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# 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 in progenitor cell adhesion in bone marrow. 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 bone marrow 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 of 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. As a further consequence, the increased adhesion on VCAM-1 allowed AML cells to bind stromal cells strongly. Thereby, the VLA-4/VCAM-1 interaction promoted activation of Akt, MAPK, NF-κB 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, enhancing AML cell retention in the supportive bone marrow microenvironment.

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## Introduction

Acute myeloid leukemia (AML) is an aggressive and difficult-to-treat hematologic 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 from chemotherapeutic drugs, thus allowing residual disease after therapy, ultimately causing 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 processing of patients' samples

Following written informed consent, BM aspirates from patients with newly diagnosed AML were collected at the Third Medical Department, Paracelsus Medical University Salzburg, Austria (Salzburg ethics committee approval number: 415-E/2009/2-2016). Normal CD34$^+$ progenitor cells from patients with myeloma or non-Hodgkin lymphoma who underwent hematopoietic stem/progenitor cell mobilization were used as non-myeloid controls (Salzburg ethics committee approval number: 415-E/1177/8-2010). Mononuclear cells were isolated using density gradient centrifugation and the viable cells were frozen until further usage. The patients' characteristics are shown in *Online Supplementary Table S1*.

The approval number for the animal experiments 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. The specificity of the blockade was confirmed by isotype control experiments in representative experiments. For homing *versus* engraftment assays (3 h and 3 days), cells were stained using the CellTrace™ Violet Cell Proliferation Kit (Thermo Fisher). Cells (0.3-1.3x10$^6$) were injected intravenously into NOD *scid* gamma (NSG) mice. After 3 h or 3 days, the mice were sacrificed, and the number of human cells that had homed to BM, spleen and peripheral blood was determined 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 days was determined on the basis of CellTrace™ dye dilution rates.[10] For long-term engraftment (28 days) shCont or shCD49d OCI-AML3 cells were injected intravenously into NSGS mice. After 28 days, the mice were sacrificed, and the number of human CD15 and CD45 double-positive cells per million measured BM cells, spleen cells or per microliter of blood was determined.

### Clustering assay

VLA-4 clustering assays were performed as described elsewhere,[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. Slides were stained with αCD49d (clone AHP1225), αCD29 (clone 12G10) primary antibodies or isotype control (not shown) followed by a 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 63×/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 at >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 and then 70,000 M2 stromal cells were seeded and cultured overnight. Primary AML (1x10$^6$ cells) or OCI-AML3 cells (0.5x10$^6$ cells) were seeded on M2 stromal cells and co-cultured for 30 min at 37°C. Cells were washed, fixed with 4% paraformaldehyde, 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 the *Online Supplementary Methods*.

## Results

### CD44 reflects and mediates leukemic infiltration of bone marrow

We measured CD44 and CD49d surface expression of BM-derived primary AML patients' samples and the AML cell line OCI-AML3 by flow cytometry. All AML patient-derived blasts, identified via CD45/side scatter 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 (*Online Supplementary Figure S1A*).

We next determined the *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 five independent experiments, total mononuclear cells from BM aspirates of five different AML patients (2 with wild-type *FLT3*, 3 with *FLT3*-ITD mutations) with a blast content of over 75% were either left untreated or treated with αCD44 Fab fragment (clone 515) or αCD49d antibody

