Integrin \( \beta 7 \) (ITGB7) mRNA is detected in multiple myeloma (MM) cells and its presence is correlated with MAF gene activation. Although the involvement of several integrin family members in MM-stoma cell interaction is well documented, the specific biologic functions regulated by integrin-\( \beta 7 \) in MM are largely unknown. Clinically, we have correlated integrin-\( \beta 7 \) expression in MM with poor survival outcomes post autologous stem cell transplantation and postsalvage therapy with bortezomib. Functionally, we have found that shRNA-mediated silencing of ITGB7 reduces MM-cell adhesion to extracellular matrix elements (fibronectin, E-cadherin) and reverses cell-adhesion-mediated drug resistance (CAM-DR) sensitizing them to bortezomib and melphalan. In addition, ITGB7 silencing abrogated MM-cell transwell migration in response to SDF1\( \alpha \) gradients, reduced vessel density in xenografted tumors, and altered MM cells in vivo homing into the BM. Mechanistically, ITGB7 knockdown inhibited focal adhesion kinase (FAK) and Src phosphorylation, Rac1 activation, and SUMOylation, reduced VEGF production in MM–BM stem cell cocultures and attenuated p65-\( \alpha B \) activity. Our findings support a role for integrin-\( \beta 7 \) in MM-cell adhesion, migration, and BM homing, and pave the way for a novel therapeutic approach targeting this molecule. (Blood. 2011;117(23):6202-6213)

**Introduction**

Multiple myeloma (MM) is a clonal disease of plasma cells that remains, for the most part, incurable despite the advent of several novel therapeutics.\(^1\) Tumor cells in this disease are cradled within the BM microenvironment by an array of adhesive interactions between the BM extracellular matrix (ECM) components such as fibronectin (FN), laminin, VCAM-1, proteoglycans, collagens and hyaluronan, and a variety of adhesion molecules on the surface of MM cells including integrins, heparan sulfate proteoglycans, and hyaluronan receptors CD44 and RHAMM. These direct adhesive interactions between the BM/ECM and MM cells transduce into cellular matrix elements (fibronectin, E-cadherin) and reverses cell-adhesion-mediated drug resistance (CAM-DR) sensitizing them to bortezomib and melphalan. In addition, ITGB7 silencing abrogated MM-cell transwell migration in response to SDF1\( \alpha \) gradients, reduced vessel density in xenografted tumors, and altered MM cells in vivo homing into the BM. Mechanistically, ITGB7 knockdown inhibited focal adhesion kinase (FAK) and Src phosphorylation, Rac1 activation, and SUMOylation, reduced VEGF production in MM–BM stem cell cocultures and attenuated p65-\( \alpha B \) activity. Our findings support a role for integrin-\( \beta 7 \) in MM-cell adhesion, migration, and BM homing, and pave the way for a novel therapeutic approach targeting this molecule. (Blood. 2011;117(23):6202-6213)
several diseases such as colitis, diabetic insulitis, and lymphoid malignancies including lymphomatous polyposis in mantle cell lymphoma, thymic lymphoma, and mucosa-associated T- and B-cell non-Hodgkin lymphomas. Together, these findings indicate that β7-integrin plays an important role for physiologic functions and pathologic alterations of the immune system. Little, however, is known about the unique biologic roles it plays in MM and whether its functions are essential or redundant to other integrins remains to be elucidated. In this study, we sought to determine the roles of integrin-β7 on MM-cell adhesion, migration, survival, and in vivo BM homing.

Methods

Cell culture and BM stem cells

The human MM cell lines H929, 8226, and U266 were purchased from the ATCC. MM1s (generated by Dr Steven Rosen, Northwestern University, Chicago, IL), OPM2, and INA6 cells were kindly provided by Drs Lawrence Boise (Emory University, Atlanta, GA) and Renate Burger (University of Erlangen-Nuernberg, Germany). Details are available in the supplemental Methods. MM tissue microarray

Myeloma tissue microarrays (TMA) were generated from formalin-fixed paraffin-embedded (FFPE) sections of pretreatment diagnostic BM biopsies (n = 79) and used to evaluate the expression of integrin-β7 (details in supplemental Methods).

