A Novel Mouse Model for Multiple Myeloma (MOPC315.BM) That Allows Noninvasive Spatiotemporal Detection of Osteolytic Disease

Peter O. Hofgaard1,*, Henriette C. Jodal1,*, Kurt Bommer2, Bertrand Huard3, Jo Caers4, Harald Carlsen5, Rolf Schwarzer6, Nicole Schünemann6, Franziska Jundt6,7, Mona M. Lindberg1, Bjarne Bogen1*

1 Centre for Immune Regulation, Institute of Immunology, University of Oslo and Oslo University Hospital, Oslo, Norway, 2 Comprehensive Cancer Centre Mainfranken and Department of Internal Medicine II, Division of Haematology and Medical Oncology, University Hospital Würzburg, Würzburg, Germany, 3 Division of Hematology, University Hospitals of Geneva and Department of Pathology-Immunology, Geneva University Medical Centre, Geneva, Switzerland, 4 Department of Hematology, University of Liège, Liège, Belgium, 5 Institute for Nutrition Research, University of Oslo, Oslo, Norway, 6 Department of Hematology, Oncology, and Tumor Immunology, Charité-Universitätsmedizin, Campus Virchow Clinic, Molekulares Krebsforschungszentrum, Berlin, Germany, 7 Max-Delbrück-Center for Molecular Medicine, Berlin, Germany

Abstract

Multiple myeloma (MM) is a lethal human cancer characterized by a clonal expansion of malignant plasma cells in bone marrow. Mouse models of human MM are technically challenging and do not always recapitulate human disease. Therefore, new mouse models for MM are needed. Mineral-oil induced plasmacytomas (MOPC) develop in the peritoneal cavity of oil-injected BALB/c mice. However, MOPC typically grow extramedullary and are considered poor models of human MM. Here we describe an in vivo-selected MOPC315 variant, called MOPC315.BM, which can be maintained in vitro. When injected i.v. into BALB/c mice, MOPC315.BM cells exhibit tropism for bone marrow. As few as 10^3 MOPC315.BM cells injected i.v. induced paraplegia, a sign of spinal cord compression, in all mice within 3–4 weeks. MOPC315.BM cells were stably transfected with either firefly luciferase (MOPC315.BM.Luc) or DsRed (MOPC315.BM.DsRed) for studies using noninvasive imaging. MOPC315.BM.Luc cells were detected in the tibiofemoral region already 1 hour after i.v. injection. Bone foci developed progressively, and as of day 5, MM cells were detected in multiple sites in the axial skeleton. Additionally, the spleen (a hematopoietic organ in the mouse) was invariably affected. Luminescent signals correlated with serum myeloma protein concentration, allowing for easy tracking of tumor load with noninvasive imaging.Affected mice developed osteolytic lesions. The MOPC315.BM model employs a common strain of immunocompetent mice (BALB/c) and replicates many characteristics of human MM. The model should be suitable for studies of bone marrow tropism, development of osteolytic lesions, drug testing, and immunotherapy in MM.

Introduction

Recent progress in unraveling the biology of MM, particularly the intracellular signaling pathways and the interactions with the bone marrow microenvironment, has resulted in development of novel targeted therapy [1]. Different animal models for MM disease have contributed to this progress, each model having advantages and disadvantages in this respect.

Xenograft models using human myeloma cell lines, or primary human myeloma have been established in immunodeficient SCID or NOD scid gamma (NSG) mice [2–8]. In particular, models have been generated where human MM cells grow in human fetal bone transplants in immunodeficient SCID mice [9]. More recently three-dimensional bone-like scaffolds were coated with mouse or human bone marrow stromal cells and implanted under the skin of SCID mice. Subsequently, injection of purified primary myeloma cells into these scaffolds gave rise to tumor formation that could be followed by measuring myeloma protein concentration [10]. Although these models allow experiments of human MM cells in vivo in mice, the models are demanding and not completely physiological.

Mouse models where MM cells can be transferred between syngeneic mice are also available. However, mouse MM models do not necessarily accurately reflect human disease. MM-like disease arises spontaneously in aged C57BL/KaLwRij mice [11–13]. The 5T2MM and the 5T33MM cell lines were established from such mice, and have been extensively used for studying homing mechanisms of MM cells to bone marrow, interaction of MM cells with the bone marrow environment, and evaluation of new therapies [14]. Both models are characterized by MM cell
infiltration restricted to bone marrow and spleen (a hematopoietic organ in mice) [15]. The 5T2MM model, but not 5T33MM, is associated with an extensive osteolysis, seen on plain radiographs of femur and tibia [15–16]. Both 5T2MM and the 5T33MM are maintained by in vivo passages and cells die after 24 to 48h in culture. However, two in vitro growing subclones of the 5T33MM model have been developed, namely the 5T33MMvt [17] and the 5TGM1 cells [18]. Intravenous injection of 5TGM1 cells give rise to MM-like osteolytic disease. 5TGM1 cells have also been labeled with luciferase [19] and green fluorescent protein (GFP) [20], and used for in vivo imaging of MM-like disease. A major drawback of the 5TMM models is the dependency on a particular mouse strain (C57BL/KaLwRij) of limited availability.

Finally, three different transgenic mice models have recently been developed based on double-transgenic Myc/Bcl-XL mice [21], the activation of MYC under the control of a light chain gene [22], or cloning of a spliced form of mouse XBP-1 downstream of the immunoglobulin VH promoter and enhancer elements [23]. Although they recapitulate several characteristics of MM, these models are time-consuming and costly, perhaps explaining their limited use thus far.

In summary, the available MM models presented above can be technically challenging and require large investments. Thus, there is a need for an MM model where MM cells can be grown in vitro and when i.v. injected in a common laboratory inbred mouse strain, such as BALB/c, faithfully duplicate the major characteristics of MM disease seen in patients.

Plasmacytomas can be experimentally induced in certain strains of mice by i.p. injection of mineral oil, adjuvants and alkanes [24]. Such mineral oil-induced plasmacytomas (MOPC) can be serially transplanted s.c. or i.p. and have been extensively used in tumor immunological studies [25–30]. However, these plasmacytomas typically grow locally at the site of injection, and only infrequently metastasize to the bone marrow [31–33]. Due to their local growth, it has been questioned if MOPC tumors represent good models for human MM that primarily affects bone marrow.

