Interactions of malignant multiple myeloma (MM) plasma cells with the microenvironment control MM plasma-cell growth, survival, drug-resistance and dissemination. As microvascular density increases in the bone marrow in MM, we investigated whether bone marrow MM endothelial cells control disease progression via the junctional adhesion molecule-A (JAM-A). Membrane and cytoplasmic JAM-A levels were upregulated in MM endothelial cells in 111 patients with newly diagnosed MM and in 201 with relapsed/refractory MM compared to the levels in patients with monoclonal gammopathy of undetermined significance and healthy controls. Elevated membrane expression of JAM-A on MM endothelial cells predicted poor clinical outcome. Mechanistically, addition of recombinant JAM-A to MM endothelial cells increased angiogenesis, whereas inhibition of this adhesion molecule impaired angiogenesis and MM growth in two-dimensional and three-dimensional in vitro cell cultures and chorioallantoic membrane assays. To corroborate these findings, we treated MM-bearing mice with a JAM-A-blocking monoclonal antibody and demonstrated impaired MM progression, corresponding to decreased MM-related vascularity. These findings support the concept that JAM-A is an important mediator of MM progression through facilitating MM-associated angiogenesis. Elevated interactions of malignant multiple myeloma (MM) plasma cells with the microenvironment control MM plasma-cell growth, survival, drug-resistance and dissemination. As microvascular density increases in the bone marrow in MM, we investigated whether bone marrow MM endothelial cells control disease progression via the junctional adhesion molecule-A (JAM-A). Membrane and cytoplasmic JAM-A levels were upregulated in MM endothelial cells in 111 patients with newly diagnosed MM and in 201 with relapsed/refractory MM compared to the levels in patients with monoclonal gammopathy of undetermined significance and healthy controls. Elevated membrane expression of JAM-A on MM endothelial cells predicted poor clinical outcome. Mechanistically, addition of recombinant JAM-A to MM endothelial cells increased angiogenesis, whereas inhibition of this adhesion molecule impaired angiogenesis and MM growth in two-dimensional and three-dimensional in vitro cell cultures and chorioallantoic membrane assays. To corroborate these findings, we treated MM-bearing mice with a JAM-A-blocking monoclonal antibody and demonstrated impaired MM progression, corresponding to decreased MM-related vascularity. These findings support the concept that JAM-A is an important mediator of MM progression through facilitating MM-associated angiogenesis. Elevated
JAM-A expression on bone marrow endothelial cells is an independent prognostic factor for the survival of both patients with newly diagnosed MM and those with relapsed/refractory MM. Blocking JAM-A restricts angiogenesis in vitro, in utero and in vivo and represents a suitable druggable molecule to halt neo-angiogenesis and MM progression.

**Introduction**

Junctional adhesion molecule-A (JAM-A), also known as JAM-1, CD321, and F11R, belongs to the immunoglobulin superfamily. In healthy tissues, JAM-A regulates cell growth and differentiation, while its aberrant expression or deregulation confers a more aggressive phenotype with poor prognosis in different types of human cancers, including multiple myeloma (MM), breast, lung, brain, and head and neck cancers. Overactivation of JAM-A results either from upregulation or aberrant dimerization, driving the receptor in a state of constitutive signal transmission, or from excessive release of JAM-A ligands by normal and tumor cells into the microenvironment. Membrane-bound JAM-A and its soluble form (sJAM-A) can form homophilic interactions and also heterophilic interactions with lymphocyte function-associated antigen 1 (LFA-1), afadin (AFDN), calcium/calmodulin-dependent serine protein kinase (CASK) and tight junction protein-1 (TJP1) with high receptor/ligand binding affinities. These interactions trigger JAM-A downstream signaling pathways involved in the regulation of tumor cell survival, growth, angiogenesis and dissemination.

JAM-A inhibition can be achieved directly by blocking the ligand-binding site on the extracellular receptor domain with monoclonal antibodies and indirectly with small-molecule inhibitors. Moreover, neutralizing the sJAM-A released into the microenvironment can prevent JAM-A activation. JAM-A plays a pivotal role in endothelial cell physiology and pathology. Although the function of JAM-A in tumorigenesis has been investigated in solid tumors, and its angiogenic role has been shown in pancreatic islet carcinoma, data on JAM-A-related angiogenesis in hematologic neoplasms remain elusive. Since bone marrow (BM) neovascularization favors the progression of MM, we investigated whether JAM-A can drive angiogenesis in MM, contributing to progression of the disease.

We quantified JAM-A surface expression on BM-derived endothelial cells (MMEC) from 312 patients with MM and demonstrated that JAM-A expression in MMEC correlates strongly with poor survival both in newly diagnosed (NDMM) and relapsed/refractory (RRMM) patients. Mechanistically, adding recombinant JAM-A protein to MM plasma cells (MM-cells) increased angiogenesis in both two-dimensional (2D) and three-dimensional (3D) models. Conversely, blocking JAM-A impaired MM-related angiogenesis. To corroborate these findings, we treated MM-bearing mice with JAM-A-blocking monoclonal antibodies and observed impaired MM progression and decreased MM vascularity.

