Clinical Cancer Research

Lenalidomide Enhances Immune Checkpoint Blockade-Induced Immune Response in Multiple Myeloma

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Abstract

Purpose: PD-1/PD-L1 signaling promotes tumor growth while inhibiting effector cell–mediated antitumor immune responses. Here, we assessed the impact of single and dual blockade of PD-1/PD-L1, alone or in combination with lenalidomide, on accessory and immune cell function as well as multiple myeloma cell growth in the bone marrow (BM) milieu.

Experimental Design: Surface expression of PD-1 on immune effector cells, and PD-L1 expression on $CD138^+$ multiple myeloma cells and myeloid-derived suppressor cells (MDSC) were determined in BM from newly diagnosed (ND) multiple myeloma and relapsed/refractory (RR) multiple myeloma versus healthy donor (HD). We defined the impact of single and dual blockade of PD-1/PD-L1, alone and with lenalidomide, on autologous anti–multiple myeloma immune response and tumor cell growth.

Results: Both ND and RR patient multiple myeloma cells have increased PD-L1 mRNA and surface expression compared with HD. There is also a significant increase in PD-1 expression on effector cells in multiple myeloma. Importantly, PD-1/PD-L1 blockade abrogates BM stromal cell (BMSC)-induced multiple myeloma growth, and combined blockade of PD-1/PD-L1 with lenalidomide further inhibits BMSC-induced tumor growth. These effects are associated with induction of intracellular expression of IFNγ and granzyme B in effector cells. Importantly, PD-L1 expression in multiple myeloma is higher on MDSC than on antigen-presenting cells, and PD-1/PD-L1 blockade inhibits MDSC-mediated multiple myeloma growth. Finally, lenalidomide with PD-1/PD-L1 blockade inhibits MDSC-mediated immune suppression.

Conclusions: Our data therefore demonstrate that checkpoint signaling plays an important role in providing the tumor-promoting, immune-suppressive microenvironment in multiple myeloma, and that PD-1/PD-L1 blockade induces anti–multiple myeloma immune response that can be enhanced by lenalidomide, providing the framework for clinical evaluation of combination therapy. *Clin Cancer Res; 21(20); 4607–18. ©2015 AACR.*

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Introduction

Multiple myeloma is a clonal B-cell malignancy associated with a monoclonal (M) protein in blood and/or urine, bone lesions, and immunodeficiency. It usually evolves from monoclonal gammopathy of undetermined significance (MGUS), with low levels of plasmacytosis and M protein without osteolytic lesions, anemia, hypercalcemia, and renal failure (1). Multiple myeloma is characterized by genetic signatures, including frequent translocations into the immunoglobulin heavy chain switch region (IgH), oncogenes, and abnormal chromosome number (2, 3). Most patients with translocations have non-hyperdiploid chromosome number (NHMM), while those patients lacking IgH translocations have hyperdiploid chromosome number (HMM) with trisomies of chromosomes 3,5,7,9,11,15,19, and 21. Importantly, patients with hyperdiploid multiple myeloma have a better outcome with prolonged survival (4, 5).

Advances in multiple myeloma biology have established that the bidirectional interaction between multiple myeloma cells, bone marrow stroma cells (BMSC), extracellular matrix, and accessory cells can induce autocrine and paracrine signaling that regulates tumor development and growth on the one hand, while transforming the BM microenvironment into an immune-suppressive milieu on the other (6, 7). We and others have extensively studied the impact of the interaction between BMSC and multiple myeloma cells on pathogenesis and cell adhesion mediated-drug resistance (CAM-DR) in order to identify and validate new

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

The interaction of tumor cells with their surrounding accessory cells and extracellular matrix provides a tumor-promoting environment while suppressing immune response. Recent studies in solid tumors have demonstrated that programmed death-1 (PD-1) signaling plays an important role in tumor-induced immune suppression and conversely, that blockade of PD-1/PD-L1 by therapeutic antibodies restores antitumor immune response. Remarkable responses have been observed to PD-1 blockade in malignant melanoma, leading to recent FDA approval of anti–PD-1 antibody therapies. Here, we assessed the impact of single and dual blockade of PD-1/PD-L1, alone or in combination with lenalidomide, on accessory and immune cell function, as well as on multiple myeloma cell growth in the BM milieu. Our study demonstrates that PD-1/PD-L1 blockade can induce anti–multiple myeloma immune responses, which are enhanced by lenalidomide. Our studies provide the preclinical rationale for evaluation of combined PD-1/PD-L1 blockade with lenalidomide to inhibit tumor cell growth, restore host immune function, and improve patient outcome in multiple myeloma.

targeted therapeutics (1). Immunomodulatory drugs thalidomide and lenalidomide, and proteasome inhibitor bortezomib are novel agents which target the tumor cell in its microenvironment and can overcome CAM-DR; they have been rapidly integrated into multiple myeloma treatment, resulting in at least a 2- to 3-fold prolongation of median survival (8–10). Even though these novel drugs have transformed the treatment paradigm and patient outcome, most multiple myeloma relapses due to minimal residual disease and drug resistance (11). Generation of more effective therapeutic strategies may therefore not only require targeting the tumor and stroma, but also overcoming blockade of antitumor immune response. Tumor-associated immune suppressor cells such as regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC) can effectively block antitumor immune responses, representing an important obstacle for immunotherapy. We have recently assessed the presence, frequency, and functional characteristics of MDSC in patients with newly diagnosed (ND-MM), responsive multiple myeloma, and relapsed, refractory multiple myeloma (RR-MM) compared with healthy donor (HD), and identified an increased MDSC population ($CD11b^+CD14^-HLA\text{-}DR^{-/low}CD33^+CD15^+$) with tumor-promoting and immune-suppressive activity in both the peripheral blood (PB) and BM of multiple myeloma patients. Moreover, we have shown that lenalidomide does not target MDSC in the BM milieu (12).

Programmed cell death-1 (PD-1, CD279), a member of the CD28 receptor family, and its ligands either PD-L1 (B7-H1, CD274) or PD-L2 (B7-DC, CD273), play a fundamental role in tumor immune escape by inhibiting immune effector functions. PD-1 gene is encoded on chromosome 2, and PD-L1 gene is on chromosome 9. PD-1 expression is induced on antigen activated T cells and exhausted T cells and B cells; PD-L1 is mainly expressed by antigen-presenting cells (APC) and various nonhematopoietic cells; and PD-L2 is found on hematopoietic cells, including dendritic cells and macrophages (13). Recent studies in solid tumors have demonstrated that expression of PD-L1 is significantly increased and associated with progressive disease in lung cancer, breast cancer, renal cell cancer, colorectal cancer, gastric cancer, esophageal cancer, and pancreatic cancer (7, 8, 14–21). Most importantly, remarkable responses have been observed to PD-1 blockade in malignant melanoma, leading to recent FDA approval of PD-1 monoclonal antibody therapies. To date, increased PD-L1 expression has been shown in multiple myeloma cells compared with HD plasma cells (13, 22–26), and increased PD-1 expression has been demonstrated on CD4T cells in multiple myeloma (11, 13, 22, 24, 25, 27). Because PD-1/PD-L1 signaling promotes tumor growth while inhibiting effector cell–mediated antitumor immune response, we here assessed the impact of single and dual blockade of PD-1/PD-L1 signaling, alone or in combination with lenalidomide, on accessory (MDSC, BMSC) and immune cell (CD4T cells, CD8T cells, NK cells, NKT cells, and monocytes/macrophages) function, as well as multiple myeloma cell growth, in the BM milieu. Our studies provided the framework for targeting PD-1 and PD-L1 in combination with lenalidomide to inhibit tumor cell growth and restore immune function in multiple myeloma.

Materials and Methods

Cell isolation

Heparinized venous blood samples and/or aspirates of BM from patients with ND-MM ($n = 6$) or RR-MM ($n = 10$) and healthy donors (HD, $n = 10$) were obtained after written informed consent per the Declaration of Helsinki and approval by the Institutional Review Board of the Dana-Farber Cancer Institute (Boston, MA).