We have previously described an in vivo-selected variant of MOPC315, MOPC315.4, which efficiently forms local tumors after s.c. injection [34]. We here show that repeated i.v. injections of MOPC315.4 cells, followed by isolation of tumor cells from femurs between passages, enriches for a stable variant (MOPC315.BM) that can be grown in vitro, has tropism for bone marrow after i.v. injection, and causes osteolytic lesions. Spatio-temporal development of disease may be monitored by serial and noninvasive measurement of the bioluminescent signal of luciferase-labeled cells.

Results

A Multiple Myeloma-like Cell Line, MOPC315.BM, which Homes to and Expands in the Bone Marrow after i.v. Injection

Previously, an in vitro-adapted MOPC315 cell line from ATCC (TIB23) was passaged s.c. twice to obtain a variant, MOPC315.4, that consistently produced s.c. extramedullary plasmacytomas [34]. This cell line has been extensively used in tumor immunological studies [26–30]. To obtain a MM-like variant cell line with tropism for the bone marrow, MOPC315.4 cells were injected i.v. at a high dosage (2 × 10⁶). Tumor cells were flushed from femurs of the first mouse that developed paraplegia, a sign of spinal cord compression. This procedure was repeated 9 times (Fig. 1A). The final cell line, MOPC315.BM, was compared with MOPC315.4 and MOPC315 (ATCC) in tumor challenge experiments in which the same number of in vitro-cultured cells...
shown in A. Data are presented as mean ± SD in Fig. S1. N.D. = Not Detected. (D) Titration of MOPC315.BM and MOPC315.4 by i.v. injection and development of paraplegia (n=3–6/group).

doi:10.1371/journal.pone.0051892.g001

(2×10⁵) were injected i.v. (Fig. 1B). Tumor development was monitored by serial measurements of M315 myeloma protein in serum (Fig. 1C, Fig. S1A). The endpoints were defined as 1) paraplegia, 2) visible extramedullary tumor growth with tumor size >1 cm in diameter, 3) distended abdomens, or 4) weight loss >10%.

All mice injected with MOPC315.BM cells developed paraplegia within 30 days. Paraplegia was also induced by MOPC315.4, but disease development was delayed by about 14 days compared to MOPC315.BM. The ATCC line was slowest at causing spinal involvement, moreover, 2 out of 12 mice died of tumor disease but without paraplegia, and one mouse did not develop tumor disease at all (Fig. 1B). Consistent with these results, mice injected with MOPC315.BM cells had a more rapid increase in serum M315 myeloma protein compared with the other two cell lines (Fig. 1C, Fig. S1A). Notably, the increase in serum M315 myeloma protein is more uniform in mice injected with MOPC315.BM, than mice injected with the MOPC315.4 or the MOPC315 (ATCC) cell lines. These results establish a hierarchy for the rapidity of spinal MM disease onset: MOPC315.BM>MOPC315.4>MOPC315 (ATCC).

Next, titrated amounts of MOPC315.BM and MOPC315.4 cells were injected i.v. (Fig. 1D). The results show that the MOPC315.BM line was more tumorigenic than parental MOPC315.4 by a factor of ~20. As few as 2000 or 400 MOPC315.BM cells caused paraplegia in respectively 66% and 33% of the mice within 50 days, while as many as 50,000 MOPC315.4 cells were required to cause paraplegia in 17% of mice within the same time frame. This finding indicates that an increased fraction of MOPC315.BM cells have the ability to establish progressive bone marrow disease after i.v. injection, compared to MOPC315.4.

Given that the MOPC315 cell line after i.v. injection appears to induce MM-like disease, we tested the sensitivity of MOPC315.BM to the MM drug bortezomib, a proteasome inhibitor, in vitro (Fig. S2). The IC₅₀ was 10–15 nM, which is in the same range as that previously reported for chemosensitive MM cell lines [35].

Tagging MOPC315.BM Cells with Luciferase Produced a Cell Line (MOPC315.BM.Luc), with a Delayed Onset of Paraplegic Disease

MOPC315.BM cells were transfected for constitutive expression of the firefly luciferase gene (MOPC315.BM.Luc). We first tested if transfection affected tumor growth. The MOPC315.BM.Luc cells were considerably slower at inducing paraplegic disease than the untransfected counterpart (Fig. 2A). This was confirmed by a delayed increase in serum myeloma protein (Fig. 2B, Fig. S1B). This finding prompted us to investigate if growth and myeloma protein secretion differed for the various cell lines in vitro (Table S1). The results show that MOPC315.BM and the MOPC315.BM.Luc cells grow at nearly equal rates and faster than both MOPC315 (ATCC) and MOPC315.4. For unknown reasons, MOPC315.BM.Luc secreted higher amounts of M315 myeloma protein than the other three cell lines. Based on these results, it appears likely, but not proven, that the slow kinetics of disease development of MOPC315.BM.Luc compared to MOPC315.BM in BALB/c mice is due to a low level immunogenicity to luciferase, resulting in slower growth but not elimination of luciferase-marked cells.

Spatiotemporal Monitoring of MM Disease using MOPC315.BM.Luc Cells by Bioluminescence Imaging (BLI)

In MOPC315.BM.Luc-injected mice, tumor burden and localization could be detected by use of a CCD camera (IVIS Spectrum), as previously described in other tumor models [36]. Repetition of this noninvasive procedure throughout the course of the experiment resulted in a spatiotemporal resolution of tumor development in single animals.

When 2×10⁵ cells were injected in BALB/c mice, signals were detected by day 10 and increased progressively (Fig. 2E,F). With time, signals from individual foci (Fig. 2C) and the overall signals (Fig. 2F) increased in strength, and an increasing number of foci could be detected (Fig. 2C,G). Typical sites of bone affection were spine > femur>shoulder region> sternum (Fig. 2D,G). In addition, signals were frequently seen corresponding to the hip region, but in BALB/c mice the level of resolution was not sufficient to pinpoint localization. Signals from the spleen were present in 100% of mice (Fig. 2G), while signals from other internal organs including liver were rarely detected.

Bioluminescence is a Valid Measurement for Tumor Load in BALB/c Mice

In the experiment of Fig. 2, both luminescence and serum myeloma protein were followed over the course of the experiment. The results show that the bioluminescence signals correlated with M315 serum concentrations in single mice (Fig. 2H, r = 0.8929, p<0.001). Moreover, the two methods for assessment of tumor load have approximately the same sensitivity although the data indicate that measurement of myeloma protein in an idotype-specific ELISA is slightly more sensitive. A significant bioluminescent signal was definitely reached when myeloma protein concentration exceeded 100 ng/ml. Because serum myeloma protein concentration is considered to be a gold standard for tumor load in MM, one may conclude that the strength of the bioluminescence signal is a valid measurement for tumor size.

