Effects of IL-8 upregulation on cell survival and osteoclastogenesis in multiple myeloma

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Abstract

Interleukin-8 (IL-8) promotes cancer cell growth, survival, angiogenesis and metastasis in several tumors. Here, we investigated the sources of IL-8 production in multiple myeloma (MM) and its potential roles in MM pathogenesis. We found that bone marrow (BM) cells from patients with MM secreted higher amounts of IL-8 than healthy donors. IL-8 production was detected in cultures of CD138+ plasma cells and CD138- cells isolated from BMs of MM patients, and in three out of seven human myeloma cell lines (HMCLs) analyzed. Interactions between MM and stromal cells increased IL-8 secretion by stromal cells through cell-cell adhesion and soluble factors. Interestingly, *IL-8* expression also increased in HMCLs, stromal cells and osteoclasts after treatment with the anti-myeloma drugs melphalan and bortezomib. In fact, the effect of bortezomib on IL-8 production was higher than that exerted by stromal-MM cell interactions. Addition of exogenous IL-8 did not affect growth of HMCLs, although it protected cells from death induced by serum starvation through a caspase-independent mechanism. Furthermore, IL-8 induced by stromal-MM cell interactions strongly contributed to osteoclast formation *in vitro*, since osteoclastogenesis was markedly reduced by IL-8-specific neutralizing antibodies. In conclusion, our results implicate IL-8 in myeloma bone disease and point to the potential utility of an anti-IL-8 therapy to prevent undesired effects of IL-8 upregulation on survival, angiogenesis and osteolysis in MM.

Keywords: multiple myeloma, interleukin-8, co-cultures, survival, osteoclastogenesis

Introduction

Multiple myeloma (MM) is the second most common hematological malignancy with an annual incidence of 4 new cases per 100,000 people. MM is characterized by the accumulation of immunoglobulin-secreting clonal malignant plasma cells within the bone marrow (BM) and the evidence of end-organ damage, including osteolytic bone lesions, hypercalcemia, renal disease and anemia. [1-3]

Interactions between MM cells and BM cells have been widely reported to increase tumor growth, survival, migration, and drug resistance of myeloma cells. [1, 4, 5] These effects are mostly mediated by several cytokines, many of them induced by the interaction of myeloma cells with BM stromal cells (BMSCs). The network of cytokines includes several interleukins (IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-11, IL-15 and IL-21), and other growth factors such as insulin-like growth factor (IGF-1), tumor necrosis factor α (TNF-α), transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF) and macrophage inflammatory protein (MIP)-1 α. Some of these soluble factors, such as TGF- β, IL-15 and IL-21, are produced and secreted by myeloma cells themselves. Others, like IGF-1, MIP-1 α, and IL-6 are mainly produced by the cells of the microenvironment. [1, 6] Finally, some cytokines like VEFG or TNF-α are produced by both types of cells.

IL-6 is considered a major growth and survival factor for myeloma cells [1, 6]. It is upregulated in BMSCs cells by either adhesion of MM cells or by soluble factors [7-9]. The function of IL-6 as a survival factor has been demonstrated by its ability to inhibit spontaneous apoptosis and that induced by different conditions, like serum deprivation, treatment with dexamethasone or Fas cell-death receptor. [10-13]

IL-8, a member of the CXC chemokine family, was originally described as a neutrophil chemoattractant. [14] This cytokine is normally produced by a wide range of cells including lymphocytes, monocytes, endothelial cells, fibroblasts, hepatocytes and

keratinocytes. The overexpression of IL-8 in many tumors and cancer cell lines has led to investigate its role in tumor progression. [15-17] IL-8 has been shown to promote tumor growth, angiogenesis and metastasis in a variety of human cancers. [16, 18-21] Moreover, production of IL-8 by breast cancer cells has been found to increase osteoclast (OC) formation probably contributing to bone metastasis. [22, 23] In MM, BMSCs and endothelial cells from patients have been shown to secrete higher amounts of IL-8 than their healthy counterparts. [24, 25] However, the contribution of myeloma cells and their interaction with the microenvironment to IL-8 production has not been deeply analyzed. Regarding the role of IL-8 in MM cell proliferation, two reports have been published with contradictory results. In one study increased MM cell proliferation mediated by the presence of the chemokine was reported [25], whereas no differences in proliferation rates were observed in the other. [26]

Here, we investigated the sources of IL-8 production in MM and the potential role of this chemokine in MM pathogenesis. Our results showed that IL-8 was majorly induced on stromal cells after the interaction with MM cells, and was also upregulated in both myeloma and stromal cells by treatment with anti-myeloma drugs. The presence of recombinant IL-8 (rIL-8) neither affected growth of human myeloma cell lines (HMCLs) nor drug resistance. However, rIL-8 protected MM cells from cell death induced by serum starvation. Importantly, we also showed that IL-8 produced by the interaction between MM cells and human mesenchymal stem cells (hMSCs) played a major role in osteoclastogenesis.

Materials and methods

Cells and culture conditions

BM aspirates from healthy donors or patients with MM were obtained after written informed consent of participants. Aspirates were subjected to lysis to remove red blood cells, and resuspended in RPMI 1640-L-Glutamine medium supplemented with 10% fetal bovine serum (FBS) and antibiotics at an initial concentration of 7.5 X 10^5 cells/ml. Cells were grown at 37ºC in a humidified atmosphere with 5% CO_2. Supernatants were collected after 48 h of culture and stored at -20ºC until use. When necessary, $CD138^+$ plasma cells were isolated (purity >95%) from the bone marrow samples using the AutoMACS automated separation system (Miltenyi-Biotec).

The HMCLs NCI-H929 and MM.1S were adquired from ATCC (American Type Culture Collection), and the rest of the HMCLs from DMSZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). HMCLs were cultured in RPMI medium as described above. The human BM mesenchymal stromal cell line-TERT (hMSC-TERT) was a generous gift from Dr Campana (Department of Oncology and Pathology, St Jude Children's Research Hospital, Memphis, TN, USA)[27]. It was cultured in DMEM low-glucose supplemented with antibiotics and 10% FBS. The MM.1S-luc cell line was kindly provided by Dr Mitsiades (Dana-Farber Cancer Institute, Boston, MA, USA), whereas RPMI-8226-luc was lentivirally transduced to express firefly luciferase [28].

Measurement of IL-8 in culture medium

MM.1S, H929, JJN3, OPM2, U266, RPMI-8226 and IM9 (2 X 10^5 cells/ml) were cultured for three days. The supernatants were collected and analyzed for IL-8 expression using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA), according to manufacturer's instructions. IL-8 concentrations below 2 pg/ml were considered undetectable.

