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Molecular Cancer Research

Proinflammatory Macrophages Promote Multiple Myeloma Resistance to Bortezomib Therapy

Ofrat Beyar-Katz[1,2], Ksenia Magidey[1], Anat Reiner-Benaim[3], Noga Barak[1], Irit Avivi[4], Yael Cohen[4], Michael Timaner[1], Shimrit Avraham[1], Michal Hayun[5], Noa Lavi[2], Marina Bersudsky[6], Elena Voronov[6], Ron N. Apte[6], and Yuval Shaked[1]

Abstract

Multiple myeloma (MM) is a plasma cell neoplasia commonly treated with proteasome inhibitors such as bortezomib. Although bortezomib has demonstrated enhanced survival benefit, some patients relapse and subsequently develop resistance to such therapy. Here, we investigate the mechanisms underlying relapse and refractory MM following bortezomib treatment. We show that bortezomib-exposed proinflammatory macrophages promote an enrichment of MM-tumor-initiating cells (MM-TIC) both *in vitro* and *in vivo*. These effects are regulated in part by IL1β, as blocking the IL1β axis by a pharmacologic or genetic approach abolishes bortezomib-induced MM-TIC enrichment. In MM patients treated with bortezomib, high proinflammatory macrophages in the bone marrow negatively correlate with survival rates (HR, 1.722; 95% CI, 1.138–2.608). Furthermore, a positive correlation between proinflammatory macrophages and TICs in the bone marrow was also found. Overall, our results uncover a protumorigenic cross-talk involving proinflammatory macrophages and MM cells in response to bortezomib therapy, a process that enriches the MM-TIC population.

Implications: Our findings suggest that proinflammatory macrophages in bone marrow biopsies represent a potential prognostic biomarker for acquired MM resistance to bortezomib therapy.

Introduction

Multiple myeloma (MM) is a plasma cell neoplasia leading to an estimated 12,770 deaths in the United States (seer.cancer.gov, accessed April 27, 2018). Bortezomib, a proteasome inhibitor, is a commonly used agent for the treatment of MM. Despite bortezomib's promising activity, some patients fail to respond due to the development of *de novo* resistance (1). We have recently demonstrated that the host, in response to almost any type of anticancer drug including chemotherapy, such as paclitaxel and gemcitabine, radiation, and even targeted drugs, generates protumorigenic and prometastatic effects that counteract the antitumor activity of the drug (2). These host responses could explain the regrowth or resistance of tumor cells to such therapies (3). It was previously shown that, despite being a targeted agent, bortezomib also induces host-mediated effects that in turn contribute to MM aggressiveness. Specifically, mice primed with bortezomib and subsequently injected with MM cells exhibited an increased mortality rate when compared with control mice implanted with MM cells. We found that plasma from mice or patients treated with bortezomib contributed to MM cell invasion and proliferation, which could explain disease progression. In addition, it was shown that proinflammatory macrophages account for MM cell aggressiveness because the depletion of macrophages in mice primed with bortezomib and subsequently injected with MM cells resulted in extended survival benefit (4, 5). This is in contrast to the major concept indicating that anti-inflammatory macrophages, also known as tumor-associated macrophages mainly in solid tumors, identified by protumorigenic activity, whereas proinflammatory macrophages are primarily considered antitumorigenic (6). However, in the case of MM, the mechanisms by which proinflammatory macrophages promote MM aggressiveness following bortezomib therapy have not been elucidated.

Cancer stem cells, also known as tumor-initiating cells (TIC), represent a small proportion of cancer cells with the ability to self-renew, therefore serving as a reservoir of cells for tumor initiation and growth (7). It has been shown that TICs resist the majority of therapies, in part due to being primarily quiescent as well as active mechanisms of DNA repair in them (8, 9). Matsui and colleagues have previously shown that MM cancer stem cells (MM-TIC) are relatively resistant to standard cytotoxic compounds and novel agents when cultured *in vitro* compared with myeloma plasma cells (10). They also demonstrated that MM cells that lack the expression of CD138 have greater clonogenic potential than those

[1]Cell Biology and Cancer Science, Rappaport Faculty of Medicine, Technion – Israel Institute of Technology, Haifa, Israel. [2]Hematology and Bone Marrow Transplantation Department, Rambam Health Care Campus, Haifa, Israel. [3]Clinical Epidemiology Unit, Rambam Health Care Campus, Haifa, Israel. [4]Hematology and Bone Marrow Transplantation Department, Ichilov Medical Center, Tel Aviv, Israel. [5]Hematology Research Center, Rambam Health Care Campus, Haifa, Israel. [6]The Shraga Segal Department of Microbiology, Immunonology and Genetics, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel.

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O. Beyar-Katz and K. Magidey contributed equally to this article.

Corresponding Author: Yuval Shaked, Technion – Israel Institute of Technology, 1 Efron Street, Bat Galim, Haifa 31096, Israel. Phone: 972-4-829-5215; E-mail: yshaked@technion.ac.il

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expressing CD138, and therefore are considered TICs (11). These CD138-negative B cells, when isolated from peripheral blood of MM patients and subsequently injected to mice, demonstrate a mature CD138-positive phenotype (10). The heterogeneity of MM cells and their clonogenic potential are key elements in explaining MM recurrence despite an initial response to therapy (10). Therefore, efforts are currently made to uncover mechanisms of MM resistance with a focus on the subpopulation of MM cells with TIC characteristics.

In the current study, we describe a unique cross-talk between proinflammatory macrophages and MM-TICs in response to bortezomib therapy. We demonstrate that proinflammatory macrophages secrete IL1β, which in turn contribute to TIC enrichment. Clinically, the levels of proinflammatory macrophages in patients treated with bortezomib correlate with worse outcome. Our results therefore suggest that characterization and enumeration of proinflammatory macrophages in the bone marrow may serve as a prognostic factor for MM progression following bortezomib therapy.

Materials and Methods

Bone marrow biopsies from MM patients

All human studies were approved by the ethics committee at the Rambam Health Care Campus (RHCC) and Tel Aviv Sourasky Medical Center (SMC), after patients signed a written informed consent. Newly diagnosed MM patients (age >18) who were scheduled to receive bortezomib-based therapy were enrolled in the study ($n = 34$). Patient characteristics are defined in Supplementary Table S1. Bone marrow (BM) biopsies were obtained at diagnosis (baseline) and several months after bortezomib therapy. For BM plasma extraction, BM aspirates were centrifuged at 1,500 × *g* for 15 minutes at 4°C. Supernatants were immediately frozen at −80°C. Mononuclear cells were separated by centrifugation over a layer of Lymphoprep (Axis-Shield PoC AS) and then stored in freezing medium in a liquid nitrogen tank. Normal BM was defined by less than 5% plasma cells following bortezomib treatment.