BLI and CT of BALB/c nu/nu Mice: Monitoring Tumor Growth and Localization to the Axial Skeleton

To increase sensitivity of detection, MOPC315.BM.Luc cells were injected i.v. at a higher dose (5×10⁶) and into BALB/c nu/nu which lack hair and are thus better suited for BLI and the 3D bioluminescence imaging technique Diffuse Luminescent Imaging Tomography (DLIT). Immediately after injection, cells were clearly observed in the lung region in all mice injected. Most importantly, a weak signal, 5–10 fold higher than the corresponding region of the control mice, could be observed in the tibiofemoral region already 1h after injection (Fig. 3B). Such tibiofemoral signals were observed in 14 out of 14 mice, average photons detected were 1.5×10⁷±6.4×10⁵ SD versus 1.9×10⁵±2.4×10⁵ SD for non-injected controls (p<0.0001), indicating that tumor cells had entered the bone marrow immediately after injection. On day 2, the signal from the lungs had disappeared and a very weak signal was located in the spleen and the liver. By day 5, signals were found in multiple foci in bones, primarily the femurs, the lower part of the spine, and the
Tumor development was followed by BLI until the mice developed paraplegia (Fig. 3A,C,D) (average time for endpoint was 33 days, n = 14, Fig. S3A). As may be seen, the average luminescence signal decreased on day 2 and 5 to about 8% of immediate post-injection values, and recovered to initial levels between days 8 and 10 (Fig. 3D). This indicates that a substantial number, perhaps 90%, of cells may die in the days following i.v. injection. Progressive tumor growth was confirmed by increasing levels of myeloma protein in sera (Fig. S3B). Luminescence signals were highly correlated with serum M315 levels (Fig. 3E, r = 0.9648, p < 0.0001). At onset of paraplegia, when the luminescence signal usually was strongest (see Fig. 3C for a typical example), DLIT and CT were performed on individual mice. Tumor tissue and the axial skeleton were co-localized in 3D (Fig. 3F). To confirm that the luminescence signal was in fact emanating from the skeleton, individual bones were dissected, or only skin was removed, before performing high resolution BLI, to more clearly expose skeletal sites (Fig. 3G–J). This procedure enhanced sensitivity, and individual foci could clearly be seen in spine (Fig. 3G), ribs (Fig. 3H), sternum (Fig. 3I) and femur and tibia (Fig. 3J). The strong signal emanating from the lower spine in most mice (e.g. Fig. 3A,C,F,G) is compatible with early occurrence of paraplegia due to compression of the spinal cord in diseased mice. In addition, internal organs were dissected and analyzed by BLI (Fig. 3K–O). A strong signal was observed from the spleen and much lower signals from other organs. Co-localization of tumor and axial skeleton is further demonstrated in two videos (full mouse in Fig. S4 and close-up in Fig. S5). Note that a strong signal corresponding to the spleen was observed, but no signals from other organs.

MOPC315.BM Cause Disease Primarily in the Bone Marrow

On autopsy, all paraplegic BALB/c mice injected with MOPC315.BM had visible tumor growth in spleens (a hematopoietic organ in the mouse), resulting in splenomegaly, but more rarely in the liver and other organs. It was macroscopically difficult to determine bone disease in paraplegic mice, even though visible tumors were sometimes seen penetrating the cortical bone. By microscopic analysis of H&E-stained sections, tumor growth was routinely found in bone marrow of femurs (Fig. 4A) and in spleens (Fig. 4B), as well as in intramedullary in the livers, fallopian tubes and subcutaneous tissues (Fig. S6). Immunohistochemistry demonstrated that MM cells expressed lambda and not kappa Ig L chains consistent with MOPC315 producing IgA L2 myeloma protein (Fig. 4A,B).

Fluorescent Protein (DsRed)-labeled MOPC315.BM Cells Preferentially Home to the Bone Marrow

To confirm the presence of MOPC315.BM cells in the bone marrow and spleen, we transfected MOPC315.BM with DsRed, and injected cells i.v. into C.B-17 SCID mice. Upon onset of
A Novel Mouse Model for Multiple Myeloma

| Days | Dorsal | Left flank | Ventral | Right flank |
|------|--------|------------|---------|-------------|
| 0    | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| 2    | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
| 5    | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| 8    | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) |
| 10   | ![Image](image17.png) | ![Image](image18.png) | ![Image](image19.png) | ![Image](image20.png) |
| 15   | ![Image](image21.png) | ![Image](image22.png) | ![Image](image23.png) | ![Image](image24.png) |
| 20   | ![Image](image25.png) | ![Image](image26.png) | ![Image](image27.png) | ![Image](image28.png) |
| 25   | ![Image](image29.png) | ![Image](image30.png) | ![Image](image31.png) | ![Image](image32.png) |

**Figure 1:**
- **A:** Time course of luminescence images showing the progression of myeloma over time.
- **B:** Representative images of mice at different time points.
- **C:** Comparison of luminescence intensity in different regions of the mouse.
- **D:** Graph showing the change in average luminescence over time.
- **E:** Scatter plot correlating luminescence with M315 level, showing a significant correlation.

**Figure 2:**
- **F:** 3D model of a mouse showing the distribution of myeloma.
- **G:** Close-up view of the bone marrow with highlighted regions of tumor.
- **H-J:** Detailed images of the tumor at different stages.
- **K-O:** Histological sections showing the infiltration of myeloma cells.

**Legend:**
- Color scale representing the intensity of luminescence and tumor burden.
paraplegia, femurs and spleens were analyzed by flow cytometry (Fig. 5A) and femurs by immunohistochemistry (Fig. 5B) for the presence of MOPC315.BM.DsRed cells. For all injected mice (n = 3), DsRed positive cells were detected in bone marrow flushed from femurs. Cells were also detected in the spleen (Fig. 5A). MOPC315.BM.DsRed cells were present in the extravascular space of femur bone marrow, as shown by microscopy of H&E stained sections from paraffin-embedded tissue (Fig. 5B). These results essentially confirm the conclusions of Luciferase-based whole body imaging, and support a bone marrow-tropism of the MOPC315.BM cell line.