Lentiviral integrin β7 (ITGB7) shRNA transduction

Lentiviral transduction particles (Sigma-Aldrich) were used to deliver shRNAs expressed from shRNAs for knockdown of ITGB7 gene (NM_000889) into MM1S, INA-6, and H929 MM cells. The sense oligonucleotide sequences for ITGB7 shRNAs and control nontarget scrambled shRNA are shown in Table 1. Lentiviral ITGB7 shRNA and nontarget control shRNA were produced in HEK293T packaging cells, concentrated at different MOIs and then individually added into MM-cell suspensions in the presence of 6 μg/mL polybrene and transduced for 24 hours followed by selection in puromycin (2 μg/mL; Invitrogen) to obtain ITGB7 silenced and control ITGB7 positive cells. The efficiency of ITGB7 silencing was monitored by flow cytometry and RT-PCR. To reduce the possibility of an off-target effect of an individual shRNA construct, puromycin-resistant cells with the most efficient ITGB7 knockdown, from 2 separate infections with individual and distinct lentiviral ITGB7 shRNAs, were selected for further studies.

Cell adhesion assay

A fluorometric-based assay has been used to evaluate the adherence of ITGB7 silenced and ITGB7 positive MM cells (5 × 10^5 cells) suspended in RPMI 1640 media were placed in the upper chambers of transwell plates (pore size 0.8 μm; Costar-Corning) with serial concentrations of SDF-1α (0-20nM) added to 500 μL of RPMI 1640 in the lower chambers. After 4 hours at 37°C, cells that migrated to the lower chambers were labeled with calcein and counted on a fluorescent plate reader (Molecular Devices).

ITGB7 blocking Ab and rescue experiments

To exclude the possibility of an off-target effect of the used shRNA constructs, blocking Ab, and rescue experiments were also performed as detailed in supplemental Methods.

VEGF and cytokine ELISA

ITGB7 silenced and control ITGB7 positive cells were added at 1 × 10^6 cells per well into 96-well plates coated with or without human BMSCs and incubated at 37°C for 48 hours. Supernatant was collected and assayed for VEGF by ELISA (R&D Systems) and profiled for their cytokines secretions using an ELISA-based assay (SA Biosciences).

Cell apoptosis assay

Survival of MM cells cultured with bortezomib or melphalan in 96-well plates under different conditions (coated with BMSCs or FN or E-CDH) was evaluated by FITC-conjugated Annexin V (Biovision) staining using flow cytometric analysis as previously described.

Immunoblotting and confocal microscopy

Whole-cell lysates were subjected to SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories). The Abs used for immunoblotting included anti-phospho- focal adhesion kinase (FAK; Tyr397), anti-FAK, anti-phospho-Src (Tyr416), anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-SUMO1, and anti-α-Tubulin (Cell Signaling Technology). A laser-scanning confocal microscope (LSM 510; Zeiss) was used to assess the distribution and localization of p-FAK. Images were acquired using a 100× Zeiss Plan-Apochromat objective and captured by Zeiss.

Table 1. Sense oligonucleotide sequences for ITGB7 shRNAs and nontargeting scrambled shRNA

<table>
<thead>
<tr>
<th>shRNA</th>
<th>Sense oligonucleotide sequence</th>
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<tbody>
<tr>
<td>ITGB7 shRNA1</td>
<td>5′-ACGGCTACTATGGTGCTCTAT-3′</td>
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<tr>
<td>ITGB7 shRNA2</td>
<td>5′-GCCTGAATAGTTGATCCTAA-3′</td>
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<td>ITGB7 shRNA3</td>
<td>5′-ACCCACCTACATCGCTTT-3′</td>
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<td>5′-GCAGAAGTGGTACACTCCCT-3′</td>
</tr>
<tr>
<td>Nontargeting scrambled shRNA</td>
<td>5′-CAACAGATGAGAAGACCAA-3′</td>
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image browser using identical laser intensity and gain parameters. ITGB7silenced and ITGB7positive cells were cultured on FN-coated plates and immunostained with anti-actin (AlexaFluor 555 phallolidin), rabbit anti-phospho-FAK (Tyr861), secondary anti-rabbit AlexaFluor 488 and DAPI (Invitrogen).