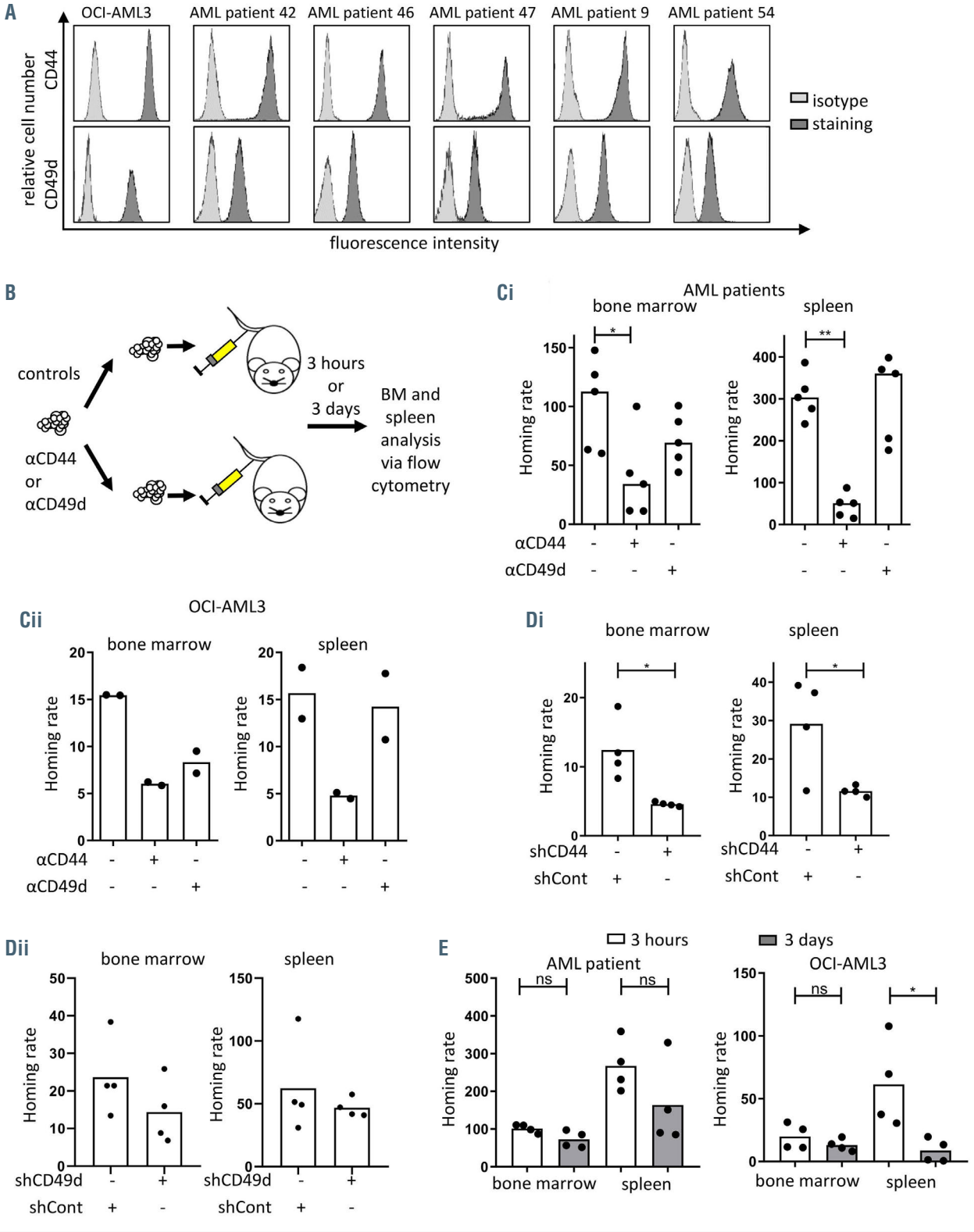


**Figure 1. CD44 and CD49d are both expressed on acute myeloid leukemia cells; CD44 has a predominant role in homing.** (A) Representative histograms of CD44 and CD49d surface expression of primary acute myeloid leukemia (AML) cells and the AML cell line OCI-AML3. (B) Mononuclear cells from bone marrow (BM) aspirates of AML patients pretreated or not with 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 $10^6$ measured cells per $10^6$ injected cells. (Ci) Homing rate to BM and spleen 3 h after injection was measured in five independent experiments using samples from five different AML patients. In each experiment, technical duplicates were performed and they were averaged for the analysis. (Cii) Homing rate to BM and spleen was measured 3 h after injection of OCI-AML3 (n=2). (Di + 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). **P*<0.05; ***P*<0.01; ns: not significant.

(clone HP2/1) and intravenously 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 the spleen and BM by human-specific antibodies (Figure 1B). Cells that had been treated with the blocking αCD44 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, αCD49d antibody treatment only slightly reduced BM homing of AML blasts and had no effect on their spleen homing (Figure 1Ci). In four of the five homing experiments we combined αCD44/αCD49d treatment, but additional CD49d blockade did not further increase the inhibitory effect above the level achieved by treatment with αCD44 alone (*Online Supplementary Figure S1B*). Comparable effects were observed when using OCI-AML3 cells (Figure 1Cii). 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 (*Online Supplementary Figure S2A*). Neither the functional inhibition of CD44 nor the inhibition of CD49d affected the general CD44 and CD49d expression of the cells (*Online Supplementary Figure S2B*). Anti-CD44 antibody treatment did not affect CD44/E-selectin-mediated cell arrest (*Online Supplementary Figure S2C*). We genetically confirmed the contribution of CD44 to homing by CD44 knockdown in OCI-AML3 cells (*Online Supplementary Figure S2Dii*), observing a significant reduction in homing of $CD44^{low}$ cells (Figure 1Di). These cells also showed reduced rolling on HA substrates under shear flow (*Online Supplementary Figure S2Di*). CD49d knockdown, which was confirmed via quantitative polymerase chain reaction (PCR), did slightly reduce homing and arrests of CD49d knockdown OCI-AML3 on VCAM-1 substrate were diminished (Figure 1Dii, *Online Supplementary Figure S2Ei+ii*). Concurrent analysis 3 days after transplantation allowed us to investigate not only homing but also early engraftment, which includes the 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 leukemic recovery in spleen 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 (*Online Supplementary Figure S2Fi+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 an αCD44 antibody, the AML pool shifted from BM to spleen 1 day after treatment, suggesting that CD44 is a BM retention factor (*Online Supplementary Figure S2G*). 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.

