

## The Phenotypic Plasticity of Myeloma Plasma Cells as Expressed by Dedifferentiation into an Immature, Resilient, and Apoptosis-Resistant Phenotype

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**Abstract Purpose:** We previously showed the ability of osteoclasts to support myeloma plasma cell survival and proliferation *in vivo* and *ex vivo*. The aim of the current study was to investigate osteoclast-induced phenotypic changes associated with long-term survival of myeloma cells in coculture. **Experimental Design:** CD138-selected myeloma plasma cells from 16 patients were cocultured with human osteoclasts for up to 20 weeks. **Results:** Precultured cells were typically CD45<sup>low/intermediate</sup> CD38<sup>high</sup> CD138<sup>high</sup>, CD19<sup>-</sup> CD34<sup>-</sup>. After >6 weeks, the phenotype of cocultured myeloma cells consistently shifted to cells expressing CD45<sup>intermediate/high</sup> CD19<sup>low</sup> CD34<sup>low</sup>. Expression of CD38 and CD138 were reduced to subpopulations with CD38<sup>intermediate</sup> and CD138<sup>low</sup> levels. Morphologically, cocultured plasma cells became plasmablastic. Blocking interleukin-6 activity did not affect the immature phenotype of myeloma cells. The effect of dexamethasone on myeloma cells cultured alone or in cocultures at baseline and after 6 weeks of coculture was determined. When baseline myeloma cells were cultured alone, dexamethasone significantly increased the percentage of apoptotic cells over the spontaneous rate. Conversely, myeloma cells recovered from cocultures had high survival rates and were resistant to dexamethasone-induced apoptosis. Long-term coculture of normal CD34-expressing hematopoietic stem cells (HSC) resulted in loss of CD34 expression, suggesting a common mechanism for osteoclast-induced myeloma and HSC plasticity. **Conclusions:** This study indicates that myeloma cells have plasticity expressed by their ability to reprogram, dedifferentiate, and acquire autonomous survival properties.

Although the classic definition of cell plasticity taken from stem cell biology implies the ability of stem cells to differentiate into various cell lineages, the term is also currently applied to the ability of a given cell type to reciprocally dedifferentiate, redifferentiate, and/or transdifferentiate in response to specific stimulation (1, 2). The plasticity potential of malignant cells has been extensively studied in epithelial tumors as a mechanism that allows the epithelial cells to transdifferentiate into mesenchymal cells and vice versa in a process that is highly controlled by the microenvironment (3).

In contrast to solid tumors, the plasticity of hematopoietic malignancies, such as multiple myeloma, has not been extensively studied. Progression of multiple myeloma is considered a multistage and dynamic process of cell differen-

tiation, survival, proliferation, and dissemination (4–6). Although the majority of multiple myeloma patients initially respond to therapy and achieve clinical remission, they subsequently relapse (7, 8). The low proliferation rate of mature multiple myeloma plasma cells and the inability to attain a cure in multiple myeloma patients led to the notion that the proliferative, self-renewal compartment subsists within the immature clonal B-cell/lymphoblastoid population. Although previous studies support this notion, the identity of the multiple myeloma “stem cell” remains elusive.

Myeloma cells from the majority of patients are phenotypically CD45<sup>low</sup> CD38<sup>high</sup> CD138<sup>high</sup> and have very low proliferative activity (<1%), as determined by the standard bromodeoxyuridine labeling index (BrdUrd LI) assay. In 1991, Caligaris-Capio et al. showed the ability of stromal cells to induce differentiation of patients' B cells into monoclonal immunoglobulin-expressing multiple myeloma plasma cells (9), suggesting that myeloma stem cells emerge from immature clonal cells. Indeed, reports from several independent laboratories have identified the myeloma clone throughout the B-cell differentiation ladder, using complementary determining region 3 (CDR3)-based PCR analysis (10–13). Pliarski et al. suggested that the myeloma clone arises from a preswitched B cell that gives rise to malignant, drug-resistant B cells with characteristics resembling stem cells, and that this clone is responsible for patient relapse (13–15). Other groups suggested that the highly proliferative myeloma cells are CD45-expressing lymphoblasts (16) rather than B cells, and upon

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cytokine stimulation, these cells differentiate into mature CD45<sup>-</sup> cells capable of long-term survival (6, 17).

Opposing these concepts, studies from our laboratory using the severe combined immunodeficient (SCID)-hu and the SCID-rab models for primary myeloma (18, 19) and other studies with the 5T murine model for myeloma (20, 21) showed that purified, mature CD45<sup>-/low</sup> CD38<sup>high</sup> and CD138-selected multiple myeloma plasma cells have proliferative potential and the ability to produce myeloma *in vivo*. Furthermore, the clonal, immature CD45<sup>high</sup> cells in those studies were incapable of engraftment (18) or had a slower rate of engraftment (20) compared with their mature counterparts. Overall, these studies show a fundamental confusion and controversy over the origin of the proliferative, self-renewing myeloma cells.

Based on their similar properties and behaviors, tumorigenic cells are often compared with normal stem cells, suggesting a theory that cancer stem cells exist in the form of a rare, primitive, subpopulation of cells capable of self-renewal, pluripotency, and longevity. In studies of hematologic malignancies, such as acute myeloid leukemia, only the rare CD34<sup>+</sup>CD38<sup>-</sup> cells were capable of producing acute myeloid leukemia in SCID mice (22). Recent work on multiple myeloma suggested that a small fraction of clonal cells expressing CD138<sup>-</sup>CD19<sup>+</sup> are the myeloma stem cells and that CD138-expressing multiple myeloma plasma cells are not clonogenic (23). Further evidence in support of the cancer stem cell theory came from breast cancer research. Clark et al. showed that only a small fraction of CD44<sup>+</sup>CD24<sup>-</sup> breast cancer cells formed tumors in nonobese diabetic/SCID mice (24).