**Rac-1 activation assay**

For the detection of activated Rac1 (GTP bound), equal amounts of protein lysates (500 μg) were obtained from ITGB7silenced and ITGB7positive cells cultured in uncoated or FN-coated plates. Lysates were then immunoprecipitated with GST-PAK1 (p21-binding domain corresponding to residues 67-150) agarose beads (20 μg; Chemicon-Millipore) for the pull-down of activated GTP-bound Rac1. Lysates and beads were incubated for 1 hour with agitation at 4°C. Beads were then pelleted by centrifugation (10 seconds, 14 000g, 4°C), washed twice with lysis buffer, resuspended and boiled in 20 μL of 2× Laemmli buffer and subjected to SDS-PAGE and PVDF membrane transfer. Activated Rac1 (GTP-Rac1 bound to PAK1 beads) was detected by immunoblotting with anti-Rac1 Abs (Chemicon-Millipore) and anti-SUMO1 (Cell Signaling Technology).

**NF-κB activity**

NF-κB activity was investigated using the TransAM NF-κB p65 kit, a DNA-binding ELISA-based assay (Active Motif) and by NF-κB p65 kit immunostaining (details in supplemental Methods).

**In vivo studies**

**Murine xenograft model of human MM.** All animal studies were conducted according to protocols and standard procedures approved by the University of Calgary Animal Care Committee. Animals were killed when their tumors reached 2 cm in largest diameter or when exhibiting any distress symptoms. CB-17 SCID-mice (Charles River Laboratories) were subcutaneously inoculated in the interscapular area with 2 × 106 ITGB7silenced or ITGB7positive MM1S cells.17 Tumor sizes were measured every 3 days in 2 dimensions using an electronic caliper, and the tumor volume was calculated using the following formula: V = 0.5 a × b2, where a and b are the long and short diameter of the tumor, respectively.

**Myeloma cell BM homing.** Mice with established MM xenografts from ITGB7silenced and ITGB7positive groups were killed whenever their tumors reached 2 cm in largest diameter. Xenografts as well as specimens from the femurs, liver, and spleen of killed animals were rinsed with PBS, dehydrated with ethanol, embedded in paraffin blocks, and sectioned. Sections from femurs, liver, and spleen were stained with Cy-3 labeled rabbit anti–human CD138 Ab (Abcam) for the fixed with 4% formaldehyde in PBS, dehydrated with ethanol, embedded in from the femurs, liver, and spleen of killed animals were rinsed with PBS, tumors reached 2 cm in largest diameter. Xenografts as well as specimens sections from femurs of examined SCID mice (n = 3 mice per condition).

**Microvessel density.** Serial sections from xenografted tumors were stained for anti–mouse CD31 (Santa Cruz Biotechnology) to evaluate the tumor microvessel density (MVD). MVD was expressed as a percentage according to the following formula: MVD (%) = CD31+ target area/total area examined. The shown MVD is the mean value of 10 randomly selected sections from ITGB7silenced (n = 3) and ITGB7positive (n = 3) xenografts.

**In vivo flow cytometry**

ITGB7silenced or ITGB7positive MM1S and H929 cells (2 × 106) labeled with Calcein-AM were injected into the tail vein of sedated BALB/c mice (n = 3 per condition) and monitored for their extravasation and homing by in vivo flow cytometry as previously described.18 Mice were anesthetized and placed on a heated stage (32°C) and a laser beam (473 nm) was focused on an appropriate arteriole in the mouse ear for the excitation of calcein-labeled circulating MM cells. Signals were detected by photomultiplier (PMT) tubes through 528 ± 19-nm bandpass filter, and then analyzed with Matlab software. Cell counts were obtained at least every 5 minutes for 40-50 minutes.

**Statistical analysis**

Statistical significances of differences were determined by using the Student t test. The minimal level of significance was P < .05.