Cell culture

CAG, U266, KMS-11, and RPMI-8226 human MM cell lines (American Type Culture Collection) were thawed from original stocks and used within 6 months of resuscitation. All cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum where RPMI-8226 cells were supplemented with 10% horse serum. All media contained 1% L-glutamine, sodium pyruvate and penicillin–streptomycin. The cells were routinely tested to be *Mycoplasma* free.

Animal tumor model

The use of animals and experimental protocols were approved by the Animal Care and Use Committee of the Technion (Haifa, Israel) as well as at the Ben-Gurion University of the Negev (Beer Sheva, Israel). Ten-week-old severe combined immune-deficient (SCID) female mice underwent whole-body radiation at a total dose of 300 rads (Department of Radiotherapy, RHCC, Haifa) 6 hours following bortezomib treatment. Bortezomib was administered intravenously at a dose of 1 mg/kg, as in refs. 4 and 12. After 24 hours, the mice were intravenously injected with RPMI-8226 (5×10^6) cells. In other experiments, 10-week-old non–tumor-bearing Balb/c mice or IL1β$^{-/-}$ Balb/c mice and their counterpart controls (13) were intravenously injected with bortezomib (1 mg/kg). Control mice were administered with a vehicle control.

Drugs and recombinant proteins

A stock of bortezomib solution (Selleckchem) was prepared to reach a final concentration of 0.1 mg/mL and was stored in aliquots at −20°C. Anakinra (Swedish Orphan Biovitrum), an IL-receptor antagonist (14), was added to cell cultures at a concentration of 100 μg/mL as previously described (15). In *in vitro* experiments, MM cells were cultured with recombinant IL1β, TREM-1, or IL16 (PeproTech) at the concentrations indicated in the text, as previously published (4), and based on the manufacturer's recommendations.

Macrophage extraction and conditioned medium preparation

Ten-week-old Balb/c mice or IL1β$^{-/-}$ Balb/c mice and their counterpart controls were intraperitoneally injected with 3 mL 4% thioglycollate. After 48 hours, the mice were treated with bortezomib or vehicle. Twenty-four hours later, macrophages were collected by peritoneal lavage. Macrophages (1×10^6 cells/mL) were cultured in serum-free medium for 48 hours, and conditioned medium (CM) was collected, and further evaluated in culture with MM cells.

Evaluation of MM-TICs and macrophages by flow cytometry

BM cells were immunostained with various antibody mixes to identify macrophages and MM-TICs. In human samples, proinflammatory macrophages were defined as CD68$^+$/CCR2$^+$ and anti-inflammatory macrophages were defined as CD163/CX3CR1$^+$, as previously described (16). MM-TICs were identified as CD34$^-$/CD138$^-$/CD20$^+$, as previously described (10). In mice, proinflammatory macrophages were defined as F4/80$^+$/CD11c$^+$, as previously described (4). In some experiments, the percentage of MM cells expressing IL1R was evaluated using a polyclonal goat anti IL1RI antibody (R&D Systems) and a secondary antibody AF488-rabbit anti-goat antibody (Jackson ImmunoResearch). All other monoclonal antibodies were purchased from BioLegend, BD Biosciences, or R&D Systems and used in accordance with the manufacturers' instructions. At least 100,000 events were acquired using a Cyan ADP flow cytometer (Buckman Coulter) or BD LSRFortessa (BD Biosciences) and analyzed using FlowJo software v.10.

Mass cytometry (CyTOF)

Balb/c mice were treated with bortezomib or vehicle control, and after 24 hours, BM cells were flushed from femurs and tibia. The cells were immunostained with a mixture of metal-tagged antibodies (listed in Supplementary Table S2), and were used as previously described (17). The analysis of data were performed using Cytobank database (with viSNE algorithm), as previously described (18), when gating on CD45$^+$ cells. Data are presented using Cytobank online software (https://www.cytobank.org/).

In vitro colony-forming assay

MM colony-forming assay was carried out as previously described with some modifications (11). Briefly, RPMI-8226 cells were seeded into 6-well plates at a density of 2,000 cells/well in MethoCult M3231 medium (Stemcell Technologies) supplemented with CM of macrophages obtained from bortezomib- or vehicle-treated mice at a dilution of 1:10. In other experiments, medium was supplemented with 100 pmol/L IL1β. After 20 days,

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colonies containing >40 cells were counted. All experiments were performed using three biological repeats.

Quantification of IL1β

The level of IL1β was measured in BM biopsies, peripheral blood samples, and macrophage CM using highly sensitive ELISA kits for human or murine IL1β (Quantikine ELISA, R&D Systems). In the case of peripheral blood samples, plasma was first concentrated (25 concentrating factor) using AmiconUltra centrifugal filters 10K (Sigma-Aldrich).

Aldehyde dehydrogenase (ALDH) activity

ALDH enzymatic activity was determined by flow cytometry using the ALDEFLOUR kit (Stemcell Technologies) in accordance with the manufacturer's instructions and as previously described (19). Briefly, MM cells were incubated with ALDH substrate (BAAA, 1 μmol/L per 1×10^6 cells) for 40 minutes at 37°C. As a negative control, samples were supplemented with 50 mmol/L diethylaminobenzaldehyde (DEAB), an ALDH inhibitor. 7-Aminoactinomycin D (7AAD) was used to distinguish between live and dead cells. At least two independent experiments using three biological repeats were carried out.