Co-cultures of HMCLs with hMSC-TERT cells

hMSC-TERT cells (1 X 10^5 cells) and HMCLs (2 X 10^5 cells) were co-cultured or cultured independently in 800 μl of RPMI medium for 24 h. In the experiments designed to identify which cell type was producing IL-8 in the co-culture system, either the h-MSC-TERT cells or the HMCLs were mildly fixed by incubation with 1% paraformaldehyde for 10 min, extensively washed with PBS and co-cultured as described above. When physical contact in the co-culture wanted to be avoided, 100 µl of medium containing 5 X 10^4 MM cells was added to the upper chamber of a 24-transwell plate (0.4 µm pore size; Costar) that contained in the lower chamber 600 µl of medium with adhered hMSC-TERT cells (1.2 X 10^5 cells). The system physically separated HMCLs from hMSC-TERT cells but allowed interaction between them by soluble factors. The supernatants were collected after 48 h of culture and measured for IL-8 secretion by ELISA. Cells were recovered and used for RNA extraction.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

To analyze *IL-8* expression, total RNA was reverse transcribed to cDNA using a cDNA Reverse Transcription Kit from Applied Byosystems (Foster City, CA, USA). qRT-PCR was performed using iQ™ SYBR® Green Supermix kit (Bio-Rad, Hercules, CA, USA), the iQ5 PCR detection system, and the following gene-specific primers: *IL-8*, forward 5' GTGCAGTTTTGCCAAGGAGT-3´ and reverse 5'- CTCTGCACCCAGTTTTCCTT-3´ *GAPDH*, forward 5'- GGGTGGAATCATATTGGAACATGTA-3' and reverse 5'-. CAGGGCTGCTTTTAACTCTGGTAA-3'. Relative expression of *IL-8* was normalized to *GAPDH* using the $2^{-\Delta Ct}$ method, where $\Delta Ct = Ct_{IL-8} - Ct_{GAPDH}$).

Reagents

rIL-8, anti-IL-8 and anti-IL-6 antibodies were purchased from *R&D* Systems*.* rIL-6 was obtained from PeproTech (London, UK). Melphalan and dexamethasone were obtained

from Sigma-Aldrich (St. Louis, MO, USA) and bortezomib (Velcade) was obtained from the Pharmacy of the University Hospital of Salamanca. Anti-Fas was obtained from Upstate-Millipore (Billerica, MA, USA). Macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-kB ligand (RANKL) were supplied by PeproTech. BAY 11-7082 and PS-1145 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell proliferation and cell viability assays

Cell proliferation in the presence of rIL-8 or IL-8 blocking antibodies, was determined by cell counting after 48 or 72 h of culture. Alternatively, cell growth was monitored by BrdU incorporation, quantitation of ATP (CellTiter-Glo, Promega, madison, WI, USA) or by measurement luminescence of RPMI-8226-luc and MM.1S-luc HMCLs. Cell viability in the presence of drugs was assessed by the MTT assay. Cell survival in the absence of serum and in the presence of rIL-8, rIL-6 or the corresponding blocking antibodies was determined by trypan blue exclusion and apoptosis by annexin V-isothiocyanate/propidium iodide (PI) double staining, according to manufacturer´s procedure (Immunostep, Salamanca, Spain). Caspase-3 activity was determined using a colorimetric assay kit provided by Promega.

In vitro osteoclast formation assay

Peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy donors after centrifugation with Ficoll-Paque™ (GE Healthcare, Uppsala, Sweden). Cells were resuspended at 1.5 X 10^6 cells/ml in alpha-minimum essential (α-MEM) medium containing 10% FBS, and cultured overnight in 96-well plates (100 µl of cell suspension per well). Adherent cells were then cultured in the same medium in the presence of M-CSF (25 ng/ml) as a negative osteoclastogenic control; addition of recombinant RANKL (rRANKL) (50 ng/ml) was assayed as a positive control for

osteoclasts formation. Conditioned medium (CM) from IM9-hMSC-TERT direct co-cultures was assayed at 1:4 dilution and rIL-8 or IL-8-blocking antibodies were assayed at 50 ng/ml and 25 µg/ml, respectively. CM contained 10 ± 0.3 ng/ml of IL-8, as measured by ELISA. Anti-IL-8 was added to some of the CM-containing wells. The concentration used, 25 µg/ml, completely blocked the IL-8 found in CM, as determined by ELISA. Medium was replaced twice a week and cultures were terminated on day 21. Plates were stained for tartrate resistance acid phosphatase (TRAP) (Sigma) and multinucleated TRAP+ cells (≥ 3 nuclei) were counted as osteoclast.

Statistical analysis

Differences between the data were assessed for statistical significance using the Student's unpaired two tailed *t*-test with the Simfit statistical software version 7.0.5 (Manchester, UK).

Results

Expression of IL-8 in MM and normal plasma cells (NPCs)

Using microarrays expression data from our previous studies (http://www.ncbi.nlm.nih.gov/geo; GEO accession number: GSE47552), *IL-8* mRNA was found to be upregulated in CD138+ PCs isolated from patients with MM compared to NPCs (Figure 1A). [29] In contrast, the expression of *IL-6* mRNA was similar in NPCs and multiple myeloma plasma cells (MMPCs). Overexpression of *IL-8* in CD138+ PCs from several MM patients was verified by qRT-PCR (Figure 1B). Then, secretion of IL-8 was measured by ELISA in culture supernatants from BM cells obtained from 15 MM patients and 10 healthy donors. We found that BM cells from MM patients secreted statistically significant higher amounts of IL-8 than those from healthy donors, whose IL-8 levels were non-detectable in most of the cases (Figure 1C, D). However, no correlation was found between levels of IL-8 and the percentage of PCs present in the BM of each patient. In fact, BMs with the highest proportion of PCs (more than 80%) secreted less amounts of IL-8 than others with lower PC infiltration. This result suggested that although high levels of IL-8 were associated with the presence of MMPCs, these cells might not be the main producers in the BM.

Direct cell contact between MMPCs and the remaining BM cells increased IL-8 secretion

Interactions between MM cells and BM cells have been shown to enhance the production of IL-6 [10] and other angiogenic factors, such as VEGF or hepatocyte growth factor (HGF). [8, 30, 31] To determine whether IL-8 was also induced as a consequence of the interaction between myeloma cells and the remaining BM cell populations, IL-8 levels were quantified by ELISA in the 24 h culture supernatant collected from the same number of CD138+ MMPCs and CD138- cell fraction cultured separately or

together (direct co-culture) (Figure 2). The assays carried out in three different patients showed that CD138- cells secreted higher amounts of IL-8 than CD138+ cells, suggesting that BM cell populations other than myeloma cells were the major IL-8 producers. In addition, IL-8 levels were further increased after co-culture of both types of cells, indicating that CD138- cells, CD138+ cells or both favored the production of IL-8 when cultured together.

Adhesion of MM cell lines to mesenchymal stromal cells triggered IL-8 secretion

ELISA quantification of IL-8 in the conditioned medium (CM) from different HMCLs showed that IL-8 production was low in IM9, JJN3 and RPMI-8226, and undetectable in the CM from all other HMCLs (Figure 3A).

