In vivo adhesion of malignant B cells to bone marrow microvasculature is regulated by $\alpha 4\beta 1$ cytoplasmic-binding proteins

Running title: $\alpha 4\beta 1$ in in vivo MM and CLL adhesion to BM

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ABSTRACT

Multiple myeloma (MM) and chronic lymphocytic leukemia (CLL) cells must attach to the BM microvasculature before lodging in the BM microenvironment. Using intravital microscopy (IVM) of the BM calvariae we demonstrate that the $\alpha_4\beta_1$ integrin is required for MM and CLL cell firm arrest onto the BM microvasculature, while endothelial P-selectin and E-selectin mediate cell rolling. Talin, kindlin-3 and ICAP-1 are $\beta_1$-integrin binding partners which regulate $\beta_1$-mediated cell adhesion. We show that talin and kindlin-3 cooperatively stimulate high-affinity and strength of $\alpha_4\beta_1$-dependent MM and CLL cell attachment, whereas ICAP-1 negatively regulates this adhesion. A functional connection between talin/kindlin-3 and Rac1 was found to be required for MM cell attachment mediated by $\alpha_4\beta_1$. Importantly, IVM analyses with talin- and kindlin-3-silenced MM cells indicate that these proteins are needed for cell arrest on the BM microvasculature. Instead, MM cell arrest is repressed by ICAP-1. Moreover, MM cells silenced for talin and kindlin-3 and cultured on $\alpha_4\beta_1$ ligands showed higher susceptibility to bortezomib-mediated cell apoptosis. Our results highlight the requirement of $\alpha_4\beta_1$ and selectins for the \emph{in vivo} attachment of MM and CLL cells to the BM microvasculature, and indicate that talin, kindlin-3 and ICAP-1 differentially control physiological adhesion by regulating $\alpha_4\beta_1$ activity.
Multiple myeloma (MM) and chronic lymphocytic leukemia (CLL) are B-cell neoplasms characterized by malignant cell trafficking to the bone marrow (BM) \(^1-^3\). Myeloma cells accumulate in the BM, leading to bone destruction, anemia, serum monoclonal gammopathy and immune suppression \(^4\), and their attachment to components of the BM microenvironment induces cell adhesion–mediated drug resistance (CAM-DR) \(^5\), \(^6\). Widespread involvement of the bone marrow and the presence of circulating MM cells in more than 70% of patients at diagnosis indicate that there is a continuous recirculation of MM cells in the peripheral blood and re-entrance to multiple areas in the bone marrow niches \(^7\), \(^8\). Homing of MM cells from the circulation into the BM milieu must begin with stepwise interactions with the BM microvasculature, involving rolling and firm attachment processes. The initial rolling step may potentially be mediated by selectins, and it was earlier shown that P-selectin glycoprotein ligand-1 (PSGL-1) contributes to the overall MM cell homing to the BM \(^9\). \textit{In vitro} data suggest that \(\alpha_4\beta_1\) and \(\alpha_L\beta_2\) integrins are relevant candidates to mediate the attachment of circulating MM cells to the BM microvasculature \(^10-^14\), but the \textit{in vivo} contribution of these integrins has not yet been fully elucidated.

The \(\alpha_4\beta_1\) integrin also plays key roles in CLL cell \textit{in vitro} migration and \textit{in vivo} homing to BM and lymph nodes (LN) \(^15-^20\). \(\alpha_4\beta_1\) expression is associated with the presence of lymphadenopathy in CLL patients \(^21\), and it was recently shown to be the strongest predictor of overall survival in CLL \(^22\). \(\alpha_L\beta_2\) displays aberrant chemokine-induced activation in CLL cells \(^23-^25\), and it also contributes to CLL cell \textit{in vitro} migration \(^21\). The role of \(\alpha_L\beta_2\) in CLL \textit{in vivo} adhesion is less clear. As in MM, CLL cell interaction with the BM microenvironment provides survival signals and induces drug resistance, thus contributing to disease progression \(^26\), \(^27\).
The α4β1 activity is tightly regulated by inside-out signalling induced upon chemokine-receptor interactions. In MM cells, the small GTPase Rac1 and its upstream activator DOCK2, as well as RhoA positively regulate this signalling in response to the chemokine CXCL12, leading to stimulation of α4β1-mediated cell adhesion. A key step in integrin activation in leukocytes is the binding of talin and the hematopoietic-specific kindlin-3 to the cytoplasmic domain of the integrin β1 subunit. The talin N-terminal FERM (protein 4.1, ezrin, radixin and moesin) region binds to the membrane-proximal NPXY/F motif of β subunit cytoplasmic tails. This causes the spatial separation of the α and β subunits and the extension of the integrin extracellular domain, leading to the generation of high-affinity integrins and upregulation of cell adhesion. Kindlin proteins also have a FERM domain which binds to a distinct NXXY/F site in the membrane-distal region of the β cytoplasmic domain. Recent data showed that talin and kindlin-3 have distinct but cooperative roles during integrin inside-out activation in leukocytes. Importantly, talin depletion leads to reduced chemokine-stimulated leukocyte adhesiveness to α4β1 and αLβ2 ligands. On the other hand, kindlin-3 regulates chemokine-stimulated leukocyte adhesion involving αLβ2, and to a lesser extent in attachment mediated by α4β1. Therefore, these observations revealed that talin and kindlin-3 are major inside-out signaling molecules for integrin activation in response to chemokines.

