Halting the vicious cycle within the multiple myeloma ecosystem: blocking JAM-A on bone marrow endothelial cells restores angiogenic homeostasis and suppresses tumor progression

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Supplementary Material

Supplementary Methods

Patients derived cells and MMECs isolation

Bone marrow (BM) primary endothelial cells from MM (MMECs) and MGUS (MGECs) patients were obtained and cultured as described.[1,2] Full BM blood served as source for MMECs identification. Endothelial cells were harvested by magnetic cell sorting with anti-CD31 micro-beads (Miltenyi Biotech, Hamburg, Germany) from adherent mononuclear cells cultured for three weeks as described.[2,3]

The NDMM patient cohort (70 male and 41 female), ages 60 to 73 (median 67 years) were newly diagnosed with MM.[4] The monoclonal (M) component was IgG (n=64), IgA (n=27), IgM (n=1) and κ or λ (n=12). The MGUS patients (23 male and 12 female), ages 42 to 79 (median 60.5 years), were IgG (n=20), IgA (n=8) and κ or λ (n=7). The validation cohort was composed of 201 relapsed/refractory patients (RRMM) defined as previously described[5] 129 male and 72 female), ages 54 to 69 (median 62 years) whose M component was IgG (n=124), IgA (n=58), IgM (n=3), IgD (n=2), κ or λ (n=13) and biclonal (n=1).

Cell separation and cultures procedures

BM mononuclear cells (BMMoCs) were obtained by centrifugation on Ficoll-Hypaque gradient of heparinized bone marrow (BM) aspirates[1] and maintained in Roswell Park Memorial Institute medium (RPMI)-1640 supplemented with 10% FBS. After seven days, media were collected and JAM-A measured by ELISA. MMECs were isolated from BMMoCs using anti-CD31 MACS beads (Miltenyi Biotech, Hamburg, Germany). Purified MMECs and MGECs were grown and expanded for four passages in fibronectin coated culture dishes (BD Falcon, Küsnacht, Switzerland) in endothelial basal medium (EBM-2 Lonza, Basel, Switzerland) supplemented with 5% FBS with 10ng/ml VEGF (Miltenyi Biotech, Hamburg, Germany) as previously described.[2,3] Cell population purity (>95%) have been determined using the FACSCanto II flow cytometry system (Becton Dickinson-BD, San Jose, CA, USA). In functional studies, MMECs have been used until the 6th passage of culture, in DMEM supplemented with 10% FBS, 2mMol L-Glutamine, 100U/ml Penicillin, 100μg/ml Streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Human RPMI-8266 MM-cells have been maintained in RPMI-1640 supplemented with 10% FBS and routinely tested for mycoplasma contamination. To obtain MMECs conditioned media, MMECs have been grown to 80% confluence in serum-free DMEM medium for 24 hours. Culture media, antibiotic/antimycotic, glutamine, trypsin/EDTA, and PBS without Ca^{2+} and Mg^{2+} have been all purchased from Sigma-Aldrich (St. Louis, MO, USA).

MGECs, MMECs and MM-PCs were obtained from BM aspirates. MM-PCs were identified as the $CD138^+$ population within the gate of live cells. MM-PCs, MM-cell lines (RPMI-8226 and OPM-2) and HUVECs were stained with an anti-JAM-A antibody (anti-JAM-A FITC, clone OV5B8 (BioLegend, San Diego, CA, USA). Full marrow blood was stained with anti-CD31 (WM-59, Thermo-Fisher, Waltham, Massachusetts, USA), anti-CD45 (HI30, Thermo-Fisher, Waltham, Massachusetts, USA) and anti-CD138 (MI15, BioLegend San Diego, CA, USA) and endothelial cells recognized from BM mononuclear cells as CD45 negative, CD138 negative, CD31 positive within the living population. Human RPMI-8226 and OPM-2 myeloma cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA), and cultured according to the manufacturer's instructions. MMECs, RPMI-8226 and OPM-2 cells were harvested for western blot, co-culture and FACS experiments. MMECs co-cultured with RPMI-8266 or OPM-2 cells were separated by immunoselection. Culture media were collected and analyzed with ELISA and an angiogenesis array (R&D Systems®, Minneapolis, Minnesota, USA). MMECs were immunomagnetically separated with anti-CD31 MACS beads (Miltenyi Biotech, Bergisch Gladbach, Germany).

Reverse transcriptase PCR, real-time RT-PCR

Isolated mRNA was reverse transcribed into cDNA with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The mean of JAM-A mRNA expression levels in MGECs or in HUVECs were used as reference values and GAPDH as housekeeping gene. Real-time PCR was performed using the "StepOne real-time RT-PCR system" (Applied Biosystems, Foster City, CA, USA). Relative quantification of the mRNA level was evaluated using the comparative Ct method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference gene and with the $2^{-\Delta\Delta CT}$ formula. qRT-PCR TaqMan probes were from Applied Biosystems (Waltham, MA, USA). Primer sequences are available in **Supplementary Table 4**. Total RNA from MMECs and MGECs was extracted using the RNeasy Mini kit (Qiagen, Milano, Italy) and the real-time was performed according to manufacturer's instructions.

Immunostaining and labeling of human tissues and cells

Consecutive sections of specimens with confluent plasma cell infiltrates were stained with the following antibodies: anti-CD138 (MI15, Dako Agilent, Waldbronn, Germany) and CD31 (ab76533, Abcam, Cambridge, UK), according to the manufacturer's instructions. Slides were evaluated by an experienced hemato-pathologist.

