Stem Cells and Development

Stem Cells and Development: http://mc.manuscriptcentral.com/scd

Efficacy assessment of interferon-alpha-engineered mesenchymal stromal cells in a mouse plasmacytoma model

Journal:	*Stem Cells and Development*
Manuscript ID:	SCD-2010-0095.R1
Manuscript Type:	Original Research Report
Date Submitted by the Author:	09-Jun-2010
Complete List of Authors:	Sartoris, Silvia; Section of Immunology, Dpt. of Pathology and Diagnostics, University of Verona Mazzocco, Marta; Section of Immunology, Dpt. of Pathology and Diagnostics, University of Verona Tinelli, Martina; Stem Cell Research Laboratory, Section of Hematology, Dpt. of Medicine, University of Verona Martini, Matteo; Section of Immunology, Dpt. of Pathology and Diagnostics, University of Verona Mosna, Federico; Stem Cell Research Laboratory, Section of Hematology, Dpt. of Medicine, University of Verona Lisi, Veronica; Stem Cell Research Laboratory, Section of Hematology, Dpt. of Medicine, University of Verona Indraccolo, Stefano; Istituto Oncologico Veneto, IRCCS Moserle, Lidia; Section of Oncology, Dept. of Oncology and Surgical Sciences Cestari, Tiziana; Section of Immunology, Dpt. of Pathology and Diagnostics, University of Verona Riviera, Anna Pia; Section of Immunology, Dpt. of Pathology and Diagnostics, University of Verona Bifari, Francesco; Stem Cell Research Laboratory, Section of Hematology, Dpt. of Medicine, University of Verona Tridente, Giuseppe; Section of Immunology, Dpt. of Pathology and Diagnostics, University of Verona Pizzolo, Giovanni; Stem Cell Research Laboratory, Section of Hematology, Dpt. of Medicine, University of Verona Krampera, Mauro; Stem Cell Research Laboratory, Section of Hematology, Dpt. of Medicine, University of Verona
Keyword:	Tissue specific stem cells, MSC, Genetic Engineering

Efficacy assessment of interferon-alpha-engineered mesenchymal stromal cells in a mouse plasmacytoma model

Silvia Sartoris[1], Marta Mazzocco[1], Martina Tinelli[2], Matteo Martini[1], Federico Mosna[2], Veronica Lisi[2], Stefano Indraccolo[3], Lidia Moserle[4], Tiziana Cestari[1], Anna Pia Riviera[1], Francesco Bifari[2], Giuseppe Tridente[1], Giovanni Pizzolo[2], and Mauro Krampera[2].

[1]Department of Pathology and Diagnostics, Section of Immunology and [2]Department of Medicine, Section of Hematology, Stem Cell Research Laboratory, University of Verona, Policlinico 'G.B. Rossi', P.le L.A. Scuro 10, 37134 VERONA, Italy;
[3]Istituto Oncologico Veneto- IRCCS; [4]Department of Oncology and Surgical Sciences, Section of Oncology, University of Padua, via Gattamelata 64, 35128 PADOVA, Italy.

S.S. and M.M. contributed equally to this study.

Running title: Efficacy assessment of engineered MSCs in myeloma

Keywords: mesenchymal stromal cells, myeloma, interferon-α

Correspondence to:

Silvia Sartoris, PhD
Sezione di Immunologia
Policlinico 'G.B. Rossi'
P.le L.A. Scuro 10
37134 VERONA - Italy
Tel. ++39 045 812.6451
Fax ++39 045 802.7468
E-mail: silvia.sartoris@univr.it

Mauro Krampera, MD, PhD
Sezione di Ematologia
Policlinico 'G.B. Rossi'
P.le L.A. Scuro 10
37134 VERONA - Italy
Tel. ++39 045 812.4173-812.4420
Fax ++39 045 802.7488
E-mail: mauro.krampera@univr.it

ABBREVIATION LIST

7-AAD = 7-Amino-Actinomycin D

Ann-V = annexin-V

BM = bone marrow

CMV = cytomegalovirus

EFS = event free survival

EGFP = enhanced green fluorescent protein

FACS = fluorescence-activated cell sorting

FBS = fetal bovine serum

G418 = geneticin sulphate

GvHD = graft-*versus*-host disease

HIV = human immunodeficiency virus

IFN = interferon

i.v. = intravenously

MGG = May Gruenwald Giemsa

MHC = major histocompatibility complex

MM = multiple myeloma

MSC = mesenchymal stromal cell

MVD = mean vascular density

NK = natural killer

OS = overall survival

s.c. = subcutaneously

α-SMA = α-smooth muscle actin

TU = transducing unit

VSV-G = protein G of vescicular stomatitis virus

vWF = von Willebrand factor

Mary Ann Liebert Inc., 140 Huguenot Street, New Rochelle, NY 10801

ABSTRACT

Bone marrow mesenchymal stromal cells (BM-MSCs) may survive and proliferate in the presence of cycling neoplastic cells. Exogenously administered MSCs are actively incorporated in the tumor as stromal fibroblasts, thus competing with the local mesenchymal cell precursors. For this reason, MSCs have been suggested as a suitable carrier for gene therapy strategies, as they can be genetically engineered with genes encoding for biologically active molecules, which can inhibit tumor cell proliferation and enhance the anti-tumor immune response.

We used BM-MSCs engineered with the murine interferon-alpha (IFN-α) gene (BM-MSCs/IFN-α) to assess in a mouse plasmacytoma model the efficacy of this approach towards neoplastic plasma cells. We found that IFN-α can be efficiently produced and delivered inside the tumor microenvironment. Subcutaneous multiple administration of BM-MSCs/IFN-α significantly hampered the tumor growth *in vivo* and prolonged the overall survival of mice. The anti-tumor effect was associated with enhanced apoptosis of tumor cells, reduction in microvessel density, and ischemic necrosis. By contrast, intravenous administration of BM-MSCs/IFN-α did not significantly modify the survival of mice, mainly as a consequence of an excessive entrapment of injected cells in the pulmonary vessels.

In conclusion, BM-MSCs/IFN-α are effective in inhibiting neoplastic plasma cell growth; however, systemic administration of engineered MSCs still needs to be improved to make this approach potentially suitable for the treatment of multiple myeloma.

INTRODUCTION

Multiple myeloma (MM) is a malignant plasma cell disorder accounting for approximately 10% of all hematological cancers [1]. Despite the fact that standard chemotherapy is usually effective in lowering the disease burden, complete remission is achievable only in a minority of patients, and responses are rarely persistent [2]. Several randomized studies have shown the improvement of both disease-free and overall survival by using autologous hematopoietic stem cell transplantation as compared with standard chemotherapy [3,4]. However, this approach is not curative, because of the persistence and ultimate recurrence of chemotherapy-resistant disease. Recent studies based on the use of biological agents, such as thalidomide, lenalidomide or bortezomib, expecially when used in combination with dexamethasone, have shown that a significantly higher number of patients may achieve clinical major responses [5,6]. Allogeneic hematopoietic stem cell transplantation is curative for a small subset of patients developing graft-*versus*-myeloma effect, but it is associated with significant morbidity and mortality as a consequence of the regimen-related toxicity and because of infections and tissue injury due to graft-*versus*-host disease (GvHD). Thus, MM still remains an uncurable disease [7]. The treatment of MM with interferon-alpha (IFN-α) has been used for years as maintenance therapy, as it may prolong survival by delaying tumor progression [8]. Mesenchymal stromal cells (MSCs) are non-hematopoietic progenitor cells endowed with multilineage differentiation potential into various cell types, including fibroblasts, adipocytes, osteoblasts, chondrocytes, skeletal and cardiac myocytes and, under specific experimental conditions, neural cells [9]. MSCs have powerful regulatory properties towards practically all immune effectors, including T, B, NK and dendritic cells, and they can modulate *in vivo* autoreactive immune responses, such as GvHD [10]. MSCs may also support tumor development, as they inhibit the specific immunity against neoplastic cells [11,12]. Studies in rodents show an initial broad bio-distribution of systemically infused MSCs, followed by a small capacity of long-term engraftment [13]. However, they may survive and proliferate in the presence of cycling neoplastic cells, and are incorporated in tumors as stromal fibroblasts [14], thus competing with the

Mary Ann Liebert Inc., 140 Huguenot Street, New Rochelle, NY 10801

local mesenchymal cell precursors [15]. At the same time, they are capable of promoting metastatic dissemination of primary tumors in some animal models [16].

MSCs can be a suitable tool for gene therapy strategies [17]. In fact, they can be genetically engineered with genes encoding for active molecules that can target specifically cancer cell proliferation, reduce MSC immune regulatory properties and eventually enhance the anti-tumor immune response. MSC transduction with cDNAs coding for factors with anti-tumor activity, such as IFN-α and –β, may represent a promising approach for cancer treatment [14,18].

In our study, we transduced mouse bone marrow derived MSCs (BM-MSCs) with IFN-α cDNA (BM-MSCs/IFN-α) and we analyzed their effects *in vivo* in the Sp6 plasmacytoma mouse model, which can be either hampered by the onset of specific immune response [19] or favoured by the injection of BM-MSCs [12].

