***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 *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.

INTRODUCTION

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 suppression⁴, 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 BM⁹. *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 microvasculature¹⁰⁻¹⁴, but the *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 *in vitro* migration and *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 patients²¹, and it was recently shown to be the strongest predictor of overall survival in CLL²². $\alpha L\beta 2$ displays aberrant chemokine-induced activation in CLL cells²³⁻²⁵, and it also contributes to CLL cell *in vitro* migration²¹. The role of $\alpha L\beta 2$ in CLL *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}.

87 The $\alpha 4\beta 1$ activity is tightly regulated by inside-out signalling induced upon
88 chemokine-receptor interactions ²⁸. In MM cells, the small GTPase Rac1 and its
89 upstream activator DOCK2, as well as RhoA positively regulate this signalling in
90 response to the chemokine CXCL12, leading to stimulation of $\alpha 4\beta 1$ -mediated cell
91 adhesion ^{29, 30}. A key step in integrin activation in leukocytes is the binding of talin and
92 the hematopoietic-specific kindlin-3 to the cytoplasmic domain of the integrin $\beta 1$
93 subunit ³¹⁻³³. The talin N-terminal FERM (protein 4.1, ezrin, radixin and moesin) region
94 binds to the membrane-proximal NPXY/F motif of β subunit cytoplasmic tails. This
95 causes the spatial separation of the α and β subunits and the extension of the integrin
96 extracellular domain, leading to the generation of high-affinity integrins and
97 upregulation of cell adhesion ^{31, 33}. Kindlin proteins also have a FERM domain which
98 binds to a distinct NXXY/F site in the membrane-distal region of the β cytoplasmic
99 domain ^{32, 33}. Recent data showed that talin and kindlin-3 have distinct but cooperative
100 roles during integrin inside-out activation in leukocytes ³⁴. Importantly, talin depletion
101 leads to reduced chemokine-stimulated leukocyte adhesiveness to $\alpha 4\beta 1$ and $\alpha L\beta 2$
102 ligands ³⁴⁻³⁸. On the other hand, kindlin-3 regulates chemokine-stimulated leukocyte
103 adhesion involving $\alpha L\beta 2$, and to a lesser extent in attachment mediated by $\alpha 4\beta 1$ ^{34, 39},
104 ⁴⁰. Therefore, these observations revealed that talin and kindlin-3 are major inside-out
105 signaling molecules for integrin activation in response to chemokines.

106 Integrin activation can be counteracted by molecules potentially competing with
107 talin and kindlin for interaction with $\beta 1$, such as ICAP-1 ⁴¹⁻⁴⁴. The ICAP-1
108 phosphotyrosine-binding domain interacts with a valine residue located at position -5
109 from the NPXY $\beta 1$ motif ^{41, 42}. Remarkably, ICAP-1-deficient cells display fibronectin
110 receptors in active conformations and increased cell adhesion to fibronectin ⁴⁵.
111 Together, these data raised the possibility that talin, kindlin-3 and ICAP-1 may

112 represent important molecules for the regulation of integrin-dependent attachment of
113 malignant B cells to the BM microvasculature and to components of the BM
114 microenvironment that potentially contribute to the trafficking and localization of these
115 cells.

116 In the present study we have used intravital microscopy to directly examine the
117 potential involvement of selectins and $\alpha 4\beta 1$ and $\alpha L\beta 2$ integrins during the early stages
118 of *in vivo* recruitment of MM and CLL cells to the BM. Moreover, using *in vivo* and *in*
119 *vitro* assays, we have further analyzed if $\alpha 4\beta 1$ -dependent MM and CLL cell adhesion is
120 regulated by talin, kindlin-3 and/or ICAP-1.

121

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, Nuaille, France). The MEC-1, MEC-2 and EHEB cell lines established from the peripheral blood of two patients with CLL⁴⁶ 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 reported¹⁶. 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 (Amaza, Cologne, Germany) using solution V and programs T-01, O-23 and T-13 for

147 NCI-H929, MM1.S and MEC-1 cells, respectively, and transfectants assayed 20 h
148 (NCI-H929) or 20-72 h (MEC-1) post-transfection. For RT-qPCR, RNA was extracted
149 using TRI-Reagent (Sigma-Aldrich), and reverse transcribed using M-MLV RT
150 (Promega, Madison, WI). Oligonucleotide sequences are provided in supplementary
151 table 2, and RT-qPCR was performed using iQ SYBR Green Supermix (Bio-Rad
152 Laboratories, Hercules, CA). Assays were performed in triplicate, and results
153 normalized according to the expression levels of TBP (TATA-binding protein) RNA
154 and expressed by using the LightCycler 480 II software (Roche).