***Wound-healing* assay**

The wound-healing assay was performed as previously described[6]. In short, MMECs were grown until confluence on fibronectin-coated (10 mg/mL) 12 well plate and the "wound" was generated by scraping the cell monolayer with a P200 pipette tip. Cells were exposed to serum free medium (SFM) alone or admixed with increasing concentrations of human sJAM-A (100 ng/mL). MMECs were also treated with 100 μg/ml neutralizing/blocking α-JAM-A mAb (10μg/mL, clone J10.4, Sigma-Aldrich, St. Louis, MO, USA). Afterward cells were fixed with 4% paraformaldehyde and stained with crystal violet. The migrating MMECs were counted in 3 different fields of the wound area at X10 magnification with EVOS digital inverted microscope (Euroclone, Pero, MI, Italy). Cell viability was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Matrigel angiogenesis assay

As the homophilic interaction between sJAM-A and the native transmembrane JAM-A has been demonstrated[7,8] a Matrigel™ angiogenesis assay was done by placing the MMECs on Matrigel™-coated plates.[9] Pictures of the skeletonized topological parameters "mesh areas", "branching points" and "vessel length" of the angiogenic network were measured by two independent observers using a computerized image analyzer[10] and ImageJ software.[11]

Matrigel (Becton Dickinson-BD, San Jose, CA, USA) 48-well plates in MMECs-derived conditioned medium (CTRL) alone or supplemented with increasing concentrations of the soluble recombinant human JAM-A (sJAM-A), up to 100 ng/mL, (Human JAM-A/F11R Protein, Sino Biological, Beijing, PRC). MMECs were also plated in CTRL in presence of 0.5 µg/mL neutralizing/blocking α-JAM-A mAb. Pictures were acquired in three randomly chosen fields with an EVOS microscope.

Western blot

Total MMECs and MGECs protein lysates were quantified with the Bradford assay (Bio-Rad, Hercules, CA, USA) and subjected to immunoblot with primary and secondary antibodies to the following: JAM-A (cod. 4267, Sigma-Aldrich, St. Louis, MO, USA) mAb; JAM-AmAb (clone OV-5B8, BioLegend San Diego, CA, USA) beta-actin (cod. A1978, Sigma-Aldrich); and mouse and rabbit horseradish peroxidase–conjugated IgG (Bio-Rad). Immunoreactive bands were visualized by enhanced chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Fisher Scientific, Waltham, MA, USA) with Gel Logic 1500 Imaging System (Eastman Kodak Co.). Bands were quantified using Kodak Molecular Imaging Software and expressed as arbitrary optical density (OD).

ELISA

Supernatants from MMECs alone or co-cultured with MM-cells were analyzed with ELISA to detect sJAM-A concentrations using a Human JAM-A ELISA Kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

Chorioallantoic membrane assay (CAM)

Fertilized white Leghorn chicken eggs were incubated at 37°C at constant humidity. On day 3, the shell has been opened and 2 to 3 mL of albumen will be removed to detach the chorioallantoic membrane (CAM). On the 8th day, the CAMs were implanted with 1 mm^3 sterilized gelatin sponges (Gelfoam, Upjohn Co, MI, USA) filled with SFM alone or with 100 ng/mL of sJAM-A, or with MMECs conditioned medium (MMECs CM) in presence or absence of 100 µg/mL of neutralizing/blocking α-JAM-A mAb, or with medium of sJAM-A-treated MMECs (JAM-A CM) with or without neutralizing/blocking anti-JAM-A mAb. On the 12th day, blood vessels entering the sponges within the focal plane of the CAMs were counted and pictures taken *in embrio* at X50 (Olympus stereomicroscope).

CAMs were examined daily until day 12 and photographed *in ovo* with a stereomicroscope. Vessels entering the sponges within the focal plane of the CAM were counted by two observers in a double-blind fashion at 50x magnification and confirmed by ImageJ software.[11,12]

In vivo experiments and immunohistochemistry on mice tissues

Intratibial xenograft MM model

Twenty mice were treated with α-JAM-A mAb (Sigma-Aldrich, St. Louis, MO, USA, mouse monoclonal clone J10.4) 5mg/kg body weight (in 100 µL PBS), or with an isotype control one day after MM-cell injection, three times per week on days 1, 4, 6, 8, 11, 13, 15 and 18.

Subcutaneous xenograft MM model

Twenty mice were treated with α-JAM-A mAb (Sigma-Aldrich, St. Louis, MO, USA, mouse monoclonal clone J10.4) 5mg/kg body weight (in 100 µL PBS), or with an isotype control (mouse IgG polyclonal antibody 12–371) three days/week for 40 days. Tumor growth was measured twice weekly, and weights (mg=mm^3) calculated as the length (mm) × width^2 (mm^2)/2. Mice were sacrificed when the tumor weight reached ~2.5 g.

The isotype control utilized for MM xenograft model was obtained from Merck Millipore, Darmstadt, Germany.