Side population assay

A side population (SP) assay was carried out using Hoechst staining as previously described (20). Briefly, 1×10^6 MM cells were stained with 6.25 μL Hoechst in 1 mL RPMI medium for 90 minutes at 37°C either alone or in the presence of 50 mol/L verapamil (Sigma-Aldrich). Verapamil is used as a negative control as it blocks SP staining. Subsequently, cells were resuspended in PBS at 4°C for reaction termination. Flow cytometry settings were set to excitation at 350 nm, emission at 405/30 nm (for Hoechst-Blue), and 670/40 nm (for Hoechst-Red). Cells negative for Hoechst-Blue and Hoechst-Red were considered positive for SP. At least two independent experiments using three biological repeats were carried out.

mRNA extraction and quantitative RT-PCR

mRNA was purified from MM cells using the High Pure RNA Isolation Kit (Roche Diagnostics), and quantified by measuring with a NanoDrop spectrophotometer (ND-1000, Nano Drop Technologies). cDNA was then synthesized using a high-capacity cDNA reverse transcription kit (iScriptTMcDNA Synthesis Kit, Bio-Rad Laboratories Inc.). Real-time PCR for human IL1 receptor (IL1R) was performed using Rotor-Gene 6000TM (Corbett) equipment with absolute blue SYBER green ROX mix (Thermo Scientific AB-4162/B) using hIL-1 receptor probe (#Hs01098710_m1, Thermo Fisher Scientific Inc.). Values were normalized to β2MG (β2 microglobulin). Primer sequences were as follows: hIL-1: F-GGCTGAAAAGCATAGAGGGAAC; R-CTGGGCTCACAATCACAGG and β2MG: F-TCTCTCTTTCTGGCCTGGAG; R-AATGTCGGATGGATGAAACC using the primer bank (https://pga.mgh.harvard.edu/primerbank/citation.html) and purchased from Sigma-Aldrich.

Western blot analysis

IL1R expression on MM cells was verified using Western blot. Briefly, CAG, U266, KMS-11, and RPMI-8226 human MM cell lysates were separated by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Immunostaining was performed using polyclonal goat anti IL1RI primary antibody (1:2000 R&D Systems). Ponceau staining served as a loading control. Following immunostaining, membranes were incubated with HRP-conjugated goat anti-mouse and donkey-anti-goat secondary antibodies (1:10,000 Sigma-Aldrich).

Statistical analysis

Data are expressed as mean ± standard error. The statistical significance for *in vitro* experiments was determined by either two-tailed Student *t* test for a comparison between two groups, or one-way ANOVA for a comparison between multiple groups, followed by Tukey post hoc statistical test, using GraphPad prism 5.0 software. For *in vivo* studies, $n = 5–7$ mice/group (as specified in the figures) were used to reach statistical significance. Differences between all groups were compared with each other, and statistical significance was set at $P < 0.05$.

For patients' statistical analysis, a multivariate Cox proportional hazard model was used for testing effects on survival, and a stepwise procedure was applied for model reduction. Kaplan–Meier curves were estimated, and the log-rank test was used for univariate assessment of the effect of categorical variables on survival. A *t* test was used for univariate assessment of the effect of continuous variables, after log transformation for normalizing the distributions. Several types of survival events were considered: death, relapse, early relapse (less than 12 months), and any event from this list. Statistical significance was set on $P < 0.05$. The analysis on patient data were carried out using the R software (www.r-project.org).

Results

Bortezomib-educated macrophages contribute to MM-TIC enrichment

A defined connection between the tumor microenvironment and TICs has been established (21). In addition, it has been shown that different host cells promote TIC enrichment in response to anticancer therapy (22). Furthermore, our previous studies have indicated that following almost any anticancer drug, the host generates protumorigenic biological processes that in turn contribute to tumor regrowth and spread (for review see ref. 2). Such protumorigenic effects have also been reported in the clinic, for example, in the case of breast cancer patients treated with neoadjuvant chemotherapy (23). Here we sought to identify the mechanisms involved in bortezomib-induced protumorigenic activities in an MM setting. To this end, we first analyzed the BM cell population of control and bortezomib-treated naïve mice using mass cytometry (CyTOF). We found that bortezomib-treated mice exhibited ∼40% and ∼20% significant increases in the percentage of proinflammatory macrophages and granulocytes, respectively, when compared with vehicle control–treated mice as demonstrated in a viSNE plot and bar chart, respectively (Fig. 1A; Supplementary Fig. S1A).

We next determined whether macrophages affect the TIC population in MM. To do this, TICs were quantified in two myeloma cell lines, CAG and RPMI-8226, exposed to CM from various macrophage cultures. First, CM obtained from naïve BM-derived macrophages enriched the TIC population in both cell lines to a greater extent than CM obtained from other BM cells, with statistical significance only in RPMI-8226 cells (Fig. 1B). Second, CM from peritoneal macrophages derived from bortezomib-treated mice caused an increase in the TIC population of RPMI-8226 but not CAG cells, in comparison with CM from macrophages derived from control mice (Fig. 1C). These results

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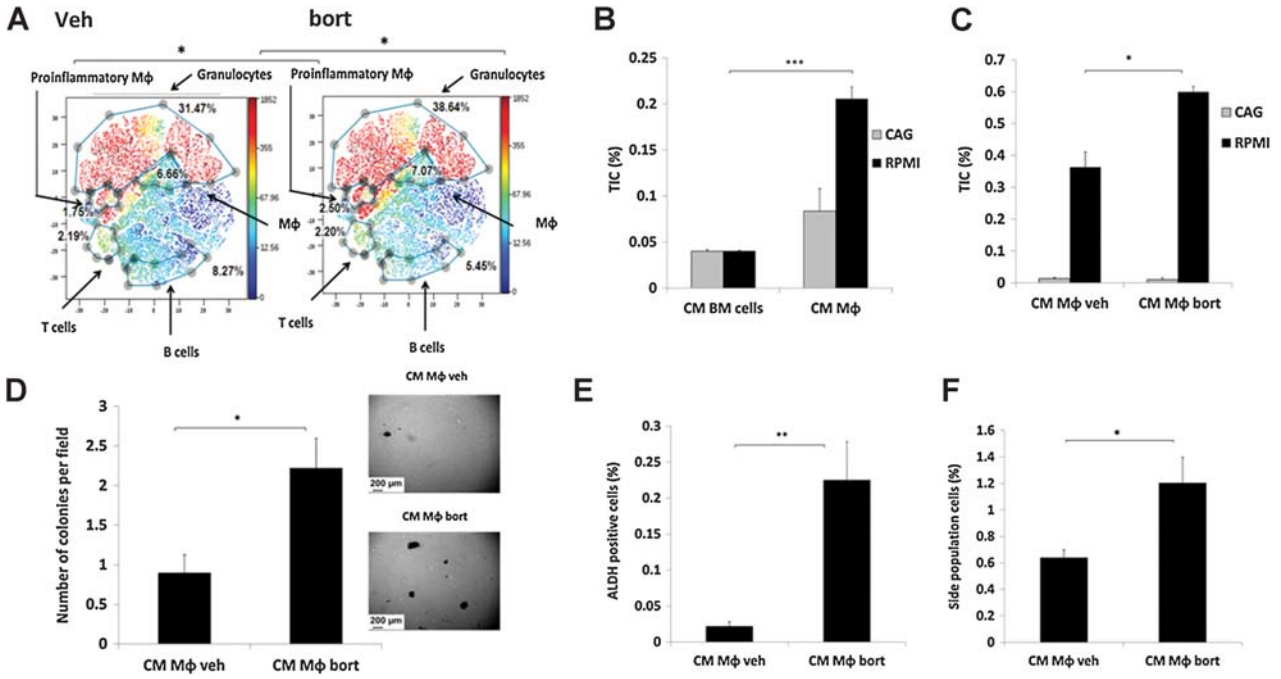

Figure 1.