155 **Cell adhesion, migration and apoptosis assays.** For static adhesion assays to $\alpha 4\beta 1$
156 ligands we followed the described method ⁴⁷. Briefly, fluorescently-labelled cells were
157 plated in triplicate on wells coated with CXCL12 or CCL21 together with FN-H89, a
158 fibronectin fragment which contains the $\alpha 4\beta 1$ -binding region called CS-1, or with
159 recombinant soluble VCAM-1, and plates were incubated for 2 min at 37°C upon a
160 short spin. MM cells were also treated with soluble chemokine (1 min) and added to
161 TNF- α -incubated HS-27A cells for 2 min at 37°C. Adhesions were quantified with a
162 fluorescence analyzer (Varioskan Flash Multimode Reader, Thermo Scientific).
163 Adhesion data is presented relative to control untreated cells, which have been given an
164 arbitrary value of 100. For flow chamber adhesion assays, we followed the described
165 protocol ⁴⁸. Briefly, cells were infused at a flow rate of 1 dyne/cm² into flow chambers
166 containing coimmobilized VCAM-1 (800 ng/ml) and CXCL12 (650 ng/ml). Rolling
167 cells that subsequently firmly attached were expressed as stable arrest, whereas
168 tethering cells that did not arrest at any moment were expressed as rolling cells. To
169 evaluate shear resistance, cells were allowed to attach, and then were subjected to
170 sequential increases of the flow rates. The number of cells remaining bound was
171 determined as the percentage of total adhered cells after the adhesion step. Cell

172 migration across TNF- α -treated HUVEC was done as described⁴⁹, and migrated cells
173 counted by flow cytometry. Cell viability was determined on a Coulter Epics XL flow
174 cytometer (Beckman Coulter, Fullerton, CA), using Annexin V- FITC (Immunostep,
175 Salamanca, Spain) and propidium iodide (Sigma-Aldrich). Bortezomib (Selleckchem
176 (Houston, TX) was used at 40 nM.

177 **Intravital microscopy.** Five week-old NSG mice prepared for intravital imaging of the
178 calvarial bone^{29, 50}, were injected with fluorescently-labelled cells through a catheter
179 placed in the left carotid artery, and images were acquired using a Leika DM6000
180 epifluorescence microscope. In the experiments in which we blocked integrins, 5×10^6
181 CFSE-labelled MM1.S, NCI-H929, RPMI 8226, MEC-1 and EHEB cells were
182 incubated for 30 minutes with 5 μ g of purified anti-human $\alpha 4$, αL or anti-human PC3
183 antibodies. Saturation with antibodies was confirmed by flow cytometry (not shown).
184 To determine endothelial selectin-mediated rolling and adhesion, mice received
185 intravenously (i.v.) 50 μ g of blocking antibodies against E-selectin and/or anti P-
186 selectin 20 min before image acquisition. For CXCR4 inhibition, cells were incubated
187 with 5 μ M of AMD3100 for 3 h, and subsequently washed and immediately i.v.-
188 injected into mice prepared for intravital imaging. For the experiments involving siRNA
189 transfectants, 5×10^6 CFSE-labelled cells were injected i.v. at the time of imaging.

190 Collecting venules (Cv) and sinusoids (S) have been anatomically defined previously,
191 and are functionally associated with early engraftment in mouse models^{50, 51}. The
192 number of rolling and adherent cells, as well as haemodynamic parameters was
193 analyzed by blind off-line video analysis. For each vessel we measured the maximum
194 blood velocity (V_{max}) from the fastest free-flowing cells, as well as the vessel diameter
195 (D_v), mean velocity (V_{mean}) and wall shear rate (WSR), by applying the following
196 formulae: $(V_{mean} - V_{max})/(2 - \epsilon^2)$, where $\epsilon = D_c/D_v$, and the cell diameter (D_c) for NCI-

197 H929 is 8 μm , $\text{WSR} = 8 (V_{\text{mean}}/D_v)$ and $V_{\text{crit}} = V_{\text{mean}} \times \varepsilon(2 - \varepsilon)$. Any cell travelling below
198 V_{crit} was considered to be rolling on the vessel wall. Cells that remained stationary for 3
199 s or more were considered arrested. The fraction of cells that rolled or arrested
200 according to these criteria were scored from the movies. An example movie with
201 MM1.S cells showing cells in free flowing, rolling or arrested is displayed in
202 Supplemental movie 1. Animal procedures followed the guidelines of the Ethical
203 Committee at CNIC.
204 **Statistical analyses.** Data were analyzed by one-way analysis of variance (ANOVA),
205 followed by Tukey-Kramer multiple comparisons. In both analyses, the minimum
206 acceptable level of significance was $p < 0.05$.