Immunohistochemical analysis followed mAb binding was visualized using 3,3'Diaminobenzidine (DAB) as a chromogenic substrate. After deparaffinization and rehydration, the BM biopsy slides were placed in a pressure cooker in 0.01M citrate buffer (pH 6.1, DAKO, Hamburg, Germany) and heated for 3 min. Incubation with anti-JAM-A mAb (clone J3F.1, Santa Cruz, Dallas, TX, USA) was

carried out at room temperature for 1 hour. Detection was performed with the DAKO Advance 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). Bone marrow sections and subcutaneous extramedullary tumors were single or double stained for Ki67 (LS-C175347, LifeSpan BioSciences, Seattle, WA, USA), CD138 (LS-B9360, LifeSpan BioSciences, Seattle, WA, USA), JAM-A (clone J3F.1, Santa Cruz, Dallas, TX, USA), CD31 (ab76533, abcam, Cambridge, UK) and CD34 (QBEND10 clone, Abcam, Cambridge, UK), according to the manufacturer's instructions. JAM-A staining was performed by incubating samples with anti-JAM-A mAb at room temperature for 1 hour. Detection was performed with the DAKO Advance system according to the manufacturer's protocol, using DAB and Magenta chromogens. BM involvement was assessed as the percentage of positive cells relative to the total cell count (for example 40% of nuclear cells); Ki67 was assessed as the mean of 5 counts of 100 cells, from separate fields; vessel density was evaluated as number of CD31+ vascular structures per mm^2. Stained bone marrow sections were analyzed by two experienced hemato-pathologists with a Zeiss Axioskop 40 microscope equipped with a Zeiss AxioCam MRc digital camera.

Mice were housed according to the Institutional Animal Care and Use Committee of the University Medical School of Bari, following guidelines published by Kilkenny *et al.*[13] Clinical signs of toxicity were monitored daily, while body weight was measured twice weekly.[14]

Immunohistochemical analysis of the mouse BM or subcutaneous mass specimens was performed with a specific antibody against JAM-A as described. Hemoglobin content of each plug was measured using Drabkin's assay (Sigma Aldrich, St. Louis, MO, USA) and normalized to its weight.[14,15] Specifically, the angiogenesis quantification in the subcutaneous xenograft model was also performed by the hemoglobin content evaluation, a well-established tool used to assess vessel density in xenograft Matrigel sponge tumor models: in detail, assay aliquots of 50 μl of reconstituted complete medium, containing 50 U/ml heparin, are added to unpolymerized Matrigel at 4°C at a final volume of 0.6 ml. The Matrigel suspension is injected subcutaneously into the flanks of mice by using a cold syringe. At body temperature, the Matrigel polymerizes to a solid gel, which becomes vascularized within 4-7 days in response to angiogenic substances. Pellets are removed at the end of the experiment, photographed, minced, and diluted in water to measure the hemoglobin content with a Drabkin reagent kit (Sigma-Aldrich, St. Louis, MO, USA)[14,16].

Measurement of cytokines and angiogenic factors

Before starting the treatment, peripheral blood from 20 mice samples were collected into EDTA-containing tubes before treatment initiation and one day before mice were sacrificed. Plasma was separated by centrifugation (2,000 rpm for 20 min at 4 °C) within 1 h from blood drawing and aliquoted into multiple cryovials. Plasma samples were stored at − 80 °C until use. Before analysis,

plasma samples were thawed slowly in an ice bath and all analyses were done from a one-off thaw sample. Cytokine and angiogenic factor (CAFs) were measured by using Q-Plex™ Array Human Angiogenesis Antigen (Quansys Biosciences, Logan, Utah) allowing the simultaneous quantification of the following factors: angiopoietin-2 (ANG-2), fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF), interleukin-8 (IL-8), platelet-derived growth factor-BB (PDGF-BB), tissue inhibitor of matrix metalloproteinase-1 and 2 (TIMP-1, TIMP-2), tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGFA), according to the manufacturer's instructions. Secreted levels of CAFs were quantified through Q-View Software (Quansys Biosciences, Logan, Utah) in triplicate samples, and the mean results were used in biomarker analysis.

Human angiogenesis array

MMECs were cultured in SFM with or without 100 ng/ml sJAM-A for 24 hours and media were collected and concentrated to be analyzed by Human Angiogenesis Array kit (R&D Systems®, Minneapolis, Minnesota, USA) according to the manufacturer's instructions. Spots were quantified with ImageJ 5.1 Software (Bio-Rad) and values were reported as mean pixel density.

***In silico* analysis**

For further validation in a larger NDMM patient cohort, we interrogated the public data set from the CoMMpass study. We considered two patient subgroups, relapsed/progressed or died *versus* ongoing for PFS and died *versus* alive for OS. In detail, a supervised analysis was performed including 125 genes known to be prognostically relevant in MM, either due to GEP phenotyping[17] or angiogenetic pathway related signature.[18]

The public data set from the CoMMpass[19] longitudinal, prospective observational study (release IA12) was interrogated, provided by the Multiple Myeloma Research Foundation and downloaded from (https://research.mmrf.org). RNAseq data from 646 NDMM patients was analyzed and the cohort stratified depending on the outcome (progression-free survival - PFS and overall survival - OS *status*). Expression profiles in patients were compared who progressed or died in comparison with patients who did not. The dataset interrogation and the relative clinical information analysis were generated as part of the Multiple Myeloma Research Foundation Personalized Medicine Initiative.

Statistical analysis

The compiled clinical data forms, together with the recoded variables, were inserted into a database built with Office Excel software and analyzed with Stata SE14 software. The median value of the variable obtained in FACS of the mean fluorescence intensity (MFI) for the expression of the JAM-A surface was used as a cutting point to recode the latter as a categorical variable (JAM-$A^{high/low}$):

the highest values equal to the median of 989 were classified as JAM-A^{high}, the lowest values as JAM-A^{low}.

Kaplan–Meier curves were used to show progression-free survival (PFS) and overall survival (OS) related to higher levels of surface JAM-A expression, and log-rank were determined to evaluate the differences. PFS refers to the time elapsed from the date of enrollment in this study and the date of relapse, disease progression or death, determined from the last follow-up visit. OS refers to the time elapsed from the date of enrollment in this study and date of death from any cause. Given the population characteristics for NDMM cohort, the scanty number of failure events we could not perform a multivariate analysis for OS. Despite the significant impact on PFS at univariate analysis none of the covariates maintained a relevant effect in the implemented multivariate model (data not shown).

