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PD-1 Blockade Reinvigorates Bone Marrow CD8$^+$ T Cells from Patients with Multiple Myeloma in the Presence of TGFβ Inhibitors

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ABSTRACT

Purpose: Immune-checkpoint inhibitors have shown therapeutic efficacy in various malignant diseases. However, anti-programmed death (PD)-1 therapy has not shown clinical efficacy in multiple myeloma.

Experimental Design: Bone marrow (BM) mononuclear cells were obtained from 77 newly diagnosed multiple myeloma patients. We examined the expression of immune-checkpoint receptors in BM CD8$^+$ T cells and their functional restoration by *ex vivo* treatment with anti–PD-1 and TGFβ inhibitors.

Results: We confirmed the upregulation of PD-1 and PD-L1 expression in CD8$^+$ T cells and myeloma cells, respectively, from the BM of multiple myeloma patients. PD-1–expressing CD8$^+$ T cells from the BM of multiple myeloma patients coexpressed other checkpoint inhibitory receptors and exhibited a terminally differentiated phenotype. These results were also observed in BM CD8$^+$ T cells specific to myeloma antigens NY-ESO-1 and HM1.24. BM CD8$^+$ T cells from multiple myeloma patients exhibited reduced proliferation and cytokine production upon T-cell receptor stimulation. However, anti–PD-1 did not increase the proliferation of BM CD8$^+$ T cells from multiple myeloma patients, indicating that T-cell exhaustion in multiple myeloma is hardly reversed by PD-1 blockade alone. Intriguingly, anti–PD-1 significantly increased the proliferation of BM CD8$^+$ T cells from multiple myeloma patients in the presence of inhibitors of TGFβ, which was overexpressed by myeloma cells.

Conclusions: Our findings indicate that combined blockade of PD-1 and TGFβ may be useful for the treatment of multiple myeloma.

Introduction

Multiple myeloma is a plasma cell malignancy and the second most common hematologic cancer (1). Although the introduction of novel therapeutic agents, such as immunomodulatory drugs (IMiD) and proteasome inhibitors, and increased use of autologous stem cell transplantation have improved survival in patients with multiple myeloma, most patients relapse after remission or are refractory to treatment (2, 3). Multiple myeloma still remains an incurable disease, and alternative therapeutic options are necessary for multiple myeloma patients.

Immune-checkpoint inhibitors (ICI), including blocking antibodies against programmed death (PD)-1 or PD-ligand 1 (PD-L1), become paradigm-shifting treatment in solid cancers. It is remarkable that, once patients achieve a clinical response with ICIs, they tend to show long-term durable disease control (4). Therefore, ICIs are attractive therapeutic options for multiple myeloma patients suffering from frequent relapses.

The rationale for targeting PD-1 and PD-L1 in multiple myeloma therapy was investigated previously. PD-L1 is expressed on malignant plasma cells from multiple myeloma patients, but not on aberrant plasma cells from patients with monoclonal gammopathy of undetermined significance (MGUS) or normal plasma cells (5, 6). PD-1 expression is also increased in CD8$^+$ and CD4$^+$ T cells from multiple myeloma patients compared with those from healthy donors (7–9). Moreover, blocking PD-1 and PD-L1 interaction restored antitumor effector functions and the cytotoxicity of T cells from multiple myeloma patients in *ex vivo* assays (10).

However, a phase I clinical trial evaluating anti–PD-1 monotherapy in patients with relapsed or refractory hematologic malignancies revealed no objective response in multiple myeloma patients (11). A recent study tried to explain the low response of anti–PD-1 monotherapy in multiple myeloma patients by showing that expanded T-cell clones in multiple myeloma patients express low levels of PD-1 and represent senescent phenotypes rather than exhaustion (12). To overcome the limitation of anti–PD-1 monotherapy in multiple

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Translational Relevance

Anti–PD-1 or anti–PD-L1 blocking antibodies are used for the treatment of various malignancies. However, clinical trials of anti–PD-1 have not shown therapeutic efficacy in patients with multiple myeloma. In the present study, we examined the expression of immune-checkpoint inhibitory receptors and the differentiation status of bone marrow–infiltrating $CD8^+$ T cells from patients with multiple myeloma. In addition, we attempted to reinvigorate bone marrow $CD8^+$ T cells by *ex vivo* treatment with anti–PD-1 blocking antibodies. Anti–PD-1 treatment restored the proliferation and effector cytokine production of bone marrow $CD8^+$ T cells in the presence of agents blocking the effect of TGFβ, which was overexpressed by myeloma cells. This study provides a rationale for combined blockade of PD-1 and TGFβ for the treatment of multiple myeloma.

myeloma, the phenotype and differentiation of $CD8^+$ T cells need to be characterized in the bone marrow (BM) of multiple myeloma patients, particularly by analyzing myeloma antigen–specific $CD8^+$ T cells. In addition, the role of immunosuppressive factors abundant in the BM of multiple myeloma patients needs to be considered, including transforming growth factor-β (TGFβ). Recent studies reported that TGFβ attenuated anti–PD-L1-induced antitumor responses in patients with metastatic urothelial cancer (13), and coinhibition of TGFβ and PD-1 triggered robust tumor regression in mouse models (13, 14).

In the present study, we investigated the expression of immune-checkpoint inhibitory receptors, including PD-1, T-cell immunoglobulin and mucin-domain containing-3 (Tim-3), lymphocyte activation gene-3 (Lag-3), and T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT), and T-cell transcription factors, including Eomesodermin (Eomes) and T-box transcription factor TBX21 (T-bet), in BM $CD8^+$ T cells from multiple myeloma patients. Moreover, we examined $CD8^+$ T cells specific to myeloma antigens, such as NY-ESO-1 and HM1.24, using major histocompatibility complex class I (MHC-I) multimers. Importantly, we evaluated the proliferation of BM $CD8^+$ T cells from multiple myeloma patients following PD-1 blockade in direct *ex vivo* assays. We found that coblockade of PD-1 and TGFβ restores the function of BM $CD8^+$ T cells, whereas PD-1 blockade alone does not.

Materials and Methods

Patients and specimens

BM aspirates and paired peripheral blood (PB) samples were collected from 77 newly diagnosed multiple myeloma patients (Supplementary Table S1) at Chungnam National University Hospital (Daejeon, Republic of Korea). BM mononuclear cells (BMMCs) and PB mononuclear cells (PBMCs) were isolated by Ficoll (GE Healthcare: 17-5442-02) density-gradient centrifugation and cryopreserved as previously described (15). BM aspirates were also collected from patients with extranodal marginal zone B-cell lymphoma (EMZL; $n = 18$; Supplementary Table S2), MGUS ($n = 10$), and smoldering multiple myeloma (SMM; $n = 7$; Supplementary Table S3). All EMZL patients were confirmed as having no BM involvement and were treated only with local treatment. To compare multiple myeloma with other BM-involving B-cell lymphomas, we also obtained BM aspirates from treatment-naïve diffuse large B-cell lymphoma (DLBCL) and Hodgkin lymphoma (HL) patients with BM involvement ($n = 10$ and $n = 2$, respectively; Supplementary Table S4). This study was approved by the institutional review boards of Chungnam National University Hospital and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Flow cytometry and immunophenotyping

Cryopreserved PBMCs and BMMCs were thawed and stained using the Live/Dead fixable cell stain kit (Invitrogen: L34975 or L34971) to exclude dead cells from the analysis. After washing with FACS staining buffer, these cells were stained with fluorochrome-conjugated antibodies for 20 minutes at room temperature. For intracellular staining, surface-stained cells were fixed and permeabilized using a Foxp3 staining buffer kit (eBioscience: 00-5523-00) according to the manufacturer's instructions. Multicolor flow cytometry was performed using an LSR II flow cytometer (BD Biosciences), and the data were analyzed by FlowJo V10 software (Treestar). Gating strategies for BM $CD8^+$ T cells (Supplementary Fig. S1), malignant and normal plasma cells (Supplementary Fig. S2), and various immune cell subsets (Supplementary Fig. S3) were provided.

Cell lines

IM-9 myeloma cells were purchased from the Korean Cell Line Bank and grown in RPMI-1640 (Welgene: LM 011-01) supplemented with 20% FBS (Sigma-Aldrich: 12003C) and 100 U/mL penicillin/streptomycin (Welgene: LS202-02). IM-9 cells express HLA-A2 (Supplementary Fig. S4A) and PD-L1 (Supplementary Fig. S4B). NY-ESO-$1_{157\text{-}165}$–specific $CD8^+$ T cells were established as described previously (16). Briefly, NY-ESO-$1_{157\text{-}165}$–specific $CD8^+$ T cells from $HLA\text{-}A2^+$ donors were expanded in RPMI-1640 media containing anti-CD3, IL2, IL7, IL15, and 10% FBS for 6 weeks using irradiated autologous PBMCs as feeder cells. Purity of NY-ESO-$1_{157\text{-}165}$–specific $CD8^+$ T cells was >95% (Supplementary Fig. S5A). When NY-ESO-$1_{157\text{-}165}$–specific $CD8^+$ T cells were cocultured with NY-ESO-$1_{157\text{-}165}$ peptide-pulsed IM-9 cells for 3 days, robust induction of PD-1 on $CD8^+$ T cells was observed (Supplementary Fig. S5B). Authentication of cell lines was not performed.

