Supplementary Materials

Cell lines and cell culture conditions

RPMI-8226, U266, OPM-2, NCI-H929 and INA-6 were maintained in a humidified incubator at 37°C and 5% CO2 in sterile tissue culture dishes in RPMI 1640 (Seromed-Biochrom, Berlin, Germany), supplemented with 20% (vol/vol) heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 100 g/mL streptomycin, 1 mM sodium pyruvate, 2 mM glutamine (all from Life Technologies, Karlsruhe, Germany). Cell media of INA-6 cells was additionally supplemented with 2 ng/mL IL-6. HUVEC were maintained as described in the suppliers' instructions (PromoCell).

Immunohistochemical analysis from patients bone marrow biopsies

Staining was performed with isoform-specific antibodies against JAM-A (monoclonal anti-human JAM-A (CD321) antibody) and binding was visualized using ACE as a chromogenic substrate. After deparaffinization and rehydration, the BM biopsy slides were placed in a pressure cooker in 0.01M citrate buffer (pH 6.0) and heated for 7 min. Incubation with αJAM-A moAb (clone J10.4, Sigma-Aldrich) was carried out at room temperature for 1 hour. Detection was performed with the DAKO EnVision system according to the manufacturer's protocol. BM involvement was assessed as the percentage of positive cells relative to the total cell count (for example 40% of nuclear cells) in 10 high power fields (HPFs) per sample.

Immunofluorescence

5×10^3 MM-PCs, RPMI-8226, and control PCs, PBMCs, HUVECs / chamber were cultured on fibronectin-coated chamber slides (LabTek, Nalge Nunc International, Naperville, IL, USA), fixed with paraformaldehyde, permeabilized with Triton X-100, and incubated with αJAM-A moAb (clone OV-5B8, FITC, BioLegend San Diego, CA, USA). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, in Vectashield® Hard_Set™ mounting medium, Vector, Burlingame, CA, USA) according to the manufacturer's instructions. Images were obtained with an AxioCamMR3 camera, mounted on an Axio Imager.Z1 microscope (Carl Zeiss, Jena, Germany) equipped with AxioVision software. The objective was a 40×/1.30 oil EC Plan-Neofluar.

Quantitative real-time PCR (SYBR Green Method)

First-strand cDNA synthesis was performed with 1 µg RNA in a 20 µl reaction mixture using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). A dissociation step was performed to generate a melting curve to confirm the specificity of the amplification. GAPDH was used as the reference gene. The relative levels of gene expression were represented as DCt= Ct gene–Ct reference and the fold changes of gene expression were calculated by the comparative $2^{-\Delta\Delta CT}$ method, where DDCt= (Ct gene–Ct reference) sample– (Ct gene–Ct reference) calibrator. Primer sequences for JAM-A were 5'-GTG AAGTTGTCCTGTGCCTACTC-3' (forward) and 5'-ACCAGTTGGCAAGAAGGTCACC-3' (reverse). Three independent experiments were conducted in triplicate under identical conditions.

Western blot

Total protein lysates (50 µg) from HUVECs, PBMCs, OPM-2, INA-6, U266 and RPMI-8226 cells were immunoblotted with anti-JAM-A (Abcam, Cambridge, MA), anti-Tubulin (Sigma-Aldrich, St. Louis, MO) and anti-JAM-A as described previously[46]. Immunoreactive bands were detected with enhanced chemiluminescence (LiteAblot; EuroClone), and Gel-Logic1500 system (Eastman Kodak Co.), and quantified as optical density units by Kodak imaging software

Small Interfering RNA treatment

Cells were seeded in a 6-well plates 24 hours before transfection experiments. The cells were transfected with 100 pmol JAM-A siRNA or negative control siRNA using Lipofectamine2000 (5 µl/well; Life Technologies Corporation, Grand Island, NY, USA) according to the manufacturer's protocol. Following transfection, the protein and mRNA levels of JAM-A were assessed 48 h later. Three independent experiments were conducted under identical conditions.

Scratch assay

After the pipette scratch, cells were left to migrate into the wound for 24 h in serum-free medium (SFM) alone (control), or spiked with αJAM-A moAb (10µg/mL, clone J10.4, Sigma-Aldrich), isotype control (IgG1, 10µg/mL), pretreated with siRNA anti-JAM-A or with control siRNA (CTRL

siRNA). Cells were counted in at least three randomly chosen ×10 magnification wound fields from three biological replicates.