**Results**

**Integrin β7 is highly expressed in MM cells and its expression correlates with poor survival**

We first screened the expression of integrin-β7 in a panel of MM cell lines (OPM2, MM1S, U266, H929, and INA6) by flow cytometry. As shown in Figure 1A, integrin-β7 was expressed by all the MM cell lines we have tested, albeit at different levels. qRT-PCR analysis also confirmed ITGB7 mRNA expression in these cell lines with the highest mRNA expression in cells harboring a t(4;14)(H929, OPM2) or t(14;16)(MM1S and 8226; Figure 1B). In primary CD138+ cells sorted from the BM aspirates of individual MM patients, ITGB7 mRNA was detectable (relative to a reference pooled human cDNA) in 24 of 26 (92.3%) of the cases (Figure 1B). A similar frequency and pattern of integrin-β7 expression was also observed in a cohort of patients treated with the Total Therapy protocol and in the MMRC myeloma cell line dataset (supplemental Figure 1A-B). To examine whether integrin-β7 had any impact on disease behavior, we correlated its expression levels with MM patients’ survival post frontline stem cell transplantation, in a TMA constructed from their diagnostic BM biopsies (n = 79). By immunohistochemical (IHC) staining, 78.5% biopsies were positive for integrin-β7 with 1 of 3 of these cases coexpressing cyclin D2 and integrin-β7 (Figure 1C, supplemental Figure 1C-D). Patients coexpressing cyclin D2 and integrin-β7 fared very poorly with a median time to progression (TTP) of 0.9 years compared with 57% relapse free at 4 years in patients expressing either one alone or none (Figure 1C). Similarly, we have also interrogated the MM dataset (GSE9782) deposited by Mulligan and colleagues38 for the prognostic impact of ITGB7 mRNA in relapsed and refractory patients treated with salvage bortezomib. High expression (top quartile) of ITGB7 (205718_at Affymetrix probe) also correlated with a significantly shorter survival in this patient cohort (supplemental Figure 2A-C).

**Integrin-β7 silencing reduces MM-cell adhesion to stromal elements, migration to SDF1α chemokine gradient, and invasion**

Integrin-mediated interactions between malignant plasma cells and BM stroma may be crucial for their adhesion and homing to BM niches. To investigate the function of integrin-β7 in MM, its expression was knocked down in 3 MM cell lines (MM1S, H929, and INA6) with lentiviral ITGB7 shRNAs. Five lentiviral ITGB7 shRNAs were generating targeting different regions in the ITGB7 mRNA. Specific ITGB7 down-regulation was confirmed by reduced integrin-β7 mRNA and protein expression in cells infected with shRNA 2 or 3 (hereafter referred to as ITGB7silenced), whereas integrin-β7 was expressed in cell lines infected with control scrambled shRNA lentiviral vector (ITGB7positive; Figures 1D-E; supplemental Figure 3).

In a calcein-AM fluorescence-based adhesion assay, we next evaluated the effects of ITGB7 silencing on MM-cell adhesion to FN, E-CDH, and human BMSCs. As shown in Figure 2A, ITGB7silenced cells exhibited 50%-70% less adhesion to BMSCs compared with their relative ITGB7positive controls. This effect was even more evident on FN- and E-CDH–coated plates, confirming
the role integrin-β7 plays in MM-cell adhesion to stromal elements. Because integrin-β1 heterodimers (in particular α4β1 and α5β1) are known to be involved in MM-cell migration to VEGF, SDF1α, and IGF1 gradients, we also sought to determine the contribution of integrin-β7 to MM-cell migration. In a transwell migration assay, SDF-1α (10-20nM) induced a 2-fold increase in the migration of \( \text{ITGB7}^{\text{positive}} \) but not \( \text{ITGB7}^{\text{silenced}} \) cells into the lower chambers, consistent with a key role for integrin-β7 in MM cells guided migration toward SDF-1α gradients (Figure 2B). Lastly, as MM cells need to actively penetrate through the subendothelial basement membrane of the BM sinus to migrate in and out of the BM, we examined whether integrin-β7 is required for MM-cell invasion. As shown in Figure 2C and D, \( \text{ITGB7} \) silencing also significantly reduced the ability of \( \text{ITGB7}^{\text{silenced}} \) MM1S and H929 cells to invade through a reconstituted membrane (Matrigel) compared with \( \text{ITGB7}^{\text{positive}} \) cells. In INA6 cells, and consistent with their low \( \text{ITGB7} \) expression, \( \text{ITGB7} \) silencing had a similar effect on these cells adhesion to BMSCs and FN, but a lesser or no effect on these cells’ adhesion to E-cadherin or transwell migration (supplemental Figure 4A-C).