To analyze whether adherence of HMCLs to stromal cells induced IL-8 secretion, four MM cell lines (IM9, RPMI-8226, JJN3 and U266) were cultured alone or together with the stromal hMSC-TERT cell line. IL-8 secretion increased approximately 3-4-fold when RPMI-8226, JJN3 or U266 were in contact with the stromal cell line, and nearly 30-fold when the IM9 cell line was co-cultured with hMSC-TERT cells compared to hMSC-TERT cells alone (Figure 3B). To determine the identity of the cells that increased IL-8 production, HMCLs or hMSC-TERT cells were fixed with paraformaldehyde before the co-cultures, according to a protocol previously described for other cytokines or growth factors [8, 32]. Fixation of hMSC-TERT cells before co-culture with RPMI-8226, JJN3 or IM9 abolished IL-8 production (Figure 3C). In contrast, fixation of RPMI-8226 or JJN3 did not significantly inhibit IL-8 overproduction induced by the co-culture. These results indicated that stromal cells were the major source of IL-8, and that IL-8 secretion, when stromal cells were co-cultured with RPMI-8226 or JJN3, was mainly induced by cell-cell adhesion. However, fixation of IM9 cells markedly reduced IL-8 production induced by the co-culture with hMSC-TERT cells, which suggested that soluble factors might also be contributing to IL-8 overexpression in addition to cell-cell adhesion.

Soluble factors contributed to IL-8 overproduction in co-cultures of IM9 with hMSC-TERT cells

To further investigate the IL-8 secretion induced by the interaction of IM9 with hMSC-TERT cells, co-cultures were established using transwell inserts, which prevent contact of both types of cells but allow the diffusion of soluble factors. Secretion of IL-8 was found highly increased when cells were co-cultured in the absence of physical contact (Figure 3D, MSC+IM9 TW), although to a lesser extent than that observed by direct cell-cell contact (Figure 3D, MSC+IM9). These results confirmed that induction of IL-8 in co-cultures of IM9 with hMSC-TERT cells was dependent on both cell-cell adhesion and soluble factors. To identify the cells responsible for the increased IL-8 production in the absence of physical contact, *IL-8* mRNA expression was quantified in IM9 and stromal cells separately recovered after the transwell co-culture (Figure 3E). *IL-8* mRNA levels were upregulated in both IM9 and hMSC-TERT cells as a result of the interaction between the two cell types in the absence of cell contact, although induction was much higher in hMSC-TERT cells.

It has been previously shown that induction of IL-6 secretion from BMSCs triggered by MM cell adhesion is mediated through NF-κB. [33] Likewise, IL-8 production by stromal cells in the presence of BM culture supernatants from MM patients was dependent on NF-kB signaling [26]. So we expected that this transcription factor would also be implicated in IL-8 overexpression observed in hMSC-TERT cells co-cultured with IM9. Thus, we found that the NF-κB inhibitor BAY 11-7082 partially blocked the *IL-8* upregulation observed in hMSC-TERT cells after the co-culture with IM9 cells, which confirmed that NF-κB was involved in the production of IL-8 in hMSC-TERT cells triggered by MM cell interactions (Figure 3F).

IL-8 secretion was induced by treatment with anti-myeloma drugs

It has been described that expression of IL-8 is induced by treatment with paclitaxel in non-small cell lung cancer (NSCLC) [34] and ovarian carcinoma cells. [35] Likewise, increased expression of *IL-8* mRNA has been recently reported after treatment of MM cells with lenalidomide [36]. These observations prompted us to investigate the effect of other anti-myeloma drugs in IL-8 secretion. We found that treatment of RPMI-8226 and IM9 cells with bortezomib or melphalan increased *IL-8* expression (Figure 4A). However, *IL-8* upregulation was found to be NF-κB-independent since treatment of these HMCLs with the NF-κB inhibitor BAY 11-7082, at the inhibitory concentration of 5 μM [37, 38], did not suppress either the bortezomib or the melphalan-induced *IL-8* expression (Figure 4B). Similar results were obtained with the specific IKKβ inhibitor PS-1145 (20 μM) [37](data not shown). We then explored whether stromal cells and osteoclasts, as components of the BM microenvironment, also increased *IL-8* expression after treatment with the anti-myeloma drugs. As shown in Figure 4B, the presence of these drugs clearly induced *IL-8* expression in the two cell types, although *IL-8* induction caused by melphalan required higher doses than those employed in MM cells. Next, we analyzed IL-8 secretion in MM cell lines and in the stromal cell line hMSC-TERT cultured alone or co-cultured in the absence or the presence of bortezomib and melphalan (Figure 4C). We observed that bortezomib increased IL-8 secretion by MM cells and stromal cells when cultured alone, whereas melphalan at 30 μM only induced IL-8 overexpression in MM cells, in accordance with the qRT-PCR data. Interestingly, we found that IL-8 secretion significantly augmented in bortezomib-treated co-cultures compared to those left untreated, indicating that the effect of this drug was higher than the effect of the co-culture on IL-8 production. Therefore, bortezomib induced IL-8 overexpression in hMSC-TERT cells and MM cells cultured separately or co-cultured. In order to give support to this finding in an *in vivo* setting, we also analyzed the effect of bortezomib on IL-8 secretion of BM cells isolated from three MM patients. BM cells clearly overexpressed IL-8 after treatment with the drug (Figure 4D).

rIL-8 protected MM cells from cell death induced by serum starvation through a caspase-independent mechanism

IL-8 has previously been reported to function as an autocrine growth factor for different normal and tumor cells. [39-41] However, the role of IL-8 in MM cell proliferation is controversial. [25, 26] We found that neither rIL-8 addition nor anti-IL-8 neutralizing antibodies affected cell proliferation of HMCLs as measured by cell counting (Figure 5A), BrdU incorporation, ATP quantification, or when the effect of rIL-8 or anti-IL-8 was assayed in RPMI-8226-luc and MM.1S-luc cells (data not shown). Similarly, addition of rIL-8 did not stimulate growth of myeloma cells even at low serum concentration (1%) (data not shown).

Previous studies have demonstrated that IL-6 protects myeloma cells from cell death induced by treatment with anti-myeloma drugs, from spontaneous and Fas-mediated apoptosis, as well as from cell death induced by serum starvation [10-13]. We first investigated the effects of IL-8 on MM cell sensitivity to melphalan in RPMI-8226 (Figure 5B), MM.1S, JJN3 and IM9 cell lines (data not shown). Addition of rIL-8 did not affect cell death induced by melphalan (Figure 5B, left panel), even when the amounts of rIL-8 added exceeded those induced by the treatment (Figure 4A). On the other hand, treatment with anti-IL-8 neutralizing antibodies had no effect on cell death induced by the drug (Figure 5B, right panel). No protection against cell death was observed after treatment with different concentrations of bortezomib, dexamethasone, Fas ligand or when spontaneous cell death was analyzed (data not shown). However, we observed that rIL-8 clearly prevented cell death induced by serum starvation in a similar way to IL-6, which was used as a positive control (Figure 5C, left panel). As expected, cell death protection was abrogated by addition of anti-IL6 or anti-IL-8 neutralizing antibodies (Figure 5C, right panel). When apoptosis was analyzed using Annexin V/PI staining, we found that addition of rIL-8 clearly protected MM cells from apoptotic cell death induced by serum starvation (Figure 5D). Interestingly, caspase 3

was activated after serum deprivation, even when rIL-8 was added to the media (Figure 5E). These results indicated that IL-8 protected HMCLs from apoptosis induced by serum deprivation via a caspase-independent mechanism.

rIL-8 stimulated growth of osteoclasts precursors and IL-8 induced by the interaction of MM cells with stromal cells strongly contributed to osteoclasts formation in vitro

To analyze the role of IL-8 in osteoclastogenesis we first investigated whether this chemokine influenced the growth of osteoclasts precursors using PBMCs cultured with M-CSF in the absence or in the presence of different concentrations of rIL-8. After 7 days of culture, cell density was increased according to the concentrations of the chemokine (Figure 6A), which was corroborated measuring proliferation by the MTT assay (Figure 6B).

