CD44 engagement enhances acute myeloid leukemia cell adhesion to the bone marrow microenvironment by increasing VLA-4 avidity


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Abstract
Adhesive properties of leukemia cells shape the degree of organ infiltration and the extent of leukocytosis. CD44 and the integrin VLA-4, a CD49d/CD29 heterodimer, are important factors of progenitor cell adhesion in bone marrow (BM). Here, we report their cooperation in acute myeloid leukemia (AML) by a novel non-classical CD44-mediated way of inside-out VLA-4 activation. In primary AML BM samples from patients and the OCI-AML3 cell line, CD44 engagement by hyaluronan induced inside-out activation of VLA-4 resulting in enhanced leukemia cell adhesion on VCAM-1. This was independent from VLA-4 affinity regulation but based on ligand-induced integrin clustering on the cell surface. CD44-induced VLA-4 activation could be inhibited by the Src family kinase inhibitor PP2 and the multikinase inhibitor midostaurin. In further consequence, the increased adhesion on VCAM-1 allowed AML cells to strongly bind stromal cells. Thereby VLA-4/VCAM-1 interaction promoted activation of Akt, MAPK, NF-kB and mTOR signaling and decreased AML cell apoptosis. Collectively, our investigations provide a mechanistic description of an unusual CD44 function in regulating VLA-4 avidity in AML, supporting AML cell retention in the supportive BM microenvironment.
Introduction

Acute myeloid leukemia (AML) is an aggressive and difficult-to-treat hematological malignancy, characterized by the accumulation of immature myeloid blasts. Within the bone marrow (BM), AML cells interact and communicate with stromal and immune cells and reprogram mesenchymal stromal cells to selectively support leukemic cells, while simultaneously suppressing normal hematopoiesis (1). These microenvironmental interactions contribute to protect leukemic stem cells (LSCs) from chemotherapeutic drugs, thus allowing residual disease after therapy, ultimately causing patient relapses (1). A better understanding of the adhesive mechanisms that facilitate the interactions between AML cells and the supportive microenvironment may pave the way for novel combination therapies antagonizing residual disease.

The glycoprotein CD44 functions by binding to its major ligand hyaluronic acid (HA), which is expressed by BM stromal cells and endothelial cells (2). In AML, targeting CD44 reduced leukemic repopulation in serial transplantations by eradication of leukemic stem cells (3). A second key orchestrator of leukemic cell-BM microenvironment interactions is the integrin VLA-4, a CD49d/CD29 heterodimer. The binding of VLA-4 to its ligand VCAM-1 is strengthened by inside-out signaling. This means that external stimuli mediate intracellular signaling triggered by other cell surface receptors resulting in a change of either the avidity or the affinity of the integrin for its ligands (4). Avidity changes occur due to cluster formation of the integrin, whereas affinity is increased by conformational changes (5). Cooperativity of CD44 and VLA-4 has previously been suggested, but little is known about the mechanism (6-8). To elucidate the mechanistic crosstalk between the two key homing factors, CD44 and VLA-4, to the BM in AML cell lines and primary AML cells, we used adoptive transplantations as well as static and shear flow adhesion assays in combination with immunofluorescence microscopy approaches. We uncovered a novel HA/CD44-induced
inside-out activation of the integrin VLA-4. This activation leads to increased avidity due to VLA-4 clusters but no alterations in affinity between VLA-4 and its ligand VCAM-1. This elevated adhesion is important for AML cell retention in the stromal niche.
Methods

Study approvals and patient sample processing

Following written informed consent, BM aspirates from patients with newly diagnosed AML were collected at the Third Medical Department, Paracelsus Medical University Salzburg, Austria; Ethics Committee Salzburg approval number: 415-E/2009/2-2016. Normal CD34+ progenitor cells from myeloma and non-Hodgkin-lymphoma patients who underwent hematopoietic stem/progenitor cell mobilization were used as non-myeloid controls; Ethics committee Salzburg approval: 415-E/1177/8-2010. Mononuclear cells were isolated using density gradient centrifugation and viably frozen until further usage. For patient characteristics, see Supplemental Table 1.

Animal experimentation approval number is BMWF-66.012/0032-WF/V/3b/2017.

Adoptive transfers

For blocking experiments, primary AML cells or OCI-AML3 cells were pretreated with αCD44 Fab fragments (clone 515, 5 µg/mL) or αCD49d (clone HP2/1, 5 µg/mL) antibodies for 15 min at 37°C, where indicated. Specificity of blockade was confirmed by isotype control experiments in exemplary experiments. For homing versus engraftment assays (3 hours (h) and 3 days (d)), cells were stained using the CellTrace™ Violet Cell Proliferation Kit (Thermo Fisher). 0.3–1.3 x 10^6 cells were injected intravenously (i.v.) into NOD scid gamma (NSG) mice. After 3 h or 3 d, mice were sacrificed, and the number of human cells that had homed to BM, spleen (SPL) and peripheral blood (PB) was detected using αCD44 (clone J.173)- and αCD49d (clone 9F10)-specific antibodies. Homing rate was calculated as the number of CD44 and CD49d double positive cells divided by the number of total measured cells divided by the number of injected AML cells (9, 10). Proliferation after 3 d was determined on basis of CellTrace™ dye dilution rates (10). For long term engraftment (28
days) shCont or shCD49d OCI-AML3 cells were injected i.v. into NSGS mice. After 28 days, mice were sacrificed, and the number of human CD15 and CD45 double positive cells per million measured BM cells, SPL cells or per µl blood was determined.

Clustering assay
VLA-4 clustering assays were performed as described (11), using 7.5 µg/mL VCAM-1/Fc. AML cells were pretreated for 10 min with 10 µg/mL HA, 60 min with 1 µM midostaurin, 30 min with 10 µM PP2 and 30 min with 10 µM cobimetinib (APExBIO, Houston, USA), where indicated. Cells were allowed to adhere for 30 min at 37°C before fixation with 4% paraformaldehyde (PFA). Slides were stained with αCD49d (clone AHP1225), αCD29 (clone 12G10) primary antibodies or isotype control (not shown) followed by secondary antibody. For CD49d cluster analysis of normal progenitor cells from patients with non-myeloid malignancies, cells were additionally stained with αCD34 antibody (clone QBEND-10). For quantification, high-resolution images were acquired on a Leica TCS SP5 II laser-scanning microscope using a 63x/1.4-NA oil-immersion objective (Leica, Wetzlar, Germany). The number of clusters was analyzed using ImageJ software by particle analysis setting the size of the particle to >2 pixels (12).

Stroma binding
Falcon culture slides were left either uncoated or coated with 20 µg/mL fibronectin for 1 h at 37°C. 70 000 M2 stromal cells were seeded and cultured overnight. Primary AML (1 x 10^6 cells) or OCI-AML3 cells (0.5 x 10^6 cells) were seeded on M2 stromal cells and co-cultured for 30 min 37°C. Cells were washed, fixed with 4% PFA, and stained using DAPI Antifade Reagent. Images were taken with an Olympus IX81 microscope (UPLSAPO 20xO/0.85
objective). Numbers of cells were determined in 12 pictures for each treatment with ImageJ software.

Additional experimental procedures are described in Supplemental Methods.
Results

CD44 reflects and mediates leukemic infiltration of BM

We measured CD44 and CD49d surface expression of BM-derived primary AML patient samples and the AML cell line OCI-AML3 by flow cytometry. All AML patient-derived blasts, identified via CD45/side scatter (SS) gating (13), and OCI-AML3 cells, expressed CD44 and CD49d (Figure 1A). We also screened various other AML cell lines, which cover most of the AML subtypes, i.e. MV4-11, KG-1a, HL-60, MOLM-13 and MOLM-14, and found a similar expression pattern (Supplemental Figure 1A).