Integrin activation can be counteracted by molecules potentially competing with talin and kindlin for interaction with β1, such as ICAP-1. The ICAP-1 phosphotyrosine-binding domain interacts with a valine residue located at position -5 from the NPXY β1 motif. Remarkably, ICAP-1-deficient cells display fibronectin receptors in active conformations and increased cell adhesion to fibronectin. Together, these data raised the possibility that talin, kindlin-3 and ICAP-1 may
represent important molecules for the regulation of integrin-dependent attachment of malignant B cells to the BM microvasculature and to components of the BM microenvironment that potentially contribute to the trafficking and localization of these cells.

In the present study we have used intravital microscopy to directly examine the potential involvement of selectins and α4β1 and αLβ2 integrins during the early stages of *in vivo* recruitment of MM and CLL cells to the BM. Moreover, using *in vivo* and *in vitro* assays, we have further analyzed if α4β1-dependent MM and CLL cell adhesion is regulated by talin, kindlin-3 and/or ICAP-1.
**MATERIALS AND METHODS**

**Patients, cell purification, and cell lines.** Samples from MM and CLL patients were obtained after informed consent and followed the guidelines from the Ethics committees of the University Hospital of Salamanca, and 12 de Octubre, Gregorio Marañón and Puerta de Hierro hospitals (Madrid). The whole study was approved by the Consejo Superior de Investigaciones Científicas Bioethics Review Board. CD138$^+$ primary myeloma cells were purified from the mononuclear fraction of BM samples from patients with active MM using CD138 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CLL cells were purified from the peripheral blood by Ficoll-Hypaque (Nycomed, Oslo, Norway) centrifugation and, if <93% CD19 expression, negative selection with anti-CD3-conjugated Dynabeads (Invitrogen Dynal AS, Oslo, Norway) was employed. The human myeloma cell lines NCI-H929, MM1.S and RPMI 8226, and BM stromal cells HS-27A (ATCC, Manassas, VA), were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) (Biowest, Nuaillé, France). The MEC-1, MEC-2 and EHEB cell lines established from the peripheral blood of two patients with CLL $^{46}$ were purchased from DSMZ (Braunschweig, Germany) and maintained in IMDM medium (Lonza) with 10% FBS. The Epstein-Barr virus (EBV)–transformed CO43 and BRO168 cell lines, established from normal B lymphocytes, have been previously reported $^{16}$. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Verviers, Belgium).

**RNA interference, transfections and RT-qPCR.** siRNAs were purchased from Sigma-Aldrich and sequences are listed in supplementary table 1. Vectors coding for GFP and GFP-fused Rac V12 were provided by Dr. Francisco Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). siRNAs and vectors were nucleofected (Amaxa, Cologne, Germany) using solution V and programs T-01, O-23 and T-13 for
NCI-H929, MM1.S and MEC-1 cells, respectively, and transfectants assayed 20 h (NCI-H929) or 20-72 h (MEC-1) post-transfection. For RT-qPCR, RNA was extracted using TRI-Reagent (Sigma-Aldrich), and reverse transcribed using M-MLV RT (Promega, Madison, WI). Oligonucleotide sequences are provided in supplementary table 2, and RT-qPCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Assays were performed in triplicate, and results normalized according to the expression levels of TBP (TATA-binding protein) RNA and expressed by using the LightCycler 480 II software (Roche).

**Cell adhesion, migration and apoptosis assays.** For static adhesion assays to α4β1 ligands we followed the described method 47. Briefly, fluorescently-labelled cells were plated in triplicate on wells coated with CXCL12 or CCL21 together with FN-H89, a fibronectin fragment which contains the α4β1-binding region called CS-1, or with recombinant soluble VCAM-1, and plates were incubated for 2 min at 37°C upon a short spin. MM cells were also treated with soluble chemokine (1 min) and added to TNF-α-incubated HS-27A cells for 2 min at 37°C. Adhesions were quantified with a fluorescence analyzer (Varioskan Flash Multimode Reader, Thermo Scientific). Adhesion data is presented relative to control untreated cells, which have been given an arbitrary value of 100. For flow chamber adhesion assays, we followed the described protocol 48. Briefly, cells were infused at a flow rate of 1 dyne/cm² into flow chambers containing coimmobilized VCAM-1 (800 ng/ml) and CXCL12 (650 ng/ml). Rolling cells that subsequently firmly attached were expressed as stable arrest, whereas tethering cells that did not arrest at any moment were expressed as rolling cells. To evaluate shear resistance, cells were allowed to attach, and then were subjected to sequential increases of the flow rates. The number of cells remaining bound was determined as the percentage of total adhered cells after the adhesion step. Cell
migration across TNF-α-treated HUVEC was done as described\textsuperscript{49}, and migrated cells counted by flow cytometry. Cell viability was determined on a Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA), using Annexin V-FITC (Immunostep, Salamanca, Spain) and propidium iodide (Sigma-Aldrich). Bortezomib (Selleckchem (Houston, TX) was used at 40 nM.