In order to ascertain the relative contribution of IL-8 induced by the interaction of MM cells with stromal cells to osteoclasts differentiation, CM from IM9-hMSC-TERT co-cultures, with and without IL-8 neutralizing antibody was added to human PBMCs cultured in the presence of M-CSF. We found that the CM from the co-culture induced osteoclasts formation (Figure 7A) as also did rIL-8 (Figure 7B), whereas anti-IL-8 antibody significantly reduced the number of osteoclasts provoked by the CM (Figure 7A). Osteoclasts induced by RANKL or IL-8 are shown in figure 7C.

Discussion

In this study we showed that interactions between myeloma and stromal cells triggered IL-8 overexpression. This chemokine, induced in stromal cells by adhesion of MM cells and also by soluble factors, increased myeloma cell survival in serum-starvation conditions and highly contributed to osteoclast formation. Moreover, we found that treatment with anti-myeloma drugs also stimulated IL-8 overexpression.

Production of IL-8 by cancer cells has been shown in melanoma, colon carcinoma, liver and pancreatic cell lines. [39, 42-44] In this study, we showed using qRT-PCR that MM cells isolated from several patients expressed higher levels of *IL-8* than those isolated from healthy donors, confirming the overexpression of this chemokine in primary MM cells previously observed by expression microarrays. [29, 45] Secretion of IL-8 was detected in PCs isolated from patients with MM and in some HMCLs, and interactions between stromal and myeloma cells significantly increased the production of this chemokine. Stromal cells were the major source of IL-8, as also described for VEGF and HGF [30, 31], which highlights the importance of these cells in producing angiogenic factors. In IM9/hMSC-TERT co-cultures, IL-8 secretion was mainly increased in stromal cells by both cell adhesion and soluble factors, which is analogous to the secretion pattern described for IL-6. [8] Levels of *IL-8* transcripts also increased in IM9 cells after co-cultures established in the absence of physical contact. These findings are in agreement with the results described in NSCLC, in which tumor/stromal co-cultures induced IL-8 production, in pulmonary fibroblast and in certain NSCLC cell lines. [46] According to previous published results for IL-6 and IL-8, we found that *IL-8* upregulation in stromal cells due to interaction with IM9 cells was partially mediated by the transcription factor NF-kB. [26, 33]

Interestingly, *IL-8* expression was also induced by treatment of MM cells, stromal cells, and osteoclasts with bortezomib or melphalan. Bortezomib-induced *IL-8* expression

was also observed in BM cells isolated from MM patients. Overexpression of IL-8 by MM cells after treatment with lenalidomine has previously been described [36], and expression of *IL-8* has also been reported to be induced by treatment with paclitaxel in solid tumors. [34, 35] A recent study also found induction of IL-8 in ovarian cells by treatment with bortezomib although this effect was not observed in HMCLs [47], in disagreement with our results. We speculate that the discrepancy could be related to the high concentrations of bortezomib used by Singha *et al*, which would result in MM cell death before IL-8 induction. *IL-8* upregulation by paclitaxel has been described to be mediated, at least in part, by the drug-mediated activation of NF-kB, a known inducer of *IL-6* and *IL-8* expression. [48, 49] NF-kB activation has also been reported when MM cells were treated with melphalan [50], and also when ovarian cancer cell lines and HMCLs were treated with bortezomib [47, 51], suggesting that IL-8 upregulation induced by treatment with these two drugs in MM could also respond to NF-kB activation. Surprisingly, we found that chemical inhibition of NF-kB did not suppress either bortezomib or melphalan-induced *IL-8* overexpression. Our results indicate that other transcriptional or post-transcriptional mechanisms must be responsible for the chemokine upregulation in MM and highlight the fact that mechanisms governing *IL-8* expression may differ among different cell types. [47, 52] In this regard, a recent study has reported that bortezomib induced *IL-8* expression in human monocytes and macrophages through a mechanism p38 MAPK-dependent and NF-kB-independent [53].

We have shown that neither rIL-8 addition nor inhibition of this chemokine by neutralizing antibodies, affected the proliferation of HMCLs, contrary to the data reported for other tumor cell lines [39, 41-44] and also in MM. [25] However, our results are in agreement with a previous study showing no effect of IL-8 on MM cell proliferation despite the presence of IL-8 receptors on different HMCLs. [26] We observed that IL-8 clearly enhanced survival of myeloma cells in the absence of serum, as also described for IL-6. [13] Neutralizing antibodies avoided the protective effect of IL-8 against cell

death induced by serum deprivation, indirectly supporting the presence of IL-8 receptors (CXCR1/CXCR2) on HMCLs. It is possible that the signaling pathways that promote IL-8 mediated-cell growth may be non-functional in MM cells, whereas those that enhance survival in the absence of growth factors could be fully operative. The effect of IL-6 in survival and protection from drug-mediated apoptosis has been attributed to the activation of JAK-STAT and PI3K/Akt signaling cascades. [1]However, its role in the protection of cell death induced by growth factor withdrawal has not been characterized. Here, we showed that protection of IL-8 against cell death induced by serum deprivation did not depend on inhibition of caspase activation, in agreement with some reports that showed the existence of caspase-independent cell death under serum free conditions. [54, 55] We found that IL-8 did not protect MM cells from apoptosis induced by chemotherapeutic agents. The fact that bortezomib and also melphalan induce caspase-dependent apoptosis in MM [56, 57] makes it tempting to speculate that IL-8 could only block myeloma apoptosis induced by caspase-independent mechanisms.

It has previously been described that rIL-8 induces osteoclasts formation. [22, 23] In this study we observed, first, that rIL-8 acts as a growth factor for osteoclasts precursors, which could eventually differentiate to functional osteoclasts in bone-resorbing areas at sites of myeloma growth; and second, that the CM from myeloma-stromal co-cultures triggered osteoclast differentiation. Neutralization of IL-8 by the correspondent antibodies markedly reduced osteoclasts formation, which revealed that the osteoclastogenic activity of the CM was mainly induced by IL-8. We found that r-IL-8 reproduced the effect of the CM, and directly stimulated osteoclastogenesis independent of RANKL, in agreement with previous reports. [22, 23]

In summary, we showed that interactions between myeloma and stromal cells, and treatment with anti-myeloma drugs triggered IL-8 overexpression. BM stromal cells were the major source of IL-8 in stromal-MM co-cultures. However, we cannot exclude

the possibility that other cells in the BM microenvironment, in addition to stromal cells, might contribute to IL-8 production. This is in fact indirectly suggested by the high content of IL-8 in the CM of BM cultures from myeloma patients (Figure 1C), and also by the elevated serum levels of IL-8 found in MM patients [58, 59]. In this study, we present evidence of osteoclastogenesis promotion by IL-8 from myeloma and stromal cultures and anti-apoptotic effects of IL-8 on serum-deprived myeloma cells. These findings, together with the angiogenic and MM cell chemotactic roles of IL-8 [25, 26], suggest that anti-IL-8 therapy might be beneficial to prevent undesired effects of IL-8 upregulation due to interactions of myeloma cells with the microenvironment or treatment with anti-myeloma drugs.