## An interaction between hyaluronic acid and CD44 triggers inside-out activation of VLA-4 in acute myeloid leukemia

To dissect the AML homing process in a mechanistic manner, we used *in vitro* flow chamber assays, as described elsewhere.[15] These assays allowed us 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 an immobilized HA substrate under shear stress. We found that the cells had a strong capacity to tether to and roll on this substrate: this capacity was completely abolished upon

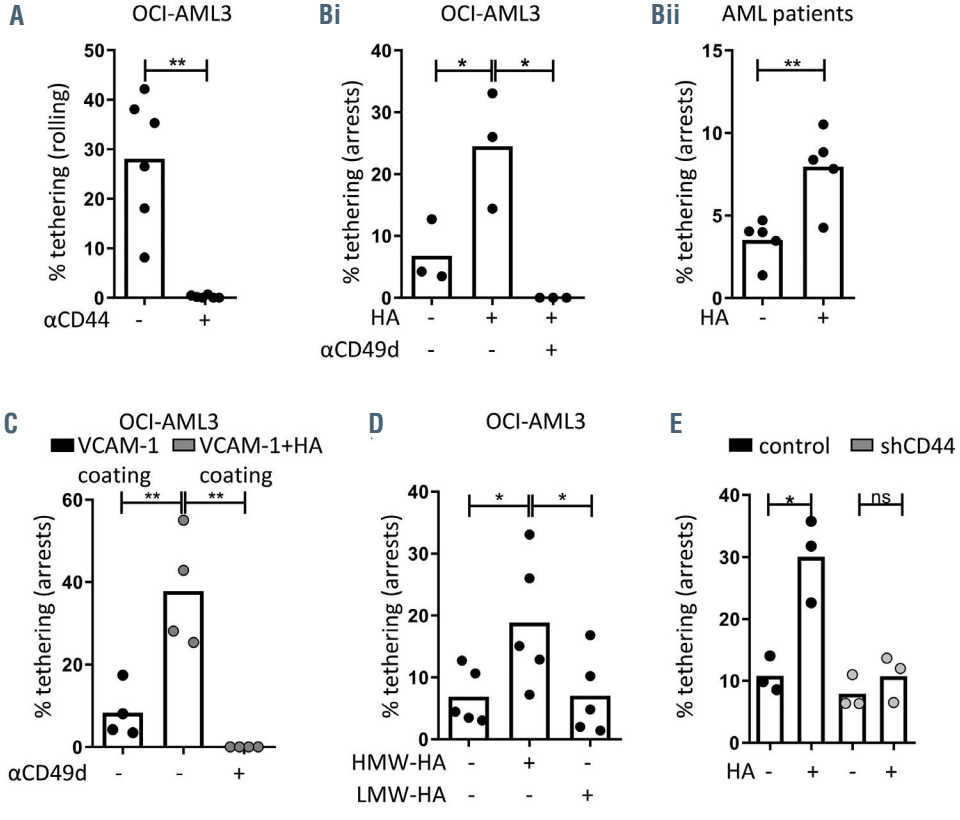


**Figure 2. Hyaluronic acid treatment increases acute myeloid leukemia cell arrests on VCAM-1 under shear flow.** (A) OCI-AML3 cells were perfused over hyaluronic acid (HA). Where indicated, cells were pretreated with blocking αCD44 antibody (clone 515). (B) OCI-AML3 (i) or primary acute myeloid leukemia (AML) cells from five 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 a 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 a paired *t*-test, three groups were compared with one-way analysis of variance with multiple comparisons. **P*<0.05; ***P*<0.01; ns: not significant.

treatment with αCD44 blocking antibody (clone 515) (Figure 2A). We further confirmed this HA-binding capacity by flow cytometry using fluorescein-labeled HA (HA-FITC) (*Online Supplementary Figure S3A*). 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 five different patients bound immobilized VCAM-1 at low adhesive strength, which was evident by the low frequency of firm adhesion on this substrate (Figure 2Bi+ii). Treatment with a blocking αCD49d 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 2Bi, *Online Supplementary Figure S3B*). 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 2Bi+ii, *Online Supplementary Figure S3C*), which suggests HA/CD44-induced inside-out VLA-4 activation. 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 depending on the molecular weight of the HA trigger.[16] Indeed, CD44-induced VLA-4 activation was only achieved by high molecular weight HA, but not by low molecular weight HA (Figure 2D). Using 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, different from the well-described, classical CXCL12/CXCR4-induced VLA-4 activation,[17] which led us to investigate the nature of this molecular crosstalk .

### Hyaluronic acid-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] (*Online Supplementary Figure S4A*). 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 a probe containing the conserved Leu-Asp-Val (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 expressed VLA-4 in an inactive conformation irrespectively of whether the cells were treated with HA or not and bound its ligand with comparable affinity (*Online Supplementary Figure S4B*).[15,20] This unexpected finding prompted us to elucidate whether 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 3Ai+ii). The mean number of clusters per cell was compared between untreated and HA-treated cells from all six patients (Figure 3Aiii). HA treatment also induced clustering of the VLA-4 β subunit CD29 (*Online Supplementary Figure S4C*). 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 cells, we confirmed that HA-induced CD49d clustering only occurred in cells that expressed CD44 (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 (*Online Supplementary Figure S4D*). We also performed an avidity-detecting shear flow assay, as described by Alon *et al.*,[21] by perfusing OCI-AML3 cells and primary cells over an αCD49d (clone HP2/1) substrate, further confirming our observations (Figure 3D, *Online Supplementary Figure S4E*). To get an insight into the lateral organization of the VLA-4 clusters on the membrane, we used methyl-beta-cyclodextrin (MßcD), which interferes with lipid structures. Although MßcD did not significantly reduce the number of HA-induced VLA-4 clusters, it abrogated their function to support cell tethering to VCAM-1 (*Online Supplementary Figure S4F*).

