Supplementary Materials and Methods

3D-light sheet fluorescence microscopy (LSFM) of bone

For LSFM analysis mice were euthanized and bones were removed and freed from soft tissue. Samples were placed and stored in 4% paraformaldehyde (PFA) for 2h at room temperature followed by an incubation in 4% PFA at 4°C over night. The next day samples were transferred to PBS and stored at 4°C until further usage. For homogenous ex situ immunofluorescence staining samples were first washed three times for 30 minutes in PBS at 4°C and then decalcified in 10% EDTA in H2O, pH 7.8 for three subsequent days. EDTA solution was replaced every day. After decalcification samples were washed once with PBS for 30 min at 4°C and blocked for 18–24 hours with 2% FBS/PBS in 0.1% Triton X-100. Sampled were then incubated with the respective antibodies for 24 hours at 4°C on a shaker, washed in PBS, followed by dehydration in a graded ethanol series (30%, 50%, 70%, 80%, 90%, 96%, and in 100%) for 2 hours each at room temperature. After the samples were rinsed for 2 hours in 100% n-hexane, the n-hexane was replaced stepwise by a clearing solution consisting of 1 part benzyl alcohol in 2 parts benzyl benzoate (Sigma-Aldrich). Air exposure was strictly avoided at this step. Tissue specimens became optically transparent and suitable for LSFM imaging after incubation in the clearing solution for at least 2 hours at room temperature.

To visualize JAM-C+ and CD138+ cells in their anatomical context to the CD144+ cells of the vascular system (i.e. directly within the MM-niche) we recorded 1500 optical sections for three individual color channels each (488, 532, and 647 nm) with an increment of 5 µm. Using higher magnification enabled us to even visualize subcellular components within the bone marrow. Moreover, tissue autofluorescence, recorded mainly in the 488 nm channel, displayed detailed microanatomical structures, which allowed for the localization of individual cells within their anatomical context and to differentiate them from MM cells.

Image analysis of LSFM acquired images and distance measurement towards blood vasculature

Multi-channel stacks of LSFM acquired images from bone marrow samples were reconstructed in 3-dimensions, processed, and analyzed with Imaris software v8.4.2 (Bitplane AG, Zurich, Switzerland). If needed, channel shift function was used to correct chromatic aberration between channels. To eliminate background signals, background subtraction with a rolling ball radius according to the diameter of cells of interest were applied. Non-specific noise was removed by applying a 2D median filter with the size of two pixels. Blood vasculature, multiple myeloma cells and JAM-C signals were segmented using “surface” module with smoothing
10% of the expected diameter of objects and manual thresholding of signals. Volume and sphericity filters were employed to remove very large and very small objects and objects with sphericity less than 0.6 with an irregular shape. The distance of MM cells and JAM-C signal from blood vessels are calculated from the distance map that is generated from blood vessel surfaces. To present the distance distribution regarding the blood vessels, the number of events is normalized to the total number of cells or signals detected in the 3D microenvironment of bone marrow image.

**Preparation of bone sections for confocal microscopy**

**Embedding**

Mice were euthanized, bones were extracted, and soft tissue was removed. Bones were placed and incubated in 4% paraformaldehyde (PFA) in PBS, pH 7.4, for at least 6h at 4°C on a shaker. Thereafter samples were washed once in PBS and transferred to 0.5 M EDTA in H2O, pH 8.0 and incubated for 3 days overnight at 4°C on the shaker. Next, EDTA solution was replaced by 20 % Sucrose/2 % PVP/H2O and samples were incubated for at least 6h at 4°C on a shaker. Afterwards, bones were dried, placed into a plastic mold and an 8% gelatin/ 20 % Sucrose/2 % PVP/H2O solution was added. Samples were left at room temperature until the solution became solid. Molds were stored at -80 °C until further usage.

**Cutting**

The mold containing the bones was placed in a -20°C cryostat (Leica CM 1950, Leica Biosystems, Wetzlar, Germany), the gelatin block was removed from the mold and excessive gelatin was cut off at the edges. Excess gelatin and all compact bone until a sizable portion of bone marrow was visible was trimmed off. 60 μm (up to 80 μm) sections were cut and placed on a SuperFrost Plus™ slide (Fisher Scientific GmbH, Schwerte, Germany). Once the section has attached completely to the slide, slides were removed and dried at RT for 15 min. Afterwards slides were stored at 4°C for at most 2 weeks or immediately stained for imaging.