**Methods**

**Patients**

Patients fulfilling the International Myeloma Working Group diagnostic criteria for NDMM (n=111), patients with RRMM (n=201) and subjects with monoclonal gammopathy of undetermined significance (MGUS) (n=35) were included in this study. The patients’ characteristics and genetic risk stratification are provided in Online Supplementary Tables S4 and S5. The study was approved by the Ethical Committees of Bari and Würzburg University Hospitals (reference numbers 5145 and 76/13), and all patients provided informed consent to participation in the study, in accordance with the Declaration of Helsinki (details are given in the Online Supplementary Methods).

**Cell lines and cultures procedures**

RFMI-8226, OFM-2 and human umbilical vein endothelial cells were cultured as described elsewhere. MM-cells were co-cultured with MMEC (4x10^5) at 1:1 and 1:5 cell ratios for 24 hours (h) with or without an inserted transwell (0.4 μm pore size; Costar, Cambridge, MA, USA). Details are provided in the Online Supplementary Methods.

**Chick chorioallantoic membrane assay**

Fertilized chicken eggs were incubated at 37°C at a constant humidity. On day 8, sterilized gelatin sponges imbued with MMEC conditioned medium or medium obtained by treatment of MMEC with sJAM-A (100 ng/mL), with or without anti-JAM-A monoclonal antibodies were implanted on the top of the chick chorioallantoic membrane (CAM) as described in more detail in the Online Supplementary Methods.

**Multiple myeloma xenograft mouse models**

Twenty female 8- to 10-week-old NOD.CB17-Prkdcscid/NGHsd mice (NOD-SCID; Envigo, Huntingdon, UK) were injected intratubially with 2x10^5 RFMI-8226 cells suspended in phosphate-buffered saline. Mice were treated with the anti-JAM-A monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA, mouse monoclonal clone J10.4) recognizing the distal membrane extracellular domain of JAM-A. Twenty female 6- to 8-week-old NOD-SCID mice were injected subcutaneously (s.c.), into the right flank, with 1x10^7 RFMI-8226 cells suspended in 200 μL RPMI-1640 medium and 200 μL Matrigel™ as described previously and detailed in the Online Supplementary Methods.

**Functional in vitro assays**

Wound-healing and Matrigel™ angiogenesis assays were performed as previously described and detailed in the Online Supplementary Methods.

**Protein expression studies and polymerase chain reaction analyses**

Western blots, enzyme-linked immunosorbent assays, human angiogenesis array and real-time reverse transcriptase polymerase chain reactions were performed according to the manufacturers’ instructions (detailed in the Online Supplementary Methods).

**Immunohistochemistry and in silico analysis**

Details of the immunohistochemical studies and the in silico analysis, using the CoMMpass study dataset, are supplied in the Online Supplementary Methods.
Statistical analysis
A descriptive analysis was carried out using the median and interquartile range for the quantitative variables and percentage values for the qualitative ones. The normality of the distribution of data was tested using the Shapiro-Wilk test. The levels of JAM-A expression on membrane MMEC, determined by mean fluorescence intensity (MFI) in fluorescence activated cell sorting (FACS), were dichotomized into two classes, JAM-Ahigh and JAM-Alow, choosing the median as the class boundary (Online Supplementary Methods). Moreover, for further confirmation, survival was analyzed using a model based on quartile ranges (Online Supplementary Methods).

Results
Elevated JAM-A expression on bone marrow primary multiple myeloma endothelial cells correlates with poor prognosis in both newly diagnosed and relapsed/refractory multiple myeloma
First, we compared JAM-A expression in MMEC and MGUS-derived endothelial cells (MGEC) (Figure 1A). JAM-A mRNA expression in MMEC significantly exceeded JAM-A levels in MGEC (1.8-fold difference, \( P < 0.0001 \)) and in endothelial cells from healthy subjects (Online Supplementary Figure S1A). Subsequent western blot analy-
sis confirmed that JAM-A protein expression was significantly upregulated in MMEC in comparison to the levels in MGEC ($P<0.0001$) (Figure 1B).