Next, we studied whether CD44-mediated inside-out activation is a general mechanism that also occurs in non-transformed progenitor cells. Using $CD34^+$ cells from four 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 (CD44v) composition, with an impact on the clinical outcome of AML patients.[22] We have analyzed the CD44v 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 (*Online Supplementary Figure S5A*). Interestingly, in OCI-AML3 CD44v6 cells, co-immunoprecipitated with CD49d, with a slight pull down increase when cells were preincubated with HA (*Online Supplementary Figure S5B*). 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 (SFK) 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 analyzed Src and Akt phosphorylation by western blot in native, control and shCD44-transduced OCI-AML3 cells (*Online Supplementary Figure S6A*). Remarkably, treating cells with the pan-SFK inhibitor PP2 abrogated the formation of HA-induced CD49d clusters on the surface of OCI-AML3 cells (Figure 4Ai) and six different AML patients' samples (Figure 4Aii + Ci), providing evidence that CD49d clustering was Src family-dependent. To start from broad but therapeutically relevant kinase inhibition, we used the multikinase inhibitor midostaurin, approved for the treatment of *FLT3*-mutated AML. We found that midostaurin is highly potent in antagonizing HA-induced CD49d cluster formation of OCI-AML3 cells (Figure 4Bi)

and six different AML patients' samples independently of their *FLT3* mutation status (*FLT3* wild-type [n=3], *FLT3*-ITD [n=3]) (Figure 4Bii + Cii).

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 (*Online Supplementary Figure S6B*). We next confirmed that PP2, midostaurin and idelalisib treatment not only inhibited cluster formation, but also reduced the binding of primary