Consistent with the \( \text{ITGB7} \) shRNA studies, blocking anti-\( \text{ITGB7} \) mAb (FIB504 clone) resulted in a significant reduction in MM-cell adhesion to stromal elements (BMSCs, FN, and E-CDH) and migration (supplemental Figure 5A-B). To further demonstrate that shRNA \( \text{ITGB7} \) silencing was responsible for the reduction of MM-cell adhesion and migration, a rescue experiment was performed by constructing a silent mutant \( \text{ITGB7}^{\text{mut}} \) that is resistant to \( \text{ITGB7} \) shRNA2 and expressing it in \( \text{ITGB7}^{\text{silenced}} \) cells (MM1S and H929). \( \text{ITGB7}^{\text{silenced}} \) shRNA2 cells infected with \( \text{ITGB7}^{\text{mut}} \) showed a restored expression of MM-cell adhesive and migratory functions that were suppressed by shRNA-mediated \( \text{ITGB7} \) silencing (supplemental Figure 7A-D).

**Integrin-β7 regulates VEGF and cytokines production in MM and BMSCs**

Direct interaction between MM cells and the BM microenvironment is known to induce MM and stromal cell secretion of several cytokines including VEGF. Therefore, we next investigated whether integrin-β7-dependent cell adhesion, like integrin-β1, mediates VEGF secretion. As shown in Figures 3, while MM cells...
and stroma cells cultured alone did not secrete any significant quantities of VEGF, cocultures of BMSCs with ITGB7-positive MM cells lead to a 3-fold increase in VEGF secretion. In contrast, cocultures of BMSCs and ITGB7-silenced cells significantly reduced levels of VEGF secretion, albeit not to baseline levels (BMSCs alone), suggesting that in addition to integrin-β7/H9252 other adhesion molecules or integrins may be involved in MM-BMSC VEGF secretion. Furthermore, ITGB7 silencing altered MM-cell production of several cytokines and growth factors in MM-BMSC cell cocultures. In particular, ITGB7-silenced cells produced significantly lower amounts of IL-1β/H9252 and MIP-1α/H9252 suggesting a possible role for integrin-β7 in MM bone disease and osteoclasts activation (supplemental Figure 8A-C).

Integrin β7 silencing restores MM drug sensitivity

To investigate the involvement of integrin-β7 in CAM-DR, ITGB7-positive and ITGB7-silenced cells were cultured in uncoated or FN-coated plates in the presence or absence of bortezomib or melphalan. As shown in Figure 4, when cultured in uncoated plates ITGB7-silenced cells were more sensitive to the cytotoxic effect of bortezomib and melphalan. In addition, while adhesion to FN protected ITGB7-positive cells from the cytotoxicity of bortezomib and melphalan, this effect was partially abrogated in ITGB7-silenced cells. Taken together, these results support a role of ITGB7 in delivering a prosurvival signal to MM cells and in conferring drug resistance through cell-adhesion dependent (in FN-coated plates) as well as independent (uncoated plates) mechanisms.

