



The involvement of stromal derived factor 1 α in homing and progression of multiple myeloma in the 5TMM model

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Background and Objectives. Multiple myeloma (MM) is a lethal plasma cell cancer characterized by the monoclonal growth of cells in the bone marrow. To reach the bone marrow, MM cells need to be attracted by chemokines. Recently, it has been shown that chemokines can also be involved in the growth of several cancer types. Stromal cell derived factor 1 α (SDF1 α) or CXCL12 is known to play an important role as a chemokine for hematopoietic progenitor cells and human MM cells. We studied the effects of SDF1 α in the 5TMM murine model.

Design and Methods. The *in vitro* effects of SDF1 α were analyzed by gelatin zymography, adhesion, migration, proliferation, and chemoinvasion assays and by blockade with the CXCR4 inhibitor, 4F-benzoyl-TN14003. *In vivo*, diseased mice were treated with either vehicle or 4F-benzoyl-TN14003.

Results. *In vitro* SDF1 α was capable of attracting both 5T2MM and 5T33MM cells and inducing a 1.6-fold increase in MMP9 production by the 5TMM cells, which was correlated with an increased invasive capacity. In addition, SDF1 α induced a 20% increase in DNA synthesis in the 5TMM cells. All these effects could be blocked by the CXCR4 inhibitor, 4F-benzoyl-TN14003. An *in vivo* study in the 5T33MM model showed that blocking CXCR4 led to a 20% reduction in bone marrow tumor load.

Interpretation and Conclusions. These data demonstrate that SDF1 α /CXCR4 is involved in the homing and the expansion of MM cells. Blocking CXCR4 could be useful in synergy with other anti-neoplastic treatments targeting the bone marrow microenvironment.

Key words: myeloma, chemokines, SDF1 α .

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Multiple myeloma (MM) is a lethal plasma cell cancer characterized by the monoclonal growth of cells in the bone marrow, where they are capable of inducing osteolysis and angiogenesis. The bone marrow micro-environment provides the MM cells with survival and growth signals.¹⁻⁴ To be able to receive these signals the MM cells must first enter and/or spread through the bone marrow. This process of extravasation from the vascular to the extravascular compartment of the bone marrow is called *homing* and has been thoroughly described for lymphocytes.⁵ Chemokines are involved in this process by attracting and activating the cells. More recently however, several chemokines have been shown to stimulate the growth of different cancer cell types.⁶

The chemokine stromal cell derived factor 1 α (SDF1 α) or CXCL12 was first cloned from mouse bone marrow stromal cells and initially identified as a growth factor for B-cell progenitors and as a chemotactic factor for T cells.⁷⁻⁹ SDF1 α belongs to the CXC subfamily of chemokines and binds to CXCR4, a seven-transmembrane G protein-coupled receptor.⁹⁻¹⁰ SDF1 α is produced by bone marrow stromal cells^{9,11-13} while its receptor is expressed by many different cell types. This chemokine plays an important role in the homing and accumulation of both hematopoietic progenitor cells¹⁴⁻¹⁵ and mature plasma cells¹⁶ in the bone marrow. Moreover, SDF1 α has been

described to be an *in vitro* chemoattractant for both human MM cell lines and human primary MM cells.^{12,17-20} Recently, it has been demonstrated by both *in vitro* and *in vivo* experiments that the SDF1 α /CXCR4 axis also plays a role in the survival and outgrowth of both primary solid tumors²¹⁻²⁴ and their metastases.^{21,25}

In this study we wanted to investigate the *in vitro* (migration, invasion, proliferation and survival) and *in vivo* (tumor promoting) role of SDF1 α in the 5T2MM and 5T33MM murine models of MM. These models originated spontaneously in aging C57BL/KaLwRij mice²⁶ and have been propagated *in vivo* by transplanting bone marrow into young syngeneic recipients through intravenous injection. The clinical characteristics of these models (selective localization in the bone marrow, associated with elevated serum M component and bone marrow angiogenesis) as well as their molecular characteristics (adhesion, chemokine and protease profile and growth factor dependency) closely resemble those of the human disease.²⁷⁻³⁰

Design and Methods

Animals

C57BL/KaLwRij mice were purchased from Harlan CPB (Horst, The Netherlands). Mice were used at 8 to 10 weeks of age. They were

housed and treated according to conditions approved by the Ethical Committee for Animal Experiments, VUB (license no. LA1230281). The animal ethics meet the standards required by the UKCCCR Guidelines (UKCCCR, 1998).