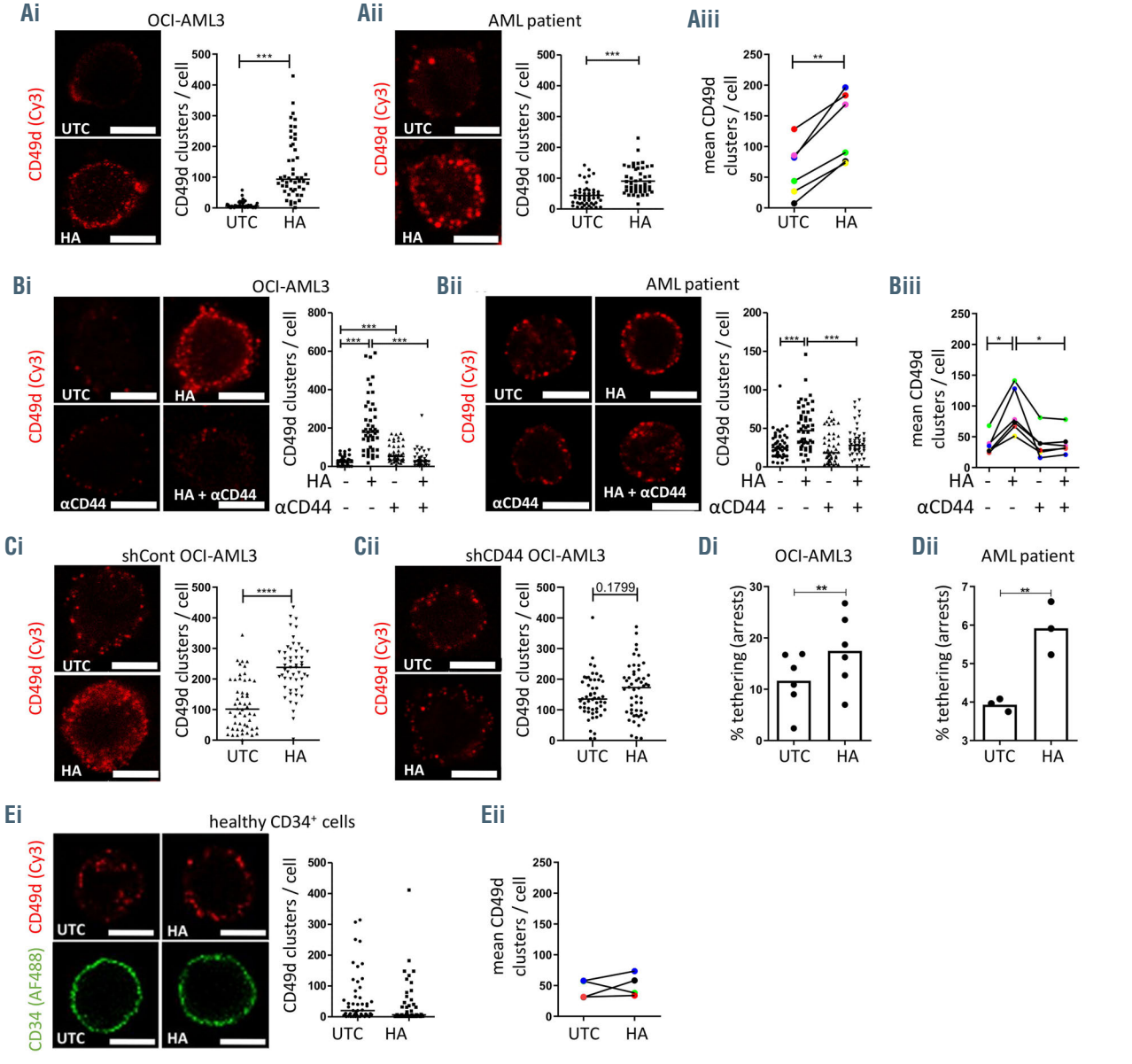


**Figure 3. Hyaluronic acid treatment induces CD49d cluster formation on acute myeloid leukemia cells.** (A) Confocal images and CD49d cluster analysis of OCI-AML3 (i) or primary acute myeloid leukemia (AML) cells from bone marrow (BM) aspirates (ii). Cells were untreated (UTC) or pretreated with hyaluronic acid (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 six different patients' 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 treatment or not with HA 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 analysis of variance [ANOVA] with multiple comparisons). Mean numbers of CD49d clusters per cell were compared from six different patients' 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). 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/cm$^2$ 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 a sample from 1 AML patient). (E) Primary CD34$^+$ cells from four different patients (3 with non-Hodgkin-lymphoma and 1 with multiple myeloma) 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's 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 four different patients' samples with/without HA treatment (paired *t*-test). Bars, 5 μm. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001.

AML cells (Figure 4D) and OCI-AML3 cells (*Online Supplementary Figure S6C*) 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.

### The hyaluronic acid-induced VLA-4/VCAM-1 interaction promotes an acute myeloid leukemia cell-stromal cell interaction leading to Akt, MAPK and NF-κB 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 four different patients were co-cultured 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 adhere firmly to 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 cells on stromal cells (Figure 5A, B). Additional controls using CD44 knockdown and control transduced OCI-AML3 cells (*Online Supplementary Figure S7A*) as well as native OCI-AML3 on αVCAM-1-antibody-treated stromal