MOPC315.BM.Luc Injected Mice Develop Osteolytic Lesions

Osteolysis is a hallmark of human MM. Mice with growth of MOPC315.BM.Luc cells in bone marrow developed osteolytic lesions by several criteria. Firstly, MOPC315.BM.Luc induced an increased number of TRAP+ osteoclasts, compared to control mice injected with RPMI 1640 tissue culture medium (Fig. 6). Secondly, transmission X-rays demonstrated osteolytic lesions of femurs (Fig. 7A,B) and tibiae (data not shown), which were confirmed by μCT (Fig. 7C,D). In addition, severity of osteolytic lesions was quantified by bone structure analysis via μCT (Fig. 7E-J, Fig. S7). Osteolysis was evident in mice 5–8 weeks after injection of MOPC315.BM.Luc (Fig. 7E-J) as well as in mice with...
paraplegic disease (6–11 weeks after injection; Fig. S7). μCT assessment of total, trabecular and cortical bone compartments revealed highly significant changes in mice after injection of MOPC315.BM.Luc cells as compared to controls. Disturbed architecture of trabecular bone was demonstrated by decrease of trabecular number and trabecular thickness, and increase of trabecular separation (Fig. 7E–G, Fig. S7A–C). Overall bone volume decreased as evidenced by reduced bone volume fraction and an increased ratio of bone surface to bone volume (Fig. 7H–I, Fig. S7D–E). Moreover, the total mineral density was significantly reduced in MOPC315.BM.Luc injected mice (Fig. 7J, Fig. S7F). Thirdly, mice injected with MOPC315.BM had increased levels of calcium (Ca²⁺) in sera. For unknown reasons, a plateau level of Ca²⁺ was obtained by day 20 (Fig. 7K). Taken together, these results demonstrate that the MOPC315.BM cell line induces osteolytic lesions, a hallmark of human MM, when delivered by i.v. injection.

Discussion

We here describe a novel variant of MOPC315, MOPC315.BM, that after i.v. injection of in vitro-cultured cells induce MM-like disease in the axial skeleton of syngeneic BALB/c mice, BALB/c nu/nu, C.B-17 SCID and NSG. Luciferase-labeled cells (MOPC315.BM.Luc) allow spatiotemporal resolution of bone disease development by repeated in vivo imaging. Injected mice develop osteolytic lesions, a hallmark of human MM. This novel mouse MM model could be useful for studies of bone marrow tropism, efficacy of drugs, mechanism of osteolysis, and immuno-therapy. An important aspect of the current MOPC315.BM model is that experiments can be performed in an immunocompetent and readily available laboratory mouse strain, BALB/c. A further advantage is that the white fur of this albino strain makes in vivo imaging more sensitive and simpler to perform than in mice with black fur.

The results demonstrate that a mineral oil-induced plasmacytoma, MOPC315 (ATCC), adapted to in vitro growth, can cause paraplegia in about 80% of mice within 100 days after i.v. injection. This finding was not anticipated since MOPCs are generally considered to be poor models of MM bone disease, even though they readily form extramedullary plasmacytomatas after local injection. It may be that MOPC315 has an increased tropism for bone marrow compared to other MOPC lines and bone marrow tropism may thereby vary between different MOPC lines. The results further demonstrate that variants of the MOPC315 (ATCC) cell line can be obtained that more rapidly induces MM-like bone disease. Thus, a cell line selected for high tumor take by s.c. injection, MOPC315.4, caused paraplegia in all i.v.-injected mice within 65 days. After 9 cycles of i.v. injection of MOPC315.4 and recovery of cells from femurs of paraplegic mice, the MOPC315.BM cell line was obtained that following i.v. injection caused paraplegia in all mice within 35 days.

It is unclear whether the consecutive s.c. and i.v. selection procedures resulted in either a gradual change of phenotype or in a selection of rare cells with more MM-like features, pre-existing in the parental plasmacytoma cell line. The fact that decreasing amounts of injected cells were needed with progressive cycles could indicate that the in vivo-selection might have enriched for a pre-existing variant. It is also unknown whether preferential growth in bone marrow was due to increased homing to the bone, or if cells simply grew better once they had settled in the bone marrow microenvironment or both.

MOPC315.BM cells labeled with firefly luciferase (MOPC315.BM.Luc) could be followed by repeated bioluminescent imaging of i.v.-injected mice. Overall, the results were similar in normal BALB/c and T cell-deficient BALB/c nu/nu mice. However, sensitivity was clearly higher in furless BALB/c nu/nu mice, the nude strain being the recommended model for DLIT (Living Image® User Manual, Caliper Life Sciences). Sensitivity was further increased by injection of a higher number of cells. Immediately after injection, cells were found primarily in the lung,

Figure 6. Osteoclasts in bone marrow of NSG mice infiltrated with MOPC315.BM.Luc. (A,B) Representative images of TRAP+ staining from the bone marrow of an age-matched control NSG mouse injected with RPMI 1640. (C–E) Representative images of TRAP+ staining from the bone marrow of an NSG animal injected with MOPC315.BM.Luc. Inset in (C) shows bone marrow localization of transplanted MOPC315.BM.Luc cells using staining for IgA. TRAP+ osteoclasts are indicated by black arrow heads. In all cases scale bar is 200 μm. (F) Quantification of osteoclasts per mm bone surface. Controls are age-matched RPMI-injected NSG mice.
doi:10.1371/journal.pone.0051892.g006
but also in the spleen and the liver. However, and importantly, a minor fraction of cells were found in the tibiofemoral region already 1 h after injection. Early invasion of bone marrow is consistent with results obtained with i.v. injection of 51Cr-labeled 5T2MM cells [37]. (However, note that detection of luciferase-labeled cells by BLI ensures that cells are alive while a detection of a 51Cr signal does not necessarily guarantee the presence of living cells). On day 5, progressively growing MM foci were seen in multiple sites of the axial skeleton, with signals first being detected in the femurs and the spine. The spleen signal progressed with time, while lung and liver signals decreased. Affection of other organs was only infrequently detected. MM growth in spleen is consistent with extramedullary hematopoiesis in this organ, and is also found in the 5TMM models [12,14].

A considerable number of the injected cells apparently died since the total bioluminescence decreased by >90% on day 2, and only recovered to initial post-injection values after day 8. Since injection of about 1000 MOPC315.BM cells caused paraplegia in
about 50% of mice, it may be roughly extrapolated that perhaps only 100 MOPC315.BM cells, surviving the days following i.v. injection, is sufficient to cause development of MM-like bone disease.

Even though relatively few bone foci were seen in BALB/c mice, the increased sensitivity in nude mice, combined with \textit{ex vivo} imaging at endpoint, revealed that MM growth was more diffuse with multiple foci in single ribs and sternum. Consistent with this, DsRed-labeled MOPC315.BM cells were detected in every flushed femur of paraplegic mice. The average is consistent with that often observed in human MM (>10% malignant plasma cells), though we only measured three paraplegic mice. Note that the endpoint, paraplegia due to compression of the lower part of the spinal cord, is observed relatively early in mice injected with MOPC315.BM. Thus, in absence of paraplegia, much higher numbers of MM cells in bone marrow of femur might have been reached. Flow cytometry data show that the infiltration of MOPC315.BM cells in tibiae equals that of vertebral bodies (data not shown), however BLI data indicate that the lower vertebralca could have a higher density of MM cell foci than other bone sites, predisposing for paraplegia.