Integrin β7-mediated adhesion to FN activates the FAK and p65 NF-κB

Adhesion of MM cells to FN is reported to increase NF-κB activity and to modulate the expression of several NFκB-dependent genes.13 Using an ELISA-based assay we measured, in nuclear fractions extracted from ITGB7-positive and ITGB7-silenced MM1S and
H929 cells, the NFκB-p65 binding to a p65-specific consensus motif. A significant reduction in NF-κB activity as measured by ELISA and decreased nuclear NFκB-p65 translocation by immunofluorescence staining were observed in ITGB7-silenced compared with ITGB7-positive cells (Figure 5), consistent with a role for p65 (RelA) in integrin-β7 downstream signaling in MM. Similar results are also shown in supplemental Figure 4C for the INA-6 cells. In addition, ITGB7 silencing significantly attenuated FAK, and Src (Tyr-416) activation in H929 and MM1S cells plated on FN-coated plates and reduced ERK phosphorylation in H929 but not MM1S cells (Figure 6A-C, supplemental 9A-C). ITGB7 knockdown also reduced the activation of Rac1 GTPase (as determined by Rac1-GTP binding to GST-PAK1) in MM1S cells cultured in uncoated (RP) as well as FN-coated plates (Figure 6D). Recent studies have indicated that Rac SUMOylation is required for optimal Rac activation, lamellipodia-ruffle formation, cell migration, and invasion.40 When cultured on FN-coated plates, we have observed a loss of lamellipodia-ruffle formation in H929 ITGB7-silenced compared with ITGB7-positive cells (Figure 6B-C). This effect was less evident in MM1S cells and was only seen when cells were cultured on poly-lysine–coated plates with FN (supplemental Figure 9A-C). We also noted in Rac1 Western blots (including IP of GTP-Rac1) an up-shifted band (∼55 kDa) consistent with the SUMOylation of Rac1 (Figure 6E, supplemental Figure 10). Therefore we next examined whether ITGB7 knockdown reduced Rac1 SUMOylation. As shown in Figure 6E and F and supplemental Figure 10, when pull-downs of GTP-Rac1 were probed for SUMO1, ITGB7 knockdown significantly reduced SUMOylated RAC1.

Integrin-β7 silencing reduces in vivo MM cells homing to the BM and decreases tumor vessel density in MM xenografts

We investigated in vivo the effect of ITGB7 silencing on tumor growth and noted no difference in the growth kinetics of the ITGB7-positive or ITGB7-silenced xenografts in SCID mice. The median time from MM-cell inoculation to mice sacrifice for ITGB7-positive was t = 24 ± 2 days compared with t = 26 ± 2 days in ITGB7-silenced xenografts, suggesting that integrin-β7 may not be required for MM-cell growth in vivo. Based on our in vitro chemotaxis and transwell migration studies, we next examined whether integrin-β7 plays a role in MM-cell BM homing in vivo. Therefore, using in vivo flow cytometry we first tested the effect of ITGB7 silencing on the number of circulating ITGB7-positive versus ITGB7-silenced MM1S and H929 cells after mouse tail vein injection. As shown in Figure
7A, 20 minutes after cell injection nearly all ITGB7positive cells exited from the circulation, whereas 50% of ITGB7silenced cells were still circulating consistent with delayed organs (including BM) homing. To further examine direct MM-cell homing to the BM, femurs were collected from killed mice and stained with fluorescent anti–human CD138 Ab. As shown in Figure 7B, mice inoculated with MM1S ITGB7silenced cells had delayed engraftment or homing into the BM, compared with ITGB7positive as indicated by the number of human CD138+ cells counted in BM sections (14% ± 2% in ITGB7positive vs 4 ± 1% in ITGB7silenced). No MM cells (human CD138+ cells) were detected in the livers or spleens of killed animals.

Finally, based on our observation that ITGB7 silencing reduced VEGF secretion in MM-BMSC cocultures, we evaluated whether ITGB7 was involved in neo-angiogenesis by CD31 immunostaining of serial sections of xenografted MM tumors. As shown in Figure 7C, the number of CD31+ cells was significantly reduced in ITGB7silenced compared with ITGB7positive tumors (CD31 target area as the percentage of the total area examined: 4.1% vs 7.7%, respectively). Taken together, these data are consistent with an essential role for integrin-β7 in MM cells in vivo homing to the BM milieu as well as myeloma-induced neo-angiogenesis.