MATERIALS AND METHODS

Cell lines

Sp6 hybridoma cells, derived from the P3/X63-Ag8 mouse plasmacytoma [20], were used for *in vitro* and *in vivo* experiments in mouse. Sp6 is a Balb/c mouse plasmacytoma induced by mineral oil and displaying prevalent peritoneal localization [21], although it can also ultimately localize into the bone-marrow (BM) after parenchymal dissemination (*SS, personal observation*), when injected intravenously (i.v.), as described for mouse S107 e X24 plasmacytomas [22]. Sp6 cells are highly tumorigenic when injected subcutaneously (s.c.) in syngeneic Balb/c mouse strain [20] and their growth is favored *in vivo* by the presence of MSCs of different sources [12]. Sp6 cells were grown *in vitro* in RPMI 1640 (Gibco Invitrogen Corporation, San Diego, CA, USA) supplemented with 10% fetal bovine serum (FBS; Euroclone, Pavia, Italy) and 5% L-glutamine (Biochrom AG, Berlin, Germany).

Mouse BM-MSCs were obtained from 7-10 week-old Balb/c (Balb/cByJIco, Charles River Italia, Calco, LC, Italy), as previously described [12,23-25].

The 293T human kidney cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and grown in DMEM supplemented with 10% FBS. 293T cells were used both as vector packaging cells and for titration experiments.

Plasmids

The human immunodeficiency virus type 1 (HIV-1)-derived lentiviral vectors used in this study were generated by exploiting the transient three-plasmid vector-packaging system based on 293T cells, as previously described [26]. The construct pRRL-SIN18-cPPT-insert-PRE, expressing either the enhanced green fluorescent protein (EGFP) or the murine IFN-α gene from an internal cytomegalovirus (CMV) promoter, was used as transfer vector [27]. The genomic RNA was packaged in lentiviral particles produced by transfection of pCMVΔR8.74, a plasmid carrying the

Mary Ann Liebert Inc., 140 Huguenot Street, New Rochelle, NY 10801

genes encoding HIV-1 Gag and Pol, and pseudotyped with protein G of vescicular stomatitis virus (VSV-G) [28].

Transduction of BM-MSCs and Sp6 cells

Lentiviral vectors were generated by transfection of 293T cells with 12 μg of pRRL-SIN18-cPPT-insert-PRE, 6 μg of pCMVΔR8.74, and 0.3 μg of the VSV-G expression construct. The day before transfection, $5.0x10^6$ 293T cells were seeded in 75-cm^2 tissue culture flasks. The cultures were transfected with plasmid DNA by means of a calcium phosphate precipitation technique. Fresh medium was added to the cultures 12-18 hours before the supernatants were collected and filtered through 0.45 μm pore filter. To determine viral titer in experiments with the EGFP-coding lentiviral vector, serial dilutions of the filtered supernatants were added to 293 target cells that had been previously seeded into 96-well culture plates the day before infection at $4x10^3$ cells per well concentration. After 6 hours at 37°C, the supernatant was replaced with fresh medium; 72 hours later, EGFP expression was detected by epifluorescence microscopy. The titer, expressed as transducing units (TU) per milliliter of supernatant, ranged between $2x10^6$ and $4x10^6$ TU/ml.

Transduction of BM-MSCs cells was performed with cell-free supernatant with either the EGFP- or the murine IFN-α-coding lentiviral vector. To this aim, variable volumes of supernatant, containing identical amounts of vector particles, were incubated with $4x10^4$ target cells in 6-well plates for 6 hours at 37°C. After 72 hours, cells were fixed with phosphate-buffered saline (PBS)-1% formaldehyde and analyzed for EGFP expression either by fluorescence-activated cell sorting (FACS) or by epifluorescence microscopy. In a set of experiments BM-MSCs were sequentially transduced by the EGFP-coding lentiviral vector followed, one week later, by transduction with the IFN-α-coding lentiviral vector.

Sp6 cells transduced through electroporation (250V and 250μF) with the cDNA coding for IFN-α (Sp6/IFN-α) were also produced and used as control to check the IFN-α production inside the tumor in the absence of BM-MSCs/IFN-α. The selection of transfectants was carried out by

Mary Ann Liebert Inc., 140 Huguenot Street, New Rochelle, NY 10801

growing cells in the presence of 0.75 mg/ml G418 (Geneticin G418 sulphate, Invitrogen Corporation, San Diego (CA) USA).

Growth capacity, immunophenotype and in vitro multilineage differentiation ability of BM-MSCs were tested either before and after transduction, according to previously described methods [29,30]. The expression of IFN-R (anti-mIFN-α/β R2, R&D, Minneapolis (MN) USA) by Sp6 cells was tested by flow cytometry on in *vitro* growing cells before and after transduction; no significant difference in expression was detected (data not shown)."

Quantification of murine IFN-α protein levels by ELISA

An ELISA assay (PBL Biomedical Laboratories, Piscataway, NY, USA) was used to quantify the levels of murine IFN-α in the supernatants of cells transduced to express the cytokine. The range of sensitivity of the assay was 12.5-500 pg/ml; according to the available literature and the Manufacturer's instructions, 10 pg of IFN-α are considered equivalent to 1 IU.

Immunofluorescence analysis and monoclonal antibodies

Cells were analyzed by immunofluorescence and flow cytometry (FACScalibur, Becton Dickinson-Pharmigen, Milan, Italy). Sp6 cells were analyzed as previously described [19]. Mouse BM-MSCs were studied for the expression of CD11c, CD90, CD105, CD44, CD14, CD80, Fas, Fas-L, CD106, MHC class I (H-2^d), and interferon-gamma (IFN-γ)-receptor (CD119). In addition, we assessed the absence of CD31, CD45, CD86, and MHC class II (I-A/I-E) expression, as previously described [23,30,31]. CD90 and CD105 were purchased from eBioscience, Frankfurt, Germany. All other antibodies were purchased from Becton Dickinson-Pharmigen, Milan, Italy. Detached cells were analyzed by flow cytometry (Software CellQuest, FACScalibur, Becton Dickinson-Pharmigen, Milan, Italy) and 10^4 cells were acquired for each sample.

Co-culture experiment and evaluation of apoptosis in vitro

Mary Ann Liebert Inc., 140 Huguenot Street, New Rochelle, NY 10801

BM-MSCs or BM-MSCs/IFN-α were co-cultured with Sp6 cells to asses their effect on Sp6 proliferation and survival *in vitro*. First of all, $75x10^3$ BM-MSCs and BM-MSCs/IFN-α were seeded in a 6-well culture plate (Multiwell™, BD, Le Pont De Claix, France) in RPMI 1640 medium supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin (all from Gibco, Milan, Italy). After 24 hours, BM-MSCs and BM-MSCs/IFN-α, now forming a homogeneous confluent monolayer, were irradiated at 6000 cGy. $75x10^3$ Sp6 cells were then seeded on top of them (1:1 ratio), and co-cultured in 4 ml medium for 5 days at 37°C in a 5% CO_2 atmosphere. Experimental conditions included: Sp6 alone, Sp6 + BM-MSCs either in direct contact or separated by a Transwell™ filter (pore size: 0.4 µm filter, BD, Le Pont De Claix, France), Sp6 + recombinant IFN-α 120 IU/ml, Sp6 + BM-MSCs + recombinant IFN-α 120 IU/ml or Sp6 + BM-MSCs/IFN-α either in direct contact or separated by a Transwell™ filter. Sp6 cells, growing in suspension, were collected and stained for Annexin-V (Ann-V), CD45 and 7-Amino-Actinomycin D (7-AAD). The fraction of apoptotic (Ann-V-positive/7-AAD-negative) or dead (Ann-V-positive/7-AAD-positive) Sp6 cells was measured after gating for the $CD45^{pos}$ cell fraction by flow cytometry. At least three independent experiments were performed for each condition.

In vivo experiments

Two months-old Balb/c mice ($H\text{-}2^d$) were injected subcutaneously (s.c.) with $0.5x10^6$ Sp6 cells ($H\text{-}2^d$), resuspended in 0.2 ml of PBS. The dose of $0.5x10^6$ cells is tumorigenic *in vivo* in 100% of syngeneic animals**.** Different conditions were performed: mice injected with $0.5x10^6$ Sp6 cells (n=15); mice injected with $0.5x10^6$ Sp6/IFN-α cells (n=15); mice injected with Sp6 and $0.5x10^6$ BM-MSCs for one (n=20) or three weekly doses (n=10); mice injected with Sp6 and either i.v., once a week, with $0.5x10^6$ BM-MSCs/IFN-α (three doses) or s.c for one (n=20), four (n=35) or 8 total doses (n=15). All s.c. injections were performed in the same anatomical quarter (usually within a radius of 0.5 cm around the first inoculum), as previously described [19]. In order to test *in vivo* engraftment of BM-MSCs inside Sp6 tumor, a subgroup of mice was injected s.c. simultaneously

Mary Ann Liebert Inc., 140 Huguenot Street, New Rochelle, NY 10801

with Sp6 and with either single-transduced BM-MSCs/EGFP or with double-transduced BM-MSCs/EGFP/IFN-α, s.c. or i.v.

All animals were then euthanized when the subcutaneously-growing tumoral mass reached a maximal diameter of 2 cm (volume = 4.187 cm^3), or became ulcerated. If signs of pain and fatigue became evident in the animals earlier, they were sacrified immediately. We evaluated tumor volumes considering Sp6 as growing subcutaneously as a sphere (mm^3). All *in vivo* experiments were part of a Research Protocol already notified to the Italian Ministry of Health on February 19th, 2008 (Protocol n.030/2006).