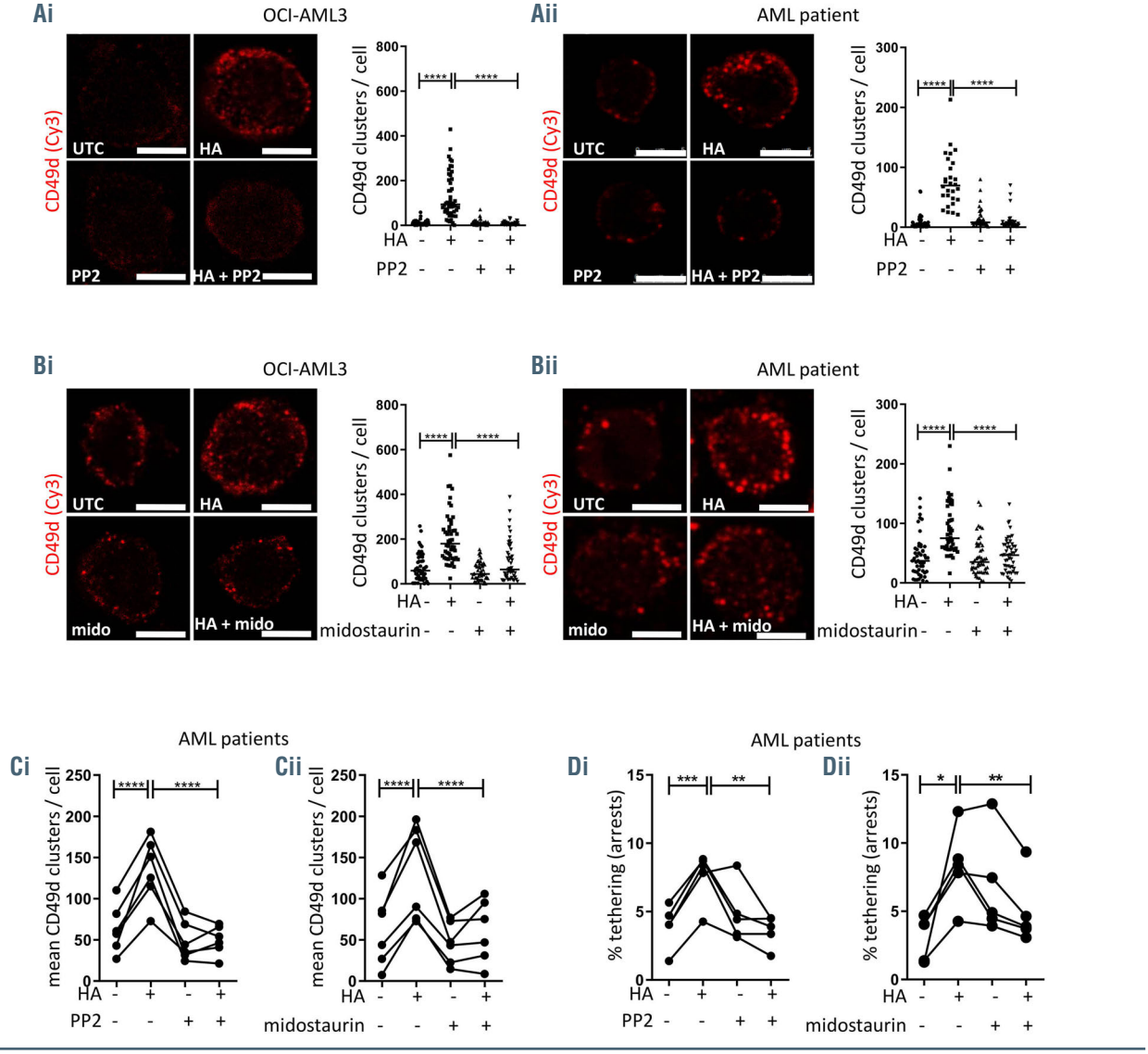


**Figure 4. Src family kinase inhibition and midostaurin treatment of acute myeloid leukemia cells inhibit hyaluronic acid-induced cluster formation.** (A) Confocal images of OCI-AML3 (i) or primary acute myeloid leukemia (AML) cells from bone marrow (BM) aspirates (ii) that were or were not pretreated with HA and/or the Src family kinase (SFK) inhibitor PP2. Cells were stained with αCD49d (red) monoclonal antibody (clone AHP1225). For OCI-AML3, one representative experiment of three is shown; for primary samples, one representative experiment of six is shown (n=50 cells). (B) Confocal images of OCI-AML3 (i) or primary AML cells from BM aspirates (ii). Cells were pretreated or not with HA and/or the multikinase inhibitor midostaurin. Cells were stained with αCD49d (red) monoclonal antibody. For OCI-AML3, one representative experiment of three is shown; for primary samples, one representative experiment of six is shown (n=50 cells). (C) Mean numbers of CD49d clusters per cell were compared from six different patients' samples with/without HA treatment and with/without PP2 treatment (i) or with/without midostaurin (ii). (D) Primary AML cells from five 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 analyses of variance with multiple comparisons were used. Bars, 5 μm. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; ns: not significant.

cells confirmed the specificity of the HA-CD44 interactions in triggering adhesion on VCAM-1 expressed by stromal cells (*Online Supplementary Figure S7B*).

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, IκB and mTOR.[1] Primary cells from five different AML patients were treated with HA and/or with VCAM-1-coated beads, where indicated. In contrast to brief HA treatment, VCAM-1-coated beads alone were sufficient to increase phosphorylation of Akt, ERK, IκB 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 (*Online Supplementary Figure S8A*). In line with this, VCAM-1 also triggered phosphorylation of ERK, IkB, FAK and paxillin (Pax) in OCI-AML3 cells (*Online Supplementary Figure S8B*).

In light of the key role of active Akt, MAPK, and NF-κB signaling in leukocyte survival, we next tested the protective effect of the 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 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 (AraC) treatment (*Online Supplementary Figure S9*). 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.

