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Tracking human multiple myeloma xenografts in NOD-Rag-1/IL-2 receptor gamma chain-null mice with the novel biomarker AKAP-4

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Abstract

Background: Multiple myeloma (MM) is a fatal malignancy ranking second in prevalence among hematological tumors. Continuous efforts are being made to develop innovative and more effective treatments. The preclinical evaluation of new therapies relies on the use of murine models of the disease.

Methods: Here we describe a new MM animal model in NOD-Rag1null IL2rgnull (NRG) mice that supports the engraftment of cell lines and primary MM cells that can be tracked with the tumor antigen, AKAP-4.

Results: Human MM cell lines, U266 and H929, and primary MM cells were successfully engrafted in NRG mice after intravenous administration, and were found in the bone marrow, blood and spleen of tumor-challenged animals. The AKAP-4 expression pattern was similar to that of known MM markers, such as paraproteins, CD38 and CD45.

Conclusions: We developed for the first time a murine model allowing for the growth of both MM cell lines and primary cells in multifocal sites, thus mimicking the disease seen in patients. Additionally, we validated the use of AKAP-4 antigen to track tumor growth *in vivo* and to specifically identify MM cells in mouse tissues. We expect that our model will significantly improve the pre-clinical evaluation of new anti-myeloma therapies.

Background

According to the American Cancer Society, more than 20,000 patients were diagnosed with multiple myeloma (MM) in the US in 2010. Among hematologic malignancies, MM ranks second in prevalence and has the shortest 5-year survival rate [1]. Multiple myeloma (MM) is an age-related cancer caused by the accumulation of antibody-producing malignant plasma cells and leads to progressive osteolysis, defective hematopoiesis and renal failure [2]. Recent progresses in understanding the molecular bases of MM have lead to the use of innovative drugs, such as bortezomib, thalidomide and lenalidomide [3]. Unfortunately, although these therapies afforded a significant improvement in the disease course, MM remains invariably fatal because of the high rate of multidrug-resistant relapse [4]. On these bases, constant efforts are dedicated to the evaluation of more effective treatment strategies [5-7].

Similarly to other malignancies [8], virtually any innovative treatment for MM requires a pre-clinical assessment, which largely relies on the use of animal models to evaluate the anti-tumor potential and possible toxicities [9-12]. To this goal, sub-lethally irradiated immunodeficient NOD/SCID mice have been extensively used since they allow for human MM cell line xenografting after intravenous injection [13-23]. More recently, it has been shown that NOD/SCID mice carrying nonfunctional IL-2 receptor gamma chain (NOD/SCID/γc^{null}, NOG) are more permissive recipients than NOD/SCID and can be easily xenografted with human MM cell lines to produce a disease similar to that seen in patients, including multiple metastatic sites and bone lesions [24,25]. A further modification of the NOD

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strain, carrying double genetic disruptions of the Rag1 and the IL-2 receptor gamma chain genes, namely NOD-Rag1^{null} IL2rg^{null} (NRG), has been reported to tolerate higher levels of radiation compared with NOD/SCID and NOG strains and to allow for efficient engraftment of human hematopoietic stem cells [26].

The development of successful animal models for MM also relies on the choice of the biomarkers used to track the disease course and to identify tumor cells in mouse tissues [27-32]. The A-kinase anchor protein 4 (AKAP-4) [33] is a scaffolding protein that participates in the intracellular signaling of protein kinase-A [34]. AKAP-4 is a cancer/testis antigen (CTA), a class of tumor associated antigens characterized by high expression in germ cells and cancer, strong immunogenicity and very low expression or absence in normal tissues [35,36]. We have previously shown that AKAP-4 is abnormally expressed at the mRNA and protein levels in MM cell lines and patients' MM primary cells, but absent in normal tissues, and therefore it is a potential novel biomarker for MM [37].

In this study, we used for the first time the NRG strain to establish an innovative model of MM, allowing for the growth and the spread of MM cell lines and primary patients' cells as well. Additionally, we provide evidence that the CTA AKAP-4 is a reliable and specific biomarker that can be used to track the growth of MM cell lines and primary cells *in vivo*.

Results

Detection of tumor growth in vivo by ELISA

Indirect ELISA was used to determine the concentration of human paraproteins (IgE and IgG) and AKAP-4 in the sera of tumor-bearing mice (Figure 1). Anti-human IgE antibodies were used to monitor the growth of U266 and H929 [38], since they are IgE-producing cell lines. For MM primary cells, IgG was used as a paraprotein marker [39]. Figure 1 shows that paraprotein and AKAP-4 levels became evident starting 21 days after injection, and that a progressive increase was detectable over time. Although AKAP-4 levels were on average 20% lower than IgE and IgG, no significant difference between AKAP-4 and paraprotein mean levels was detected at any time analyzed point (two-way ANOVA and Bonferroni's post-test $p > 0.05$).