Because JAM-A had been previously proven to be a prominent adhesion molecule on MM cells, we investigated whether JAM-A expression in the vascular microenvironment affects disease outcome. To this end, we enrolled 312 patients, 111 with NDMM and 201 with RRMM. Employing flow cytometry on MMEC we divided the patients with NDMM on the basis of JAM-A$^{\text{high}}$ and JAM-A$^{\text{low}}$ MMEC surface expression (Figure 1C). Immunohistochemical analyses of BM trephines corroborated the findings (Figure 1D, Online Supplementary Figure S1B and C). Notably, overall survival was significantly shorter in patients with JAM-A$^{\text{high}}$ MMEC than in patients with JAM-A$^{\text{low}}$ MMEC: median not reached versus 78 months, respectively (hazards ratio [HR]=9.14, 95% confidence interval [95% CI]: 2.80-29.76; $P<0.001$; $\chi^2_{LR}=20.11$; $P<0.0001$, upper panel). Uni- and multivariate analysis (lower panel). BM: bone marrow; MMEC: endothelial cells from patients with multiple myeloma; MGEC: endothelial cells derived from patients with monoclonal gammopathy of undetermined significance; NDMM: newly diagnosed multiple myeloma; Pts: patients; OS: overall survival; NR: not reached. R-ISS: Revised International Staging System; Hb: hemoglobin.

Next, we performed flow cytometry on samples from a cohort of 201 patients with RRMM. Within these patients, JAM-A$^{\text{high}}$ MMEC represented an independent poor prognostic factor for both overall survival and progression-free survival (Figure 2A and B). Survival differed significantly in patients according to the level of JAM-A expression on

**Cox model for Overall Survival**

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<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<td>HR (95%CI)</td>
<td>P value</td>
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<td>MMECs JAM-A surface expression (JAM-A$^{\text{high}}$ vs JAM-A$^{\text{low}}$)</td>
<td>9.14 (2.80-29.76)</td>
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<tr>
<td></td>
<td>9.11 (2.79-29.76)</td>
<td>$&lt;0.001$</td>
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<tr>
<td>Bone Lesion (Yes vs No)</td>
<td>1.31 (0.60-2.89)</td>
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<tr>
<td>Hb (&lt;10 vs ≥10 g/dL)</td>
<td>1.44 (0.75-2.77)</td>
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<td>R-ISS</td>
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<tr>
<td>R-Stage I</td>
<td>1.37 (0.55-3.39)</td>
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<tr>
<td>R-Stage II</td>
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Figure 1. (continued from the previous page). (E) Kaplan-Meier estimator of OS, by level of surface MMEC JAM-A expression. The median OS estimated in subjects with JAM-A$^{\text{high}}$ MMEC cells at FACS was not reached whereas in subjects with JAM-A$^{\text{low}}$ MMEC, the median OS was 78 months (HR=9.14, 95% CI: 2.8-29.76; $P<0.0001$; $\chi^2_{LR}=20.11$; $P<0.0001$, upper panel). Uni- and multivariate analysis (lower panel). BM: bone marrow; MMEC: endothelial cells from patients with multiple myeloma; MGEC: endothelial cells derived from patients with monoclonal gammopathy of undetermined significance; NDMM: newly diagnosed multiple myeloma; Pts: patients; OS: overall survival; NR: not reached. R-ISS: Revised International Staging System; Hb: hemoglobin.
MMEC. The median overall survival was 130 months in patients with JAM-A<sup>high</sup> MMEC and was not reached in those with JAM-A<sup>low</sup> MMEC (HR=2.96, 95% CI: 1.36–6.37, \( P < 0.006; \chi^2_{LR} = 8.52; \ P = 0.0035 \)) (Figure 2A).

In patients with JAM-A<sup>low</sup> MMEC cells, the estimated median progression-free survival was 27 months, while, in subjects with JAM-A<sup>high</sup> MMEC the median progression-free survival was only 18.3 months (HR=1.41, 95% CI: 1.05-1.88; \( P = 0.019; \chi^2_{LR} = 5.78; \ P = 0.0162 \)) (Figure 2B).

Multivariate analyses confirmed that JAM-A<sup>high</sup> MMEC was an independent significant risk factor for both overall survival (HR=1.41, 95% CI: 1.05-1.88; \( P = 0.019; \chi^2_{LR} = 5.78; \ P = 0.0162 \)) (Figure 2C).