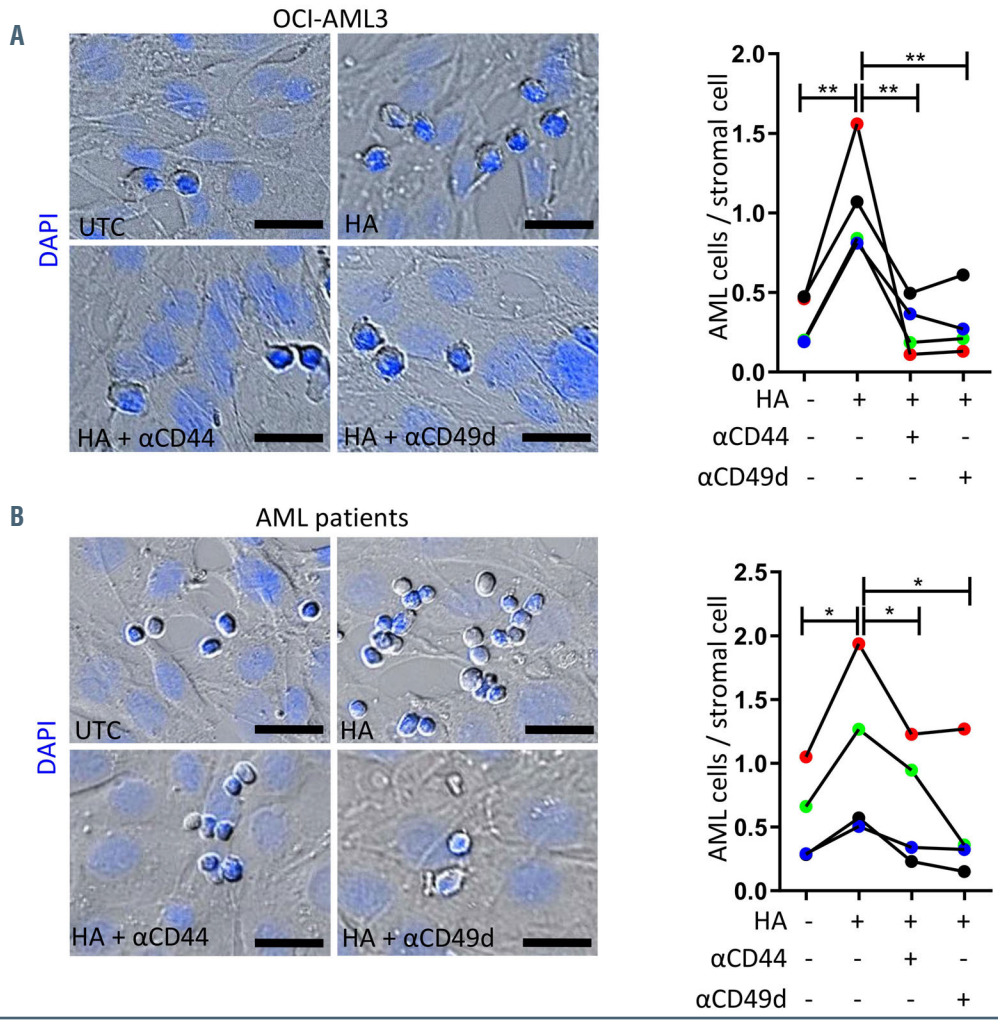


**Figure 5. Hyaluronic acid treatment leads to strong interaction between acute myeloid leukemia cells and stromal cells.** OCI-AML3 cells (n=4) (A) or primary cells from bone marrow (BM) aspirates from four different patients with acute myeloid leukemia (AML) (B) were pretreated or not with HA and/or 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 bound to the stromal cells was counted on bright field images with additional DAPI staining by fluorescence microscopy. One-way analyses of variance with multiple comparisons were used. Images were taken at 20x magnification. Bars, 20 μm. *$P$<0.05; **$P$<0.01.

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 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 to be 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,

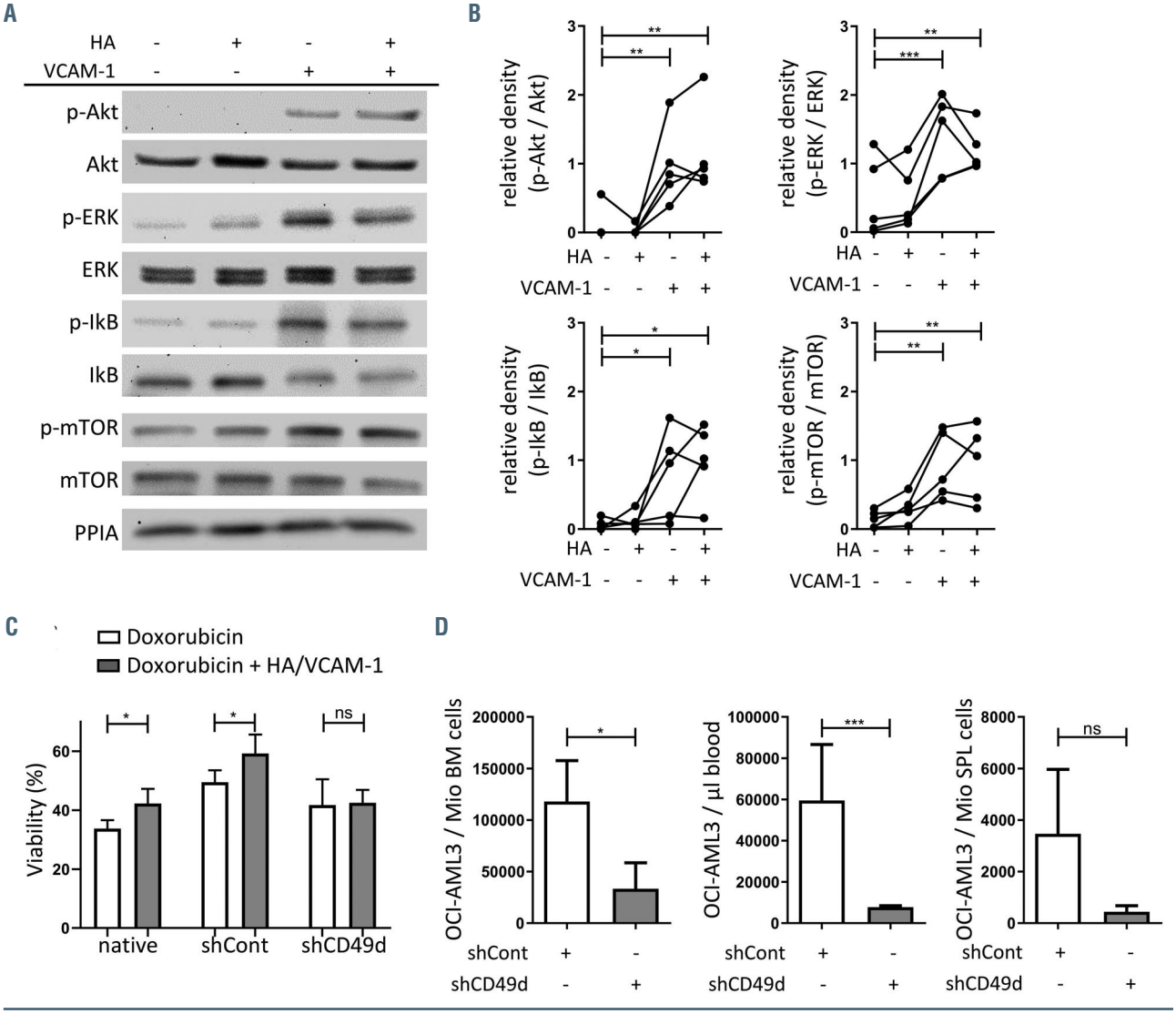


Figure 6. VLA-4 engagement triggers the phosphorylation of Akt, ERK, IκB and mTOR and contributes to acute myeloid leukemia progression. (A) Protein lysates of primary acute myeloid leukemia (AML) cells from bone marrow (BM) aspirates were treated or not with hyaluronic acid (HA) and/or VCAM-1-coated beads and tested for their ERK, phospho-ERK, Akt, phospho-Akt, IκBα, phospho-IκBα, mTOR and phospho-mTOR content by western blot. One representative experiment of five is shown. (B) Five independent experiments with five different AML patients' samples were quantified. Expression intensities were quantified with ImageJ software and phosphorylation was normalized to total protein content. One-way analyses of variance 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 of one representative experiment of two independent experiments are shown. (D) Number of shCont or shCD49d OCI-AML3 cells in BM, blood, and spleen (SPL) of NSG mice, 28 days after intravenous injection (n=7 per group). *$P$<0.05; **$P$<0.01; ***$P$<0.001; ns: not significant.