207

RESULTS

The $\alpha 4\beta 1$ 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 $\alpha 4\beta 1$ and $\alpha L\beta 2$ integrins in the initial adhesive steps of circulating MM and CLL cells to the BM endothelium. Preliminary analyses indicated that $\alpha 4\beta 1$ 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- $\alpha 4$ ALC 1/63 mAb (Supplementary Figure 1A, B). NCI-H929 and MEC-1, but not MM1.S cells express $\alpha L\beta 2$ (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- $\alpha 4$ 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- $\alpha 4$ 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 4×10^6 cells/condition/mouse, well above the cell number available from MM-BM sources. Opposite to the anti- $\alpha 4$ 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 $\alpha 4$ integrins (Supplementary Figure 2). These results indicate that $\alpha 4\beta 1$ is

233 a main integrin mediating *in vivo* attachment of circulating MM and CLL cells to the
234 BM microvasculature, an early and limiting step in their homing to the bone marrow.

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

236 Using a flow cytometry-based assay to measure binding of soluble P- or E-selectin
237 chimeras (P- or E-selectin/hIgM), which identifies the presence of functional selectin
238 ligands (Supplementary Figure 3), we found that MM1.S cells express only ligands for
239 P-selectin, whereas NCI-H929 and MEC-1 cells express ligands for both P- and E-
240 selectin (Figure 2A). We addressed the potential selectin involvement in *in vivo* MM
241 and CLL cell tethering onto the BM microvasculature by exposing mice to anti-P- or
242 anti-E-selectin blocking antibodies before performing IVM analyses. The data revealed
243 that both antibodies significantly inhibited the rolling and subsequent firm arrest of MM
244 and CLL cells to the BM endothelium (Figure 2B, C), thus indicating that P- and E-
245 selectin ligands also contribute to the interaction of these cells with the BM
246 microvasculature. Of note, whereas anti-selectin antibodies inhibited to similar levels
247 both rolling and firm arrest of MM and CLL cells, anti- $\alpha 4$ mAb impaired their stable
248 adhesion to a higher extent than rolling, indicating that selectins participate in firm
249 arrest of MM and CLL cells by mediating rolling, while $\alpha 4\beta 1$ function is important for
250 both their rolling and firm arrest.

251 **Talin and kindlin-3 positively regulate $\alpha 4\beta 1$ -dependent MM cell attachment to the**

252 **BM microvasculature and to BM stromal cells.** Having identified a central role for
253 $\alpha 4\beta 1$ in the early adhesive steps of MM and CLL cell migration to the BM, we next
254 examined if its activity can be regulated by $\beta 1$ -binding proteins in these cells. The
255 integrin $\beta 1$ subunit cytoplasmic domain contains docking sites for talin and kindlin-3³¹.
256 RT-qPCR experiments revealed heterogeneous levels of talin and kindlin-3 mRNAs
257 amongst different primary MM BM samples (Figure 3A). Additionally, talin and

258 kindlin-3 were detected in the MM1.S, NCI-H929 and RPMI 8226 cell lines (Figure 3B,
259 left; Supplementary Figure 4A). Co-immunoprecipitation experiments indicated that $\beta 1$
260 associates with talin and kindlin-3 in NCI-H929 cells, and that the chemokine CXCL12
261 gradually increases this association (Figure 3B, right). Moreover, talin- $\beta 1$ assembly was
262 also detected in MM1.S and RPMI 8226 cells (Supplementary Figure 4B).

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

276 To identify mechanisms that are associated with the defective adhesion of MM
277 cells silenced for talin and kindlin-3, we first analyzed if their $\alpha 4\beta 1$ affinity was altered.
278 For this purpose, we performed flow cytometry with the $\beta 1$ activation-reporter mAb
279 HUTS-21, which detects $\alpha 4\beta 1$ high-affinity conformations⁵². Control siRNA
280 transfectants displayed moderate but significantly induced HUTS-21 mAb binding in
281 response to CXCL12, whereas binding was inhibited when both talin and kindlin-3 were
282 silenced (Figure 3D). Next, we performed adhesion assays under flow conditions

283 measuring the cells that rapidly (less than 20 sec) and stably adhere under a shear stress
284 of 1 dyne/cm² to VCAM-1 co-immobilized with CXCL12. Of note, talin- and kindlin-3-
285 knockdown MM transfectants displayed a significant decrease in stable cell arrest
286 compared with control siRNA transfectants (Figure 3E, left). To analyze the $\alpha 4\beta 1$ -
287 dependent adhesion strength of these MM transfectants, we subjected cells that had
288 been attached to VCAM-1/CXCL12 at 1 dyne/cm², to detachment at sequential
289 increases of shear stress. Transfectants knock down for both proteins were found to
290 develop lower resistance to detachment at high shear stress than control siRNA
291 transfectants (Figure 3E, right).