Cell lines

MM1.S, U266, and H929 multiple myeloma cells were purchased from ATCC; plasma cell leukemia (PCL) cells OPM1 and OPM2 were provided by Dr. Edward Thompson (University of Texas Medical Branch, Galveston, TX). Cell lines have been tested and authenticated by STR DNA fingerprinting analysis (Molecular Diagnostic Laboratory, DFCI), and used within 3 months after thawing. All cell lines were maintained in RPMI-1640 (Bio Whittaker) containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies).

Reagents and compounds

Functional grade PD-1 and PD-L1 blocking antibodies, anti-human PD-1 (clone J116) and anti-human PD-L1 (clone MIH1), were obtained from eBiosciences. Immunomodulatory drug lenalidomide (10 mmol/L) was dissolved in DMSO and stored at −20°C. Anti-CD3 and anti-CD28 MAbs (10 μg/mL; Becton Dickinson Biosciences) were used to stimulate cells.

Cell phenotyping

Cell surface expression of PD-1 (CD279) on $CD4^+$T cells, $CD8^+$T cells, $CD56^+$ NK cells and $CD3^+CD8^+CD56^+$NKT cells, and PD-L1 (CD274) on $CD138^+$ multiple myeloma cells, $CD14^+$ monocytes/macrophages, $CD11b^+CD14^+HLA\text{-}DR^+$ APCs, $CD11b^+CD14^-HLA\text{-}DR^{-/low}CD33^+CD15^+$ nMDSC, and $CD11b^+CD14^+HLA\text{-}DR^{-/low}$ mMDSC was determined on PBMCs or BMMCs from multiple myeloma patients or healthy donors by multiparameter flow-cytometric analysis. Cells were stained with CD11b APCCy7, CD14 Pacific blue, HLA-DR PECy7, CD33 PECy5, and CD15 FITC conjugated MAbs (BD

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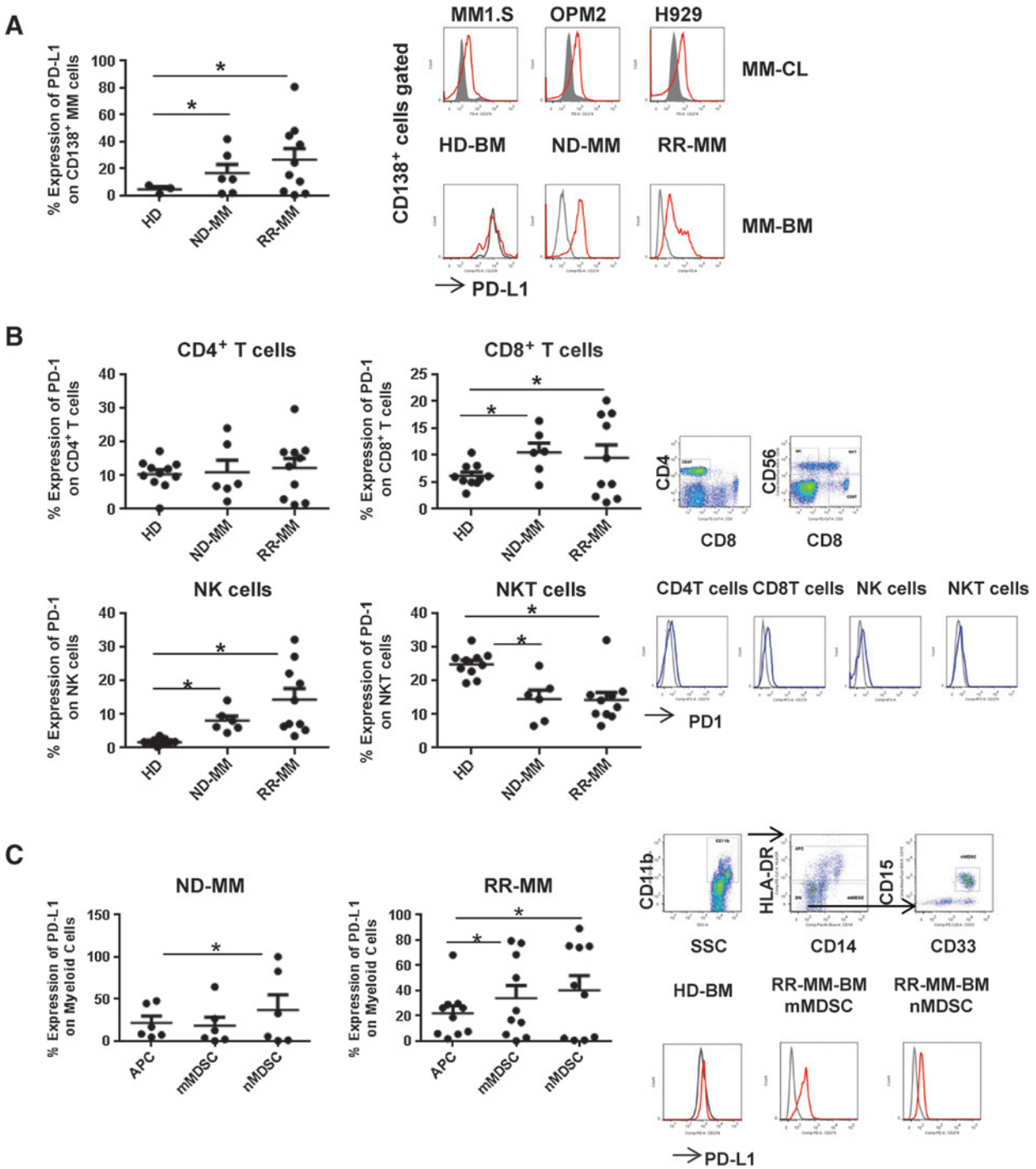

Figure 1.
Increased frequency of PD-1 and PD-L1 expression in multiple myeloma BM microenvironment. Cell surface expression of PD-1 (CD279) and PD-L1 (CD274) is shown on CD138$^+$ multiple myeloma cells, immune effector cells, and immune-suppressive MDSC in ND-MM and RR-MM. A, cell surface expression of PD-L1 is quantitated on patient multiple myeloma cells obtained from patients with ND-MM ($n = 6$) and RR-MM ($n = 10$), and compared with healthy donor plasma cells (HD, $n = 3$). Data represent percentage of PD-L1–expressing CD138$^+$ multiple myeloma cells. Representative histogram plots of PD-L1 expression (red) relative to control (gray) is shown on a gated population of CD138$^+$ plasma cells. Top panel demonstrates PD-L1 expression on multiple myeloma cell lines (MM1.S, OPM2, H929), and bottom panel represents PD-L1 expression on BM CD138$^+$ plasma cells from patients with ND-MM and RR-MM, as well as HD-BM. B, cell surface expression of PD-1 is quantitated on immune effector cells (CD4T cells, CD8T cells, NK cells, and NKT cells) from patients with ND-MM ($n = 6$) and RR-MM ($n = 10$) compared with healthy donors (HD, $n = 10$). Data represent percentage of PD-1–coexpressing CD4T cells (top left), CD8T cells (top right), NK cells (bottom left), and NKT cells (bottom right) in BM of patients with ND-MM and RR-MM compared with HD-PBMC. Representative histogram plots of PD-1 expression (blue) versus control (gray) on BM immune effector cells: CD4T cells, CD8T cells, NK cells, and NKT cells with gating strategy are shown by multiparameter dot plots (right). C, the frequency of PD-L1 cell surface expression is shown in monocytic MDSC (mMDSC) and neutrophilic MDSC (nMDSC) compared with APCs from BM of patients with ND-MM (left) and RR-MM (right). Representative flow-cytometric histogram of PD-L1 expression (red) versus control (gray) on MDSC in healthy donor and RR-MM BM (right) with gating strategy for mMDSC and nMDSc is shown by multiparameter dot plots (right). *, $P < 0.05$.