Discussion

Adhesion molecules mediate plasma cell interaction with BM-ECM components, transducing “outside-in” and “inside-out” signals that regulate MM-cell behavior and gene expression.41 As such, integrins are reported to play a cardinal role in the physiology of malignant plasma cells, including their survival, proliferation, homing into and egress from the BM niches and particularly their resistance to variable therapeutics. To date, functional studies of integrins involved in MM-BM-ECM interactions have predominantly focused on integrins-β1 (α4β1 and α5β1)5,11-14 as well as β2 and β3 (αvβ3)15 and far less integrin-β7.21 Interest in the defined roles integrin-β7 plays in MM pathogenesis remerged with the molecular classification of the disease and the detection of high levels of ITGB7 mRNA in “high-risk” MS and MF myeloma subgroups.23,24 In addition, integrin-β7 was recently identified as a target gene of the oncogene C-MAF which is overexpressed in nearly 50% of MM patients as a result of IgH translocation with t(14;16) or through cytokines (BAFF and APRIL) binding to TACI.21,22

In the current study, we have first demonstrated that integrin-β7 mRNA and protein are expressed in MM cell lines with and without
t(14;16) as well as primary CD138+/H11001 MM cells in which integrin-/H9252 ITGB7 mRNA and protein were detectable in 92.3% and 78.5% of the cases, respectively. These results are partially consistent with the previous work by Hurt and colleagues who reported a C-MAF–driven expression of ITGB7 mRNA in 64.3% and 50% of MM cell lines and MM patients, respectively.21 Interrogation of the gene dataset GEO-GSE4581 identified ITGB7 mRNA in 124 of 130 (95.4%) MM patients even in the absence of detectable calls with the C-MAF probes (supplemental Figure 1A). Therefore, these results suggest that in addition to the C-MAF other oncogenes or cytokine-activated transcription factors drive the expression of integrin-/H9252 ITGB7 in MM cells. As such, the ITGB7 promoter is reported to be responsive to other MM oncogenes like MAFB42 as well to MM cytokines like TGF-/H92521.43 Functionally and through ITGB7 knockdown, we have demonstrated that integrin-/H9252 ITGB7 plays a major role in MM-cell adhesion to FN, E-CDH, and to human BMSCs. This reduction in adhesion of ITGB7-silenced cells was particularly evident on E-CDH and to a lesser extent on FN-coated plates. Previous work relying on α4β7 blockade with Act-1 Ab that binds to an unique α4β7, but not to αEβ7 or β7 epitopes, reported a minor role for integrin-β7 in MM-cell adhesion to FN.14 In contrast, studies relying on blocking Abs that bind to a β7 unique epitope (Fib 504 clone) regardless of its heterodimer partners, resulted in a significant reduction in MM-cell adhesion to E-CDH and BMSCs.21 In sum, these results along with our findings suggest that (1) β7-integrin is responsible for MM-cell adhesion to E-CDH and (2) MM-cell adhesion to FN and BMSCs is dependent on several integrins (α4β1, α5β1, etc) including β7 (α4β7, αEβ7). Whether these integrins contribute simultaneously or act individually through a regulated temporal-spatial expression to regulate MM-cell adhesion to BM-ECM components remains to be determined.