A hallmark of human MM is osteolytic lesions. The MOPC315.BM induced osteolytic lesions were detected by histology (staining for TRAP+ osteoclasts), transmission X-rays and \textit{µCT} of isolated bones, and elevated Ca$^{+}$ levels in serum. Thereby, \textit{ex vivo} \textit{µCT} analysis of distal femurs demonstrated highly significant changes in trabecular and cortical architecture in MOPC315.BM injected mice compared to controls. However, the osteolytic lesions were difficult to detect by standard X-rays of living mice. Again, if mice could have been observed beyond development of paraplegia, osteolytic lesions might have become more pronounced.

Lynch and co-workers have previously described that MOPC315 grown in diffusion chambers i.p. have a preferential loss of cells which are of plasmacytoid appearance, resulting in enrichment of cells which secrete less myeloma protein, but have increased expression of surface BCR [38]. They have also described a variant, MOPC315$^{5}$, that when injected i.v. readily gave rise to splenic tumor foci [39]. The bone marrow-homing MOPC315.BM appears distinct from these previously described variants since its production of secreted myeloma protein and BCR is similar to that of MOPC315.4, and since it has tropism for bone in addition to spleen.

It is generally believed that MM cells represent malignant counterparts of plasma cells that at the earlier B cell stage have been through a germinal center reaction[40–42]. However, it is unclear where the neoplastic process initially takes place. One possibility is that MM originates from plasma cells malignantly transformed within the bone marrow, and that MM cells later metastasize to other bones. Another possibility is that neoplastic cells originate in an extramedullary site, and then seed multiple bones where they are exposed to a microenvironment conducive to growth. Several pieces of evidence support the latter possibility. Firstly, MM has been associated with less differentiated clonogenic precursors found in blood[43–45]. Secondly, extramedullary plasmacytomas in humans can metastasize to bone [46]. Thirdly, ileocecal plasmacytoma in an aged gonadectomized mouse [31], as well as transplanted MOPC tumors [32,33], can metastasize to the bone marrow. The present data are compatible with the idea that MM could originate from cells undergoing malignant transformation outside the bone marrow and that variants with increased bone marrow tropism seed multiple bones.

It is of great interest to find out why MM cells home to and grow in the bone marrow. A comparison of MOPC315.BM and MOPC315.4 at the protein and gene expression levels might offer clues as to why the former variant more rapidly causes bone disease. A number of factors could contribute, such as CD44 splice variants, chemokine and cytokine receptors, integrins, metallo-proteases, and responsiveness to growth signals in the bone marrow microenvironment, including cytokines like IL-6 [47]. In a global approach to this issue, repeated and independent gene expression profiling of MOPC315.BM and MOPC315.4, and extensive flow cytometric and cytokine secretion analysis, has revealed stable differences (H. C, Jodal et al., proposals for further investigation). In addition, since the MOPC315.BM model was established in the BALB/c strain it might be possible, by use of available knock-out mice on this background, to study the influence of genes on MM cell homing and growth in the bone marrow.

In summary, MOPC315.BM and the 5TMM tumors [8,12,14] appear to share the ability to home to the bone marrow and cause osteolytic lesions. Similar to the 5TGM1 cell line [18], MOPC315 can be cultured \textit{in vitro}, injected i.v., and causes MM-like disease. However, the MOPC315.BM has certain advantages for experimental manipulation since we here show that it may be readily labeled by luciferase or DsRed for tracking by \textit{in vivo} imaging, and since it reproducibly causes bone disease in a common strain of laboratory mice, BALB/c. The model should be valuable for studying mechanisms of homing of MM cells to bone marrow and drug treatment. Moreover, since so much is known about idioype-specific T cell immunotherapy of s.c. extramedullary MOPC315 plasmacytomas [26–30], the present model should allow the application of T cell immunotherapy to MM cells in the bone marrow (Holgado and Bogen, proposals for further investigation).

**Materials and Methods**

**Ethics Statement**

This study was carried out in accordance with the 3Rs principle. Experiments were approved by the National Committee for Animal Experiments (Oslo, Norway), application no. 2153. All surgery and euthanization was performed under isoflurane (2.5–5% inhalation) or midazolam and fentanyl (both 5 mg/ml, i.p. injection) anesthesia, and all efforts were made to minimize suffering.

**Mice**

BALB/c, BALB/c nu/nu and C.B-17 SCID mice were obtained from Taconic (Ry, Denmark). NOD scid gamma (NSG) were obtained from Charles River (Germany).

**The MOPC315.BM Cell Line**

MOPC315 [48] was obtained as an \textit{in vitro}-adapted cell line from ATCC (Manassas, Virginia). This cell line was cycled between \textit{in vitro} culture and \textit{in vivo} growth s.c.; the resulting cell line, MOPC315.4, has been used in our previous s.c. tumor challenge experiments [26,27,34,49]. To obtain a variant of MOPC315 that homes to the bone marrow and grows in that site, a large number of MOPC315.4 cells ($2\times10^6$) were injected i.v. into five BALB/c mice. The first mouse to develop paraplegia was euthanized, bone marrow cells were flushed from the femurs, and cultured in RPMI 1640 GlutaMAX (Gibco) supplemented with 1% MEM NEAA 100x (Gibco), 1% sodium pyruvate (Gibco), 0.005% 1M 1-thioglycerol (Sigma) solution, 0.03% Genaminycin 40 mg/ml (Sanofi aventis) and 10% fetal bovine serum (Biochrom AG). Normal bone marrow cells eventually died and the culture was taken over by expanding MOPC315.4 cells, which were expanded and injected i.v. into new BALB/c mice. With
consecutive passages, mice developed paraplegia more rapidly, and the number of MOPC315.4 cells injected was eventually reduced to $2.5 \times 10^5$. After the third and the ninth passages, MOPC315.4 was cloned \textit{in vitro} by limiting dilution, and clones that grew well and produced M315 myeloma protein were selected for further use. After 9 passages, the resulting cell line, MOPC315.BM, was frozen in aliquots.