Immunohistochemistry and immunofluorescence

Tumors developing in the mice were excised and snap-frozen in liquid nitrogen in an OCT compound (Tissue-Tek, Elkhart, IN, USA). Sections of 5-8 μm thickness were then cut using a cryostat, fixed with cold aceton for 5 minutes, air-dried and stored at -80 °C. In the case of May-Gruenwald Giemsa (MGG), staining was then performed on PBS-rehydrated slides as per Manufacturer's instructions. In the case of immunofluorescent staining, slides were rehydrated in PBS, and antigen retrieval was done by microwaving (400 W for four minutes) in sodium-citrate/citric acid buffer (pH=6). Primary antibodies were used as follows: α-smooth muscle actin, α-SMA (1:400, Sigma-Aldrich, USA), von Willebrand factor, vWF (1:100, DAKO, Milan, Italy), anti-mouse CD4 (1:50, Becton Dickinson-Pharmigen, Milan, Italy), anti-mouse CD8 (1:50, Becton Dickinson-Pharmigen, Milan, Italy), anti-asialo GM1 (1:100, Wako Chemical GmbH, Neuss, Germany), anti-mouse CD45 (1:40, Becton Dickinson-Pharmigen, Milan, Italy), anti-mouse CD90 (1:50, Becton Dickinson-Pharmigen, Milan, Italy), anti-IFN-α (1:50, PBL Biomedical Laboratories, Piscataway, NY, USA), anti-cleaved caspase 3 (Asp 175) (1:100, Cell Signaling, Beverly, MA) and anti-IFN-R-type I (CD118) (1:50, Abcam, Cambridge, UK).

To track BM-MSCs into the tumors after s.c. or i.v. injection, we used double-transduced BM-MSCs/EGFP/IFN-α. Transfection efficiency and EGFP-espression, measured *in vitro*, were

checked after the transduction and immediately before the injection. To detect EGFP-producing cells, tumor sections were stained with a primary rabbit polyclonal anti-EGFP antibody, (1/40, Invitrogen Corporation, San Diego, CA, USA), followed by a donkey anti-rabbit FITC-conjugated secondary Ab, (1/50, Jackson ImmunoResearch, West Grove, PA, USA). Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole), 1 ng/ml, and slides were finally treated with Sudan Black, 1:1000 in a 70% ethanol solution, to increase contrast. Images were acquired using an automated epifluorescent Zeiss Axiovert™ Z1 microscope and analyzed by the imaging software Zeiss Axiovision™.

Morphometry

Tumor sections of 5-8 μm were processed for immunohistochemistry by standard procedures. Tumor vessels were labeled using immunofluorescent staining for vWF and α-SMA. Negative controls were performed in all cases by omitting the primary antibody. Mean vascular density (MVD) was measured by using a score based on the total sum of the ratios of the two diameters of each vessel (Max_{Diam}/Min_{Diam}) on the total observed area (samples from three independent experiments/group) . Anti-vWF and anti-α-SMA stainings were both used to correctly delimit the vessels. Vasculature was assessed randomly in 9 fields at high magnification (200x) for each animal; three different animals in each treatment group have been used (Sp6 alone, Sp6 + BM-MSCs, Sp6 + BM-MSCs/IFN-α one dose, Sp6 + BM-MSCs/IFN-α 8 doses).

To detect any difference in IFN^{pos} areas in tumors from animals treated with either single or multiple doses of BM-MSCs/IFN-α, the volume of IFN-α producing areas formed by engrafting BM-MSCs/IFN-α was assessed by means of sequential digital pictures mapping a total area of 10 mm^2 (approximately 20 sequential fields at 200x) in slides stained for IFN-α. Numerical codes were used to blindly identify groups of treatments, and areas were manually highlighted by two independent operators (MT and FM) and measured using the tools provided by the digital imaging software Zeiss Axiovision™. Five different animals in each treatment group have been used (Sp6 +

Mary Ann Liebert Inc., 140 Huguenot Street, New Rochelle, NY 10801

BM-MSCs/IFN-α one dose, Sp6 + BM-MSCs/IFN-α four doses and Sp6 + BM-MSCs/IFN-α 8 doses). Mice injected with Sp6 cells alone or with one dose of BM-MSCs were also stained for IFN-α, and used as negative controls.

Statistics

Data are expressed as mean value ± standard deviation (SD). Statistical comparison between multiple groups has been carried out using one-way ANOVA and Holm-Sidak test to evaluate internal differences between multiple groups. Survival analysis has been carried out using the method developed by Kaplan and Meiers, and the log-rank test to compare survival. Specific time points were selected as particularly relevant on the basis of biological events or study protocol; at these time points differences in tumor incidence were also assessed using χ^2 test with Yates correction. Differences were considered statistically significant for p values ≤ 0.05.

All statistical calculations were performed using Stata™ IC v.10.0 (StataCorp, Texas - USA) for Microsoft Windows®.

RESULTS

BM-MSCs transduced with IFN-α cDNA secrete IFN-α *in vitro*

The immunophenotype of BM-MSCs/IFN-α was characterized by the expression of CD106, CD90, CD105, CD44 and MHC class I molecules, and the absence of leukocyte (CD45, CD14, CD11c) and endothelial cell markers (CD31), as well as of CD86 and MHC class II molecules, as previously described for unmanipulated BM-MSCs [23,30-32]. There was no significant difference in the immunophenotype of BM-MSCs before and after IFN-α transfection, with the exception of the down-modulation of CD80 observed in BM-MSCs/IFN-α (data not shown). Comparison of proliferation rate of BM-MSCs and BM-MSCs/IFN-α showed only negligible variations (data not shown). When we compared untransduced BM-MSCs and BM-MSCs/IFN-α in terms of *in vitro* differentiation and capability of inhibiting immune responses, we found a comparable multilineage differentiation potential in both cell types, as assessed by Oil Red, Alkaline Phosphatase and Toluidine Blue staining (**Supplementary Figure 2**); furthermore, BM-MSCs/IFN-α could inhibit specific immune responses mediated by activated T lymphocytes as efficiently as BM-MSCs (**Supplementary Figure 3**).

IFN-α production by BM-MSCs and BM-MSCs/IFN-α was evaluated by ELISA in culture supernatants (n=3). In the case of BM-MSCs, the levels of IFN-α were below the detection limit of the assay (12.5 pg/ml) in all samples, whereas IFN-α level in the supernatants of BM-MSCs/IFN-α, tested in the same conditions, was 1270.86 ± 308.69 pg/ml. Repeated sampling after four weeks of culture showed equivalent levels of IFN-α. On the other hand, the level of IFN-α in the supernatants of Sp6/IFN-α cells was 1803.91±400.83 pg/ml."

BM-MSCs/IFN-α inhibit the survival of Sp6 cells *in vitro*

Aim of our *in vivo* study was to have a persistent source of IFN-α inside Sp6 growing tumor; thus, we compared the effects of the IFN-α levels reached in the supernatants of *in vitro* BM-MSCs/IFN-α + Sp6 cell co-culture with those obtained with comparable concentrations of recombinant IFN-α exogeneously added. Supernatant IFN-α mean value was 1155.849±109.93 pg/ml that, according to the available literature on the topic, corresponds approximately to 120 IU/ml. This level was associated to a theoretical 73% Sp6 viability in our dose-effect curve (**Supplementary Figure 1**).

In vitro, BM-MSCs/IFN-α inhibit the survival of Sp6 cells, either in direct contact and in Transwell™ conditions (**Figure 1**). As compared to Sp6 cultured alone (87.81±2.55% n=5), only the co-culture with BM-MSCs/IFN-α was capable of exerting statistically significant pro-apoptotic effects, either in direct contact (65.72±2.47%, n=6; $P<0.001$) or in Transwell™ (58.41±7.58%, n=6; $P<0.001$). All other culture conditions were not statistically different from Sp6 cultured alone. In details: Sp6 + BM-MSCs contact 82.19±5.06% (n=6); Sp6 + BM-MSCs Transwell™: 82.16±9.41% (n=6); Sp6 + recombinant murine IFN-α 120 IU/ml 84.23±0.15% (n=3); Sp6 + BM-MSCs + recombinant murine IFN-α 120 IU/ml 77.60±2.34% (n=3). As compared to the addition of recombinant IFN-α, the use of BM-MSCs/IFN-α managed to obtain a significantly higher inhibition of Sp6 survival *in vitro,* either in direct contact ($P=0.002$) or in Transwell™ ($P<0.001$) (**Figure 1**).

BM-MSCs are efficient tools for gene delivery inside growing tumors

To assess whether Sp6 cells could be targeted by different kind of transduced BM-MSCs *in vivo*, BM-MSCs were either single-transduced with EGFP or double-transduced with both EGFP and IFN-α, and injected s.c. or i.v. into Balb/c mice together with Sp6 cells at the same time. The localization of BM-MSCs inside growing tumors was then analyzed using immunofluorescent staining for EGFP and IFN-α. Scattered cells and small clusters of $EGFP^{pos}$ BM-MCSs were visible

within Sp6 growing masses starting from 14-24 days after the injection (data not shown); the same occurred with double-transduced EGFP^{pos}/IFN-α^{pos} BM-MSCs, which were associated to longer survival of the mice (**Figure 2**). We further tested the dissemination of both cell types in samples taken from the spleen, the liver, the central nervous system and lungs upon either s.c. or i.v. administration. We did not detect any EGFP^{pos}/IFN-α^{pos} cell outside the tumors after s.c. administration. By contrast, following i.v. aministration, most BM-MSCs/EGFP/IFN-α remained entrapped inside the pulmonary microcirculation and, to a minor extent, into the spleen, with poorer evidence of intratumoral engraftment and therapeutical efficacy (data not shown). For this reason, we focused our attention only on the efficacy of BM-MSCs/IFN-α administered s.c. in the site of developing Sp6 tumors.