MHC-I multimers

To detect tumor antigen–specific $CD8^+$ T cells, we used phycoerythrin (PE)-conjugated HLA-A*0201 dextramers, including NY-ESO-$1_{157\text{-}165}$ (SLLMWITQC) and HM1.$24_{22\text{-}30}$ (LLLGIGILV) dextramers (Immudex: WB2696 and custom ordered). A dextramer stain protocol was applied to detect tumor antigen–specific $CD8^+$ T cells (17). Briefly, BMMCs were incubated with 50 nmol/L dasatinib (Axon Medchem: 1392) in 1% FBS-PBS for 30 minutes at 37°C, and then stained with dextramer without washing for 15 minutes to prevent T-cell receptor downregulation following MHC-I multimer binding (18). After washing two times with FACS staining buffer, anti-PE antibody (BioLegend: 408101; clone PE001) was incubated for 20 minutes on ice. After washing with FACS staining buffer, dextramer-stained cells were processed sequentially in the Live/Dead cell stain, surface stain, and intracellular stain steps as described previously. Among 77 patients with multiple myeloma, 40 patients were $HLA\text{-}A2^+$, and we applied HLA-A*0201 NY-ESO-$1_{157\text{-}165}$ and HM1.$24_{22\text{-}30}$ dextramers to BMMCs from 29 and 12 $HLA\text{-}A2^+$ patients with multiple myeloma, respectively. BM $CD8^+$ T cells from 20 patients had > 40 of NY-ESO-$1_{157\text{-}165}$ $dextramer^+$ cells, and BM $CD8^+$ T cells from 4 patients had >40 of HM1.$24_{22\text{-}30}$ $dextramer^+$ cells. To detect human cytomegalovirus (HCMV) pp65-specific $CD8^+$ T cells, PE-conjugated HLA-A*0201 dextramer loaded with HCMV pp$65_{495\text{-}503}$ (NLVPMVATV) was used (Immudex: WB2132).

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Antibodies

The following fluorochrome-conjugated monoclonal antibodies were used for multicolor flow cytometry: anti-CD3-Brilliant Violet (BV) 786 (563800; clone SK7), anti-CD4-BV605 (562658; clone RPA-T4), anti-CD8-AlexaFluor700 (557945; clone RPA-T8), anti-CD11c-BV650 (563404; clone B-ly6), anti-CD14-PE-Cy7 (562698; clone M5E2), anti-CD15-APC (555401; clone HI98), anti-CD19-BV605 (562653; clone SJ25C1), anti-CD30-FITC (341644; clone Ber-H83), anti-CD38-Brilliant Blue 515 (564498; clone HIT2), anti-CD45-BV786 (563716; clone HI30), anti-CD56-BV711 (563169; clone NCAM16.2), anti-HLA-A2 (551285; clone BB7.2), anti-Ig, κ Light Chain-BV510 (563213; clone G20-193), anti-Ig, λ Light Chain-FITC (555796; clone JDC-12), anti–PD-L1-BV421(563738; clone MIH1; all from BD Biosciences); anti–PD-1-BV421 (329920; clone EH12.2H7), anti–Tim-3-PE (345012; clone F38-2E2), anti–Tim-3-PE/Dazzle594 (345034; clone F38-2E2; all from BioLegend); anti-HLA-DR-APC-eFluor780 (47-9956-42; clone LN3), anti-Lag-3-APC (17-2239-42; clone 3DS223H), anti-TIGIT-PerCP-eFluor710 (46-1550-42; clone MBSA43; all from eBioscience). For intracellular staining, anti-T-bet-PE-Cy7 (25-5825-82; clone 4B10) and anti-Eomes-FITC or -eFluor660 (11-4877-42 or 50-4487-42; clone WD1928; all from eBioscience); anti-TCF-1/TCF-7 (2203; clone C63D9; from Cell Signaling Technology) were used.

Ex vivo stimulation of T cells and intracellular cytokine staining

Cryopreserved BMMCs were thawed and incubated overnight in complete RPMI-1640 medium. The 96-well flat-bottom plate was precoated with 1 μg/mL anti-CD3 (eBioscience: 16-0037-81; clone OKT3) and 1 μg/mL anti-CD28 (BioLegend: 302933; clone CD28.2). After incubating for 24 hours, Brefeldin A (BD Biosciences: 555029) and monensin (BD Biosciences: 554724) were added for intracellular accumulation of cytokine protein. After another 6 hours of incubation, cells were stained using a Live/Dead fixable cell stain kit and then stained with fluorochrome-conjugated antibodies against surface markers, including anti-CD3-BV786 and anti-CD8-BV711 (BD Biosciences: 563677; clone RPA-T8). Surface-stained cells were fixed and permeabilized using the Foxp3 staining buffer kit and stained with anti-TNF-FITC (BD Biosciences: 554512; Mab11) and anti-IFNγ-PE-Cy7 (BD Biosciences: 557844; 4S.B3).

TGFβ1 intracellular cytokine staining

For enumeration of TGFβ1-producing $CD38^+CD319^+$ plasma cells, including myeloma cells, 1×10^6 BMMCs were cultured in a 96-well round-bottom plate in RPMI-1640 containing 10% FBS. Brefeldin A and monensin were added 1 hour after incubation. After another 5 hours of incubation, the BMMCs were harvested and counted using an automatic cell counter (Cellometer Auto 2000; Nexcelom). The harvested BMMCs were stained using the Live/Dead fixable cell stain kit and then stained with fluorochrome-conjugated antibodies against surface markers. Surface-stained cells were fixed and permeabilized using the Foxp3 staining buffer kit and stained with anti-BV421-TGFβ1 (BD Biosciences: 562962; TW4-9E7).

T-cell proliferation assay

To evaluate functional restoration of T cells, we performed T-cell proliferation assay with anti–PD-1 blocking antibodies and/or TGFβ inhibitors (19, 20). Cryopreserved BMMCs were thawed and incubated overnight in complete RPMI-1640 medium. The BMMCs were labeled with CellTrace Violet (CTV; Invitrogen: C34557) in PBS containing 5% FBS for 20 minutes at 37°C and stimulated with 1 ng/mL of anti-CD3. After 108 hours, the BMMCs were harvested and stained with Live/Dead cell stain and surface antibodies for flow-cytometric analysis. For tumor antigen peptide stimulation, NY-ESO-$1_{157\text{-}165}$ (SLLMWITQC) and HM1.$24_{22\text{-}30}$ (LLLGIGILV) epitope peptides (customized from Peptron) were added to CTV-labeled BMMCs at 1 μg/mL and incubated in RPMI-1640 containing 10% FBS for 6 days. Media containing 5 μg/mL of anti–PD-1 (BioLegend: 329943; EH12.2H7), anti-TGFβ1 (BioLegend: 521703; 19D8), or mouse IgG_1 isotype control (Miltenyi Biotec: 130-106-545; clone IS5-21F5) antibody, or 1 μmol/L of galunisertib, a TGFβ receptor-1 inhibitor (LY2157299, purchased from Selleckchem Chemicals) was added to the culture.

To measure T-cell proliferation accurately, the mitotic index (21) was calculated as follows: Mitotic index = (Total number of mitotic events)/(Absolute number of precursor cells) = $\sum_0^n \left(\frac{X_n(T)-X_n(T)}{2^n}\right) / \sum_0^n \left(\frac{X_n(T)}{2^n}\right)$, where $X_n(T)$ is the absolute number of daughter T cells in each division peak n.

Ex vivo expansion of antigen-specific BM $CD8^+$ T cells

BMMCs from HLA-$A2^+$ multiple myeloma patients were stimulated with NY-ESO-$1_{157\text{-}165}$, HM1.$24_{22\text{-}30}$, or HCMV $pp65_{495\text{-}503}$ peptides in RPMI-1640 supplemented with IL2, IL7, IL15, and 10% FBS in the presence of anti–PD-1, TGFβ inhibitors such as anti–TGFβ1-neutralizing antibody or galunisertib, or isotype control for 3 weeks, and the frequency of antigen-specific $CD8^+$ T cells was examined by MHC-I multimer staining.

In vitro cytotoxicity assay and intracellular cytokine staining

IM-9 target cells were labeled with PKH26 dye (Sigma-Aldrich: PKH26GL) according to the manufacturer's instructions and pulsed with 10 μg/mL of NY-ESO-$1_{157\text{-}165}$ peptide for 1 hour at 37°C in a 5% CO_2 incubator. Then target cells were cocultured with the same number of NY-ESO-$1_{157\text{-}165}$–specific PD-1^+CD8^+ T cells (effector to target ratio 1:1) in the absence or presence of anti–PD-1 blocking antibodies and/or TGFβ inhibitors. After 6 hours, cells were harvested and stained with TO-PRO-3 (Thermo Fisher Scientific: T3605) to detect dead target cells. For intracellular cytokine staining, brefeldin A and monensin were added during 6 hours of incubation.