Recent studies using global microarray profiling to identify common stem cell markers and signaling pathways in tumor cells further fuel the cancer stem cell theory and have provided new insights into our understanding of tumorigenesis. Several stem cell-associated signaling pathways, including *Wnt*/ $\beta$ -catenin, Notch, and HoxB, are activated in tumor cells (2). Other genes involved in cell proliferation (*Nucleostemin*; ref. 25) and prevention of senescence (*BMI-1*; ref. 26) were highly expressed by both tumor cells and undifferentiated stem cells. Although these findings may help identify potential targets for cancer stem cells, they also indicate that each tumor cell expresses, to a varying degree, a range of stem cell-like genes and therefore could potentially acquire the characteristics of a stem cell.

Myeloma cells from the majority of patients typically reside in the bone marrow and alter this microenvironment to their advantage. By inhibiting osteoblastogenesis and inducing osteoclastogenesis, angiogenesis, and immunosuppression, multiple myeloma cells protect themselves from spontaneous and drug/immune-induced apoptosis, thereby ensuring their continued growth. In the majority of patients with myeloma, growth of multiple myeloma cells in the bone marrow is associated with induction of osteolytic bone disease (7, 27, 28). Myeloma plasma cells contribute to bone destruction through their interactions with bone marrow stromal cells, directly (29–32) and indirectly (33–35) stimulating differentiation of bone-destroying osteoclasts. Multiple myeloma cells also prevent differentiation of bone-building osteoblasts through secretion of the *Wnt*-signaling antagonist DKK1 (36). This indicates that multiple myeloma cells uncouple the processes of osteoclastic bone resorption and osteoblastic bone formation,

resulting in disturbed bone remodeling in multiple myeloma patients.

Clinical observations and experimental studies using osteoclast inhibitors highlighted the interdependence between myeloma bone disease and tumor progression. It seems that bisphosphonates, in addition to preserving bone, are also effective antitumor agents (37, 38). Studies using a murine model for myeloma and our studies with the myelomatous SCID-hu mice showed that inhibition of myeloma-associated bone disease by bisphosphonates or by inactivation of the receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) halts progression of bone resorption and also has a profound antimyeloma effect. These findings suggest that osteoclasts facilitate survival and growth of myeloma cells in bone marrow (33, 39–41). This notion is further supported by our findings that osteoclasts alone support survival and continued proliferation of purified primary multiple myeloma cells *ex vivo* (32). Together, these studies strongly suggest that osteoclasts play a critical role in regulation of myelomagenesis.

The aim of this study was to determine the osteoclast-induced phenotypic changes associated with survival of multiple myeloma cells in long-term coculture. We showed that purified, mature multiple myeloma plasma cells, cocultured with osteoclasts for up to 20 weeks, gradually lost their mature phenotype and dedifferentiated to an immature, resilient, apoptosis-resistant phenotype. This indicates that multiple myeloma cells have a plasticity that allows them to be reprogrammed and acquire autonomous survival properties upon long-term, direct contact with the osteoclasts they promote.

## Materials and Methods

**Reagents and kits.** Neutralizing polyclonal antibodies against human interleukin-6 (IL-6) and human IL-6 receptor (IL6R) were obtained from R&D Systems, Inc. (Minneapolis, MN). Anti-human BrdUrd was obtained from DAKO Corp. (Carpinteria, CA). Monoclonal antibodies to human CD138 (phycoerythrin), CD38 (phycoerythrin), CD45 (FITC), CD19 (FITC), and CD34 (phycoerythrin) for fluorescence-activated cell sorting analysis were from BD Biosciences (San Jose, CA).  $\alpha$ -MEM and antibiotic cocktail containing penicillin, streptomycin, and neomycin were from Life Technologies (Grand Island, NY). Fetal bovine serum was from Hyclone (Logan, UT). Recombinant human macrophage colony-stimulating factor and RANKL were from RDI (Flanders, NJ). Dexamethasone, BrdUrd, fluorodeoxyuridine, and PKH26 red fluorescent were from Sigma (St. Louis, MO). Anti-human CD138 and anti-CD34 antibodies for immunomagnetic bead separation were from Miltenyi Biotec (Auburn, CA). Cell culture plates were from Becton Dickinson (Franklin Lakes, NJ). Annexin V/propidium iodide detection kit for flow cytometry was from Caltag Laboratories (Burlingame, CA).

**Preparation of myeloma and hematopoietic stem cells.** Myeloma plasma cells were obtained from heparinized bone marrow aspirates from 16 patients with active myeloma during scheduled clinical visits. Adult hematopoietic stem cells (HSC) were obtained from peripheral blood mononuclear cells of three donors. All patients signed Institutional Review Board-approved informed consent forms. The bone marrow samples were separated by density centrifugation using Ficoll-Paque (specific gravity, 1.077 g/mL), and the proportion of multiple myeloma plasma cells in the light-density cell fractions was determined by CD38/CD45 flow cytometry. Multiple myeloma plasma cells and HSCs were isolated using CD138 and CD34 immunomagnetic bead selection, respectively, and the autoMACs automated separation system (Miltenyi Biotec). Plasma cell purity was

determined by CD38/CD45 flow cytometry to be routinely  $\geq 95\%$ . HSC purity was  $>95\%$  as determined by CD34/CD38 flow cytometry. Myeloma cell viability was determined by trypan blue exclusion, and apoptotic cells were enumerated using an Annexin V/propidium iodide kit. In three experiments, the CD138-selected multiple myeloma cells were cocultured with osteoclasts for 24 to 36 hours and then further depleted of CD45<sup>+</sup> cells using immunomicrobead-negative selection. The remaining multiple myeloma cells were then labeled with PKH26 red fluorescent as a tracing marker and incubated for  $>4$  weeks.