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Biosciences) for MDSC; as well as CD138 APC for multiple myeloma cells, and CD4 PE-Cy5, CD8 PE-Cy7, CD56 FITC for effector cells (BD Biosciences).

Intracellular cytokine analysis

Autologous effector cells and CD138$^+$ multiple myeloma cells or MDSCs were cocultured in the absence or presence of anti-human PD-1 (10 μg/mL) and anti-human PD-L1 blocking Ab (10 μg/mL) alone or in combination with or without addition of lenalidomide (1 μmol/L) for 16 hours to 4 days. Of note, 5 μg/mL of brefeldin-A solution (eBiosciences) was added during the last 2 hours of incubation. Cells were then fixed in 4% paraformaldehyde-PBS and stained with PE-conjugated IFNγ and FITC-conjugated granzyme B (Gzm-B) MAbs (Becton Dickinson Biosciences) in permeabilization buffer (0.5% saponin-PBS). Intracytoplasmic cytokines in T cells, NK cells, NKT cells, and monocytes/macrophages was detected by flow cytometry using BD-LSR Fortessa (Becton Dickinson Biosciences) and analyzed using FlowJo software (TreeStar).

Cell proliferation assay

CD138$^+$ multiple myeloma cells were isolated by FACSAria IIu sorter, labeled with CFSE, and cultured either alone or with BMSC generated from BM aspirates of multiple myeloma patients, or autologous MDSC, with or without anti-human PD-1 and anti-human PD-L1 Ab alone or in combination for 24 hours to 4 days (2:1 ratio). Multiple myeloma cell growth was measured following propidium iodide addition (PI, 1 μg/mL) by CFSE/PI flow-cytometric analysis using BD-LSR Fortessa (Becton Dickinson Biosciences), and data were analyzed using FlowJo software (TreeStar).

MDSC and CD3$^+$T cells were isolated from PB or BM aspirates of multiple myeloma patients by FACS-sorting, and MDSC were cocultured for 4 days with CFSE-labeled autologous T cells (MDSC:T cell ratio 1:4) in the absence or presence of checkpoint blockade antibodies with lenalidomide (1 μmol/L).

Cytotoxicity assay

CD138$^+$ multiple myeloma cells and autologous effector cells (CD3T cells and NK cells) were isolated by FACS sorting from multiple myeloma BM. CD138$^+$ multiple myeloma cells were labeled with CFSE and cultured with each effector cell population in the absence or presence of anti–PD-1 or anti–PD-L1, alone or in combination, for 4 hours. PI (1 μg/mL) was added before analysis. Apoptotic/dead multiple myeloma cells

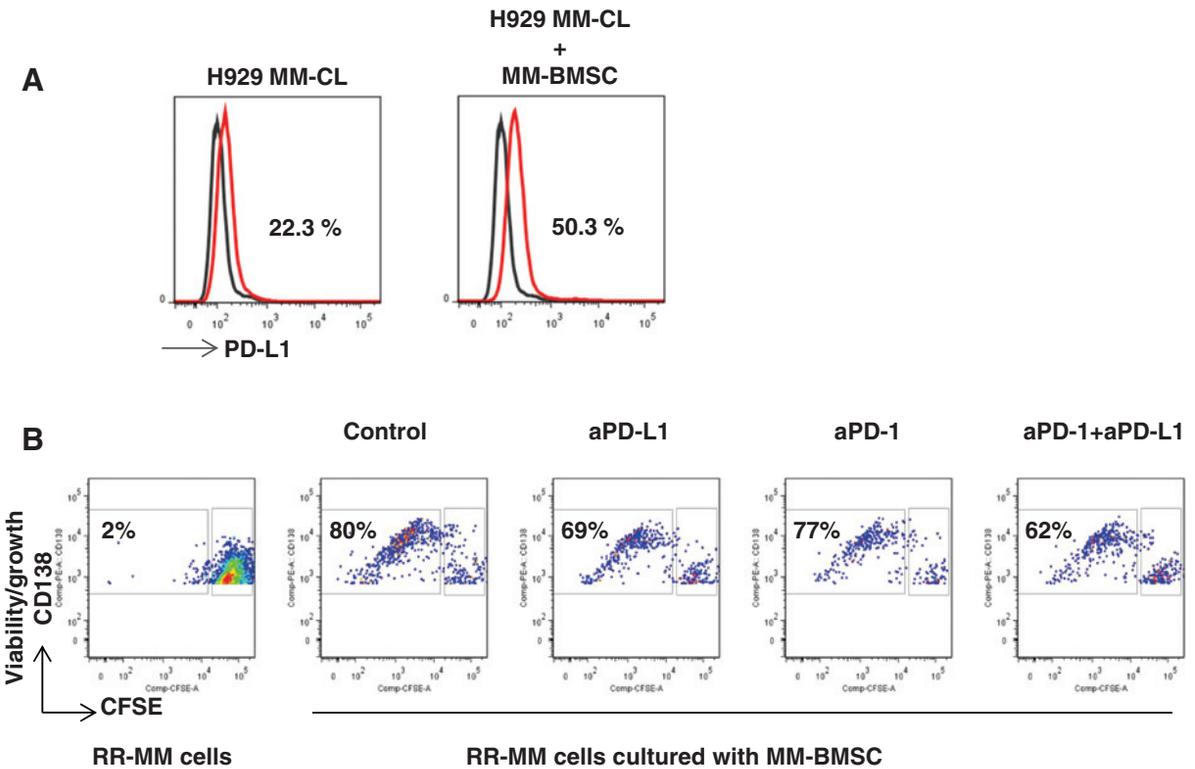

Figure 2.
Checkpoint blockade overcomes BM stroma-mediated multiple myeloma growth. PD-1/PD-L1 signaling role in the bidirectional interaction between multiple myeloma cells and BM stromal cells (BMSC) is shown in coculture of BMSC and multiple myeloma cells. A, BMSC effect on PD-L1 expression in multiple myeloma cells is demonstrated by multiparameter flow-cytometric analysis of multiple myeloma cell–BMSC cocultures. Representative histogram plot for PD-L1 expression in CD138$^+$ multiple myeloma cell population (red) versus control (gray) is demonstrated in multiple myeloma cell line (H929) alone and cultured with BMSC. B, checkpoint blockade effect on BMSC-mediated multiple myeloma growth is demonstrated by CFSE-flow analysis in cocultures of CD138$^+$ multiple myeloma cells from patient with RR-MM with BMSC. Shown are multiparameter dot plots for multiple myeloma cells alone and cultured with BMSC with or without single and dual blockade of PD-1 and PD-L1. CD138$^+$CFSE^{low} cell population represents live/growth multiple myeloma cells (large gated box), and CD138$^+$CFSE^{high} cell population represents non-dividing/dead multiple myeloma cells (small gated box).

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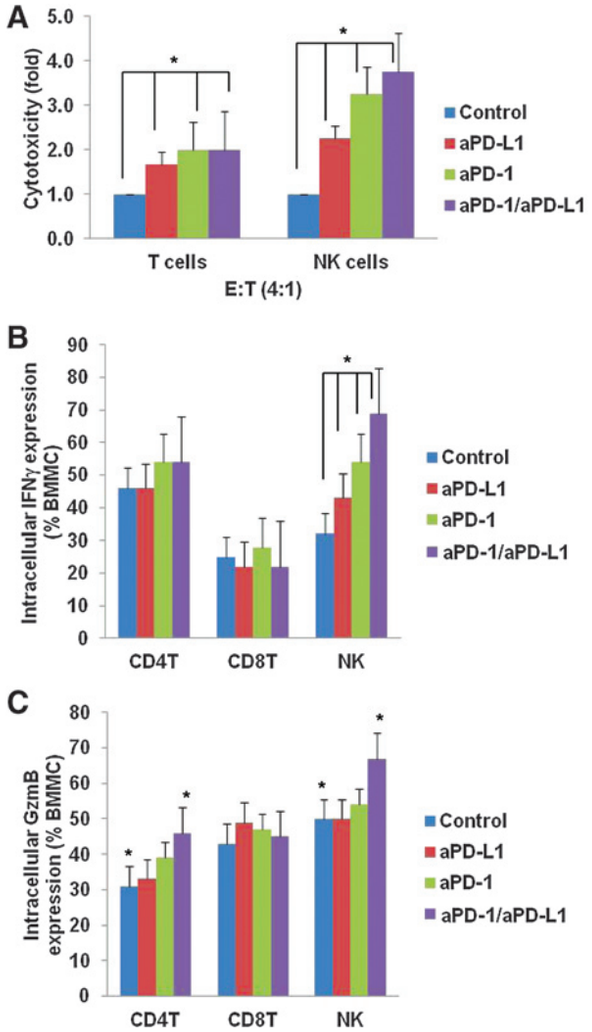