Analysis of KRAS mutation in multiple myeloma specimens and measurement of T-cell response to KRAS G12D mutation

Multiple myeloma cells were isolated from BM samples with CD138 microbead (Miltenyi: 130-051-301) and subjected to Sanger sequencing after DNA isolation and amplification (forward sequence: CCTGACATACTCCCAAGGAAA; backward sequence: CTTAAGC-GTCGATGGAGGAG) to investigate KRAS mutation. CTV-labeled BMMCs from multiple myeloma patients with or without KRAS G12D mutation were stimulated with a mixture of 9 overlapping peptides (9-mer) spanning KRAS codon 12 of wild-type (YKLVVV-GAGGVGKSALT; customized from Peptron) or mutant-type (YKLVVVGADGVGKSALT; customized from Peptron) at 1 μg/mL for each peptide and incubated in RPMI-1640 containing 10% FBS. Cellular proliferation was measured with CTV dilution among $CD8^+$ T cells upon stimulation with a mixture of overlapping peptides after 144 hours.

In vitro treatment of TGFβ1

BM $CD8^+$ T cells were purified with CD8 microbead (Miltenyi: 130-045-201) and stimulated with plate-bound anti-CD3 (1 μg/mL), anti-CD28 (1 μg/mL), and PD-L1 (10 μg/mL, Sino Biological: 10084-H02H) in the presence or absence of TGFβ1 (50 ng/mL, PeproTech: 100-21). After 30 hours, the expression of PD-1 and T-bet in $CD8^+$

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T cells was investigated. In addition, BMMCs were stimulated with anti-CD3 (1 ng/mL) in the presence or absence of anti-TGFβ antibody. After 36 hours, the expression of PD-1, T-bet, and TCF-1 in $CD8^+$ T cells was investigated.

In vivo mouse model

MOPC315.BM murine myeloma cells were grown in RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, and 2 mmol/L L-glutamine (Thermo Fisher Scientific: 25030081) as previously described (22). Balb/c mice were purchased from Orient and 6-to 8-week-old mice were used for all experiments. To establish an *in vivo* plasmacytoma model, 1×10^6 MOPC315.BM myeloma cells were subcutaneously injected. To test the therapeutic effect, isotype control antibodies (Bio X Cell: BE0089), anti–PD-1 antibodies (Bio X Cell: BE0146, 200 μg/mouse), anti-TGFβ antibodies (Bio X Cell: BE0057, 200 μg/mouse), or a combination of anti–PD-1 with anti-TGFβ antibodies was administered once every 3 days from 11 days after tumor injection. This study was approved by the Institutional Animal Care and Use committee of Inje University College of Medicine.

Statistical analysis

Statistical analyses were performed using Prism software version 7.0 (GraphPad). The nonparametric Mann–Whitney *U* test was used to compare two groups. Paired values were compared using the nonparametric Wilcoxon matched-pairs signed rank test.

Results

Increased expression of PD-1 on BM $CD8^+$ T cells from multiple myeloma patients

In the flow-cytometric analysis of BMMCs from multiple myeloma patients, we gated $CD8^+$ T cells (Supplementary Fig. S1) and examined the expression of PD-1. The BM $CD8^+$ T cells had a significantly higher frequency of PD-1^+ cells than paired PB $CD8^+$ T cells (**Fig. 1A**). The frequency of PD-1^+ cells among BM $CD8^+$ T cells from multiple myeloma patients was significantly higher than the frequency among BM $CD8^+$ T cells from patients with EMZL without BM involvement (**Fig. 1B**). In addition, the frequency of PD-1^+ cells among BM $CD8^+$ T cells from multiple myeloma patients was significantly higher than the frequency among BM $CD8^+$ T cells from patients with MGUS and SMM, which are premalignant or precursors of multiple myeloma (**Fig. 1C**). Taken together, the results confirm that BM $CD8^+$ T cells in multiple myeloma express increased levels of PD-1.

Next, we analyzed the coexpression of other immune-checkpoint receptors, such as Tim-3, Lag-3, and TIGIT, on PD-1–expressing $CD8^+$ T cells from the BM of multiple myeloma patients. Among BM $CD8^+$ T cells, PD-1^+ cells had significantly higher frequencies of Tim-3^+, Lag-3^+, or $TIGIT^+$ cells than PD-1^- cells (**Fig. 1D** and **E**). We also examined the expression of T-cell transcription factors, such as T-bet and Eomes, which are related to T-cell differentiation (23). In particular, an $Eomes^{hi}T\text{-}bet^{lo}$ phenotype represents a terminally differentiated status for $CD8^+$ T cells, which is associated with poor reinvigoration of $CD8^+$ T cells upon PD-1 blockade. In BMMCs from multiple myeloma patients, PD-1^+CD8^+ T cells had a higher percentage of $Eomes^{hi}T\text{-}bet^{lo}$ cells than PD-1^-CD8^+ T cells (**Fig. 1F** and **G**). In addition, TCF-1 expression was lower in PD-1^+ cells than PD-1^- cells among BM-resident ($CD69^+$) $CD8^+$ T cells (**Fig. 1H**). These data demonstrate that PD-1–expressing $CD8^+$ T cells from the BM of multiple myeloma patients exhibit a terminally differentiated phenotype with coexpression of multiple immune-checkpoint inhibitory receptors.

Increased expression of PD-1 on myeloma antigen–specific $CD8^+$ T cells

To examine the characteristics of myeloma antigen–specific $CD8^+$ T cells, we used HLA-A*0201 dextramers loaded with NY-ESO-$1_{157\text{-}165}$ (24) or HM1.$24_{22\text{-}30}$ (25) peptides. NY-ESO-$1_{157\text{-}165}$– and HM1.$24_{22\text{-}30}$–specific $CD8^+$ T cells were successfully detected in BMMCs from multiple myeloma patients (**Fig. 2A**). The frequency of NY-ESO-$1_{157\text{-}165}$– (Supplementary Fig. S6A) and HM1.$24_{22\text{-}30}$ (Supplementary Fig. S6B)–specific cells was higher in BM $CD8^+$ T cells from multiple myeloma patients than in BM $CD8^+$ T cells from EMZL patients without BM involvement, or patients with MGUS and SMM. NY-ESO-$1_{157\text{-}165}$– and HM1.$24_{22\text{-}30}$–specific BM $CD8^+$ T cells from multiple myeloma patients had significantly more PD-1^+ cells than total BM $CD8^+$ T cells (**Fig. 2B** and **C**). In NY-ESO-$1_{157\text{-}165}$– or HM1.$24_{22\text{-}30}$–specific $CD8^+$ T cells, PD-1^+ cells had significantly higher percentages of Tim-3^+, Lag-3^+, or $TIGIT^+$ cells than PD-1^- cells (**Fig. 2D** and **E**). In an analysis of the differentiation status of myeloma antigen–specific $CD8^+$ T cells, PD-1^+ cells expressed a higher percentage of $Eomes^{hi}T\text{-}bet^{lo}$ cells than PD-1^- cells from the BM of multiple myeloma patients (**Fig. 2F**). Taken together, the results indicate that myeloma antigen–specific $CD8^+$ T cells also overexpress PD-1 and present a terminally differentiated phenotype.

Expression of PD-L1 on malignant plasma cells and immune cells in BM from multiple myeloma patients

We also analyzed the expression of PD-L1 on BM cells from multiple myeloma patients by gating malignant plasma cells ($CD38^+CD319^+CD45^-CD19^-$) and normal plasma cells ($CD38^+CD319^+CD45^+CD19^+$; refs. 26, 27). Malignant plasma cells had a single type of immunoglobulin light chain (kappa or lambda), but normal plasma cells did not (Supplementary Fig. S2). The percentage of malignant plasma cells among BMMCs was 15.49% ± 10.17% (mean ± standard deviation; **Fig. 3A**). Malignant plasma cells had a significantly higher frequency of PD-$L1^+$ cells than paired normal plasma cells from multiple myeloma patients (**Fig. 3B** and **C**). Malignant plasma cells from multiple myeloma patients also had a significantly higher frequency of PD-$L1^+$ cells than normal plasma cells from EMZL patients without BM involvement (**Fig. 3D**). However, malignant plasma cells had a lower frequency of PD-$L1^+$ cells than BM malignant cells in DLBCL ($CD45^+CD19^+CD20^+\kappa$ or λ LC^+ cells) or HL ($CD15^+CD30^+$ Reed–Stenberg cells; Supplementary Fig. S7A). We also analyzed the expression of PD-L1 on $CD11c^+$ dendritic cells ($CD45^+CD14^-CD3^-CD11c^+HLA\text{-}DR^+$), B cells ($CD45^+CD14^-CD3^-CD19^+HLA\text{-}DR^+$), and monocytes ($CD45^+CD3^-CD14^+$) from the BM of multiple myeloma, EMZL, DLBCL, and HL patients. The frequencies of PD-$L1^+$ cells in all three subsets from the BM of multiple myeloma patients were significantly higher than the frequency in tumor-free BM aspirates from EMZL patients (**Fig. 3E**) and were comparable with those from the BM of DLBCL and HL patients (Supplementary Fig. S7B). These results demonstrate that PD-L1 is upregulated in the BM microenvironment of multiple myeloma patients in addition to the upregulation of PD-1 on BM $CD8^+$ T cells.