MM models

The 5T33MM cells originated spontaneously in elderly C57BL/KaLwRij mice and have since been propagated, *in vivo*, by intravenous transfer of diseased marrow into young syngeneic mice. The paraprotein was assessed by protein electrophoresis of the serum samples. When the serum concentration reached 10 mg/mL, the mice were sacrificed and the bone marrow was flushed out of the femora and tibiae and crushed out of the vertebrae. The bone marrow cells were suspended in serum-free medium (RPMI 1640 (GIBCO, Life Technologies, Ghent, Belgium), supplemented with penicillin-streptomycin, glutamine, and MEM NEAA-pyruvate (GIBCO). The cells were then purified by Lympholyte M (Cedarlane, Hornby, Canada) gradient centrifugation at 1000 g for 20 min. The cell band on top of the gradient contained enriched 5T2MM or 5T33MM cells, with a purity reaching 85%, as measured by flow cytometric analysis. More than 95% of the cells were viable.

Bone marrow stromal cells and endothelial cells

Bone marrow stromal cell cultures were established by flushing out the content of the femora of the C57BL/KaLwRij mice into Dulbecco's modified Eagle's medium (DMEM), followed by preparation of a mononuclear cell suspension by Lympholyte M centrifugation. The mononuclear cells were plated out at a concentration of 1×10^6 cells/mL in RPMI 1640 (GIBCO) medium supplemented with 12.5% bovine serum (Fetal Clone I, Hyclone, Logan, UT, USA), 12.5% horse serum (Global Pharm, Surrey, UK), 10^{-6} mol/L hydrocortisone (Sigma, St. Louis, MO, USA), penicillin-streptomycin, glutamine, and MEM. The cells were incubated at 37°C in 5% CO₂. After three trypsinizations, cells were cultured until confluence. For conditioned medium, culture medium was removed and the cells were held for 48 h in serum-free conditions in complemented RPMI medium, when a monolayer was formed. This medium was harvested and concentrated five times with Centriprep-3 (Millipore, Bedford, MA, USA). The murine bone marrow sinusoidal endothelial cell line STR4 (BEC STR4) was originally established by transfecting primary endothelial cell cultures with SV40. The cells of the BEC STR4 line were maintained in culture in RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, MEM (GIBCO) and 10% bovine serum (Fetal Clone I, Hyclone). Conditioned medium was collected as described above.

Reagents

The inhibitor for CXCR4 (4F-benzoyl-TN14003) was obtained from Dr. Tamamura, Japan.^{31,32} The inhibitor was dissolved in MilliQ for *in vitro* use; for *in vivo* use, the inhibitor was dissolved in saline (for use in Alzet pumps). Dose-response experiments showed that the effective dose of 4F-benzoyl-TN14003 for 5T2MM was higher than that for 5T33MM, and higher for the migration experi-

ments than for the proliferation experiments. Recombinant mouse SDF1α was obtained from R&D systems (Abingdon, UK) and used at 200 ng/mL.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from 5×10^6 cells was isolated using the SV total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The concentration and purity of RNA were determined by spectrophotometric measurement (Gene Quant II: Pharmacia Biotech, Cambridge, UK). Total RNA was converted into cDNA by the superscript first-strand synthesis system (GIBCO) using random hexamers as primers. The PCR mix contained PCR buffer, 2 mM magnesium chloride, 0.2 mM dNTP, 0.5 µM of each primer and 0.625 U Taq polymerase. The sense and antisense primers used for the amplification of mouse CXCR4 were: 5'-CATGGAACCGATCAGTGTGA-3' and 5'-GGCAGAGCTTTTGAACTTGG-3'. Amplification was performed, for 35 cycles, as follows: denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and polymerization at 72°C for 1 min. The predicted size of the amplified product was 960bp.

Flow cytometric analysis

CXCR4 expression on the 5TMM cells was assessed by double staining with CXCR4-FITC (Becton Dickinson, BD, CA, USA) and tumor-specific anti-idiotypic monoclonal antibodies. Irrelevant isotype-matched antibodies were used as negative controls. Samples were acquired on a FACSCalibur flow cytometer (BD) and CellQuest software was used for analysis.

Enzyme-linked immunosorbent assay (ELISA)

The SDF1α content in the serum and conditioned medium of the several cell types was measured with an ELISA kit for mouse SDF1α (R&D systems). The ELISA was performed according to manufacturers' instructions. The cut-off value of the assay was 0.044 ng/mL.

Thymidine incorporation assays

Cells (1×10^6 /mL) were pretreated with the CXCR4 inhibitor, 2 µM (5T2MM) or 1 µM (5T33MM), and then incubated in serum-free medium with or without SDF1α for 17 hours. Sixteen hours before harvesting, cells were pulsed with 1 µCi (methyl-³H) thymidine (Amersham, Roosendaal, The Netherlands). Cells were harvested by a cell harvester (Inotech, Wohlen, Switzerland) onto paper filters (Filtermat A, Wallac, Turku, Finland). Filters were dried for 1 hour in a 60°C oven and sealed in sample bags (Wallac) containing 4 mL Optiscint Scintillation Liquid (Wallac). Radioactivity was counted using a 1450 Microbeta Liquid Scintillation Counter (Wallac). Results are expressed as the relative DNA synthesis in treated cells compared to in untreated cells.