For RRMM cohort univariate and multivariate Cox proportional hazard models were performed to detect significant predictors (covariates chosen on the basis of the statistical significance (univariate analysis, $P \leq .05$) and of the clinical judgment; age and sex were used as adjusting variables) for OS and PFS in the patient cohort. The collinearity between the nominal covariates was tested using the Cramér's V measure. For the covariates that did not satisfy the hypothesis of proportional hazards we proceeded with stratified Cox model (with interaction and with no interaction including the Likelihood Ratio Test used to estimate model parameters).

In vitro, *in embrio* and *in vivo* experimental results were expressed as individual data or as the mean ± SD and analyzed using Wilcoxon signed-rank test, Mann-Whitney U test and One-Way Anova test. Statistical analysis was performed using GraphPad Prism6 software (La Jolla, CA, USA). Analysis performed on the *in silico* data was conducted using t-test and fold change.

For *in vivo* experiments, sample size was calculated using G*Power software version 3.1.9.2 (power of 80% and 0.05 statistical level). Assuming an effect-size of 0.4 with statistical significance of $\alpha < .05$ and a power of 80%. Values <.05 deemed as statistically significant. Statistical analyses were performed with STATA/SE for Windows, version 15.

Supplementary Tables

Supplementary Table 1.

Patients characteristics, NDMM cohort.

Variable	N. patients (%)	Median Values
Median Age	111/111 (100)	67 years (60 - 73)
Sex	111/111 (100)	
Male:	70/111 (63)	
Female:	41/111 (37)	
R-ISS	111/111 (100)	
Stage I	23/111 (20.7)	
Stage II	65/111 (58.6)	
Stage III	23/111 (20.7)	
Type of MM	104/111 (93.7)	
IgG	64/104 (61.5)	
IgA	27/104 (26)	
IgM	1/104 (1)	
Light chain	12/104 (11.5)	
Genetic risk*:	106/111 (95)	
Standard risk:	57/106 (53.8)	
High risk:	49/106 (46.2)	
Hemoglobin	110/111 (99)	10.2 g/dL (9 – 11.8)
Hb <10 g/dL	51/110 (46.3)	
Kidney failure	107/111 (96.3)	
Yes:	49/107 (45.8)	
No:	58/107 (54.2)	
Bone lesions	111/111 (100)	
Yes:	78/111 (70.3)	
No:	33/111 (29.7)	

NDMM: newly diagnosed MM; R-ISS: Revised-international staging system.

*Evaluated in patients with appropriate genetic risk assessed according to Sonneveld P, *et al.*,[20] when appropriate material was available.[19]

Supplementary Table 2.

Patients characteristics, RRMM cohort.

Variable	N. patients (%)	Median Values
Median Age	201/201 (100)	62 years (54 - 69)
Sex	201/201 (100)	
Male:	129/201 (64.2)	
Female:	72/201 (35.8)	
R-ISS	201/201 (100)	
Stage I	61/201 (30.3)	
Stage II	117/201 (58.2)	
Stage III	23/201 (11.5)	
Type of MM	201/201 (100)	
IgG	124/201 (61.7)	
IgA	58/201 (28.8)	
IgM	3/201 (1.5)	
IgD	2/201 (1)	
Light chain	13/201 (6.5)	
Biclonal	1/201 (0.5)	
Genetic risk*:	183/201 (91)	
Standard risk:	115/201 (62.8)	
High risk:	68/201 (37.2)	
Hemoglobin	201/201 (100)	10.2 g/dL (9.7 – 12.8)
Hb <10 g/dL	55/201 (27.4)	
Kidney failure	201/201 (100)	
Yes:	64/201 (31.8)	
No:	137/201 (68.2)	
Bone lesions	201/201 (100)	
Yes:	131/201 (65.2)	
No:	70/201 (34.8)	
Extra medullary disease	201/201(100)	
Yes:	66 (32.8)	
No:	135 (67.2)	
PD at sampling	105/201 (52.2)	
Previous transplant	201/201 (100)	
Yes:	149/201 (74.1)	

No:	52/201 (25.9)	
IMiDs resistant	201/201 (100)	
Yes:	97/201 (48.3)	
No:	104/201 (51.7)	
PI resistant	201/201 (100)	
Yes:	89/201 (44.3)	
No:	112/201 (55.7)	
Previous Immunotherapy	16/201 (7.9)	

RRMM: relapsed/refractory MM; ISS: international staging system; PD: progressive disease; R-ISS: Revised-international staging system; IMiDs: immunomodulatory drugs; PI: proteasome inhibitor.
*Evaluated in patients with appropriate genetic risk according to Sonneveld P, *et al.*[20] when appropriate material was available[21]. PD: progressive disease, as defined by Rajikumar *et al.*[5]

Supplementary Table 3. Gene Expression profile summary comparison between survival characteristics from 646 patients divided in high- and low-expressers from CoMMpass longitudinal, prospective observational study (release IA12).

	PFS		OS	
Gene	**t-test; *P* value**	**Fold change**	**t-test; *P* value**	**Fold change**
ENO-1	5.97; <.0001	1.39	4.57 <.0001	1.43
AURKA	5.31; <.0001	1.87	4.11 <.0001	2
VEGFA	4.63; <.0001	1.31	2.43 .02	1.2
JAM-A	2.29; 0.02	1.13	2.64; <.01	1.2
TJP1	ns	ns	-2.59; <.01	0.8

PFS: progression free survival; OS: overall survival. ENO1: Enolase 1; AURKA: Aurora Kinase A; VEGFA: Vascular Endothelial Growth Factor A; JAM-A: Junctional adhesion molecule-A; TJP1: Tight junction protein-1. ns: not significant.