Flow-cytometry identification of MM cells from mouse tissues

Six weeks after initiation of tumor challenging, tumor-bearing and healthy mice were euthanized, and tissues were processed as described in the Methods section. Flow cytometry analysis was performed to detect the presence of MM cell lines or primary cells in the bone marrow, blood and spleen (Figures 2, 3 and 4; Tables 1, 2, 3). Exponentially growing U266 and H929 cell lines or primary cells from bone marrow aspirate were used as positive controls (Figures 2 and 3; Tables 1, 2). IgE was used as a marker for U266 and H929 [38], while primary MM cells were identified by CD38 and CD54 [39]. AKAP-4 was expressed by MM cell lines (Figure 2; Tables 1, 2) and primary MM cells (Figure 3; Table 3); therefore it was tested for the detection of both cell types. Results showed that IgE$^+$ U266 and H929 were present in mouse bone marrow, blood and spleen (Figure 2; Tables 1, 2). Similarly, primary CD38$^+$ and CD54$^+$ primary MM cells were detected in bone marrow, blood and spleen (Figure 3; Table 3). The expression pattern of AKAP-4 was comparable to that of IgE, CD38 and CD54 (Figures 2 and 3; Tables 1, 2, 3). The specificity of the assay was confirmed by the failure to detect positive cells in tumor-free mice (Figure 4).

Analysis of AKAP-4 expression at the mRNA and protein levels in MM xenografts

RT-PCR was performed to evaluate AKAP-4 mRNA expression in tumor-challenged or tumor-free mice. Results (Figure 5) show that the AKAP-4 transcript was present in MM cell lines, primary MM cells, bone

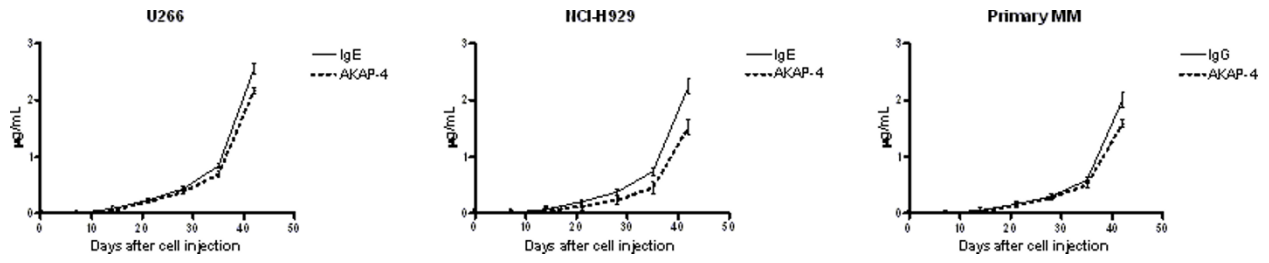

Figure 1 Measurement of circulating paraproteins and AKAP-4 levels. Mice were bled once a week as described in the Methods section. The assay was run in triplicate for each time point. Graphs display mean protein levels obtained from each tumor group and error bars indicate SEM. No statistically significant difference was evidenced between IgE or IgG and AKAP-4 levels at any of the analyzed time points as evaluated by two-way ANOVA (Bonferroni's post-test $p > 0.05$ for all comparisons).