In vitro migration assay

The *in vitro* chemotactic activity of SDF1α was measured in an 8 µm (5T33MM) or a 5 µm (5T2MM) pore size Transwell® system (Elscolab, Kruikebeke, Belgium). Fibroblast conditioned medium (5× concentrated) was placed in the lower compartment of the system and 5×10^4

5TMM cells in 200 μ L were added in the upper compartment. When indicated, the cells were pre-incubated with either 10 μ M (5T2MM) or 5 μ M (5T33MM) CXCR4 inhibitor for 30 min. The cells were allowed to migrate for 2 hours. After the assay, the migrated cells were quantified by flow cytometry. A known number of Sphero calibration beads (BD) was used as the internal standard.

Chemoinvasion assay

The *in vitro* chemoinvasion activity of SDF1 α was measured in an 8 μ m (5T33MM) or a 5 μ m (5T2MM) pore size Transwell[®] system. Transwells were coated with 32 μ g Matrigel (BD). SDF1 α was placed in the lower compartment of the system and 2×10^5 cells were added in 200 μ L in the upper compartment. The cells were allowed to invade overnight. Quantification and pre-incubation with inhibitor were performed in the same way as for the *in vitro* migration assays.

Gelatin zymography

Cells were plated at $2 \times 10^5/300\mu$ L serum-free medium. The supernatant was harvested after 28 h and electrophoresed under non-reducing conditions using 10% sodium dodecylsulfate-polyacrylamide gels containing 1 mg/mL gelatine. After electrophoresis, the gel was washed in 2% Triton X-100. After overnight incubation in 0.05 M Tris buffer (pH 7.6) containing 10 mM CaCl₂ and 3 mM NaN₃, gels were stained with Coomassie brilliant blue and destained. Gelatinolytic activity was identified as a clear band on a blue background. Supernatant from human fibrosarcoma HT1080 cells was used as a positive control for the detection of the 92 kDa matrix metalloproteinase (MMP9). The inactive form of mouse MMP9 has a molecular weight of 110 kDa and is thus located higher in the gel. The optical density of the MMP9 band was measured with the NIH 1.62 image program. This program has been explained extensively by Leber *et al.*³³ Briefly, a rectangle was drawn around the MMP9 band and another around the background above and below the band. Subsequently, the area and pixel intensity were calculated for each rectangle (band) and the background subtracted.

In vivo immediate homing

In vivo bone marrow homing was analyzed by tracing radioactively-labeled cells. One group of four naive mice was injected intravenously with either 5×10^5 ⁵¹Cr-labeled 5T2MM²⁷ or 5T33MM cells while the other group was injected with MM cells pre-incubated with 10 μ M (5T2MM) or 5 μ M (5T33MM) CXCR4 inhibitor. After 18 h, the animals were sacrificed and the radioactivity in the ribs, vertebrae, hind legs and forelegs was measured and related to injected radioactivity.

In vivo study

Two days before injection with 5TMM cells, Alzet osmotic pumps (duration 28 days; 0.25 μ L/hr, model 2004, ALZA, Mountain View, CA, USA) containing 80mg/mL of 4F-benzoyl-TN14003 or vehicle were implanted subcutaneously into the mice. Two groups of eight mice were injected with 5T33MM and one group of naive mice was added as a negative control. One group

was treated with the inhibitor, the other with the vehicle. At week 3, when the vehicle controls showed signs of morbidity, the mice were sacrificed. Bone marrow was isolated from the hind legs to determine the proportion of tumor cells (FACS staining) and one leg was fixed for CD31 staining. The liver and spleen from all animals were removed and weighed. Blood samples were also obtained to determine serum paraprotein concentrations.

Assessment of microvessel density

Microvessel density (MVD) was determined by CD31 staining as previously described.³⁴ Briefly, one leg was fixed in zinc fixative for 48 h, decalcified for 48 h and embedded in paraffin. After blocking with normal goat serum, sections were incubated with a rat anti-CD31 antibody (PECAM-1, 1/10, Pharmingen, LA, USA) overnight at 4°C. As a secondary antibody, a biotin-conjugated goat anti-rat antibody was used (1/75, Pharmingen). A streptavidin-horseradish peroxidase conjugate in combination with tyramide signal amplification (NEN Life Science Products, MA, USA) was used for detection. In the area with the highest blood vessel density (hot spot), the number of blood vessels per 0.22 mm² was counted.

Statistical analysis

The Mann Whitney test was used for statistical analysis of the *in vitro* data, whereas Student's T-test was used for the *in vivo* data. $p \leq 0.05$ was considered statistically significant.