Interestingly, only JAM-A<sup>high</sup> MMEC remained significant in the multivariate model. Moreover, we found a significant association in the setting of RRMM between JAM-A<sup>high</sup> MMEC and R-ISS stage II and III disease (\( \chi^2 = 7.04, \ P = 0.008 \)). Thus, JAM-A surface expression on BM endothelial cells derived from MM patients exerted a strong and independent effect, with a linear trajectory, on overall survival in both cohorts and an additive poor prognostic impact on progression-free survival in the RRMM cohort. Additionally, we divided MMEC JAM-A surface expression of the entire cohort (Online Supplementary Figure S1D) into quartiles (JAM-A<sup>D1</sup> to JAM-A<sup>D4</sup>) and then compared the outcomes of patients in the lowest quartile (JAM-A<sup>D4</sup>) to those of patients in the highest quartile (JAM-A<sup>D1</sup>), and then compared the outcomes of patients in the lowest quartile (JAM-A<sup>D4</sup>) to those of patients in the highest quartile (JAM-A<sup>D1</sup>). Strikingly, overall survival differed significantly: the median overall survival was 88 months in JAM-A<sup>D4</sup> patients and was not reached in JAM-A<sup>D1</sup> patients (HR=8.24, 95% CI: 3.2-20.9, \( P < 0.0001; \chi^2_{LR} = 28.15; \ P < 0.0001 \)). Interestingly, JAM-A<sup>D4</sup> MMEC maintained significance in the multivariate Cox-model (HR=6.36, 95% CI: 2.30-17.63; \( P < 0.001 \)). This comparison further corroborated the role of JAM-A-positive vs. JAM-A-negative MMEC in predicting poor clinical outcome in our cohort (Online Supplementary Figure S1E, upper and lower panels). The absence of a statistically significant impact on progression-free survival of patients with NDMM is likely due to a more pronounced effect of JAM-A MMEC expression in more advanced stages of MM. This suggests the importance of JAM-A within the BM microenvironment during disease progression.
Figure 3. Bone marrow primary multiple myeloma endothelial cells enhance JAM-A expression on multiple myeloma cells. (A, B) Experimental design depicted at the top. RPMI-8226 cells were cultured alone or co-cultured with MMEC at a 1:5 ratio (RPMI-8226:MMEC) in inserted transwells and analyzed for JAM-A expression by western blotting (A) and flow cytometry (B). (C) Experimental design shown at top; RPMI-8226 cells were maintained for 24 h in CM from MMEC or MGEC. Cells were harvested and lysed and the extracted proteins immunoblotted for JAM-A expression. Overall densitometric analyses are reported. (D) RPMI-8226 cells were also analyzed by FACS after culture for 24 h in MGEC or MMEC CM. Results are presented as mean ± standard deviation (MGEC from 24 patients with MGUS; MMEC from 24 patients with NDMM). **P < 0.0001, Mann-Whitney test. BM: bone marrow; MMEC: endothelial cells from patients with multiple myeloma; MGEC: endothelial cells derived from patients with monoclonal gammopathy of undetermined significance; NDMM: newly diagnosed MM; CM: conditioned medium; MFI: mean fluorescence intensity.
Figure 4. Legend on next page.
Multiple myeloma endothelial cells enhance JAM-A expression on multiple myeloma plasma cells

To address how interactions with MMEC functionally influence MM-cell biology, we performed indirect and direct co-culture experiments of MMEC with MM-cell lines. JAM-A expression levels increased on MM-cells when co-cultured with MMEC (Figure 3A and B). We next exposed MM-cells to primary MMEC- or MGEC-derived culture media. JAM-A protein expression was higher on MM-cells after exposure to MMEC CM alone (CTRL) or previously supplemented for 12 h with sJAM-A at 100 ng/mL and treated with isotype control (+sJAM-A +ISO) or anti-JAM-A (+sJAM-A +α-JAM-A) blocking antibody. Lower panel: Migrating cells in each wound were counted. Counts of proliferating and migrating cells of six independent experiments. ***P<0.0001, Mann-Whitney test. (B) Photographs at 3 hours of newly-formed capillary networks after MMEC were seeded on a Matrigel® layer. Direct comparison of MMEC in CM vs. MWE treated with 100 ng/mL sJAM-A (upper left panel). Independent experiment to assess JAM-A inhibition in MMEC treated with an anti-JAM-A blocking antibody or isotype control antibody (upper right panel). Independent experiment to assess JAM-A knock-down in MMEC comparing treatment with JAM-A specific siRNA vs. non-specific scrambled siRNA without addition of sJAM-A (lower left panel). Independent experiment to assess the effect of blocking JAM-A after addition of 100 ng/mL of sJAM-A by comparing capillary formation after MMEC treatment with sJAM-A and anti-JAM-A blocking antibody vs. sJAM-A and isotype control antibody (lower right quadrant). Representative pictures of three biological replicates. Skeletonization of the meshes were analyzed and branching points measured. Data are normalized to control. Scale bar=100 μm. (C) Chorioallantoic membrane assay with the gelatin sponge loaded with MMEC CM alone (CTRL) or with MMEC CM supplemented with sJAM-A (+sJAM-A), in the presence or absence of 0.5 μg/mL anti-JAM-A monoclonal antibody. On day 12, pictures were taken in ovo. One representative experiment is shown at 50X magnification. Newly formed vessels were counted. Mann-Whitney test. (D) An array of 55 human angiogenesis-related proteins was performed on MMEC CM after sJAM-A treatment without and with blocking with the anti-JAM-A monoclonal antibody. Array spots were analyzed with ImageJ Lab v. 1.51 software and normalized to positive control signal intensities. Graph bars represent the pixel density of the detected angiogenesis-related cytokines in two independent experiments. Values are expressed as mean ± standard deviation of ten independent experiments. Mann-Whitney test. *P<0.05; ***P<0.0001, versus SFM as control. See Online Supplementary Figure S3 and the main text for more details. MMEC: bone marrow primary multiple myeloma endothelial cells; α-JAM-A: monoclonal antibody against JAM-A; sJAM-A: soluble JAM-A; CM: conditioned medium; CTRL: control; SFM: serum-free medium; n.s.: not significant. CAM: chick chorioallantoic membrane. ADAMTS1: human metalloproteinase with thrombospondin type 1 motifs; PLG: plasminogen; FGF-2: fibroblast growth factor-2; IGFBP1: insulin-like growth factor binding protein 1; IL8: interleukin-8; TIMP1: tissue inhibitor matrix metalloprotease 1; VEGFA: vascular endothelial growth factor A; VEGFC: vascular endothelial growth factor C.
progression of MM may enhance angiogenesis. To study this, we treated MMEC with increasing concentrations of human recombinant sJAM-A and measured different parameters of angiogenesis. To examine whether JAM-A directly affects spontaneous MMEC migration, we performed experiments in two-dimensional (2D) and three-dimensional (3D) environments. Enhanced spontaneous MMEC migration was observed after 12 h of sJAM-A treatment in a 2D scratch assay in which migrating MMEC were counted (Figure 4A, upper and lower panels). Blocking JAM-A abolished the enhanced MMEC migration (Figure 4A, upper panel) and reduced the numbers of migrating MMEC (Figure 4A, lower panel). In a 2D angiogenesis assay, sJAM-A treatment increased endothelial structural complexity in terms of branching points and vessel length, which are parameters of angiogenesis. Three hours after seeding, sJAM-A treatment resulted in a structured capillary network, while the control remained in a rudimentary stage of organization with small clumps of cells distributed on the Matrigel™ layer (Figure 4B, upper left quadrant). Furthermore, we tested the effect of JAM-A inhibition in MMEC by both siRNA and an antibody blocking JAM-A without adding sJAM-A (Figure 4B, lower left and upper right quadrants, respectively). Consistently, blocking JAM-A with a monoclonal antibody impaired the capillary network formation and resulted in poorly skeletonized structures (Figure 4B, lower right quadrant). The observed down-modulation of MMEC migration, reduced number of branching points and shorter vessel length occurred independently of cytotoxic