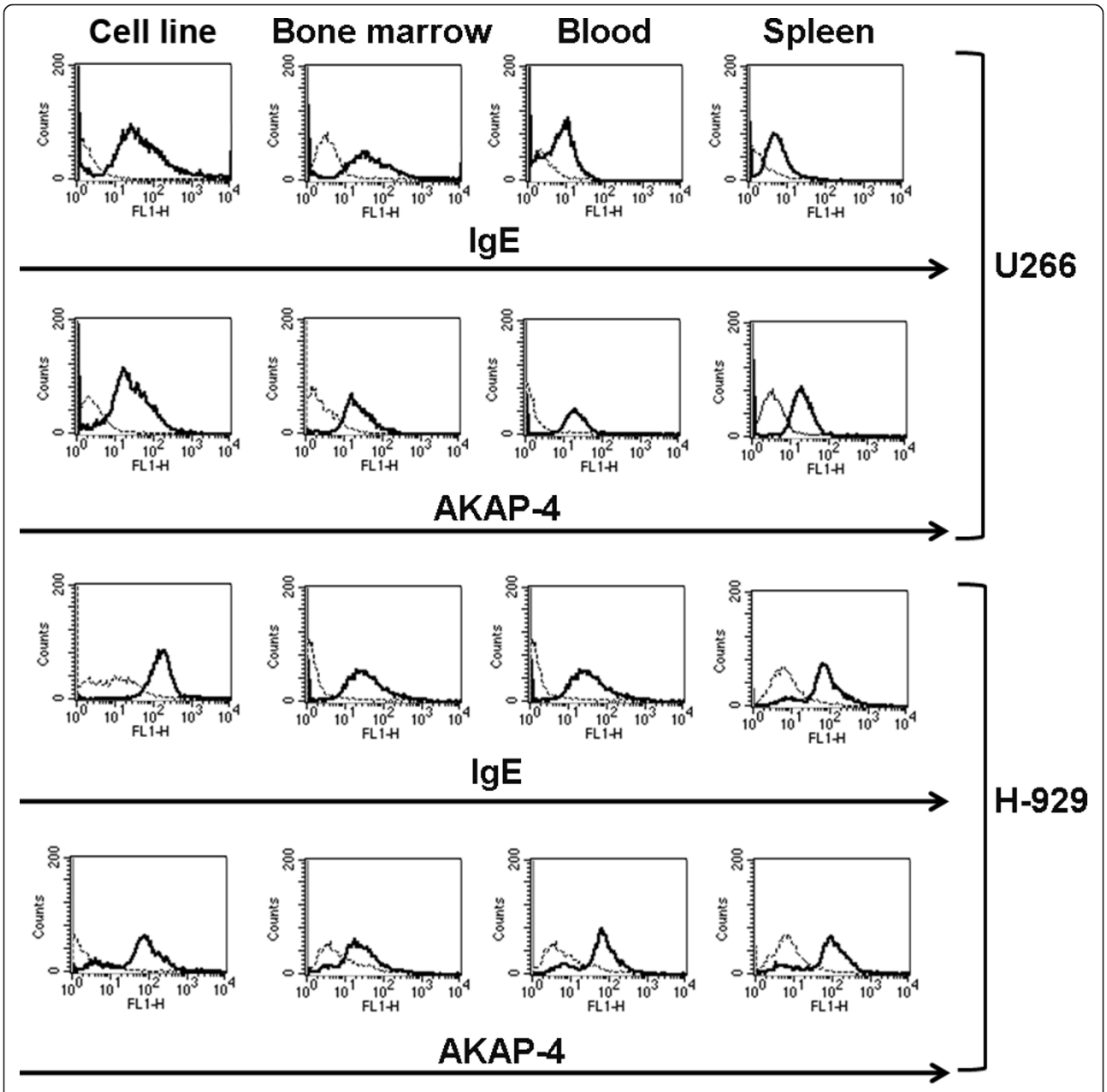

Figure 2 Detection of MM cell lines in tumor-bearing mice. U266 and H929 cell lines, or tissues derived from U266 and H929- injected mice analyzed by flow-cytometry. Histograms show the fluorescence intensity measured with the indicated specific antibody (bold lines) or with the corresponding isotypic control (dotted lines). Graphs are representative of comparable results obtained from 5 U266- and 5 H929-challenged mice.

marrow, peripheral blood and spleens of tumor-bearing mice, but undetectable in tumor-free mice (healthy controls). Specificity of results was also confirmed by PCR reactions carried out without cDNA template or without retrotranscribed RNA.

AKAP-4 protein was detected by Western blot analysis (Figure 5) in MM cell lines, primary MM cells, bone marrow, peripheral blood and spleens of tumor-bearing mice, but not in tumor-free mice (healthy controls).

Discussion

This study was aimed to establish and characterize a new murine model of disseminated MM, allowing for the engraftment of human MM cell lines and primary tumor cells derived from MM patients. To this goal, we used the NOD-Rag1^{null} IL2rg^{null} (NRG) murine strain, intravenously injected with MM cell lines or with primary MM cells. The lacking of a functional IL-2 receptor makes IL2rg^{null} mice better xenograft recipients then