Results

Expression of SDF1 α and CXCR4

The expression of CXCR4/ SDF1 α was determined in the 5TMM models. We found by RT-PCR that both 5T2MM and 5T33MM cells express CXCR4; bone marrow stromal cells on the other hand, do not express CXCR4 (Figure 1A). Flow cytometric analysis confirmed the membrane expression of CXCR4 on the 5TMM cells but not on the bone marrow stromal cells (Figure 1B). Using an ELISA assay, we found that SDF1 α was present in the serum of both 5TMM models as well as in healthy mice, reaching levels of up to 4 ng/mL (Figure 1C). Furthermore, we determined that the cells of the bone marrow micro-environment, namely the bone marrow stromal cells and bone marrow endothelial cells secrete SDF1 α while the myeloma cells themselves do not. Bone marrow stromal cells also secreted more SDF1 α (3 ng/mL) than STR4 bone marrow endothelial cells (1 ng/mL) (Figure 1D). This demonstrates that there is a CXCR4/SDF1 α axis between the MM cells and the bone marrow micro-environment.

In vitro effects of SDF1 α

The effects of SDF1 α on MM cells were determined *in vitro*. We first examined the known chemotactic activity of SDF1 α . To study this, we let the cells migrate towards fibroblast conditioned medium (5 \times), since this is, in addition to other chemokines, also a source of SDF1 α (Figure 1D). Both 5T2MM and 5T33MM cells (Figure 2A and B) showed a large migratory capacity (30 \times unstimulated

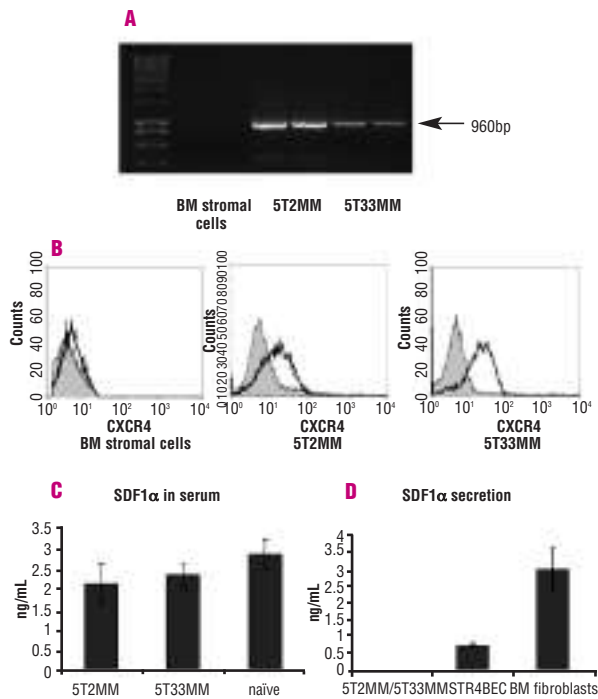


Figure 1. Expression of CXCR4 and SDF1 α . **A.** RT-PCR for CXCR4, which can be detected as a band of 960bp. Both 5T2MM and 5T33MM are positive for CXCR4. Two different samples are shown. **B.** Flow cytometric analysis for CXCR4. The gray filled histogram is the control, the black line CXCR4 staining. The CXCR4 staining is shown on gated α -idiotypic positive cells. 5T2MM and 5T33MM stain positive for CXCR4, whereas bone marrow stromal cells are negative. One experiment, representative of three, is shown. **C.** Concentration of SDF1 α in the sera of healthy and diseased mice measured by ELISA. **D.** Concentrations of SDF1 α in the conditioned media of 5TMM cells, bone marrow endothelial cells (STR4 EC) and bone marrow fibroblasts. The mean value \pm SD of three independent experiments is given.

migration) towards the conditioned medium. To demonstrate that the SDF1 α in the medium can attract MM cells, the cells were pre-incubated with the CXCR4 inhibitor 4F-benzoyl-TN14003. In a first experimental set-up we determined the dose-response curve for the inhibitor with 5T33MM cells (Figure 2A). The optimal dose proved to be 5 μ M (as also determined in the invasion assays, Figure 3A). For 5T2MM cells, slightly higher doses (10 μ M) were needed (*data not shown*). Figure 2B illustrates that 50% inhibition of the migration occurred with 10 μ M (5T2MM) and 5 μ M (5T33MM) of the inhibitor, demonstrating the functional role of CXCR4/SDF1 α in the migration of 5TMM cells towards fibroblast conditioned medium.