BM-MSCs/IFN-α inhibit *in vivo* Sp6 cell growth and increase survival

Groups of Balb/c mice were injected with Sp6 cells alone or with Sp6 + BM-MSCs/IFN-α s.c. (ratio 1:1) in the same anatomical quarter (one, four or 8 weekly doses). On day +14 after treatment, all animals injected with Sp6 cells alone or co-injected with different doses of BM-MSCs developed tumors. In details: after a single dose of BM-MSCs, tumor incidence was 100%, with mean event free survival (EFS) of 12 days (range 10-14 days); after three doses of BM-MSCs, tumor incidence was 100%, with mean EFS of 10 days (range 7-13 days). In this subset, we could not administer more than three doses of BM-MSCs, similarly to BM-MSCs/IFN-α, because of the early appearance of s.c. rapidly growing tumors: in fact, at day +19, all mice were euthanized because the tumors had reached the critical diameter of 2 cm or had become ulcerated. By contrast, mice co-injected with Sp6 and weekly doses of BM-MSCs/IFN-α s.c. (one, four or 8 total doses) showed a significant delay in the onset of palpable tumors and a trend towards slower tumor growth, thus resulting in prolonged EFS (P<0.001) and better overall survival (OS) (P<0.001) (**Figure 3**). In fact, median EFS was 17 days in mice treated with one single dose of BM-

Mary Ann Liebert Inc., 140 Huguenot Street, New Rochelle, NY 10801

MSCs/IFN-α s.c. vs 20 days in mice receiving four doses of BM-MSCs/IFN-α s.c. and 64 days in mice treated with 8 doses of BM-MSCs/IFN-α s.c. As expected, direct IFN-α transduction of Sp6 (Sp6/IFN-α) increased median EFS (21 days) as compared to controls, but less efficiently than the treatment with 8 doses of BM-MSCs/IFN-α s.c. As a consequence of the effects on tumor onset, median OS was also significantly improved by the administration of BM-MSCs/IFN-α. Median OS was 19 days in the control animals (range: 18-24 days, n=15) and 17 days in animals receiving either one or three doses of unmanipulated BM-MSCs (one dose: range: 14-25 days; three doses: range: 13-19 days). It improved up to 30-31 days in mice treated with one or four doses of BM-MSCs/IFN-α s.c. (range: 21-150 days for one dose; range: 20-150 days for four doses) and up to 77 days in mice treated with 8 weekly s.c. doses (range: 34-150 days) (**Figure 3C**). All mice developed a palpable tumor at day +90. We observed a trend towards slower tumor growth in the group of mice coinjected with Sp6 and BM-MSCs/IFN-α s.c. (one, four and 8 doses), where Sp6 mean volume at day +10 was 0.37±0.25 cm^3 (n=19), 0.37±0.28 cm^3 (n=14) and 0.34±0.36 cm^3 (n=10), respectively. By comparison, mean volume at the same time point was 1.14±0.28 cm^3 (n=13) in the case of Sp6 injected alone. Sp6 transfection with IFN-α or co-administration of one to three doses of unmanipulated BM-MSCs resulted in comparable tumor volumes at day 10 from onset (Sp6/IFN-α: 1.44±1.55 cm^3, n=13; +1 dose BM-MSCs: 2.00±1.61 cm^3, n=10; +3 doses BM-MSCs: 1.19±0.64 cm^3, n=10).

Considered together, these data suggest that BM-MSCs transduction with IFN-α gene is capable of interfering with BM-MSCs-mediated support of Sp6 tumor development in a statistically significant manner ($P<0.001$) (**Figure 3**).

BM-MSCs/IFN-α alter tumor architecture

We studied tumor architecture by standard staining and immunohistochemistry in treated and untreated mice. Tumors developing in Sp6-injected untreated mice were easily identifiable from surrounding subcutaneous tissue as masses of densely packed, dysplastic, middle-sized, homogeneous cells with a central nucleus and high nucleus/cytoplasmic ratio (**Figure 4A**). These masses were limited by a highly vascularized connective capsule containing high numbers of $CD90^{pos}$ cells, an antigen expressed by mesenchymal cells at different levels of differentiation (**Figure 4D**).

Regardless of treatment, Sp6 tumors appeared homogenously formed by $CD45^{pos}$ cells (**Figure 4G, J and M**), as already known from flow cytometry analysis (data not shown). Scattered $CD90^{pos}$ cells, most probably tumor-associated fibroblasts, are evident in untreated Sp6 tumors (**Figure 4D and H**), but clusters of $CD90^{pos}$ elements could be identified only after BM-MSCs or BM-MSCs/IFN-α s.c. injection (**Figure 4E, F, K and N**). Only in the latter group, these clusters were associated with areas, clearly visible also at a MGG staining (**Figure 4C**, arrow), characterized by the loss of homogeneous tumoral architecture due to internal necrosis and to the accumulation of cellular debris. All these features were evident also in mice treated with just one dose of BM-MSCs/IFN-α s.c., even though they were more common and evident in mice treated with 8 weekly doses.

IFN-α^{pos} cells, detected as either scattered elements or as clusters, were virtually absent in Sp6 tumors injected alone or together with unmanipulated BM-MSCs (**Figure 4P and Q**). By contrast, they were detectable by immunofluorescence in animals treated with either one, four or 8 weekly doses of BM-MSCs/IFN-α (**Figure 4R**), with no statistically significant difference between the study groups (data not shown). In treated mice, clusters of IFN-α producing cells were surrounded by Sp6 cells undergoing apoptosis, as shown by their positivity for activated caspase-3 (**Figure 4R**). Sp6 cells were recognizable from surrounding environment on the basis of their characteristic large dysplastic nucleus with broadly dispersed chromatin pattern.

We assessed the expression of IFN-R type I by Sp6 cells after treatment with BM-MSCs/IFN-α as a potential escape mechanism during tumor development. However, IFN-R type I did not appear significantly downregulated on the surface of Sp6 cells following BM-MSCs/IFN-α administration (data not shown).

We then analyzed the tumor vasculature using a double staining for vWF and α-SMA to identify endothelial and perivascular cells. Complex vasculo-stromal axis were evident only in untreated mice (**Figure 4I and L**); on the contrary, mice treated with BM-MSCs/IFN-α s.c. displayed a very simple vasculature, consisting mostly of microvascular structures often lacking evident pericytic walls (**Figure 4O**). Tumors developing in mice treated with BM-MSCs/IFN-α s.c. displayed lower mean vasculature density (MVD) as compared to controls: in particular, MVD was 170.66±75.74 n/mm2 in mice injected once ($P<0.001$) vs 87.17±53.96 n/mm2 in mice treated with 8 weekly injections ($P<0.001$). Corresponding values of MVD in untreated mice and mice injected with one dose of BM-MSCs were 290.98±98.42 n/mm2 and 249.92±116.86 n/mm2, respectively (P=NS). Complete statistical evaluation is reported in **Table 1**. Overall, these findings show that repeated doses of BM-MSCs/IFN-α result in a proportional inhibition of tumor angiogenesis, and, subsequently, in a corresponding reduction of *in vivo* tumor growth.

Finally, we assessed the presence of $CD8^{pos}$, $CD4^{pos}$ T and NK infiltrates inside the Sp6 tumors, in treated and untreated mice. In all cases, there was no evidence of infiltration by $CD8^{pos}$ or $CD4^{pos}$ lymphocytes, or NK cells (data not shown).

DISCUSSION

Different animal models have previously shown that i.v. administered MSCs specifically engraft inside the tumor architecture and may support cancer cell growth [14,18]. When engineered to produce anti-cancer molecules, MSCs may significantly inhibit tumor development *in vitro* and *in vivo* [14,18,33-35]. This result has been obtained by injecting MSCs either i.v. in lung cancers or metastases or into the feeding arteries of solid tumors (i.e. gliomas) [33-35].

Myeloma is a systemic and multifocal disease that develops inside the BM. Neoplastic plasma cells are sensitive to IFN-α, which has pleiotropic effects in many tumors, such as melanoma, Kaposi's sarcoma, and renal cancer [36-39], but its systemic effects limit the clinical use. For all these reasons we studied in our mouse model of plasmacytoma the effect of intra-tumor delivery of BM-MSCs/IFN-α to assess their *in situ* anti-cancer effect and their efficacy in terms of increase of EFS and OS. Unfortunately, i.v. administration was hampered by the important entrapment of MSCs inside the lung vessels. Consequently, a scarce number of MSCs could be detected inside Sp6 tumor architecture and no therapeutic effect was evident.