292 To further investigate mechanisms linked to the deficient adhesion of talin- and
293 kindlin-3-knockdown cells, we analyzed the transfectants for a potentially altered
294 function of the GTPase Rac1, as we and others have shown that Rac1 was required for
295 CXCL12-stimulated, $\alpha 4\beta 1$ -dependent myeloma cell adhesion^{29, 30}. To address this
296 question, we transfected GFP-fused constitutively active forms of Rac (GFP-Rac V12)
297 together with siRNA for both talin and kindlin-3, and then tested the transfectants in
298 adhesion assays to VCAM-1 co-immobilized with CXCL12. We found that GFP-Rac
299 V12 expression reversed the inhibitory effects of talin/kindlin-3 siRNA on the
300 stimulation of cell adhesion to VCAM-1/CXCL12 (Figure 3F; Supplementary Figure 6).

301 Importantly, combined talin and kindlin-3 depletion significantly impaired the *in*
302 *vivo* MM cell rolling and firm arrest on the BM microvasculature, as evidenced in IVM
303 analyses (Figure 3G). Together, these results indicate that both talin and kindlin-3 are
304 needed for optimal $\alpha 4\beta 1$ -dependent attachment of circulating MM cells to the BM
305 microvasculature, and reveal that these two proteins cooperate to enhance the affinity
306 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 $\alpha 4\beta 1$.

We next analyzed the potential involvement of talin and kindlin-3 in MM cell transendothelial migration (TEM) using TNF- α -treated HUVEC. Anti- $\alpha 4$ 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 $\alpha 4\beta 1$.

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- $\alpha 4$ 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 $\alpha 4\beta 1$ -dependent adhesion of MM cells to the BM microenvironment is positively regulated by talin and kindlin-3.

As $\alpha 4\beta 1$ -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 $\alpha 4\beta 1$ ligands observed with talin/kindlin-3 MM transfectants and MM cell apoptosis mediated by the proteasome inhibitor bortezomib

332 (BTZ). Apoptosis levels increased from 30% to 45% in NCI-H929 cells knock down for
333 both talin and kindlin-3 when they were exposed to BTZ in FN-H89-coated dishes, as
334 compared with control siRNA transfectants incubated under the same conditions
335 (Figure 4D). These data indicate that inhibition of positive regulators of $\alpha 4 \beta 1$ -
336 dependent MM cell adhesion such as talin and kindlin-3 render myeloma cells more
337 susceptible to BTZ-mediated cell apoptosis.

338 **ICAP-1 negatively regulates $\alpha 4 \beta 1$ -dependent MM cell attachment to the BM**
339 **microvasculature.** ICAP-1 binds to the $\beta 1$ integrin cytoplasmic domain, in possible
340 competition with kindlin-3⁴⁴. Real-time PCR experiments revealed heterogeneous
341 levels of ICAP-1 mRNA in primary MM bone marrow cells, as well as in the NCI-
342 H929, MM1.S and RPMI 8226 cell lines (Figure 5A, left). Using several commercially-
343 available anti-ICAP-1 antibodies we were unable to detect ICAP-1 protein by
344 immunoblotting using NCI-H929 cell lysates (not shown). However, anti- $\beta 1$ mAb co-
345 immunoprecipitated a 23-25 kD protein from these cells which was specifically detected
346 by anti-ICAP-1 antibodies (Figure 5A, right). Cell exposure to CXCL12 did not
347 significantly alter the amount of ICAP-1 co-precipitated with $\beta 1$ (not shown). These
348 results suggest that a large portion of ICAP-1 is bound to $\beta 1$ integrins in NCI-H929
349 myeloma cells.

350 Next, we knocked down ICAP-1 in MM cells (Figure 5B, left), and tested
351 transfectants in adhesion assays to purified $\alpha 4 \beta 1$ ligands or to HS-27A BM stromal
352 cells. Of note, ICAP-1-depleted cells displayed a significant increase in adhesion to
353 VCAM-1, FN-H89 and HS-27A cells compared to control siRNA-transfectants (Figure
354 5B, middle and right; and Figure 5C). No alterations in $\alpha 4 \beta 1$ expression (not shown) or
355 in survival (Supplementary figure 5A) were detected in ICAP-1-knockdown MM cells.
356 Moreover, ICAP-1-depleted cells exhibited a significant increase in HUTS-21 binding

357 compared to control transfectants (Figure 5D), revealing that $\alpha 4\beta 1$ switched to high-
358 affinity conformations upon reducing ICAP-1 expression. Importantly, ICAP-1
359 knocking down in MM cells led to upregulated cell firm arrest onto BM
360 microvasculature compared to control transfectants, as assessed in IVM analyses
361 (Figure 5E; and Supplementary video 6). We did not observe any obvious change in cell
362 rolling in ICAP-1-silenced cells. These data indicate that ICAP-1 represses the $\alpha 4\beta 1$ -
363 dependent adhesion of MM cells.