**Preparation of osteoclasts.** Cultures of multinucleated bone-resorbing osteoclasts were prepared as previously described (32). Briefly, peripheral blood mononuclear cells were obtained from multiple myeloma patients and from healthy subjects. Signed Institutional Review Board-approved informed consent forms are kept on record. The cells were cultured at  $2.5 \times 10^6/\text{mL}$  in  $\alpha$ -MEM supplemented with 10% fetal bovine serum, antibiotics, RANKL (50 ng/mL), and macrophage colony-stimulating factor (25 ng/mL) for 10 to 14 days, at which time they contained large numbers of multinucleated osteoclasts with bone-resorbing activity (32).

**Cocultures of osteoclasts with multiple myeloma plasma cells and hematopoietic stem cells.** Osteoclasts were washed thrice with PBS to detach and remove the nonadherent cells. Purified multiple myeloma plasma cells ( $0.5 \times 10^6/\text{mL}$ ) in osteoclast medium supplemented with macrophage colony-stimulating factor and RANKL were added to osteoclasts in six-well plates (3 mL/well). After each 3- to 4-week period of culturing, the myeloma cells were transferred into plates containing freshly prepared osteoclasts. At the end of each experiment, portions of multiple myeloma plasma were analyzed by flow cytometry; other portions were processed for assessing BrdUrd LI and Annexin V binding. In five experiments, dexamethasone ( $10^{-7}$  mol/L was added to cultures of multiple myeloma plasma cells ( $0.5 \times 10^6$  per well, duplicates) alone, and to cocultures of multiple myeloma plasma cells and osteoclasts at the initiation of the study (baseline) and after 6 weeks of coculturing. The effect of dexamethasone on multiple myeloma cell apoptosis was determined after 3 days using an Annexin V/propidium iodide kit. Values are expressed as the mean  $\pm$  SE, and the Student's paired *t* test was used to evaluate the effect of dexamethasone on multiple myeloma plasma cell survival.

Adult CD34-selected HSCs were cocultured with osteoclasts for up to 3 months in medium lacking any cytokines. Because osteoclasts did not survive long without macrophage colony-stimulating factor and RANKL, the HSCs were transferred to fresh osteoclast cultures every 6 to 10 days. HSC viability was determined biweekly using trypan blue exclusion. A portion of cocultured HSCs were taken every 4 weeks for assessment of CD34/CD38 expression by flow cytometry.

**Bromodeoxyuridine labeling index.** BrdUrd and fluorodeoxyuridine (20 and 2  $\mu\text{mol/L}$  final concentrations, respectively) were added to cultures of myeloma cells alone or to cocultures of myeloma cells with osteoclasts. After 2 hours, myeloma cells were collected, and cytospin slides were prepared (40,000 per slide) and air-dried. Duplicate slides were treated with 5 N HCl for 15 minutes and with 3% hydrogen peroxide for 10 minutes before incubation with monoclonal antibody against BrdUrd (5  $\mu\text{g/mL}$ ). BrdUrd incorporation was detected using Vector's immunoperoxidase kit, and cells were lightly counterstained with hematoxylin. BrdUrd LI was calculated as the percentage of positive cells out of 500 cells in each of the duplicate slides.

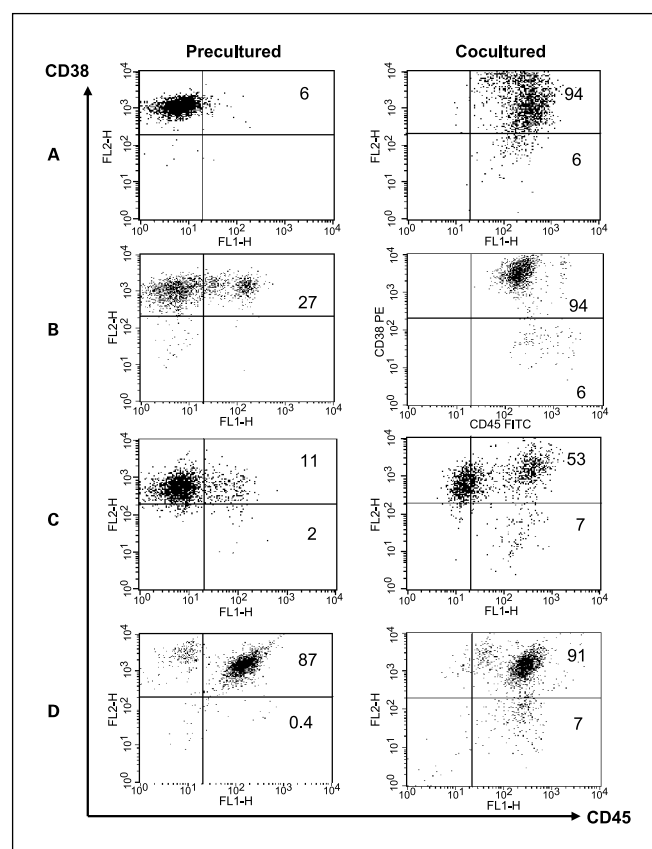
**Quantitative real-time PCR.** First-strand cDNA was synthesized from 1  $\mu\text{g}$  of purified RNA with avian myeloblastosis virus reverse transcriptase XL (Takara Mirus Bio, Madison, WI) using random 9-mer primers provided by the manufacturer. Following reverse transcription, cDNA samples were diluted to a final volume of 480  $\mu\text{L}$ , and 5- $\mu\text{L}$  samples were used for each quantitative PCR analysis. Gene expression was quantified by the SYBR Green method using the Taqman 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Taqman PCR Universal Master Mix (Applied Biosystems) was used to carry out the PCR reaction. The relative quantity of specific mRNA is

calculated based on glyceraldehyde-3-phosphate dehydrogenase level versus the gene of interest using the  $\Delta\Delta C_t$  method.

