

Modeling multiple myeloma-bone marrow interactions and response to drugs in a 3D surrogate microenvironment

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Supplementary Methods

Bioreactor RCCS™. 3-D dynamic culture was performed using the RCCS™ bioreactor RCCS-1 equipped with four rotating 10 ml-HARV culture vessels (Synthecon Inc., Houston TX, USA). Vessels are provided with a gas exchange membrane made of silicon rubber, that allows an optimal diffusion of O₂. Bioreactor was kept inside an incubator, with humidified atmosphere at 37°C and 95% air 5% CO₂. During the experimental procedures, RCCS™ operational conditions were settled and constantly monitored in order to keep samples in a “free fall” condition, that minimize scaffold sedimentation and maximize mass transfer.¹⁶ As a control, when indicated, parallel cultures in 2D static conditions were performed in plates and maintained under the same culture conditions as for cultures in Bioreactor.

Scaffold preparation. Scaffold discs were cut from Spongostan sheets (Ethicon, Inc. USA) using a sterile 4 mm biopsy punch and then pre-seeded with stromal cells (Fig.1A). Briefly, scaffolds were incubated for one-hour with stromal cells (200.000/scaffold) in 96 well-plates, then transferred to 10ml High Aspect Ratio Vessels (HARV) in 1ml TCM and cultured overnight in bioreactor. Finally, MM cells were added to the vessels filled with 10 ml TCM. At the end of the culture period, MM cells and stromal cells were recovered from scaffolds by means of liberase (Roche) (25µg/ml) treatment. Alternatively, scaffolds were formalin-fixed and submitted to IHC.¹⁶

MM cells and BMSC isolation. Bone marrow mononuclear cells were separated by Ficoll-Hypaque. Primary MM cells from patients were obtained through positive selection with anti-CD138 coated magnetic nanoparticles (Robosep, Stemcell Technologies, Canada) using an EasySep™ magnet according to the Manufacturer’s Protocol. BMSCs were obtained by culturing CD138-negative mononuclear cells in DMEM medium with FBS, antibiotics and 2 mM L-glutamine (all from Lonza).

BMSC osteogenic differentiation. BMSC from normal donors were cultured for 16 days in osteogenic medium. Osteogenic differentiation medium consisted of DMEM-FCS supplemented with 10 mM β -glycerophosphate, 0.1 μ M dexamethasone, and 200 μ M ascorbic acid-2-phosphate (all from Sigma). Differentiation was verified by morphological analysis, including the acquisition of polygonal shape and the mineralization, alizarin (2%) staining and release of alkaline phosphatase activity as in¹⁸.

2D and 3D co-cultures. HS-5 stromal cells, L-VCAM1 and the WT counterpart (kind gift from prof. V. Gattei, Aviano, Italy) were seeded in 6-well plates at 2×10^5 cells per well and co-cultured with MM cell lines at 5×10^5 cells per well. For 3D cultures, MM cell lines (500×10^3 /scaffold) were cultured with HS-5 cells or HUVEC or with L-VCAM1 and its WT counterpart (200×10^3 /scaffold).

Primary cells: scaffolds were pre-seeded with pooled allogeneic BMSC and HUVEC (100×10^3 each/scaffold) and then co-cultured with MM cells from patients (200×10^3 /scaffold). In single cases, parallel 2D/3D co-cultures of MM cells with either BMSC or HUVEC were performed.

Assessment of IL-6 mediated drug resistance. To assess the role of IL-6 in dexamethasone-induced apoptosis, MM1.S cells were treated with the drug (20 μ M) with/without IL-6 (10ng/ml) for 48hrs and then assessed by MTT assay.

MTT assay. Experiments were performed in parallel 2D and in 3D cultures. Cells were plated in a 12-well plate at a density of 200×10^3 per well with media containing the drug (20 μ M) with or without IL-6 (10ng/ml). In parallel 200×10^3 MM1.S cells were cultured with the same treatments in bioreactor. After incubation for 48 hours, cells were retrieved from both types of cultures and collected in 1.5ml vials. Media was then removed, 100 μ l of MTT solution (1 mg/mL; Sigma) was added and incubated for 4 hours in a humidified incubator at 37°C with 5% CO₂. After that, MTT was removed and 100 μ L of dimethyl-sulfoxide (DMSO, Sigma) was added to each sample and

transferred in a 96-well plate. The absorbance was measured using a microplate reader at 580 nm (Bio-Rad, Hercules, CA).

Western Blot analysis. Scaffolds were lysed with NP40 (Invitrogen, USA) and run on 12% SDS page as in.²⁰ The inhibitory effect exerted by the anti-IL6-receptor (IL-6R) mAb tocilizumab (TCZ, Roche) treatment on IL-6 signaling was evaluated pre-treating MM1.S cells with TCZ (10 μ g/ml) for 30' and then adding IL-6 (10ng/ml) for 5'.