Proinflammatory macrophages enrich the MM TIC population. **A,** 8–10-week-old Balb/c mice were intravenously injected with bortezomib (1 mg/kg, bort) or vehicle control (veh). After 24 hours, femurs were removed and flushed to obtain BM cells. The indicated BM cell types were identified by CyTOF mass cytometry. Results are presented as viSNE plots. **B,** Femurs from 8-week-old naïve Balb/c mice were flushed to obtain BM cells. Cells were immunostained and macrophages were sorted out. Macrophages and BM cells were then cultured separately for 48 hours to generate conditioned medium (CM). CAG and RPMI-8226 cells were cultured for 4 days in the presence of macrophage or BM cell CM. The percentage of TICs was evaluated by flow cytometry. **C,** Peritoneal macrophages were harvested from mice sequentially treated with thioglycollate followed by bortezomib (bort) or vehicle control (veh). Macrophages were cultured for 48 hours, and CM was collected. CAG and RPMI-8226 cells were cultured for 4 days in the presence of macrophage CM. The percentage of TICs was assessed by flow cytometry. **D,** In parallel, RPMI-8226 cells were cultured with MethoCult M3231 at a density of 2000 cells/well in the presence of macrophage CM. After 20 days, colonies were counted. Colony scoring (left) and representative micrographs (right) are presented. **E–F,** RPMI-8226 cells were cultured for 4 days in the presence of macrophage CM. Aldehyde dehydrogenase (ALDH) activity was assessed by flow cytometry. Quantifications of data are shown (**E**). In a parallel experiment, a side population assay was performed. Quantifications of data are shown (**F**). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ using a two-tailed Student *t* test.

suggest that macrophages from BM or peritoneal lavage enrich TICs in a similar manner. To further confirm the validity of the phenotypic-based results, we evaluated TIC enrichment using a range of functional assays. Clonogenic potential, ALDH activity and SP assays all demonstrated that culturing RPMI-8226 cells in the presence of CM from macrophages derived from bortezomib-treated mice resulted in TIC enrichment (Fig. 1D–F; Supplementary Fig. S1B–A1C). Lastly, coculturing RPMI-8226 cells with macrophages from bortezomib-treated mice resulted in a similar effect on TIC enrichment (Supplementary Fig. S1D–S1F). These collective results suggest that cell–cell contact between macrophages and TICs is not necessary for TIC enrichment. Rather, secreted factors from macrophages account for bortezomib-induced TIC enrichment. Of note, TIC enrichment and increased ALDH activity were also observed in KMS-11 and U266 MM cells when using the same experimental conditions as above (Supplementary Fig. S1G–S1H). Overall, our findings thus far suggest that bortezomib-educated macrophages promote MM-TIC enrichment in different cell lines.

Bortezomib contributes to an increased percentage of proinflammatory macrophages and TICs *in vivo*

Our finding that bortezomib-educated macrophages enrich MM-TICs *in vitro* prompted us to evaluate the *in vivo* effects of bortezomib on macrophages and TICs using the RPMI-8226 MM model. To this end, 10-week-old SCID mice underwent whole-body radiation (300 rad) 6 hours following treatment with bortezomib or vehicle control. Twenty-four hours later, mice were injected i.v. with RPMI-8226 (5×10^6) cells. After 14 days, BM was harvested from bones and analyzed by flow cytometry for the percentage of macrophages and MM-TICs. The level of proinflammatory macrophages was substantially increased in the BM of bortezomib-treated mice in comparison with control mice (Fig. 2A). In addition, mice treated with bortezomib exhibited a significant increase in the percentage of TICs (Fig. 2B). Importantly, in a parallel experiment, bortezomib-treated mice that were subsequently injected with MM cells exhibited an increased mortality rate in comparison with vehicle control–treated mice injected with MM cells (Fig. 2C). These results demonstrate that bortezomib therapy increases the percentage of proinflammatory macrophages and TICs in the BM niche, an effect that could explain decreased survival of mice preconditioned with bortezomib.

IL1β secreted by bortezomib-educated macrophages accounts for MM-TIC enrichment

In a previous study, we demonstrated that several factors were upregulated and secreted from bortezomib-treated macrophages

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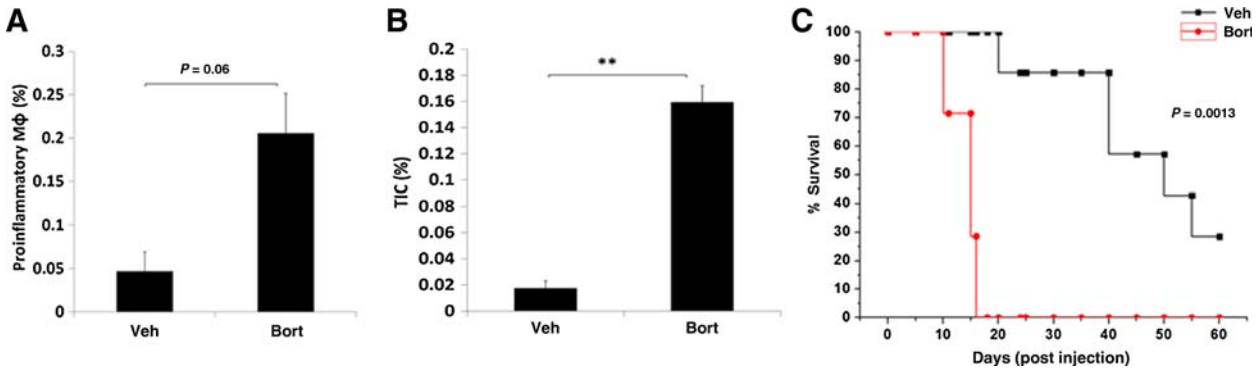

Figure 2.