364 **Role of talin, kindlin-3 and ICAP-1 in $\alpha 4\beta 1$ -dependent CLL cell adhesion and**
365 **transendothelial migration.** Real-time PCR experiments showed heterogeneous levels
366 of talin, kindlin-3 and ICAP-1 mRNAs amongst different primary CLL samples, as well
367 as in the MEC-1, MEC-2 and EHEB CLL cell lines, and EBV-transformed CO43 and
368 BRO168 cells established from normal B lymphocytes (Figure 6A; Supplementary
369 Figure 4D). In addition, talin and kindlin-3 proteins were observed in all these CLL
370 cells (Figure 6B, left). Co-immunoprecipitation experiments indicated that association
371 of the $\beta 1$ subunit with talin and kindlin-3 was enhanced upon incubation for 1.5 min of
372 MEC-1 cells with the chemokine CCL21 (Figure 6B, right). Importantly, combined
373 silencing of talin and kindlin-3 led to a significant reduction in MEC-1 cell adhesion to
374 VCAM-1, both under basal conditions and in the presence of CCL21 (Figure 6C, left
375 and middle; Supplementary figures 4E and 5B). Furthermore, talin and kindlin-3
376 depletion partially impaired MEC-1 cell migration across HUVEC using CCL21 as
377 stimulus (Figure 6C, right). In contrast, ICAP-1-knockdown MEC-1 cells displayed
378 significantly increased adhesion to VCAM-1 (Figure 6D; and Supplementary figure
379 5B). Altogether these data indicate that CLL cell adhesion and transendothelial
380 migration involving $\alpha 4\beta 1$ are differentially regulated by talin, kindlin-3 and ICAP-1.

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DISCUSSION

Trafficking of MM and CLL cells to the BM and disease progression are connected by integrin $\alpha 4\beta 1$ activity^{53-55 17, 18, 20}. Previous *in vitro* data led to the proposal that $\alpha 4\beta 1$ could participate in the initial adhesive steps of MM and CLL cell migration into the BM¹³⁻¹⁵. Using intravital microscopy of the BM calvariae of NSG mice, we demonstrate that $\alpha 4\beta 1$, but not $\alpha L\beta 2$, is a major integrin mediating firm arrest of circulating MM and CLL cells to the BM microvasculature. In addition, we found that $\alpha 4\beta 1$ also contributes to the preceding MM cell rolling step. Furthermore, our data reveal that selectin ligands (most likely PSGL-1⁹) on MM and CLL cells mediate interactions with P-selectin and E-selectin, which are constitutively expressed by the BM endothelium⁵⁶, 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 $\alpha 4\beta 1$ 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 data⁵⁷, the present results strongly suggest that CXCL12 exposed on BM endothelial cells stimulates $\alpha 4\beta 1$ 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 $\beta 1$ subunit cytoplasmic domain and stimulate the affinity and avidity of leukocyte integrins³¹⁻³³. We show here that talin and kindlin-3 bind to $\beta 1$ 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

407 stimulation of adhesion by these positive regulators is based on induction of $\alpha 4\beta 1$ high-
408 affinity conformations, and also on boosting the strength to the adhesions to VCAM-
409 1/CXCL12. This later response was especially detected in adhesion assays under flow
410 conditions, providing high relevance to the finding. Further, a functional link between
411 talin/kindlin-3 and Rac1 during chemokine-stimulated MM cell adhesion to VCAM-1
412 was found. Characterization of this link should provide additional mechanistic insights
413 on the regulated MM cell adhesion. These *in vitro* results are strengthened by intravital
414 imaging analyses with talin- and kindlin-3-silenced MM cells, which indicate that these
415 proteins are required for efficient cell arrest on the BM microvasculature. Therefore, our
416 data support a cooperative model between talin and kindlin-3 for efficient stimulation of
417 $\alpha 4\beta 1$ -dependent MM cell attachment onto the BM endothelium (Figure 7), similar to
418 the cooperation between these proteins during $\alpha 4\beta 1$ - and $\alpha L\beta 2$ -mediated leukocyte
419 adhesion^{39, 40}. As CCL21 is mainly expressed in lymph nodes, whereas no or low
420 CCL21 has been detected in BM⁵⁸, our data with CLL cells depict a putative role for
421 talin and kindlin-3 in supporting $\alpha 4\beta 1$ -dependent CLL cell attachment to and migration
422 across endothelium in the trafficking of these cells to lymph nodes.