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![Diagram](image_url)
effects, since JAM-A neutralization did not affect MMEC survival (Online Supplementary Figure S3A, left and right panels).

Based on the 2D observations, we investigated whether JAM-A could influence structured MM-associated angiogenesis in a 3D CAM assay. CAM were implanted with gelatin sponges imbued with either MMEC conditioned medium as the control (CTRL) or MMEC conditioned medium with sJAM-A (+sJAM-A), in the presence or absence of a monoclonal antibody blocking JAM-A. MMEC conditioned medium stimulated new vessel formation in CAM, and this effect was markedly enhanced by the addition of sJAM-A. The effect could be selectively inhibited by treatment with a sJAM-A blocking antibody as treatment with a cocktail containing an isotype IgG1 control antibody (sJAM-A + ISO, middle panel) did not reduce vessel formation (Figure 4C).

To explore potential factors that enhance JAM-A-mediated MM angiogenesis, we compared conditioned media from MMEC supplemented with sJAM-A before and after treatment with the JAM-A blocking antibody with an angiogenesis array (Figure 4D, Online Supplementary Figure S3B). sJAM-A strongly reduced anti-angiogenic and increased pro-angiogenic factors secreted by MMEC, such as plasminogen (PLG), fibroblast growth factor 2 (FGF-2), insulin-like growth factor binding protein 1 (IGFBP1) and vascular endothelial growth factors A and C (VEGFA, VEGFC). Reverse transcriptase polymerase chain reaction analysis corroborated the proteomic findings and revealed sJAM-A-induced transcriptional upregulation of these factors and ligands (PLG and ENO1, JAM-A with LFA-1 and TJP1) (Online Supplementary Figure S3C-L). Moreover, because JAM-A can form homophilic interactions with JAM-A itself as well as heterophilic interactions with LFA-1, TJP1, CAV1 and CASK, we investigated whether the expression of these ligands correlated with MM-MMEC interactions. Direct co-culture of RPMI-8226 cells and MMEC significantly increased LFA-1 and CAV1