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Figure legends

Figure 1. Expression and secretion of IL-8 in MM. **A:** *IL-8* and *IL-6* gene expression by microarray analysis in PCs derived from healthy donors or MM patients (CD138+-isolated cells). Gene expression was determined by microarray analysis. Data are the average ± SD of 5 NPCs and PCs from 74 MM samples. (*** p<0.001). **B:** Expression levels of *IL-8* quantified by qRT-PCR. Expression of *IL-8* was normalized to *GAPDH* and presented as $2^{-\Delta Ct}$. **C:** IL-8 levels, assessed by ELISA, in culture supernatants of BM cells obtained from healthy donors and patients with MM. Percentage of PC infiltration for each sample is indicated. **D:** Box plot representation of IL-8 levels showed in C. (*** p<0.001).

Figure 2. Interactions between MM cells and stromal cells induce IL-8 overproduction. CD138+ PCs and CD138- cells were isolated from three patients with MM using inmunomagnetic beads. CD138+ cells (1 X 10^5) and CD138- cells (1 X 10^5) were cultured independently or together in 200 μl of culture medium. IL-8 was measured after 24 h of culture by ELISA. Data are shown as mean ± SD from duplicates. (** p<0.01, * p<0.05).

Figure 3. Interactions between HMCLs and stromal cells increase IL-8 secretion. **A:** IL-8 levels in culture supernatants of HMCLs measured by ELISA. **B:** Cells from the indicated HMCLs and hMSC-TERT cells (MSC) were cultured independently or together in 800 μl of culture medium. IL-8 levels were determined after 24 h of culture by ELISA. Data shown are mean ± SD from three independent experiments. (*** p<0.001; ** p<0.01 compared to MSC). **C:** Same as (B) but with hMSC-TERT cells or HMCLs mildly fixed with paraformaldehyde (indicated as F). Data are the mean of three independent experiments (*** p<0.001 compared to MSC). **D:** IL-8 levels in CM from hMSC-TERT cells, IM9 cells or direct or transwell (TW) co-cultures. **E:** *IL-8* expression levels in hMSC-TERT or IM9 cells quantified by qRT-PCR in the specified conditions.

F: hMSC-TERT cells ($4x10^5$) per well were plated in 6-well dishes and allowed to form an adherent monolayer over 24 h. Then hMSC-TERT cells and IM9 cells (6×10^5) were pretreated 3 h with non-lethal concentrations of BAY 11-7082 (5 µM and 3 µM, respectively) and cultured independently or co-cultured with 3 µM BAY 11-7082 for an additional 24 h in RPMI medium. Finally, MM cells were mechanically detached from the hMSC-TERT, leaving the monolayer undisturbed and *IL-8* expression was quantified in hMSC-TERT cells by qRT-PCR. Data in D, E and F are means ± SD from three independent experiments (*** $p<0.001$; ** $p<0.01$).

Figure 4. Upregulation of *IL-8* by treatment with bortezomib and melphalan. **A:** *IL-8* expression in RPMI-8226 and IM9 cells pretreated or not 12 h with BAY 11-7082 before 24 h incubation with the indicated doses of bortezomib and melphalan. BAY 11-7082 was used at 5 µM in RPMI-8226 cells and at 3 µM in IM9 cells **B:** *IL-8* expression in hMSC-TERT cells (MSC) and osteoclasts after 24 h of culture in the absence (UT) or the presence of the indicated drugs. Osteoclasts were obtained from PBMCs after 21 days of culture in osteogenic medium containing M-CSF (25 ng/ml) and RANKL (50 ng/ml). **C:** HMCLs and hMSC-TERT cells (MSC) were cultured independently or co-cultured in 800 µl of culture medium in the absence (UT) or the presence of the indicated drugs. IL-8 levels were determined after 24 h of culture by ELISA. Data are means ± SD from three independent experiments (*** $p<0.001$ compared to UT cells; ** $p<0.01$; * $p<0.05$). **D:** IL-8 secretion by BM cells isolated from three MM patients treated or not (UT) with bortezomib for 24 h.

Figure 5. rIL-8 affects neither the growth rate of MM cells nor their resistance to anti-myeloma drugs, but protects them from cell death induced by serum starvation. **A:** Cell growth of IM9 and JJN3 cell lines. Cells were plated at 5×10^4 cells/ml in 24-well plates and cultured with medium alone or medium containing rIL-8 (100 ng/ml) or anti-IL-8 (5 µg/ml). Cell number was determined after 48 and 72 h of culture. Values correspond to mean cell number ± SD of triplicates. **B:** Survival of RPMI-8226 cells to melphalan after

72 h of culture in the presence of IL-8 (panel 1) or anti-IL-8 neutralizing antibodies (panel 2) assessed by MTT. **C:** Cells were plated at 1 X 10^5 cells/ml in serum-free medium and survival was determined at indicated times by trypan blue exclusion. Recombinant IL-6, rIL-8 or the corresponding neutralizing antibodies were used when indicated at 100 ng/ml (IL-6 and IL-8) or 5 µg/ml (IL-6 or IL-8 neutralizing antibodies). **D:** Cell death in JJN3 and H929 cells determined by Annexin/PI double staining after 72 h of culture in serum-free medium and in the same medium supplemented with rIL-8 (100 ng/ml). Percentages of annexin-/PI- (live cells) correspond to the mean ± SD of three independent experiments (** $p< 0.01$ compared to untreated cells). Representative experiments are shown. **E:** Caspase 3 activation in JJN3 and H929 at the indicated times of culture in serum-free medium in the absence or in the presence of rIL-8 (100 ng/ml).

Figure 6. rIL-8 stimulates the growth of osteoclasts precursors. **A:** Representative micrographs of osteoclasts precursors stained with hematoxylin/eosin (HE*).* PBMCs were cultured in osteogenic medium containing M-CSF (25 ng/ml) and the indicated concentrations of rIL-8 for 7 days. **B:** Cell viability was evaluated at the indicated conditions by the MTT assay (* $p< 0.05$ compared to M-CSF-treated cells).