Figure 3.
Checkpoint blockade enhances immune effector cell–mediated anti–multiple myeloma responses in multiple myeloma BM. A, impact of checkpoint blockade on immune effector cell–mediated anti–multiple myeloma response is demonstrated by a multiparameter CFSE/PI flow-cytometric analysis. CFSE-labeled target CD138$^+$ multiple myeloma cells and autologous immune effector cells (CD3T cells and NK cells) were cultured with or without anti–PD-1 and anti–PD-L1, alone or in combination. Effector cell–mediated cytotoxicity was determined by CD138$^+$CFSE$^+$PI$^+$ apoptotic/dead multiple myeloma cells. Fold change is relative to control (effector cell–mediated multiple myeloma cytotoxicity without checkpoint blockade). Impact of checkpoint blockade on effector cytokines IFNγ (B) and Gzm-B (C) mediating cytotoxicity against multiple myeloma is shown in effector cells in cocultures of CD138$^+$ multiple myeloma cells and autologous effector cells. *, $P < 0.05$.

were identified as CD138$^+$CFSE$^+$PI$^+$ by multiparameter flow-cytometric analysis using BD-LSR Fortessa (Becton Dickinson Biosciences), and data were analyzed using FlowJo software (TreeStar).

To determine the effect of checkpoint blockade with lenalidomide on anti–multiple myeloma cytotoxicity in the BM, BM mononuclear cells (BMMC) from patients with RR-MM were labeled with CFSE and cultured in the absence or presence of anti–PD-1 (10 μg/mL) and anti–PD-L1 (10 μg/mL), alone or in combination, or with the addition of lenalidomide (1 μmol/L). PI (1 μg/mL) was added before analysis. Immune complex with Apoptotic/dead multiple myeloma cells was identified as CD138$^+$CFSE$^+$PI$^+$ by multiparameter flow-cytometric analysis using BD-LSR Fortessa (Becton Dickinson Biosciences), and data were analyzed using FlowJo software (TreeStar). Minimum of 10,000 live events per sample were acquired using multiparameter flow cytometry.

Statistical analysis

All *in vitro* experiments were performed in triplicate and repeated at least three times. Statistical significance was determined by nonparametric *t* test, two-tailed distribution, with minimal significance level *, $P < 0.05$. Statistical analysis was performed using GraphPad Prism (v6) software.

Results

Increased PD-L1 gene expression in multiple myeloma

We first assessed expression and frequency of PD-L1 gene in the BM CD138$^+$ multiple myeloma cells from patients with multiple myeloma at diagnosis [ND-MM; Intergroupe Francophone du Myelome (IFM), $n = 170$] versus normal BM plasma cells (HD, $n = 6$; Supplementary Fig. S1A). Exon array profiling analysis of CD138$^+$ plasma cells demonstrated that there was a significant increase in PD-L1 mRNA expression in multiple myeloma cells from patients with ND-MM compared with HD ($P = 0.0064$). Furthermore, 45% patients had increased copy number of PD-L1 gene in their tumor clone (Supplementary Fig. S1B). Expression and copy number of PD-L1 gene were significantly correlated in clonal multiple myeloma cells (Pearson correlation, $R^2 = 0.37$ and $P = 5.5e\text{-}07$; Supplementary Fig. S1C). Because PD-L1 gene is encoded on chromosome 9, we next compared PD-L1 gene expression among normal, hyperdiploid multiple myeloma (HMM) and non-hyperdiploid multiple myeloma (NHMM) groups. PD-L1 gene expression was significantly upregulated in NHMM ($P = 0.03$), and even more highly expressed in HMM ($P = 0.0007$), compared with normal plasma cells. In addition, there was a significant difference between HMM and NHMM subgroups ($P = 1.75e\text{-}09$; Supplementary Fig. S1D). In contrast, there was no significant association between PD-L1 expression and chromosomal abnormalities in multiple myeloma, including *t*(4;14), del (17p), del (1q), *t*(11;14), del(13), and *t*(14;16; data not shown).

Increased frequency of PD-1 and PD-L1 surface expression in multiple myeloma BM microenvironment

We next assessed surface expression of PD-L1 by multiparameter flow cytometry in BM CD138$^+$ multiple myeloma cells from patients with ND-MM ($n = 6$), RR-MM ($n = 10$), and normal plasma cells ($n = 3$); as well as in a panel of multiple myeloma and PCL cell lines (MM1.S, OPM1, OPM2, U266, and H929; Fig. 1). PD-L1 surface expression was significantly increased in multiple myeloma cells from patients with ND-MM (mean = 16.5 ± 6.5) and RR-MM (mean = 26.6 ± 8.2) compared with normal plasma cells (mean = 4.7 ± 1.8; $P < 0.05$; Fig. 1A, left and bottom right), and was detectable only in MM1.S, OPM2, and H929 multiple myeloma cell lines (Fig. 1A, top right). There was no significant expression of PD-L2 on multiple myeloma cells (data not shown).

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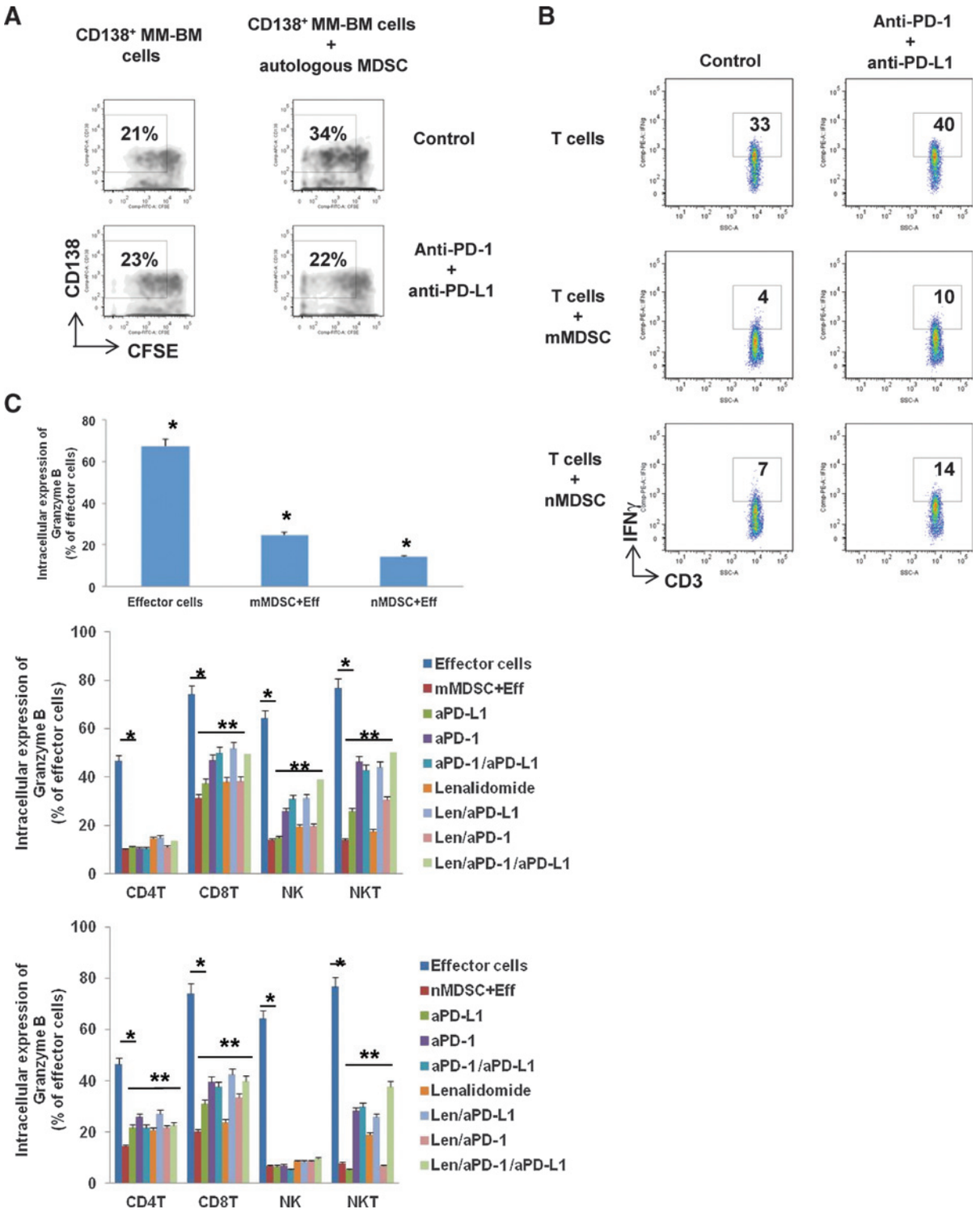