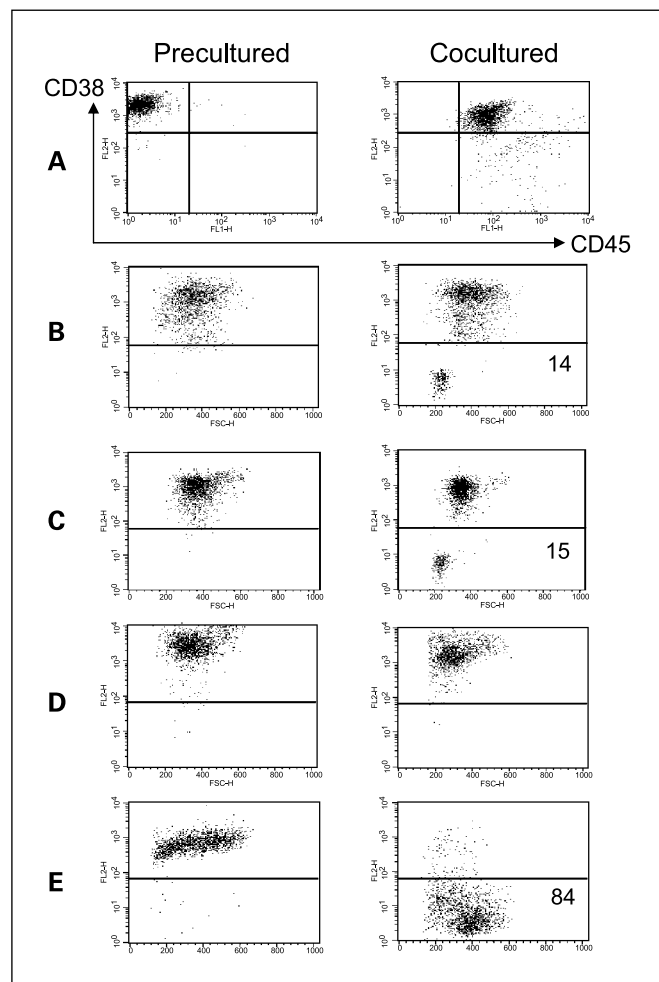
## Results

**Osteoclasts induce phenotypical and morphologic changes in myeloma cells.** CD138-selected multiple myeloma plasma cells from 16 patients were cocultured with osteoclasts for 6 to 20 weeks. Although multiple myeloma cells interacted with the adherent osteoclasts in a cell-cell contact manner, they did not firmly adhere to osteoclasts and all multiple myeloma plasma cells could be easily recovered from the cocultures as previously shown (32). During the course of the experiment, multiple myeloma cells had a BrdUrd LI of  $2.5 \pm 2.0$  and their viability was  $97 \pm 1\%$ .

We initially analyzed the changes in CD45 and CD38 expression on cocultured multiple myeloma cells by flow cytometry, because these markers are typically used to identify terminally differentiated multiple myeloma plasma cells. CD138-selected multiple myeloma cells freshly obtained from the majority of patients expressed low to intermediate levels of CD45 and high levels of CD38. After  $>6$  weeks in coculture, the phenotype of cocultured myeloma cells consistently shifted to a less mature phenotype. In all cases in which



**Fig. 1.** Osteoclasts induce dedifferentiation of multiple myeloma cells. CD138-selected multiple myeloma cells from 16 patients were cocultured with osteoclasts for up to 20 weeks. A-D, four experiments showing CD45/CD38 expression on precultured and cocultured myeloma cells. Note an up-regulation of CD45 in multiple myeloma cells whose initial phenotype was CD45<sup>-/low</sup> (A-C) but no changes in cells predominantly expressing CD45 before coculture (D). In all experiments, subpopulations of multiple myeloma cells had reduced CD38 expression.



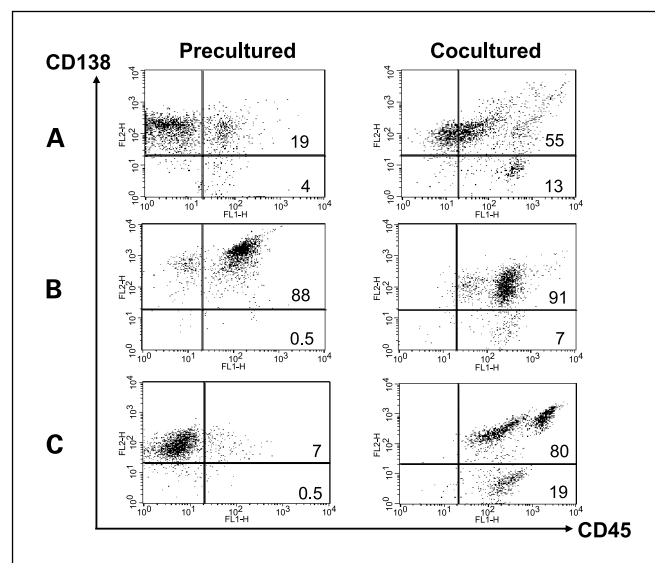
**Fig. 2.** Multiple myeloma cells maintain their low proliferative activity during long-term coculture with osteoclasts. CD138-selected multiple myeloma cells were depleted of CD45-expressing cells and labeled with the cytoplasmic marker, PKH26. *A*, CD45/CD38 expression on precultured and cocultured primary multiple myeloma cells. *B-D*, experiments showing PKH26 intensity on precultured and cocultured multiple myeloma cells from three patients. *E*, PKH26 fluorescent intensity on CAG multiple myeloma cell line before and after 2 weeks of culturing was used as comparator. Although PKH26 fluorescent intensity was reduced on CAG cells due to high proliferation, the fluorescent intensity on the majority of cocultured primary multiple myeloma cells remained high, indicating that these cells did not proliferate during the experimental period.