However, the specific engraftment of BM-MSCs was proved by using double-transduced BM-MSCs (BM-MSCs/EGFP/IFN-α) injected s.c. together with the inoculation of Sp6 cells. BM-MSCs/EGFP/IFN-α were able not only to localize specifically into the tumor, but also to produce *in situ* IFN-α, even after the withdrawal of BM-MSCs inoculation. The persistence of BM-MSCs/IFN-α and the *in situ* production of IFN-α led to longer EFS, slower tumor growth rate and a dose-dependent effect on the OS, which was up to 3-fold increased in mice treated with 8 total doses of BM-MSCs/IFN-α, as compared to controls that all rapidly developed tumors after receiving Sp6 cells alone or Sp6 co-injected with unmanipulated BM-MSCs. These findings suggest that a cell therapy approach based on the use of BM-MSCs/IFN-α may be effective in MM, once the problem of i.v. administration is solved or, at least, tempered.

According to literature, direct induction of tumor cells apoptosis, inhibition of local angiogenesis, and enhancement of NK infiltration are some of the possible explanation for the anti-tumor effect observed with IFN-α-based gene therapies [40,41]. In a co-culture system, i*n vitro*, BM-MSCs/IFN-α affect Sp6 cell survival and proliferation. In fact, after 5 days of co-culture, the number of viable Sp6 cells was significantly lower in the presence of BM-MSCs/IFN-α, thus showing Sp6 sensitivity to IFN-α. Similarly, we could assess *in vivo* a focal increase of apoptosis of Sp6 cells surrounding IFN-α-producing clusters of engrafted BM-MSCs/IFN-α. Angiogenesis is another factor playing a pivotal role in tumor growth, and a potential target for therapy [42,43]. In Sp6 tumors, the role of angiogenesis is shown by the presence of complex vasculo-stromal structures, provided of a complete endothelial layer surrounded by pericytes. By contrast, developing tumors in BM-MSCs/IFN-α-treated mice displayed a diffuse microvasculature rather than a complex vascularization, as shown by the significant MVD reduction; this finding was associated with the presence of necrotic areas inside the tumors. On the other hand, no clear evidence was observed of a role of immune effector cells, such as NK cells, $CD4^{pos}$ and $CD8^{pos}$ T cells, in the anti-tumor effect *in vivo*. In other words, the therapeutic effect of BM-MSCs/IFN-α seems to be predominantly related to the direct pro-apoptotic and anti-angiogenic effect of IFN-α, rather than to the triggering of innate or adaptive immunity.

Although we could never prevent or eradicate completely Sp6 tumors in our model, the growth of tumors was significantly delayed by the use of BM-MSCs/IFN-α. Moreover, treated animals eventually developing a mass showed a significantly lower tumor growth rate. This is particularly relevant if we consider that Sp6 plasmacytoma grows and disseminates more quickly than human MM [21]. The mechanisms by which Sp6 tumor eventually develops once BM-MSCs/IFN-α administration is withdrawn, must be further investigated to significantly improve the results obtained. However, the use of combined therapeutic approaches including other effective drugs could improve the results obtained with BM-MSCs/IFN-α administration.

ACKNOWLEDGEMENTS

No competing financial interests exist.

We thank Nadia Brutti for her excellent technical support.

This work was supported by:

- Italian Ministry of University and Scientific Research (PRIN 2005, PRIN 2007);

- Fondazione CARIVERONA, Bando 2003 - Integrazione tra tecnologia e sviluppo di settore; linea di ricerca: “Basi molecolari del danno cellulare e nuovi approcci metodologici per la caratterizzazione di eventi molecolari complessi”, progetto di ricerca: “Microambiente e progressione neoplastica: il network di regolazione indotto da cellule stromali. Biologia e applicazioni terapeutiche”;

- Fondazione CARIVERONA, Bando 2008 – Tecnologie al servizio della salute, linea di ricerca: “Terapie rigenerative dei tessuti (strutturali e funzionali) mediante l’impiego di cellule staminali di diversa origine”, progetto di ricerca: “Utilizzo delle capacità rigenerative ed immunoregolatorie delle cellule staminali mesenchimali per il trattamento di patologie ossee, neurologiche e cardiologiche”.

REFERENCES

1. Kyle RA and SV Rajkumar . (2006). Monoclonal gammopathy of undetermined significance. Br J Haematol 134:573-89.

2. Alexanian R and M Dimopoulos . (1994). The treatment of multiple myeloma. N Engl J Med 330:484–489.

3. Attal M, JL Harousseau, AM Stoppa, JJ Sotto, JG Fuzibet, JF Rossi, P Casassus, H Maisonneuve, T Facon, N Ifrah, C Payen and R Bataille. (1996). A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. Intergroupe Francais du Myelome. N Engl J Med 335:91–97.

4. Kyle RA and SV Rajkumar. (2004). Multiple myeloma. N Engl J Med 351:1860–1873.

5. Richardson PG, P Sonneveld, MW Schuster, D Irwin, EA Stadtmauer, T Facon, JL Harousseau, D Ben-Yehuda, S Lonial, H Goldschmidt, D Reece, JF San-Miguel, J Bladé, M Boccadoro, J Cavenagh, WS Dalton, AL Boral, DL Esseltine, JB Porter, D Schenkein and KC Anderson. (2005). Assessment of Proteasome Inhibition for Extending Remissions (APEX) Investigators. Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. N Engl J Med 352:2487–2498.

6. Barlogie B, J Shaughnessy, G Tricot, J Jacobson, M Zangari, E Anaissie, R Walker and Crowley J. (2004). Treatment of multiple myeloma. Blood 103:20–32.

7. Rosenblatt J and D Avigan. (2008). Cellular immunotherapy for multiple myeloma. Best Pract Res Clin Haematol 21:559-577.

8. Schaar CG, HC Kluin-Nelemans, C Te Marvelde, S Ie Cessie, WP Breed, WE Fibbe, WA van Deijk, MM Fickers, KJ Roozendaal and PW Wijermans; Dutch-Belgian Hemato-Oncology Cooperative Group HOVON. (2005). Interferon-alpha as maintenance therapy in patients with multiple myeloma. Ann Oncol 16:634-639.

9. Kassem M. (2004). Mesenchymal stem cells: biological characteristics and potential clinical applications. Cloning Stem Cells 6:369-374.

10. Krampera M, A Pasini, G Pizzolo, L Cosmi, S Romagnani and F Annunziato. (2006). Regenerative and immunomodulatory potential of mesenchymal stem cells. Curr Opin Pharmacol 6:435-441.

11. Zhao RC, L Liao and Q Han. (2004). Mechanisms of and perspectives on the mesenchymal stem cell in immunotherapy. J Lab Clin Med 143:284-91.

12. Krampera M, S Sartoris, F Liotta, A Pasini, R Angeli, L Cosmi, A Andreini, F Mosna, B Bonetti, E Rebellato, MG Testi, F Frosali, G Pizzolo, G Tridente, E Maggi, S Romagnani and F Annunziato. (2007). Immune regulation by mesenchymal stem cells derived from adult spleen and thymus. Stem Cells Dev 16:797-810.

13. Pereira RF, KW Halford, MD O'Hara, LB Leeper, BP Sokolov, MD Pollard, O Bagasra and DJ Prockop. (1995). Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci USA 92:4857-4861.

14. Studeny M, FC Marini, RE Champlin, C Zompetta, IJ Fidler and M Andreeff. (2002). Bone marrow-derived mesenchymal stem cells as vehicles for interferon-β delivery into tumours. Cancer Res 62:3603-3608.

15. Rønnov-Jessen L, OW Petersen, VE Koteliansky and MJ Bissell. (1995). The origin of the myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. J Clin Invest 95:859-873.

16. Karnoub AE, AB Dash, AP Vo, A Sullivan, MW Brooks, GW Bell, AL Richardson, K Polyak, R Tubo and RA Weinberg. (2007). Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 449:557-563.

17. Bartholomew A, S Patil, A Mackay, M Nelson, D Buyaner, W Hardy, J Mosca, C Sturgeon, M Siatskas, N Mahmud, K Ferrer, R Deans, A Moseley, R Hoffmann and SM Devine. (2001). Baboon mesenchymal stem cells can be genetically modified to secrete human erythropoietin in vivo. Hum Gene Ther 12:1527-1541.

18. Studeny M, FC Marini, JL Dembinski, C Zompetta, M Cabreira-Hansen, BN Bekele, RE Champlin and M Andreeff. (2004). Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. J Natl Cancer Inst 96:1593-1603.

19. Sartoris S, MG Testi, E Stefani, R Chignola, C Guerriero, A Matucci , T Cestari, A Scarpa , AP Riviera, G Zanoni, G Tridente and G Andrighetto. (2003). Induction of an anti-tumour adaptive immune response elicited by tumour cells expressing de novo B7-1 mainly depends on the anatomical site of their delivery. The dose applied regulates the expansion of the response. Immunology 110:474-481.

20. Köhler G and C Milstein. (1976). Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. Eur J Immunol 6:511-519.

21. Potter M. (2003). Neoplastic development in plasma cells. Immunol Rev 194:177-195.

22. Roschke V, P Hausner, E Kopantzev, JG Pumphrey, M Riminucci, DM Hilbert and S Rudikoff. (1998). Disseminated growth of murine plasmacytoma: similarities to multiple myeloma. Cancer Res 58:535-541.

23. Glennie S, I Soeiro, PJ Dyson, EW Lam and F Dazzi. (2005). Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. Blood 105:2821-2827.

