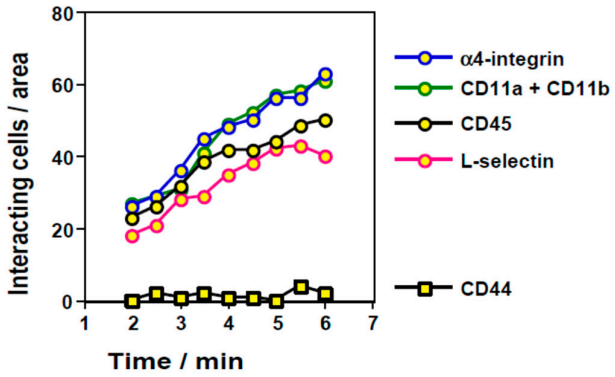
stresses between 0.7 and 2 dyn/cm². Under identical flow conditions, bEnd.3 monolayers supported more interacting cells across the full range tested, whereas interactions on ECV304 monolayers were consistently lower. Data shown are representative of three independent experiments.

2.3. 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.

(a)



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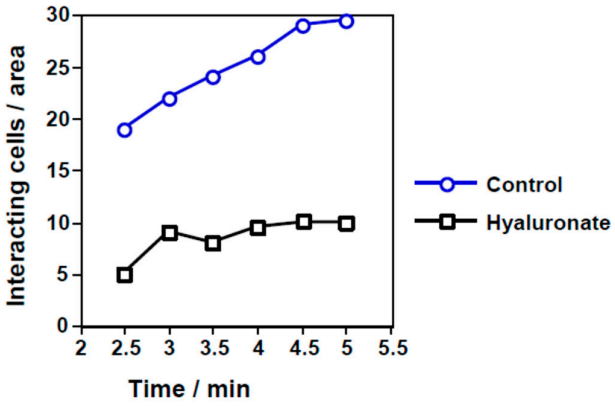

Figure 4. CD44-mediated rolling and arrest of WEHI 78/24 cells at low shear stress. **(a)** WEHI 78/24 cells were analyzed on ECV304 monolayers in parallel-plate flow chamber assays at 1 dyn/cm² following pretreatment with function-blocking antibodies. Anti-CD44 markedly reduced rolling and firm arrest, 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.

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

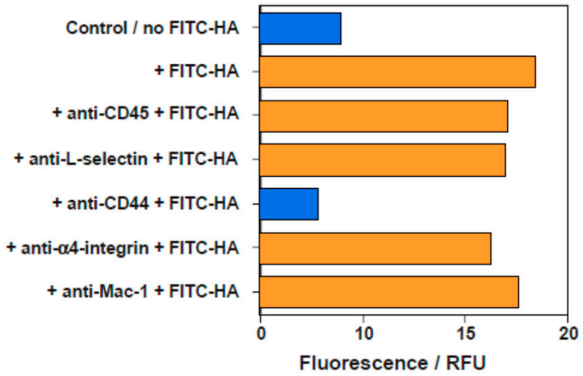

Figure 5. CD44 mediates soluble hyaluronan binding by WEHI 78/24 cells. Flow cytometric analysis demonstrated robust binding of FITC-labelled hyaluronan (FITC-HA) to WEHI 78/24 cells. Binding was abolished by a function-blocking anti-CD44 antibody, whereas control antibodies had no detectable effect.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

2.5. 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.

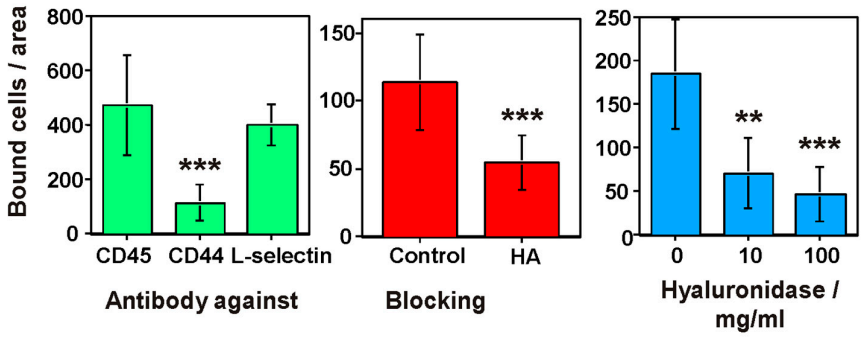

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Figure 6. CD44-hyaluronan-dependent adhesion of WEHI 78/24 cells to ECV304 monolayers. Adhesion of WEHI 78/24 cells to ECV304 monolayers was assessed in static assays following pretreatment with function-blocking anti-CD44 antibodies (left), soluble 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 independent experiments. **p < 0.01, ***p < 0.001 versus control.

2.6. 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.