**Staining and imaging**

Slides were stained in a Shandon Chamber with corresponding antibodies (dilution 1:10 - 1:100) for 4h at RT. Afterwards slides were washed three times with PBS, covered with Vectashield® mounting medium containing DAPI (Vector Laboratories, Burlingame CA, USA) and encircled with Entellan® (Merck KGaA, Darmstadt, Germany). Slides were stored at 4°C in the dark until further usage or immediately imaged. Pictures were taken with a multiphoton laser scanning microscope (LSM 780) using the ZEN Black Software (Carl Zeiss AG, Oberkochen, Germany).
Image analysis of LSM acquired images and fluorescence intensity measurements

JAM-C$^{\text{high}}$ and CD138$^{\text{high}}$ expressing regions were segmented based on intensity of respective antibody signal after removing the background with gaussian filter with filter size of 0.5 µm using surface module in Imaris software. Detection cell only mode was selected with smallest cell diameter of 8 µm. Manual thresholding was employed to keep all pixels which are higher than 80 in intensity and objects bigger than 10 µm$^2$ to remove small objects and smaller cell types. After segmentation of objects, all identified JAM-C$^{\text{high}}$ and CD138$^{\text{high}}$ cells, were measured for CD144 signal intensity. The results are presented as bar graph showing the mean values of three individual animals.

Immunostaining and labeling of human bone marrow biopsies

4-µm-thick sections were cut from bone marrow biopsies of MM patients, which were formalin fixed, paraffin embedded and EDTA decalcified. Immunostainings were performed on a Dako OMNIS platform. CD31/CD38 and CD31/JAM-C double stains were performed revealing the first antibody in brown (DAB Chromogen) and the second one in red (HRP Magenta).

Antibodies

The following antibodies were used in experiments: anti-mouse JAM-C, AF555 and AF647 (BIOSS, Woburn, MA, USA) dilution: 1:100 for FACS, 1:10 for confocal microscopy and 15µg/100µl in PBS for LSFM; anti-mouse CD138, clone 300506, AF488 (Novus Biologicals, Centennial, CO, USA) dilution 1:200 for FACS, 1:100 for confocal microscopy and 15µg/100µl in PBS for LSFM and clone B239442, PerCP-Cy5.5 (BioLegend, San Diego, CA, USA), dilution 1:200 for FACS; anti-mouse CD144 (VE-Cadherin) clone BV13, AF647 (BioLegend, San Diego, CA, USA) dilution 1:100 for confocal microscopy and 15µg/100µl in PBS for LSFM. Anti-human CD31-Alexa Fluor 700 (clone: WM59) dilution: 1:200, CD38-APC/Fire 750 (clone: HB-7) dilution: 1:100, CD56-PE/Cy7 (clone 5.1H11) dilution: 1:100 and CD138-PerCP/Cy5.5 (clone: MI15) dilution: 1:50 were from Biolegend; CD19-PE (clone: HIB19) dilution: 1:1600 and CD45 Pacific Orange (clone: HI30) dilution: 1:50 from Thermo Fisher; anti human JAM-C (clone: REA429) was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany), dilution: 1:20. LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit from Thermo Fisher was used to distinguish between live and dead cell populations according to manufacturer’s instructions.

Immunostainings of human bone marrow biopsies were performed with the following antibodies: CD31 (Mouse monoclonal [JC70A], #GA610, Dako, Agilent Technologies Inc., Santa Clara, CA, USA); CD38 (Rabbit monoclonal [EPR4106], #ab108403, Abcam,
Cambridge, UK), dilution for both: 1:100; JAM-C (Rabbit polyclonal, #ab224327, Abcam, Cambridge, UK), dilution: 1:50.

Monoclonal anti-mouse JAM-C antibody for in vivo treatment and corresponding isotype control antibody were produced by Davids Biotechnologie GmbH (DAVIDS-BIO, Regensburg, Germany). Screening for JAM-C-binding capacity of different clones was performed as described below. One of our positively screened antibodies, as well as a matched isotype control, were used in this study with a concentration of 100 µg/20 g body weight per i.p. injection or 20 µg/mL in co-culture experiments.