We then performed chemoinvasion assays to address the question of whether SDF1 α could also stimulate the invasive capacity of 5TMM cells. Both 5T2MM and 5T33MM cells (Figure 3A and B) were placed in Transwell inserts coated with Matrigel and allowed to invade overnight towards 200 ng/mL SDF1 α . The invasion in the negative control was between 5%-10%. SDF1 α could stimulate chemoinvasion in the 5T33MM cells up to twice control levels, and in 5T2MM cells even up to four times control levels (30%-40% invasion). The optimal dose of the CXCR4 inhibitor was determined for

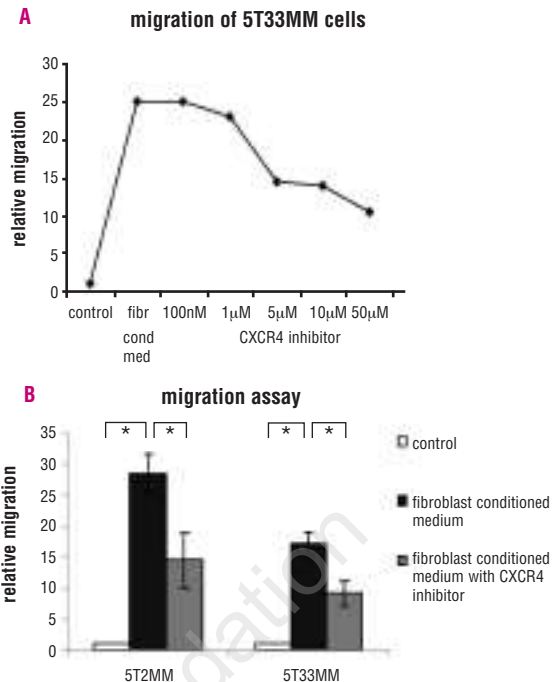


Figure 2. *In vitro* migration of 5TMM cells towards fibroblast conditioned medium. **A.** 5T33MM cells were pre-incubated with different concentrations of the inhibitor for 30 min to determine optimal concentrations (fibr cond med=fibroblast conditioned medium). One experiment, representative of three, is shown. **B.** Cells (5T2MM and 5T33MM) were pre-incubated with either 10 or 5 μ M CXCR4 inhibitor for 30 min. Migration was towards conditioned medium, 5 times concentrated. The percentage migration is shown as a relative value compared to the control value. The mean value \pm SD of three independent experiments is given, * p <0.05.

5T33MM cells (Figure 3). Again, 5 μ M was an optimal dose for 5T33MM cells, with higher doses becoming aspecific. Blocking the CXCR4 receptor with 10 μ M (5T2MM) and 5 μ M (5T33MM) of the inhibitor completely inhibited the chemoinvasion in both models (Figure 3B). Tests with the human MM cell line, Karpas, showed similar results (*data not shown*).

To make certain that this effect was due to stimulation of invasion and not solely to the chemotactic response, we analyzed the effect of SDF1 α on the production of MMP9 by the MM cells. MMP9 is known to be involved in the invasion process of both 5T33MM cells (*unpublished data*) and human cells.³⁵ Using gelatin zymography (Figure 3C) we found that SDF1 α had no effect on MMP9 production by naive bone marrow cells, but that it could induce a 1.6-fold increase in MMP9 production by 5T2MM cells and a 1.3-fold increase by 5T33MM cells. These increases could be almost completely blocked by the CXCR4 inhibitor, suggesting that the stimulatory effect of SDF1 α on the chemoinvasion of 5TMM cells is due to both upregulation of MMP9 and increased migration. Finally, we examined the capacity of SDF1 α to stimulate proliferation and inhibit apoptosis in 5TMM cells. When MM cells from both models (Figure 4) were incubated with 200 ng/mL SDF1 α , their DNA synthesis