Impaired proliferation and cytokine production of BM $CD8^+$ T cells from multiple myeloma patients

As the expression of PD-1 and PD-L1 was upregulated in BM cells from multiple myeloma patients, we investigated the proliferation and cytokine production of BM $CD8^+$ T cells from multiple myeloma patients. The BM $CD8^+$ T cells from multiple myeloma patients

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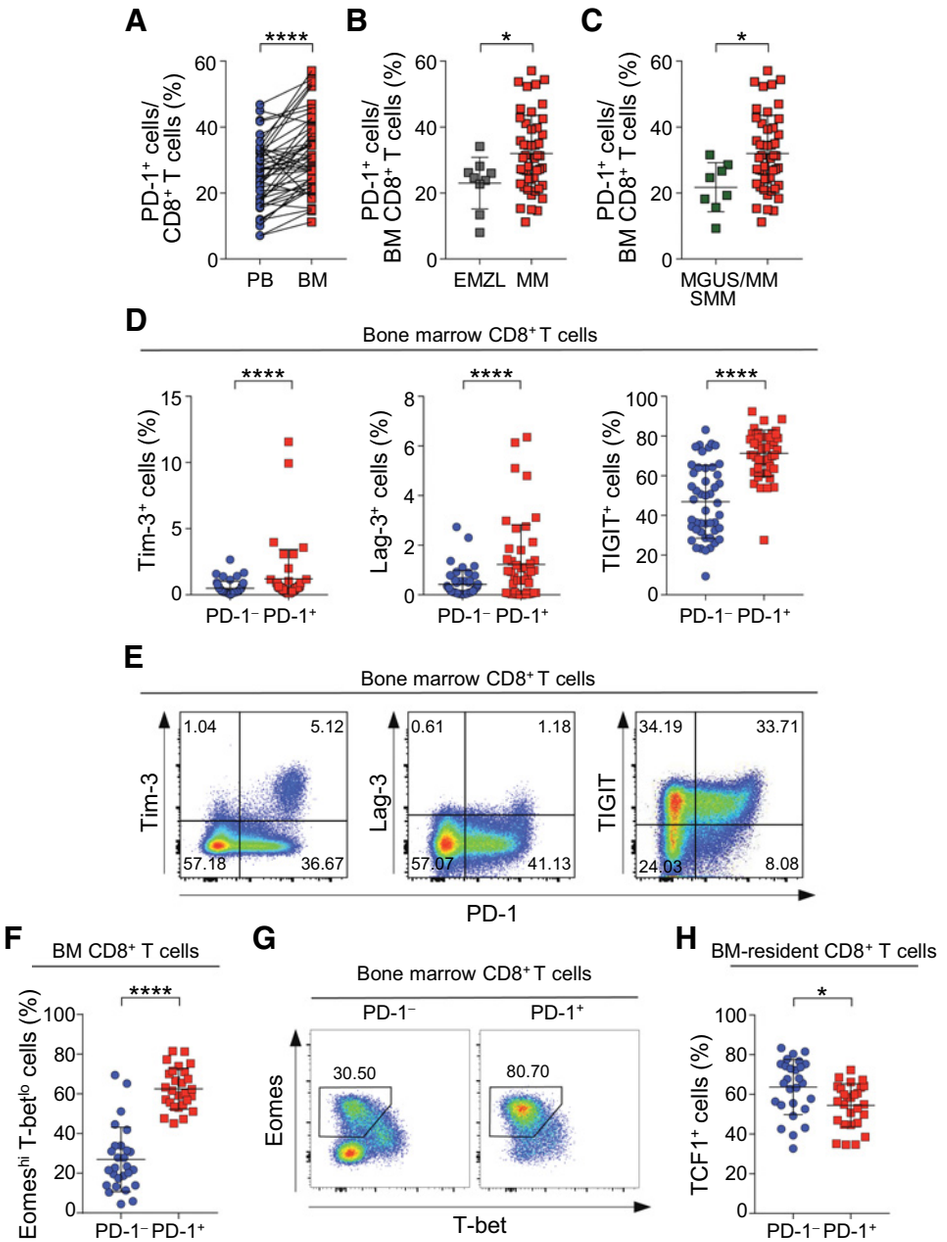

Figure 1.

Phenotypic characteristics of BM CD8$^+$ T cells from multiple myeloma (MM) patients. **A–C,** PBMCs and BMMCs from 47 newly diagnosed multiple myeloma patients, and BMMCs from 9 EMZL patients without BM involvement, 4 MGUS patients, and 4 SMM patients were analyzed by flow cytometry. The relative frequency of PD-1$^+$ cells among BM CD8$^+$ T cells from multiple myeloma patients was compared with paired PB CD8$^+$ T cells (**A**), BM CD8$^+$ T cells from EMZL patients (**B**), and BM CD8$^+$ T cells from MGUS or SMM patients (**C**). **D** and **E,** The relative frequency of Tim-3$^+$, Lag-3$^+$, or TIGIT$^+$ cells among PD-1$^+$ cells was compared with the frequencies among PD-1$^-$ cells in BM CD8$^+$ T cells from multiple myeloma patients (**D**). Representative figures from a single multiple myeloma patient are presented (**E**). **F** and **G,** The relative frequency of Eomes^{hi}T-bet^{lo} cells among PD-1$^+$ cells was compared with the frequency among PD-1$^-$ cells in BM CD8$^+$ T cells from multiple myeloma patients ($n = 23$; **F**). Representative figures from a single multiple myeloma patient are presented (**G**). **H,** The relative frequency of TCF-1$^+$ cells among PD-1$^+$ cells was compared with the frequency among PD-1$^-$ cells in BM CD69$^+$CD8$^+$ T cells from multiple myeloma patients ($n = 27$). Statistical analysis was performed using the Mann–Whitney *U* test or Wilcoxon matched-pairs signed rank test. Error bars, SD. *, $P < 0.05$; ****, $P < 0.0001$. MM, multiple myeloma.

exhibited significantly diminished anti-CD3–induced proliferation compared with PB CD8$^+$ T cells from the healthy donors (Supplementary Fig. S8A) or multiple myeloma patients (Supplementary Fig. S8B) or BM CD8$^+$ T cells from EMZL patients without BM involvement (Supplementary Fig. S8C) or MGUS/SMM patients (**Fig. 4A** and **B**). In addition, we examined effector cytokine production by BM CD8$^+$ T cells from multiple myeloma patients. The production of IFNγ and TNF was analyzed by intracellular cytokine staining and flow cytometry in response to anti-CD3 stimulation. The percentage of IFNγ$^+$, TNF$^+$, or double-positive (IFNγ$^+$TNF$^+$) cells among BM CD8$^+$ T cells was significantly lower in multiple myeloma patients than in MGUS or SMM patients (**Fig. 4C**).

We also compared the functions of BM CD8$^+$ T cells from multiple myeloma patients with those from patients with other B-cell lymphomas with BM involvement (DLBCL and HL). Interestingly, BM CD8$^+$ T cells from multiple myeloma patients proliferated significantly less than those from DLBCL or HL patients (**Fig. 4D** and **E**). Moreover, BM CD8$^+$ T cells from multiple myeloma patients exhibited a significantly lower frequency of TNF$^+$ or IFNγ$^+$TNF$^+$ cells than those from DLBCL or HL patients (**Fig. 4F**). Taken together, the results indicate that BM CD8$^+$ T cells from multiple myeloma patients are functionally impaired.