**Intravital microscopy.** Five week-old NSG mice prepared for intravital imaging of the calvarial bone\textsuperscript{29, 50}, were injected with fluorescently-labelled cells through a catheter placed in the left carotid artery, and images were acquired using a Leika DM6000 epifluorescence microscope. In the experiments in which we blocked integrins, 5×10\textsuperscript{6} CFSE-labelled MM1.S, NCI-H929, RPMI 8226, MEC-1 and EHEB cells were incubated for 30 minutes with 5 μg of purified anti-human α4, αL or anti-human PC3 antibodies. Saturation with antibodies was confirmed by flow cytometry (not shown). To determine endothelial selectin-mediated rolling and adhesion, mice received intravenously (i.v.) 50 μg of blocking antibodies against E-selectin and/or anti P-selectin 20 min before image acquisition. For CXCR4 inhibition, cells were incubated with 5 μM of AMD3100 for 3 h, and subsequently washed and immediately i.v.-injected into mice prepared for intravital imaging. For the experiments involving siRNA transfectants, 5×10\textsuperscript{6} CFSE-labelled cells were injected i.v. at the time of imaging. Collecting venules (Cv) and sinusoids (S) have been anatomically defined previously, and are functionally associated with early engraftment in mouse models\textsuperscript{50, 51}. The number of rolling and adherent cells, as well as haemodynamic parameters was analyzed by blind off-line video analysis. For each vessel we measured the maximum blood velocity ($V_{\text{max}}$) from the fastest free-flowing cells, as well as the vessel diameter ($D_v$), mean velocity ($V_{\text{mean}}$) and wall shear rate (WSR), by applying the following formulae: $(V_{\text{mean}} - V_{\text{max}})/(2 - \varepsilon^2)$, where $\varepsilon = D_c/D_v$, and the cell diameter ($D_c$) for NCI-
H929 is 8 µm, WSR = 8 \((V_{mean}/D_v)\) and \(V_{crit} = V_{mean} \times \varepsilon(2 - \varepsilon)\). Any cell travelling below \(V_{crit}\) was considered to be rolling on the vessel wall. Cells that remained stationary for 3 s or more were considered arrested. The fraction of cells that rolled or arrested according to these criteria were scored from the movies. An example movie with MM1.S cells showing cells in free flowing, rolling or arrested is displayed in Supplemental movie 1. Animal procedures followed the guidelines of the Ethical Committee at CNIC.

**Statistical analyses.** Data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparisons. In both analyses, the minimum acceptable level of significance was \(p<0.05\).
RESULTS

The α4β1 integrin is required for in vivo MM and CLL cell attachment to the BM microvasculature. We used intravital imaging (IVM) of the calvarial bone of NSG mice to determine the in vivo role of α4β1 and αLβ2 integrins in the initial adhesive steps of circulating MM and CLL cells to the BM endothelium. Preliminary analyses indicated that α4β1 is expressed in MM1.S, NCI-H929 and RPMI 8226 myeloma cells, as well as in primary CLL cells and in the CLL cell lines MEC-1 and EHEB (not shown). In addition, we found that cell adhesion to recombinant VCAM-1 is blocked by the anti-α4 ALC 1/63 mAb (Supplementary Figure 1A, B). NCI-H929 and MEC-1, but not MM1.S cells express αLβ2 (not shown), and cell attachment to ICAM-1 was inhibited by the anti-αL R7-1 mAb (Supplementary Figure 1A, B).

IVM analyses showed that pre-incubation with anti-α4 mAb led to a dramatic inhibition of the firm arrest of fluorescently-labelled MM1.S, NCI-H929, RPMI 8226, MEC-1 and EHEB cells, as well as of primary CLL cells, to the microvasculature of the calvarial BM, as compared with attachment of cells incubated with control mAb (Figure 1A-C; Supplementary movies 1-6). Cell rolling was reduced by anti-α4 mAb in MM cells, whereas decreased rolling was especially detected on EHEB and primary CLL cells and to a lower extent in MEC-1 cells (Figures 1A-C). We were unable to perform IVM analyses with primary bone marrow MM cells, as drawing solid and statistically significant conclusions from these analyses require at least 4x10^6 cells/condition/mouse, well above the cell number available from MM-BM sources. Opposite to the anti-α4 mAb, blockade of αL did not inhibit the rolling or firm arrest of MM or CLL cells to the BM microvasculature (Figures 1A-C). The CXCR4 inhibitor AMD3100 impaired MM cell attachment to the BM endothelium to an extent similar to that obtained by inhibition of α4 integrins (Supplementary Figure 2). These results indicate that α4β1 is
a main integrin mediating *in vivo* attachment of circulating MM and CLL cells to the
BM microvasculature, an early and limiting step in their homing to the bone marrow.

**Role of selectins on the *in vivo* MM and CLL cell attachment to BM endothelium.**

Using a flow cytometry-based assay to measure binding of soluble P- or E-selectin chimeras (P- or E-selectin/hIgM), which identifies the presence of functional selectin ligands (Supplementary Figure 3), we found that MM1.S cells express only ligands for P-selectin, whereas NCI-H929 and MEC-1 cells express ligands for both P- and E-selectin (Figure 2A). We addressed the potential selectin involvement in *in vivo* MM and CLL cell tethering onto the BM microvasculature by exposing mice to anti-P- or anti-E-selectin blocking antibodies before performing IVM analyses. The data revealed that both antibodies significantly inhibited the rolling and subsequent firm arrest of MM and CLL cells to the BM endothelium (Figure 2B, C), thus indicating that P- and E-selectin ligands also contribute to the interaction of these cells with the BM microvasculature. Of note, whereas anti-selectin antibodies inhibited to similar levels both rolling and firm arrest of MM and CLL cells, anti-α4 mAb impaired their stable adhesion to a higher extent than rolling, indicating that selectins participate in firm arrest of MM and CLL cells by mediating rolling, while α4β1 function is important for both their rolling and firm arrest.