We next determined the \textit{in vivo} contribution of CD44 and CD49d to homing of AML cells by performing short-term adoptive transfer experiments of primary human AML cells as well as OCI-AML3 cells in immunodeficient NSG mice. In 5 independent experiments, total mononuclear cells from BM aspirates of 5 different AML patients (2 patients with wild-type FLT3, 3 patients with FLT3-ITD mutations) with a blast content of over 75% were either left untreated or treated with \(\alpha\)CD44 Fab fragment (clone 515) or \(\alpha\)CD49d antibody (clone HP2/1) and intravenously (\textit{i.v.}) injected into NSG mice. Mice were sacrificed after 3 h (short-term homing, allowing leukemia cell entry into organs but no proliferation), and transplanted cells were flow cytometrically identified in spleen and BM by human-specific antibodies (Figure 1B). Cells that had been treated with the blocking \(\alpha\)CD44 Fab fragment had a lower capacity to home to BM within 3 h compared to untreated cells. The homing of primary human AML cells to the spleen was strongly diminished upon CD44 blockade. In contrast, \(\alpha\)CD49d antibody treatment only slightly reduced BM homing of AML blasts and had no effect on their spleen homing (Figure 1C i). In 4 of the 5 homing experiments we combined \(\alpha\)CD44/\(\alpha\)CD49d treatment, but additional CD49d blockade did not further elevate the inhibitory effect above the level achieved by single \(\alpha\)CD44 treatment (Supplemental Figure 1B). Comparable effects were observed when using OCI-AML3 cells (Figure 1C ii). The
significantly reduced recovery of αCD44-treated cells was not due to toxicity of the antibody, as in vitro treatment for 3 h had no effect on cell viability (Supplemental Figure 2A). Neither the functional inhibition of CD44 nor the inhibition of CD49d did affect the general CD44 and CD49d expression of the cells (Supplemental Figure 2B). Anti-CD44 antibody treatment did not affect CD44/E-selectin mediated cell arrest (Supplemental Figure 2C). We genetically confirmed the CD44 contribution to homing by a CD44 knockdown in OCI-AML3 (Supplemental Figure 2D ii), observing a significant reduction in homing of CD44 low cells (Figure 1D i). These cells also showed reduced rolling on HA substrates under shear flow (Supplemental Figure 2D i). CD49d knockdown, which was confirmed via qPCR, did slightly reduce homing and arrests of CD49d knockdown OCI-AML3 on VCAM-1 substrate were diminished (Figure 1D ii and Supplemental Figure 2Ei+ii). Concurrent 3 days post-transplantation analysis allowed us to investigate not only homing but also early engraftment that includes first proliferation events (14). We noted equal numbers of primary AML cells and OCI-AML3 cells at 3 h and 3 days in BM while in spleen leukemic recovery was diminished after 3 days (Figure 1E). In concordance, recovered AML cells had undergone more cell divisions in BM than in spleen at this time (Supplemental Figure 2Fi+ii), indicating that the BM rather than the spleen microenvironment provides supportive signals for leukemic engraftment. Furthermore, when NSGS mice were engrafted with human AML cells and afterwards treated with a αCD44 antibody, the AML pool shifted from BM to spleen one day after treatment, suggesting CD44 as a BM retention factor (Supplemental Figure 2G). In summary, we found that CD44 plays a key role in homing of AML cells to murine BM and spleen, with the BM providing a favorable environment for early engraftment of AML.
**HA/CD44 interaction triggers an inside-out activation of VLA-4 in AML**

To dissect the AML homing process in a mechanistic manner, we used *in vitro* flow chamber assays, as described (15). These assays allowed to study the individual and combined interactions of CD44 and VLA-4 expressed on AML cells with the respective ligands HA and VCAM-1. First, we perfused OCI-AML3 cells over immobilized HA substrate under shear stress. We found a strong capacity of the cells to tether and roll on this substrate, which was fully abolished upon treatment with $\alpha$CD44 blocking antibody (clone 515) (Figure 2A). We further confirmed this HA binding capacity by flow cytometry using fluorescein-labeled HA (HA-FITC) (Supplemental Figure 3A). Next, we tested whether AML cells were capable of tethering to the VLA-4 ligand VCAM-1 under shear flow conditions. Unstimulated OCI-AML3 and primary AML cells from 5 different patients bound immobilized VCAM-1 at low adhesive strength, which was evident by low frequency of firm adhesion on this substrate (Figure 2Bi+ii). Treatment with a blocking $\alpha$CD49d antibody abrogated all interactions of the OCI-AML3 and primary cells with the immobilized VCAM-1, confirming the VLA-4 dependency of this process (Figure 2B i, Supplemental Figure 3B). Notably, pre-treating AML cells with the CD44 ligand HA and then perfusing the cells over a VCAM-1 substrate increased adhesion, without changing CD44 or CD49d surface expression (Figure 2B i+ii and Supplemental Figure 3C), which suggests HA/CD44-induced inside-out VLA-4 activation. A co-immobilization of both ligands HA and VCAM-1 resulted in strong CD49d-dependent adhesive capacity of the leukemia cells, suggesting inside-out activation rather than mere additive effects in adhesion (Figure 2C). CD44-mediated inside-out signaling is well known to vary by high versus low molecular weight HA triggering (reviewed in (16)). Indeed, CD44-induced VLA-4 activation was only achieved by high molecular weight HA (HMW-HA), but not by low molecular weight HA (Figure 2D). Using the shCD44-transduced OCI-AML3 cells confirmed that the increased VLA-4/VCAM-1 binding upon HA treatment is CD44
dependent (Figure 2E). These data indicated an unusual integrin activation in contrast to the well-described classical CXCL12/CXCR4 induced VLA-4 activation (17), which led us to investigate the nature of this molecular cross talk.

**HA induced inside-out signaling to VLA-4 results in CD49d cluster formation but not in VLA-4 affinity modulation**

VLA-4-dependent adhesion is controlled by either affinity changes, gained by several conformational states of the integrin (18), or avidity changes due to clustering of the molecule on the cell surface (19) (Supplemental Figure 4A). To investigate the alterations in VLA-4 conformational states upon HA treatment, we used the αCD29 antibody (clone HUTS-21) that binds solely to the ligand-occupied state of VLA-4 (20). To mimic VLA-4 ligand binding, we used the small molecule (LDV), a probe containing the conserved LDV sequence, specific for the VLA-4 binding site. Manganese was used as a positive control as it induces the maximal extent of VLA-4 activation, which is not achieved under physiological conditions (20). Surprisingly, OCI-AML3 cells express VLA-4 with an inactive conformation irrespectively whether cells were treated with HA or not and bound its ligand with comparable affinity (Supplemental Figure 4B) (15, 20). This unexpected finding prompted us to elucidate if the HA-induced AML cell arrests on the substrate are based on increased avidity rather than affinity of VLA-4 to VCAM-1. We performed immunofluorescence microscopy and found increased CD49d cluster formation on AML cells upon treatment with HA. This was quantified by counting the number of the clusters on the individual OCI-AML3 and patient AML cells (1 representative of 6 patients shown) (Figure 3A i+ii). The mean number of clusters per cell was compared between untreated and HA-treated cells from all 6 patients (Figure 3A iii). HA treatment also induced clustering of the VLA-4 beta subunit CD29 (Supplemental Figure 4C). Pretreatment with blocking αCD44 (clone 515) inhibited cluster
formation on OCI-AML3 as well as primary AML cells (Figure 3B). Using CD44 knockdown and control transduced OCI-AML3, we confirmed that HA-induced CD49d clustering only occurs in CD44 expressing cells (Figure 3C). This cluster formation translated into enhanced adhesive capacity, as we confirmed in an additional static cell adhesion assay, using an alternative colorimetric method for cell counting (Supplemental Figure 4D). We also performed an avidity-detecting shear flow assay as described (21), by perfusing OCI-AML3 cells and primary cells over an \( \alpha \text{CD49d} \) (clone HP2/1) substrate, further confirming our observations (Figure 3D + Supplemental Figure 4E). To get an insight into the lateral organization of the VLA-4 clusters on the membrane, we used methyl-beta-cyclodextrin (M\(\beta\)cD), which interferes with lipid structures. Albeit M\(\beta\)cD did not significantly reduce the number of HA induced VLA-4 clusters, it abrogated their function to support cell tethering to VCAM-1 (Supplemental Figure 4F).