\section*{Labeling of MOPC315.BM with Luciferase and DsRed}

Luciferase labeling: MOPC315.BM cells were co-transfected by electroporation with the pGL3-Control vector containing the luciferase gene (Promega), and the pcDNA 3.1(+) vector containing the gene for neomycin resistance (Invitrogen). For DsRed labeling, MOPC315.BM cells were co-transfected with the pCMV-DsRed-Express vector (Clontech), and the pcDNA 3.1(+) vector. Clones with high expression were re-cloned by limiting dilution, selected for high expression, frozen in aliquots, and used for tumor challenge experiments.

\section*{Tumor Challenges. Measurement of Myeloma Protein}

MOPC315 (ATCC), MOPC315.4, MOPC315.BM, MOPC315.BM.Luc, and MOPC315.BM.DsRed cells were cultured \textit{in vitro} (37°C, 5% CO$_2$) and harvested when growing exponentially, then centrifuged (300 g, 7 min) and resuspended in PBS (Gibco-BRL, Carlsbad CA). Cells were counted and injected i.v. in the tail vein. Injection dosage used is indicated in the figure legend or text. Endpoints were 1) paraplegia, 2) visible extramedullary tumor disease with tumor size $>1$ cm in diameter, 3) distended abdomens, or 4) weight loss $>10\%$. The mice were sacrificed on the same day the endpoint was reached. Disease was verified by measurement of M315 myeloma protein in ELISA [26].

\section*{Whole Body in vivo Imaging of Tumor Cells}

Imaging of luciferase activity \textit{in vivo} was performed essentially as described [36]. Mice were injected i.p. with D-luciferin (150 mg/kg body weight) and imaged using either the IVIS Spectrum or IVIS Spectrum CT imaging systems (Caliper Life Sciences, Hopkinton, Massachusetts). Images were acquired from 10 to 20 minutes post substrate injection. Data were analyzed using LivingImage® (Caliper Life Sciences) software. Each image included a non-tumor bearing control mouse also administered D-luciferin. Luminescence for each side or region of tumor bearing mice was quantified using average photons/second/cm$^2$/steradian, with the respective area on the non-tumor bearing control mice subtracted. Diffuse Light Imaging Tomography (DLIT) and Computer Tomography (CT) modes were performed on an IVIS Spectrum CT (Caliper Life Sciences). For the DLIT mode, four spectral images were acquired at 580 nm, 600 nm, 620 nm, and 640 nm. Auto exposure was set at 10,000 photons. Sequence acquisition was performed between 5 and 40 minutes post i.p. injection of D-luciferin. For CT, the standard one mouse or medium resolution settings were used.

To increase sensitivity of detection of MOPC315.BM.Luc, BALB/c nu/nu mice, which lack hair and are thus better suited for BLI and the 3D bioluminescence imaging technique DLIT, were used in some experiments (Fig. 3). However, BALB/c nu/nu mice also lack a thymus and thus T cells, which may affect the immune response and tumor growth.

\section*{Flow Cytometry}

DsRed is, in contrast to luciferase, immunogenic in BALB/c mice, and immunodeficient mice thus have to be used when injecting DsRed-transfected cells. C.B-17 SCID mice lack both B and T cells due to a defect in V(D)J recombination. This may affect tumor growth to some degree as an immune response towards the tumor cells will be lacking. C.B-17 SCID mice i.v. injected with MOPC315.BM DsRed cells were followed until endpoint and then euthanized. Bone marrow was flushed with complete medium, using a needle and syringe, from femurs. Spleens were dissected from individual mice and placed in complete medium. Spleens were then minced with a scissor and gently pushed through a sieve (Sigma). The cell suspensions were centrifuged (300 g 7 min), resuspended in erythrocyte lysis solution (TRIS 17 mM, NH$_4$Cl 140 mM, pH 7.2), then washed with PBS and thereupon fixed (1% paraformaldehyde (PFA)). Bone marrow was gently pipetted to obtain a cell suspension, centrifuged, then resuspended in PBS with 5% BSA, and fixed in PFA. The samples were run and analyzed on the FL2 channel of a FACSCalibur flow cytometer using Cell Quest Pro™ and WEASEL softwares. To determine the percentage of tumor cells in a sample, a marker was placed in front of the negative controls (femur and spleen cells from a normal mouse) such that $>98\%$ of the cells were to the left of that marker. Using this marker, all cells to the right were judged to be positive. The mean and standard deviation from three mice were then calculated. The backgrounds in the negative controls (percentage of the cells to the right of the marker) are indicated in the figure legend.

\section*{Histochemistry and Microscopy}

When MOPC315.BM-injected BALB/c mice developed para-plegia, samples were taken from limbs, liver, spleen, lungs, gastrointestinal tract, kidneys and heart, and fixed in 4% PFA before embedding in paraffin. 5-μm sections throughout the organs were made with a rotation microtome (Leica RM2135). One slide was stained with haematoxylin and eosin and two additional slides were immunostained for light chain kappa and lambda in order to visualise the MOPC315.BM cells. For immunostaining, the endogenous peroxysase was quenched by incubating the slides with a 50-mL methanol, 0.5-mL H$_2$O$_2$ (3%) (Merck, Darmstadt, Germany) solution for 20 min. Autoclave heating was used for antigen retrieval by immersing the slides in a autoclavable tray, containing 5 mL of Target Retrieval Solution (Dako, Glostrup, Denmark) and 45 mL of deionised water and autoclaving at 120°C for 10 minutes. Slides were blocked using a blocking buffer (Universal Blocking Reagent, Bio Genex) to reduce non-specific background staining. The horseradish peroxidase coupled anti-mouse lambda and kappa antibodies (Rockland, Gilbertsville, PA, USA) were incubated for 2 h at room temperature at a dilution of 1:1000. Chromogenic visualisation was accomplished through the use of a DAB reaction (3,3-diaminobenzidine hydrochloride kit; DAB; Dako), followed by counterstaining with haematoxylin.

For immunohistochemistry related to Fig. 6, the tissue was fixed in 4% PFA, decalcified in 10% EDTA (pH 7.4), embedded in paraffin before being sectioned (4 μm) and rehydrated. After HEIR (5 mM citrate buffer pH 7), CD138 mAb (Clone 281–2, BD Pharmingen) and an AP labeled anti-IgA (Sigma, St. Louis, Missouri) were used to identify MOPC315.BM.Luc cell infiltration. All sections were analyzed using a Nikon Eclipse E 800, and digital images processed with AnalySIS Soft Imaging System, or images were captured using a Nikon Eclipse TE 2000-U with either a DS-Qi1Mc (5.03) or Dxm1200 digital camera, with NIS-Elements BR version 3.0 software.