precultured myeloma cells expressed CD45<sup>low</sup>, the majority of cocultured cells expressed CD45<sup>intermediate/high</sup>, whereas in cases where precultured myeloma cells predominantly expressed CD45<sup>intermediate/high</sup>, the level of this marker remained high after coculture (see Figs. 1, 2, and 6). In all cocultures, CD38 expression in a subpopulation of cells gradually reduced from CD38<sup>high</sup> to CD38<sup>intermediate</sup> (Fig. 1). To test whether CD45<sup>+</sup> multiple myeloma cells originated from the terminally differentiated CD45<sup>-</sup> multiple myeloma cells and not from a minor population of CD45-expressing cells, CD138-selected multiple myeloma cells were further depleted of CD45<sup>+</sup> and labeled with cytoplasmic marker, PKH26. After coculture, the CD138<sup>+</sup>CD45<sup>-</sup> multiple myeloma cells reexpressed CD45. In contrast to the highly proliferating multiple myeloma cell line, PKH26 fluorescent intensity remained high on the majority of cocultured primary multiple myeloma cells, indicating that these cells were originated from nonproliferating precultured

CD45<sup>-</sup> cells and not due to proliferation of contaminated immature multiple myeloma cells (Fig. 2).

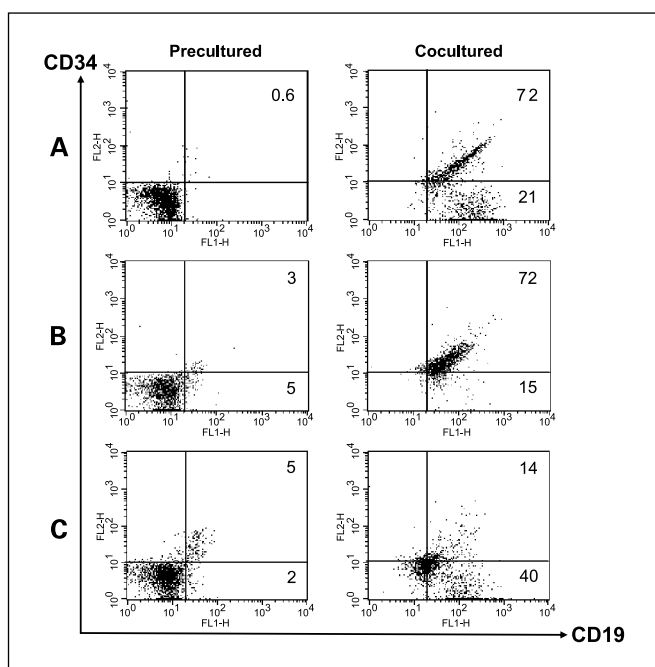
We also determined the changes in CD138, CD19, and CD34 expression in cocultured myeloma cells. Precultured multiple myeloma cells from the majority of patients expressed CD138 but not CD19 or CD34. Following coculture with osteoclasts, a subset of tumor cells lost expression of CD138 (Fig. 3). Cocultured myeloma cells also gained expression of CD19 and various degrees of CD34 (Fig. 4). Of note, all those phenotypical markers gradually changed in cocultured myeloma cells and, evidently, were not detected by flow cytometry during the first 4 weeks of coculture with osteoclasts. The significant up-regulation of CD45 and CD19 in cocultured multiple myeloma cells was further confirmed at the gene expression level using quantitative reverse transcription-PCR (Fig. 5A). Morphologically, in contrast to the heterogeneous population of precultured cells, the cocultured multiple myeloma cells uniformly gained plasmablastic characteristics, thus further supporting the dedifferentiation phenomena seen with the phenotype analysis (Fig. 5B-E).

**Role of interleukin-6 in osteoclast-induced myeloma cell plasticity.** Previous studies using myeloma cell lines suggested that IL-6 is important for maintaining CD45 expression on myeloma cells and that IL-6 is a survival and growth factor for immature but not mature myeloma plasma cells (42, 43). We have previously shown that IL-6 is highly produced by osteoclasts (32). To study the possible role of IL-6 in osteoclast-induced myeloma cell dedifferentiation, multiple myeloma plasma cells from two patients were cocultured with osteoclasts, and dedifferentiation of multiple myeloma cells, as determined by increased CD45 expression, was allowed to take place for 6 weeks (Fig. 6). The cocultures were then treated with neutralizing antibodies against IL-6 plus IL-6R (5 µg/mL, each) for >4 weeks. We previously showed that at this concentration, these neutralizing antibodies inhibited growth of the IL-6-dependent ANBL6 cell line and partially attenuated the



**Fig. 3.** Loss of CD138 expression in a subpopulation of cocultured myeloma cells. Precultured and cocultured multiple myeloma cells were stained for CD138/CD45. *A-C*, three experiments showing that in addition to up-regulation of CD45, a subpopulation of multiple myeloma cells lost CD138 expression after long-term coculture with osteoclasts.