Failure of anti–PD-1-induced reinvigoration of BM CD8$^+$ T cells from multiple myeloma patients

Next, we tested whether PD-1 blockade restores the function of BM CD8$^+$ T cells from multiple myeloma patients. CTV-labeled BMMCs were stimulated with anti-CD3 in the presence of anti–PD-1 blocking or isotype control antibodies, and the proliferation of CD8$^+$ T cells was evaluated. Anti–PD-1 did not significantly restore the proliferation of

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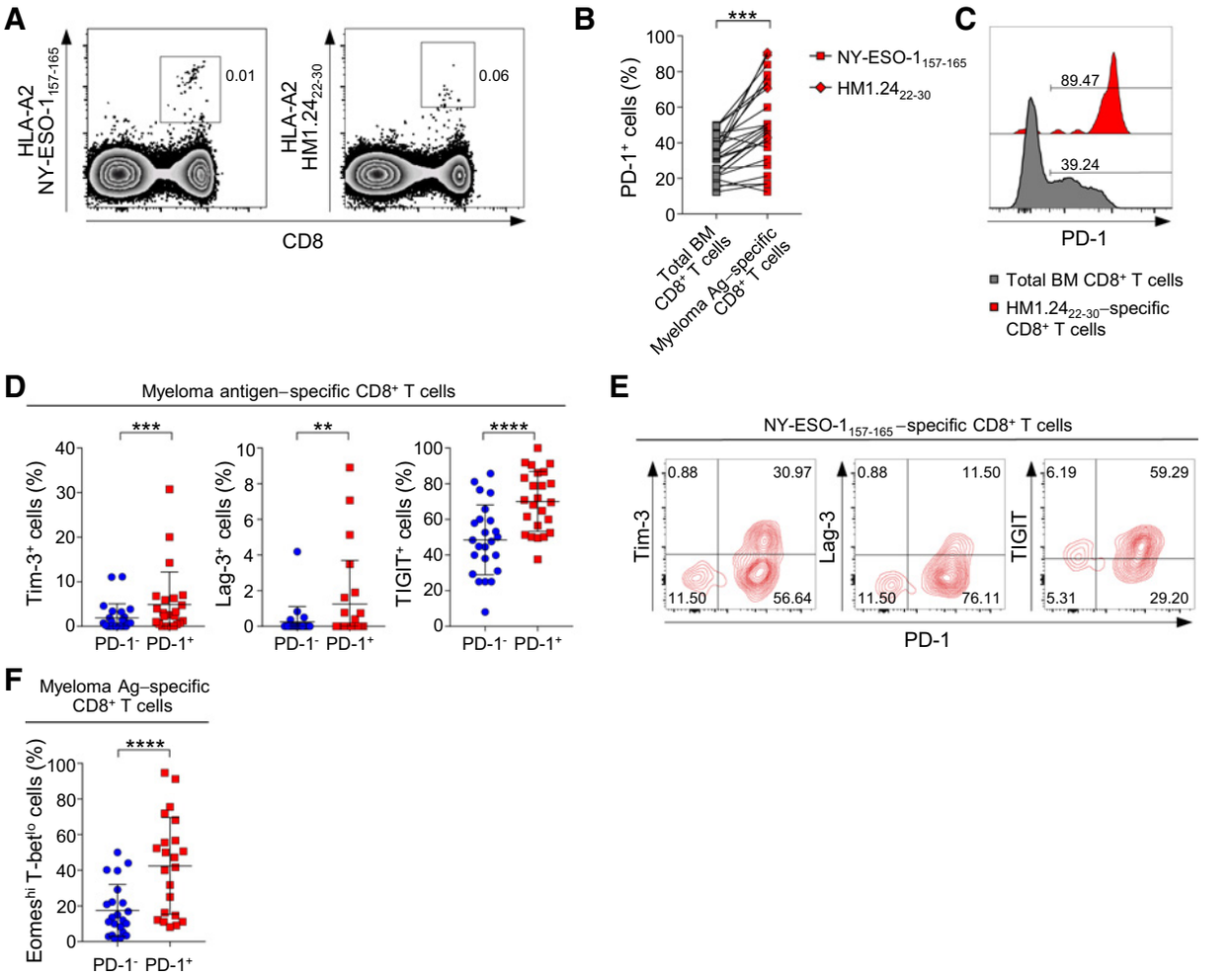

Figure 2.

Phenotypic characteristics of myeloma antigen–specific CD8$^+$ T cells from the BM of multiple myeloma patients. **A–E,** BMMCs from newly diagnosed HLA-A2$^+$ multiple myeloma patients were stained with HLA-A*0201 NY-ESO-1$_{157\text{-}165}$ (SLLMWITQC) and HM1.24$_{22\text{-}30}$ (LLLGIGILV) dextramers and analyzed by flow cytometry. Representative figures are presented (**A**). The relative frequency of PD-1$^+$ cells in NY-ESO-1$_{157\text{-}165}$ dextramer$^+$ ($n = 20$) or HM1.24$_{22\text{-}30}$ dextramer$^+$ ($n = 4$) CD8$^+$ T cells was compared with total BM CD8$^+$ T cells (**B**). Representative figures are presented (**C**). Among NY-ESO-1$_{157\text{-}165}$ dextramer$^+$ or HM1.24$_{22\text{-}30}$ dextramer$^+$ CD8$^+$ T cells, the relative frequency of Tim-3$^+$, Lag-3$^+$, or TIGIT$^+$ cells among PD-1$^+$ cells was compared with the frequency among PD-1$^-$ cells (**D**). Representative figures are presented (**E**). Among NY-ESO-1$_{157\text{-}165}$ dextramer$^+$ ($n = 18$) or HM1.24$_{22\text{-}30}$ dextramer$^+$ ($n = 4$) CD8$^+$ T cells, the relative frequency of Eomes^{hi}T-bet^{lo} cells in PD-1$^+$ cells was compared with PD-1$^-$ cells (**F**). Error bars represent SD. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

BM CD8$^+$ T cells from multiple myeloma patients (**Fig. 5A** and **B**), whereas the proliferation of BM CD8$^+$ T cells from DLBCL or HL patients was significantly restored by anti–PD-1 (**Fig. 5A**). Combined blockade of PD-1 and other immune-checkpoint receptors such as Tim-3, Lag-3, or TIGIT did not significantly increase the anti–CD3-stimulated proliferation of BM CD8$^+$ T cells from multiple myeloma patients (Supplementary Fig. S9A and S9B). We confirmed failure of anti–PD-1-induced reinvigoration of CD8$^+$ T cells by stimulation of BMMCs from multiple myeloma patients with HLA-A*0201–restricted myeloma antigen peptides, including NY-ESO-1$_{157\text{-}165}$ and HM1.24$_{22\text{-}30}$ peptides. Anti–PD-1 did not significantly restore the proliferation of BM CD8$^+$ T cells from HLA-A2$^+$ multiple myeloma patients (**Fig. 5C** and **D**). When this assay was performed with BMMCs from HLA-A2$^+$ patients with EMZL, MGUS, or SMM, proliferation of BM CD8$^+$ T cells was not enhanced by PD-1 blockade (Supplementary Fig. S10A–S10D). These data demonstrate that blocking PD-1 is not sufficient to restore the function of BM CD8$^+$ T cells from multiple myeloma patients.

Reinvigoration of BM CD8$^+$ T cells from multiple myeloma patients by PD-1 blockade in the presence of TGFβ inhibitors

We hypothesized that blockade of an additional T-cell inhibitory factor is required to restore the function of BM CD8$^+$ T cells, even if PD-1/PD-L1 interaction is blocked. It has been known that TGFβ, which is actively secreted by malignant plasma cells and BM stromal cells (28), can inhibit T-cell responses (29). We confirmed that the major source of TGFβ1 is plasma cells including myeloma cells among BMMCs from multiple myeloma patients, and the number of TGFβ1-producing plasma cells, including myeloma cells, is increased in the BM of multiple myeloma patients compared with

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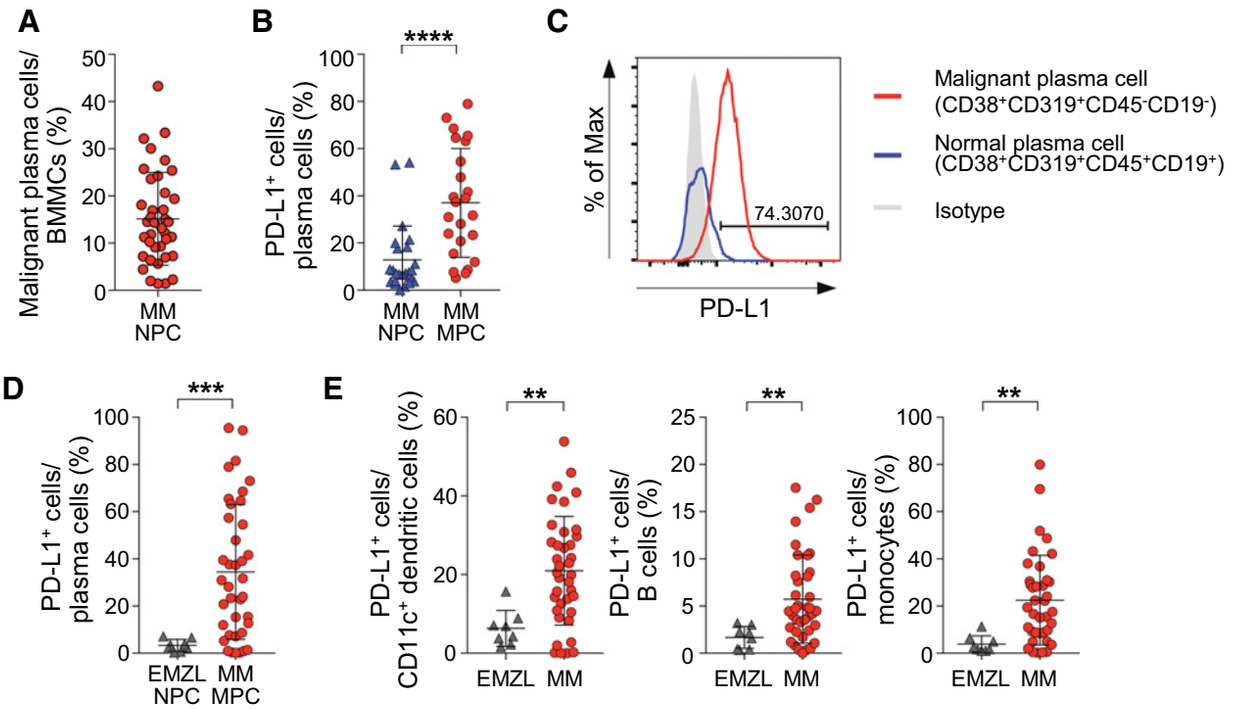

Figure 3.