Next, we studied if the CD44-mediated inside-out activation is a general mechanism that also occurs in non-transformed progenitor cells. Using CD34\(^+\) cells from 4 different patients harboring a non-myeloid, i.e. lymphoid malignancy occurring at later differentiation states (non-Hodgkin-lymphoma (n = 3) and multiple myeloma (n = 1)), we did not find HA-induced CD49d clustering, suggesting that induced cluster formation is a specific feature of transformed myeloid progenitor cells (Figure 3E).

Transformed myeloid progenitors may differ in their CD44variant composition, with an impact on the clinical outcome of AML patients (22). We have analyzed the CD44variant composition of several cell lines as well as primary AML samples and normal CD34\(^+\) cells by reverse transcription PCR and found differences in the length of CD44v6 containing transcripts among the different primary samples (Supplemental Figure 5 A). Interestingly, in OCI-AML3 CD44v6 co-immunoprecipitated with CD49d, with a slight pull down increase when cells were preincubated with HA (Supplemental Figure 5 B). In conclusion, our data
demonstrate that HA/CD44 binding induces CD49d cluster formation in AML, but not normal CD34+ progenitor cells, without changing the conformation of the VLA-4 heterodimer.
**Src family kinase inhibition and midostaurin treatment interfere with the CD44-VLA-4 activation axis**

Src family kinases (SFKs) are important downstream molecules of HA/CD44 (23) and likely candidates for integrin activation (24). To confirm Src and PI3K activation upon HA treatment, we have analyzed Src and Akt phosphorylation by Western blot in native, control and shCD44 transduced OCI-AML3 cells (Supplemental Figure 6A). Remarkably, treating cells with the pan SFK inhibitor PP2 abrogated HA-induced CD49d clusters on the surface of OCI-AML3 cells (Figure 4A i) and 6 different AML patient samples (Figure 4A ii + C i), providing evidence that CD49d clustering was Src family-dependent. To start from a broad but therapeutically relevant kinase inhibition, we used the multikinase inhibitor midostaurin, approved for treatment of FLT3 mutated AML. We found that midostaurin is highly potent in antagonizing HA-induced CD49d cluster formation of OCI-AML3 cells (Figure 4B i) and 6 different AML patient samples independent of their FLT3 mutation status (FLT3 wild-type n = 3, FLT3-ITD n = 3) (Figure 4B ii + C ii).

Additional experiments, using the MEK inhibitor cobimetinib and the PI3Kδ inhibitor idelalisib, further suggested that PI3K, but not MAPK pathways are involved in CD44-triggered inside-out CD49d cluster formation (Supplemental Figure 6B). We next confirmed that PP2, midostaurin and idelalisib treatment not only inhibited cluster formation, but also reduced the binding of primary AML cells (Figure 4D) and OCI-AML3 cells (Supplemental Figure 6C) to VCAM-1 under shear flow conditions. Collectively, our findings point to a Src family- and PI3K-dependent signaling pathway that is initiated upon HA/CD44 engagement leading to CD49d cluster formation.
HA-induced VLA-4/VCAM-1 interaction promotes AML cell-stromal cell interaction leading to Akt, MAPK and NF-kB pathway activation

To further identify the impact of the CD44-mediated VLA-4 activation on pathophysiologically relevant processes in the BM microenvironment, we used a static adhesion assay combined with microscopy of AML cells on a stromal cell layer. Untreated or HA pretreated OCI-AML3 cells or primary AML cells from 4 different patients were cocultured with stromal cells for 30 min. After extensive washing, cell nuclei were visualized via DAPI staining. We found that HA pretreated cells had a much higher ability to firmly adhere on stromal cells. We confirmed that this adhesion was dependent on the HA-induced interaction of VLA-4 expressed by the AML cells and the VCAM-1 expressed by the stromal cell, as additional pretreatment with the αCD44 antibody as well as pretreatment with the αCD49d antibody inhibited the HA-induced adhesion of OCI-AML3 on stromal cells (Figure 5A+B). Additional controls using CD44 knockdown and control transduced OCI-AML3 cells (Supplemental Figure 7A) as well as native OCI-AML3 on VCAM-1-antibody-treated stromal cells confirmed the specificity of the HA-CD44 interactions in triggering adhesion on VCAM-1 expressed by stromal cells (Supplemental Figure 7B).

We next investigated downstream signaling of this VLA-4/VCAM-1 interaction in AML cells by Western blotting and quantified phosphorylation levels of previously shown signaling molecules that are important for AML cell survival, namely Akt, ERK, IkB and mTOR (1). Primary cells from 5 different AML patients were treated with HA and/or with VCAM-1-coated beads, where indicated. In contrast to short HA treatment, VCAM-1-coated beads alone were sufficient to increase phosphorylation of Akt, ERK, IkB and mTOR in primary AML cells (Figure 6A+B). This can be attributed to the experimental three-dimensional nature of this system, which allows a lot more cells to bind to the VCAM-1-coated beads than immobilized VCAM-1 used for microscopy (Supplemental Figure 8A). In line, VCAM-1 also
triggered phosphorylation of ERK, IkB, FAK and paxillin (Pax) in OCI-AML3 cells (Supplemental Figure 8B).

In light of the key role of active Akt, MAPK, and NF-kB signaling in leukocyte survival, we next tested the protective effect of CD44-VLA-4 dependent cell adhesion in the context of chemotherapy. We found that OCI-AML3 cells adherent to a co-immobilized substrate of HA and VCAM-1 underwent less doxorubicin-induced apoptosis than cells lacking such a substrate. CD49d expression was thereby mandatory for the protective effect as CD49d knockdown cells were not protected by HA/VCAM-1 (Figure 6C). The importance of CD49d in leukemic progression was also confirmed by long-term in vivo engraftment experiments in NSGS mice. AML progression was decelerated upon engraftment of CD49d knockdown (shCD49d) OCI-AML3 cells as compared to engraftment of control cells (shCont) (Figure 6D). In a xenotransplant model anti-CD49d antibody treatment altered the organ-specific localization of engrafted MOLM-13 cells, but did not significantly prolong the overall survival of mice undergoing cytarabine treatment (AraC, Supplemental Figure 9). At this point we were not successful in establishing a model for testing standard induction therapy (combined AraC- doxorubicin) and anti-CD49d treatment in immunodeficient mice, as doxorubicin requires careful further dosing studies to avoid severe toxicities.

In conclusion, we demonstrate that HA-induced VLA-4 cluster formation is critical for direct cell-cell contact of human AML cells with stromal cells, thereby contributing to supportive signaling pathways in AML cells (Figure 7).
**Discussion**

The BM microenvironment plays a decisive role in the tumor evolution and persistence of AML (1). Adhesive processes are mandatory to signal perception and leukemia cell-microenvironment communication by facilitating the retention of the tumor cells to protective cues. Moreover, CD44 has been reported as a marker of primary human AML cancer stem cells and its blockade revealed a potential for differentiation in human AML cell lines (3, 25, 26). Here, we identified a novel non-classical HA/CD44-triggered way of inside-out activation of the integrin VLA-4, leading to VLA-4 cluster formation and increased adhesive strength on VCAM-1, important for the direct interaction of AML cells with supportive stromal cells.

In short-term adoptive transfers of human primary AML cells to immunodeficient mice, we observed that CD44 had a key function in rapid tumor cell homing to BM and spleen, reflecting the biology of normal cellular counterparts as well as malignant cells, e.g. chronic myeloid leukemia-initiating cells (27, 28). However, BM engraftment of malignant cells is dependent not only on homing events but even more on retention of the cells in distinct supportive zones of this organ (1). The VLA-4 integrin is known for being key for the retention of progenitor cells in BM (29). The strength of binding of the integrin to its ligand VCAM-1, which is presented by stromal cells, is thereby regulated by signaling cascades inducing increased affinity due to conformational changes of the integrin, the so called inside-out signaling (30). In contrast to the well-described classical chemokine induced VLA-4 inside-out activation by conformational affinity alterations, less is known about chemokine-independent alternative integrin activation (14, 31). We have previously observed that in B cell malignancies, signals via the B cell receptor can induce not only affinity but also avidity changes of the VLA-4 receptor (11). Here, we identified an AML-specific HA/CD44-mediated VLA-4 activation via integrin cluster formation without obvious conformational
modulation, further promoting strong VLA-4/VCAM-1 binding. This chemokine bypass from CD44 towards VLA-4 is somewhat reminiscent of the previously observed E-selectin/HCELL-VLA-4 interaction in mesenchymal stem cells (32), but it clearly differs in the receptor-ligand couple (HA-CD44) and is for the first time observed in a leukocyte. Nevertheless, the reminiscence may point to a more general mechanism and is also interesting in light of the relevant role of E-selectin in AML and other hematological malignancies (e.g. reported by (33-38)). Notably, we could not observe CD44-VLA-4 inside-out activation in normal CD34+ mobilized progenitors, suggesting this is a transformation-related and tumor-acquired feature for increasing integrin-mediated retention of AML cells.