Figure 6. JAM-A inhibition restricts angiogenesis and tumor growth in subcutaneous multiple myeloma xenograft model. NOD/SCID mice (n=20) bearing RPMI-8226 subcutaneous xenografts were repetitiously treated with a JAM-A blocking monoclonal antibody (α-JAM-A), or isotope control IgG (ISO) or with vehicle only for 40 days for 3 days/week. (A) Immunohistochemistry staining: JAM-A (red) reactivity is stronger in the smaller neoplastic cells whereas it is lower in the more pleomorphic/anaplastic components. The opposite staining distribution is observed for Ki67 nuclear staining (brown), which is more clear-cut in the larger cells. CD31 staining shows focal positivity in the control group and is absent in the group treated with the JAM-A blocking antibody. (B) Treatment was continued for 3 days/week for 40 days and tumor volumes were measured every 2 days with a caliper. (C) Hemoglobin values, Ki-67 positivity, vessel area and number of vessels expressed as mean ± standard deviation of three independent experiments. *P<0.05 versus vehicle-treated control. Scale bar=50 μm.****P<0.0001 versus controls; Mann-Whitney test.
on MM-cells, whereas TJP1, CASK and ADAM17 expression levels decreased (Online Supplementary Figure S3G-K). We therefore investigated whether the induced gene expression was non-random, by studying other molecules involved in neoplastic angiogenesis processes, namely VEGFA, VEGFC, HGF, FGF16 and Aurora kinase A (AURKA); also in this case we found significant VEGFA and AURKA gene upregulation after MM-MMEC co-culture (Online Supplementary Figure S3F and L).

These data support the concept that MM-MMEC interactions enhance angiogenesis. Thus, we investigated whether MM cells participate actively in the angiogenesis program in a reciprocal interaction with the BM microenvironment in patients and whether a pro-angiogenic gene signature could identify patients with worse progression-free and overall survival. We therefore studied 646 NDMM patients enrolled in the CoMMpass trial, comparing two different cohorts, based on survival outcome (alive vs. dead for overall survival and progressed vs. ongoing disease for progression-free survival) performing a supervised analysis based on the gene expression of the pro-angiogenic factors contained in the angiogenesis array and other well-known JAM-A interactors. Strikingly, these two cohorts differed significantly: JAM-A, ENO-1, VEGFA and AURKA were all overexpressed in patients experiencing shorter progression-free and overall survival. Conversely, reduced TJP1 expression in patients correlated with poor survival (Online Supplementary Table S3). These gene expression data confirmed the protein expression results from our cohort of patients. Exogenous JAM-A modulated the secretory profile of MMEC, favoring angiogenesis, and highlighted the tight connection with an angiogenic environment, comprising key angiogenic factors, such as PLG, FGF-2, IGFBP1, VEGFA and VEGFC.

**JAM-A inhibition impairs angiogenesis and inhibits tumor growth in vivo**

To investigate whether JAM-A inhibition may affect in vivo angiogenesis and in turn MM-cell growth, we employed two different mouse models. To mimic advanced MM we injected RPMI-8226 cells intratibially into NOD-SCID mice and analyzed bone specimens after anti-JAM-A treatment. Blocking JAM-A reduced MM-cell proliferation and angiogenesis (Figure 5A). The difference was statistically significant regarding numbers and percentages of Ki-67 high proliferating MM-cells (79.87±1.242 and 35.38±0.345 in the groups treated with isotype control and anti-JAM-A, respectively; P<0.0001) and vessels/mm² field (9.3 and 7.1 in the groups treated with isotype control and anti-JAM-A, respectively P<0.0001) (Figure 5B). The group treated with the antibody against JAM-A expressed lower JAM-A levels (79.78±1.443 and 36.98±0.466 in the groups treated with isotype control and anti-JAM-A, respectively; P<0.0001), lower CD31%(5.58±1.34 and 3.48±0.646 in the groups treated with isotype control and anti-JAM-A, respectively; P<0.0001) and...
lower vessel density (calculated as number of vessels/mm²) than the group treated with the isotype control, which had a higher number of vessels with well-lit lumina (9.77±2.63 and 6.48±0.63 in the groups treated with isotype control vs. anti-JAM-A, respectively; P<0.0001).

To assess the activity of the anti-JAM-A blocking antibody on angiogenesis on a solitary plasmacytoma in vivo and to monitor MM-cell growth at extra-osseous sites non-invasively with a caliper, we employed a subcutaneous MM xenograft model. This approach enables dissection of the endothelial bystander effect on BM-independent extramedullary MM.21 Thus, we employed a second in vivo xenograft model engrafting RPMI-8226 cells subcutaneously into the flanks of NOD/SCID mice.22 The animals were randomized at day 3 after engraftment and treated with either anti-JAM-A monoclonal antibody or a non-specific isotype control antibody intraperitoneally for 3 days/week for 40 days. Subsequently, we measured the vascular area, tumor volume and hemoglobin content of the MM mass. Blocking JAM-A reduced the vascular area in the soft tissue MM masses compared to that in animals treated with an isotype control (difference between medians -0.0155; P<0.0001). No adverse events occurred upon continuous anti-JAM-A treatment.