**Screening of monoclonal anti-JAM-C antibodies**

To analyze binding affinities of different hybridoma-derived monoclonal antibodies to the extracellular domain of murine Jam-C we performed cell-free equilibrium binding studies. For this, the different monoclonal antibodies (5 µg/ml in 0.1 M carbonate buffer) were immobilized overnight at 4°C on black high binding ELISA plates (Greiner, Frickenhausen, Germany). Humira (aTNF-antibody) served as negative control. On the next day plates were washed three times with PBS-Tween and then plates were treated with 10%-FCS in PBS to block free, unspecific binding sites (1 h at room temperature). After an additional washing step, the incubation (1.5 h at room temperature) of the antibodies with increasing concentrations of the GpL-fusion protein mJamC(ed)-Flag-Fc-GpL (GpL = *Gaussia princeps* luciferase) followed. Subsequently plates were washed five times with PBS to remove unbound GpL-fusion protein and 50 µl media (RPMI1640 containing 0.5 % FCS) were filled in each well. Finally, the well-associated luciferase activity, which is a degree for binding properties, was determined. For this, the GpL-substrate Coelenterazine (1.5 µM in PBS) was added and the light emission immediately detected and quantified using a LUmio-luminometer (Anthos Labtec Instruments).

**Flow cytometry**

Expression of described murine and human cell surface markers was performed using antibodies described above. Briefly, cell populations (flushed cells from bone marrow, peripheral blood cells, or cell populations from in vitro experiments) were harvested by centrifugation and washed once with PBS. Afterwards cells were stained for 30 min at 4°C in the dark including a LIVE/DEAD™ Fixable Violet Dead Cell Stain (molecular probes by life technologies; Fisher Scientific GmbH, Schwerte, Germany) to discriminate live from dead cells in further analysis. After another washing/centrifugation step to remove unbound antibody, samples were acquired using a FACS Canto II cytofluorimeter (BD Biosciences, San Jose, CA, USA) or an Attune NxT Flow Cytometer (Fisher Scientific GmbH, Waltham, MA USA). Data
were analyzed using FlowJo single cell analysis software v10.5.3 (Tree Star, Inc., Ashland, OR, USA). Results are displayed as representative histograms, dot or contour plots showing gate frequencies and summarized as graphs showing statistical evaluation. Mean fluorescence intensities (MFI) are only shown when being significantly different between experimental groups.

Human MM-PCs samples were obtained from bone marrow samples from volunteers. Total bone marrow samples were frozen and kept in liquid nitrogen until use. After thawing, cells were washed once with complete medium and treated in analogy to murine samples.

Quantitative Real-time PCR (qRT-PCR)

Total RNA from pelleted cells was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA synthesis was performed with 1 μg RNA in a 20 μl reaction mixture using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories GmbH, Feldkirchen, Germany) according to manufacturer’s instructions. qRT-PCR reactions were performed using the SsoAdvanced Universal SYBR Green Supermix on a CFX Connect Real-Time PCR Detection System (both Bio-Rad). GAPDH and ACTB were used as the reference genes. The relative normalized expression levels were calculated by the comparative \(2^{-\Delta\Delta CT}\) method and displayed as fold-change using the Bio-Rad CFX Manager software Version 3.1. Primer sequences were the following: JAM-C: 5’-CAGAATTCCAGCCGCTGTAC-3’ (forward) and 5’-TGGCTCTGGATCTGTAGGC-3’ (reverse); CD138: 5’-AGGCAGCTGTACATCTCAT-3’ (forward) and 5’-TTTGATGACAGAAGTGCCGC-3’ (reverse); GAPDH: 5’-AGTATGACAGACGGTCCGC-3’ (reverse); ACTB: 5’-AGGGGAATGGGTCAGAAGGA-3’ (forward) and 5’-CTTCTCCATGTCGTCCCAGT-3’ (reverse). Measurements were performed in duplicates using RNA/cDNA of 4 independent experiments under identical conditions.

Bioluminescence imaging (BLI)

BLI was performed on mice and ex vivo on prepared bones with an IVIS Spectrum (Caliper-Xenogen, Alameda, CA, USA) imaging system as previously described. Briefly, mice were anesthetized and the substrate for firefly luciferase D-Luciferin (150 mg/kg) was injected intraperitoneally. BLI measurements on mice were started exactly 10 min after injection. For ex vivo imaging mice were euthanized 10 min after substrate injection, bones were removed, cleaned from soft tissue, and used for BLI measurement.
Statistical Analysis

Statistics were performed using Graphpad Prism 7 (Graphpad, La Jolla, CA, USA). To determine statistical significance of differences between experimental groups tests were used as stated in figure legends. p-values <.05 were considered as significant.

References


Supp. Fig. 1 MM cells interact with the vascular endothelium within the 3D bone marrow microenvironment

(A) 3D-reconstruction of an image stack of BM bearing MOPC-315.BMP2.FUGLW with light sheet fluorescence microscopy (LSFM). Blood vessels (BV) are depicted in red (CD144-AF647), MM cells in green (CD138-AF488). (B) Computational segmentation of BV and MM cell signals by extracting cell and BV surfaces (scale bar in A and B is 200µm). (C+D) example of visualizing and segmenting MM and BV signals at cellular resolution. (E) Measuring distance of a single MM cell to surrounding BV (scale bar in C, D and E is 20µm). (F) Normalized number of MM cells in direct contact/co-localization to BV within the bone marrow.