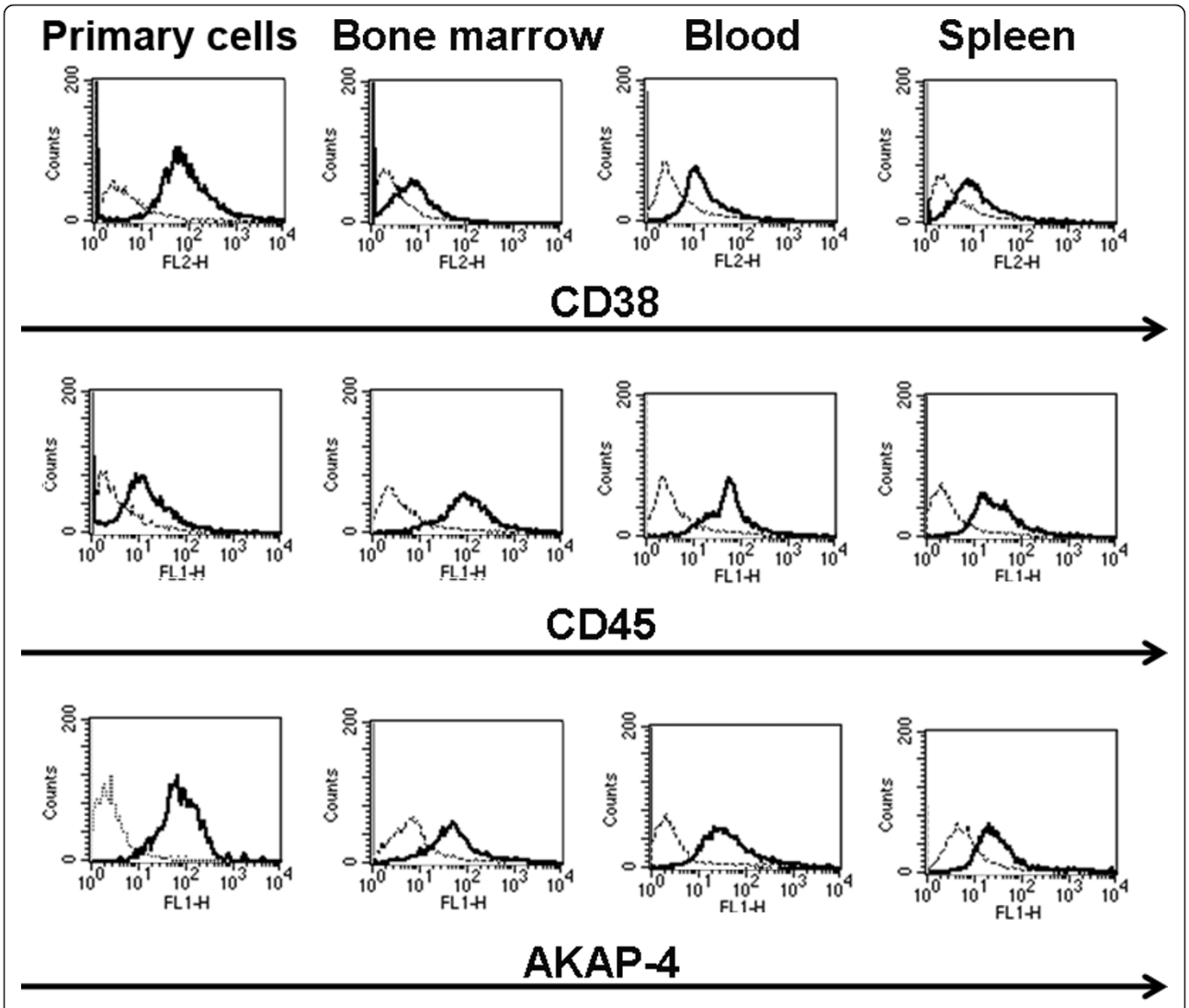

Figure 3 Detection of primary MM cells in tumor-bearing mice. Ficoll-hypaque isolated cells from primary bone aspirate, or cells derived from primary MM-injected mice were analyzed by flow-cytometry. Histograms show the fluorescence intensity measured with the indicated specific antibody (bold lines) or with the corresponding isotypic control (dotted lines). Histograms are representative of comparable results obtained from 5 primary MM-challenged mice.

NOD/SCID animals, because of the absence of NK cells [38]. In addition, compared with NOD/SCID or NOD/SCID/γc^{null} (NOG) strains, NRG mice tolerate significantly higher levels of radiation. Differently from SCID mice, the NRG strain carries a functional Prkdc gene, which is essential for the repair of DNA damage induced by radiation in many tissues [26].

ELISA for serum MM paraproteins showed that xenografted animals supported the growth of both MM cell lines and primary tumor cells. Importantly, AKAP-4 was detectable in the sera of tumor-challenged mice and its levels increased over time, similarly to those of IgE and IgG. This indicates that AKAP-4 is a suitable biomarker for tracking MM progression in murine xenografts. Different techniques have been described to monitor the MM burden in animal models, such as fluorescent tagging of tumor cells [40-45] or measurement of MM-derived paraptrotein in the serum [29-32]. In the clinic, better methods for staging and monitoring the aggressiveness of MM, especially in assessing relapse, are thought to be critical to improve patients' outcomes and develop personalized therapies [46]. A number of methods are under investigation, including mass spectrometry for the quantification of serum immunoglobulins [46], and immunohistochemistry for the expression of FGFR3 and cyclin D1 (reported in 15%, and 50% of patients with MM, respectively) [46,47]. In this context, the identification of novel