Supplementary Table 4. Primer sequences.

Oligo Name	Sequence 5' to 3'
AURKA f	GAAATTGGTCGCCCTC
AURKA r	TGATGAATTTGCTGTGATCC
CASK f	ATTCTCCATATCCATGTCTCCG
CASK r	TGGAAGAAATTTCATGTTACCC
ENO-1 f	GCCTCCTGCTCAAAGTCAAC
ENO-1 r	AACGATGAGACACCATGACG
F11R f	AAGTTGTCCTGTGCCTACTC
F11R r	ACCAGTTGGCAAGAAGGTCACC
LFA1 f	CACGAAGTTCAAGGTCAGCA
LFA1 r	TTGTGGTCTTCCTGGGTTTC
MLLT4 f	GCCAAGTGACAAAGGGAT
MLLT4 r	TAACTGAAGGCGGTAAAG
TJP1 f	TGCCTCCGAGAGAGATGACA
TJP1 r	CGCCAGCCACAAATATTCCG

AURKA: Aurora Kinase A; LFA-1: Lymphocyte function-associated antigen 1; CASK: Calcium/Calmodulin Dependent Serine Protein Kinase; ENO1: Enolase 1; F11R, alias for JAM-A, Junctional adhesion molecule-A; LFA-1: Lymphocyte function-associated antigen 1; MLLT4, alias for Afadin, Adherens Junction Formation Factor TJP1: Tight junction protein-1.

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Supplementary Figure legends

Supplementary Figure 1

JAM-A is overexpressed in MM derived endothelial cells. (A) Basal mRNA expression level of JAM-A have been detected on MMECs by real time RT-PCR. The mean of JAM-A mRNA expression levels in MGECs or in HUVECs were used as reference values and GAPDH as housekeeping gene. **(B)** Absolute JAM-A expression across the entire cohort measured with flow cytometry and displayed as mean fluorescence intensity (MFI) values. **(C)** Anti-CD34 and anti-JAM-A staining on a representative MGUS BM sample (compare to Figure 1D and main text for details). Magnification x 200, Scale bar=50 μm. **(D)** Anti-CD31/anti-JAM-A double immunohystochemistry staining on BM trephine: within the control BM lacunae (see $CD31^+$ megakaryocytes) the vessels are more distended and endothelia display a thin, uncospicuous cytoplasmic rim. CD31 (brown) highlights endothelia lining thin walled microvessels. Lumina appear to be only slightly dilated. Anti-JAM-A (in red) stains a fraction of neoplastic plasma cells, with a cytoplasmatic pattern. Magnification x 200. Scale bar = 50 μm. Red insert: magnification x 2. BM: bone marrow; MMECs: BM primary MM endothelial cells; MGECs: MGUS derived endothelial cells; MFI: mean fluorescence intensity.

Supplementary Figure 2

Primary MM derived endothelial cells enhance sJAM-A levels and JAM-A-expression on MM after direct or indirect coculture. Experimental designs depicted on the left. **(A)** MMECs directly co-cultured with RPMI-8226 cells at 1:5 ratio (RPMI8266:MMECs) or cultured alone for 24hrs. After removing tumor cells, MMECs were harvested and JAM-A post transcription level quantified using western blotting for RPMI-8226 cells. **(B)** MMECs cells were cultured alone or co-cultured with RPMI-8226 at 1:5 ratio (RPMI-8266:MMECs) directly co-cultured and MMECs cells analyzed for JAM-A expression with flow cytometry.

(**C**) sJAM-A concentration was measured in MMECs CM with ELISA. Values represent mean ± SD. n = 12 for each group. sJAM-A concentration was measured in MM CM by ELISA. Values represent mean ± SD. n = 12 for each group; cells were directly co-cultured with RPMI-8226 cells at 1:5 ratio (RPMI8266:MMECs) or cultured alone for 24hrs. Co-culture medium and medium of MMECs cultured alone were collected and sJAM-A concentration measured by ELISA. **(D)** OPM-2 cells were cultured alone or directly co-cultured with MMECs at 1:5 ratio (OPM-2:MMECs) and analyzed for JAM-A expression by WB. Results are presented as mean ± SD (n=24 NDMM derived MMECs), Mann-Whitney test. *** $P< .001$; **** $P< .0001$. MMECs: bone marrow primary MM endothelial cells; CM: conditioned medium; NDMM: newly diagnosed MM.