SFKs are crucial downstream signaling molecules of CD44 in hematopoietic cells of healthy and sick (39-41). They can contribute to enrichment of CD44/beta 1 integrin complexes in lipid rafts (42) and also function immediately downstream of integrins, in concert with focal adhesion kinases (43). Employing the SFK inhibitor PP2, we identified SFK within CD44-mediated VLA-4 inside-out activation cascade, and propose that a further stabilization of VLA-4 clusters involves Src-FAK signaling. This could have therapeutic relevance as PP2 administration was reported to attenuate progression of a FLT3-mutated AML model (44). However, in light of the complexity of the Src kinase family, the particular kinase responsible for the HA/CD44-mediated inside-out signaling needs to be elucidated by a genetic screening approach.

We investigated the therapeutically relevant drug midostaurin, which is approved for treatment of FLT3-mutant AML (45), and currently under clinical investigations in non-FLT3 mutated AML cases (NCT03512197). Midostaurin is a broad multikinase inhibitor, and more selective inhibitors such as gilteritinib and quizartinib for FLT3 and dasatinib for Src kinases recently entered clinical stage (46-48). Notably, kinase signals via FLT3-ITD can increase the affinity of VLA-4 to soluble VCAM-1 (49). Thus, the here reported CD44-dependent
mechanism of VLA-4 activation may be an alternative pathway used by FLT3 wild-type AML cells to increase their adhesion to protective stromal cells. As FLT3 mutation status is an important prognostic marker in AML, it is interesting that the observed CD44/VLA-4 crosstalk was independent from the patient FLT3 mutation status, suggesting involvement of alternative compensatory kinases.

Further downstream of SFK, we supposed PI3K to mediate signaling (50-52) and indeed observed diminished HA-induced CD49d cluster formation in presence of the PI3Kδ inhibitor idelalisib. PI3K has been described to promote human AML survival and BM stromal cell-mediated protection (53), giving a rationale to further investigate PI3K inhibitors in AML. In further functional consequence, the VLA-4-mediated AML adhesion triggered Akt, MAPK, mTOR and NF-kB pathway activation, which are known important mediators of AML survival (53, 54). However, in a first xenograft model we could not establish a treatment regime of combined VLA-4 inhibition and induction chemotherapy with a survival benefit for the mice. While this argues for kinase inhibition as the preferential cytarabine combination partner, it does not exclude a role of VLA-4 inhibition in other treatment schedules or at other disease points, e.g. during graft-versus-host management.

Taken together, we suggest that HA binding to CD44 triggers a signaling axis via SFKs and PI3K to rapidly trigger VLA-4 avidity and hence support AML cell retention in their preferential niches (Figure 7). Compensatory survival mechanisms of malignant cells still comprise a major challenge in current AML therapy that may be tackled by the usage of combinatorial therapies administrating kinase inhibitors which may help to interfere with cellular position as well as growth signaling accompanied by drugs inducing apoptosis, as a step forward in improving current treatment modalities.
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**Competing Interests:** The authors have declared that no conflict of interest exists.
Reference List

**Figure Legends**

**Figure 1. CD44 and CD49d are both expressed on AML cells; CD44 has a predominant role in homing.** (A) Representative histograms of CD44 and CD49d surface expression of primary AML cells and the AML cell line OCI-AML3 are shown. (B) Mononuclear cells from BM aspirates of AML patients pretreated with/without αCD44 antibody clone 515 (αCD44) or αCD49d clone HP2/1 (αCD49d) were injected into the tail veins of NSG mice. After 3 h the number of AML cells that had homed to BM and spleen of the recipients was determined by flow cytometry using human-specific αCD44 and αCD49d antibodies. The homing rate was defined as the number of measured leukemic cells per 1 million measured cells per 1 million injected cells. (C i) Homing rate to BM and spleen 3 h after injection was measured in 5 independent experiments using samples from 5 different AML patients. In each experiment, technical duplicates were performed and they were averaged for the analysis. (C ii) Homing rate to BM and spleen was measured 3 h after injection of OCI-AML3 (n = 2). (D i + ii) Homing rate to BM and spleen was measured 3 h after injection of OCI-AML3 cells transduced with shCD44 or shCD49d or control shRNA (shCont) (n = 4, unpaired t-test) (E) Mononuclear cells from the BM aspirate of one AML patient and OCI-AML3 cells were injected into the tail veins of NSG mice. After 3 h and 3 days the number of AML cells that had homed or engrafted to BM and spleen of the recipient mice was determined by flow cytometry using human-specific αCD44 and αCD49d antibodies (n = 4, unpaired t-test).

**Figure 2. HA treatment increases AML cell arrests on VCAM-1 under shear flow.** (A) OCI-AML3 cells were perfused over HA. Where indicated, cells were pretreated with blocking αCD44 antibody (clone 515). (B) OCI-AML3 (i) or primary AML cells from 5 different patients (ii) were perfused over VCAM-1. Where indicated, cells were pretreated with soluble HA or αCD49d antibody (clone HP2/1) (abrogating VLA-4-mediated
interactions). (C) OCI-AML3 cells were perfused over VCAM-1 or VCAM-1/HA substrate upon pretreatment with αCD49d antibody (clone HP2/1), where indicated. (D) OCI-AML3 cells were perfused over VCAM-1. Where indicated, cells were pretreated with low molecular weight HA (LMW-HA) or high molecular weight HA (HMW-HA). (E) OCI-AML3 cells transduced with shCD44 or control shRNA were perfused over VCAM-1; cells were pretreated with soluble HA, where indicated. Categories of interaction (tethers) are expressed as frequencies of cells in direct contact with the substrate. Two groups were compared with paired t-test, three groups were compared with one-way ANOVA with multiple comparisons.

Figure 3. HA treatment induces CD49d cluster formation on AML. (A) Confocal images and CD49d cluster analysis of OCI-AML3 (i) or primary AML cells from BM aspirates (ii). Cells were pretreated with/without HA and then settled on immobilized VCAM-1, followed by fixation and staining with αCD49d (red) monoclonal antibody (AHP1225) where indicated (1 representative of 6 different patients is shown). CD49d clusters for each treatment were quantified using ImageJ software (n = 50 cells, unpaired t-test). Mean numbers of CD49d clusters per cell were compared from 6 different patient samples with/without HA treatment (iii, paired t-test). (B) Confocal images and CD49d cluster analysis of OCI-AML3 (i) and primary AML cells (ii). Cells were pretreated with/without αCD44 antibody (clone 515) before HA treatment and then settled on immobilized VCAM-1, followed by fixation and staining with αCD49d (red) monoclonal antibody. CD49d clusters were quantified for each treatment using ImageJ software (n = 50 cells, one-way ANOVA with multiple comparisons). Mean numbers of CD49d clusters per cell were compared from 6 different patient samples with/without HA treatment and with/without αCD44 treatment (iii, one-way ANOVA with multiple comparisons). (C) Confocal images and CD49d cluster analysis of control shRNA (shCont) (i) or shCD44 transduced OCI-AML3 (ii) were performed. Cells were pretreated
with/without HA and then settled on immobilized VCAM-1, followed by fixation and staining with αCD49d (red) monoclonal antibody where indicated (1 of 2 replicates is shown). CD49d clusters for each treatment were quantified using ImageJ software (n = 50 cells, unpaired t-test). (D) OCI-AML3 (i) or primary cells (ii) were perfused over an αCD49d (clone HP2/1) substrate for 1 min at 0.5 dyn/cm² with/without HA pretreatment. Categories of interaction (tethers) are expressed as frequencies of cells in direct contact with the substrate (6 replicates were performed with OCI-AML3, 3 replicates were performed with one AML patient sample). (E) Primary CD34⁺ cells from 4 different patients (non-Hodgkin-lymphoma (n = 3) and multiple myeloma (n = 1)) were pretreated with/without HA and then settled on immobilized VCAM-1, followed by fixation and staining with αCD49d (red) and αCD34 (green, clone QBEND-10) monoclonal antibodies (1 representative patient sample is shown). CD49d clusters were quantified for each treatment using ImageJ software (n = 50 cells, unpaired t-test). (ii) Mean numbers of CD49d clusters per cell were compared from 4 different patient samples with/without HA treatment (paired t-test). Bars, 5 μm.