**Talin and kindlin-3 positively regulate α4β1-dependent MM cell attachment to the BM microvasculature and to BM stromal cells.** Having identified a central role for α4β1 in the early adhesive steps of MM and CLL cell migration to the BM, we next examined if its activity can be regulated by β1-binding proteins in these cells. The integrin β1 subunit cytoplasmic domain contains docking sites for talin and kindlin-3. RT-qPCR experiments revealed heterogeneous levels of talin and kindlin-3 mRNAs amongst different primary MM BM samples (Figure 3A). Additionally, talin and
kindlin-3 were detected in the MM1.S, NCI-H929 and RPMI 8226 cell lines (Figure 3B, left; Supplementary Figure 4A). Co-immunoprecipitation experiments indicated that β1 associates with talin and kindlin-3 in NCI-H929 cells, and that the chemokine CXCL12 gradually increases this association (Figure 3B, right). Moreover, talin-β1 assembly was also detected in MM1.S and RPMI 8226 cells (Supplementary Figure 4B).

To analyze the role of talin and kindlin-3 in α4β1-mediated MM cell attachment to the BM microvasculature, we silenced them by RNA interference in NCI-H929 cells (Figure 3C, left and middle panels; Supplementary figure 4C), and first tested the knockdown transfectants in static adhesion assays to VCAM-1. Control flow cytometry experiments indicated that α4, β1 or CXCR4 expression was not significantly altered by talin or kindlin-3 knocking down (not shown), and that cell viability was similar among the different transfectants (Supplementary figure 5A). Depletion of either talin or kindlin-3, or their combined silencing caused a significant reduction in cell attachment to VCAM-1, compared with control transfectants (Figure 3C, right). CXCL12 upregulated the attachment of control siRNA transfectants to co-immobilized VCAM-1, whereas the adhesion of single talin or kindlin-3 knockdown transfectants was approximately half of control transfectant adhesion. Furthermore, depletion of both proteins completely abolished the CXCL12-stimulated attachment.

To identify mechanisms that are associated with the defective adhesion of MM cells silenced for talin and kindlin-3, we first analyzed if their α4β1 affinity was altered. For this purpose, we performed flow cytometry with the β1 activation-reporter mAb HUTS-21, which detects α4β1 high-affinity conformations. Control siRNA transfectants displayed moderate but significantly induced HUTS-21 mAb binding in response to CXCL12, whereas binding was inhibited when both talin and kindlin-3 were silenced (Figure 3D). Next, we performed adhesion assays under flow conditions
measuring the cells that rapidly (less than 20 sec) and stably adhere under a shear stress of 1 dyne/cm² to VCAM-1 co-immobilized with CXCL12. Of note, talin- and kindlin-3-knockdown MM transfectants displayed a significant decrease in stable cell arrest compared with control siRNA transfectants (Figure 3E, left). To analyze the $\alpha_4\beta_1$-dependent adhesion strength of these MM transfectants, we subjected cells that had been attached to VCAM-1/CXCL12 at 1 dyne/cm², to detachment at sequential increases of shear stress. Transfectants knock down for both proteins were found to develop lower resistance to detachment at high shear stress than control siRNA transfectants (Figure 3E, right).

To further investigate mechanisms linked to the deficient adhesion of talin- and kindlin-3-knockdown cells, we analyzed the transfectants for a potentially altered function of the GTPase Rac1, as we and others have shown that Rac1 was required for CXCL12-stimulated, $\alpha_4\beta_1$-dependent myeloma cell adhesion \cite{29, 30}. To address this question, we transfected GFP-fused constitutively active forms of Rac (GFP-Rac V12) together with siRNA for both talin and kindlin-3, and then tested the transfectants in adhesion assays to VCAM-1 co-immobilized with CXCL12. We found that GFP-Rac V12 expression reversed the inhibitory effects of talin/kindlin-3 siRNA on the stimulation of cell adhesion to VCAM-1/CXCL12 (Figure 3F; Supplementary Figure 6).

Importantly, combined talin and kindlin-3 depletion significantly impaired the \textit{in vivo} MM cell rolling and firm arrest on the BM microvasculature, as evidenced in IVM analyses (Figure 3G). Together, these results indicate that both talin and kindlin-3 are needed for optimal $\alpha_4\beta_1$-dependent attachment of circulating MM cells to the BM microvasculature, and reveal that these two proteins cooperate to enhance the affinity and the strength of $\alpha_4\beta_1$-dependent adhesion. Furthermore, the data identify key
functional connections between Rac1 and talin/kindlin-3 during chemokine-stimulated MM cell adhesion involving α4β1.

We next analyzed the potential involvement of talin and kindlin-3 in MM cell transendothelial migration (TEM) using TNF-α-treated HUVEC. Anti-α4 mAb inhibited by 60% the CXCL12-upregulated migration of NCI-H929 cells across HUVEC (Figure 4A, left). Similarly, single talin or double talin-kindlin-3 knockdown transfectants displayed 50-60% impairment in their TEM, whereas silencing kindlin-3 alone reduced migration to a lower degree (25%) (Figure 4A, right). These data indicate that talin and to a lower extent kindlin-3 are needed for MM cell TEM involving α4β1.

Once MM cells cross the BM microvasculature, they establish contacts with extracellular matrix proteins, including fibronectin, as well as with stromal cells of the BM microenvironment. Depletion of either talin or kindlin-3 caused a significant reduction in basal and CXCL12-stimulated MM cell attachment to the CS-1/fibronectin fragment FN-H89, whereas cells silenced for both proteins showed a full blockade of chemokine-activated adhesion (Figure 4B). Moreover, under conditions where the adhesion of NCI-H929 cells to HS-27A BM stromal cells was reduced by 50% by anti-α4 mAb (Figure 4C, left), CXCL12-upregulated attachment was significantly inhibited by single talin or kindlin-3 knocking down, and inhibition was further increased upon silencing both proteins (Figure 4C, right). These results indicate that α4β1-dependent adhesion of MM cells to the BM microenvironment is positively regulated by talin and kindlin-3.