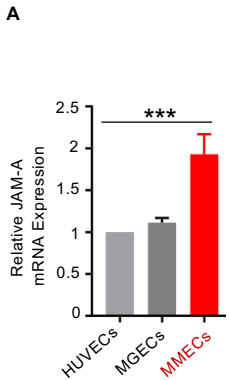
Supplementary Figure 3

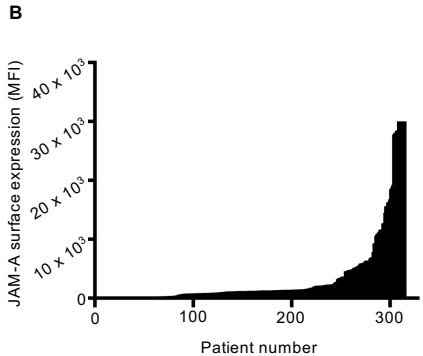
JAM-A neutralization impair MM endothelial cells function, without affecting MMECs viability. (A) Lack of anti-JAM-A-dependent induction of cytotoxicity against the MMECs measured with flow cytometry (Vivid, left panel). Cell viability did not differ in terms of % living cells from nine independent experiments, t-Student test. **(B)** Array of 55 human angiogenesis related proteins (left), and protein list analyzed. Relative mRNA expression level of PLG, ENO-1, FGF2, VEGFA, LFA-1, TJP1, CAV1, CASK, ADAM17, AURKA **(C-L)** were investigated comparing MMECs to MGECs with real time RT-PCR to validate the proteome profiling results, (n=24 MGUS patients derived MGECs and 24 NDMM derived MMECs). Results are presented as mean ± SD, Mann-Whitney test. **** $P< .0001$. The mean of mRNA expression levels of JAM-A in HUVECs was used as reference sample and GAPDH as housekeeping gene. Graph compares the mRNA expression of each ligand between MGECs and MMECs and it shows the most expressed ligand in each cell type setting with the lower as unit. MMECs: bone marrow primary MM endothelial cells; PLG:

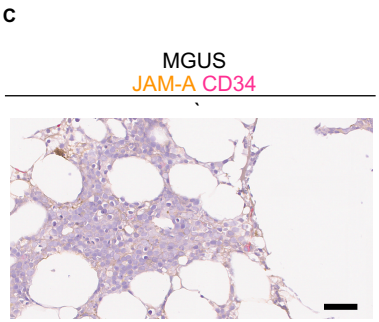
plasminogen; ENO1: Enolase 1; FGF-2: Fibroblast growth factor-2; VEGFA: Vascular Endothelial Growth Factor A; LFA-1: Lymphocyte function-associated antigen 1; TJP1: Tight junction protein-1; CAV1: Caveolin1; CASK: Calcium/Calmodulin Dependent Serine Protein Kinase; ADAM17: ADAM metallopeptidase domain 17; AURKA: Aurora Kinase A; MGECs: MGUS derived endothelial cells; NDMM: newly diagnosed MM. See results and discussion for additional details.

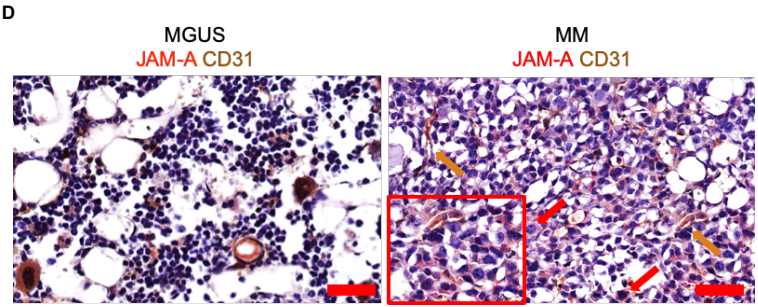
Supplementary Figure 4

Effect of anti-JAM-A treatment on systemic sJAM-A, FGF2 and VEGF levels. (A) Representative extracted tumor masses after treatment from subcutaneous tumor xenograft model. **(B)** s-JAM-A (297,6 ± 15,57 and 71.89 ± 9,24 in the ISO Control and in the α-JAM-A-treated group respectively, t-student test, *P*< .0001)**, (C)** FGF-2 (median 1500 and 253.89 in the ISO Control and in the α-JAM-A-treated group respectively, Mann-Whitney test, *P*< .0001) and **(D)** VEGF-A (42470 ± 2694 and 4070 ± 815,6 in the ISO Control and in the α-JAM-A-treated group respectively, t-student test, *P*< .0001)**,** significantly decreased in MM bearing mice after anti-JAM-A blocking antibody treatment, evaluated with ELISA. **** *P*< .0001. For one mouse there was not sufficient biological material for ELISA test available. FGF-2: Fibroblast growth factor-2; VEGFA: Vascular Endothelial Growth Factor A; ISO: isotype control.