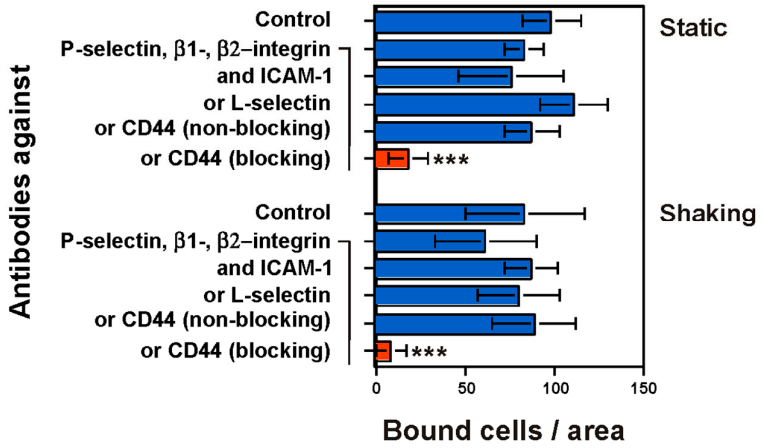

Figure 7. CD44-dependent adhesion of primary human monocytes to ECV304 monolayers. Human monocyte adhesion to ECV304 monolayers was assessed under static and shaking conditions. A function-blocking anti-CD44 antibody (9B5) produced near-complete inhibition of adhesion, whereas the non-blocking anti-human 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 independent experiments. ***p < 0.001 versus control.

2.7. 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 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.

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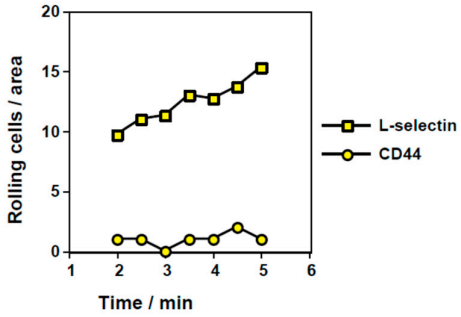

Figure 8. CD44 mediates rolling of primary human monocytes under low shear. Primary human monocytes were analyzed on ECV304 monolayers in parallel-plate flow chamber assays at 1 dyn/cm². A function-blocking anti-CD44 antibody markedly reduced rolling interactions, whereas control antibodies had no detectable 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.

3. Discussion

CD44–hyaluronan interactions have previously been implicated in leukocyte adhesion and rolling under inflammatory and tissue-specific conditions [9,21–23]. Here, we asked whether CD44–hyaluronan interactions can support monocyte rolling under shear flow when dominant α4-integrin-dependent adhesion is absent or reduced. The data show that CD44-dependent rolling can be separated, or unmasked, from stronger α4-integrin-mediated adhesion in a cell-based flow chamber system. While α4-integrin ligands supported stronger adhesive interactions on endothelial monolayers, CD44-dependent rolling was detected on hyaluronan-presenting ECV304 monolayers.

ECV304 cells were originally introduced as endothelial-like cells but are now recognized as derivatives of the T24 bladder carcinoma line. In the present study, they were intentionally used as a hyaluronan-presenting cellular monolayer without dominant α4-integrin-dependent adhesion. The weaker rolling interactions observed on ECV304 compared with bEnd.3 monolayers are consistent with the transient nature of CD44-mediated adhesion under flow [14,26]. Their inhibition by CD44 blockade, soluble hyaluronan competition, and hyaluronidase treatment supports a hyaluronan-dependent mechanism.

One plausible explanation for the low-shear dependence of CD44-mediated rolling relates to receptor topography. As von Andrian and colleagues showed, efficient tethering and rolling under flow depend on the spatial presentation of adhesion receptors on the leukocyte surface, particularly their localization on microvilli [27]. 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 topographical mechanism 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 [18,21,22]. 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 present findings are consistent with in vivo observations demonstrating impaired

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macrophage recruitment and endothelial interactions in CD44-deficient mice during inflammatory airway disease[19]. Our flow chamber system extends these observations by experimentally separating CD44-mediated rolling from dominant α4-integrin-dependent adhesion pathways.

CD44 has been reported to interact with non-hyaluronan ligands, including selectins, and cholesterol-dependent redistribution of CD44 has been shown to modulate monocyte rolling in the context of E-selectin [28]. 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.

Activated ECV304 cells have been reported to express VCAM-1 under certain conditions [29]. In our assays, however, α4-integrin blockade did not reduce adhesion or rolling on ECV304 monolayers, whereas CD44 blockade, soluble hyaluronan, and hyaluronidase treatment did. Thus, the observed interactions were functionally CD44–hyaluronan-dependent rather than α4-integrin-dependent.

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 significant 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.

The physiological significance of this phenomenon 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 mechanism 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 [30]. 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 [20,23,24]. In addition, endothelial changes that promote monocyte adhesion have been described in experimental models 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

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blood flow, and regions of markedly reduced shear stress [31–33]. 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.

Tumor-associated vasculature and abnormal vessel-like structures, including vasculogenic mimicry, can create non-canonical cellular interfaces and altered shear conditions [34–36]. Although the present study does not model tumor vasculature directly, the use of a T24-derived ECV304 monolayer may be relevant for analyzing monocyte interactions with tumor-associated cellular surfaces under flow. Such surfaces may differ from intact vascular endothelium in ligand composition, glycocalyx organization, and local shear conditions.