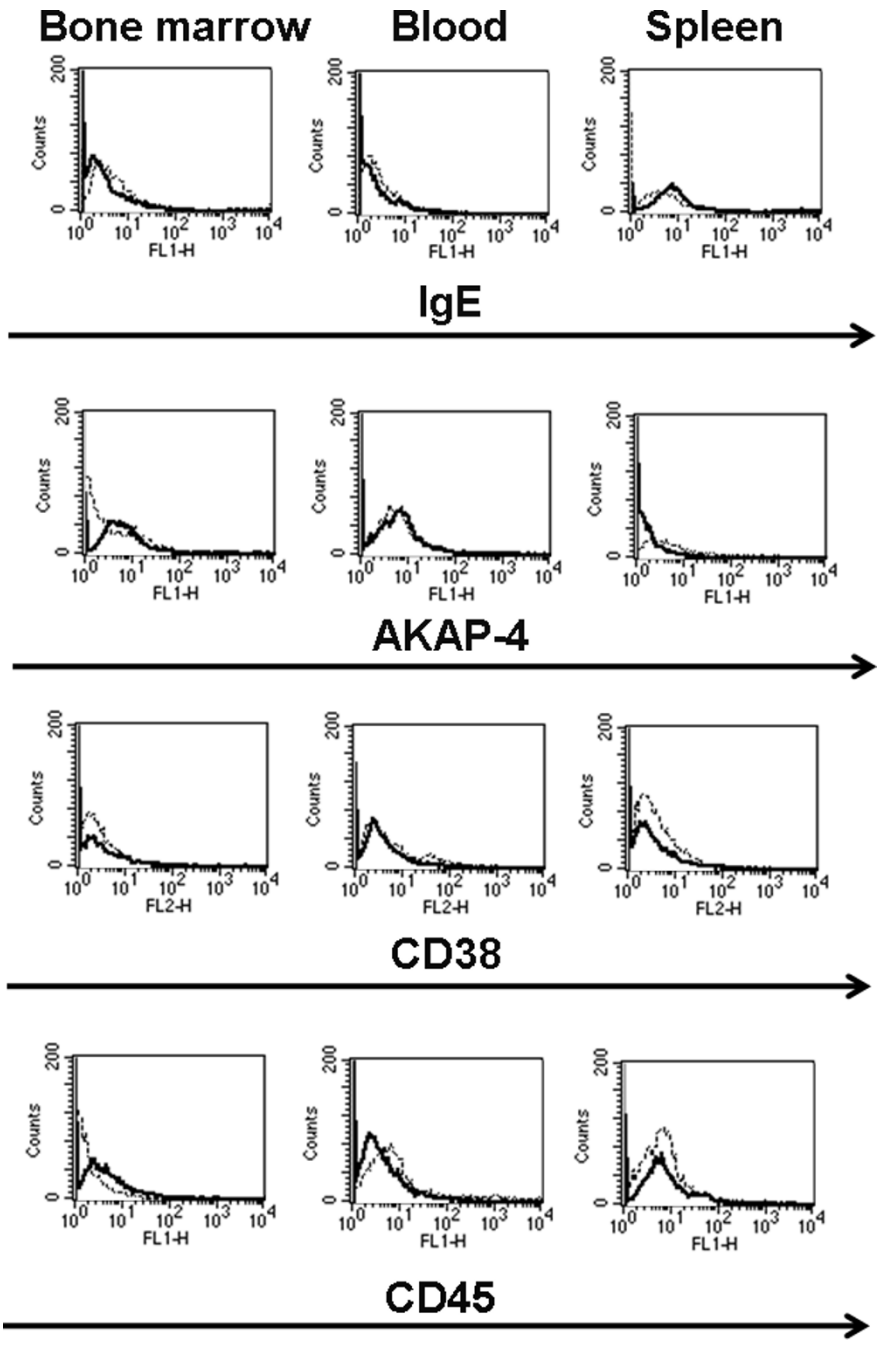

Figure 4 Flow-cytometry evaluation of tumor-free mice. Six weeks after irradiation, healthy control mice were euthanized and processed in parallel with tumor-bearing mice. Histograms show the fluorescence intensity measured with the indicated specific antibody (bold lines) or with the corresponding isotypic control (dotted lines). Histograms are representative of comparable results obtained from 5 tumor-free mice.

tumor antigens in the sera could be instrumental for a more sensitive detection of disease progression [36]. Here we showed for the first time the use of AKAP-4 as a novel serum biomarker in MM animal models. Further investigations are warranted to evaluate AKAP-4 serum levels in MM patients and the correlation with treatment outcome.

Flow-cytometry analysis confirmed the presence of MM cell lines and primary patient's cells in the bone marrow, blood and spleen of tumor challenged mice, indicating that intravenously injected tumor cells were able to systemically disseminate *in vivo*. The specificity of this finding was confirmed by the failure to detect paraprotein- or AKAP-4- positive cells in tumor-free

Table 1 Mean fluorescence intensity for U266 cells

Marker	Cell line	Bone marrow	Blood	Spleen
IgE	56.3[a] (5.4)[b]	48.7 (3.8)	25.4 (3.2)	19.7 (4.5)
AKAP-4	54.9 (3.3)	47.8 (7.6)	52.1 (6.4)	38.7 (4.5)

Mean fluorescent intensities (a) and SEM (b) were calculated by flow cytometry from cells and tissues of 5 U266-challenged mice.

mice. Additionally, we showed that AKAP-4 was expressed in the same tissues at the transcriptional and protein levels in tumor-bearing animals, but absent in healthy controls. Collectively, these results indicate that our model is suitable for the growth and systemic dissemination of human MM cell lines and primary tumors. Currently available murine models for MM include immunocompetent mice, such as the 5TMM series [48,49] and genetic models of MM [50-52], or immunocompromised mice, namely NOD/SCID [13-23], SCID-hu [53-56], and NOG [24,25,38,57]. The 5TMM and the genetic models of MM have the advantage of affording pre-clinical studies in immunocompetent hosts, where possible effects of the therapy on the interaction between tumor cells and the immune system can be evaluated. However, molecular and biological differences exist between murine and human MM cells [11]. Additionally, the number of available murine genetic models of MM and of 5TMM cell lines is extremely restricted and do not represent the heterogeneity of the human disease [50-52,58,59]. Therefore, it is evident that pre-clinical studies on MM cells of human origin are essential [9], but they are only feasible by using immunodeficient murine xenografts. Among these, subcutaneous inoculation of human MM cells has been extensively described [13,17,45,60-62]. This model affords the possibility to directly assess changing in tumor growth induced by therapies. Yet, tumor cells growing subcutaneously do not interact with the bone marrow microenvironment, which largely accounts for MM drug resistance [61,63]. Because we described the presence of tumor cells in the bone marrow of tumor-challenge mice, we propose that our model is suitable to evaluate the protective role played by the bone niche against anti-tumor therapies. Some concerns have been raised about the possibility that the interactions between MM cells and the bone stroma may be partially species-specific [11,64]. To address this potential difficulty, the SCID-hu model was developed, in which MM cells are located

Table 2 Mean fluorescence intensity for H929 cells

Marker	Cell line	Bone marrow	Blood	Spleen
IgE	135.7[a] (12.8)[b]	49.6 (5.3)	46.2 (7.3)	128.9 (14.3)
AKAP-4	131.5 (7.6)	45.2 (8.1)	128.7 (5.7)	131.2 (6.4)

Mean fluorescent intensities (a) and SEM (b) were calculated by flow cytometry from cells and tissues of 5 H929-challenged mice.