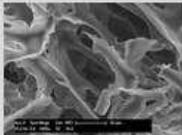
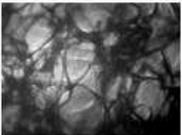
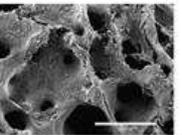
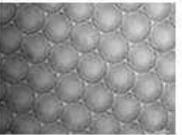
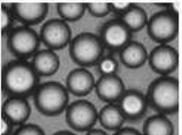
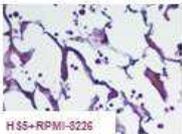
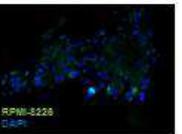
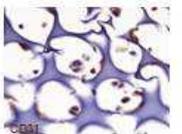
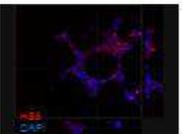
	Spongostan (Gelatin)	Aviten Ultra-Foam (Collagen)	Orthoss (Hydroxyapatite)	Go Matrix (Gelatin)	Microcarrier Beads (Gelatin coated)
Stiffness*	±	+	+++	±	+++
Ultrastructure					
Microscopic analysis feasibility					
Seeding efficiency**	257000±10000	212000±9000	≤ 10000	70000±5000	180000±10000
MM cell recovery***	999800±93196	753800±151179	≤ 10000	not done	not done

Fig. S1 -Selection of the scaffold. Gelatin (Spongostan, Go Matrix), Collagen (Aviten), Hydroxyapatite (Orthoss) scaffolds and gelatin-coated microcarrier beads were compared in terms of physical and ultrastructural features and also of efficiency to serve as support for the generation of 3D MM microenvironment.

*Young's modulus ≤0.1 MPa = ±; 0.1-1MPa = +; 1-10=MPa = ++; ≥10MPa = +++.

**200.000 HS-5 cells were seeded over scaffolds and counted after 18hrs. Data are means ±sd of 3 experiments.

*** 500.000 MM1.S cells were added to HS-5 pre-seeded scaffolds, recovered upon enzymatic digestion after 18 hrs of culture and counted. Data are means ±SD of 3 experiments.

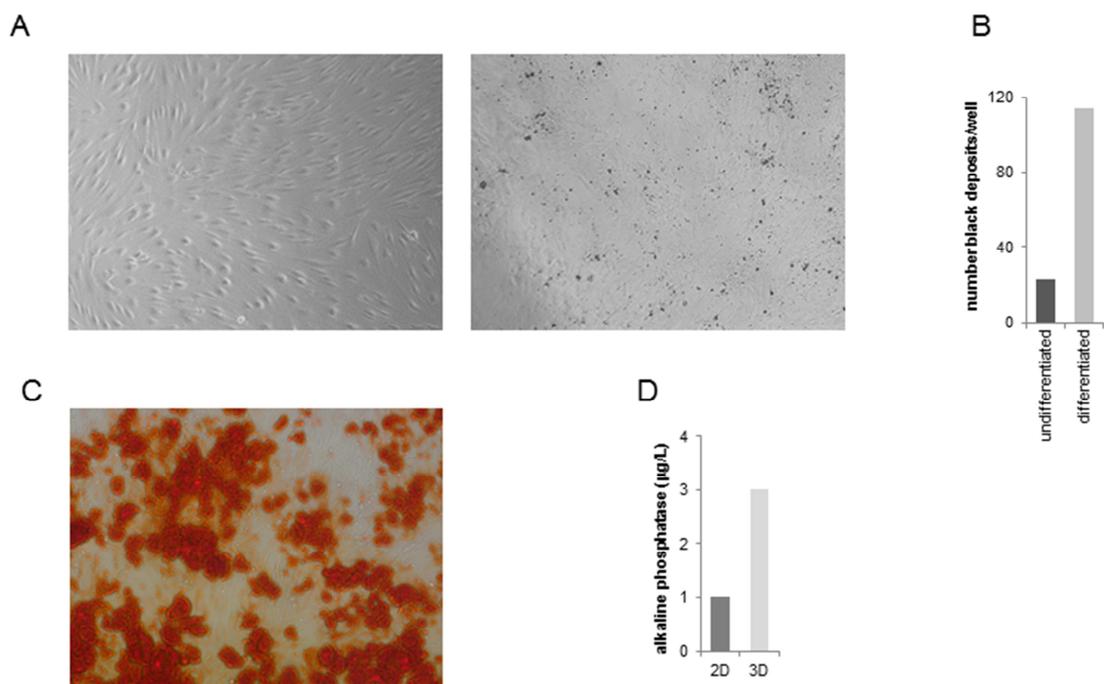


Fig.S2. Osteogenic differentiation of Bone Marrow Stromal Cells. A: Phase contrast micrographs of BMSC cultured w/o (left) or with (right) osteogenic differentiation medium on day 16; the latter showed increased mineralization, in terms of black deposits, as quantified in B. C: Alizarin staining of bone-differentiated BMSC. D: concentrations of alkaline phosphatases released in supernatants from 2D and 3D cultures