Figure 7. IL-8 induced by interactions between HMCLs and hMSC-TERT cells strongly contributes to osteoclasts formation. **A:** PBMCs were cultured in osteoclastogenic medium with M-CSF (25 ng/ml), or in the same medium containing either RANKL (50 ng/ml) or CM from IM9-hMSC-TERT cultures at 1:4 dilution. Anti-IL-8 was added to some of the CM-containing wells. Percentage of TRAP+ multinucleated cells (≥ 3 nuclei) is indicated. A total of 400 cells were counted per treatment group. The experiment was performed in triplicate. (* $p<0.05$, compared to wells containing only M-CSF). **B:** Osteoclasts formation induced by rIL-8 or RANKL. The experiment was performed as described in A, but with rIL-8 (50 ng/ml). **C:** Representative micrographs of osteoclasts induced by RANKL or rIL-8.

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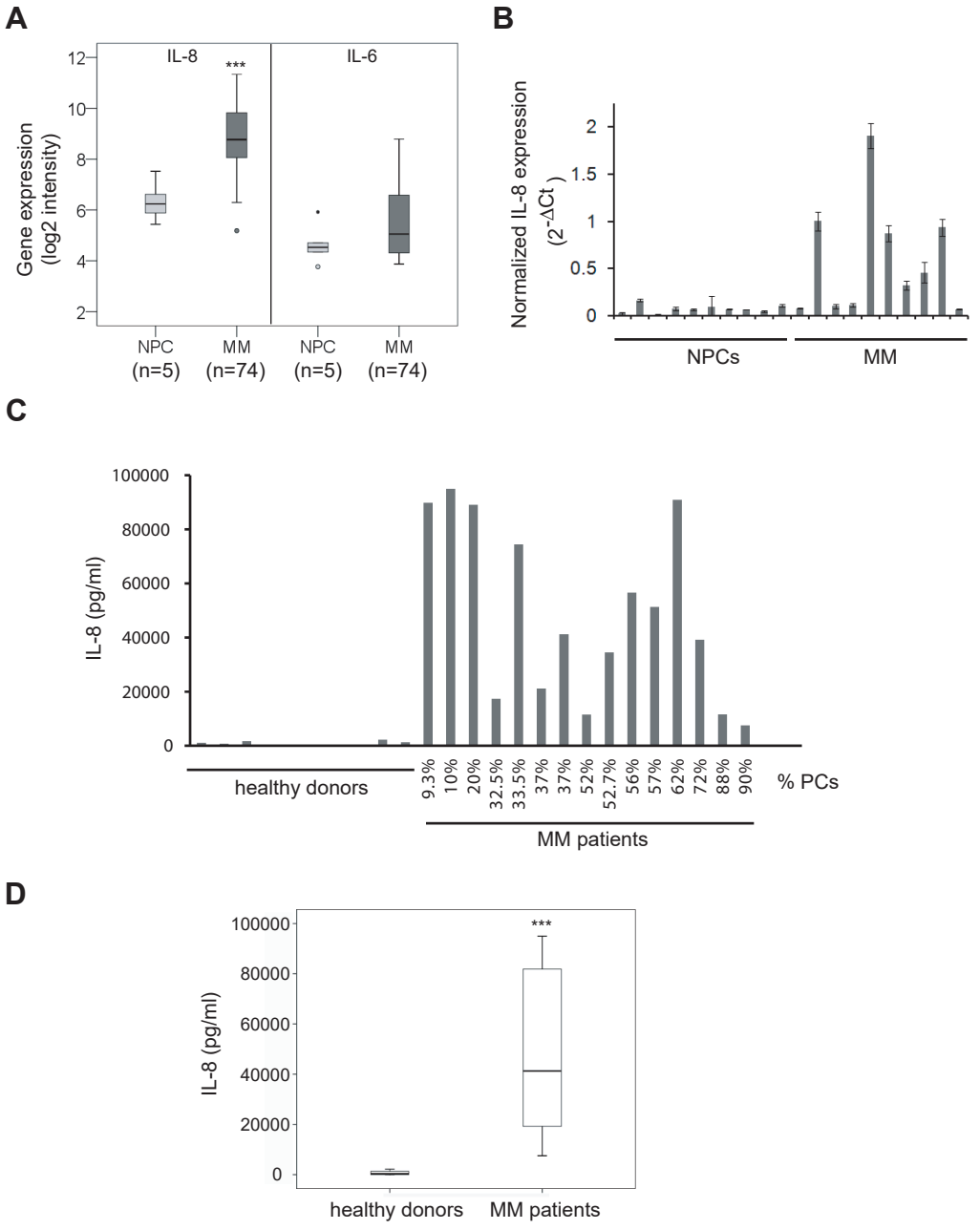

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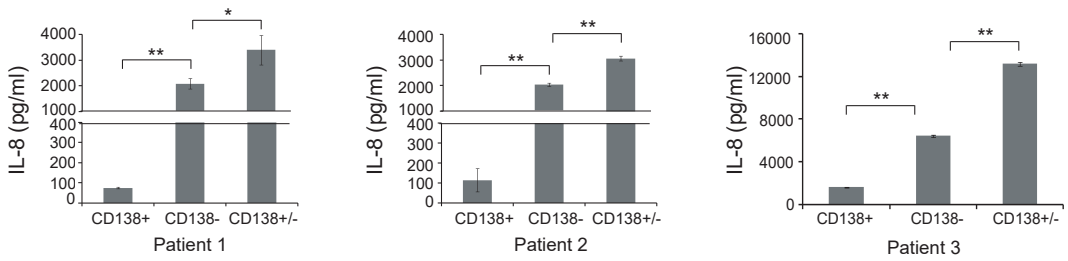