Table 3 Mean fluorescence intensity for primary MM cells

Marker	Cell line	Bone marrow	Blood	Spleen
CD38	98.9[a] (11.2)[b]	22.7 (6.7)	27.8 (8.4)	25.4 (4.7)
CD45	26.7 (3.1)	97.6 (7.6)	87.2 (4.1)	38.7 (2.1)
AKAP-4	98.6 (8.7)	88.4 (7.6)	33.2 (2.4)	47.6 (5.3)

Mean fluorescent intensities (a) and SEM (b) were calculated by flow cytometry from cells and tissues of 5 primary MM-challenged mice.

in subcutaneously implanted human bone chips [53-56]. Although SCID-hu mice allow for the growth of MM cells in a bone niche of human origin [53-56], they do not reproduce the pattern of dissemination and multifocal spread seen in MM patients. Here we described for the first time a murine model supporting the engraftment human MM cells, and allowing for the development of a disease involving multiple sites, similar to that observed in patients [65-73]. This is an important advantage, because the effect of bone resorption in multiple osteolytic lesions is a critical factor for the survival of MM patients [6,7]. Pre-clinical studies basing on immunocompromised xenograft models have previously described metastatic spread involving multiple bones, yet they have been limited to human MM cell lines [40,41,43,44,74-76]. In this study, we show that our model supports the metastatic growth of primary human MM cells. MM cell lines do not adequately represent the heterogeneity of the human disease because they are established from late stage disease and frequently present mutations not seen in patients [9]. Therefore, the possibility to study not only cell lines, but also primary MM cells in murine models is relevant.

Conclusions

We presented here the proof-of-principle for the use of NRG mice as a new model supporting the metastatic growth of human MM cell lines and primary cells. Additionally, we propose the use of AKAP4 as a universal biomarker to track tumor cells *in vivo*. We foresee that our results will significantly contribute to the improvement of the pre-clinical evaluation of new anti-myeloma therapies. Because our model sustains the growth of primary MM cells, further investigations are warranted to study the suitability of this system to assess the efficacy of personalized therapies directly on patient's cells.

Methods

Animals

Six-week-old female NOD.Cg-Rag1^{tm1Mom} IL2rg^{tm1Wjl}/SzJ (NRG) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.). All mice were maintained in filtered-air laminar-flow cabinets under specific pathogen-free conditions. Treatment and care of the