As α4β1-dependent MM cell adhesion has been linked to cell adhesion-mediated drug resistance \(^5\), \(^6\), we searched for potential connections between the inhibition of cell adhesion to α4β1 ligands observed with talin/kindlin-3 MM transfectants and MM cell apoptosis mediated by the proteasome inhibitor bortezomib.
(BTZ). Apoptosis levels increased from 30% to 45% in NCI-H929 cells knock down for both talin and kindlin-3 when they were exposed to BTZ in FN-H89-coated dishes, as compared with control siRNA transfectants incubated under the same conditions (Figure 4D). These data indicate that inhibition of positive regulators of α4β1-dependent MM cell adhesion such as talin and kindlin-3 render myeloma cells more susceptible to BTZ-mediated cell apoptosis.

ICAP-1 negatively regulates α4β1-dependent MM cell attachment to the BM microvasculature. ICAP-1 binds to the β1 integrin cytoplasmic domain, in possible competition with kindlin-3. Real-time PCR experiments revealed heterogeneous levels of ICAP-1 mRNA in primary MM bone marrow cells, as well as in the NCI-H929, MM1.S and RPMI 8226 cell lines (Figure 5A, left). Using several commercially-available anti-ICAP-1 antibodies we were unable to detect ICAP-1 protein by immunoblotting using NCI-H929 cell lysates (not shown). However, anti-β1 mAb co-immunoprecipitated a 23-25 kD protein from these cells which was specifically detected by anti-ICAP-1 antibodies (Figure 5A, right). Cell exposure to CXCL12 did not significantly alter the amount of ICAP-1 co-precipitated with β1 (not shown). These results suggest that a large portion of ICAP-1 is bound to β1 integrins in NCI-H929 myeloma cells.

Next, we knocked down ICAP-1 in MM cells (Figure 5B, left), and tested transfectants in adhesion assays to purified α4β1 ligands or to HS-27A BM stromal cells. Of note, ICAP-1-depleted cells displayed a significant increase in adhesion to VCAM-1, FN-H89 and HS-27A cells compared to control siRNA-transfectants (Figure 5B, middle and right; and Figure 5C). No alterations in α4β1 expression (not shown) or in survival (Supplementary figure 5A) were detected in ICAP-1-knockdown MM cells. Moreover, ICAP-1-depleted cells exhibited a significant increase in HUTS-21 binding
compared to control transfectants (Figure 5D), revealing that $\alpha_4\beta_1$ switched to high-affinity conformations upon reducing ICAP-1 expression. Importantly, ICAP-1 knocking down in MM cells led to upregulated cell firm arrest onto BM microvasculature compared to control transfectants, as assessed in IVM analyses (Figure 5E; and Supplementary video 6). We did not observe any obvious change in cell rolling in ICAP-1-silenced cells. These data indicate that ICAP-1 represses the $\alpha_4\beta_1$-dependent adhesion of MM cells.

**Role of talin, kindlin-3 and ICAP-1 in $\alpha_4\beta_1$-dependent CLL cell adhesion and transendothelial migration.** Real-time PCR experiments showed heterogeneous levels of talin, kindlin-3 and ICAP-1 mRNAs amongst different primary CLL samples, as well as in the MEC-1, MEC-2 and EHEB CLL cell lines, and EBV–transformed CO43 and BRO168 cells established from normal B lymphocytes (Figure 6A; Supplementary Figure 4D). In addition, talin and kindlin-3 proteins were observed in all these CLL cells (Figure 6B, left). Co-immunoprecipitation experiments indicated that association of the $\beta_1$ subunit with talin and kindlin-3 was enhanced upon incubation for 1.5 min of MEC-1 cells with the chemokine CCL21 (Figure 6B, right). Importantly, combined silencing of talin and kindlin-3 led to a significant reduction in MEC-1 cell adhesion to VCAM-1, both under basal conditions and in the presence of CCL21 (Figure 6C, left and middle; Supplementary figures 4E and 5B). Furthermore, talin and kindlin-3 depletion partially impaired MEC-1 cell migration across HUVEC using CCL21 as stimulus (Figure 6C, right). In contrast, ICAP-1-knockdown MEC-1 cells displayed significantly increased adhesion to VCAM-1 (Figure 6D; and Supplementary figure 5B). Altogether these data indicate that CLL cell adhesion and transendothelial migration involving $\alpha_4\beta_1$ are differentially regulated by talin, kindlin-3 and ICAP-1.
DISCUSSION

Trafficking of MM and CLL cells to the BM and disease progression are connected by integrin α4β1 activity. Previous in vitro data led to the proposal that α4β1 could participate in the initial adhesive steps of MM and CLL cell migration into the BM. Using intravital microscopy of the BM calvariae of NSG mice, we demonstrate that α4β1, but not αLβ2, is a major integrin mediating firm arrest of circulating MM and CLL cells to the BM microvasculature. In addition, we found that α4β1 also contributes to the preceding MM cell rolling step. Furthermore, our data reveal that selectin ligands (most likely PSGL-1) on MM and CLL cells mediate interactions with P-selectin and E-selectin, which are constitutively expressed by the BM endothelium, thus representing the main adhesive events accounting for the rolling of these cells onto the BM microvasculature. To our knowledge, this is the first in vivo demonstration showing the involvement of P- and E-selectin and α4β1 in the rolling and firm arrest steps of circulating MM and CLL cells on the BM endothelium. Together with our intravital microscopy analyses revealing that CXCR4 blockade inhibits the MM cell attachment to the BM endothelium, in line with previous data, the present results strongly suggest that CXCL12 exposed on BM endothelial cells stimulates α4β1 activity of tethered MM cells, together crucially contributing to the initial steps of MM cell adhesion onto the BM microvasculature.