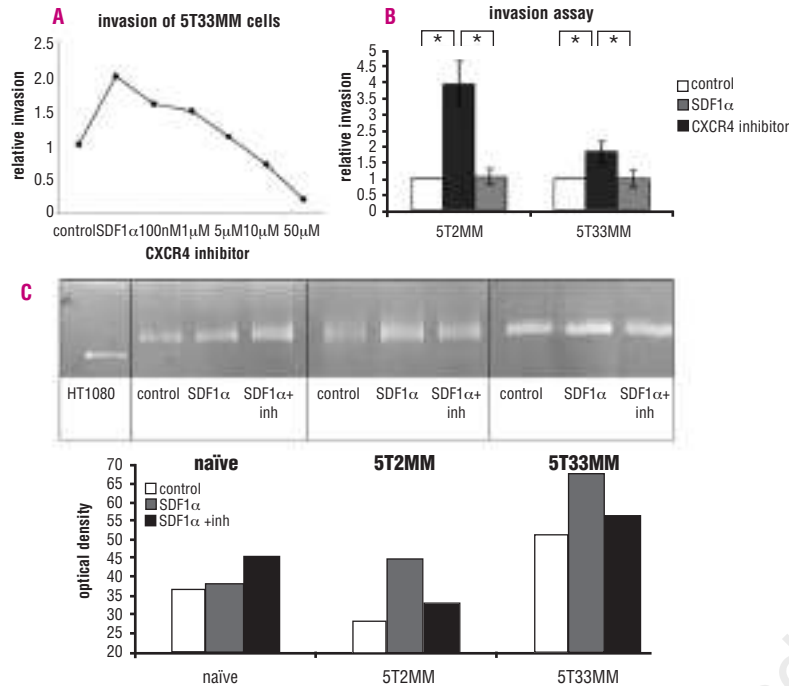


Figure 3. Chemoinvasion of 5TMM cells towards SDF1 α and their MMP9 secretion. **A.** 5T33MM cells were pre-incubated with different concentrations of the inhibitor for 30 min to determine optimal concentrations. Invasion was towards 200 ng/mL SDF1 α , overnight. One experiment, representative of three, is shown. **B.** Cells (5T2MM and 5T33MM) were pre-incubated with either 10 or 5 μ M CXCR4 inhibitor for 30 min. Invasion was towards 200 ng/mL SDF1 α , overnight. The percentage invasion is shown as a relative value compared to the control. The mean value \pm sd of three independent experiments is given, $*p < 0.05$. **C.** The top panel shows a gelatin zymography for MMP9 while the bottom panel shows the density of the MMP9 bands. The positive control (supernatant from human fibrosarcoma HT1080) for human MMP9 is at 92 kDa, while mouse MMP9 is 110 kDa. SDF1 α induces an increase in MMP9 activity. One experiment, representative of three, is shown, $p > 0.05$ for naive cells and $p < 0.03$ for 5T2MM and 5T33MM.

increased by 20%, and this could be completely blocked by 2 μ M (5T2MM) or 1 μ M (5T33MM) of the CXCR4 inhibitor, demonstrating that SDF1 α does, indeed, have a proliferative effect in MM, although this effect is more modest than its chemotactic function. We also studied the influence of SDF1 α on apoptosis by caspase 3 staining but found no significant effect in either mouse model (*data not shown*).

Effect of SDF1 α on immediate bone marrow homing

As SDF1 α has a strong chemotactic effect *in vitro*, we wanted to investigate whether it is involved in the homing of MM cells towards the bone marrow *in vivo*. We injected ^{51}Cr -labeled 5TMM cells, pre-incubated with either 4F-benzoyl-TN14003 or vehicle, into naive mice. After 18 h, the bone marrow homing of 5T2MM cells was reduced by 40% and the homing of 5T33MM cells by 25% after incubation with the inhibitor (Figure 5). This confirms that SDF1 α is indeed involved in the bone marrow homing of MM cells.

In vivo effects of SDF1 α in the 5T33MM model

Since SDF1 α has multiple *in vitro* effects on both the homing capacity and the proliferative capacity of 5TMM cells, we investigated the role of the chemokine in the development of MM *in vivo*. To this end, 16 mice were treated with either 80mg/mL 4F-benzoyl-TN14003 or the vehicle (saline) from injection with 5T33MM onward. The mice treated with the inhibitor had a 20% reduction in tumor load (Figure 6A), and a correlated 30% reduction in paraprotein levels (Figure 6B), demonstrating that SDF1 α is indeed involved in MM development. Recently it has been shown that SDF1 α could be involved in neo-vascularization in solid tumors.^{36,37} To study this, we examined microvessel density. With this technique, we have previously shown that 5T33MM cells induce angio-

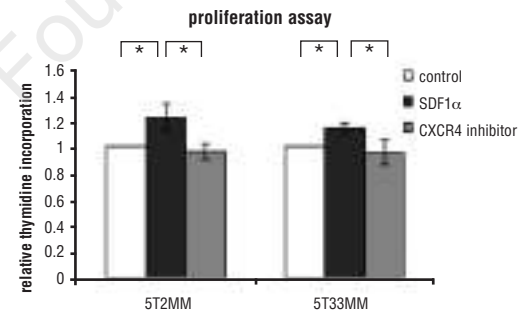


Figure 4. Proliferation of 5TMM cells induced by SDF1 α . Cells (5T2MM and 5T33MM) were pre-incubated with either 2 or 1 μ M CXCR4 inhibitor for a half hour, followed by incubation with 200 ng/mL SDF1 α . The percentage proliferation is shown as a relative value compared to the control value. The mean value \pm SD of three independent experiments is given, $*p < 0.05$.

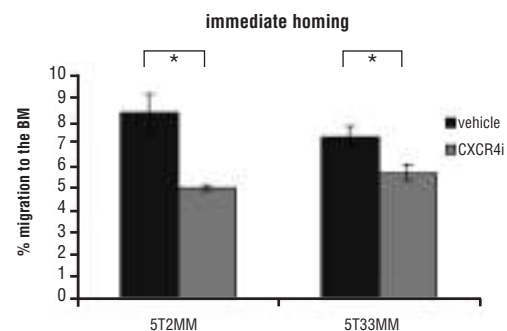


Figure 5. *In vivo* effect of SDF1 α on the homing of 5TMM cells. Cells (5T2MM and 5T33MM) were labeled with ^{51}Cr and then pre-incubated with either 10 or 5 μ M CXCR4 inhibitor (CXCR4i) for a half hour and then injected into naive mice ($n = 4$). After 18 h the radioactivity in the bone marrow was measured and related to the total radioactivity; this percentage is shown. One experiment, representative of two, is shown. $*p < 0.05$.