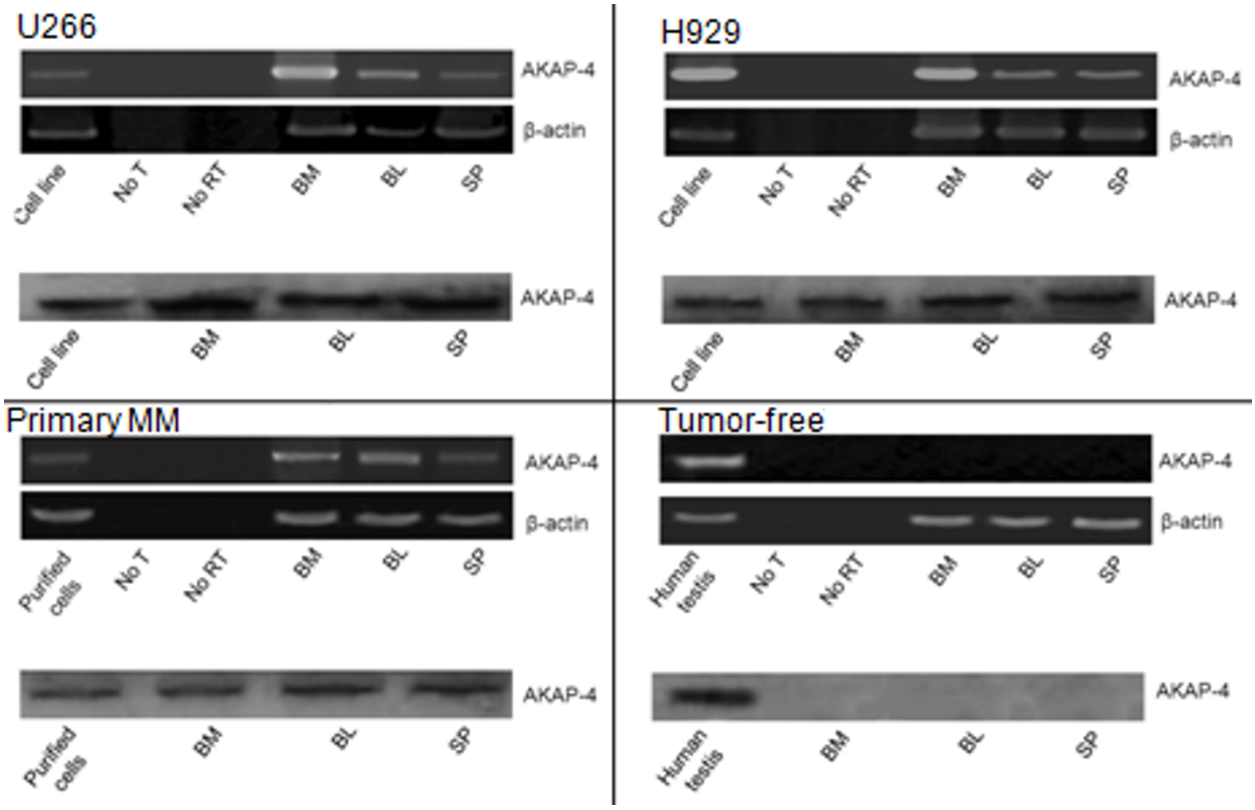

Figure 5 RT-PCR/Western blot analyses of AKAP-4 mRNA/protein expressions and. RT-PCR analysis of mouse tissues was performed to evaluate AKAP-4 expression. AKAP-4 was detected in bone marrow (BM), blood (BL) and spleen (SP) of mice injected with MM cell lines (U266, H929) and primary tumor cells, but not in tumor-free mice. Positive controls were RNA isolated from exponentially *in vitro* growing cell lines, or from primary cells after ficoll purification. For the evaluation of healthy mice, RNA isolated from human testis was used as a positive control. Analysis of β-actin transcript served to confirm RNA integrity. Negative controls were PCR performed without template (No T), or with RNA not subjected to the reverse transcription step (No RT). Proteins extracted from MM cells and mouse tissues were analyzed for the presence of AKAP-4 protein by Western blot. AKAP-4 protein was detected in MM cell lines (U266 and H929), primary MM cells, and in the bone marrow, blood and spleen of tumor-challenged mice, but not in healthy controls (tumor-free). Total protein extracts from human testis were used as a positive control for the analysis of tumor-free mice. The pictures are representative of comparable results independently obtained from 5 mice in each group.

animals were in accordance with the Institutional Guidelines and the Animal Welfare Assurance Act. The mice were checked daily and euthanized 6 weeks after tumor challenge or if they showed signs of excessive discomfort (hind leg paralysis, inability to move, eat or drink).

Human MM cell lines

The human MM cell lines U266 and H929 were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.), and cultured in RPMI-1640 medium, supplemented with 10% V/V fetal bovine serum (FBS) and penicillin/streptomycin mix (10 mg/mL each) in 95% air and 5% CO_2 at 37°C. Prior to injection, cells were washed once in PBS and then resuspended at 10^8 cells/mL in pre-warmed PBS prior to injection.

Primary MM cells

Human material was obtained under informed consent and with the approval from the local ethics committee. Bone marrow aspirate was obtained from a MM patient at diagnosis (Durie-Salmon stage III) from the hip bone. Light density cells were separated by ficoll hypaque centrifugation (Histopaque; Pharmacia, Uppsala, Sweden) [39], washed twice in PBS, counted and adjusted at the final concentration of 10^8 cells/mL in pre-warmed PBS prior to injection.

Xenografts

20 mice were sub-lethally irradiated with a total dose of 550 cGy at 139 cGy/min rate [26]. After 6 hours, mice were assigned to the following groups (5 mice/group): group 1 received U266 cells, group 2 received H929, group 3 was given primary MM cells, while group 4 was

left tumor-free and served as a negative control. Each mouse received 10^7 cells by a single intravenous injection in the lateral tail vein (100 μL/mouse).

ELISA for the measurement of serum paraprotein and AKAP-4 concentration

Blood (50 μL) was collected weekly from each mouse. Serum was prepared by centrifugation in the absence of anticoagulants and stored at -20°C until use. An enzyme-linked immunosorbent assay (ELISA) was performed on mouse sera for the determination of human paraprotein (IgE and IgG) [39] or AKAP-4 concentration. Antibodies were purchased from BD Biosciences (San Diego, CA, U.S.A.). 96-well polystyrene plates were coated with serum (50 μL/well diluted 1:10 in carbonate coating buffer), and incubated overnight at 4°C. Plates were washed three times in PBS containing 0.05% (V/V) Tween-20 (PBS/Tween) and then incubated in 1% bovine serum albumin (BSA) in PBS for 1 h at RT to block unspecific sites. After washing three times with PBS/Tween, plates were incubated at 37°C with 50 μL/well of primary anti-human IgE, IgG, or AKAP-4 antibodies (5 μg/mL in PBS) for 1 h. After washing twice with PBS/Tween, HRP-linked secondary antibody (1:4,000 dilution in PBS, 50 μL/well, Santa Cruz Biotechnology, CA, U.S.A.) was added and allowed to bind for 60 minutes at RT. After washing trice with PBS/Tween, 100 μL/well of TMP substrate (Abcam, Carpinteria, CA, U.S.A.) was added. The reaction was stopped 15 minutes later by adding 50 μL/well H_2SO_4 solution. Optical density (OD) was measured with a Victor^2 plate reader (PerkinElmer, Waltham, MA) at 450 nm. All samples were analyzed in triplicates. Quantification of the target antigens was made by interpolation of the mean OD for each sample using a standard curve obtained by 13 serial 3-fold dilutions (from 2,400 to 1.5 × 10^{-3} ng/mL) of purified human paraproteins (GenWay Biotech, Inc., San Diego, CA, U.S.A.), or human AKAP-4 (Abnova, CA, U.S.A.).