Supporting its role in CAM-DR, we have observed that increased integrin-β7 expression on plasma cells correlated with poor survival outcomes in newly diagnosed MM patients treated with high-dose melphalan or in relapsed patients receiving salvage bortezomib in the APEX trial. Similarly, recent updates from the Total Therapy 3 trial reported poor survival in the MF molecular subgroup (characterized by high integrin-β7 expression) despite treatment with the combination of bortezomib and immunomodulatory drugs (IMiDs).44 In line with these clinical observations, we confirmed in vitro the contribution of integrin-β7 to CAM-DR by...
demonstrating a partial loss of the protective effect mediated by FN adhesion in integrin-\( \beta7 \) silenced cells treated with bortezomib or melphalan. Seminal studies by Damiano and colleagues,\(^5\) which focused on the role of integrin-\( \beta4 \) in CAM-DR, also showed an increase in the expression of integrin-\( \beta7 \) (but not \( \beta5 \)) in melphalan-resistant MM cells. Also of note, the integrin-\( \beta4 \) blocking Abs used in their study equally affects CAM-DR mediated by integrin-\( \beta4 \) as well as integrin-\( \beta4 \) and integrin-\( \beta7 \) heterodimers. Mechanistically, integrin-\( \beta7 \) silencing partially attenuated NF-\( \kappaB \) activation induced by adhesion to FN as previously reported with integrin-\( \beta4 \)\(^11\) and significantly reduced the levels of VEGF and several other cytokines produced in MM-BMSC cocultures. Of interest, among these cytokines affected by integrin-\( \beta7 \) silencing, several (\( \text{IL-1} \), MIP-1\( \text{a} \), MIP-1\( \text{b} \)) are implicated in osteoclast-osteoblast-MM-cell interaction leading us to speculate that integrin-\( \beta7 \) might be involved in MM bone disease. Lastly, bortezomib was recently reported to partially overcome CAM-DR by downregulating \( \alpha4 \) expression in MM cells,\(^45\) no such effect was observed on integrin-\( \beta7 \) expression (data not shown). Of note, \( ITGB7 \) silencing in the H929 cell line (and to a lesser extent in MM1S cells) did potentiate the cytotoxic effect of bortezomib in cells cultured in uncoated plates suggesting that an autocrine soluble or a MM cell-surface ligand may be contributing to the integrin-\( \beta7 \)-mediated drug resistance. In summary, based on the results of the current study and others, CAM-DR in MM appears to be mediated through a collaborative effect of several adhesion molecules including integrin-\( \beta7 \). Future treatments aiming at reversing CAM-DR shall take into account this coordinated protective effect of adhesion molecules.

Circulating plasma cells are documented in 35%–70% of MM patients and reported to predict poor survival outcomes.\(^46\) In addition, residual focal bone lesions in patients in hematologic remission were recently correlated with disease relapse highlighting the ability of MM cells to spread and migrate in and out of the BM.\(^47\) Similarly, previous works by the Plarski group and others did identify a circulating clonal B-cell population within the peripheral blood of MM patients who was implicated in the migratory spread of the disease.\(^48\) Of interest, the adhesive and migratory properties of these clonal B cells were described as CD44 and \( \alpha4 \)-integrin dependent. In the current work, we have first demonstrated in vitro the role of the \( \beta7 \)-integrin in MM-cell
transwell migration and matrigel basal membrane invasion toward SDF-1α chemokine gradients. We have also shown an integrin-β7-mediated activation of FAK, Src, and the Rho-like GTPase Rac-1 (including Rac1-SUMOylation). More importantly, we have demonstrated in vivo the role of integrin-β7 in MM cells homing to the BM. SCID mice xenografted with ITGB7silenced cells developed
localized subcutaneous tumors with growth kinetics similar to mice engrained with ITGB7positive cells but displayed a significant reduction in their homing to the BM milieu. Measuring the number of circulating MM cells with in vivo flow cytometry in BALB/c mice, we have also shown that ITGB7 silencing resulted in a significant delay in the time needed for MM cells to exit the circulation and home to the BM following tail vein injection. These results demonstrate the direct involvement of integrin-β7 in MM cells homing to the BM and suggest that SDF-1α guided chemotaxis of MM cells to the BM requires integrin-β7 for adhesion to endothelial cells, endothelial basement membrane invasion, and possibly retention of MM cells into the BM niches. Further studies to determine which integrin-β7 heterodimer (α4β7 or αEβ7) and what ligands (VCAM-1, E-cadherin, etc) are involved in these processes are ongoing. Lastly, and consistent with a possible role for integrin-β7 in MM-induced BM neo-angiogenesis, we observed a reduction in VEGF secretion in ITGB7silenced MM-BMSC cocultures and shown a significant decrease in the microvessel density of ITGB7silenced compared with ITGB7positive xenografts.

In conclusion, we have demonstrated that integrin-β7 plays a critical role in MM-cell adhesion, migration, invasion, BM homing, and CAM-DR. Humanized mAbs blocking integrin-β7 are currently investigated for the treatment of chronic colitis and gastrointestinal GVHD and may warrant further testing in MM.

References


Integrin β7-mediated regulation of multiple myeloma cell adhesion, migration, and invasion

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