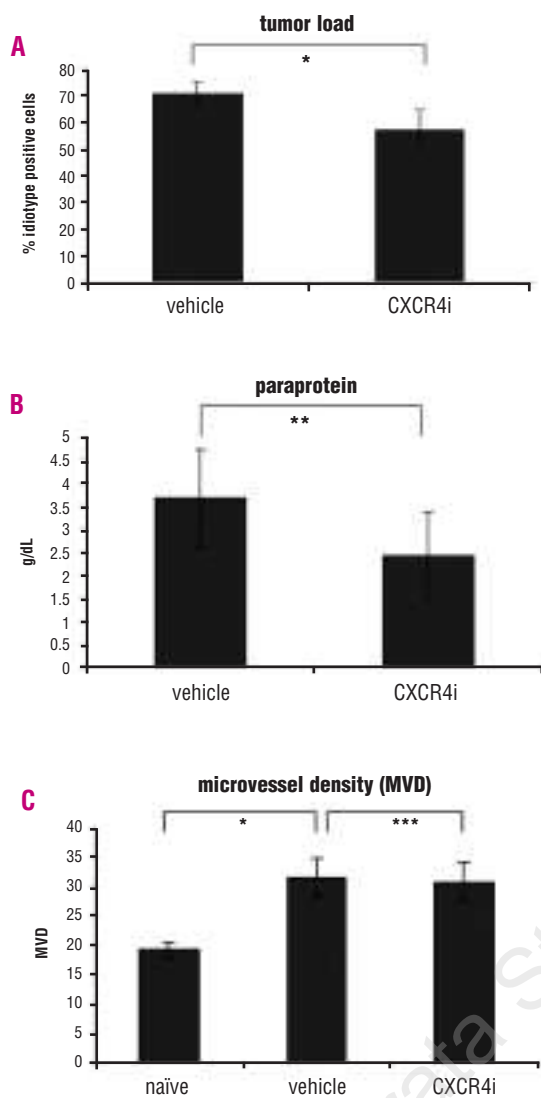


Figure 6. *In vivo* effect of inhibiting CXCR4. Pumps implanted in the mice ($n = 8$) secreted 0.25 μL of a 80mg/L solution of 4F-benzoyl-TN14003 daily. **A.** The effect of 4F-benzoyl-TN14003 on the tumor load, measured as the number of idiotype positive cells in the bone marrow. **B.** The effect on serum paraprotein concentration, measured by electrophoresis. **C.** The effect on microvessel density (MVD) given by the number of microvessels in the tibiae and femora of the mice, counted by CD31 staining. The mean value \pm SD is given, * $p < 0,001$, ** $p < 0,03$, *** $p > 0,05$.

genesis *in vivo*.³⁴ Here, immunohistochemical staining for CD31 showed an almost 2-fold increase in tumor bearing mice, compared to naïve mice. However, mice treated with 4F-benzoyl-TN14003 had only a slight, but not significant, reduction in microvessel density, suggesting that SDF1 α is not involved in MM-induced angiogenesis (Figure 6C).

Discussion

MM still remains a deadly malignancy, despite emerging therapies and extensive research. One of the main characteristics of the disease is the localization of the malignant plasma cells in the bone marrow. This is most

likely due to a combination of selective homing and selective survival of the MM cells in the bone marrow.²⁷ The process of homing of MM cells is thus an important event in the development of the disease and is dependent on several different chemokines. SDF1 α is a known chemokine for hematopoietic progenitor cells and human MM cells.^{14,17} Our group has recently found that in 80 bone marrow samples from MM patients, 59% were positive for CXCR4.³⁸ Moreover, Pellegrino *et al.*³⁹ demonstrated that bone marrow endothelial cells of MM patients secrete higher levels of SDF1 α than do healthy human umbilical vein endothelial cells. In the present study, we wanted to determine both the *in vitro* and *in vivo* effects of SDF1 α in the 5T2MM and 5T33MM mouse models. We first demonstrated the secretion of SDF1 α by both the bone marrow stromal cells and endothelial cells. We found no SDF1 α secretion by the 5TMM cells although they were positive for SDF1 α by RT-PCR analysis (*data not shown*). It could be that 5TMM cells secrete amounts of SDF1 α that are lower than the detection limit of the available ELISA or it could be that there was no translation of the SDF1 α mRNA. On the other hand, 5TMM cells did express CXCR4 on their membrane whereas bone marrow stromal cells did not, creating an SDF1 α /CXCR4 axis.