TRAP staining was done according to Acid Phosphatase, Leukocyte (TRAP) Kit from Sigma (St. Louis, Missouri).
without HEIR. These sections were then photographed, regions of bone marked and the osteoclasts counted by three individuals, then averaged and divided by bone surface length (mm) (Fig. 6F).

For detection of MOPC315.BM.DsRed cells, femurs were fixed in 4% PFA, decalcified, embedded in paraffin, and de-waxed. Sections were analyzed by fluorescence microscopy (Nikon Eclipse E 800) or H&E stained and analyzed by light microscopy (Leitz Dialux), and digital images processed with AnalySIS Soft Imaging System.

Transmission X-ray and μCT

NSG mice were injected with a MOPC315.BM.Luc clone which expresses a doxycycline inducible non-functional control shRNA (Fig. 7A–D). Explanted bones were fixed in 4% PFA and X-rayed in an experimental μCT facility[50–51] (Fraunhofer Development Center for X-ray Technology EZRT, Fürth, and Institute for X-ray Microscopy, University Würzburg, Germany). X-ray source from Excillum, using a liquid metal jet (Galinstan, GalInSn alloy) anode with a 15 μm spot size at 70 kV and 80 Watt. Images were detected using a Gadolinium oxysulphide scintillator and a CMOS camera with 6.5 m pixel size. Isosurface from μCT data was computed using Image J (version 1.47c) and the BoneJ plugin (version 1.34) [32].

For bone structure analysis, BALB/c mice were injected with MOPC315.BM.Luc (Fig. 7E–J, Fig. S7). Total femur length was measured using digital calipers on freshly isolated bones. At the distal femur, endocortical contours were drawn for a region with a height equivalent to 5% of the femur length, beginning at 850 μm. Mc315 secretion was measured by adding 10 ng/ml (solid lines of individual mice). Lower detection limit of 10 ng/ml.

Figure S4 Video of DLIT and CT of a whole BALB/c nu/nu mouse. Shows the co-localization of MOPC315.BM.Luc and the axial skeleton. Note the presence of tumor cells in the spleen, but no other organs.

(AVI)

Figure S5 Close-up video of DLIT and CT of a BALB/c nu/nu mouse. Shows the co-localization of MOPC315.BM.Luc and the axial skeleton, as shown as a whole mouse in Fig. S4. Note the presence of tumor cells in the spleen, but no other organs.

(AVI)

Figure S6 Infrequent examples of microscopically detected MOPC315.BM tumors. As shown in Fig. 2 in paper version, spleen and bone marrow were almost always affected, while affection of other organs was rarer. Shown are infrequent examples of microscopic growth in liver (A) and fallopian tube (B), and subcutaneous macroscopic growth (C).

(TIF)

Figure S7 Osteolysis induced by MOPC315.BM.Luc at paraplegia. BALB/c mice were injected with MOPC315.BM.- Luc (2×10^6 cells) i.v. and distal femurs were analyzed by μCT at paraplegia (6–11 weeks after injection). Non-injected 10–11 weeks old BALB/c mice served as controls.

(TIF)

Table S1 In vitro growth rate and in vitro M315 secretion. All cell lines were cultured as previously described in this article. Cells growing exponentially were then harvested, counted, resuspended in medium and kept on ice until use. In vitro growth was estimated by an assay where 8 parallel samples of 50 μl at a concentration of 1×10^5 cells/ml (5000 cells) of a cell line was added to 200 μl of warm medium (37°C) in 8 wells of a 96 well, flat bottom Costar® plate (Corning Inc., USA). After standard incubation (37°C, 5% CO2) for 48 hours, and while the cultures were growing exponentially, the dish was placed on ice and the cells in each well were counted. The website (http://www.doubling-time.com/compute.php) was used to calculate doubling times. In vitro M315 secretion was measured by adding 5 parallel samples of 50 μl at a concentration of 1×10^5 cells/ml (5×10^5 cells) of a cell line to 200 μl of warm medium (37°C) in 3 wells of a 24 well, flat bottom Costar® plate and incubated for 1 hour under standard conditions. The cultures were then immediately centrifugated and supernatants were stored at −70°C. For each well, the M315 concentration was determined from the mean of two independent M315 ELISAs. The average secretion rate was obtained using the added cell number (5×10^6).

The unpaired t test was used to calculate p values. *p<0.05 compared to MOPC315 (ATCC) and MOPC315.4. **Significantly higher than the other cell lines p<0.05.

(PPT)
Acknowledgments

The authors thank Hilde Omholt (Institute of Immunology, Oslo, Norway), Eleonore Ojtcasques (Department of Hematology, Liège, Belgium) and Kathryn S. Bommer (University Hospital Würzburg, Germany) for their expert help. The outstanding technical help from the staff of the Department of Comparative Medicine, Rikshospitalet, Oslo University Hospital and University of Oslo is gratefully acknowledged.