Figure 4. Src family kinase (SFK) inhibition and midostaurin treatment of AML cells inhibit HA-induced cluster formation. (A) Confocal images of OCI-AML3 (i) or primary AML cells from BM aspirates (ii) that were pretreated with/without HA and/or with/without the SFK inhibitor PP2. Cells were stained with αCD49d (red) monoclonal antibody (clone AHP1225). For OCI-AML3 1 representative of 3 experiments is shown, for primary samples 1 representative of 6 experiments is shown (n = 50 cells). (B) Confocal images of OCI-AML3 (i) or primary AML cells from BM aspirates (ii). Cells were pretreated with/without HA and/or with/without the multikinase inhibitor midostaurin. Cells were stained with αCD49d (red) monoclonal antibody. For OCI-AML3 1 representative experiment of 3 is shown, for primary samples 1 representative experiment of 6 is shown (n = 50 cells). (C) Mean numbers
of CD49d clusters per cell were compared from 6 different patient samples with/without HA treatment and with/without PP2 treatment (i) or with/without midostaurin (ii). (D) Primary AML cells from 5 different patients with/without HA treatment and with/without PP2 treatment (i) or with/without midostaurin (ii) were subjected to shear flow analyses over VCAM-1. One-way ANOVAs with multiple comparisons were used. Bars, 5 μm.

**Figure 5. HA treatment leads to strong interaction between AML cells and stromal cells.** OCI-AML3 cells (n = 4) (A) or primary cells from BM aspirates from 4 different AML patients (B) were pretreated with/without HA and with/without αCD44 (clone 515) or αCD49d (clone HP2/1) and allowed to adhere to M2 stromal cells for 30 min. The number of AML cells that had bound to stromal cells was counted on bright field images with additional DAPI staining by fluorescence microscopy. One-way ANOVAs with multiple comparisons were used. Images were taken at 20x magnification. Bars, 20 μm.

**Figure 6. VLA-4 engagement triggers the phosphorylation of Akt, ERK, IkB and mTOR and contributes to AML progression.** (A) Protein lysates of primary AML cells from BM aspirates were treated with/without HA and with/without VCAM-1 coated beads and tested for their ERK, phospho-ERK, Akt, phospho-Akt, IkB alpha, phospho-IkB alpha and mTOR, phospho-mTOR content by western blot. One representative of five experiments is shown. (B) Five independent experiments with 5 different AML patient samples were quantified. Expression intensities were quantified with ImageJ software and phosphorylation was normalized to total protein content. One-way ANOVAs with multiple comparisons were used. (C) Apoptosis of native, shCont or shCD49d OCI-AML3 cells was induced with 0.5 μM of doxorubicin, additionally cells were treated with immobilized HA and VCAM-1 where indicated. Cell viability was determined using trypan blue. Four replicates are shown of one
representative experiment of 2 independent experiments. (D) Number of shCont or shCD49d OCI-AML3 cells in BM, SPL and blood of NSG mice, 28 days post i.v. injection (n = 7 per group).

Figure 7. Schematic overview of the suggested CD44-VLA-4 activation axis and downstream consequences. Binding of AML cells to the BM stromal component HA is dependent on CD44 and enhances adhesion of AML cells to the VLA-4 substrate VCAM-1, a second important adhesion factor displayed on stromal cells. Mechanistically, this enhanced adhesion is based on inside-out activation of VLA-4, without altering the conformation of the integrin. The signaling downstream of CD44 involves several kinases (e.g. src family kinases (SFKs)) causing clustering of the integrin, thereby stabilizing adhesion strength that facilitates direct interaction with stromal cells. This AML cell-stromal cell interaction leads to survival signaling involving Akt, MAPK and NF-kB pathway activation.
Supplemental Methods

Cell lines
Murine BM stromal cells (M2-10B4, ATCC-CRL-1972) were obtained from the ATCC (Manassas, Virginia, USA). AML cell lines were purchased from DSMZ (Braunschweig, Germany): OCI-AML3 (ACC-582), KG-1a (ACC-421), HL-60 (ACC-3), MOLM-13 (ACC-554), MOLM-14 (ACC-777), MV4-11 (ACC-102). Cell lines were cultured for less than 15 passages or authenticated by DNA fingerprinting and STR-technology (1).

Antibodies
Antibodies are listed in Supplemental Table 2.

Flow cytometry
Whole BM aspirates and AML cells were stained with monoclonal antibodies (Supplemental Table 2) or corresponding isotype controls. Classical blast gating was performed, based on side scatter properties and (low) CD45 expression (2). αCD13, αCD14, αCD33, αCD34, and αCD117 were used to further discriminate various AML subtypes and other cell types (3), in combination with CD44 and CD49d determination. Measurements were performed using the Gallios system (Beckman Coulter, Brea, California, USA). For cell line experiments, CD44 and CD49d expression were determined using a FC-500 system (Beckman Coulter). Viability was assessed using Annexin V-FITC and 7AAD.

RNA interference and lentiviral transduction
RNA interference and lentiviral transduction experiments were performed as described (4). The following short hairpin RNA (shRNA) constructs selected from the Mission TRC shRNA
library (Sigma-Aldrich, St. Louis, Missouri, USA) were used: shRNA CD44 (CCGCGCTTGAAACATAACCATTACTCGAGTAATGGTTATGTTTCCAACCGGTTTTTGTG), shRNA CD49d (CCGGGCTCCGTGTATCAAGATTATCTCAGATAATCTTGGATAACACGGAGCTTTT) and scrambled control shRNA (CCGCAACAAGATGAAGACCAACTCGAGTTGGTGGCTCTTCATCTTTGTGTGT). Transduced cells were selected for puromycin resistance prior to further analysis.

**Shear flow assay**

Shear flow assays were conducted as described (5). µ-slides (Ibidi, Martinsried, Germany) were coated with protein A/VCAM-1/Fc (R&D Systems, Minneapolis, Minnesota, USA) or HA or HA followed by protein A/VCAM-1/Fc. Mononuclear cells from BM aspirates of AML patients (blast content: 75-95%, determined by flow cytometry) were preincubated with 10 μg/mL HA (Sigma-Aldrich), with 2 μg/mL αCD49d (clone HP2/1), with 5 μg/mL αCD44 Fab fragments (clone 515) for 10 min, where indicated. Src family kinase inhibition was performed using 10 μM PP2 (Merck, Darmstadt, Germany) for 30 min. Midostaurin was used at 1 μM (Selleckchem, Houston, USA) for 60 min. The cells were allowed to accumulate at subphysiological shear stress (0.5 dyn/cm²), and then subjected to physiological shear stress (2 dyn/cm²). Frequencies of adhesive categories were determined as percentages of cells flowing immediately over the substrate using customized image analysis software (Wimasis, Córdoba, Spain) (6-8).

**Western blotting**

AML cells were analyzed as described (9) using antibodies against phosphorylated and non-phosphorylated IkB, Akt, ERK, FAK, paxillin and PPIA. For VCAM-1/Fc treatment protein A Dynabeads™ (Invitrogen, Carlsbad, California, USA) were incubated for 1 h at room
temperature with VCAM-1/Fc (3.5 µg/mL) (R&D Systems). 3 x 10⁶ cells were either untreated or pretreated with high molecular weight HA (10 µg/mL) (Sigma-Aldrich) for 2 min and then incubated with VCAM-1/Fc coated Dynabeads™ (2 x 10⁶ cells per 15 µl beads) for 20 min at 37°C. Cells were lysed and used for western blotting.