We next investigated the *in vitro* effects of SDF1 α on 5TMM cells. The adhesion to endothelial cells, the first step in the process of homing, was studied first. Although several studies in solid tumors and lymphocytes have shown that SDF1 α enhanced adhesion^{32,40-41} to extracellular matrix proteins, we found that SDF1 α had no effect on the adhesion of the 5TMM cells to STR4 bone marrow endothelial cells (*data not shown*). We then performed migration and chemoinvasion assays for the other processes involved in homing. Migration of the 5TMM cells towards bone marrow stromal cell conditioned medium was reduced by 50% with the CXCR4 inhibitor, 4F-benzoyl-TN14003, and exogenously added SDF1 α could induce a 4-fold increase in invasion. Similar results were obtained with the human MM cell line, Karpas (*data not shown*). This increase in invasion was correlated to an increased secretion of MMP9, showing that SDF1 α is capable of inducing MMP9 expression by MM cells. These data confirm and extend work in the SCID/hu model mentioned by J. Epstein.⁴² SDF1 α has also been described to induce MMP9 secretion in other tumor models.^{43,44} For the definitive test of whether SDF1 α is really involved in the process of homing of MM cells *in vivo*, we performed an immediate homing analysis. 4F-benzoyl-TN14003 blocked the immediate homing of 5T2MM cells by 40% and that of 5T33MM cells by 25%, confirming the important role of SDF1 α in the homing of MM cells to the bone marrow. The CXCR4 inhibitor 4F-benzoyl-TN14003 has been shown to be specific for the CXCR4 receptor by Tamamura *et al.* in calcium fluorimetry experiments with CXCR4-transfected and non-transfected cells.⁴⁵ Since it has recently been shown^{20,22,23} that SDF1 α is capable of inducing proliferation of cancer cells, we further investigated the role of this chemokine on 5TMM cell survival and proliferation. SDF1 α induces a 20% increase in DNA synthesis in 5TMM cells. Compared to the increase caused by the known growth factor, IGF-1,⁴⁶

this is only a modest increase, but is in agreement with the study performed by Hideshima *et al.*²⁰ on human MM cells, which also demonstrated a modest SDF1 α -induced increase in proliferation. However, in contrast to others (studies on stem cells⁴⁷ and MM cells⁴⁸) we found no significant effect of SDF1 α on basal or dexamethasone induced apoptosis of 5TMM cells (*data not shown*). The study by Hideshima²⁰ also demonstrated only a modest inhibition of dexamethasone-induced apoptosis by SDF1 α in human MM cells. These discrepancies between the different studies are probably due to the different cell lines used.

We used the 5T33MM model to study the effects of blocking the SDF1 α /CXCR4 axis *in vivo* on the development of MM. This model develops bone marrow-restricted MM in 3-4 weeks and is thus suitable for studying the direct effects of the CXCR4 inhibitor on the tumor load. Mice that were continuously treated with 4F-benzoyl-TN14003 showed a 20% lower tumor load compared to that of vehicle-treated mice. This demonstrates for the first time that SDF1 α is indeed involved in MM development. However, compared to treatment studies targeting growth factors such as IGF-1,⁴⁹ this effect is of a smaller scale. Since the effects of SDF1 α on migration and invasion are more pronounced than its effects on proliferation, and since blocking SDF1 α reduces immediate homing by 25%, SDF1 α is probably more involved in the homing of the MM cells toward the bone marrow than in their survival within this compartment. Moreover, a study from our group has previously shown that when the immediate homing of 5TMM cells becomes impaired, for example, by blocking an adhesion molecule (CD44v10) involved in the adhesion of MM cells to bone marrow endothelial cells, this leads to a similar decrease in tumor load at the end stage of the disease.⁵⁰

Recent studies^{36,37} have shown that SDF1 α can also be involved in angiogenesis in solid tumors. Martin *et al.*⁵¹ showed that SDF1 α alone could not stimulate angiogene-

sis, but was able to do so in synergy with RANKL. RANKL is also expressed in the 5T33MM model, making this a suitable model for studying such an interaction.⁵² We compared microvessel density between 4F-benzoyl-TN14003-treated mice and vehicle-treated mice. We did not, however, find any significant difference, suggesting that SDF1 α does not have a direct effect on the induction of angiogenesis in the 5T33MM model. The fact that no expression of CXCR4 on bone marrow stromal cells or STR4 bone marrow endothelial cells (*data not shown*) was found by RT-PCR supports this finding.

In summary, we conclude that SDF1 α plays an important role in the immediate homing and spreading of MM cells through the bone marrow. In contrast to its role in certain solid tumors, the role of SDF1 α in tumor survival of MM is less pronounced. Nonetheless, treating mice with 4F-benzoyl-TN14003 *in vivo* did lead to a 20% reduction in tumor load. Targeting CXCR4 in the treatment of MM could thus be useful in synergy with other anti-neoplastic treatments acting on the bone marrow microenvironment.

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