Notably, after 40 days, the vascular area increased significantly in tumors and MM disease progressed more in controls than in the group treated with the monoclonal antibody against JAM-A (Figure 6A, CD31 staining, and 6B). In isotype-treated control mice, tumors grew exponentially, contrasting with the only limited tumor growth in anti-JAM-A-treated animals (Figure 6B, Online Supplementary Figure S4A). Lower hemoglobin content confirmed poor MM vascularity in the anti-JAM-A-treated mice (8.4±0.04 in the isotype-treated control mice vs. 5.5±0.04 in the anti-JAM-A-treated group; P<0.0001, 95% CI: -3.02 to -2.8 (Figure 6C). Ki-67-staining, vascular area and vessel count confirmed that blocking JAM-A strongly reduced MM vascularity and disease progression. Furthermore, JAM-A blocking significantly reduced pro-angiogenic factors such as FGF-2 and VEGF-A in the peripheral blood of MM-bearing mice (Online Supplementary Figure S4B-D).

Discussion

Angiogenic switching is a key process during transition from premalignant asymptomatic MGUS to full-blown MM. Angiogenic parameters in the BM at the time of diagnosis were widely considered to be able to predict MM progression.22 In solid tumors, such as breast, lung, head and neck, and brain cancers, JAM-A activation promotes tumor progression, while its inhibition by anti-JAM-A agents reduces tumor growth.23 We demonstrated in four independent experimental settings that JAM-A essentially stimulates MM-associated angiogenesis. In the CAM assay, a monoclonal antibody neutralizing JAM-A caused a strong reduction of the number of vessels, implying that JAM-A exerts an essential angiogenic stimulus that could not be replaced by any other compensating factor contained in the MMEC conditioned medium.24 Our new findings pinpoint JAM-A as an attractive target in MM patients.

JAM-A appears pivotal in MM evolution, which can be explained by several angiogenic mechanisms.24,25 First, we demonstrated significantly higher JAM-A levels on MMEC from NDMM patients than on MEGEC. Furthermore, we could link the high JAM-A surface expression on MMEC with a significantly shorter overall survival in both NDMM and RRMM and, at even more advanced disease stages, higher JAM-A expression levels also correlated with reduced progression-free survival. We therefore examined the pathophysiological basis responsible for favoring MM progression. As already described for the HGF/CMET axis,26,27 JAM-A acts within the BM microenvironment, sustaining the neoplastic clone and promoting MM-related angiogenesis both directly and indirectly by priming MMEC. Thus, JAM-A and its soluble isofrom sJAM-A appear to feed into a vicious cycle involving MMEC, generating a malignant environment favorable for MM progression. Although JAM-A is expressed in several solid cancers, to our knowledge this is the first report of the role of endothelial JAM-A expression in the MM tumor microenvironment. Homophilic interactions between recombinant sJAM-A and membrane JAM-A have been demonstrated biochemically.28 Homophilic JAM-A interactions can be inhibited by an anti-JAM-A monoclonal antibody that binds to an epitope close to the N-terminus of the mature protein29 as well as by a peptide that corresponds to the N-terminal 23 residues of the mature protein.30 This suggests that the homophilic trans-interaction is mediated through the membrane-distal V-type Ig-like JAM-A domain at the N-terminus of the molecule. Targeting this domain of the JAM-A molecule on MMEC in our in vitro co-culture systems suggested that this type of interaction mediates the MM-MMEC crosstalk. In line with previous reports about MMEC sustaining MM growth,26,31 our disease models showed that during the transition from the pre-angiogenic to the angiogenic phase, proliferation of tumor cells and neovascularization intensify involve over-expression of JAM-A on MMEC. MMEC were responsive to the presence of sJAM-A in the surrounding microenvironment, which increased their JAM-A surface protein expression. sJAM-A directly and indirectly upregulated JAM-A on the bystander MM-cells, independently of their basal JAM-A expression status. These observations support the concept that cellular components of MM BM, including MMEC, can release JAM-A to sustain disease progression and prepare a tumor-“friendly” niche, exerting significant modulation on FGF-2, VEGF-A and PLCE1 downstream effects.

JAM-A has been described to interact with CD9, a well-known driver of MM-related drug resistance32 and clinical prognosis.33 We found significant expression of FGF-2, a potent stabilizer and activator of a ternary complex involving JAM-A, CD9 and ß3 integrin, a novel potential therapeutic target.34 Peddibhotla et al.35 described that the aggregation of this ternary complex can activate downstream pathway cascades to induce proliferation, migration and an angiogenic stimulus to endothelial cells.36 Our in silico validation shed more light on this pathophysiological process. ENO1 encodes α-enolase, which in the cytoplasm works as a plasminogen receptor and has been described to show upregulated membrane expression in several types of cancer.37,38 Of note, plasminogen upregulation had been correlated with tumor invasion and angiogenesis;39 its activation, derived from the interaction with α-enolase, prompted activation of downstream signaling such as the MEK-ERK pathway, which was able to promote cell invasion and angiogenesis further. α-enolase can also modulate antimor immune responses. Cappello et al.39 described that α-enolase40 myeloid-derived suppressor cells could not
adhere to tumor necrosis factor-α-primed endothelial cells in the presence of an anti-ENO-1 monoclonal antibody. Consequently, decreased migration of the myeloid-derived suppressor cells reduced in situ immunosuppression, enhancing T-cell-mediated immunity against malignant cells.4,13,39