Bortezomib preconditioning increases mortality rate in a murine MM model. **A–C,** Systemically irradiated SCID mice were treated with bortezomib (bort) or vehicle control (veh). After 24 hours, mice were intravenously injected with RPMI-8226 cells (5×10^6/mouse). Two weeks later, BM cells were obtained from femurs ($n = 4$ mice/group), and the percentages of proinflammatory macrophages (**A**) and TICs (**B**) were assessed by flow cytometry. **C,** In a parallel experiment, survival was monitored ($n = 7$ mice/group) and a Kaplan–Meier survival curve was plotted. **, $P < 0.01$, using a two-tailed Student *t* test.

including TREM-1, IL16, and IL1β (4). We therefore inquired whether such factors are involved in TIC enrichment induced by bortezomib-educated macrophages. Phenotypic and functional TIC assays revealed an enrichment in the TIC population of RPMI-8226 cells cultured in the presence of recombinant IL1β but not TREM-1 or IL16, suggesting that IL1β may account for MM stemness (Fig. 3A–D; Supplementary Fig. S2A–S2B). However, the TIC population of CAG cells cultured in the presence of IL1β was not enriched (Supplementary Fig. S2C). We therefore evaluated the expression of the receptor for IL1β, namely, IL1 receptor (IL1R), on the different MM cells. RNA levels of IL1R were approximately 4-fold higher in RPMI-8226 cells in comparison with CAG cells (Fig. 3E). U266 and KMS-11 cells also expressed significantly higher levels of IL1R in comparison with CAG cells (Supplementary Fig. S2D). Expression levels of IL1R were confirmed at the protein level using flow cytometry and Western blot analyses (Fig. 3F; Supplementary Fig. S2E). These findings suggest that TIC enrichment is dependent on the IL1β–IL1R axis. This may explain why CAG cells, which do not express high levels of IL1R, do not respond to IL1β or to bortezomib-educated macrophages, with respect to TIC enrichment.

Next, to further evaluate the contribution of the IL1β–IL1R axis to TIC enrichment in our experimental setting, we investigated the effects of disrupting the IL1β–IL1R axis using both pharmacologic and genetic approaches. To this end, RPMI-8226 cells were cultured with CM obtained from bortezomib-educated or vehicle-treated control macrophages in the presence or absence of Anakinra, an IL1R antagonist. After 5 days, the percentage of TICs was evaluated by flow cytometry. The addition of Anakinra to CM of bortezomib-educated macrophages abolished TIC enrichment of RPMI-8226 cells phenotypically as well as functionally, as assessed by surface marker expression, ALDH and SP assays (Fig. 3G). In a genetic approach, macrophages were harvested from wild-type or IL1β$^{-/-}$ mice treated with bortezomib or vehicle control. CM from macrophage cultures of each group was added to RPMI-8226 cells, and the percentage of TICs was assessed. As expected, CM of macrophages obtained from wild-type bortezomib-treated mice significantly increased the percentage of MM-TICs in comparison with the control group. In contrast, CM of macrophages obtained from bortezomib-treated IL1β$^{-/-}$ mice had no effect on the TIC population (Fig. 3H). However, when using CAG cells in the same experimental setting, no significant differences were found between the different groups (Fig. 3H). These results were also confirmed using other MM cells (Supplementary Fig. S2F). Collectively, these findings demonstrate that IL1β, which is secreted by bortezomib-educated macrophages, plays a key role in promoting TIC enrichment in MM cells expressing its receptor.

Increased percentage of proinflammatory macrophages in MM patients treated with bortezomib correlates with increased mortality rate

To investigate the clinical relevance of our findings, we obtained BM samples from newly diagnosed MM patients ($n = 34$) at the time of diagnosis and several months following bortezomib treatment. Proinflammatory macrophages and TICs were identified in the BM samples of these patients as shown in Fig. 4A from a representative individual. We then assessed potential confounding factors in multivariate analysis including residual MM in the BM biopsy performed after treatment, number of treatments administered overall, stage at diagnosis (ISS), and TICs in the BM before and following treatment. The multivariate Cox regression found a significant effect of proinflammatory macrophages and survival with no effect of other factors tested. A low percentage of proinflammatory macrophages in the BM following bortezomib treatment was significantly correlated with patients' overall survival (hazard ratio = 1.722; 95% CI, 1.138–2.608; $P = 0.0102$; Fig. 4B). In addition, the percentage of proinflammatory macrophages before and after treatment was also significantly correlated with event-free survival (hazard ratio = 2.2698; 95% CI, 1.3224–3.8957; $P = 0.00294$; Fig. 4C; Supplementary Fig. S3A). In agreement, patients who displayed low proinflammatory macrophages in the BM niche both at diagnosis and following bortezomib treatment demonstrated enhanced event-free survival compared with patients who displayed high proinflammatory macrophages at diagnosis or following treatment. The Kaplan–Meier survival curve estimates are based on cutoffs of 0.125% and 0.0625% for macrophages before and after treatment, respectively. The value of proinflammatory macrophages measured at baseline or after therapy was determined based on the subgroups shown in Supplementary Fig. S3B–S3C.

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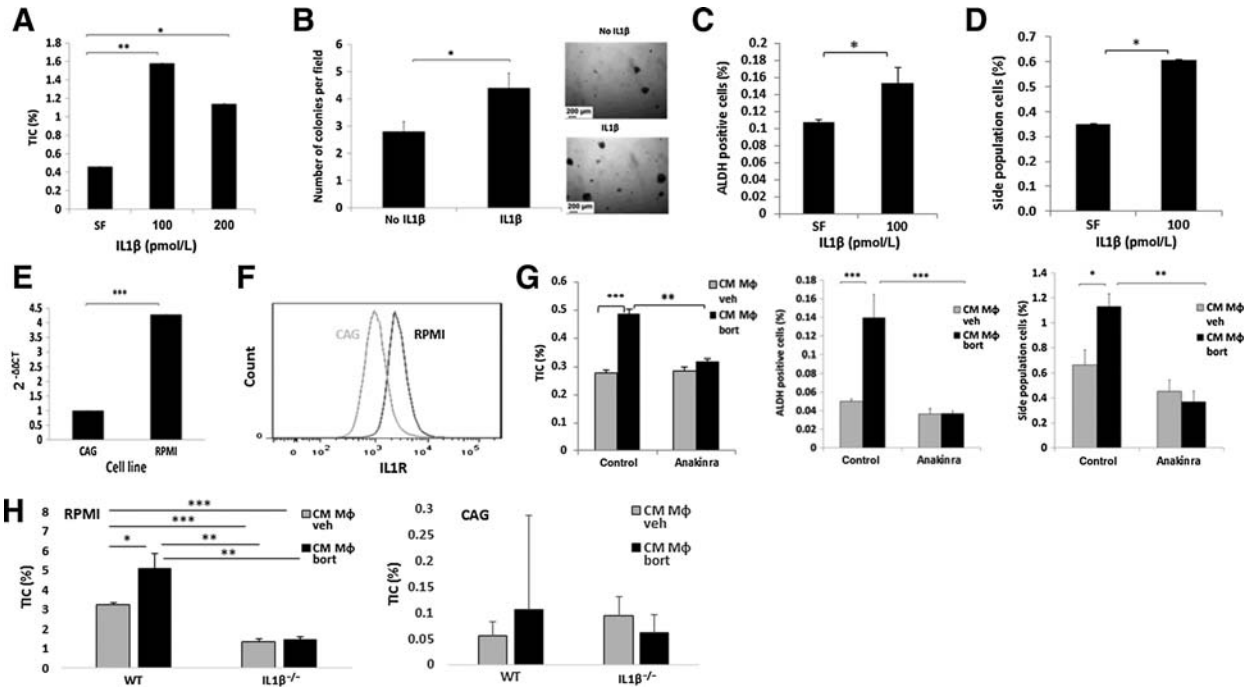