Preparation of tissues

Femurs, hips, sternums, and spleens were mechanically disrupted in serum-free RPMI-1640 medium. Minced organs were placed into 250 mL flasks containing 3 mL of enzyme solution (0.14% collagenase type I; Sigma Aldrich, MO, U.S.A.) and 0.01% DNase (2000 kU/mg; Sigma Aldrich) in RPMI-1640 and incubated on a magnetic stirring apparatus at 37°C for 30 min. Then, cells were washed in PBS and filtered through a nylon mesh with 150 μm pores to generate single-cell suspensions. Blood (500 μL) was taken by retro-orbital venipuncture immediately after the euthanasia procedure and placed in a heparin-coated tube. Cells were harvested by centrifugation and washed twice in PBS before analyses.

Flow-cytometry

The expression of human IgE, CD38, CD45 and AKAP-4 was analyzed by flow-cytometry 6 weeks after tumor injection as previously described [77]. Specifically, IgE was used to identify U266 and H929 cells [38], while CD38 and CD45 were used as markers for human MM primary cells [39]. AKAP-4 was analyzed in both cell lines and primary cells [37]. U266, H929, primary MM cells and cells obtained from mouse bones, spleen and blood were fixed with 2% W/V buffered PFA (Sigma-Aldrich, MO, U.S.A) in PBS for 5 minutes at RT. After washing with PBS, cells were permeabilized with 0.3% saponin (Sigma-Aldrich, MO, U.S.A.) in PBS for 5 minutes at RT. After washing twice with PBS, cells were incubated on ice with monoclonal antibodies raised against human IgE, CD38, CD45 or AKAP-4 (Santa Cruz Biotechnology, CA, U.S.A.) or isotypic controls for 1 hour. After washing twice with PBS, cells were incubated with FITC-conjugated secondary antibodies (for IgE, AKAP-4 and CD45), or PE-conjugated secondary antibody (for CD38) (BD Biosciences, NJ, U.S.A.) for 1 h on ice. Analysis was performed using a BD FACScan (BD Biosciences, NJ, U.S.A), after 3 final washing steps with PBS.

RT-PCR and immunoblot

Total RNA was extracted from bone, spleen, blood, or from MM cell lines and primary MM cells by Trizol-reagent (Sigma, St Louis, MO, U.S.A.). Purified total RNA was treated with 5 μg RNase-free DNase I (Promega, Madison, WI, U.S.A.) at 37°C for 2 h. mRNA was then isolated using Oligotex mRNA Mini Kit (QIAGEN, Valencia, CA, U.S.A.). First-strand cDNA synthesis was performed using oligo (dT) 15-mers primers. PCR primers for AKAP-4 were as follows: forward 5'-GCGTACTCTGATACTACAATGATG -3' and reverse 5'- GGG GTTTTGGGTAAAGTCA- 3' [78]. PCR was performed by 35 amplification cycles at 59°C annealing temperature. For each sample, RNA integrity was checked by amplification of the β-actin cDNA. Successful removal of genomic DNA contamination was confirmed by amplification of the RNA without prior reverse-transcription reaction. All results were confirmed in four independent RT-PCR tests. Immunoblots for AKAP-4 were performed using standard methods, as previously described [78]. Positive controls for immunoblots were proteins extracted from injected MM cells. For healthy mice, protein extracts from human testis were used as positive controls (Applied Biosystems, Foster City, CA, USA) [37].

Statistical analysis

All data are expressed as mean values ± SEM (Standard Error of the Mean). Results were analyzed using

GraphPad Prism software (GraphPad Software, Inc., CA, USA.). Statistical analyses were performed by the two-way ANOVA test. A p value < 0.05 was considered statistically significant.

List of abbreviations used

MM: multiple myeloma; AKAP-4: A kinase anchor protein; RT-PCR: reverse-transcription polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay; Ig: immunoglobulin; NOD/SCID: nonobese diabetes/severe combined immunodeficiency; NRG: NOD-Rag1^{null} IL2rg^{null}; NOG: NOD/SCID/γc^{null}; SCID-hu: humanized SCID; CTA: cancer/testis antigen; BM: bone marrow; BL: blood; SP: spleen; RT: room temperature.

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Authors' contributions

LM performed flow-cytometry analyses. YY performed ELISA, RT-PCR experiments, and established the MM model. MJ and CJ participated in study design and coordination, and revised the manuscript. RC analyzed the data and revised the manuscript. EC participated in study design and coordination, and revised the manuscript. MCI and LM carried out the study design, analyzed the data, wrote, and revised the manuscript.
All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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