Talin and kindlin-3 bind to the β1 subunit cytoplasmic domain and stimulate the affinity and avidity of leukocyte integrins. We show here that talin and kindlin-3 bind to β1 in MM and CLL cells, and that this binding is activated by chemokines. Moreover, the in vitro adhesion data obtained both under static and flow conditions reveal that talin and kindlin-3 positively regulate the attachment of these cells to VCAM-1, both under basal and chemokine-stimulated conditions. Our results show that...
stimulation of adhesion by these positive regulators is based on induction of α4β1 high-
affinity conformations, and also on boosting the strength to the adhesions to VCAM-1/CXCL12. This later response was especially detected in adhesion assays under flow conditions, providing high relevance to the finding. Further, a functional link between talin/kindlin-3 and Rac1 during chemokine-stimulated MM cell adhesion to VCAM-1 was found. Characterization of this link should provide additional mechanistic insights on the regulated MM cell adhesion. These in vitro results are strengthened by intravital imaging analyses with talin- and kindlin-3-silenced MM cells, which indicate that these proteins are required for efficient cell arrest on the BM microvasculature. Therefore, our data support a cooperative model between talin and kindlin-3 for efficient stimulation of α4β1-dependent MM cell attachment onto the BM endothelium (Figure 7), similar to the cooperation between these proteins during α4β1- and αLβ2-mediated leukocyte adhesion. As CCL21 is mainly expressed in lymph nodes, whereas no or low CCL21 has been detected in BM, our data with CLL cells depict a putative role for talin and kindlin-3 in supporting α4β1-dependent CLL cell attachment to and migration across endothelium in the trafficking of these cells to lymph nodes.

Examination of the role of talin and kindlin-3 on CXCL12-stimulated migration of MM cells across endothelium involving α4β1 revealed that this process is highly dependent on talin and to a lesser extent on kindlin-3. These results pose that talin is crucial for both α4β1-dependent MM cell attachment to and migration across endothelium, whereas kindlin-3 is important especially at the attachment step. Notably, cooperation between talin and kindlin-3 was also observed during attachment of MM cells to components of the BM microenvironment, as the α4β1-mediated, CXCL12-stimulated MM cell adhesion to CS-1/fibronectin and to BM stromal cells required both
talin and kindlin-3. These data strongly suggest that once MM cells cross the BM microvasculature, their lodging in the BM mediated by α4β1 needs talin and kindlin-3 function. Furthermore, our data raise the possibility that talin- and kindlin-3-supported MM cell adhesion to BM stroma involving α4β1 might contribute to MM disease progression and possibly to CAM-DR responses. An experimental insight into this later process revealed that talin/kindlin-3-knockdown MM cells exposed to α4β1 ligands were more sensitive to cell death induced by bortezomib, highlighting that activators of α4β1-dependent MM cell adhesion contribute to CAM-DR. Therefore, the molecules that regulate α4β1-mediated MM cell adhesion to the BM microenvironment might constitute targets whose therapeutic relevance deserves to be explored.

In the present study we also addressed whether ICAP-1, a protein thought to compete with talin and kindlin-3 for binding to the β1 cytoplasmic domain, thus acting as a putative integrin repressor, could modulate MM and CLL α4β1-dependent cell adhesion. We found that ICAP-1 was expressed in primary MM and CLL cells, and importantly, ICAP-1 silencing in MM and CLL cell lines led to remarkable increases in α4β1-dependent adhesion involving stimulation of the integrin high-affinity conformations. Moreover, highlighting the regulatory role of ICAP-1, its silencing in MM cells resulted in enhanced attachment of circulating cells onto the BM microvasculature during IVM studies. Overall, these results indicate that ICAP-1 negatively regulates α4β1 function in MM cells (Figure 7). Our data represent the first identification of an endogenous molecule strongly opposing α4β1 activation and adhesion in MM and CLL cells. Thus, these findings provide an attractive scenario to be investigated in light of the importance of α4β1 in the progression of these B-cell malignancies.
The proposed model (Figure 7) suggests that the global level of cell adhesion is likely the balance between the positive signals from talin and kindlin-3 and the inhibitory actions of ICAP-1. Following talin and kindlin-3 silencing, inactive α4β1 conformationss accumulate, perhaps due to enhanced ICAP-1 binding to β1, leading to reduced α4β1-dependent MM and CLL cell attachment. When ICAP-1 is knocked down, the balance between positive and negative signals tilts towards higher stimulation of α4β1 activity by talin and kindlin-3, resulting in enhanced MM and CLL cell adhesion to the BM endothelium.

The distinct regulation by the β1-interacting partners talin, kindlin-3 and ICAP-1 on MM and CLL cell adhesion involving α4β1 could account for mechanisms associated with drug resistance and tumor progression in human B-cell malignances. Thus, targeting intracellular regulators of the α4β1-integrin in combination with present therapies might exert beneficial effects to overcome the deleterious emergence of constitutive or transient integrin activation in clonal derivatives of drug-resistant malignant B-cells.
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CONFLICT-OF-INTEREST

The authors declare no conflict of interest.