**Fig. 4.** Osteoclasts induce upregulation of CD19 and CD34 on myeloma cells. *A-C*, representative of three experiments showing CD19/CD34 expression on precultured and cocultured multiple myeloma cells by flow cytometry.

supportive effect of osteoclasts on the survival but not proliferation of primary multiple myeloma plasma cells (32). Blocking IL-6 activity in this experiment did not reverse the upregulation of CD45 on myeloma cells. Intriguingly, IL-6 inhibition was considerably associated with reduced CD38 expression in multiple myeloma cells (Fig. 6).

**Osteoclasts induce apoptosis-resistant phenotype in myeloma cells.** To test whether myeloma cell plasticity is associated with the acquisition of an apoptosis-resistant phenotype, we conducted five experiments to determine the effect of dexamethasone ( $10^{-7}$  mol/L) on tumor cells, cultured alone or cocultured with osteoclasts, at baseline (preculture) and after 6 weeks of coculture. When baseline multiple myeloma cells were cultured alone, dexamethasone significantly increased the percentage of apoptotic cells compared with multiple myeloma cells alone (Fig. 7) as determined by Annexin V/propidium iodide flow cytometry. In contrast, when multiple myeloma cells recovered from the cocultures

after 6 weeks were cultured alone, they had a lower percentage of apoptotic cells compared with cultures of multiple myeloma cells alone at baseline. Furthermore, multiple myeloma cells cultured alone after long-term exposure to osteoclasts had survival rates similar to cocultured cells and were resistant to dexamethasone-induced apoptosis. As previously reported (32), osteoclasts supported survival of myeloma cells at both baseline and after 6 weeks; and similar to other stromal cells (44), osteoclasts also protected myeloma cells from dexamethasone-induced apoptosis (Fig. 7). Overall, this experiment showed the ability of multiple myeloma cells to gain autonomous properties following long-term direct interaction with osteoclasts.

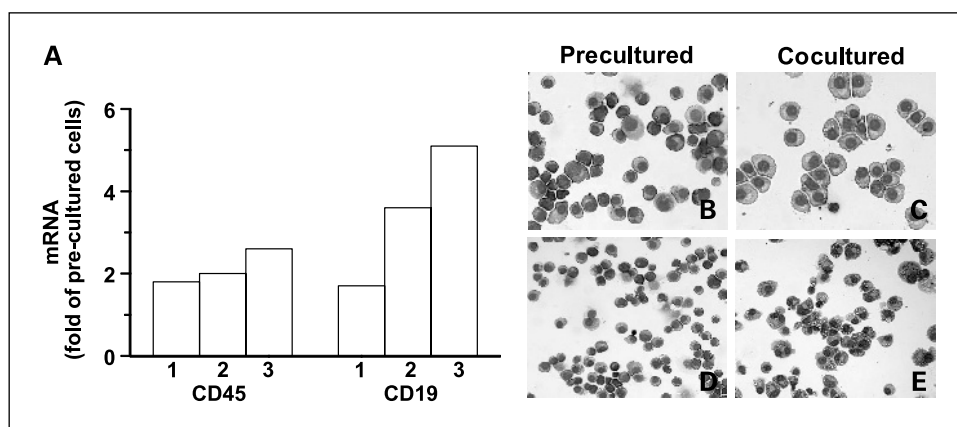
**Osteoclasts promote hematopoietic stem cell plasticity.** As discussed in the Introduction, tumor cells are often compared with normal stem cells. To test whether multiple myeloma cell phenotypic plasticity resembles that of stem cells, we examined the fate of HSCs in our coculture system and evaluated the possible involvement of osteoclasts on HSC biology. Although the plasticity potential of HSCs is a matter of continuing debate (1, 2), it has been suggested that the primitive, quiescent HSC with self-renewal potential is CD34<sup>-</sup>. Reciprocal expression of CD34 on HSCs was previously reported in murine but not human HSCs (45).

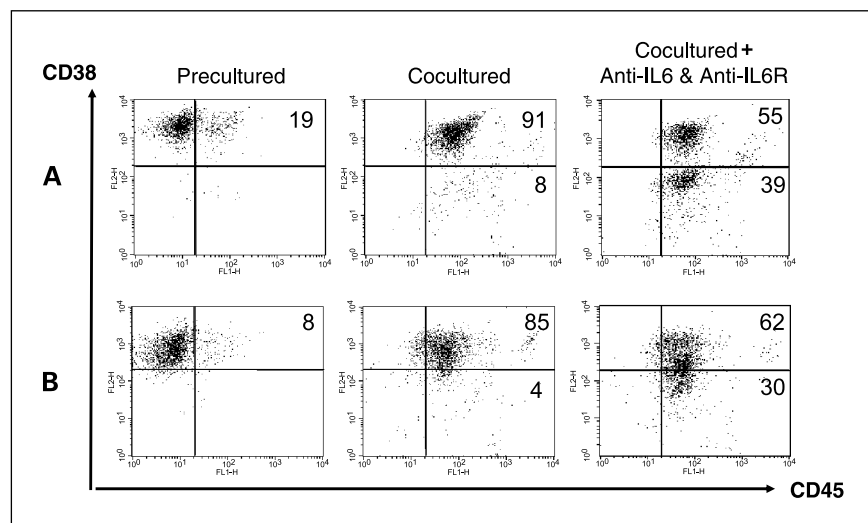
Like multiple myeloma cells, HSCs did not adhere to the osteoclasts and were easily recovered from cocultures by gentle pipetting. Following 1 to 3 weeks of coculture, committed CD34-selected HSCs rapidly differentiated into various hematopoietic cell lineages followed by phagocytosis of terminally differentiated hematopoietic cells by the osteoclasts. The number of cocultured cells was reduced, and the remaining HSCs were highly viable (>90% by trypan blue exclusion) and gradually lost their CD34 expression, so that the cultures contained subpopulations of HSCs expressing CD34<sup>-/low</sup> CD38<sup>+</sup> and CD34<sup>-/low</sup> CD38<sup>-</sup> (Fig. 8). This indicates that osteoclasts are an important bone marrow component regulating human HSC plasticity and fate.