Figure 4.

Checkpoint blockade partially reverses MDSC-mediated multiple myeloma growth and immune suppression in multiple myeloma BM. A, impact of checkpoint blockade on MDSC-mediated tumor growth is demonstrated in the BM of patients with RR-MM by CFSE-flow cytometric analysis. CFSE-labeled $CD138^+$ multiple myeloma cells and autologous MDSC were cultured in the absence or presence of anti–PD-1 and anti–PD-L1, alone or in combination. Viability/growth of $CD138^+$ multiple myeloma cells ($CD138^+CFSE^{low}$) is shown by representative histogram plots. B, effect of checkpoint blockade on MDSC-mediated immune suppression is shown by intracellular effector cytokine analysis in RR-MM BM. Autologous T cells cultured either alone or with mMDSC and nMDSC in the absence or presence of anti–PD-1 and anti–PD-L1, alone or in combination. (*Continued on the following page.*)

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We next evaluated surface expression of PD-1 in immune effector cells in both BM and peripheral blood of patients with active multiple myeloma using multiparameter flow cytometry (Fig. 1B). Even though CD4T cells from several patients with ND-MM and RR-MM expressed high levels of PD-1, there was no significant difference in PD-1 expression on CD4T cells in multiple myeloma BM, either ND-MM (mean = 10.8 ± 3.4) or RR-MM (mean = 12 ± 2.8), compared with normal CD4T cells (mean = 10.2 ± 1.3; Fig. 1B, top left). In contrast, there was a significant increase in expression of PD-1 on CD8T cells in ND-MM (mean = 10.4 ± 1.7) and RR-MM (mean = 9.4 ± 2.3) compared with normal CD8T cells (mean = 6.1 ± 0.6; Fig. 1B, top right). Likewise, PD-1 expression was higher in BM NK cells in patients with ND-MM (mean = 7.9 ± 1.4) and RR-MM (mean = 14.2 ± 3.2) compared with normal NK cells (mean = 1.6 ± 0.2; Fig. 1B, bottom left). In contrast, PD-1 expression was significantly lower in BM $CD8^+CD56^+$NKT cells of ND-MM (mean = 14.3 ± 2.6) and RR-MM (mean = 14.1 ± 2.2) than normal NKT cells (mean = 24.7 ± 1.1; Fig. 1B, bottom right). Increased expression of PD-1 is demonstrated by histogram of PD-1 coexpressing effector cells (CD8T cells and NK) in RR-MM (Fig. 1B, right). Similar changes in PD-1 expression were observed in effector cells in the PBMC of ND-MM and RR-MM (data not shown).

We have recently characterized the neutrophil-like $CD11b^+CD14^-HLA\text{-}DR^{low/-}CD33^+CD15^+$ MDSC population with tumor-promoting and immune-suppressive activity in the multiple myeloma BM (12). To determine whether MDSC-induced multiple myeloma cell growth and immune effector cell suppression are mediated by the PD-1/PD-L1 pathway, we next assessed cell surface expression and frequency of PD-L1 in the BM MDSC of patients with ND-MM and RR-MM. Because MDSCs are either absent or present at a very low numbers in healthy donors, we first evaluated the frequency of PD-L1 expressing within myeloid cell subpopulations in ND-MM and RR-MM (Fig. 1C). Myeloid cell subpopulations in each multiple myeloma patient BM were phenotypically characterized as $CD11b^+CD14^+HLA\text{-}DR^+$ APCs, $CD11b^+CD14^+HLA\text{-}DR^{low/-}$ monocytic myeloid-derived suppressor cells (mMDSC), and $CD11b^+CD14^-HLA\text{-}DR^{low/-}CD33^+CD15^+$ neutrophilic myeloid-derived suppressor cells (nMDSC). Multiparameter flow-cytometric analysis showed that PD-L1 expression was increased in nMDSC (mean = 37.8 ± 18) compared with APCs (mean = 21.5 ± 7.9) in the BM of patients with ND-MM (Fig. 1C, left). Of note, there was a more significant increase in PD-L1 in mMDSC (mean = 33.9 ± 10) and nMDSC (mean = 40.1 ± 11.5) compared with APC (mean = 21.8 ± 6) in the BM of patients with RR-MM. PD-L1–expressing mMDSC increased in RR-MM (mean = 33.9 ± 10) versus ND-MM (mean = 22 ± 9.4), but there was no significant change in PD-L1–expressing nMDSC with disease progression (Fig. 1C, right). Shown is a representative histogram of PD-L1 expression in mMDSC and nMDSC of RR-MM and HD (Fig. 1C, right panel). Thus, PD-L1 in multiple myeloma cells and MDSC, along with PD-1 in immune effector (CD8T cells and NK) cells, were increased in BM of ND-MM and RR-MM.

Checkpoint blockade overcomes BM stroma-mediated multiple myeloma growth

We next investigated whether PD-1/PD-L1 signaling plays a role in BMSC-mediated multiple myeloma cell growth in cocultures of multiple myeloma cells and BMSC from patients with RR-MM (Fig. 2). BMSCs were generated from multiple myeloma BM and cultured either with multiple myeloma cell lines or autologous $CD138^+$ multiple myeloma cells with or without anti–PD-1 and anti–PD-L1, alone or in combination. Multiple myeloma cell viability/growth was measured by CFSE-flow cytometry analysis or 3H-thymidine cell proliferation assays. We first determined whether BMSC affects expression of PD-L1 on multiple myeloma cells. BMSC significantly induced PD-L1 expression on H929 multiple myeloma cells (>2-fold increase), as shown by multiparameter flow-cytometric analysis (Fig. 2A). The regulatory role of PD-1/PD-L1 in BMSC-mediated multiple myeloma growth is further evidenced in the cocultures of CFSE-labeled $CD138^+$ multiple myeloma cells from RR-MM with BMSC, with or without single and dual PD-1/PD-L1 signaling blockade. BMSC significantly increased $CD138^+CFSE^{low}$ multiple myeloma cell viability/growth ($CD138^+CFSE^{low}$ cells:80% of $CD138^+$ cells in BM) compared with multiple myeloma cells alone ($CD138^+CFSE^{low}$ cells:2% of $CD138^+$ cells in BM); and blockade of PD-L1 ($CD138^+CFSE^{low}$ cells:69% of $CD138^+$ cells in BM), PD-1 ($CD138^+CFSE^{low}$ cells:77% of $CD138^+$ cells in BM), or the combination ($CD138^+CFSE^{low}$ cells:62% of $CD138^+$ cells in BM) overcame BMSC-mediated multiple myeloma cell growth (Fig. 2B). Therefore, the PD-1/PD-L1 pathway may play an important role in stroma-mediated tumor growth, independent of immune effector cell function.