**Quantitative real time PCR (qPCR)**

RNA isolation and cDNA synthesis were performed as described (9). qPCR was performed on the ViiA 7 System (Applied Biosystems, Foster City, California, USA) using TaqMan Gene Expression Assays. Results were quantified by normalization to 18S rRNA or GAPDH expression using the ΔCT method.

**Viability assay**

OCI-AML3 cells were seeded into 24-well plates at 2 x 10⁵ cells/well and cultured on wells coated with or without VCAM-1 (7.5 µg/ml). Cells were previously stimulated with 10 µg/ml HA for 10 minutes at 37°C and then treated with 0.1 and 0.5 µM doxorubicin final concentration for 24 hours. Viable cell count was performed with TC10™ Trypan Blue dye (0.4 %) and evaluated with TC20™ automated cell counter according to the manufacturer’s instructions (Bio-Rad, USA).

**Flow cytometric HA-binding assay**

To detect HA binding, OCI-AML3 cells were incubated with HA-FITC (AbLab, Vancouver, Canada) for 15 min at room temperature with/without pretreatment with αCD44 (clone 515) or soluble HA (Sigma) for 10 min.
In vivo engraftment and mobilization

10 NSG5 mice were engrafted with secondary (previously expanded in NSG mice) human AML cells originally derived from Patient 46. One week after i.v. injection of the cells, 5 mice were treated with 100 µg αCD44 antibody (clone 515) and the other 5 mice with the same volume of PBS i.v. One day after treatment with the αCD44 antibody mice were sacrificed and blood, BM and spleen were analyzed for human cell content via anti-human CD45 (clone J33), CD34 (clone 581) and CD38 (clone LS198-4-3) antibody staining.

Xenotransplant treatment study

5 million MOLM-13 cells were injected i.v. into the tail vein of NOD/SCID mice. Mice were randomized into 4 groups (a 7 mice): control, cytarabine (AraC), anti-CD49d or AraC + anti-CD49d combination. 3 days after injection treatment was started. Control group 1 received iNvivoPure Dilution Buffer 5 ml/kg/day ip (Days 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, 51, 54, 57). Group 2 was given with 5 mg/kg/day iv Day(s) 0-42 AraC. Group 3 was given 10 mg/kg/day ip (Days: 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, 51, 54, 57) anti-CD49d (clone PS2) antibody. Group 4 received AraC and anti-CD49d (clone PS2) in combination. The treatment study was conducted at Charles River Laboratory. The experiment was terminate at day 74 after cell injection. At this time, all mice of the control group had died, 1 mouse was still alive in AraC group, 2 mice were alive in PS2 group and 1 mouse was still alive in combination group. Blood, spleen, lymph node and bone marrow were harvested to measure number of human CD45 positive cells by flow cytometry. Counting beads were used to determine absolute numbers. Significant outliers were removed.
Supplemental shear flow assay

Shear flow assays were conducted as described in the main manuscript. µ-slides (Ibidi, Martinsried, Germany) were coated with HA, protein A/VCAM-1/Fc or HA followed by protein A/VCAM-1/Fc. OCI-AML3 cells were treated with/without low molecular weight HA (Cat# 40583, Sigma) and high molecular weight HA (Cat# 41897, Sigma) or with/without 15 mM methyl-beta-cyclodextrin (MβCD) (Sigma-Aldrich) and HA for 10 min at 37 °C where indicated. For avidity assay 6-channel µ-slides (Ibidi, Martinsried, Germany) were coated with αCD49d (clone HP2/1) overnight at 4 °C.

Determination of VLA-4 conformation

VLA-4 conformation assay has been described in (10). Shortly, AML cells were resuspended in HBSS (10 mM HEPES, 0.2% BSA, 1 mM CaCl₂, 1 mM MgCl₂). Viability, CD44, CD44v6, CD49d, and CD29 expression were cytometrically determined prior to each experiment. Cells were incubated with LDV and with/without HA in the presence of an excess of αCD29 mAb (clone HUTS-21, detecting the ligand occupied conformation of VLA-4) for 30 min at 37 °C. Subsequently, the mean fluorescence intensity (MFI) of labeled HUTS-21 mAb was cytometrically determined. EC₅₀ values for HUTS-21 binding report LDV ligand binding affinity, as described (11).

Static cell adhesion assay

Flat 96-well plates were coated with 1μg/mL recombinant human VCAM-1-Fc (R&D systems) over night at 4°C. OCI-AML3 cells were treated with either αCD49d (5 μg/mL, clone HP2/1) antibody or αCD44 antibody (5 μg/mL, clone 515). Afterwards the cells were treated with/without HA (AMO Germany) for 30 min and aliquoted into the coated plates at a final concentration of 1x10⁶/mL. Plates were incubated for 1 h or 10 min at 37 °C followed by
fixation and crystal violet staining. Absorbance was determined at 595 nm (MolecularDevices SpectraMax iD3 microplate reader). Results were shown as mean OD values of triplicate wells ± SD.

**E-Selectin adhesion assay**

The wells of a 96 well plate were coated over night with 0.5 μg/ml E-selectin-Fc on 2 μg/ml ProteinA at 4°C. OCI-AML3 cells (1 million/ml) were pre-incubated or not for 10 minutes at 37°C with anti-CD44 (clone 515, 5 μg/ml), and then allowed to adhere to the E-selectin coating at 37°C for 30 min (100 μl cell suspension/well). Adhesion was quantified using CyQuant Cell Proliferation Kit (Thermo Fisher). Each condition was assessed in technical triplicates.

**Supplemental VLA-4 cluster assay**

Mononuclear cells from BM aspirates of AML patients (blast content 75-95%) were pretreated for 10 min with 10 μg/mL HA and/or for 24 h with 10 μM cobimetinib (APExBIO, Houston, USA), and/or for 90 min with 5 μM idelalisib (APExBIO) and allowed to adhere for 30 min at 37 °C before fixation with 4% paraformaldehyde (PFA). Slides were stained with primary αCD49d antibody (clone AHP1225) and Cy3-conjugated secondary anti–rabbit antibody. OCI-AML3 cells were pretreated for 10 min with 10 μg/mL HA and/or for 30 min with 7.5 μM MßcD and allowed to adhere for 30 min at 37°C before fixation with 4% PFA. Slides were stained with primary αCD49d antibody (clone AHP1225) and Cy3-conjugated secondary anti-rabbit antibody.
Supplemental Stroma binding

Falcon culture slides were coated with 20 μg/mL fibronectin for 1 h at 37 °C. 70 000 M2 stroma cells were seeded and cultured overnight. M2 stroma cells were pretreated with 20 μg/mL αVCAM-1 antibody (clone 429) for 20 min before OCI-AML3 cells (0.5-1.0 x 10^6 cells) were seeded on M2 stromal cells and co-cultured for 30 min at 37°C. Cells were washed, fixed with 4% PFA, and stained using DAPI Antifade Reagent. Images were taken with an Olympus IX81 microscope (UPLSAPO 20xO/0.85 objective). Numbers of cells were determined in 10-12 pictures for each treatment with ImageJ software.

Immunofluorescence microscopy

Falcon culture slides were coated with 7.5 μg/mL VCAM-1 for 3 hours at room temperature and overnight at 4 °C. OCI-AML3 were treated with/without 10 μg/mL HA for 10 min, and allowed to adhere for 30 min at 37°C before fixation with 4% PFA. Slides were stained with primary αCD49d antibody (clone AHP1225) and αCD29 antibody (clone 12G10) and Cy3-conjugated secondary anti-rabbit and AF488-conjugated anti-mouse antibody. Images were taken with an Olympus IX81 microscope.

Reverse transcription PCR

For detection of CD44v transcription by reverse transcription PCR (RT-PCR), cDNA from AML samples was amplified by panCD44 or CD44 variant exon specific primers and visualized by agarose gels, as described in (12).