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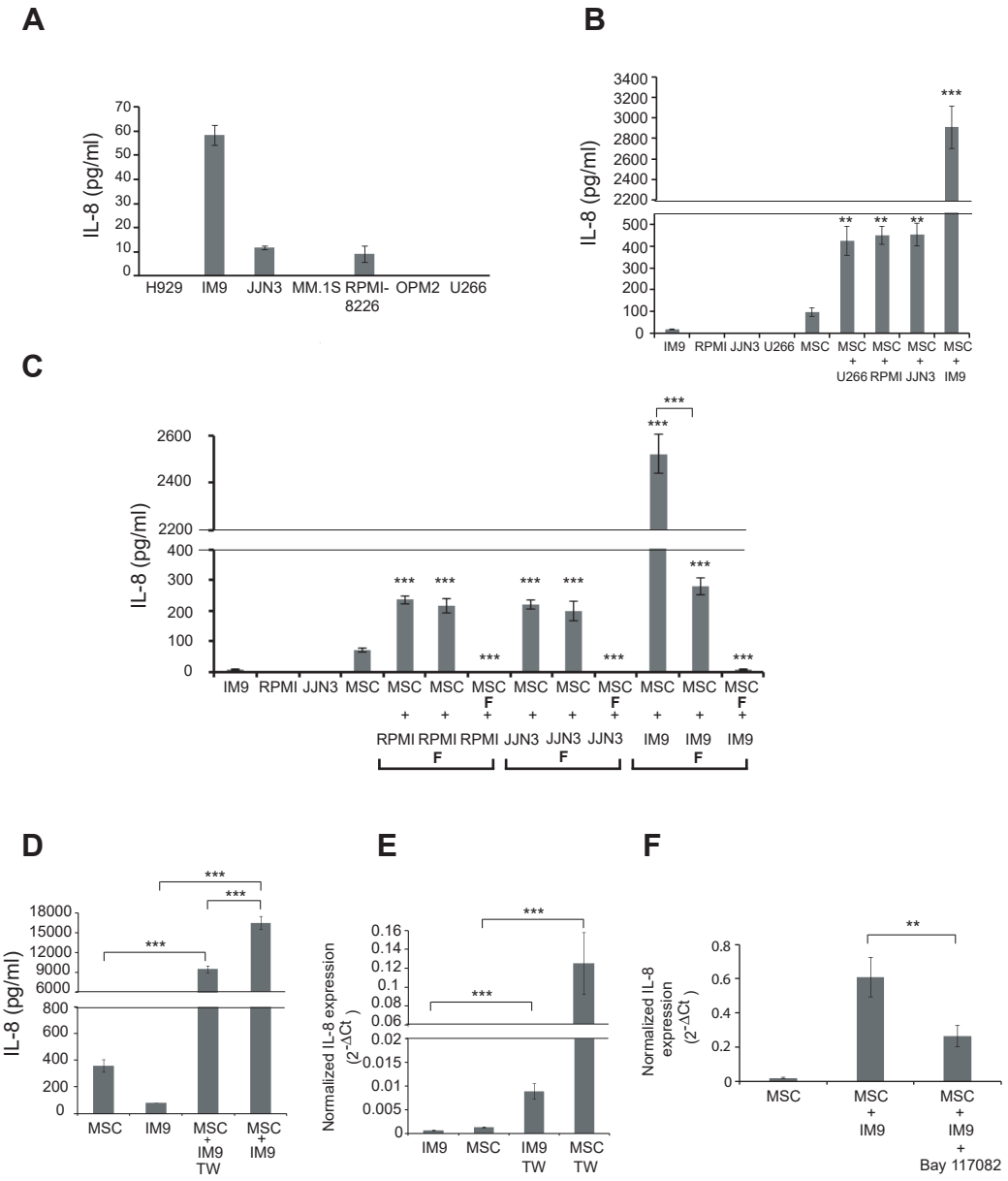

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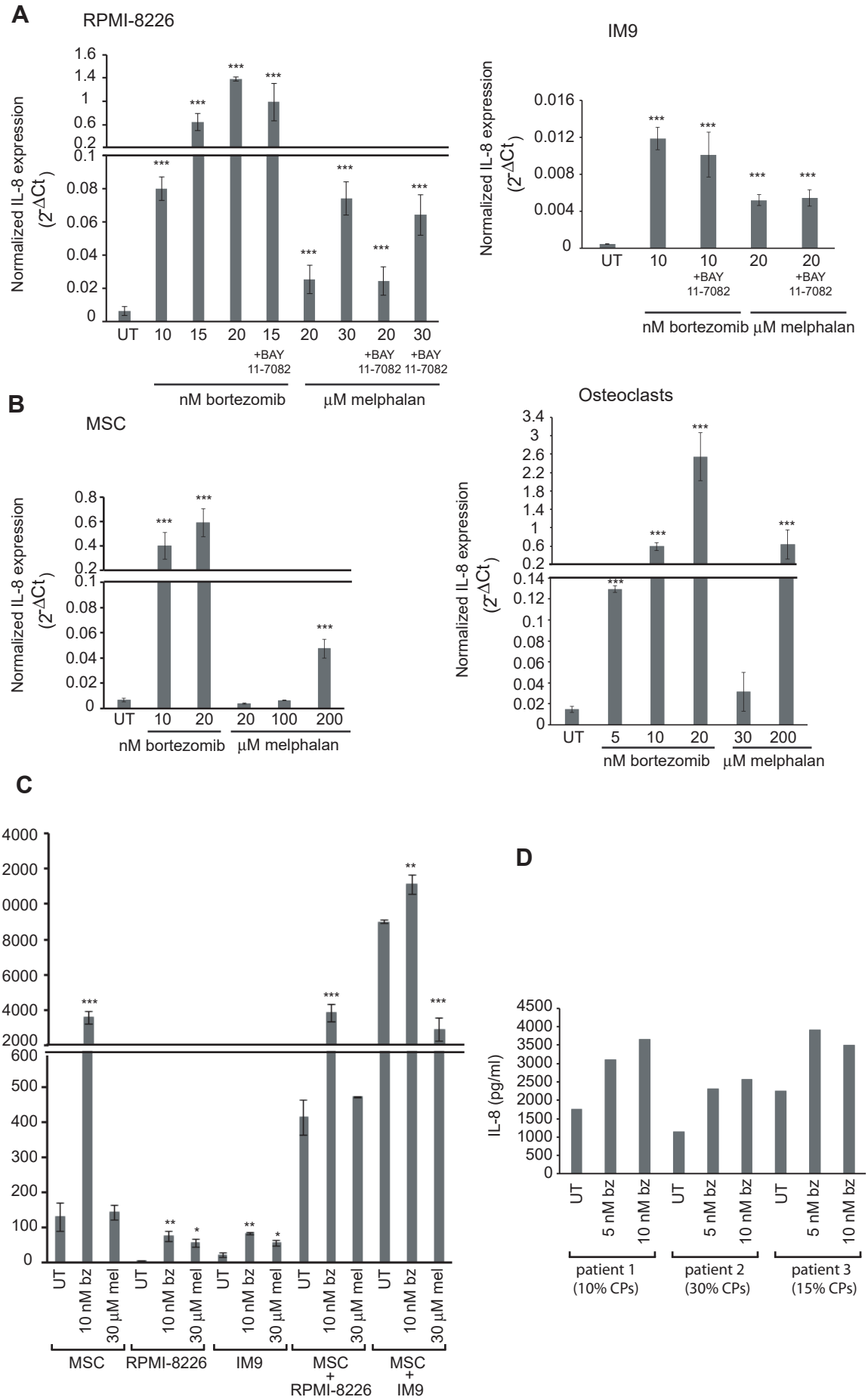