References

1. Anderson KC (2007) Targeted therapy of multiple myeloma based upon tumor-microenvironmental interactions. Exp Hematol 35: 155–162.
2. Fero-Zuppardi FJ, Taylor CW, Iwato K, Lopez MH, Grogan TM, et al. (1992) Long-term engraftment of fresh human myeloma cells in SCID mice. Blood 80: 2453–2500.
3. Huang YW, Richardson JA, Tong AW, Zhang BQ, Stone MJ, et al. (1993) New xenograft model of multiple myeloma and efficacy of a humanized antibody against human interleukin-6 receptor. Blood 90: 2437–2444.
4. Hjorth-Hansen S, Seifert MF, Borse T, A Peter H, Ostlie A, et al. (1999) Marked osteoblastopenia and reduced bone formation in a model of multiple myeloma bone disease in severe combined immunodeficiency mice. J Bone Miner Res 14: 256–263.
5. Pihlström LM, Hiippersen G, Seeherberger K, Pruski E, Coupland RW, et al. (2000) Multiple myeloma progenitors in the blood of patients with aggressive or minimal disease: engraftment and self-renewal of primary human myeloma in the bone marrow of NOD/SCID mice. Blood 95: 1056–1063.
6. Miyakawa Y, Ohnishi Y, Tomisawa M, Monna M, Kohamura K, et al. (2004) Establishment of a new model of human multiple myeloma using NOD/SCID/gamma(null) (NOG) mice. Biochem Biophys Res Commun 313: 258–262.
7. Minnaden CS, Anderson KC. Carrasco DR (2007) Mouse models of human myeloma. Hematol Oncol Clin North Am 21: 1051–69.
8. Yacoby S, Barlogie B, Epstein J (1998) Primary myeloma cells growing in NOD/SCID gamma(null) (NOG) mice. Blood 91: 2941–1945.
9. Calimeri T, Battista E, Conforti F, Neri P, Di Martino MT, et al. (2011) A novel myeloma model. Am J Pathol 139: 593–597.
10. Carral J, De Gloop DR, Sturh RT, Zurcher C (1979) Idiopathic paraproteinemia. II. Transplantation of the paraprotein-producing clone from old to young C57BL/12xRij mice. J Immunol 122: 609–613.
11. Carral J, Croce JW, Zurcher C, Van den Enden Vierven MH, de Leeuw AM (1988) Animal model of human disease. Multiple myeloma. Am J Pathol 132: 593–593.
12. Vanderkerken K, Assoung K, Croucher P, Van CB (2003) Multiple myeloma biology: lessons from the 5TMM models. Immunol Rev 194: 196–206.
13. Vanderkerken K, De RH, Goes E, Van MS, Radl J, et al. (1997) Organ inflammation and lymph node adhesion protein expression profile of 5T2 and 5T33 myeloma cells in the C57BL/12xRij mice. Br J Cancer 76: 451–460.
14. Vanderkerken K, Goes E, De RH, Radl J, Van CB (1996) Follow-up of bone lesions in an experimental multiple myeloma mouse model: description of an in vivo technique using radiography dedicated for mammography. Br J Cancer 73: 1463–1463.
15. Manning LS, Berger JD, O’Donoghue HL, Sheridan GN, Claringbold PG, et al. (1992) A model of multiple myeloma: culture of 5T33 myeloma cells and evaluation of tumorigenicity in the C57BL/12xRij mouse. Br J Cancer 66: 1099–1093.
16. Vandenberk K, De GC, Assoung K, Arteta B, De VM, et al. (2000) Selective initial in vivo homing pattern of 5T2 multiple myeloma cells in the C57BL/12xRij mouse. Br J Cancer 82: 649–657.
17. Roher JW, Vasa K, Lynch RG (1997) Myeloma cell immunoglobulin expression during in vivo growth in diffusion chambers: evidence for repetitive cycles of differentiation. J Immunol 119: 861–866.
18. Dubey MJ, Bridges SH, Lynch RG (1976) Plasmacytoma spleen colonization: a sensitive, quantitative in vivo assay for idiotype-specific immune suppression of MOPC-315. J Immunol Methods 24: 47–56.
19. Poter M (1972) Immunoglobulin-producing tumors and myeloma proteins of mice. Rev Physiol 52: 631–636.
20. Lynch RG, Graff RJ, Sirinith S, Simms ES, Eisen RN (1972) Myeloma proteins as tumor-specific transplantation antigens. Proc Natl Acad Sci U S A 69: 1540–1544.
21. Oyajobi BO, Weiss S, Dembic Z, Bogen B (1972) Naive idiotype-specific CD4+ T cells and immunosurveillance of B-cell tumors. Proc Natl Acad Sci U S A 69: 5700–5704.
22. Dembic Z, Schenck K, Bogen B (2000) Dendritic cells purified from myeloma are primed with tumor-specific antigen (idiotype) and activate CD4+ T cells. Proc Natl Acad Sci U S A 97: 6097–6092.
23. Vanderkerken K, Skovsheim DK, Lundin KU, Roes J, Omholt H, et al. (2005) Primary antitumor immune response mediated by CD4+ T cells. Immunology 22: 371–383.
24. Vanderkerken K, Lundin KU, Lorvink KB, Holggaard PO, Bogen B (2009) Secretion of tumor-specific antigen by myeloma cells is required for cancer immunosurveillance by CD4+ T cells. Cancer Res 69: 5901–5907.
25. Haebeth OA, Lorvink KB, Harmanstorp C, Donaldson IM, Haraldsen G, et al. (2011) Inflammation driven by tumour-specific Th1 cells protects against B-cell lymphoma. Nat Commun 2: 675.
26. Poter M, Fehrey JL, Pilgrim H (1975) Abnormal serum protein and bone destruction in transmissible mouse plasma cell neoplasm (multiple myeloma). Proc Soc Exp Biol Med 94: 327–333.
27. Kohayashi H, Poter M, Dunn TB (1962) Bone lesions produced by transplanted plasma-cell tumors in BALB/c mice. J Natl Cancer Inst 23: 1088–1093.
28. Corthay A, Lundin KU, Lorvick KB, Hofgaard PO, Bogen B (2009) Liver metastasis of cancer facilitated by chemokine receptor CCR6. Scand J Immunol 77: 533–544.
29. Lauritzsen GF, Bogen B (1993) Liver metastasis of cancer facilitated by chemokine receptor CCR6. Scand J Immunol 73: 1463–1465.
30. Haabeth OA, Lorvick KB, Harmanstorp C, Donaldson IM, Haraldsen G, et al. (2011) Inflammation driven by tumour-specific Th1 cells protects against B-cell lymphoma. Nat Commun 2: 675.
31. Lauritzsen GF, Bogen B (1993) The role of idiotype-specific, CD4+ T cells and immunosurveillance of B-cell tumors. Proc Natl Acad Sci U S A 90: 2437–2444.
32. Kobayashi H, Potter M, Dunn TB (1962) Bone lesions produced by transplanted plasma-cell tumors in BALB/c mice. J Natl Cancer Inst 23: 1088–1093.
48. Eisen HN, Simms ES, Potter M (1968) Mouse myeloma proteins with antihapten antibody activity. The protein produced by plasma cell tumor MOPC-315. Biochemistry 7: 4126–4134.

49. Bogen B, Munthe L, Sollien A, Hofgaard P, Omholt H, et al. (1995) Naive CD4+ T cells confer idiotype-specific tumor resistance in the absence of antibodies. Eur J Immunol 25: 3079–3086.

50. Salamon M, Hanke R, Krüger P, Uhlmann N, Voland V (2008) Realization of a computed tomography setup to achieve resolutions below 1 micrometer. Nuclear Instruments and Methods in Physics Research A 591.

51. Nachtrab F, Salamon M, Burtzlaff S, Porsch F, Johannson W, et al. (2008) Progress in sub-micrometer resolution computed tomography. Nuclear Science Symposium Conference Record, 2008 NSS ’08 IEEE. 532–535.

52. Doube M, Kłosowski MM, Arganda-Carreras I, Cordeilieres FP, Dougherty RP, et al. (2010) BoneJ: Free and extensible bone image analysis in ImageJ. Bone 47: 1076–1079.