24. Krampera M, A Pasini, A Rigo, MT Scupoli, C Tecchio, G Malpeli, A Scarpa, F Dazzi, G Pizzolo and F Vinante F. (2005). HB-EGF/HER-1 signalling in bone marrow mesenchymal stem cells: inducing cell expansion and reversibly preventing multi-lineage differentiation. Blood 106:59-66.

25. Krampera M, L Cosmi, R Angeli, A Pasini, F Liotta, A Andreini, V Santarlasci, B Mazzinghi, G Pizzolo, F Vinante, P Romagnani, E Maggi, S Romagnani and F Annunziato. (2006).

Role of the IFN-γ in the immunomodulatory activity of human mesenchymal stem cells. Stem cells 24:386-398.

26. Schnell T, P Foley, M Wirth, J Munch and K Uberla. (2000). Development of a self-inactivating, minimal lentivirus vector based on simian immunodeficiency virus. Hum Gene Ther 11:439-447.

27. Indraccolo S, V Tisato, V Tosello, W Habeler, G Esposito, L Moserle, L Stievano, L Persano, L Chieco-Bianchi and A Amadori. (2005). Interferon-α gene therapy by lentiviral vectors contrasts ovarian cancer growth through angiogenesis inhibition. Hum Gene Ther 16:957-970.

28. Follenzi A, LE Ailles, S Bakovic, M Geuna and L Naldini. (2000). Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 *pol* sequences. Nat Genet 25:217-222.

29. Pittenger MF, AM Mackay, SC Beck, RK Jaiswal, R Douglas, JD Mosca, MA Moorman, DW Simonetti, S Craig and DR Marshak. (1999). Multilineage potential of adult human mesenchymal stem cells. Science 284:143-147.

30. Krampera M, S Glennie, J Dyson, D Scott, R Laylor, E Simpson and F Dazzi. (2003). Bone marrow mesenchymal stem cells inhibit the response of naïve and memory antigen-specific T cells to their cognate peptide. Blood 101:3722-3729.

31. Peister A, JA Mellad, BL Larson, BM Hall, LF Gibson and DJ Prockop. (2004). Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. Blood 103:1662-1668.

32. Sudres M, F Norol, A Trenado, S Grégoire, F Charlotte, B Levacher, JJ Lataillade, P Bourin, X Holy, JP Vernant, D Klatzmann and JL Cohen. (2006). Bone marrow mesenchymal stem cells suppress lymphocyte proliferation in vitro, but fail to prevent graft-versus-host disease in mice. J Immunol 176:7761-7767.

33. Ren C, S Kumar, D Chanda, J Chen, JD Mountz and S Ponnazhagan. (2008). Therapeutic potential of mesenchymal stem cells producing interferon-α in a mouse melanoma lung metastasis model. Stem Cells 26:2332-2338.

34. Loebinger MR, A Eddaoudi, D Davies and SM Janes. (2009). Mesenchymal stem cell delivery of TRAIL can eliminate metastatic cancer. Cancer Res 69:4134-4142.

35. Nakamizo A, F Marini, T Amano, A Khan, M Studeny, J Gumin, J Chen, S Hentschel, G Vecil, J Dembinski, M Andreeff and FF Lang. (2005). Human Bone Marrow-Derived Mesenchymal Stem Cells in the Treatment of Gliomas. Cancer Res 65:3307-18.

36. Belardelli F, M Ferrantini, E Proietti and JM Kirkwood. (2002). Interferon-α in tumor immunity and immunotherapy. Cytokine Growth Factor Rev 13:119-134.

37. Brassard DL, MJ Grace and RW Bordens. (2002). Interferon-α as an immunotherapeutic protein. J Leukoc Biol 71:565-581.

38. De Palma M, R Mazzieri, LS Politi, F Pucci, E Zonari, G Sitia, S Mazzoleni, D Moi, MA Venneri, S Indraccolo, A Falini, LG Guidotti, R Galli and L Naldini. (2008). Tumor-targeted interferon-α delivery by Tie2-expressing monocytes inhibits tumor growth and metastasis. Cancer Cell 14:299-311.

39. Pfeffer LM, CA Dinarello, RB Herberman, BR Williams, EC Borden, R Bordens, MR Walter, TL Nagabhushan, PP Trotta and S Pestka. (1998). Biological properties of recombinant α-interferons: 40th anniversary of the discovery of interferons. Cancer Res 58:2489-2499.

40. Lokshin A, JE Mayotte and ML Levitt. (1995). Mechanism of interferon β-induced squamous differentiation and programmed cell death in human non-small-cell lung cancer cell lines. J Natl Cancer Inst 87: 206-212.

41. Qin XQ, N Tao, A Dergay, P Moy, S Fawell, A Davis, JM Wilson and J Barsoum. (1998). Interferon- β gene therapy inhibits tumor formation and causes regression of established tumors in immune-deficient mice. Proc Natl Acad Sci U.S.A. 95: 14411-14416.

42. Ozawa S, H Shinohara, HO Kanayama, CJ Bruns, CD Bucana, LM Ellis, DW Davis and IJ Fidler. (2001). Suppression of angiogenesis and therapy of human colon cancer liver metastasis by systemic administration of interferon-α. Neoplasia 3:154-164.

43. Indraccolo S, E Gola, A Rosato, S Minuzzo, W Habeler, V Tisato, V Roni, G Esposito, M Morini, A Albini, DM Noonan, M Ferrantini, A Amadori and L Chieco-Bianchi. (2002). Differential effects of angiostatin, endostatin and interferon-α_1 gene transfer on in vivo growth of human breast cancer cells. Gene Ther 9: 867-878.

FIGURE LEGENDS

Figure 1. BM-MSCs/IFN-α inhibit the survival of Sp6 cells *in vitro*. Mean percentage of viable Sp6 cells (Ann-V-negative/7-AAD-negative) at day +5 (mean ± standard deviation). As compared to Sp6 cultured alone, only the co-culture with BM-MSCs/IFN-α was capable of exerting a statistically significant pro-apoptotic effect, either in direct contact (P<0.001) or in Transwell™ (P<0.001). Respect to the addition of recombinant IFN-α, the use of BM-MSCs/IFN-α managed to obtain a significantly higher inhibition of Sp6 survival *in vitro*, either in direct contact (P=0.002) or in Transwell™ (P<0.001).

Figure 2. Double-transduced BM-MSCs efficiently engraft inside growing tumors and maintain a long-lasting production of IFN-α. Tumors of mice injected with Sp6 and BM-MSCs/EGFP/IFN-α s.c. were analyzed by immunofluorescence for EGFP and IFN-α. IFN-α-producing cells were detectable within the tumors only in mice treated s.c. with BM-MSCs/EGFP/IFN-α, and IFN-α was detected within the cells up to 10 weeks after the last administration of BM-MSCs/EGFP/IFN-α. The Figure shows a representative case injected s.c. with BM-MSCs/EGFP/IFN-α. Nuclei were counterstained with DAPI. (A) blue channel (DAPI) (B) green channel (FITC; EGFP). (C) red channel (Cy3; IFN-α). (D) merge (RBG). Bars represent 100 μm.

Figure 3. Treatment with BM-MSCs/IFN-α s.c. *in vivo* reduces tumor growth and delays the onset of palpable tumors, prolonging the overall survival of mice. Balb/c mice were injected s.c. with 0.5×10^6 cells of Sp6, Sp6+BM-MSCs either once or for three doses, Sp6 +BM-MSCs/IFN-α

Mary Ann Liebert Inc., 140 Huguenot Street, New Rochelle, NY 10801

(either once or for four or 8 doses) or Sp6/IFN-α transfectant. (A) EFS. (B) tumor growth rate after onset (mean volumes). (C) OS.

Figure 4. BM-MSCs/IFN-α presence determines areas of necrosis inside the tumors and induces apoptosis in Sp6 cells. Immunohistochemistry and immunofluorescent analysis of Sp6 tumors (A, D, G, H, I, P), tumors from mice injected with one dose of BM-MSCs s.c. (B, E, J, K, L, Q) and tumors from mice treated with 8 doses of BM-MSCs/IFN-α s.c. (C, F, M, N, O, R).

(A, B, C) May Grunwald-GIEMSA (MGG) staining. Upon s.c. injection, Sp6 tumors are densely packed masses of dysplastic, middle-sized, homogeneous cells with high nucleus/cytoplasmic ratio. Necrotic areas within the tumors are present only in mice treated with BM-MSCs/IFN-α s.c. (as showed by arrow).

(D, E, F, H, K, N) Staining with anti-CD90. Scattered $CD90^{pos}$ cells, probably tumor-associated fibroblasts, are visible also in untreated Sp6 tumors (D, H), but significant clusters of CD90 cells are detected only in mice injected with either BM-MSCs (E, K) or BM-MSCs/IFN-α (F, N). These clusters are more frequent in the latter group (F), and areas of intratumoral necrosis are evident only in this latter case (C, F, N).

(G, J, M) Staining with anti-CD45. Regardless of treatment, Sp6 tumors are formed by homogeneously $CD45^{pos}$ cells.

(I, L, O) Staining with anti-vWF (green) and anti-α-SMA (red). Complex vasculostromal axis, formed by vWF^{pos} endothelial cells surrounded by SMA^{pos} pericytes, can be recognized only in Sp6 tumors either injected alone or together with one dose of BM-MSCs (I, L). On the contrary, vasculature consists almost exclusively of very simple vWF^{pos} capillary networks in mice treated with BM-MSCs/IFN-α s.c. (O).