Figure 3.

IL1β secreted by bortezomib-educated macrophages enriches the MM-TIC population in mice. **A,** RPMI-8226 cells were incubated in serum-free (SF) medium in the presence of increasing concentrations of IL1β for 4 days. The percentage of TICs was analyzed by flow cytometry. **B,** RPMI-8226 cells were cultured with MethoCult M3231 at a density of 2000 cells/well in the presence or absence of 100 pmol/L IL1β. Colonies were counted following 20 days of incubation. Colony scoring (left) and representative micrographs (right) are presented. **C** and **D,** RPMI-8226 cells were incubated in the presence or absence of 100 pmol/L IL1β for 4 days. Aldehyde dehydrogenase (ALDH) activity (**C**) and side population assay (**D**) were performed by flow cytometry. Quantifications of data are shown. **E,** mRNA was extracted from CAG and RPMI-8226 cells, and the level of IL1 receptor mRNA was assessed by real-time PCR. Values were normalized to β2MG. **F,** CAG and RPMI-8226 cell were analyzed for IL1R expression using flow cytometry. A histogram plot is shown. **G,** Peritoneal macrophages were harvested from mice sequentially treated with thioglycollate followed by bortezomib (bort) or vehicle control (veh). Macrophages were cultured for 48 hours and conditioned medium (CM) was collected. RPMI-8226 cells were cultured for 4 days with macrophage CM in the presence or absence of Anakinra (100 μg/mL). The percentage of TICs, ALDH activity, and side population were assessed by flow cytometry. **H,** Peritoneal macrophages were harvested from wild-type (WT) or IL1β$^{-/-}$ mice sequentially treated with thioglycollate followed by bortezomib (bort) or vehicle control (veh). Macrophages were cultured for 48 hours, and CM was collected. RPMI-8226 and CAG cells were cultured for 4 days with macrophage CM and the percentage of TICs was analyzed by flow cytometry. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, using a two-tailed Student *t* test when comparing between two groups and one-way ANOVA followed by Tukey post hoc statistical test when comparing between more than two groups.

Furthermore, a higher percentage of TICs after bortezomib therapy was associated with poor survival in MM patients (Fig. 4D; Supplementary Fig. S3D). Importantly, a positive correlation between the levels of proinflammatory macrophages and TICs before and following bortezomib treatment was found (Fig. 4E). Taken together, these results suggest that bortezomib treatment causes a detrimental host–cell mediated effect in patients, similar to that observed in mice.

IL1β correlates with increased percentage of TICs and residual MM in patients treated with bortezomib

We next asked whether correlations exist between IL1β levels, TIC enrichment and clinical outcome in MM patients. To this end, we quantified the level of IL1β in BM samples taken from MM patients after they received bortezomib treatment. A high level of IL1β in the BM samples was associated with residual MM and death (Fig. 5A). In addition, IL1β levels were positively correlated with the percentage of TICs (Fig. 5B). Of note, BM cells from MM patients express IL1R (Fig. 5C). Lastly, plasma, which was extracted from BM samples of 6 selected patients who demonstrated high IL1β (before or after bortezomib therapy), was added to RPMI-8226 cultures in the presence or absence of Anakinra, and the percentage of TICs was assessed. Cultures treated with Anakinra displayed a reduced, albeit not statistically significant percentage of TICs (Fig. 5D). Taken together, similar to the mouse data, our results suggest that an increased level of IL1β in BM samples of MM patients who underwent bortezomib therapy contributes to MM-TIC enrichment and may serve as an indication of an aggressive disease.

Discussion

Bortezomib is among the most widely used and effective agents for the treatment of MM (24). Its mechanism of action includes induction of apoptosis, inhibition of tumor growth as well as amending cellular interactions and cytokine secretion in the BM microenvironment (25). Bortezomib is known to alter the immune balance within the host: it directly impairs B-cell function, inhibits inflammation in macrophages, and depletes T regulatory cells (26). Regardless, disease progression is common, and once a patient is refractory to treatment, prognosis is very poor (27).