## Discussion

In this study, we showed for the first time the ability of recognizable terminal-differentiated primary human myeloma plasma cells to reprogram and dedifferentiate into an immature phenotype and to acquire autonomous survival properties, such as resistance to spontaneous and drug-induced apoptosis.

**Fig. 5.** Up-regulation of CD45 and CD19 gene expression and morphological changes in cocultured myeloma cells. *A*, quantitative reverse transcription-PCR of RNA extracted from precultured and cocultured multiple myeloma plasma cells from three patients. *B-E*, cytospin slides of precultured and cocultured multiple myeloma cells from two experiments stained for Giemsa. Note a uniform plasmablastic morphology in cocultured cells (*C* and *E*) compared with their precultured counterparts (*B* and *D*).





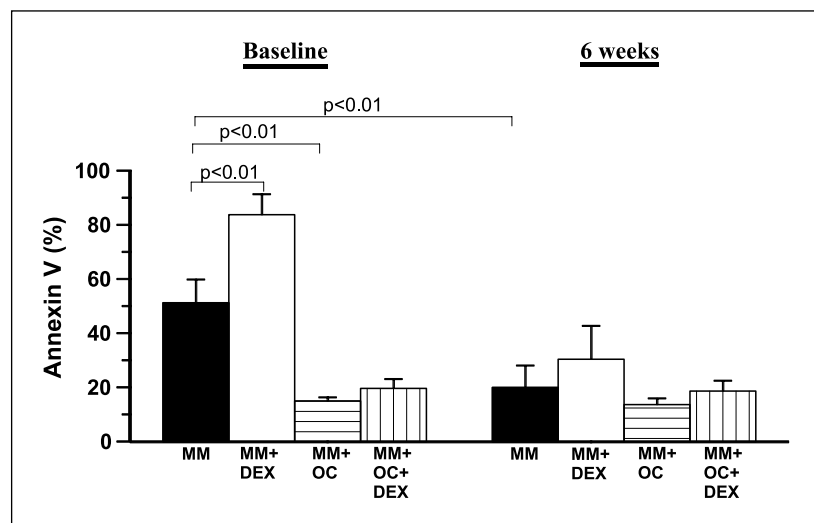
**Fig. 6.** A-B, blocking IL-6 activity did not reverse the immature phenotype of cocultured myeloma cells. CD45/38 flow cytometry of multiple myeloma plasma cells from two patients before culture (precultured), after 6 weeks in culture with osteoclasts (cocultured), and after 4 additional weeks in culture with osteoclasts, anti-IL6, and anti-IL6R. Note an up-regulation of CD45 expression by osteoclasts that was not reversed by inhibition of IL-6 activity.

This phenomenon is closely regulated by osteoclasts, which are considered a key microenvironmental component in myelomatous bones (39), directly promoting survival and proliferation of myeloma cells (32, 46).

Primary multiple myeloma plasma cells expressing high levels of CD138 and CD38 and low to intermediate levels of CD45 shifted and expressed high levels of CD45, with subpopulations of cells losing CD38 and CD138 expression upon long-term interaction with osteoclasts. Furthermore, cocultured myeloma cells gained expression of CD19, a B-cell marker, and CD34, a hematopoietic stem cell marker. Primary multiple myeloma cells, which are often spontaneously apoptosed when growing without appropriate cytokine and cellular support, also had higher survival rates and were resistant to dexamethasone-induced apoptosis when recovered from cocultures and grown alone. These findings suggest that mature multiple myeloma cells have plasticity that allows them to be reprogrammed, enter dormancy, turn senescent/quiescent, and possess other features typically characteristic of stem cells.

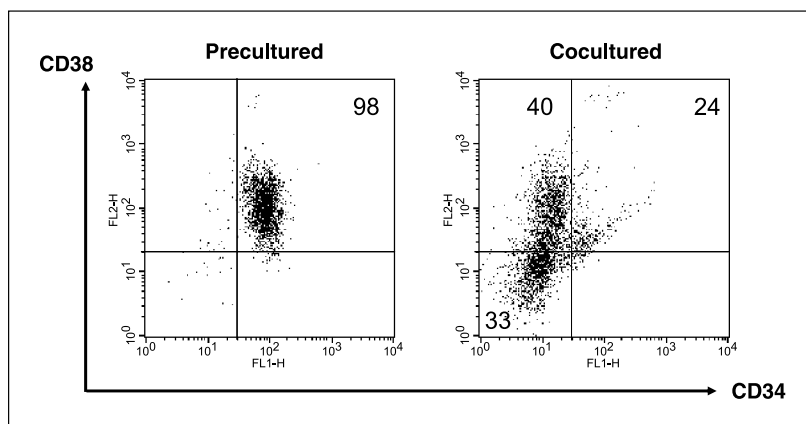
A line of evidence is emerging that authenticates the results of this study. First, during the long-term coculture, myeloma cells kept their very low proliferative activity (BudR LI of  $2.5 \pm 2.0$ )