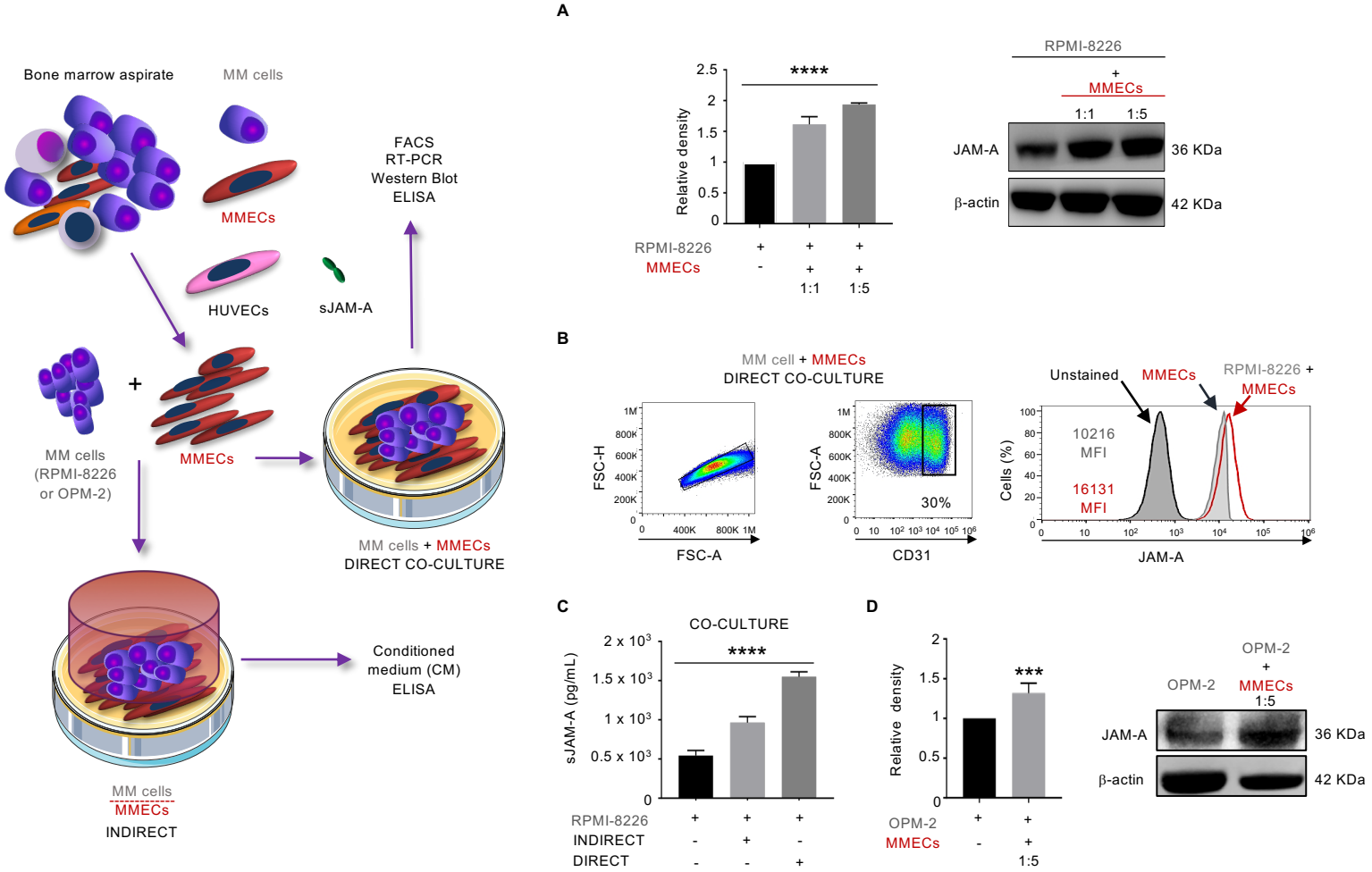




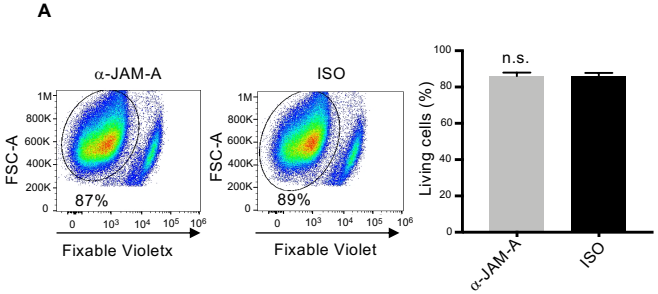


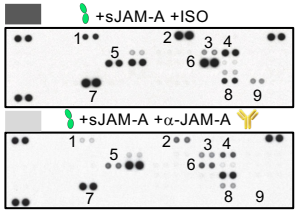


Supplementary Figure 1

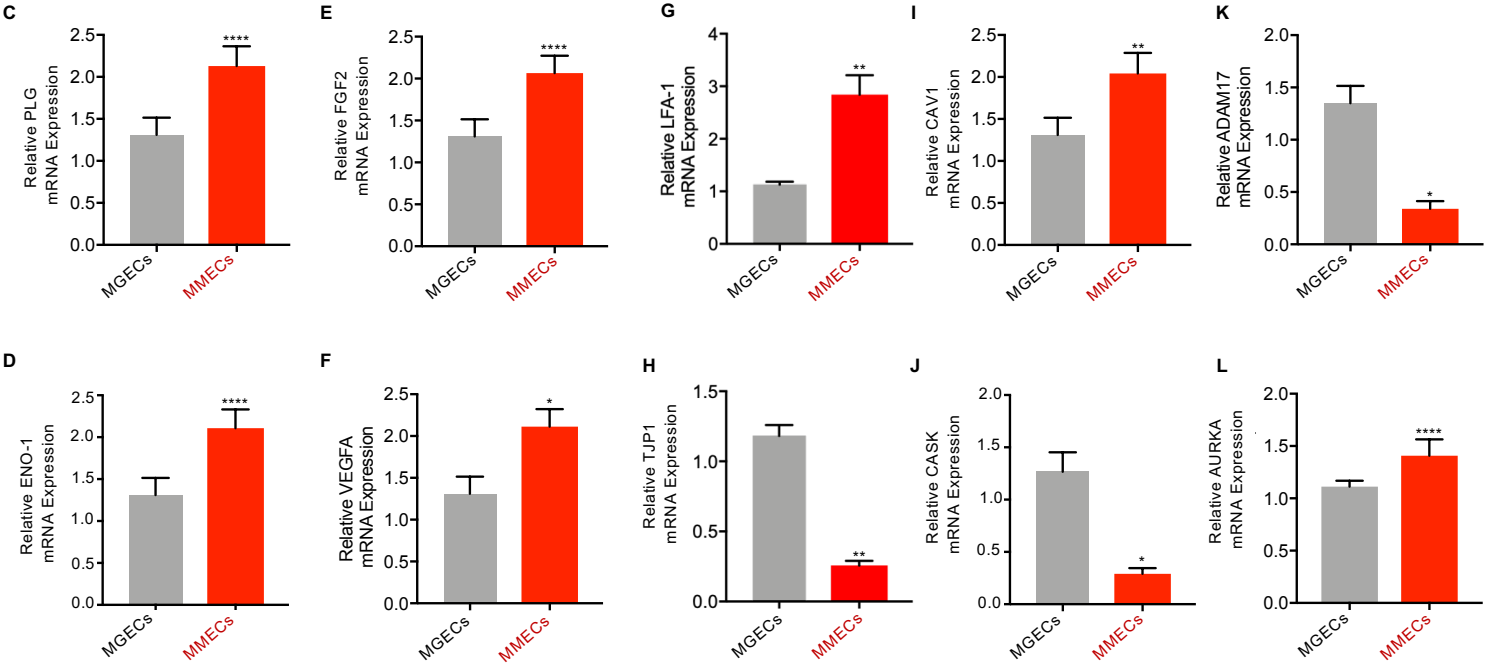


Supplementary Figure 2

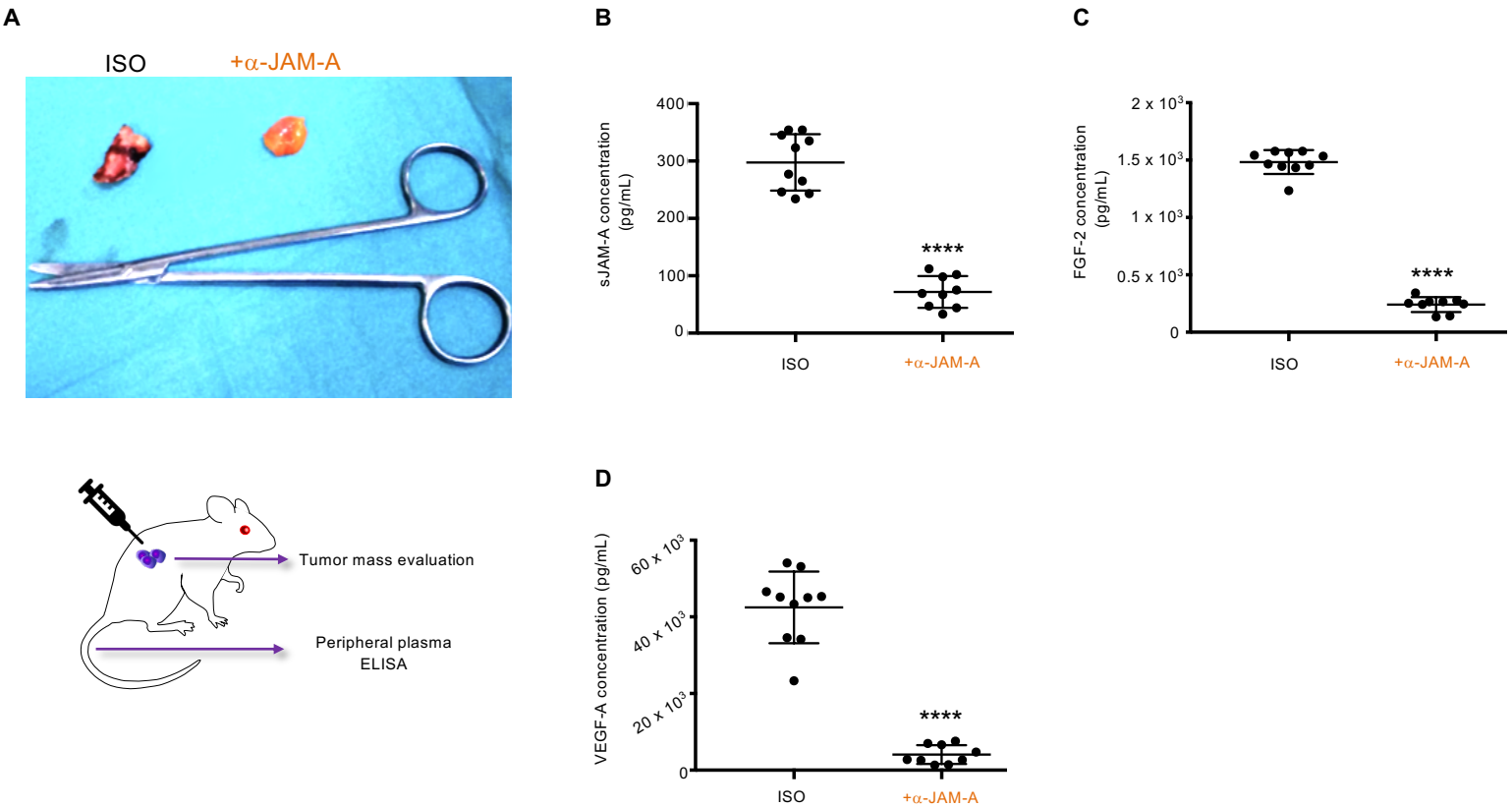




Protein	Information: Alternate Nomenclature	Spot position
ADAMTS-1	Human Metalloproteinase with Thrombospondin Type 1 Motifs	1
PLG	Plasmninogen	2
FGF	Fibroblast growth factor	3
FGF-2	Fibroblast growth factor basic	4
IGFBP1	Insulin Like Growth Factor Binding Protein 1	5
IL8	CXCL8	6
TIMP1	TIMP Metallopeptidase Inhibitor 1	7
VEGFA	Vascular Endothelial Growth Factor A	8
VEGFC	Vascular Endothelial Growth Factor C	9



Supplementary Figure 3



Supplementary Figure 4