Expression of PD-L1 on malignant plasma cells and immune cells from the BM of multiple myeloma patients. **A,** The relative frequency of malignant plasma cells (MPC; $CD38^+CD319^+CD45^-CD19^-$) among BMMCs from multiple myeloma patients ($n = 38$) was analyzed. **B** and **C,** The relative frequency of PD-L1$^+$ cells was analyzed in both malignant plasma cells ($CD38^+CD319^+CD45^-CD19^-$) and normal plasma cells (NPC; $CD38^+CD319^+CD45^+CD19^+$) in BMMCs from multiple myeloma patients who had detectable numbers of normal plasma cells ($n = 24$). Representative histograms are presented (**C**). **D,** The relative frequency of PD-L1$^+$ cells was analyzed in malignant plasma cells among BMMCs from multiple myeloma patients ($n = 38$) and normal plasma cells from EMZL patients without BM involvement ($n = 8$). **E,** The relative frequency of PD-L1$^+$ cells was analyzed in $CD11c^+$ dendritic cells ($CD45^+CD14^-CD3^-CD11c^+HLA\text{-}DR^+$, left), B cells ($CD45^+CD14^-CD3^-CD19^+HLA\text{-}DR^+$, middle), and monocytes ($CD45^+CD3^-CD14^+$, right) among BMMCs from multiple myeloma patients ($n = 38$) and EMZL patients without BM involvement ($n = 8$). Statistical analysis was performed using the Mann–Whitney *U* test or Wilcoxon matched-pairs signed rank test. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. MM, multiple myeloma.

that from EMZL, MGUS, and multiple myeloma patients (**Fig. 6A**). Next, we examined the effect of TGFβ1 or TGFβ1 blocking antibodies on the phenotype and differentiation of BM CD8$^+$ T cells from multiple myeloma patients. TGFβ1 increased PD-1 expression but reduced T-bet expression in BM CD8$^+$ T cells (Supplementary Fig. S11A and S11B). Anti-TGFβ1 blocking antibodies decreased the expression of PD-1 in BM CD8$^+$ T cells from multiple myeloma patients (**Fig. 6B**) and increased the expression of T-bet (**Fig. 6C**) and TCF-1 (**Fig. 6D**).

We investigated whether blocking TGFβ signaling enhances reinvigoration of BM CD8$^+$ T cells from multiple myeloma patients. CTV-labeled BMMCs were stimulated with anti-CD3 in the presence of anti–PD-1 and/or anti-TGFβ1 blocking antibodies, and the proliferation of CD8$^+$ T cells was evaluated. The combined blockade of PD-1 and TGFβ significantly increased the proliferation of BM CD8$^+$ T cells from multiple myeloma patients (**Fig. 6E–G**). However, the mitotic index of multiple myeloma-derived BM CD8$^+$ T cells in the presence of anti–PD-1 antibody and TGFβ inhibitors was not at the level of BM CD8$^+$ T cells from EMZL, MGUS, or SMM patients or PB CD8$^+$ T cells from multiple myeloma patients or healthy donors in the absence of anti–PD-1 antibody and TGFβ inhibitors (Supplementary Fig. S12A–S12D). In addition, combined blockade of PD-1 and TGFβ did not increase the proliferation of BM CD8$^+$ T cells from EMZL, MGUS, and SMM patients (Supplementary Fig. S13A–S13D) and PB CD8$^+$ T cells from normal healthy donors or multiple myeloma patients (Supplementary Fig. S13E and S13F). When stimulated with anti-CD3, the production of IFNγ and TNF by BM CD8$^+$ T cells was also rescued by combined blockade of PD-1 and TGFβ (Supplementary Fig. S14A–S14C).

In addition, combination of anti–PD-1 antibody and TGFβ inhibitors increased proliferative responses of BM CD8$^+$ T cells from HLA-A2$^+$ multiple myeloma patients stimulated with a mixture of HLA-A*0201–restricted myeloma antigen peptides (NY-ESO-$1_{157\text{-}165}$ and HM1.$24_{22\text{-}30}$ peptides; **Fig. 6H–J**). However, PD-1 and/or TGFβ blockade–induced increase in CD8$^+$ T-cell proliferation was not observed in the absence of antigen stimulation (Supplementary Fig. S15A and S15B). When BMMCs from HLA-A2$^+$ patients with EMZL, MGUS, or SMM were stimulated with a mixture of HLA-A*0201–restricted myeloma antigen peptides, PD-1 and/or TGFβ blockade did not enhance CD8$^+$ T-cell proliferation in BMMCs from EMZL, MGUS, and SMM patients (Supplementary Fig. S16A–S16D).

Enhancement in proliferative response of BM CD8$^+$ T cells to neoantigen was also investigated for KRAS mutation. The KRAS G12D mutation was identified in two multiple myeloma patients, and their BMMCs were stimulated with a mixture of 9 overlapping peptides (9-mer) spanning KRAS codon 12 of wild-type (YKLVVVGAGGVGKSALT; from YKLVVVGAG to GGVGKSALT) or

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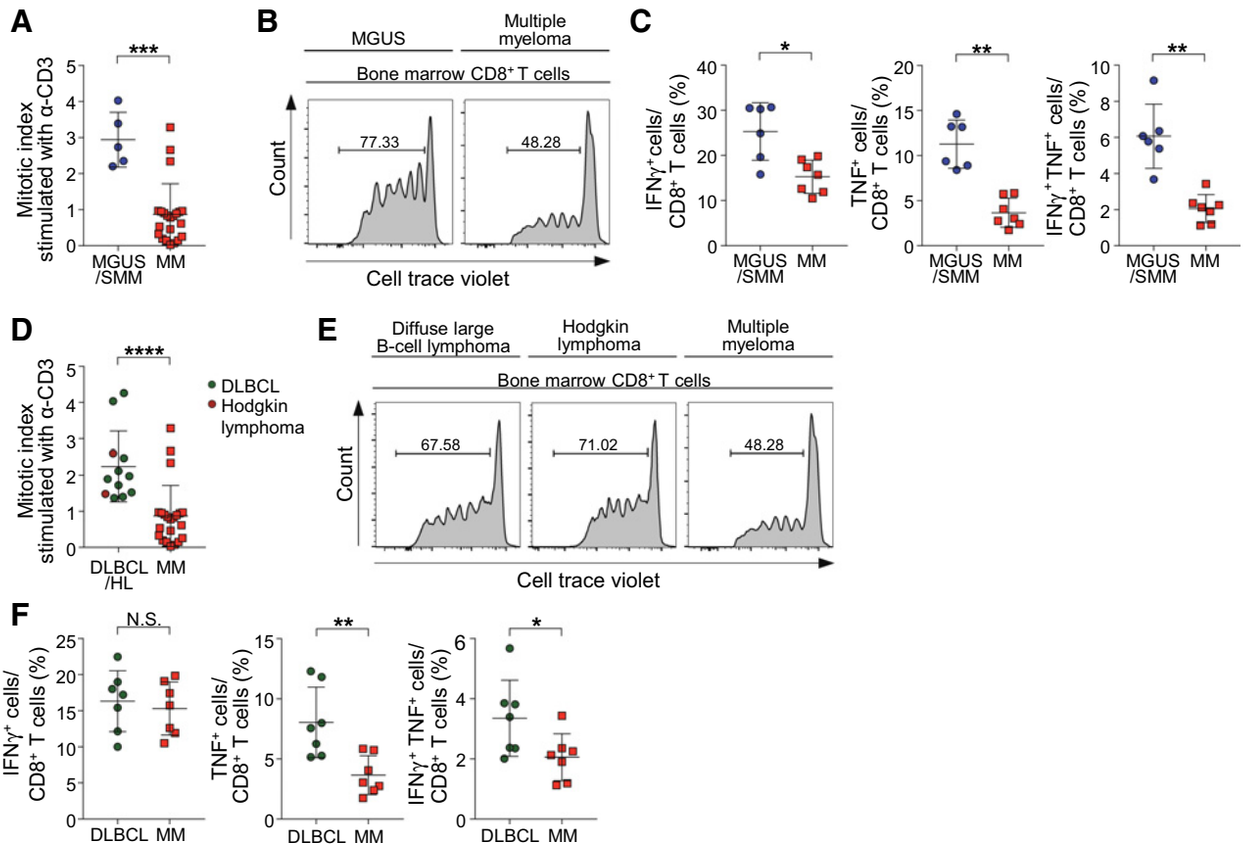

Figure 4.