Supp. Vid. 1 MM cell signal co-localizes with BV signal in BM

Representative 3D-reconstructed light sheet fluorescence microscopy image stack of intact long bones of a MM bearing mouse; blood vessels (BV) are depicted in red (CD144-AF657), MM cells in green (CD138-AF488) and autofluorescence (AF) background signal in blue.

Supp. Vid. 2 JAM-C signal co-localizes with MM cells and BV

Representative reconstructed light sheet fluorescence microscopy movie (Z-stack) of intact long bones of a MM bearing mouse; blood vessels (BV) are depicted in red (CD144-AF647), MM cells in green (CD138-AF488) and JAM-C signal in blue (AF555). Scale bar is 150µm.

Supp. Fig. 2 JAM-C signal is enhanced on human MM cells in perivascular subniches

Myeloma cells, highlighted by CD38 staining, constitute roughly 30% if the infiltrate, growing in a perivascular and interstitial fashion (A, CD31brown/CD38red, 20x). JAM-C is mildly expressed, with higher intensity in the perivascular compartment and in scattered circulating cells (B, CD31brown/JAM-Cred, 20x).

Supp. Fig. 3 CD138low/neg JAM-C+ MM cells are less sensitive to Bortezomib treatment and show lower proliferation

(A) Representative FACS plots of surface CD138- and JAM-C expression on single living MM cells after depicted treatment and corresponding histograms regarding Ki-67-staining in displayed populations (B). (C) Graphical and statistical display of...
fold increase in percentages compared to isotype ctrl. treatment calculated from flow
cytometric analyses in (A). Bzmb was used with a final concentration of 2.5 ng/ml. All
experiments were independently repeated three times. Unpaired, two-tailed Student t-tests
were used. Errors reflect SD.

**Supp. Fig. 4: Screening of murine αJAM-C antibodies using cell-free equilibrium binding**

**studies (A+B)** Binding properties of the indicated antibodies were evaluated by cell-free
equilibrium binding studies using plastic-immobilized antibodies and the indicated
concentrations of muJAM-C(ed)-F-Fc-GpL. Binding was indicated by light emission (RLU). The
specific binding of antibody #42 to muJAM-C(ed)-F-Fc-GpL were calculated by subtraction of
the unspecific binding (binding of αTNF antibody Humira to muJAM-C(ed)-F-Fc-GpL) from total
binding (binding of antibody #42 to muJAM-C(ed)-F-Fc-GpL). Graphic illustration of the data is
shown in (C+D). Representative example of a tested clone (#238) showing no binding capacity
(B+D), that served as matched isotype control.

**Supp. Fig. 5:** JAM-C is upregulated on CD138(low/neg) cells in BM of MM patients

Representative FACS plots of surface JAM-C-expression on living human MM cells gated
initially on single CD45- CD56+ cells and afterwards divided in CD38Dim+/ CD138(high and
CD38Dim+/ CD138(low/neg) in BM at time of diagnosis (T0) and n+1 relapse (T2).

**Supp. Fig. 6:** Percentage of JAM-C+ population increases within CD138low/neg MM
cells in BM of MM patients

Representative FACS plots of surface CD138- and JAM-C-expression on living human MM
cells gated on single CD38Dim/+ CD45- CD56+ cells in BM at time of diagnosis (T0) and n+1
relapse (T2).
Supp. Fig. 1 MM cells interact with the vascular endothelium within the 3D bone marrow microenvironment
Supp. Fig. 2 JAM-C signal is enhanced on MM cells in perivascular subniches
Supp. Fig. 3 CD138<sub>low/neg</sub> JAM-C<sup>+</sup> MM cells are less sensitive to Bortezomib treatment and show lower proliferation
Supp. Fig. 4: Screening of murine αJAM-C antibodies using cell-free equilibrium binding studies

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#### Diagrams

**C)**

- Graph showing specific binding at different concentrations.

**D)**

- Graph showing specific binding at different concentrations.
Supp. Fig. 5: JAM-C is upregulated on CD138\textsuperscript{low/neg} cells in BM of MM patients
Supp. Fig. 6: Percentage of JAM-C+ population increases within CD138low/neg MM cells in BM of MM patients