Checkpoint blockade enhances immune effector cell–mediated anti–multiple myeloma response in multiple myeloma BM

We next investigated the immune-suppressive role of PD-1/PD-L1 signaling in the multiple myeloma microenvironment, and determined whether blockade of PD-1/PD-L1 signaling can reverse tumor-induced immune suppression in the multiple myeloma BM microenvironment (Fig. 3). Immune effector cell–mediated multiple myeloma cytotoxicity was measured by CFSE/PI apoptotic/dead cell detection assays in cocultures of effector cells (autologous T cells and NK cells) and target cells ($CD138^+$multiple myeloma cells) from RR-MM. $CD3^+$T cells, $CD56^+$NK cells, and $CD138^+$ multiple myeloma cells were isolated by FACS-sorting from BM of patients with RR-MM. $CD138^+$ target multiple myeloma cells were then labeled with CFSE and cocultured for 4 hours with each autologous effector cell population in the absence or presence of anti–PD-1 and anti–PD-L1, alone or in combination. Apoptotic/dead $CD138^+$ multiple myeloma cells were characterized as $CD138^+CFSE^+PI^+$ multiple myeloma cells using CFSE/PI-flow cytometry analysis. Blockade of PD-1 and PD-L1, alone and more significantly in combination, induced effector cell–mediated multiple myeloma cytotoxicity. Within the effector cell populations, NK cells (4-fold, $P < 0.05$) demonstrated more pronounced anti–multiple myeloma cytotoxicity than T cells

(*Continued.*) Gated boxes demonstrate percent intracellular IFNγ expression in T cells cultured alone (top), with mMDSC (middle), and with nMDSC (bottom) with or without dual PD-1 and PD-L1 blockade. C, impact of checkpoint blockade with lenalidomide on MDSC-mediated immune suppression is shown in RR-MM BM by flow-cytometric intracellular cytokine analysis. A representative bar graph shows percent intracellular Gzm-B expression in all effector cells cultured alone or with autologous mMDSC and nMDSC (top). A representative bar graph of intracellular Gzm-B expression is shown in each effector cell population from the coculture of effector cells with autologous mMDSC (middle) and with autologous nMDSC (bottom). *, $P < 0.05$; **, $P < 0.05$.

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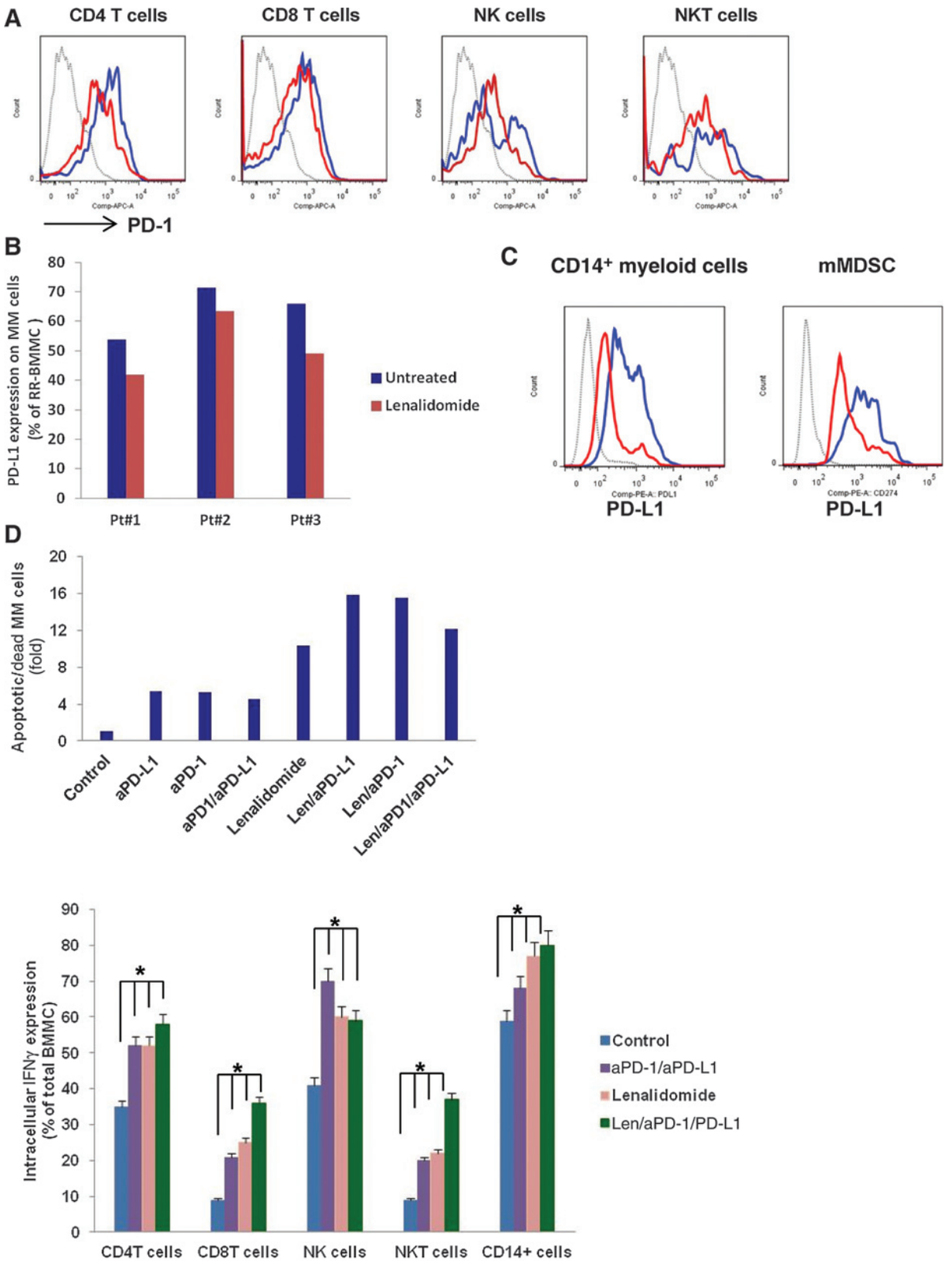

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(2-fold increase, $P < 0.05$) with blockade of PD-1 (2-fold, $P < 0.05$) and PD-L1 (3-fold, $P < 0.05$) alone, or in combination (4-fold, $P < 0.05$; Fig. 3A).

To define the impact of checkpoint blockade on effector cytokine pattern mediating cytotoxicity, we next evaluated intracellular production of effector cytokines (IFNγ and Gzm-B) in CD4T cells, CD8T cells, and NK cells cocultured with autologous $CD138^+$ multiple myeloma cells from RR-MM BM (Fig. 3B and C). Intracellular cytokine analysis by multiparameter flow cytometry demonstrated that checkpoint blockade induced higher IFNγ production in NK cells (anti–PD-L1:1.3-fold, anti–PD-1: 1.7-fold, anti–PD-1/PD-L1: 2.2-fold) than T cells during anti–multiple myeloma cytotoxicity (Fig. 3B), along with increased production of Gzm-B (Fig. 3C). Even though checkpoint blockade modulates effector cytokine production, there was no associated change in effector cell proliferation (data not shown), indicating that checkpoint molecules regulate effector cell function. Of note, checkpoint blockade-induced cell activation varies in each effector cell subpopulation, indicating a differential antitumor response in patients.