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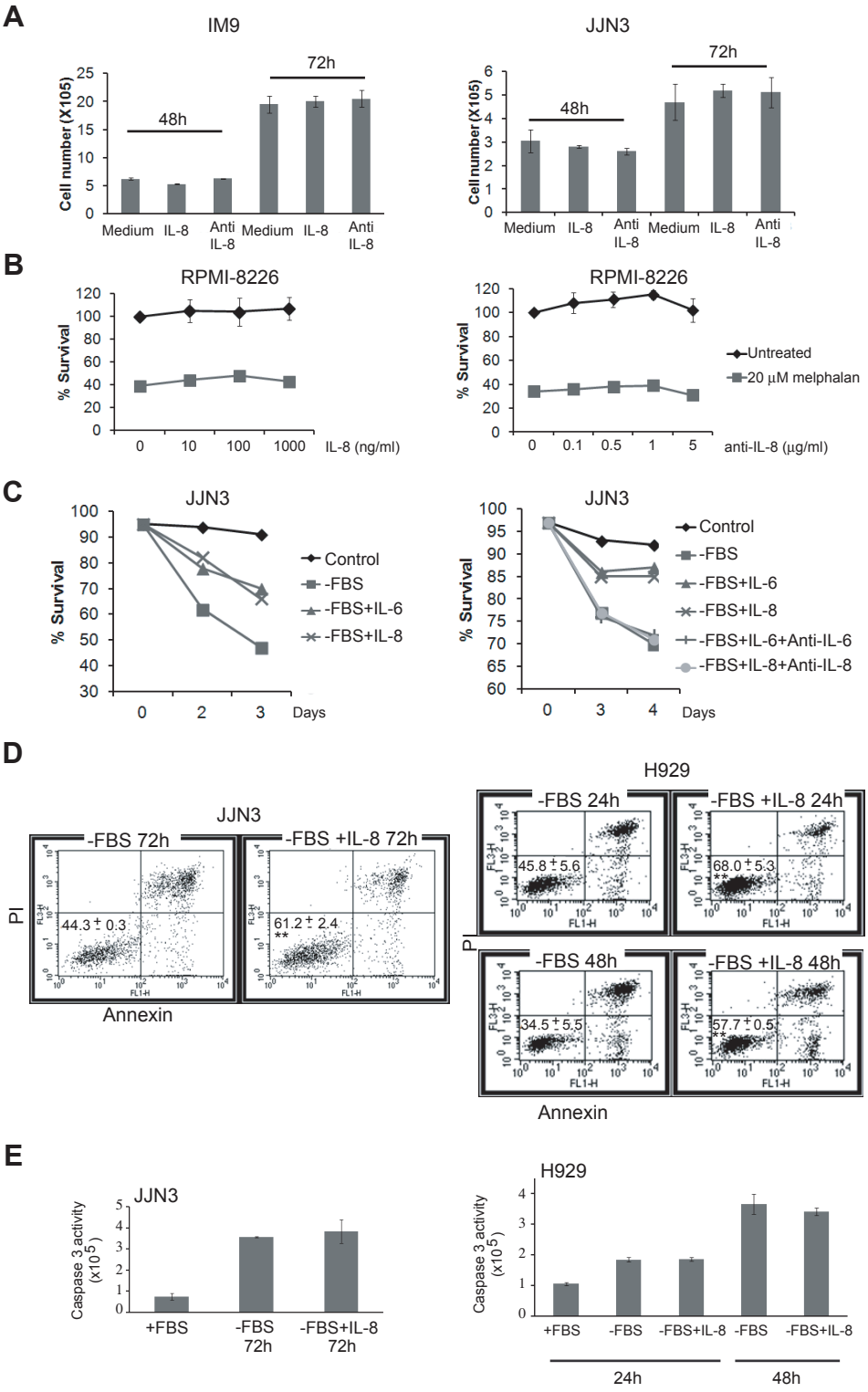

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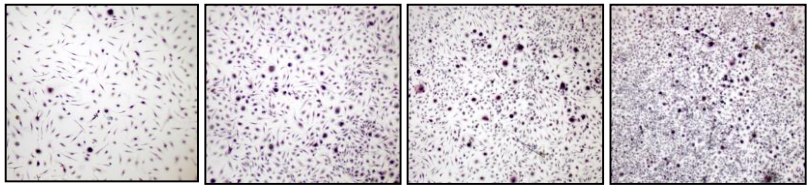
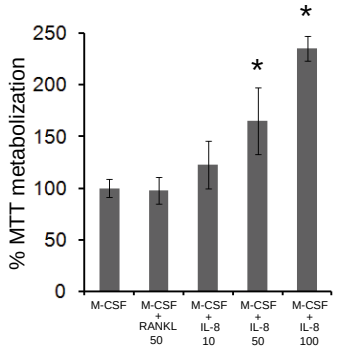
A



0 10 50 100 IL-8 (ng/ml)

B



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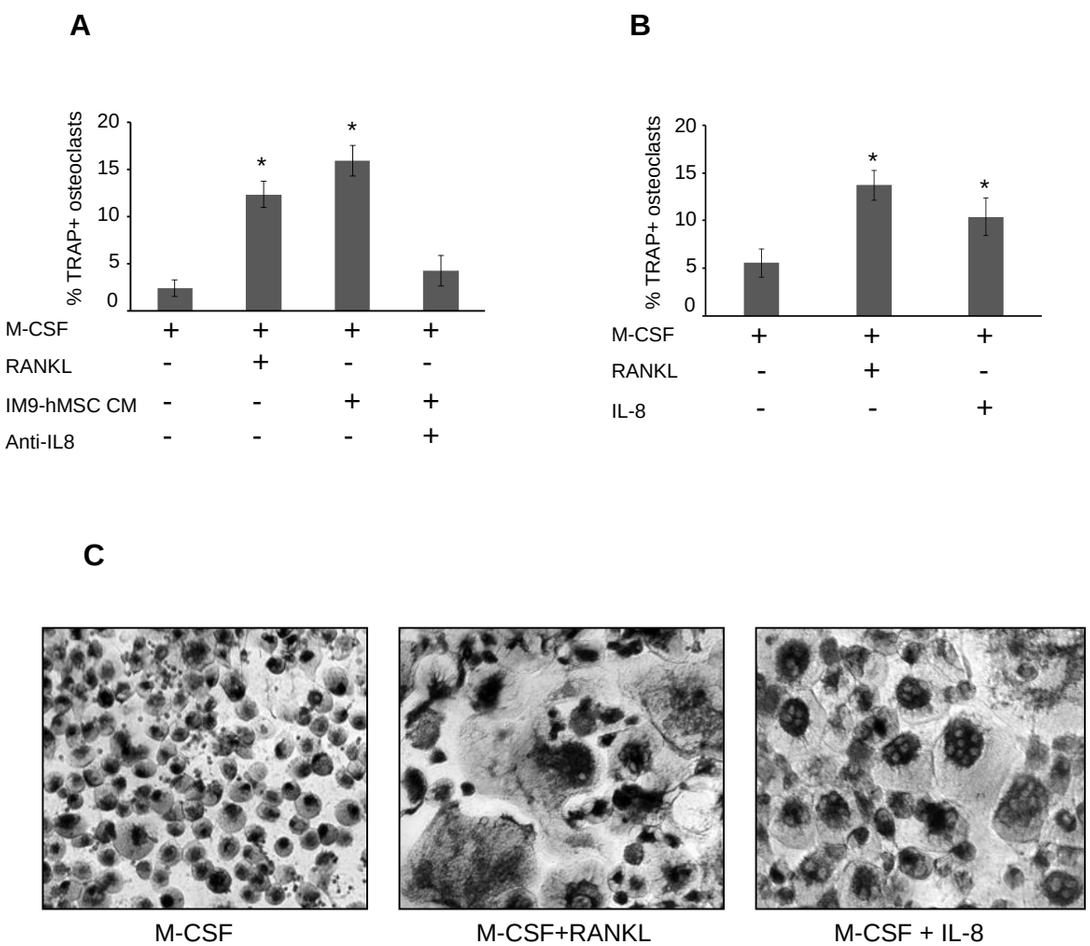

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