(P, Q, R) Staining for Caspase-3 (green) and IFN-α (red). The engraftment of BM-MSCs/IFN-α directly induces apoptosis (Caspase-3^{pos} cells) in surrounding Sp6 cells, recognizable on the basis of their characteristic large dysplastic nucleus (R).

Mary Ann Liebert Inc., 140 Huguenot Street, New Rochelle, NY 10801

(A, B, C, D, E, F) Magnification 100x, bars represent 200 μm. (G, H, J, K, M, N) Magnification 400x, bars represent 200 μm. (I, L, O) Magnification 100-200x, bars represent 200 μm. (P, Q, R) Magnification 200x, bars represent 40 μm. Insets represent details at 400x.

Supplementary Figure 1. Sp6 viability at day 5 with increasing doses of recombinant murine IFN-α added to culture medium. Mean value ± SD (n=3).

Supplementary Figure 2. BM-MSCs and BM-MSCs/EGFP/IFN-α in vitro differentiation. A-D: adipogenic differentiation: differentiating cells are stained by Oil-Red-O in red, and, as expected, represent a minority of cultured cells; the same cell types, at a earlier differentiation step (preadipoblasts, characterized by more frequent, smaller neutral lipidi vacuoles) are clearly visible also when treating BM-MSCs/EGFP/IFN-α with induction medium; E-H osteogenic differentiation; ALP staining is coloured in faint red; I-L: chondrogenic differentiation. Differentiation is induced on cell pellets; proteoglycan and glicosaminoglycan produced by differentiatin chondroblasts are coloured in blue. A-H: magnification 100x. I-L: magnification 200x.

Supplementary Figure 3. Cytotoxity assay performed in presence of BM-MSCs and BM-MSCs/IFN-α. According to the method previously described in *Krampera M. et al. Stem Cell Dev 2007;16:797-810*, splenocytes were collected from mice immunized with 5x106 Sp6/B7 cells and co-cultured with their target cells (irradiated Sp6/B7 cells) in presence of either BM-MSCs or BM-MSCs/IFN-α. Both cell types exerted a comparable reduction of immunological CTL-mediated specific lysis (as assessed by ^{51}Cr release).

TABLE LEGENDS

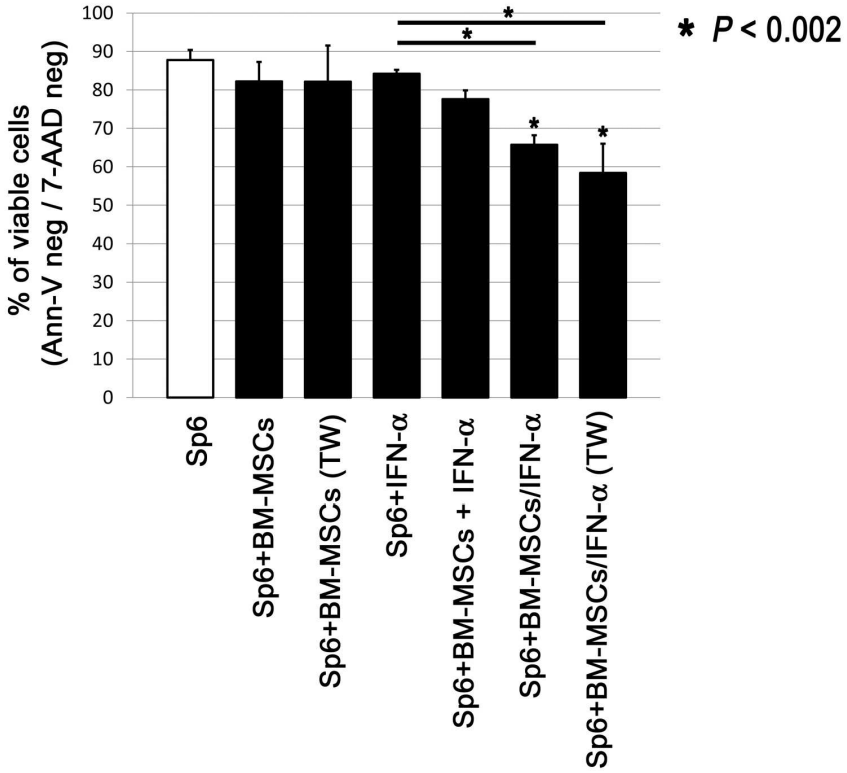
Table 1. Mean Vascular Density (MVD) in the study groups. MVD was measured by using a score based on the total sum of the ratios of the two diameters of each vessel (Max_{Diam}/Min_{Diam}) on the total observed area (samples from three independent experiments/group). Anti-vWF and anti-α-SMA stainings were both used to correctly delimit the vessels. Increasingly frequent doses of BM-MSCs/IFN-α corresponded to increasingly lower MVD ($P<0.001$).

Table 1. Mean vascular density in the study groups.

Groups of injected mice (n=3 in each group)	MVD (n/mm^2)	Statistics
Sp6 only	290.98 ± 98.42	P = NS; P < 0.001; P < 0.001
Sp6 + 1 dose of BM-MSCs	249.92 ± 116.86	
Sp6 + 1 dose of BM-MSCs/IFN-α	170.66 ± 75.74	P = 0.023
Sp6 + 8 doses of BM-MSCs/IFN-α	87.17 ± 53.96	

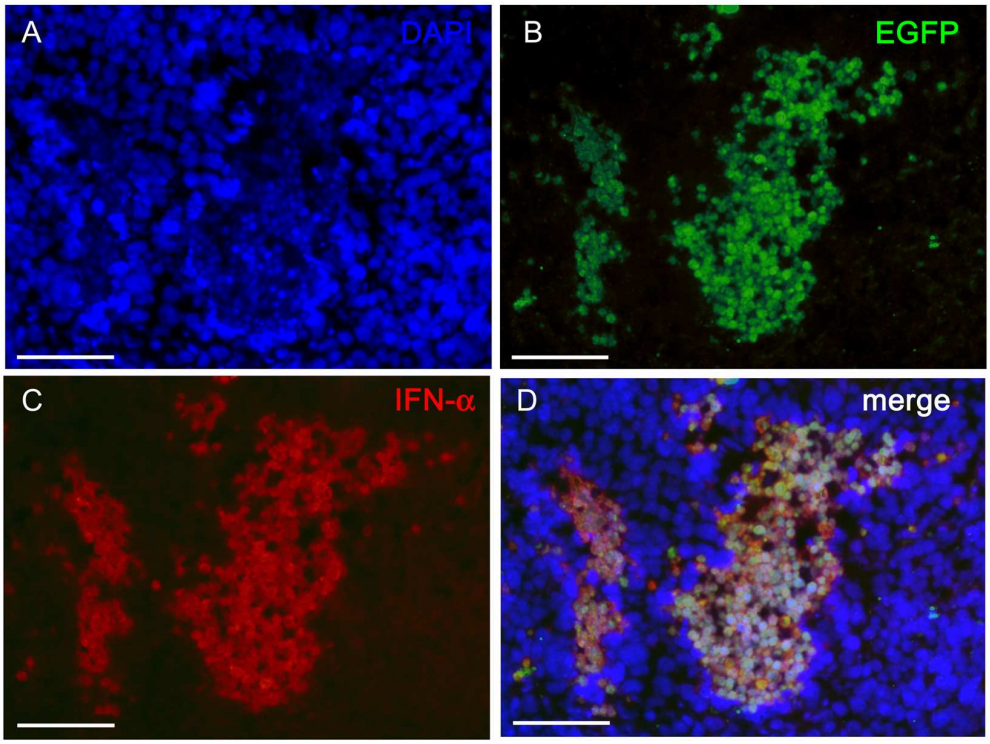
119x71mm (300 x 300 DPI)

Figure 1. BM-MSCs/IFN-α inhibit the survival of Sp6 cells *in vitro*.



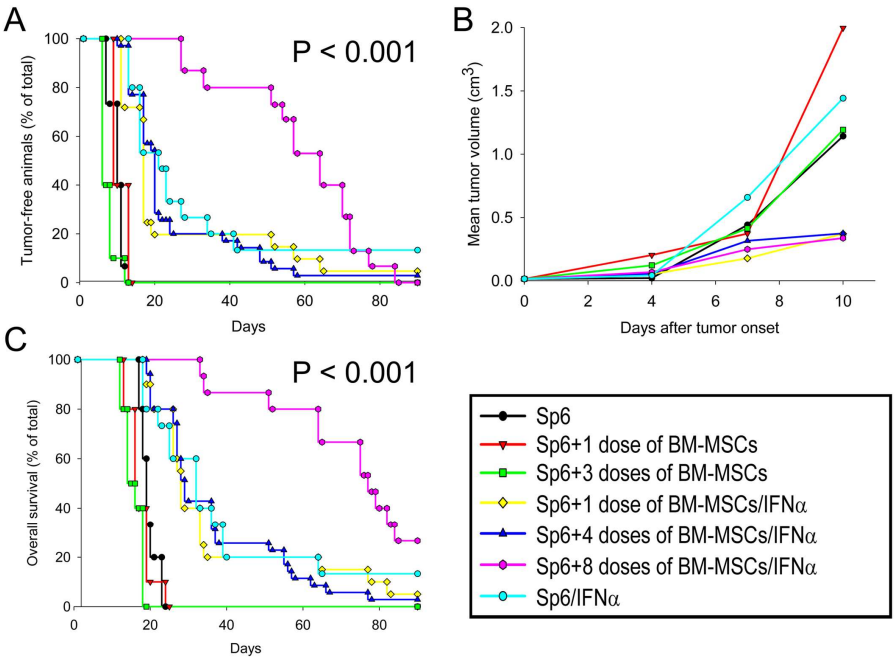
150x132mm (300 x 300 DPI)

Figure 2. Double-transduced BM-MSCs efficiently engraft inside growing tumors and maintain a long-lasting production of IFN-α.