Co-Immunoprecipitation

OCI-AML3 cells were stimulated with HA where indicated. For immunoprecipitation (IP) of CD49d, cells were lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM aprotinin and
1mM leupeptin. The cleared lysates were incubated with antibody-coated protein A/G agarose beads (Merck). The beads were washed in lysis buffer, then subjected to western blot analysis.

**Statistical analysis**

Statistics were performed using GraphPad Prism 8. Normal distribution was determined using the Kolmogorov–Smirnov test. Two normally distributed groups were compared using t-test. Nonparametric data sets were analyzed by Wilcoxon signed-rank tests for paired analysis or Mann-Whitney test for unpaired analysis. Three or more groups of data were analyzed with the one-way ANOVA with post-hoc tests. Results were considered significantly different when P < 0.05, with values at P < 0.05 marked as *, P < 0.01 as **, P < 0.001 as *** and P < 0.0001 as ****.
Supplemental Figure Legends

Supplemental Figure 1.
(A) Representative histograms of CD44 and CD49d surface expression of the cell lines HL-60, MV4-11, MOLM-13, MOLM-14 and KG-1a are shown. (B) Mononuclear cells from BM aspirates of AML patients pretreated with/without αCD44 antibody clone 515 (αCD44) and/or αCD49d clone HP2/1 (αCD49d) were injected into the tail veins of NSG mice. After 3 h the number of AML cells that had homed to BM and spleen of the recipients was determined by flow cytometry using human-specific αCD44 (clone J.173) and αCD49d (clone 9F10) antibodies. The homing rate was defined as the number of measured leukemic cells per 1 million measured cells per 1 million injected cells (data for double treatment from 4 of the 5 primary samples shown in Figure 1 C i).

Supplemental Figure 2.
(A) In 4 of 5 homing experiments shown in Figure 1C i, the viability of patient cells treated with/without αCD44 (clone 515) for 3 h in vitro was determined in parallel via AnnexinV/7AAD staining. (B) In 4 of 5 experiments shown in Figure 1C i, the percentage of CD44 and CD49d positive patient cells with and without corresponding blocking antibody (αCD44 (clone 515) and αCD49d (clone HP2/1)) treatment was determined before injection into recipient mice. (C) OCI-AML3 cells pretreated with/without αCD44 (clone 515) were allowed to adhere to E-Selectin. (D i) OCI-AML3 cells transduced with shCD44 or control shRNA (shCont) were perfused over HA for 1 min at 0.5 dyn/cm². Three runs were averaged per experiment and 3 independent experiments were performed. (ii) CD44 mRNA expression was determined via qPCR from OCI-AML3 cells transduced with shCD44 or control shRNA (shCont) (n = 3). (E i) OCI-AML3 cells transduced with shCD49d or control shRNA (shCont)
were perfused over VCAM-1 for 1 min at 0.5 dyn/cm². Five runs were performed. (ii) CD49d (ITGA4) mRNA expression was determined via qPCR from OCI-AML3 cells transduced with shCD49d or control shRNA (shCont) (n = 3). Unpaired t-tests were used in C+D. (F i) In the experiment shown in Figure 1 E proliferation of primary AML cells after 3 h and 3 d was determined using CellTrace™ dye dilution (n = 4). One-way ANOVA with multiple comparisons was used. (ii) Representative histograms of CellTrace™ dye intensity are shown in AML cells found in BM and spleen after 3 h or 3 d. (G) Human AML cells in murine organs (bone marrow: BM, blood and spleen: SPL) were determined as CD45+/CD34+ cells one day after aCD44 antibody or PBS treatment of the mice (n = 5 per group).

Supplemental Figure 3.
(A) HA binding capacity of the OCI-AML3 cell line was determined by flow cytometry using fluorescein-labeled HA (HA-FITC). OCI-AML3 were pretreated with a blocking aCD44 antibody (clone 515) or soluble HA (n = 5). One-way ANOVA with multiple comparisons was used. Representative histograms are shown in ii. (B) Primary AML cells from 1 patient were perfused over VCAM-1. Where indicated, cells were pretreated with soluble HA or/and αCD49d antibody (clone HP2/1, abrogating VLA-4-mediated interactions). Runs were performed in triplicates. (C) OCI-AML3 (n = 14) (i) and AML patient samples (n = 7) (ii) were pretreated with/without HA (10 µg/mL) for 10 min. CD49d and CD44 surface expression was determined via flow cytometry. Relative mean fluorescence intensity ratio = MFIR. Paired t-tests were used.

Supplemental Figure 4.
(A) VLA-4 activation by inside-out signaling. VLA-4 binding strength to its ligand VCAM-1 can be modulated by either increased avidity mediated by receptor clustering or increased
affinity by distinct conformational states. (B) OCI-AML3 cells with/without HA or Mn^{2+} (positive control) were incubated with the indicated LDV concentrations in presence of an excess of HUTS-21 mAb. The HUTS-21 antibody detects the epitope on the hybrid domain of the CD29 subunit only exposed in the ligand-occupied conformation. Fitting of the data was done using the sigmoidal dose-response equation with variable slope using GraphPad Prism 8 software. Each point represents the mean of triplicates. The EC_{50} values calculated for control and HA treatment indicate a resting state of the VLA-4 integrin. (C) Confocal images and CD29 cluster analysis of OCI-AML3 and of primary AML cells from BM aspirates were performed. Cells were pretreated with/without HA and then settled on immobilized VCAM-1, followed by fixation and staining with αCD29 (green) monoclonal antibody (clone 12G10) where indicated (1 representative of 2 different patients is shown). CD29 clusters for each treatment were quantified using ImageJ software (n = 50 cells, unpaired t-test). (D) OCI-AML3 cells with/without αCD44 or αCD49d were cultured on VCAM-1 for 1 h. Absorbance was determined at 595 nm (n = 3, one-way ANOVA with multiple comparisons). (E) OCI-AML3 cells were perfused over an anti-CD49d (clone HP2/1) substrate or an isotype control substrate for 1 min at 0.5 dyn/cm² with/without HA pretreatment. Categories of interaction (tethers) are expressed as frequencies of cells in direct contact with the substrate. (F i) Confocal images of OCI-AML3 cells that were pretreated with HA and with/without methyl-beta-cyclodextrin (MβcD). Cells were stained with αCD49d (red) monoclonal antibody (clone AHP1225). (ii) CD49d clusters were quantified for each treatment using ImageJ software (n = 50 cells). (iii) OCI-AML3 cells were perfused over VCAM-1, HA or VCAM-1 + HA substrate for 1 min at 0.5 dyn/cm². Where indicated, cells were pretreated with/without HA and/or with/without MβcD. Arrests are expressed as frequencies of cells in direct contact with the substrate. One-way ANOVAs with multiple comparisons were used.
**Supplemental Figure 5.**  
(A) RT-PCR analysis of CD44 variant transcripts. 5 forward primer (5 fw) and v2, v3, v4, v5, v6, v7, v8, v9, v10 reverse (rv) primer combination were used in cDNA from HL60, MV4-11, MOLM13, MOLM14, OCI-AML3 cell line as well as from 6 different primary AML samples and 2 normal CD34+ samples. (B) Co-Immunoprecipitation (IP) of CD49d or isotype control following western blot (WB) determination of CD44v6 and CD49d in the pull-down of OCI-AML3 cell lysates. HA treatment where indicated.

**Supplemental Figure 6.**  
(A) Protein lysates from native, control shRNA (shCont) and shCD44-transduced OCI-AML3 cells were treated with/without 10 µg/ml HA for 10 min and tested for their Src, phospho-Src, Akt, phospho-Akt and PPIA content by western blot. (B) Confocal images of OCI-AML3 that were pretreated with/without HA and/or (i) with/without the MEK inhibitor cobimetinib (cobi) or (ii) with/without the PI3K inhibitor idelalisib (idela). Cells were stained with aCD49d (red) monoclonal antibody (clone AH1225). CD49d clusters were quantified for each treatment using ImageJ software (n = 50 cells). (C) OCI-AML3 cells were perfused over VCAM-1 substrate for 1 min at 0.5 dyn/cm². Where indicated, cells were pretreated with/without HA and/or with/without the Src kinase inhibitor PP2 and/or with/without the multikinase inhibitor midostaurin (mido) and/or with/without the PI3K inhibitor idelalisib (idela). Arrester were expressed as frequencies of cells in direct contact with the substrate. One-way ANOVAs with multiple comparisons were used.