AUTHORSHIP

M.M-M, M.L and N.A-M performed experiments and analyzed data. S.S-M, S.I.V and N.A-S performed some experiments, analyzed data and prepared valuable reagents. N.G, R.M., J.M-L, I.B. and J.A.G-M collected and provided primary cells from MM and CLL patients. J.T., A.G-P, A.H and P.S-M designed the research and wrote the manuscript. All authors reviewed and approved the manuscript.

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58. Davids MS, Burger JA. Cell Trafficking in Chronic Lymphocytic Leukemia. *Open J Hematol* 2012; 3(S1).
Figure 1. The α4β1 integrin is required for in vivo MM and CLL cell attachment to the BM microvasculature. MM cells (NCI-H929, MM1.S and RPMI 8226) (A), and CLL cells (MEC-1, EHEB and primary cells) (B, C) were fluorescently labeled and pre-incubated with control, anti-α4 ALC 1/63 or anti-αL R7-1 mAb, and subsequently injected into NSG mice prepared for intravital imaging of the calvarial bone marrow. Micrographs are representative images of the collecting venules (Cv) and sinusoids (S) that irrigate the BM from each experimental group. Free flowing (arrowhead), rolling (·) or arrested cells (*) can be visualized against the auto-fluorescent bone and dark vessels. Bar graphs show the percentage of passing cells that rolled (defined as those moving below the Vcrit, as described in the Methods section), or arrested for 3 sec or longer. Bars represent mean ± SEM values obtained from the analysis of 16-20 venules from 4 mice. ***p<0.001, **p<0.01 (one-tailed t test); n.s., non-significant.

Figure 2. Role of P-selectin and E-selectin on the in vivo MM and CLL cell attachment to BM endothelium. (A) Cells were incubated with fluorescently-labelled P- or E-selectin/hIgM chimera in the absence or presence of EDTA, and samples subjected to flow cytometry. Selectin ligands were detected with Cy5-labelled anti-human IgM. (B, C) Cells were analyzed by IVM using NSG mice that had been pre-treated with control antibodies, or with antibodies to E-/and or P-selectin. Micrographs are representative images of collecting venules and sinusoids irrigating the BM. Bar graphs show the percentage of rolling or arrested cells. Bars represent mean ± SEM values obtained from the analysis of 16-20 venules from 4 mice. ***p<0.001, **p<0.01, * p<0.05; n.s., non-significant.

Figure 3. Role of talin and kindlin-3 in α4β1-dependent MM cell attachment. (A) Primary CD138+ BM samples from MM patients were analyzed by RT-qPCR for talin
and kindlin-3 mRNA levels (normalized to the reference gene TBP). (B, left) The indicated MM cell lines were analyzed by immunoblotting for talin or kindlin-3 expression. Loading control was assessed with anti-vinculin antibodies. (Right) Cells were serum-starved and incubated for different times in the absence or presence of CXCL12, and subsequently subjected to immunoprecipitation and immunobloting with the antibodies shown. (C, left) Cells were nucleofected with the indicated single or combined siRNA, and transfectants analyzed by western blotting. (Medium) Densitometric analyses of protein gel bands show the mean ±SD of four independent experiments. (Right) The different control, talin and kindlin-3 siRNA transfectants were analyzed in adhesion assays to VCAM-1 co-immobilized with or without CXCL12. (D) Transfectants were tested by flow cytometry for binding of the anti-β1 HUTS-21 mAb, in the absence or presence of CXCL12. Adhesions and binding (n= 4) were significantly inhibited, ***p<0.001, *p<0.05. (E, left) NCI-H929 cells transfected with both talin and kindlin-3 or with control siRNA were perfused in flow chambers coated with VCAM-1 co-immobilized with CXCL12, and analyzed for rolling and stable cell arrest (n=3). Data is presented as mean ± SD of cell percentages from the total cell population. Adhesions were significantly inhibited in comparison with those of control siRNA transfectants, *p<0.05. (Right) The indicated siRNA transfectants pre-attached onto co-immobilized VCAM-1 and CXCL12 in flow chambers, were subjected to cell detachment after sequential increases of shear stress. Data show mean ±SD of cell percentages from the initial number of bound cells remaining attached at the indicated shear stresses (n=3). (F) NCI-H929 cells were transfected with the indicated siRNA and GFP vector combinations, and analyzed in adhesion assays to VCAM-1 immobilized with CXCL12. Adhesion was significantly inhibited, *p<0.05, or rescued, Δp<0.05. (G) NCI-H929 cells were transfected either with control or with both talin and kindlin-3
siRNA, and fluorescently-labeled transfectants were subsequently injected into NSG mice prepared for IVM of the calvarial BM. Bar graphs show the percentage of passing cells that rolled or arrested, and represent the mean ± SEM values obtained from the analysis of 16-20 venules from 4 mice. (**p<0.001).

**Figure 4. Role of talin and kindlin-3 in α4β1-dependent MM cell transendothelial migration and in adhesion to fibronectin and BM stroma.** (A) NCI-H929 cells pre-incubated with control or anti-α4 mAb (left), or the indicated NCI-H929 siRNA transfectants (right), were subjected to transendothelial migration towards CXCL12 across TNF-α-incubated HUVEC. Migration was significantly inhibited, ***p<0.001 or **p<0.01 (n= 6). (B) Control, talin and kindlin-3 siRNA transfectants were analyzed in adhesion assays to FN-H89 co-immobilized with or without CXCL12. (C) NCI-H929 cells pre-incubated with control or anti-α4 mAb (left), or the indicated NCI-H929 siRNA transfectants (right), were subjected to adhesion to HS-27A BM stromal cells. Adhesions were significantly inhibited, ***p<0.001, **p<0.01 or *p<0.05 (n= 4). (D) Cell apoptosis was measured in control and talin/kindlin-3 siRNA NCI-H929 transfectants incubated for 36 h in FN-H89-coated dishes in the absence or presence of bortezomib (40 nM). (ΔΔp<0.01, Δp<0.05, n=4).