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 changes not only in the affinity but also the avidity 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 observed for the first time 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 hematologic malignancies.[33-38] Notably, we did 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.

SFK are crucial downstream signaling molecules of CD44 in hematopoietic cells of healthy and sick individuals.[39-41] They can contribute to enrichment of CD44/β1 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 the 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 the 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 the 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 patients' *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 the 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 investigate PI3K inhibitors further in AML. As another functional consequence, the VLA-4-mediated AML adhesion triggered activation of Akt, MAPK, mTOR and NF-κB pathways, which are known important mediators of AML survival.[53,54] However, in a first

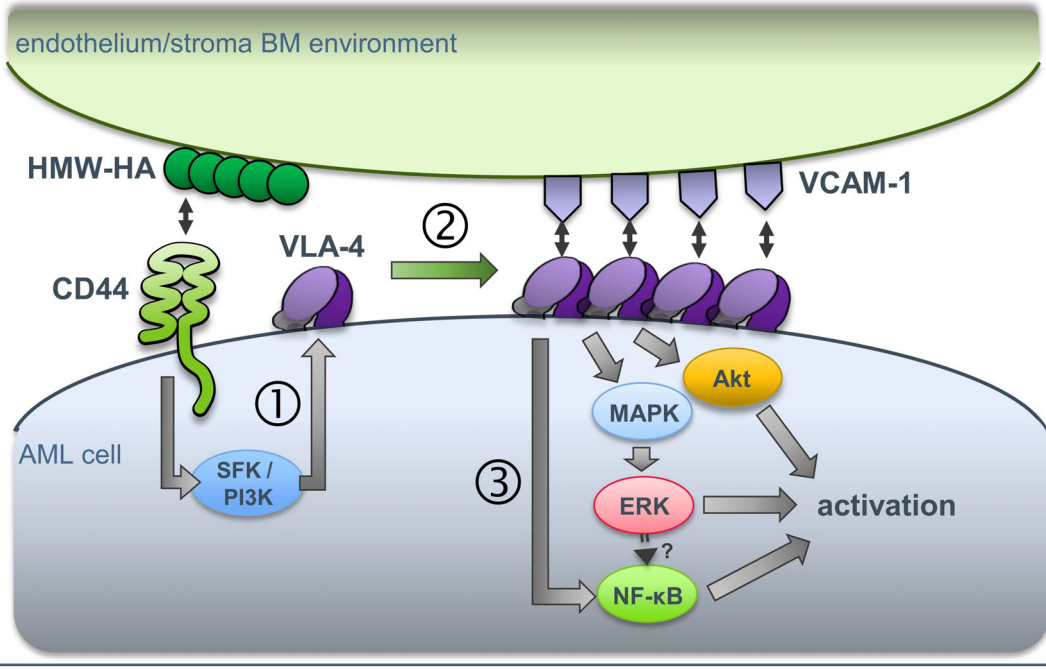


**Figure 7. Schematic overview of the suggested CD44-VLA-4 activation axis and downstream consequences.** Binding of acute myeloid leukemia (AML) cells to the bone marrow (BM) stromal component hyaluronic acid (HA) is dependent on CD44 and enhances adhesion of AML cells to the VLA-4 substrate VCAM-1, 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 [SFK]) 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 activation of the Akt, MAPK and NF-κB pathways.

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.

Taking all our data together, we suggest that HA binding to CD44 triggers a signaling axis via SFK and PI3K to rapidly trigger VLA-4 avidity and hence support the retention of AML cells in their preferential niches (Figure 7). Compensatory survival mechanisms of malignant cells still comprise a major challenge in current AML therapy which may be tackled by the use of combinatorial therapies, administering kinase inhibitors that 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.

### Disclosures

*No conflicts of interest to disclose.*

### Contributions

*JCG and TNH conceived and designed the study and wrote the manuscript. JCG, EB, XY, JML, AR, AC and DN developed the methodology. JCG, EB, XY, JML, JPH, GA, TR, AS, ES, TH, AH, ST, SP, AR, MA, AC and DN acquired data. JCG, EB, XY, JML, GA, TR, AS, AH, AC, DN, DFL; VO-R, RG and TNH analyzed and interpreted the data. JCG, EB, XY, JML,, JPH, GA, ST, TR, AS, ES, VD-O, AR, AH, NZ, AC, FA, DN and DFL reviewed and/or revised the manuscript. RG, LP, DN, DFL, VO-R. FA and TNH provided administrative, technical, or material support. TNH supervised the study.*

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