Impaired proliferation and cytokine production by BM CD8$^+$ T cells from multiple myeloma patients. **A** and **B,** BMMCs from multiple myeloma ($n = 22$), MGUS ($n = 4$), or SMM ($n = 1$) patients were labeled with CTV and stimulated with anti-CD3 (1 ng/mL) for 108 hours. The mitotic index of CD8$^+$ T cells was analyzed (**A**). Representative figures are presented (**B**). **C,** BMMCs from multiple myeloma ($n = 7$), MGUS ($n = 4$), or SMM ($n = 2$) patients were stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) for 30 hours, and intracellular cytokine staining for IFNγ and TNF was performed. The percentages of IFNγ$^+$ cells (left), TNF$^+$ cells (middle), and double-positive (IFNγ$^+$TNF$^+$) cells (right) among BM CD8$^+$ T cells were analyzed. **D** and **E,** BMMCs from multiple myeloma ($n = 22$), DLBCL with BM involvement ($n = 10$), or HL with BM involvement ($n = 2$) patients were labeled with CTV and stimulated with anti-CD3 (1 ng/mL) for 108 hours. The mitotic index of CD8$^+$ T cells was analyzed (**D**). Representative figures are presented (**E**). **F,** BMMCs from multiple myeloma ($n = 7$) or DLBCL with BM involvement ($n = 7$) patients were stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) for 30 hours, and intracellular cytokine staining for IFNγ and TNF was performed. The percentages of IFNγ$^+$ cells (left), TNF$^+$ cells (middle), and double-positive (IFNγ$^+$TNF$^+$) cells (right) among BM CD8$^+$ T cells were analyzed. Error bars, SD. Statistical analysis was performed using the Mann–Whitney *U* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. MM, multiple myeloma; N.S., not significant.

mutant-type (YKLVVVGADGVGKSALT; from YKLVVVGAD to DGVGKSALT). As a result, the proliferation of BM CD8$^+$ T cells was efficiently increased by the combined blockade of PD-1 and TGFβ in the presence of mutant-type KRAS peptides. When BM CD8$^+$ T cells were stimulated by wild-type KRAS peptides, their proliferation was ignorable and not enhanced by the combined blockade of PD-1 and TGFβ (**Fig. 6K**). Importantly, there was no considerable proliferation of BM CD8$^+$ T cells from multiple myeloma patients without KRAS G12D mutation following stimulation with wild-type or G12D mutant–type peptides (Supplementary Fig. S17A), and their proliferation was not enhanced by the blockade of PD-1 and/or TGFβ (Supplementary Fig. S17B–S17E). When BMMCs from HLA-A2$^+$ multiple myeloma patients were stimulated with NY-ESO-1$_{157\text{-}165}$ and HM1.24$_{22\text{-}30}$ peptides for 3 weeks, combined blockade of PD-1 and TGFβ significantly increased the frequency of NY-ESO-1$_{157\text{-}165}$ or HM1.24$_{22\text{-}30}$ HLA-A*0201 multimer$^+$ CD8$^+$ T cells, although PD-1 blockade alone was not sufficient to increase their frequency (Supplementary Fig. S18A–S18F). In contrast, the frequency of HCMV pp65$_{495\text{-}503}$–specific CD8$^+$ T cells was not influenced by PD-1 or TGFβ blockade (Supplementary Fig. S18G–S18I). Combined blockade of PD-1 and TGFβ robustly enhanced the target cell killing capacity (Supplementary Fig. S19A–S19C), as well as the production of IFNγ and TNF (Supplementary Fig. S19D–S19F) in coculture assays using NY-ESO-1$_{157\text{-}165}$–specific PD-1$^+$CD8$^+$ T-cell lines. In line with this, combined treatment with anti–PD-1 and anti-TGFβ significantly reduced the tumor size in a mouse model of MOPC315.BM plasmacytoma (**Fig. 6L**; Supplementary Fig. S20). Thus, PD-1 blockade reinvigorates BM CD8$^+$ T cells from multiple myeloma patients in the presence of TGFβ inhibitors.

Discussion

PD-1/PD-L1 blockade that reinvigorates exhausted T cells has been approved for the treatment of various solid tumors or hematologic malignancies. In addition, the combination strategy of PD-1/PD-L1

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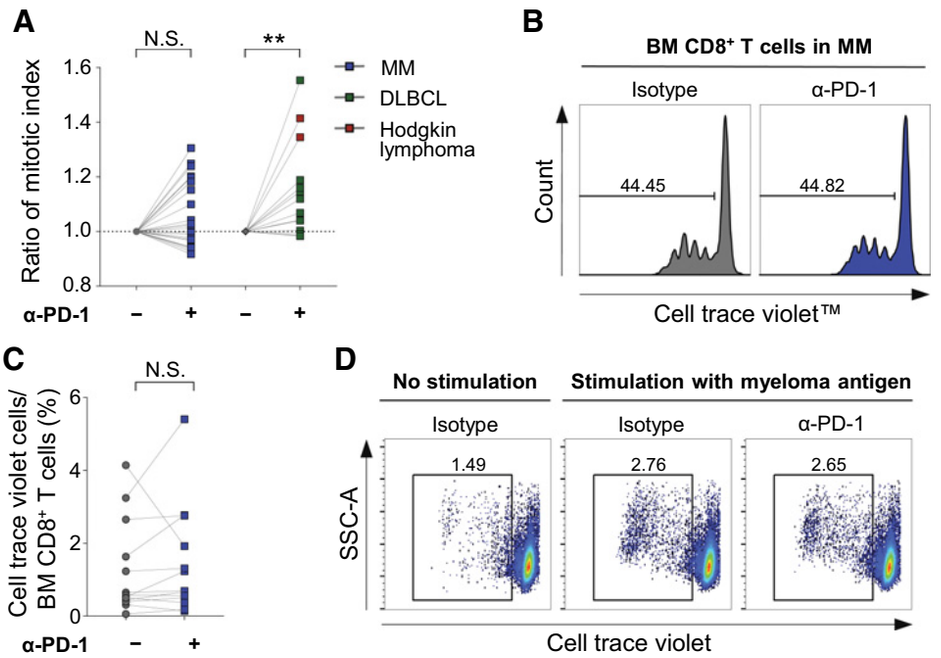

Figure 5.

Effect of PD-1 blockade on the proliferation of BM CD8$^+$ T cells from multiple myeloma patients. **A** and **B,** BMMCs from multiple myeloma ($n = 22$), DLBCL with BM involvement ($n = 10$), or HL with BM involvement ($n = 2$) patients were labeled with CTV and stimulated with anti-CD3 (1 ng/mL) in the presence of anti–PD-1 or isotype control antibody for 108 hours. The ratio of mitotic index with and without anti–PD-1 (mitotic index with anti–PD-1/mitotic index with isotype control antibody) was assessed (**A**). Representative figures are presented (**B**). **C** and **D,** BMMCs from HLA-A*0201$^+$ multiple myeloma patients ($n = 13$) were labeled with CTV and stimulated with a mixture of NY-ESO-$1_{157\text{-}165}$ (SLLMWITQC) and HM1.$24_{22\text{-}30}$ (LLLGIGILV) peptides in the presence of anti–PD-1 or isotype control antibody for 144 hours. The ratio of mitotic index with and without anti–PD-1 (mitotic index with anti–PD-1/mitotic index with isotype control antibody) was calculated as follows: (mitotic index with anti–PD-1)/(mitotic index with controls) (**C**). Representative figures are presented (**D**). Error bars represent SD. Statistical analysis was performed by Wilcoxon matched-pairs signed rank test. **, $P < 0.01$. MM, multiple myeloma; N.S., not significant.

blockade with conventional chemotherapy or targeted therapy has enhanced clinical efficacy in clinical trials for patients with non–small cell lung cancer (30) or renal cell carcinoma (31). However, in a clinical trial of multiple myeloma patients, anti–PD-1 monotherapy did not result in a clinical response (11). Furthermore, clinical trials of combining PD-1 blockade with immunomodulatory drugs (32) or anti-CD38 monoclonal antibody (33) failed to demonstrate clinical benefits in multiple myeloma patients. To enhance the clinical efficacy of ICIs in multiple myeloma, an elaborate characterization of BM CD8$^+$ T cells in multiple myeloma patients is essential.

In the present study, we demonstrated that BM CD8$^+$ T cells from multiple myeloma patients express increased levels of PD-1 and a terminally differentiated phenotype with impaired proliferation and effector functions. Using NY-ESO-$1_{157\text{-}165}$ and HM1.$24_{22\text{-}30}$ multimers, we demonstrated that myeloma antigen–specific CD8$^+$ T cells also overexpress PD-1. Although PD-1 blockade alone did not reinvigorate BM CD8$^+$ T cells, the combined blockade of PD-1 and TGFβ successfully rescued the cells from exhaustion.