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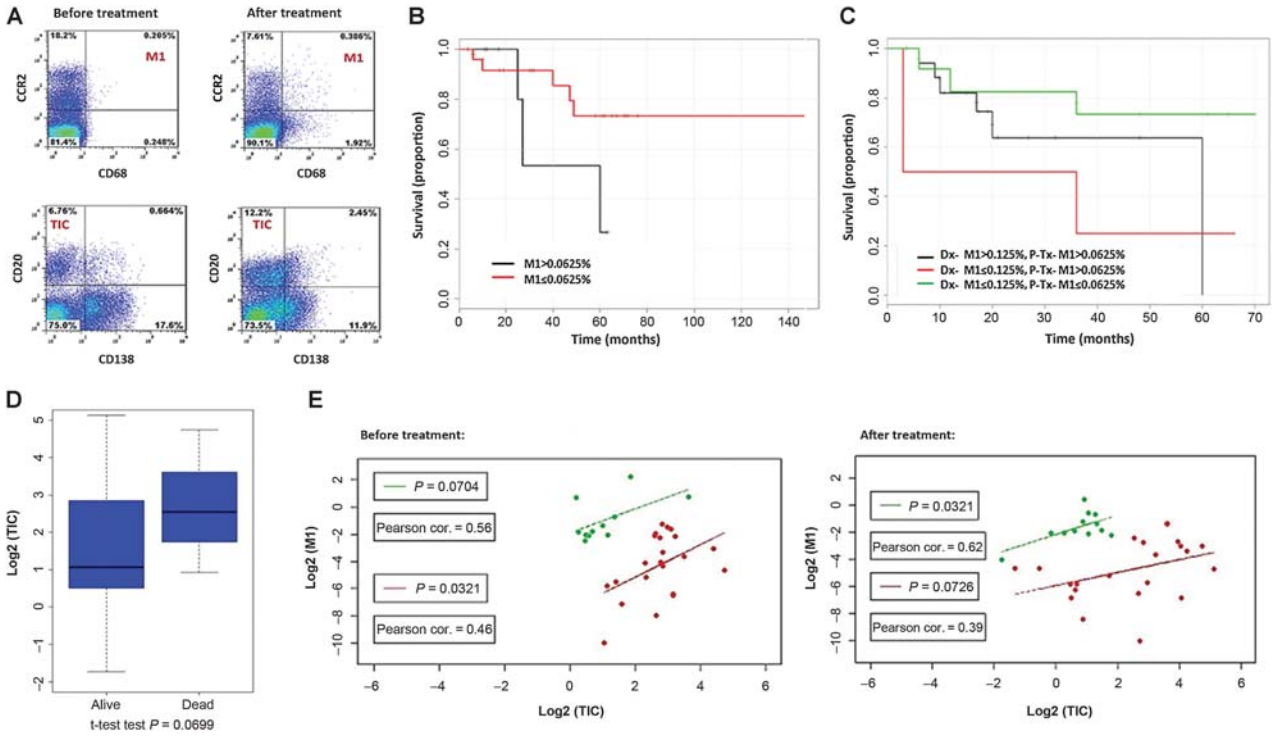

Figure 4.

The percentage of proinflammatory macrophages in the BM correlates with disease outcome in MM patients. BM samples were obtained from newly diagnosed MM patients ($n = 34$) immediately before and several months after receiving bortezomib treatment. **A,** The levels of M1-proinflammatory macrophages and TICs were quantified by flow cytometry. Representative flow cytometry plots from one MM patient are shown. **B,** Patients were grouped according to the level of M1-proinflammatory macrophages prior to therapy (M1-high>0.125%, and M1-low$\leq$ 0.125%) and after therapy (M1-high>0.0625%, and M1-low$\leq$0.0625%). Kaplan–Meier curve of overall survival of the 2 patient groups is presented. **C,** Kaplan–Meier curve of event-free survival is presented based on the percentage of M1-proinflammatory macrophages at diagnosis (Dx) and at posttreatment (P-Tx). **D,** The correlation between TICs following bortezomib treatment and MM-related death is presented. **E,** The correlation between TICs and M1-proinflammatory macrophages before and following bortezomib treatment is presented. Two separate clusters (green and red) were identified based on TIC and M1 values. The correlation was performed separately for each cluster.

Compelling data have shown that the major therapies for cancer, including chemotherapy, radiotherapy, and surgery, induce host effects (indirect effects) that may lead to enhanced tumor aggressiveness and subsequent relapsed disease (3, 28, 29). For example, several chemotherapeutic drugs have been shown to support the emergence of a tumor cell metastatic phenotype in lung carcinoma murine models, mainly by inducing the secretion of matrix-metalloproteinases from bone marrow–derived cells (30). Yet, the effects of targeted drugs, presumably with minimal off-target effects, are called into question. Remarkably, we have previously reported that bortezomib induces comparable protumorigenic effects to those described for other anticancer agents (4). Specifically, we demonstrated that plasma obtained from bortezomib-treated mice induced a significant increase in MM cell migration, viability, and proliferation *in vitro*. Furthermore, mice initially treated with bortezomib and only subsequently were injected with MM cells succumbed to extensive disease earlier than mice treated with a vehicle control. These effects were associated with proinflammatory macrophages because depletion of macrophages by chlodronate resulted in survival rates that were similar to control untreated mice (4). Mechanisms to explain these results were not previously reported.

Formally, macrophages are subdivided into antitumoral (M1) and protumoral (M2) subtypes. The protumoral subtype is considered as tumor-associated macrophages (TAM), which has been shown to support the growth and spread of tumor cells especially in solid tumors (31). However, in recent years, it has been realized that macrophages in tumor represent a continuum of phenotypes among the M1 and M2 extremes, depending on local signals and their proinflammatory and anti-inflammatory roles, respectively (32). It is now widely appreciated that TAMs belong to several subtypes, and their plasticity in the tumor microenvironment can alter tumor fate (33). Importantly, among the diverse TAM subtypes, there is also a distinct group of TIC-promoting macrophages (32). These studies therefore prompted us to evaluate the contribution of macrophages to TIC enrichment in our MM setting.

In the current study, we demonstrate that culturing RPMI-8226 cells with CM obtained from macrophages from bortezomib-treated mice induces TIC enrichment *in vitro*, both phenotypically and functionally. In our study, the phenotypic characterization of TICs is based on CD20 and CD138 surface markers, similar to most studies (11). It should be noted, however, that we have not evaluated TIC markers described in other studies (34, 35). Nevertheless, the functional assays carried out in our experimental setting further confirm that MM-TICs are enriched in response to CM of macrophages obtained from bortezomib-treated mice. Additionally, we show that IL1β (either secreted by proinflammatory macrophages following bortezomib treatment or when

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A

IL1β value after therapy	Patients (*n*)	Normal BM after therapy (%)	Death from MM (%)
High IL1β	7	0	42.8
Low IL1β	6	66.6	33.3