**Supplemental Figure 7.**  
(A) OCI-AML3 cells transduced with shCD44 or control shRNA (shCont) were pretreated with/without HA and allowed to adhere to M2 stromal cells for 30 min. (B) Native OCI-AML3
were pretreated with/without HA and allowed to adhere to αVCAM-1 antibody (clone 429) pretreated M2 stromal cells for 30 min where indicated. The number of AML cells that had bound to stromal cells was counted on 10-12 bright field images with additional DAPI staining by fluorescence microscopy. One-way ANOVAs with multiple comparisons were used. Images were taken at 20x magnification. Bars, 20 μm.

**Supplemental Figure 8.**

(A) For VCAM-1 treatment, protein A Dynabeads™ (Invitrogen, Carlsbad, California, USA) were incubated for 1 h at room temperature with VCAM-1/Fc (3.5 μg/mL) (R&D Systems). 3 x 10^6 OCI-AML3 cells were either untreated or pretreated with high molecular weight HA (10 μg/mL) (Sigma-Aldrich) for 2 min and then incubated with VCAM-1 coated dynabeads (2 x 10^6 per 15 μL beads) for 20 min at 37 °C. Cells were analyzed via flow cytometry. (B) Protein lysates from OCI-AML3 cells treated with/without HA- and with/without VCAM-1-coated beads were tested for their PPIA, IκB alpha, phospho-IκB alpha, ERK, phospho-ERK, focal adhesion kinase (FAK), phospho-FAK, paxillin (Pax) and phospho-Pax content by western blot. Expression intensities were quantified with ImageJ software and phosphorylation was normalized to total protein content. Bottom: 3 independent experiments were quantified, using one-way ANOVAs with multiple comparisons. Top: individual experiment.

**Supplemental Figure 9**

Total number of CD45+ cells in untreated, cytarabine, anti-CD49d (clone PS2) antibody treated or cytarabine + anti-CD49d treated MOLM-13 xenotransplants were determined in bone marrow (A), spleen (B), lymph-node-like structures (C) and blood (D) by flow cytometry (n = 5-7; mean ± sem.). Data of mice that were euthanized due to experiment end at day 74 are
shown in red. (E) Overall survival was determined (7 mice per group). Mice that were still alive at experiment end were censored.
Supplemental References

10. Chigaev A, Blenc AM, Braaten JV et al. Real time analysis of the affinity regulation of alpha 4-integrin. The physiologically activated receptor is intermediate in affinity between resting and Mn (2+) or antibody activation. J Biol Chem. 2001; 276:48670-48678.
Supplemental Table 1: AML patient characteristics.

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**Supplemental Table 2: List of used antibodies.** FACS: flow cytometry; WB: Western blotting; IF: immunofluorescence microscopy; Blocking: Blocking experiment

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Supplemental Figure 1

A

CD44

relative cell number

CD49d

fluorescence intensity

HL-60  
MV4-11  
MOLM-13  
MOLM-14  
KG-1a

isotype  
staining

B

AML patients

ebone marrow

Homing rate

αCD44 -  + - +  
αCD49d - - + +  

spleen

Homing rate

αCD44 -  + - +  
αCD49d - - + +  

*  ****  **
Supplemental Figure 2

A

\[ \begin{align*}
\% \text{ viable cells} \\
\alpha\text{CD44} & - & + \\
\end{align*} \]

B

\[ \begin{align*}
\% \text{ CD44}^+ \text{ cells} \\
\alpha\text{CD44} & - & + \\
\% \text{ CD49d}^+ \text{ cells} \\
\alpha\text{CD44} & - & + \\
\end{align*} \]

C

\[ \begin{align*}
\% \text{ arrests on E-selectin} \\
\alpha\text{CD44} & - & + \\
\end{align*} \]

D i

\[ \begin{align*}
\% \text{ rolling on HA} \\
\alpha\text{CD44} & - & + \\
\end{align*} \]

D ii

\[ \begin{align*}
\text{CD44 mRNA normalized to GAPDH} \\
\alpha\text{CD44} & - & + \\
\end{align*} \]

E i

\[ \begin{align*}
\% \text{ arrests on VCAM-1} \\
\alpha\text{CD44} & - & + \\
\end{align*} \]

E ii

\[ \begin{align*}
\text{CD49d mRNA normalized to 18s} \\
\alpha\text{CD44} & - & + \\
\end{align*} \]

F i

AML patient

\[ \begin{align*}
\text{Average number of division cycles per cell} \\
\text{3 hours} & & \text{3 days} \\
\text{bone marrow} & & \text{spleen} \\
\end{align*} \]

F ii

\[ \begin{align*}
\text{3 hours} \\
\text{3 days} \\
\text{cell count} \\
\text{cell trace violet} \\
\end{align*} \]

G

\[ \begin{align*}
\text{AML cells/measured BM cells} \\
\alpha\text{CD44} & - & + \\
\text{AML cells/measured blood cells} \\
\alpha\text{CD44} & - & + \\
\text{AML cells/measured SPL cells} \\
\alpha\text{CD44} & - & + \\
\end{align*} \]
Supplemental Figure 3

A i

OCI-AML3

soluble HA - + - -

αCD44 - - + +

A ii

B

AML patient

HA-FITC

% tethering (arrests)

HA - + + +

αCD49d - - + +

C i

OCI-AML3

CD49d (MFIR)

CD44 (MFIR)

HA - +

HA - +

C ii

AML patients

CD49d (MFIR)

CD44 (MFIR)

HA - +

HA - +

ns

ns

ns

ns
Supplemental Figure 4

**A**

- Microenvironmental signal
- Clustering (conformational change)
- VLA-4
- Receptor

**B**

- OCI-AML3
- EC$_{50}$ values:
  - Control: $1.889 \times 10^{-8}$
  - +HA: $1.582 \times 10^{-8}$
  - +Mn2+: $5.767 \times 10^{-9}$

**C**

- CD29 (AF488) for OCI-AML3 and AML patient

**D**

- Optical density (595 nm)
- CD29 clusters / cell

**E**

- % tethering (arrests)

**F**

- CD49d (Cy3) coating:
  - VCAM-1
  - VCAM-1 + HA

- HA
- MßcD
Supplemental Figure 5

A

HL60

MV4-11

MOLM13

MOLM14

ID 42

ID 46

ID 47

normal CD34⁺ 1

ID 9

ID 16

ID 36

OCI-AML3

ID 42

ID 46

ID 47

normal CD34⁺ 2

B

IP: CD49d

cont  hHA

IgGRat

cont  hHA

WB

CD44v6

170kD

130kD

CD49d

170kD

130kD
Supplemental Figure 6

A

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B i

![Graph showing CD49d clusters/cell](image1)

B ii

![Graph showing CD49d clusters/cell](image2)

C

![Graphs showing % tethering (arrests)](image3)

OCI-AML3

![Immunoblots of P-Src, Src, P-Akt, Akt, and PPIA](image4)
Supplemental Figure 7

A

shCont  shCD44

UTC  UTC

HA  HA

B

UTC  αVCAM-1

HA  HA+αVCAM-1

AML cells / stromal cell

UTC

αVCAM-1

AML cells / stromal cell

UTC

αVCAM-1

AML cells / stromal cell
Supplemental Figure 8

A

UTC

HA

VCAM-1

HA + VCAM1

forward scatter

side scatter

B

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Relative density

(p-ERK / ERK)

(p-IkB / IkB)

(p-FAK / FAK)

(p-Pax / Pax)

HA - - + + VA CAM-1 - - + +
Supplemental Figure 9

A. Bone marrow

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B. Spleen

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C. Blood

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</table>

E. Survival

- Control
- AraC
- αCD49d
- AraC + αCD49d

Days after first treatment