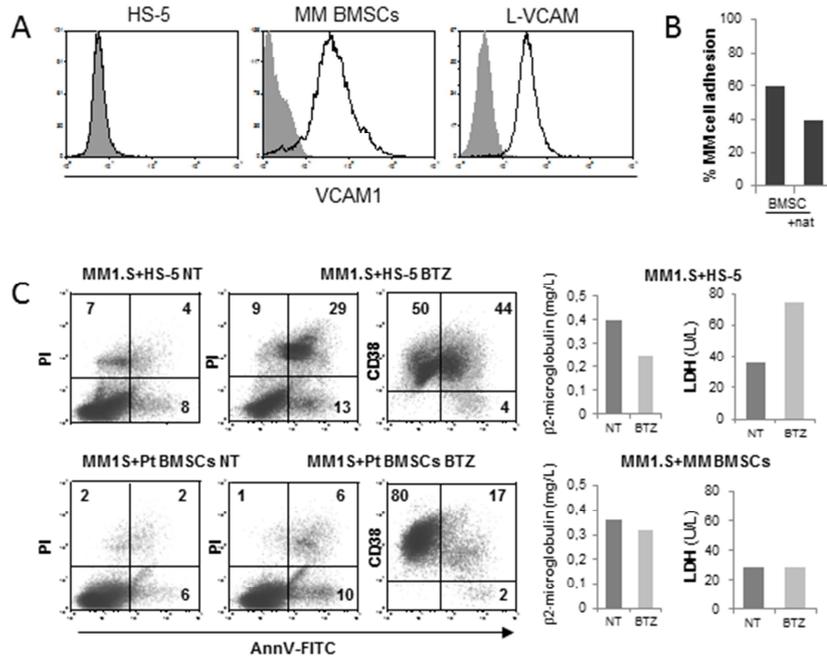


Fig.S3. (A) VCAM1 expression by HS-5 cells, primary MM BMSC and L-VCAM was determined by FACS analysis. Grey histograms represent isotype controls. B: MM1.S cell adhesion to primary MM BMSC is partially inhibited by the addition of the specific $\alpha 4$ inhibitor natalizumab. C: Resistance to bortezomib (BTZ)-induced apoptosis in vitro by HS-5 cells (upper) and primary MM BMSC (lower). MM1.S apoptosis was measured as CD38+/annexin V(AnnV)+ cells at FACS analysis (left) and $\beta 2$ -microglobulin and LDH levels in supernatants (right). Results are representative of two independent experiments. NT= not treated.

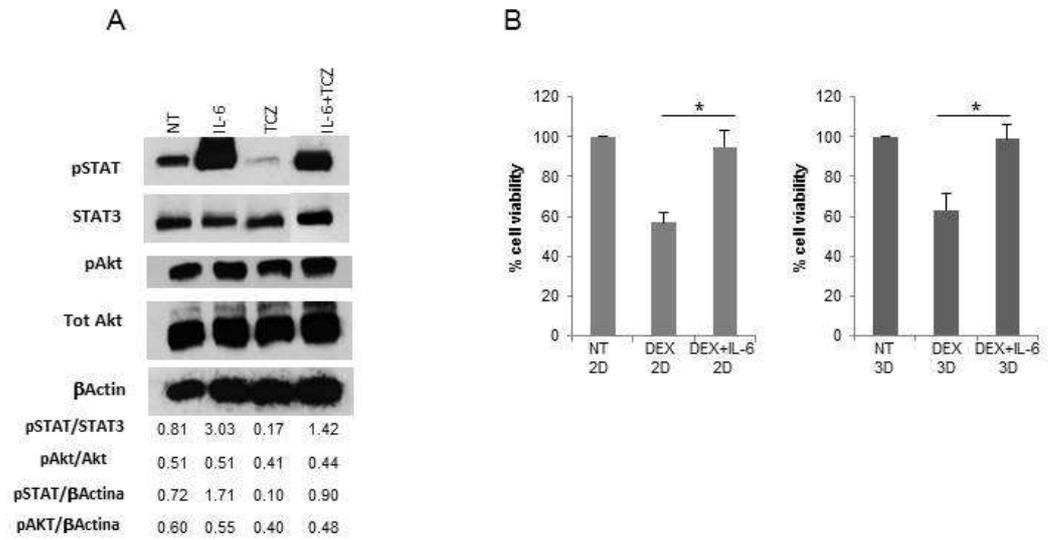


Fig.S4. A: IL-6 signaling and its inhibition exerted by tocilizumab (TCZ) treatment were evaluated in MM1.S cells. MM1.S cells, either pre-treated (30') or not with TCZ (10μg/ml), were stimulated with IL-6 (10ng/ml) for 5', lysed and submitted to WB analysis. B: viability of MM1.S.cells, either untreated or treated with dexamethasone (DEX, 20μM) in the presence/absence of IL-6 (10ng/ml) was evaluated in 2D (left) and 3D (right) conditions by MTT assay. Data are presented as % viability compared to untreated controls and are means ±SD of four independent experiments.*p ≤ 0.05.