423 Examination of the role of talin and kindlin-3 on CXCL12-stimulated migration
424 of MM cells across endothelium involving $\alpha 4\beta 1$ revealed that this process is highly
425 dependent on talin and to a lesser extent on kindlin-3. These results pose that talin is
426 crucial for both $\alpha 4\beta 1$ -dependent MM cell attachment to and migration across
427 endothelium, whereas kindlin-3 is important especially at the attachment step. Notably,
428 cooperation between talin and kindlin-3 was also observed during attachment of MM
429 cells to components of the BM microenvironment, as the $\alpha 4\beta 1$ -mediated, CXCL12-
430 stimulated MM cell adhesion to CS-1/fibronectin and to BM stromal cells required both

431 talin and kindlin-3. These data strongly suggest that once MM cells cross the BM
432 microvasculature, their lodging in the BM mediated by $\alpha 4\beta 1$ needs talin and kindlin-3
433 function. Furthermore, our data raise the possibility that talin- and kindlin-3-supported
434 MM cell adhesion to BM stroma involving $\alpha 4\beta 1$ might contribute to MM disease
435 progression and possibly to CAM-DR responses. An experimental insight into this later
436 process revealed that talin/kindlin-3-knockdown MM cells exposed to $\alpha 4\beta 1$ ligands
437 were more sensitive to cell death induced by bortezomib, highlighting that activators of
438 $\alpha 4\beta 1$ -dependent MM cell adhesion contribute to CAM-DR. Therefore, the molecules
439 that regulate $\alpha 4\beta 1$ -mediated MM cell adhesion to the BM microenvironment might
440 constitute targets whose therapeutic relevance deserves to be explored.

441 In the present study we also addressed whether ICAP-1, a protein thought to
442 compete with talin and kindlin-3 for binding to the $\beta 1$ cytoplasmic domain ⁴¹⁻⁴³, thus
443 acting as a putative integrin repressor ⁴⁴, could modulate MM and CLL $\alpha 4\beta 1$ -dependent
444 cell adhesion. We found that ICAP-1 was expressed in primary MM and CLL cells, and
445 importantly, ICAP-1 silencing in MM and CLL cell lines led to remarkable increases in
446 $\alpha 4\beta 1$ -dependent adhesion involving stimulation of the integrin high-affinity
447 conformations. Moreover, highlighting the regulatory role of ICAP-1, its silencing in
448 MM cells resulted in enhanced attachment of circulating cells onto the BM
449 microvasculature during IVM studies. Overall, these results indicate that ICAP-1
450 negatively regulates $\alpha 4\beta 1$ function in MM cells (Figure 7). Our data represent the first
451 identification of an endogenous molecule strongly opposing $\alpha 4\beta 1$ activation and
452 adhesion in MM and CLL cells. Thus, these findings provide an attractive scenario to be
453 investigated in light of the importance of $\alpha 4\beta 1$ in the progression of these B-cell
454 malignancies.

455 The proposed model (Figure 7) suggests that the global level of cell adhesion is
456 likely the balance between the positive signals from talin and kindlin-3 and the
457 inhibitory actions of ICAP-1. Following talin and kindlin-3 silencing, inactive $\alpha 4\beta 1$
458 conformations accumulate, perhaps due to enhanced ICAP-1 binding to $\beta 1$, leading to
459 reduced $\alpha 4\beta 1$ -dependent MM and CLL cell attachment. When ICAP-1 is knocked
460 down, the balance between positive and negative signals tilts towards higher stimulation
461 of $\alpha 4\beta 1$ activity by talin and kindlin-3, resulting in enhanced MM and CLL cell
462 adhesion to the BM endothelium.

463 The distinct regulation by the $\beta 1$ -interacting partners talin, kindlin-3 and ICAP-1
464 on MM and CLL cell adhesion involving $\alpha 4\beta 1$ could account for mechanisms
465 associated with drug resistance and tumor progression in human B-cell malignances.
466 Thus, targeting intracellular regulators of the $\alpha 4\beta 1$ -integrin in combination with present
467 therapies might exert beneficial effects to overcome the deleterious emergence of
468 constitutive or transient integrin activation in clonal derivatives of drug-resistant
469 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.