Colony forming assay

RPMI-8226 cells ($1x10^4$ per dish) were plated into methylcellulose medium (Stem Cell Technologies, Cologne, Germany) for 14 days and were either left untreated or exposed to the neutralizing αJAM-A moAb (10 μg/mL, clone J10.4, Sigma-Aldrich). MM cell colonies that consisted of >40 cells[41] were counted under an inverted microscope. Three independent experiments were conducted in triplicate under identical conditions.

Chemotaxis

$1×10^5$ RPMI-8226 cells/well were seeded in triplicate on the upper compartment of the chamber, exposed to the neutralizing αJAM-A moAb (10 μg/mL, clone J10.4, Sigma-Aldrich, St. Louis, MA, USA), and IgG isotype control or treated with siRNA and non-coding sequence control, and left to migrate towards DMEM with 1.5% fetal bovine serum (FBS) (negative control) or supplemented with hepatocyte growth factor (HGF) (10 ng/mL, Sigma-Aldrich, St. Louis, MA, USA) and basic fibroblast growth factor (FGF-2) (10 ng/mL, Peprotech Inc., Rocky Hill, NJ,USA) (positive control) in the lower compartment. After 8 h at 37°C, the migrated cells were fixed, stained and counted on 5-6 ×400 fields/membrane using an AxioCamMR3 camera, mounted on an Axio Imager.Z1 microscope (Carl Zeiss, Jena, Germany) equipped with AxioVision software.

Ki-67 assay

RPMI-8226 were left untreated, or treated with iso CTRL, (10 μg/mL), with Anti-JAM-A moAb (purified monoclonal, unlabeled, 10 μg/mL), 3 x 10^4 cells per condition were used. All the experiments were performed in triplicates, for each of the 3 time points: 6h, 12 h and 24h treatment. RPMI-8226 cells were harvested, counted and pelleted following standard procedures. While vortexing, 5 ml cold 70% - 80% ethanol were added dropwise into the cell pellet (1-5 x 10^7 cells). The next steps were conducted according to the Flow Cytometry Staining Protocol for Detection of Ki-67 as indicated in the suppliers' instructions (BD Biosciences, San Jose, CA, USA).

Apoptosis assay

3 x 10^4 RPMI-8226 cells (American Type Culture Collection, ATCC Boulevard Manassas, USA) were left untreated, or treated with iso CTRL, (10 μg/mL), with Anti-JAM-A moAb (purified monoclonal, unlabeled, 10 μg/mL), All experiments were performed in triplicates for each of the 3 time points: 6h, 12 h and 24h treatment. Apoptosis was assessed in RPMI-8226 cells using Annexin-V-PE/7aminoactinomycin-D (Becton Dickinson-BD, San Jose, CA, USA). Cell apoptosis was determined with a FACScanto II flow cytometer (BD) and data were analyzed with FACSDiva software (BD).

Multiple myeloma tumor model and moAb treatment

RPMI-8226 luc^+ cells were suspended (2 x 10^5 cells) in PBS and injected intra-tibially (i.t.) into 8–10 weeks old NSG mice (Charles River, Sulzfeld, Germany). One day after the injection, animals were imaged by *in vivo* bioluminescence (BLI) and randomized to three experimental groups. After BLI, the mice were treated with αJAM-A moAb (day 1 after MM injection), isotype control, or left untreated. Tumor-bearing mice were treated with αJAM-A moAb (N=12) or an IgG1 isotype moAb (N=10) or were left completely untreated (N=5). Mice received treatment three times a week intra-peritoneally (i.p.). Changes in tumor load were monitored with *in vivo* bioluminescence imaging (BLI) and was performed immediately before the first treatment (day 1 of MM injection), before every additional treatment, and immediately before euthanizing the animals (day 22).

Bioluminescence imaging (BLI)

BLI was performed on mice with an IVIS Spectrum (Caliper-Xenogen, Alameda, CA, USA) imaging system as previously described.[42,43] Briefly, mice were anesthetized i.p. with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) in PBS. D-Luciferin (150 mg/kg) was coinjected, and BLI measurements were started exactly 10 min later. To confirm localization of light emitting foci, at the end of the experiment mice were euthanized, organs were prepared, and *ex vivo* imaging was performed. Imaging data were analyzed with Living Image 4.0 (Caliper-Xenogen) and Prism 6 software (GraphPad, La Jolla, CA, USA). Animals were also analyzed to determine the average

number of skeletal foci per mouse and the percentage of mice that displayed signals in liver and spleen.