and high cytoplasmic PKH26 fluorescent intensity, eliminating the possibility of a selection process in which only immature, proliferating tumor cells remained in culture. Second, in some experiments, the entire population of multiple myeloma cells that expressed CD45<sup>-</sup>/CD19<sup>-</sup> completely transformed to become CD45<sup>high</sup> (e.g., Fig. 1A) and express CD19 (Fig. 3). Third, depletion of CD45-expressing multiple myeloma cells before long-term coculture resulted in similar phenotypical shift. Fourth, long-term coculture of normal CD34-selected HSCs with osteoclasts resulted in loss of CD34 expression on these cells, suggesting a common mechanism for the osteoclast-induced plasticity of multiple myeloma cells and HSCs. Finally, the phenotypical changes seen in cultures could not be accounted for by possible cell contamination, because the majority of dedifferentiated multiple myeloma cells still expressed high levels of the myeloma cell markers CD138 and CD38 and kept a plasmablastic morphology. In addition, the reprogramming process took place gradually and was not evident during the first 4 weeks of coculture, indicating that multiple myeloma plasma cells, which were highly viable throughout the experimental period ( $97 \pm 1\%$ ), dedifferentiated and acquired a stem cell phenotype.



**Fig. 7.** Induction of myeloma cell apoptosis resistance by osteoclasts. Multiple myeloma (MM) plasma cells from five patients were incubated with and without osteoclasts and dexamethasone (DEX,  $10^{-7}$ ) for 3 days at initiation (baseline) and after 6 weeks of coculture. Multiple myeloma cell apoptosis was determined using Annexin V/propidium iodide flow cytometry. Note that multiple myeloma cells cultured alone at baseline but not after recovery from a 6-week coculture were susceptible to spontaneous and dexamethasone-induced apoptosis. As reported, osteoclasts supported survival of multiple myeloma cells at baseline and after 6 weeks of coculture and protected multiple myeloma cells from dexamethasone-induced apoptosis.

**Fig. 8.** Long-term coculture of HSCs with osteoclasts is associated with loss of CD34 expression. CD34-selected adult HSCs were cocultured with osteoclasts in medium lacking any growth factors for >8 weeks. Note reduced CD34 and CD38 expression in subsets of cells.



There is little evidence of the ability of multiple myeloma plasma cells to dedifferentiate. We previously showed the ability of purified, mature human CD45<sup>low</sup> CD38<sup>high</sup> and CD138-selected multiple myeloma plasma cells (18, 19) to proliferate and produce myeloma in experimental models. Indeed, a small fraction of myeloma cells recovered from those *in vivo* studies had an immature phenotype, further validating our *ex vivo* studies. It has also been shown that some of the mouse CD45<sup>-</sup> 5T multiple myeloma cells can reexpress CD45 (47), and that murine CD45<sup>high</sup> multiple myeloma cells are quiescent, lowly proliferative, and resistant to drug-induced apoptosis but highly invasive (20, 48). Using the U266 myeloma cell line, which harbors two distinct CD45<sup>+</sup> and CD45<sup>-</sup> fractions, Kwano et al. showed that the CD45-expressing cells are highly proliferative and that IL-6 induces CD45 expression in the CD45<sup>-</sup> fraction (42, 49). However, in our study, blocking IL-6 activity did not attenuate CD45 expression on cocultured multiple myeloma cells, suggesting that factors other than IL-6 are involved in osteoclast-induced multiple myeloma cell phenotypic plasticity.

Although myeloma cells from the majority of patients express CD45<sup>-/low</sup>, small fractions of CD45<sup>intermediate/high</sup> and CD138<sup>low</sup> multiple myeloma cells are often detected in their bone marrow. These patients have shorter survival rates than those whose multiple myeloma cells are predominantly CD45 positive (50), leading to the development of a "one-way" cellular model. This model suggests that multiple myeloma cells lose CD45 expression as the disease aggressively progresses (6). The current study illuminates a novel angle suggesting that the CD45-expressing multiple myeloma cells from the majority of patients are derived from both immature clones that normally differentiate thorough the B-cell pathway and from mature CD45<sup>-</sup> multiple myeloma plasma cells that possess a plasticity machinery.

Our data also suggest that a portion of malignant cells closely resides with osteoclasts in lytic bone lesions of patients with

myeloma, and that these cells become quiescent and apoptosis resistant and survive conventional high-dose chemotherapy and autologous transplantation. Whether these cells are responsible for a patient's relapse is a matter of continuing research. That the majority of remitted patients subsequently relapse (7, 8) may suggest that these cells enter dormancy for a durable period that could last several years and eventually, in response to an as yet unknown stimuli, rapidly emerge as an aggressive clone. We further speculate that the plasticity potential of multiple myeloma cells changes at this stage, resulting in a population of cells that predominantly possess a permanent stem cell-like phenotype, often characterizing extramedullary disease and cell lines. These cells are autonomous, highly proliferative, resistant to apoptosis, and no longer require plasticity machinery for disease progression.

In conclusion, this study suggests that myeloma cells have plasticity, expressed by the ability of terminally differentiated tumor cells to reprogram and dedifferentiate into an immature, resilient phenotype and become autonomous after coculture with osteoclasts. We hypothesize that these myeloma cells are dormant, resistant to spontaneous and drug-induced apoptosis, and could be responsible for relapse. Future studies to unravel molecular mechanism of myeloma cell plasticity and identify factors responsible for redifferentiation of these cells will help develop novel interventions to cure myeloma. This work also further emphasizes the linkage between myeloma bone disease and tumor progression and the critical role of bone marrow microenvironment in the pathogenesis of the disease.

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