Checkpoint blockade partially reverses MDSC-mediated multiple myeloma growth and immune suppression in multiple myeloma BM

Because both mMDSC and nMDSC express PD-L1 in RR-MM BM, we next assessed whether blockade of PD-1/PD-L1 signaling can reverse MDSC-mediated tumor growth and immune suppression in multiple myeloma BM (Fig. 4). The impact of checkpoint blockade on MDSC-mediated multiple myeloma growth was analyzed in autologous cocultures of $CD138^+$ multiple myeloma cells and mMDSC and nMDSC from RR-MM BM. $CD138^+$ multiple myeloma cells, $CD11b^+CD14^+HLA\text{-}DR^{low/neg}$ mMDSC, and $CD11b^+CD14^-HLA\text{-}DR^{low/neg}CD33^+CD15^+$ nMDSCs were isolated from RR-MM BM by FACS-sorting. CFSE-labeled $CD138^+$ multiple myeloma cells were cultured for 2 days with autologous mMDSC or nMDSC with or without anti–PD-1 and anti–PD-L1, alone or in combination. $CD138^+CFSE^{low}$ multiple myeloma cells were assessed by CFSE-flow analysis. As shown in Fig. 4A, mMDSC induced $CD138^+$ multiple myeloma cell growth (21% to 34% $CD138^+CFSE^{low}$ cells of $CD138^+$multiple myeloma cells); importantly, dual blockade of PD-1 and PD-L1 abrogated MDSC-mediated multiple myeloma cell growth in RR-MM BM (34%–22% $CD138^+CFSE^{low}$ cells of $CD138^+$ multiple myeloma cells). In contrast, there was no significant change in MDSC-mediated multiple myeloma growth by either PD1 or PD-L1 single blockade (data not shown).

The impact of checkpoint blockade on MDSC-mediated immune suppression was next assessed in RR-MM BM. mMDSC, nMDSC, and autologous effector cells were isolated by FACS-sorting from RR-MM BM. MDSC and autologous effector cells (T cells, NK cells, and NKT cells) were cultured with or without anti–PD-1 and anti–PD-L1, alone or in combination, and intracellular production of cytokines IFNγ and Gzm-B was determined in effector cells (Fig. 4B and C). As shown by representative multiparameter dot plots of intracellular IFNγ expression in gated CD3T cells, both mMDSC and nMDSC significantly suppressed IFNγ production in T cells, and only combined blockade of PD-1 and PD-L1 overcame this suppression (Fig. 4B).

We next tested whether targeting PD-1/PD-L1 inhibitory signaling using checkpoint blockade antibodies while inducing immune effector cell activity with lenalidomide can reverse MDSC-mediated immune-suppression in RR-MM BM (Fig. 4C). Intracellular cytokine analysis in effector cells cultured with either autologous mMDSC or nMDSC demonstrated that both mMDSC and nMDSC induced suppression of intracellular Gzm-B production in all effector cells (Fig. 4C, top). Specifically, checkpoint blockade in cocultures of mMDSC and autologous effector cells induced Gzm-B production in CD8T cells, NK, and NKT cells; and the combination of lenalidomide with checkpoint blockade further enhanced Gzm-B production, particularly in CD8T cells, NK, and NKT cells (Fig. 4C, middle). Similarly, checkpoint blockade in cocultures of nMDSC with autologous effector cells from RR-MM BM significantly increased intracellular Gzm-B production in T cells and NKT cells, but not NK cells (Fig. 4C, bottom). Of note, checkpoint blockade, either alone or with lenalidomide, was not able to enhance effector cell proliferation in the presence of MDSC (data not shown).

Lenalidomide reduces expression of PD-1 and PD-L1 in BM cells and enhances checkpoint blockade-induced multiple myeloma cytotoxicity

The impact of lenalidomide on surface expression of PD-1 and PD-L1 in multiple myeloma cells and BM accessory cells was next defined in RR-MM BM. BMMCs from patients with RR-MM were cultured with lenalidomide (1 μmol/L); and cell surface expression of PD-1 in effector cells (CD4T cells, CD8T cells, NK cells, and NKT cells), as well as surface expression of PD-L1 in $CD138^+$multiple myeloma cells, MDSC, and $CD14^+$ monocytes/macrophages, was then determined by multiparameter flow cytometry analysis (Fig. 5). Lenalidomide significantly reduced PD-1 surface expression on CD4T cells, CD8T cells, and NK cells in RR-MM BM (Fig. 5A). Lenalidomide modestly decreased surface expression of PD-L1 on $CD138^+$ multiple myeloma cells (Fig. 5B), and more significantly downregulated PD-L1 expression on monocytes/macrophages and mMDSC in the BM from RR-MM (Fig. 5C).

Because lenalidomide downregulates surface expression of checkpoint molecules in multiple myeloma cells and accessory cells in multiple myeloma BM, we next investigated anti–multiple myeloma cytotoxic activity of lenalidomide in

Figure 5.

Lenalidomide reduces expression of PD-1 and PD-L1 and enhances checkpoint blockade-induced multiple myeloma cytotoxicity in multiple myeloma BM. Impact of lenalidomide on cell surface expression of PD-1 on effector cells (A) and PD-L1 on $CD138^+$ multiple myeloma cells (B), as well as $CD14^+$ myeloid cells and MDSCs (C) in RR-MM BM is shown by representative histogram plots. The percentage of PD-1 expression on the effector cells and PD-L1 on the $CD14^+$ myeloid cells and MDSCs of untreated BM cells (blue) and lenalidomide-treated BM cells (red) is shown relative to control (gray). D, impact of lenalidomide on checkpoint blockade-induced multiple myeloma cytotoxicity is shown in a representative graph of RR-MM BM. Mononuclear cells from patient with RR-MM BM were labeled with CFSE and cultured in the absence or presence of anti–PD-1 and anti–PD-L1, alone or in combination, and with or without addition of lenalidomide. Shown is a percentage of apoptotic/dead ($CD138^+CFSE^+PI^+$) multiple myeloma cells in BMMC (top). Impact of lenalidomide on checkpoint blockade-induced effector cell activity is shown in RR-MM BM by intracellular effector cytokine analysis. RR-MM BM cells were cultured with anti–PD-1 and anti–PD-L1 alone, or in combination, or with the addition of lenalidomide. Representative bar graph shows intracellular expression of IFNγ in each effector cell population in RR-MM BM (bottom). *, $P < 0.05$.

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combination with checkpoint blockade in RR-MM (Fig. 5D). To mimic the BM microenvironment, all BM cells were labeled with CFSE and cultured with anti–PD-1 anti–PD-L1, alone or together, and with lenalidomide. Apoptotic/dead $CD138^+$ $CFSE^+PI^+$ multiple myeloma cells were identified by multiparameter CFSE/PI-flow cytometry analysis. There was a significant increase in $CFSE^+PI^+$ apoptotic/dead $CD138^+$ multiple myeloma cells in BM cultured with anti–PD-1 and anti–PD-L1; and lenalidomide further enhanced checkpoint blockade-mediated multiple myeloma cytotoxicity in RR-MM BM (Fig. 5D, top). We further analyzed the effect of checkpoint blockade, alone or with lenalidomide, on cytokine production in BM effector cells. BM cells were cultured with anti–PD-1, anti–PD-L1, or anti–PD-1/PD-L1, alone or with lenalidomide. Intracellular expression of effector cytokines (IFNγ and Gzm-B) was then measured in CD4T cells, CD8T cells, NK cells, NKT cells, and monocytes/macrophages by multiparameter flow-cytometric analysis (Fig. 5D, bottom). Intracellular cytokine analysis of effector cells in RR-MM BM demonstrated that dual blockade of PD-1 and PD-L1 significantly induced IFNγ production in all effector cells (Fig. 5D, bottom); as well as Gzm-B production in NK cells and NKT cells (data not shown). Importantly, lenalidomide further enhanced dual checkpoint blockade-induced IFNγ production in all effector cells (Fig. 5D, bottom).