**Figure 5. Increased α4β1-dependent adhesion of ICAP-1-depleted MM cells.** (A, left) Primary CD138⁺ BM samples from MM patients, or the indicated MM cell lines were analyzed by RT-qPCR for ICAP-1 mRNA levels (normalized to the reference gene TBP). (Right) Cells were subjected to immunoprecipitation and immunobloting with the antibodies shown. (B, left) NCI-H929 cells were nucleofected with the indicated siRNA and transfectants analyzed by RT-qPCR. Bars represent relative mRNA expression levels (mean ±SD). Expression was significantly reduced, ***p<0.001 (n=4). NCI-H929 siRNA transfectants were analyzed in adhesion assays to VCAM-1 (n=4; middle) and
FN-H89 (n=3; right) co-immobilized with or without CXCL12. (C) NCI-H929 siRNA transfectants were pre-incubated with or without CXCL12 and subjected to adhesion assays to TNF-α-treated HS-27A stromal cells (n=4). Adhesions were significantly stimulated, ΔΔΔp<0.001, ΔΔp<0.01 or Δp<0.05. (D) The same siRNA transfectants were exposed to CXCL12 and tested by flow cytometry for binding of the anti-β1 HUTS-21 mAb (n=5; Δp<0.05). (E) NCI-H929 cells were transfected with control or ICAP-1 siRNA, and labeled cells subsequently injected into mice prepared for IVM.

Micrographs are representative images of collecting venules and sinusoids irrigating the BM. Bar graphs show the percentage of passing cells that rolled or arrested, and represent the mean ± SEM values obtained from the analysis of 16-20 venules from 4 mice. (ΔΔΔp<0.001)

Figure 6. Talin, kindlin-3 and ICAP-1 regulate α4β1-dependent CLL cell adhesion and transendothelial migration. (A) Analyses by RT-qPCR showing talin, kindlin-3 and ICAP-1 mRNA levels in primary CLL samples (top), as well as in the MEC-1 CLL cell line and in EBV–transformed CO43 and BRO168 cells (normalized to the reference gene TBP) (n=4). (B, left) The indicated CLL cells were analyzed by immunoblotting for talin and kindlin-3 expression. Loading control was assessed with anti-vinculin antibodies. (Right) Cells were serum-starved and incubated for different times in the absence or presence of CCL21, and subsequently subjected to immunoprecipitation and immunoblotting with the antibodies shown. (C, left) MEC-1 cells were nucleofected with both talin and kindlin-3 siRNA, and upon 48 h transfectants were analyzed by western blotting. Transfectants were subjected to adhesion assays to VCAM-1 co-immobilized with or without CCL21 (middle), or to transendothelial migration towards CCL21 across TNF-α-treated HUVEC (right). Adhesions and migrations were significantly inhibited, ***p<0.001 and **p<0.01 (n=4). (D, left) MEC-1 cells were
nucleofected with control or with the indicated ICAP-1 siRNA, and transfectants analyzed by RT-qPCR. Bars represent relative mRNA expression levels (mean ±SD). Expression was significantly reduced, *p<0.05 (n=3). (Right) siRNA transfectants were analyzed in adhesion assays to VCAM-1 (n=3) co-immobilized with or without CXCL12. Adhesions were significantly stimulated, ^ΔΔΔ^p<0.001.

**Figure 7. Working model of regulation of α4β1-dependent MM and CLL adhesion by talin, kindlin-3 and ICAP-1.** After selectin-mediated cell rolling, inside-out signalling from CXCR4 leads to α4β1-dependent cell arrest on the BM microvasculature, followed by chemokine-guided cell extravasation and lodging onto the BM stroma involving α4β1 function (Bottom). A final step of this signalling involves stimulation of talin and kindlin-3 binding to the β1 cytoplasmic domain, which results in the generation of α4β1 high-affinity conformations and upregulation of cell attachment. Rac1 functional links to talin/kindlin-3 further contributes to enhanced cell adhesion. On the other hand, ICAP-1 represses the adhesion, which might involve competitive binding and displacement of the talin/kindlin-3 module. The effects of the silencing of this module or of ICAP-1 in expression of different α4β1 conformations and on cell adhesion is also shown (top)
Figure 1

A

NCI-H929

Control Ab  Anti-α4  Anti-αL

Cells (%)  Rolling cells  Adherent cells

MM1.S

Control Ab  Anti-α4

Cells (%)  Rolling cells  Adherent cells

RPMI 8226

Control Ab  Anti-α4

Cells (%)  Rolling cells  Adherent cells

B

MEC-1

Control Ab  Anti-α4  Anti-αL

Cells (%)  Rolling cells  Adherent cells

EHEB

Control Ab  Anti-α4

Cells (%)  Rolling cells  Adherent cells

C

Primary CLL

Control Ab  Anti-α4

Cells (%)  Rolling cells  Adherent cells
Figure 4
Figure 5
ICAP-1 knockdown

High affinity

α4β1-mediated MM/CLL cell adhesion to BM microvasculature

CXCL12/CXCR4

Talin
Kindlin

ICAP1

Rac 1

Talin/kindlin-3 knockdown

Low affinity

Inhibition of α4β1-mediated MM/CLL cell adhesion to BM microvasculature

Figure 7