Hyaluronan is a dynamically regulated component of the luminal glycocalyx and can be organized into adhesive structures that support leukocyte interactions under flow [23,24,37,38]. Altered hyaluronan synthesis and accumulation are also common features of many tumors [39]. In this broader context, CD44–hyaluronan interactions may contribute not only to leukocyte recruitment but also to transient adhesive contacts relevant to tumor-associated inflammation and dissemination [40–43]. Other non-classical adhesion pathways, including L1-dependent binding via VLA-5 integrins, further illustrate that tumor and immune cell interactions can involve adhesion mechanisms beyond the classical selectin/integrin paradigm [44]. Experimental modulation of vascular adhesion molecules can alter metastatic patterns in vivo, supporting the idea that adhesive properties of cellular surfaces influence dissemination routes [45]. These concepts also intersect with cancer stem cell and liquid biopsy frameworks, which emphasize rare circulating or dissemination-competent tumor cell populations [46–52]. The present flow-based model may therefore provide a simplified experimental system for analyzing monocyte interactions with tumor-associated cellular surfaces under shear conditions.

In summary, this study shows that CD44–hyaluronan interactions can support monocyte rolling under defined low-shear conditions when dominant α4-integrin–ligand interactions are absent or reduced. By comparing murine monocytoid cells and primary human monocytes, the data identify CD44-mediated rolling as a weaker but reproducible adhesion mechanism that can be separated experimentally from VLA-4-dependent adhesion. This pathway may be most relevant at inflammatory or tumor-associated cellular surfaces where local flow and ligand presentation differ from intact vascular endothelium.

4. Materials and Methods

4.1. Cells and Reagents

bEnd.3 cells (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 [53], 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 (ATCC CRL-1998) were originally described as spontaneously transformed human endothelial cells [54], but were later shown to be derived from the T24 bladder carcinoma line [55–58]. 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].

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HMEC-1 human dermal microvascular endothelial cells (ATCC CRL-3243) [59] 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 [60] 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 (Sigma; St. Louis, MO, USA; cat. no. H5388), tested (Figure S1) and stored in aliquots as described previously [61].

Antibodies used in this study were as follows: HERMES-3 (mouse IgG2a, anti-human CD44) [62]; 84H10 (anti-human ICAM-1); L133 (anti-human CD31); TY1138 (anti-human VCAM-1); WAPS1.2 (anti-human P-selectin) [63]; DREG56 (mouse IgG1, anti-human L-selectin) [63]; 9B5 (anti-human CD44); IM7.8.1 or TJB1.7 (anti-mouse CD44, depending on experiment); Mel-14 (anti-mouse L-selectin) [64]; PS/2 (rat IgG2b, anti-mouse α4 integrin) [65]; MI/70 (anti-mouse αM/Mac-1) [66]; TIB213 (anti-mouse αL); and 30G12 (rat IgG2a, anti-mouse CD45) [67]. Additional antibodies used in specific experiments are indicated in the corresponding figure legends.

4.2. Monolayer Activation

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 18 h before use in adhesion assays.

4.3. 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.

4.4. 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.

4.5. 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. 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 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.

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4.6. 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.

4.7. Laminar Flow Assays

Parallel-plate laminar flow assays were performed using a custom-made flow chamber (Figure 1a) based on established flow-chamber designs [68-70]. 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. The chamber was mounted on the stage of an inverted phase-contrast microscope. WEHI 78/24 cells or primary human monocytes were resuspended[68–70 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.

4.8. Statistics

Data are presented as mean ± SD from at least three independent experiments unless otherwise indicated. Technical replicates within individual experiments were averaged before statistical analysis. Comparisons involving more than two experimental groups were analyzed using one-way ANOVA followed by appropriate post hoc testing. Pairwise comparisons were analyzed using Student's t-test where applicable. A p value < 0.05 was considered statistically significant. Representative images and flow cytometry plots are shown from at least three independent experiments repeated with comparable results.

4.9. 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.

4.10. AI Tool Disclosure

AI-assisted tools (ChatGPT, OpenAI; GPT-3.5 and 5.5) were used for language editing and 3D-refinement of two 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.

5. Conclusions

This study identifies CD44–hyaluronan interactions as a distinct mechanism supporting monocyte rolling and transient adhesion under low-shear flow when VLA-4–dependent adhesion is

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absent or reduced. In contrast to endothelial monolayers, where VLA-4-mediated adhesion predominated, ECV304 monolayers supported detectable CD44-dependent rolling. The same principle was observed with primary human monocytes. Inhibition by CD44 blockade, soluble hyaluronan, and hyaluronidase supports a hyaluronan-dependent mechanism. These findings separate CD44-mediated rolling from dominant VLA-4 adhesion and suggest that this pathway may contribute to monocyte interactions with inflammatory or tumor-associated cellular surfaces.

Supplementary Materials:

Author Contributions: M.H. designed, performed, and analyzed experiments and contributed to manuscript preparation. R.H.E. designed and performed experiments, analyzed data, collected human samples, wrote the initial manuscript, and finalized writing.

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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.

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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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