Discussion

The multiple myeloma microenvironment is transformed in the presence of tumor cells to promote multiple myeloma development/growth while allowing tumor escape from immune surveillance due to suppression of anti–multiple myeloma immune effector responses. As a result, infections remain a major cause of death (28). Recent studies have defined immune checkpoint receptor PD-1/PD-L1 signaling as a key pathway regulating the critical balance between immune activation and tolerance (23, 28–31). Binding of PD-1 on effector cells to PD-L1 or PD-L2 on non-hematopoietic cells triggers inhibitory signaling in effector cells, leading to induction and maintenance of tolerance (32). Recent studies in solid tumors have demonstrated that PD-1/PD-L1 signaling allows for escape from immune surveillance, transforming the tumor microenvironment into a tumor-protective, immune-suppressive milieu (7, 8, 16, 18, 33–36). Specifically, PD-L1 expression on tumor cells inhibits T-cell activation and CTL-mediated tumor lysis. Importantly, recent studies have shown increased expression of PD-L1 on lung, skin, renal, gastric, pancreatic, colorectal, breast, and ovarian cancers (8, 15, 16, 20, 35, 37–40). Moreover, blockade of PD-1/PD-L1 signaling using clinically relevant anti–PD-1 monoclonal antibodies restored immune responses and achieved remarkable clinical responses in solid tumors, including melanoma and lung cancer, providing a very promising novel immunotherapeutic strategy.

Studies in hematologic malignancies have shown increased expression of PD-L1 in B-cell lymphomas, chronic lymphocytic leukemia, acute myeloid leukemia, and multiple myeloma (24, 25, 33, 41–45). In the present study, we investigated the role of PD-1/PD-L1 inhibitory signaling in the bidirectional interaction between tumor, stroma, and immune accessory cells in the multiple myeloma BM microenvironment. Importantly, we assessed the impact of single and dual blockade of PD-1/PD-L1 signaling, alone or in combination with lenalidomide, on the tumor-promoting, immune-suppressive multiple myeloma microenvironment. Previous studies have demonstrated that PD-L1 is not expressed on normal plasma cells, but is expressed on multiple myeloma cell lines and primary multiple myeloma cells (25–27). Here, we showed that both mRNA and cell surface expression of PD-L1 is increased on $CD138^+$ multiple myeloma cells from ND-MM and further elevated in RR-MM, compared with normal BM plasma cells. However, within a broad panel of multiple myeloma cell lines, constitutive PD-L1 expression is limited to MM1.S, OPM2, and H929 cells, suggesting that PD-L1 expression is induced on multiple myeloma cells by the bidirectional interaction between tumor and accessory cells. PD-L1 gene is encoded on chromosome 9, which is increased in copy number in HMM. Importantly, patients with HMM have a better prognosis and outcome than patients with NHMM. Analysis of PD-L1 gene expression in tumor cells from patients with ND-MM demonstrated that the expression of PD-L1 gene was significantly correlated with the copy number in most patients' tumor clones. Although expression of PD-L1 gene was significantly increased in NHMM relative to normal donor plasma cells, it was even higher in HMM. Enhancing anti–multiple myeloma immune response by targeting checkpoint molecules may therefore improve outcome even in HMM.

PD-1 expression is increased on CD4T cells from patients, and returns to levels in normal CD4T cells following autologous transplant (25). Benson and colleagues (22). have shown that PD-1 is expressed on NK cells from multiple myeloma patients, but not normal NK cells; that blockade of PD-1 signaling by anti–PD-1 antibody induces cytolytic activity of NK cells; and that lenalidomide further induces NK-mediated antitumor responses. Here, we extended these studies to determine the impact not only of PD-1 blockade but also of dual blockade of PD-1 and PD-L1, alone or with lenalidomide, on the functional sequelae in multiple myeloma cells, stroma, immune effector cells (CD4T cells, CD8T cells, NK cells, NKT cells, and monocytes/macrophages) and immune suppressor MDSCs in the multiple myeloma BM.

We first determined that PD-1 expression is significantly increased on effector immune cells, particularly on CD8T cells and NK cells, whereas PD-L1 is expressed by myeloid effector cells monocytes/macrophages. Previous studies have shown that MDSCs express high levels of B7 in murine models of solid tumor (46–48). Moreover, It has been recently demonstrated that PD-1 and PD-L1 are expressed at low levels in MDSC of patients with multiple myeloma (49). Here, we compared PD-L1 expression on myeloid cell subpopulations, including APCs, mMDSC, and nMDSC in the BM of patients with multiple myeloma. PD-L1 expression is significantly higher in MDSC in RR-MM than ND-MM. Increased expression of PD-1 on immune effector cells, and increased PD-L1 on both multiple myeloma cells and immune suppressor MDSC, indicate that PD-1/PD-L1 inhibitory signaling plays an important role in providing a tumor-promoting, immune-suppressive microenvironment in multiple myeloma BM.

Extensive studies focusing on the interaction of BMSC with multiple myeloma cells have demonstrated that BMSCs promote multiple myeloma cell growth and drug resistance. Tamura and colleagues (26) have demonstrated that BMSC also upregulates PD-L1 expression on multiple myeloma cells. To delineate whether PD-1/PD-L1 plays a role in BMSC-mediated multiple myeloma growth, we assessed the impact of

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single and dual blockade of PD-1/PD-L1 signaling in cocultures of tumor cells from patients with RR-MM and BMSC. Importantly, BMSC markedly induced PD-L1 expression in multiple myeloma cells, and BMSC-mediated multiple myeloma cell growth was abrogated by blockade of PD-1 and PD-L1, suggesting that checkpoint blockade may have a direct effect on BMSC-induced multiple myeloma growth, independent of its immune accessory cell activity.

Immunomodulatory drug lenalidomide not only targets the multiple myeloma cell directly, but also induces anti–multiple myeloma activity of immune effector cells. We have recently shown that lenalidomide does not alter MDSC-mediated tumor growth and immune suppression in multiple myeloma (14). However, lenalidomide reduces PD-1 expression on NK cells and PD-L1 expression on tumor cells from patients with multiple myeloma (22). Here, we found that lenalidomide decreased PD-1 expression in all effector cells (CD4T cells, CD8T cells, NK cells, and NKT cells), as well as PD-L1 expression in multiple myeloma cells, MDSC, and monocytes/macrophages. We characterized the immunomodulatory effects of PD-1/PD-L1 blockade with lenalidomide in autologous cocultures of immune effector cells with multiple myeloma cells from patients with RR-MM. Even though there was no change in effector cell proliferation, PD-1/PD-L1 blockade significantly induced cytotoxic activity of autologous T cells, NK cells, and monocytes/macrophages against multiple myeloma cells; and lenalidomide further enhanced effector cell–mediated cytotoxicity. PD-1/PD-L1 blockade also induced intracellular expression of cytotoxic cytokines IFNγ and Gzm-B in CD4T cells, CD8T cells, NK cells, and monocytes/macrophages in RR-MM. Furthermore, MDSC-mediated multiple myeloma cell growth was significantly decreased by PD-1/PD-L1 blockade. Finally, PD-1/PD-L1 blockade induced intracellular expression of IFNγ and Gzm-B in T cells, NK cells, and NKT cells cultured with autologous MDSC; and lenalidomide further enhanced this effector cell activation. Of note, checkpoint blockade induced response in each effector cell population regardless of PD-1 expression level.

Our data therefore demonstrate that immune checkpoint signaling plays an important role conferring the tumor-promoting, immune-suppressive microenvironment in multiple myeloma BM. Importantly, blockade of PD-1 or PD-L1, alone and in combination, induces anti–multiple myeloma immune responses, which can be further enhanced by lenalidomide. Targeting checkpoint signaling using PD-1 and PD-L1–blocking antibodies, particularly in combination with lenalidomide, therefore represents a promising novel immune-based therapeutic strategy to inhibit tumor cell growth, restore host immune function in multiple myeloma, and improve patient outcome in multiple myeloma.

Disclosure of Potential Conflicts of Interest

J.P. Laubach reports receiving commercial research grants from Celgene, Millennium, Novartis, and Onyx. N.C. Munshi, P.G. Richardson, and K.C. Anderson are consultants/advisory board members for Celgene. No potential conflicts of interest were disclosed by the other authors.

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