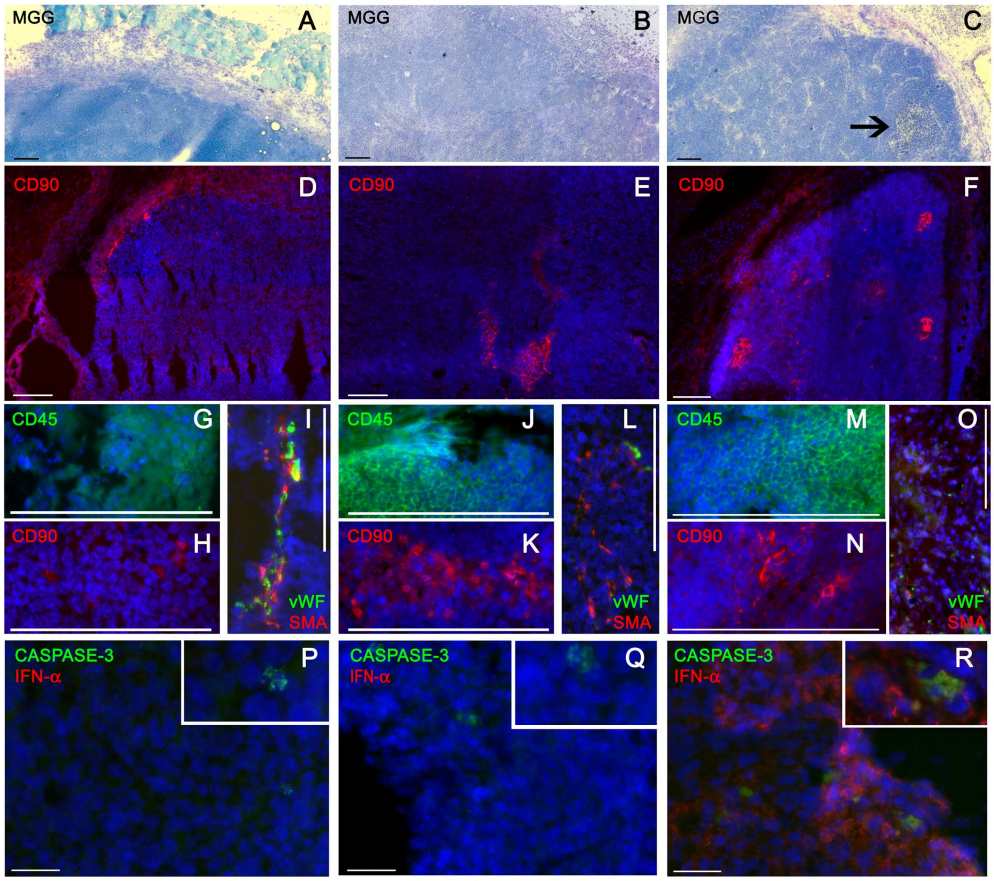
140x118mm (300 x 300 DPI)

Figure 3. Treatment with BM-MSCs/IFN-α s.c. *in vivo* reduces tumor growth and delays the onset of palpable tumors, prolonging the overall survival of mice.



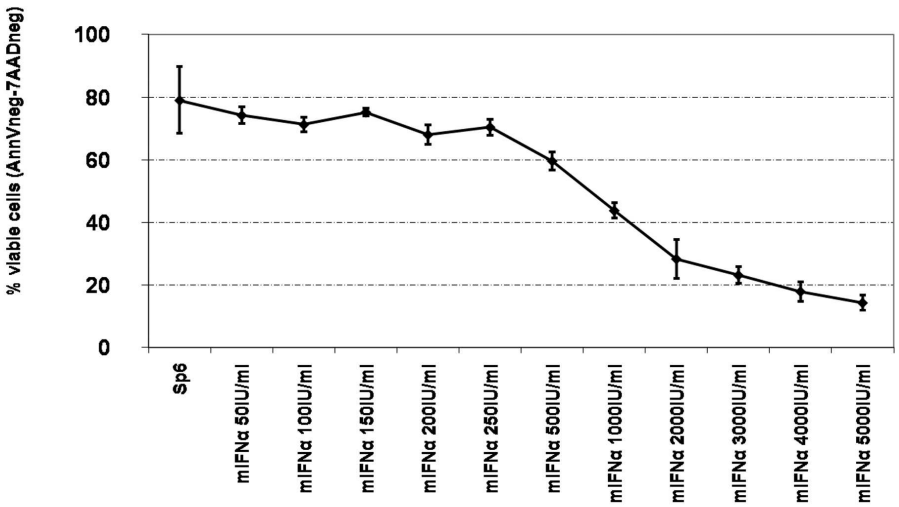
199x155mm (300 x 300 DPI)

Figure 4. BM-MSCs/IFN-α produce areas of necrosis inside the tumors and induce apoptosis in Sp6 cells.



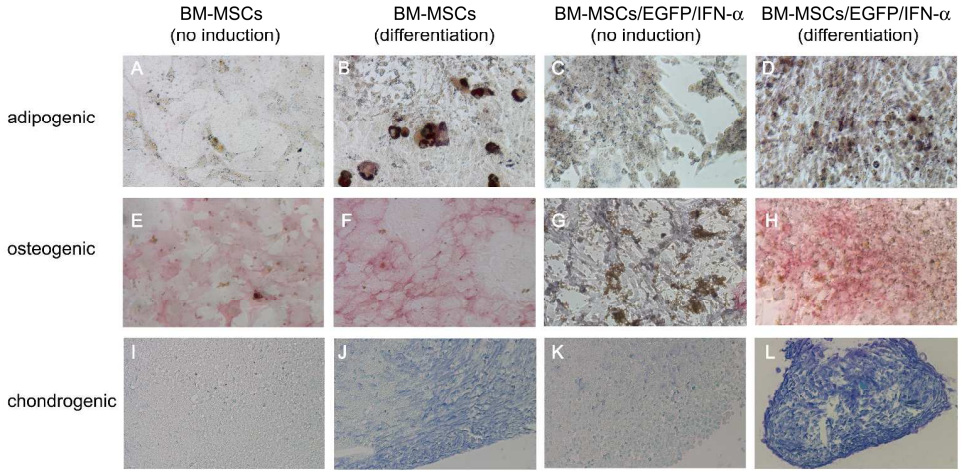
199x193mm (300 x 300 DPI)

Supplementary Figure 1. Sp6 viability at day 5 with increasing doses of recombinant murine IFN-α added to culture medium.



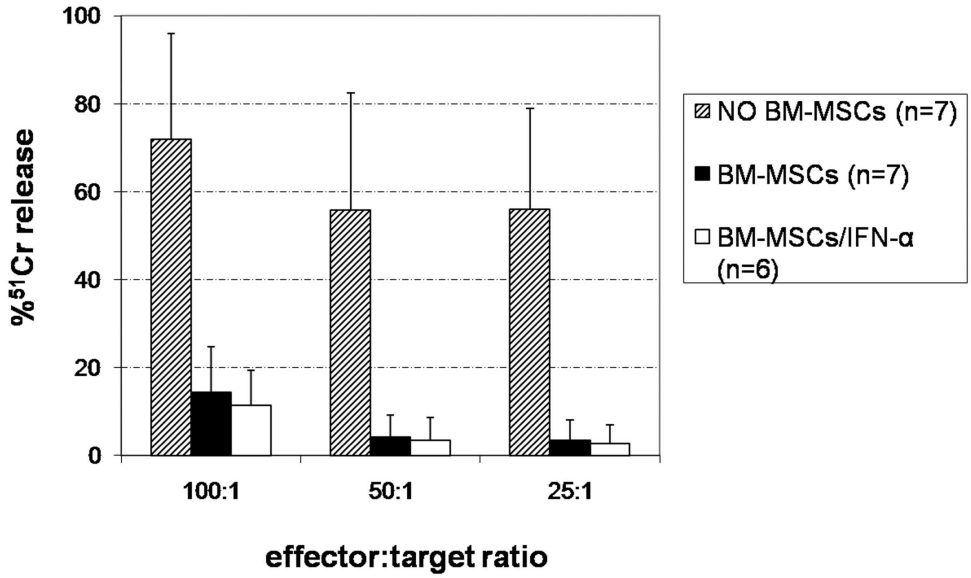
171x114mm (300 x 300 DPI)

Supplementary Figure 2. BM-MSCs and BM-MSCs/EGFP/IFN-α *in vitro* differentiation.



270x144mm (300 x 300 DPI)

Supplementary Figure 3. Cytotoxicity assay performed in presence of BM-MSCs and BM-MSCs/IFN-α.



208x152mm (300 x 300 DPI)