Ki-67 staining

1 μm thick sections from formalin-fixed, paraffin-embedded, and decalcified (for bone tissues only) tissue blocks were deparaffinized in xylene and boiled in 1mM EDTA (pH=8) prior to staining with goat anti-Ki67 antibody (sc-7846; Santa Cruz Biotechnology, Dallas, TX, USA). This was followed by incubation with an alkaline phosphatase-conjugated isotype-specific secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA) and developed with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT&BCIP; Roche).

Antibodies

The following antibodies were used in experiments: anti-human JAM-A, clone OV-5B8, FITC (BioLegend San Diego, CA, USA) dilution: 1:200; anti-human JAM-A, clone M.Ab.F11, PE (BD Biosciences San Jose, CA, US), dilution: 1:100; anti-human JAM-A, mouse monoclonal, clone J10.4, purified (Sigma-Aldrich, St. Louis, MA, USA), dilution: 1:100 for histology, 20 μg/20 μl PBS for LSFM stainings and 100 μg/20 g bodyweight for treatment. Anti-human CD138 (clone MI15), APC (BD Biosciences San Jose, CA, USA), dilution: 1:600; anti-mouse CD31, Biotin (BioLegend, San Diego, CA, USA), dilution: 10μg / 20μl in PBS for LSFM; anti-mouse Ki-67 Antibody Rat IgG2a, κ (clone 16A8), purified (BioLegend San Diego, CA, USA), dilution: 1:100; mouse IgG1 κ isotype Ctrl Antibody (clone MOPC-21), FITC (BioLegend San Diego, CA, USA), dilution: 1:200; mouse IgG polyclonal antibody control 12-371, unconjugated (Merck Millipore, Darmstadt, Germany), dilution: 1:100.

Preparation of mouse specimens.

Mice were anesthetized by intraperitoneal injection of ketamine-xylazine and transcardially perfused with 20 ml ice-cold PBS, followed by 40 ml of 4% paraformaldehyde (pH 7.4). Following the perfusion, bones were removed. The samples were placed and stored in 4% paraformaldehyde for at least 2 hours at 4°C until use in further procedures. Hemoglobin-rich organs were bleached

for 30 minutes in 15% hydrogen peroxide/methanol. For homogeneous ex situ immunofluorescence staining of large specimens, the tissue samples were blocked for 18–24 hours with 2% FBS/PBS in 0.1% Triton X-100 and then incubated with the respective antibodies for 24 hours at 4° C on a shaker, washed in PBS, incubated with streptavidin for 24 hours, and washed again 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 had been 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 in 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.

TUNEL staining

1 μm thick slices were cut from each tibia bone block, deparaffinized in xylene, and hydrated in graded concentrations of ethanol. After being washed with 100 mM Tris-HCl at pH 7.8, sections were immersed in methanol containing 0.3% H_2O_2 for 20 minutes to inhibit endogenous peroxidase activity. The sections were rinsed three times for 2 minutes with distilled water each time, and then treated with 10 μg/ml proteinase K in lysis buffer at 37°C for 10 minutes. The sections were then immersed in terminal deoxynucleotidyl transferase (TdT) (0.3e.u./ml) (GIBCO, Grand Island, NY, USA) and biotinylated dUTP (0.04mmol/ml) (GIBCO) in TdT buffer (0.1M potassium cacodylate at pH 7.2, 2 mM cobalt chloride, 0.2mM dithiothreitol) (GIBCO), and left to react in a humid atmosphere at 37 °C for 90 minutes. The reaction was terminated by transferring the sections to stopping buffer (300 mM NaCl, 30 mM sodium citrate) for 15 minutes at RT.

After three washes for 2 minutes with PBS, sections were incubated in Avidin Biotin Complex (ABC) reagent (Vector Laboratories) for 1 hour. The color reaction was developed in 2% 3-3'-diaminobenzidine (Sigma) and 0.3% H_2O_2 in Tris buffer, and the sections were counterstained with methyl green. Cancer cells were morphologically distinguished from BMSCs by microscopic examination (x100; x400). TUNEL positivity indicates the apoptotic cells.