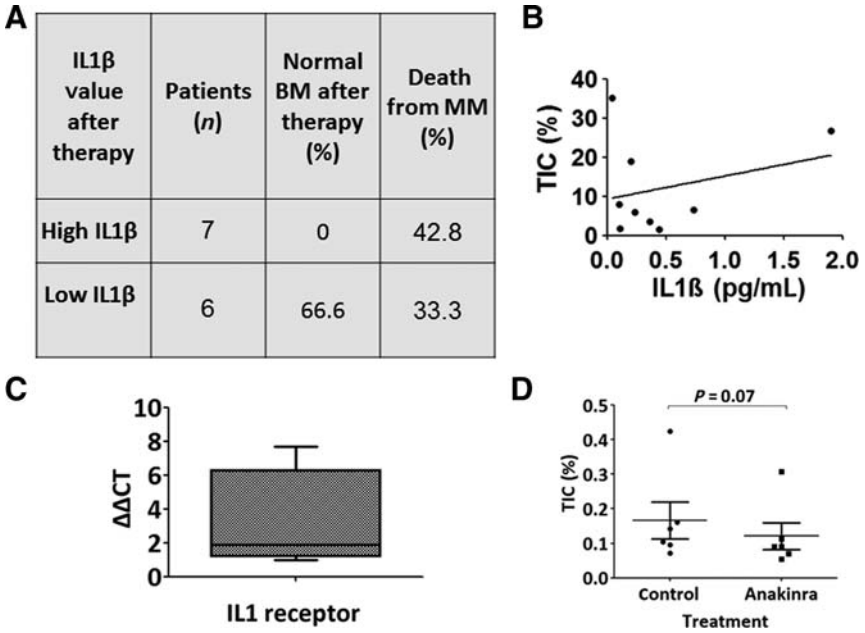

Figure 5.

The level of IL1β correlates with MM patient survival and BM TIC levels. **A,** BM samples were obtained from newly diagnosed MM patients ($N = 13$) immediately before and several months after receiving bortezomib treatment. IL1β concentration in BM plasma extracts was assessed by ELISA. Median value of IL1β following bortezomib treatment was calculated and indicated as IL1β > 0.158 (pg/mL; high) versus IL1β < 0.158 (pg/mL; low). Patient BM response and survival a few months after therapy are shown with respect to IL1β levels. **B,** The percentage of TICs in available BM samples (9 of 13) was assessed by flow cytometry. A correlation between TICs and IL1β levels following bortezomib treatment is presented. **C,** RNA was extracted from BM of 4 newly diagnosed MM patients. The level of IL1 receptor mRNA was assessed by real-time PCR. Values were normalized to β2MG. **D,** Plasma was extracted from BM samples of 6 MM patients who underwent bortezomib treatment. RPMI-8226 cells were cultured with the plasma samples for 4 days in the presence or absence of Anakinra (100 μg/mL). The percentage of TICs was assessed by flow cytometry. Statistical analysis was performed using the Student *t* test.

added as a recombinant protein) increases the percentage of TICs in MM cultures. Previous studies have demonstrated a link between IL1 signaling and tumor cell stemness. For example, IL1 signaling was shown to be increased in BM from chronic myeloid leukemia patients and its blockade resulted in reduced leukemic stem cell growth (36). In acute myeloid leukemia (AML) patients, IL1 receptor–associated protein (IL1RAP) was found to be upregulated in all leukemic stem cells and associated with poor prognosis (37). Knockdown of IL1RAP resulted in reduction in clonogenicity and is studied as a potential stem cell target in AML (38, 39). In addition, it has been shown that IL1β promotes stemness and tumor cell invasion in colon cancer (40). Thus, our results further support the notion that the IL1β–IL1R axis contributes to MM stemness, similar to its reported involvement in other tested tumor types.

In addition to our murine models, we evaluated the effect of bortezomib on macrophages and TICs in newly diagnosed MM patients treated with a bortezomib containing regimen. We demonstrate that overall survival is significantly correlated with a low percentage of proinflammatory macrophages in the BM following bortezomib treatment. Remarkably, event-free survival significantly correlates with proinflammatory macrophages both prior to and following bortezomib treatment. Congruently, poor survival is associated with a higher percentage of TICs following bortezomib treatment. Thus, the results obtained in cell culture and in the murine model suggest the notion that bortezomib treatment of MM patients may induce relapse through host effects. We also acknowledge that by defining TICs as $CD138^{-}CD20^{+}$ we may have included also mature B lymphocytes residing in the BM. Further studies in this direction should enroll a larger patient cohort and a more detailed phenotype of TICs to confirm the validity of the clinical results.

Currently, based on the scant and confounding knowledge on TICs in MM, it is difficult to draw a definite conclusion on the specific characteristics of MM-TICs in MM patients. However, our study is the first and largest demonstrating the connection between proinflammatory macrophages, IL1β, and TICs in MM patients. Previous studies have shown that host-derived IL1β promotes the malignant process (review in ref. 41), mainly through TAMs activated to secrete cytokines/mediators that increase invasiveness and also display an immunosuppressive phenotype (13). Our findings also provide further rationale for ongoing clinical trials assessing IL1β blockade as a treatment strategy for MM. Treatment with IL1 inhibitors was evaluated in 47 patients with smoldering or indolent MM at risk for progression. Among these patients, 3 patients achieved a minor response to IL1 inhibition alone and 9 patients achieved minor/partial response after adding dexamethasone (42). Based on these results, Anakinra is currently being evaluated in a phase I/II trial including early-stage MM patients treated with lenalidomide and dexamethasone (NCT02492750, www.clinicaltrials.gov). It will be of interest to evaluate whether lenalidomide and dexamethasone contribute to MM-TIC enrichment similar to the effect of bortezomib demonstrated in the current study.

At present, with the wide repertoire of treatment options for MM patients, it is imperative to incorporate biomarkers that

would preclude one treatment modality over the other in clinical decision-making. Based on our findings, we propose that macrophage state and TIC levels in the BM compartment of newly diagnosed MM patients can be used as a predictive tool for relapse. Further analysis should be performed on a larger sample size to strengthen this conclusion. Nevertheless, our findings provide a mechanistic explanation for MM resistance to bortezomib, paving the way toward the development of novel prognostic biomarkers for resistance to bortezomib therapy.

Disclosure of Potential Conflicts of Interest

Y. Shaked is an advisor at OncoHost. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: O. Beyar-Katz, K. Magidey, I. Avivi, R.N. Apte, Y. Shaked

Development of methodology: O. Beyar-Katz

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O. Beyar-Katz, K. Magidey, N. Barak, I. Avivi, Y. Cohen, M. Timaner, S. Avraham, M. Hayun, N. Lavi, M. Bersudsky, E. Voronov

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O. Beyar-Katz, K. Magidey, A. Reiner-Benaim, Y. Cohen, Y. Shaked

Writing, review, and/or revision of the manuscript: O. Beyar-Katz, I. Avivi, Y. Cohen, R.N. Apte, Y. Shaked

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): O. Beyar-Katz, I. Avivi, Y. Cohen, N. Lavi

Study supervision: Y. Shaked

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Ofrat Beyar-Katz, Ksenia Magidey, Anat Reiner-Benaim, et al.

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