Supplementary information is available at Leukemia's website

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FIGURE LEGENDS

Figure 1. The $\alpha 4\beta 1$ 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- $\alpha 4$ 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 ($\cdot$) 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 $\pm$ 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 $\pm$ 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 $\alpha 4\beta 1$ -dependent MM cell attachment. (A) Primary CD138⁺ BM samples from MM patients were analyzed by RT-qPCR for talin

758 and kindlin-3 mRNA levels (normalized to the reference gene TBP). (B, left) The
 759 indicated MM cell lines were analyzed by immunoblotting for talin or kindlin-3
 760 expression. Loading control was assessed with anti-vinculin antibodies. (Right) Cells
 761 were serum-starved and incubated for different times in the absence or presence of
 762 CXCL12, and subsequently subjected to immunoprecipitation and immunoblotting with
 763 the antibodies shown. (C, left) Cells were nucleofected with the indicated single or
 764 combined siRNA, and transfectants analyzed by western blotting. (Medium)
 765 Densitometric analyses of protein gel bands show the mean $\pm$ SD of four independent
 766 experiments. (Right) The different control, talin and kindlin-3 siRNA transfectants were
 767 analyzed in adhesion assays to VCAM-1 co-immobilized with or without CXCL12. (D)
 768 Transfectants were tested by flow cytometry for binding of the anti- β 1 HUTS-21 mAb,
 769 in the absence or presence of CXCL12. Adhesions and binding (n= 4) were significantly
 770 inhibited, *** p<0.001, * p<0.05. (E, left) NCI-H929 cells transfected with both talin and
 771 kindlin-3 or with control siRNA were perfused in flow chambers coated with VCAM-1
 772 co-immobilized with CXCL12, and analyzed for rolling and stable cell arrest (n=3).
 773 Data is presented as mean $\pm$ SD of cell percentages from the total cell population.
 774 Adhesions were significantly inhibited in comparison with those of control siRNA
 775 transfectants, * p<0.05. (Right) The indicated siRNA transfectants pre-attached onto co-
 776 immobilized VCAM-1 and CXCL12 in flow chambers, were subjected to cell
 777 detachment after sequential increases of shear stress. Data show mean $\pm$ SD of cell
 778 percentages from the initial number of bound cells remaining attached at the indicated
 779 shear stresses (n=3). (F) NCI-H929 cells were transfected with the indicated siRNA and
 780 GFP vector combinations, and analyzed in adhesion assays to VCAM-1 immobilized
 781 with CXCL12. Adhesion was significantly inhibited, * p<0.05, or rescued, Δ p<0.05. (G)
 782 NCI-H929 cells were transfected either with control or with both talin and kindlin-3

783 siRNA, and fluorescently-labeled transfectants were subsequently injected into NSG
784 mice prepared for IVM of the calvarial BM. Bar graphs show the percentage of passing
785 cells that rolled or arrested, and represent the mean $\pm$ SEM values obtained from the
786 analysis of 16-20 venules from 4 mice. (** $p < 0.001$).

787 **Figure 4. Role of talin and kindlin-3 in $\alpha 4\beta 1$ -dependent MM cell transendothelial**
788 **migration and in adhesion to fibronectin and BM stroma.** (A) NCI-H929 cells pre-
789 incubated with control or anti- $\alpha 4$ mAb (left), or the indicated NCI-H929 siRNA
790 transfectants (right), were subjected to transendothelial migration towards CXCL12
791 across TNF- α -incubated HUVEC. Migration was significantly inhibited, *** $p < 0.001$ or
792 ** $p < 0.01$ ($n = 6$). (B) Control, talin and kindlin-3 siRNA transfectants were analyzed in
793 adhesion assays to FN-H89 co-immobilized with or without CXCL12. (C) NCI-H929
794 cells pre-incubated with control or anti- $\alpha 4$ mAb (left), or the indicated NCI-H929
795 siRNA transfectants (right), were subjected to adhesion to HS-27A BM stromal cells.
796 Adhesions were significantly inhibited, *** $p < 0.001$, ** $p < 0.01$ or * $p < 0.05$ ($n = 4$). (D) Cell
797 apoptosis was measured in control and talin/kindlin-3 siRNA NCI-H929 transfectants
798 incubated for 36 h in FN-H89-coated dishes in the absence or presence of bortezomib
799 (40 nM). ($\Delta\Delta p < 0.01$, $\Delta p < 0.05$, $n = 4$).