We found that JAM-A overexpression in MMEC strongly correlated with the expression of ADAMTS1, a regulator of angiogenesis42 and immune-surveillance,40 which appears to play a central role in preparing a favorable BM milieu.40 We also identified that the expression of JAM-A on the surface of MMEC was inversely correlated with ADAM17 expression. Conversely, sJAM-A release correlated directly with ADAM17 upregulation, a mechanism described for endothelial cells in inflammation.42 ADAM17 upregulation has also been observed in MM in the context of fractalkine release,42 which identifies this system as a potential novel therapeutic target in MM patients to disrupt a vicious circle enhancing the MM niche.

JAM-A levels also correlated strongly with AURKA levels in MM patients. This finding may link JAM-A-mediated cell adhesion to MM resilience and drug resistance.20 Indeed, proteasome engulfment-derived proteotoxicity44 and invasiveness through epithelial-mesenchymal-transition and cell adhesion6 are complex biological events that affect prognosis.40 We also demonstrated that the interaction between MMEC and MM-cells affects the expression of JAM-A and other fundamental molecules, such as TJP1 and LFA-1. This reciprocal “education” parallels the invasive behavior of the MM-cells towards the endothelial counterpart, instructing the vasculature to interact actively with the malignant cells, potentially driving their survival and drug resistance.7,40 In line with this, increased JAM-A endothelial levels correlated strongly with unfavorable and resistant MM stages such as high R-ISS disease stages and the risk of extramedullary development.

A network of interactions between JAM-A and α-enolase46 emphasizes the strong communication with the MM niche environment to allow persistence and sustaining proangiogenic signaling. Therefore, JAM-A may represent a key factor in the nurturing substrate49 supporting the evolution of MM. Moschetta et al.56 previously described the interdependency of endothelium and MM-cells: endothelial progenitor cell trafficking enhances MM progression, particularly at an early disease stage. Rajkumar et al.53 highlighted a progressive increase in BM angiogenesis along the spectrum of plasma cell disorders from MGUS to advanced MM.7,11 Integrating the prognostic relevance of JAM-A expressed by MMEC and our experimental data led us to propose JAM-A as a key player in coordinating the interactions with the MM milieu enabling a permissive BM ecosystem during the aggressive disease evolution from NDMM to RRMM.

Indeed, the anti-MM effect of blocking JAM-A also relies on a complex antiangiogenic effect, especially in critical transition phases of MM progression, such as the passage from MGUS to symptomatic MM and from responsive to drug-refractory disease, interfering with a main proangiogenic factor and with MM-cell proliferation. Our 2D and 3D models showed that, mechanistically, JAM-A drives MM-associated angiogenesis via a homophilic interaction and through identified downstream targets, namely FGF2, VEGF-A and PLG/ENO1, in the BM microenvironment. Moreover, the clinical impact demonstrated in a large cohort of consecutive individuals pinpoints the JAM-A axis as a new player in MM-associated angiogenesis able to refine the prognostic stratification of patients, especially those with more advanced disease.

The close link between MM and the BM microenvironment appears paradigmatic for MM evolution and disease progression. We connected the interaction of MMEC with MM-cells via the adhesion molecule JAM-A. Our data point towards a vicious cycle of JAM-A overexpression on MMEC reflected by a higher JAM-A expression on the tumor counterpart. Shed from the cell surface, sJAM-A enhances the establishment of homophilic JAM-A complexes fostering MM niche formation. Finally, our results may lead to the development of JAM-A-based therapeutic strategies directed against MM-interactions with the tumor microenvironment (Figure 7A-D). Clearly, these findings need to be confirmed in a larger population of patients in a carefully designed, prospective, clinical study.

Disclosures
No conflicts of interest to disclose.

Contributions
AGS, MCDV, VR, HE, AV and ABe designed and performed research, analyzed and interpreted data and wrote the manuscript; AGS, MCDV, PL, GC, PTG, GDL, ABr, EH, HR-W, DR performed research and analyzed data: AGS, MCDV, AA, PB, GC, FBP, AM, SDS, TS, ABe, MAF, WK, AR and ST analyzed data; AGS, HE, RR, LR, PD, KMK, VR and AV provided patients’ samples; AGS, MCDV, CT, MC, VR, AV, HE and ABe designed research, interpreted data, and edited the manuscript; all authors revised the manuscript and approved its submission. VR and HE contributed equally to this study; AV and ABe contributed equally to the conception and design of this study.

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