Statistical analysis

To evaluate relations between categorical data such as ISS, genetic risk and disease stage and surface JAM-A and sJAM-A concentrations (both continuous data), the latter were dichotomized into two classes, choosing the median as class boundary.

Variables entered in the Cox model were: age (as continuous adjusting covariate), gender, disease stage (NDMM vs MM after optimal response vs MM after suboptimal response), ISS (stage III vs stage I, stage II vs stage I), type of Ig (IgA vs IgG), genetic risk (high vs low, medium vs low), surface JAM-A expression at t0, at t1 and at t2 (higher than each patient's own median vs lower), sJAMA concentration at t0, at t1 and at t2 (higher than each patient's own median vs lower).

Supplementary table and figure legends

Supplementary Figure 1. JAM-A surface expression: impact on OS. Kaplan-Meier curves to compare OS between patients JAM-A surface. Expression at relapse higher vs lower than the median (1236). For 5 patients (out of 147 cases) JAM-A surface expression data was unobtainable: for 1 patient complete information regarding the date of disease progression and death was not available.

Supplementary Figure 2. (**A**) Differences in the JAM-A expression between MM-PCs from MM vs PCs obtained from healthy adults and MGUS patients determined by FACS analysis. *P*<.0001 assessed by Mann-Whitney test. (**B**) Differences in the serum sJAM-A concentration between MM patients and controls (healthy adults and MGUS patients) determined by ELISA. *P*<.0001 assessed by Mann-Whitney test.

Supplementary Figure 3. JAM-A overexpression in MM patients at relapse. (**A**) t2: expression of membrane JAM-A from MM patients cohort at relapse shows a direct statistically significant correlation with proportion of BM PCs infiltration at relapse (rs=0.25; p=0.0051); (**B**) t2: statistically significant direct correlation between serum levels of JAM-A at relapse with BM PCs infiltration (rs=0.21, p=0.0192). ; t2 = at disease relapse.

Supplementary Figure 4. JAM-A is differentially expressed in MM cell lines. (**A**) Relative expression of JAM-A mRNA in a panel of myeloma cell lines assessed by real-time PCR. The relative quantity of JAM-A mRNA, normalized to GAPDH, was compared to PBMCs and HUVECs treated with TNFα. Columns: mean of triplicate values in a representative experiment; bars: max/min RQ. Experiments were independently repeated three times and conducted in triplicates with similar results. (**B**) Immunofluorescence staining of RPMI-8226 cells. Scale bar, 50 μm. **(C)** Surface expression of JAM-A in RPMI-8226 cells and in a panel of myeloma cell lines assessed with flow cytometry. Data are shown as representative histograms, mean fluorescence intensity (MFI) of triplicate values in a representative experiment, (columns: mean; bars: SD). Experiments were independently repeated 3 times in triplicate, yielding similar results. (**D**) JAM-A was measured by immunoblotting of protein extracts from MM cell lines and normal PBMCs and HUVECs (treated with TNFα). Anti-tubulin staining served as loading control. TNFα treated HUVECs served as positive control.

Supplementary Figure 5. *In vivo* concentration of sJAM-A. Circulating sJAM-A increased in MM bearing mice (8 MM bearing mice, 3 healthy control mice, unpaired t-test; $P < .0003$ considered significant. 95% CI=154.6 to 365.8).

Supplementary Table 1. Clinical characteristics of multiple myeloma patients. The patient cohort, comprising subjects at different disease stages, was evaluated for JAM-A surface expression on MM-PCs, and sJAM-A concentration at the time of recruitment (t0), after treatment (at least 4 cycles of therapy, t1), at disease relapse (t2) and until death, when applicable.

Supplementary Table 2. Characteristics of controls.

Supplementary Table 3. JAM-A surface expression and sJAM-A concentration change from the time of recruitment, after therapy to disease relapse. Univariate and multivariate analysis - *Cox regression*: Progression Free Survival.

Supplementary Table 4. JAM-A surface expression and sJAM-A concentrations changes from the time of recruitment, after therapy to disease relapse. Univariate and multivariate analysis - *Cox regression*: Overall Survival.

Supplementary Table 5. Correlation coefficient (P-values) to evaluate relation between each parameter (on the left column) and JAM-A surface expression (left table) or sJAM-A concentration (right table).

Supplementary Table 6. Relation between JAM-A surface expression (upper tables), sJAM-A concentration (lower tables) and ISS, genetic risk and stage of disease; *P*-values resulted from chi-square test.