800 **Figure 5. Increased $\alpha 4\beta 1$ -dependent adhesion of ICAP-1-depleted MM cells.** (A,
801 left) Primary CD138⁺ BM samples from MM patients, or the indicated MM cell lines
802 were analyzed by RT-qPCR for ICAP-1 mRNA levels (normalized to the reference gene
803 TBP). (Right) Cells were subjected to immunoprecipitation and immunoblotting with the
804 antibodies shown. (B, left) NCI-H929 cells were nucleofected with the indicated siRNA
805 and transfectants analyzed by RT-qPCR. Bars represent relative mRNA expression
806 levels (mean $\pm$ SD). Expression was significantly reduced, *** $p < 0.001$ ($n = 4$). NCI-H929
807 siRNA transfectants were analyzed in adhesion assays to VCAM-1 ($n = 4$; middle) and

808 FN-H89 (n=3; right) co-immobilized with or without CXCL12. (C) NCI-H929 siRNA
 809 transfectants were pre-incubated with or without CXCL12 and subjected to adhesion
 810 assays to TNF- α -treated HS-27A stromal cells (n=4). Adhesions were significantly
 811 stimulated, $^{\Delta\Delta\Delta}p<0.001$, $^{\Delta\Delta}p<0.01$ or $^{\Delta}p<0.05$. (D) The same siRNA transfectants were
 812 exposed to CXCL12 and tested by flow cytometry for binding of the anti- $\beta 1$ HUTS-21
 813 mAb (n=5; $^{\Delta}p<0.05$). (E) NCI-H929 cells were transfected with control or ICAP-1
 814 siRNA, and labeled cells subsequently injected into mice prepared for IVM.
 815 Micrographs are representative images of collecting venules and sinusoids irrigating the
 816 BM. Bar graphs show the percentage of passing cells that rolled or arrested, and
 817 represent the mean $\pm$ SEM values obtained from the analysis of 16-20 venules from 4
 818 mice. ($^{\Delta\Delta\Delta}p<0.001$)

819 **Figure 6. Talin, kindlin-3 and ICAP-1 regulate $\alpha 4\beta 1$ -dependent CLL cell adhesion**
 820 **and transendothelial migration.** (A) Analyses by RT-qPCR showing talin, kindlin-3
 821 and ICAP-1 mRNA levels in primary CLL samples (top), as well as in the MEC-1 CLL
 822 cell line and in EBV-transformed CO43 and BRO168 cells (normalized to the reference
 823 gene TBP) (n=4). (B, left) The indicated CLL cells were analyzed by immunoblotting
 824 for talin and kindlin-3 expression. Loading control was assessed with anti-vinculin
 825 antibodies. (Right) Cells were serum-starved and incubated for different times in the
 826 absence or presence of CCL21, and subsequently subjected to immunoprecipitation and
 827 immunoblotting with the antibodies shown. (C, left) MEC-1 cells were nucleofected
 828 with both talin and kindlin-3 siRNA, and upon 48 h transfectants were analyzed by
 829 western blotting. Transfectants were subjected to adhesion assays to VCAM-1 co-
 830 immobilized with or without CCL21 (middle), or to transendothelial migration towards
 831 CCL21 across TNF- α -treated HUVEC (right). Adhesions and migrations were
 832 significantly inhibited, $^{***}p<0.001$ and $^{**}p<0.01$ (n=4). (D, left) MEC-1 cells were

833 nucleofected with control or with the indicated ICAP-1 siRNA, and transfectants
834 analyzed by RT-qPCR. Bars represent relative mRNA expression levels (mean $\pm$ SD).
835 Expression was significantly reduced, * $p < 0.05$ (n=3). (Right) siRNA transfectants were
836 analyzed in adhesion assays to VCAM-1 (n=3) co-immobilized with or without
837 CXCL12. Adhesions were significantly stimulated, $\Delta\Delta p < 0.001$.

838 **Figure 7. Working model of regulation of $\alpha 4\beta 1$ -dependent MM and CLL adhesion**
839 **by talin, kindlin-3 and ICAP-1.** After selectin-mediated cell rolling, inside-out
840 signalling from CXCR4 leads to $\alpha 4\beta 1$ -dependent cell arrest on the BM
841 microvasculature, followed by chemokine-guided cell extravasation and lodging onto
842 the BM stroma involving $\alpha 4\beta 1$ function (Bottom). A final step of this signalling
843 involves stimulation of talin and kindlin-3 binding to the $\beta 1$ cytoplasmic domain, which
844 results in the generation of $\alpha 4\beta 1$ high-affinity conformations and upregulation of cell
845 attachment. Rac1 functional links to talin/kindlin-3 further contributes to enhanced cell
846 adhesion. On the other hand, ICAP-1 represses the adhesion, which might involve
847 competitive binding and displacement of the talin/kindlin-3 module. The effects of the
848 silencing of this module or of ICAP-1 in expression of different $\alpha 4\beta 1$ conformations
849 and on cell adhesion is also shown (top)

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A **B****MM1.S** **C****MEC-1** **MM1.S****NCI-H929****MEC-1** **Figure 2**

Figure 3

Figure 4

A **B** **C** **D** **E** **Figure 5**

Figure 6

Figure 7