Several studies have reported the expression of immune-checkpoint inhibitory receptors in T cells from multiple myeloma patients. The expression of PD-1 has been reported to be significantly upregulated on BM CD8$^+$ T cells from multiple myeloma patients (7–9). Moreover, CD4$^+$ and CD8$^+$ T cells from relapsed multiple myeloma patients expressed significantly higher levels of PD-1 than those from MGUS or newly diagnosed multiple myeloma patients (8). However, a recent study reported that multiple myeloma patients have clonally expanded T cells with low levels of PD-1 expression (12). In the present study, the expression of PD-1 was upregulated in total CD8$^+$ T cells from the BM of multiple myeloma patients, corroborating the results from earlier studies (7–9). In addition, we showed for the first time that the expression of PD-1 was upregulated in myeloma antigen–specific CD8$^+$ T cells, using MHC-I multimers loaded by myeloma antigenic peptides.

Two heterogeneous subsets of exhausted T cells were identified according to the expression of T-bet and Eomes in a mouse model of chronic lymphocyte choriomeningitis virus infection (34). During chronic infection, T-bet^{hi}Eomes^{lo}CD8$^+$ T cells maintain a proliferative potential and cytokine secretion, whereas Eomes^{hi}T-bet^{lo}CD8$^+$ T cells exhibit poor proliferative potential and cytokine secretion, indicating that they are terminally differentiated cells. Moreover, Eomes^{hi}T-bet^{lo}CD8$^+$ T cells have limited potential with anti–PD-1-induced reinvigoration (20, 23). In our data, BM PD-1$^+$CD8$^+$ T cells from multiple myeloma patients had skewed expression toward Eomes^{hi}T-bet^{lo}. BM CD8$^+$ T cells in multiple myeloma patients also exhibited reduced cytokine secretion and proliferation upon activation of T-cell receptor signals. Moreover, proliferation of BM CD8$^+$ T cells from multiple myeloma patients was not reinvigorated by PD-1 blockade.

The TGFβ signaling pathway contributes to tumor progression by diverse mechanisms, including proliferation and differentiation, epithelial–mesenchymal transition, invasion and migration, and the production of mitogenic growth factors in tumor cells (35). In

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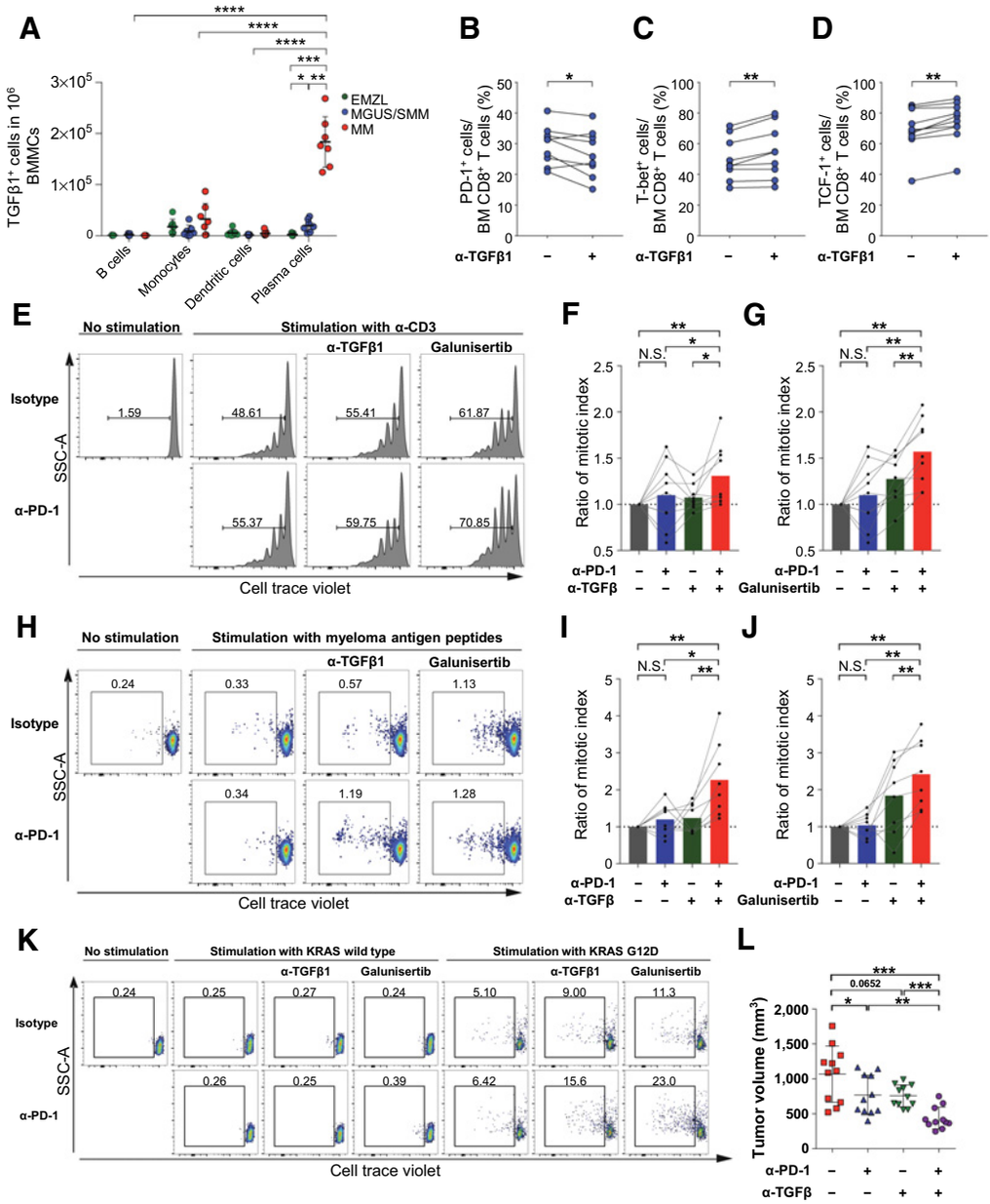

Figure 6.

Reinvigoration of BM CD8$^+$ T cells from multiple myeloma patients by PD-1 blockade in the presence of TGFβ inhibitors. **A,** The number of TGFβ1-producing cells was analyzed in BMMCs from multiple myeloma patients ($n = 7$), MGUS ($n = 4$), SMM ($N = 3$), or EMZ ($n = 7$) by intracellular cytokine staining. **B–D,** BMMCs from multiple myeloma patients ($n = 9$) were cultured for 36 hours in the presence of anti-CD3 (1 ng/mL) and/or anti-TGFβ1, and the frequency of PD-1$^+$ (**B**), T-bet$^+$ (**C**), or TCF-1$^+$ (**D**) cells among BM CD8$^+$ T cells was analyzed. **E–G,** BMMCs from multiple myeloma patients ($n = 9$) were labeled with CTV and stimulated with anti-CD3 (1 ng/mL) in the presence of anti–PD-1, TGFβ inhibitors such as anti–TGFβ1-neutralizing antibody (**F**) or galunisertib (**G**), or isotype control antibody for 108 hours. Representative histograms are presented (**E**). **H–J,** BMMCs from HLA-A2$^+$ multiple myeloma patients ($n = 8$) were labeled with CTV and stimulated with a mixture of NY-ESO-1$_{157\text{-}165}$ (SLLMWITQC) and HM1.24$_{22\text{-}30}$ (LLLGIGILV) peptides in the presence of anti–PD-1, TGFβ inhibitors such as anti–TGFβ1-neutralizing antibody (**I**) or galunisertib (**J**), or isotype control antibody for 144 hours. Representative figures are presented (**H**). The ratio of mitotic index with and without anti–PD-1 and TGFβ inhibitors (mitotic index with anti–PD-1 and TGFβ inhibitors/mitotic index with isotype control antibody) was calculated as follows: (mitotic index with anti–PD-1 and TGFβ inhibitors)/(mitotic index with controls). Lines represent the data from each patient, and bars represent mean values. **K,** BMMCs from a multiple myeloma patient harboring KRAS G12D mutation were labeled with CTV and stimulated with a mixture of 9 overlapping peptides (9-mer) spanning KRAS codon 12 of wild-type (YKLVVVGAGGVGK-SALT) or G12D mutant-type (YKLVVVGADGVGKSALT) in the presence of anti–PD-1, TGFβ inhibitors such as anti–TGFβ1-neutralizing antibody or galunisertib, or isotype control for 144 hours. **L,** MOPC315.BM myeloma cells were subcutaneously injected into Balb/c mice ($n = 11$ for each group), and isotype control antibodies, anti–PD-1 antibodies, anti-TGFβ antibodies, or a combination of anti–PD-1 with anti-TGFβ antibodies were administered every 3 days from 11 days after tumor injection. Tumor volume was measured 20 days after tumor injection. Error bars, SD. Statistical analysis was performed using the Mann–Whitney *U* test. *P* values were indicated. Statistical analysis was performed using the Mann–Whitney *U* test or Wilcoxon matched-pairs signed rank test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. MM, multiple myeloma; N.S., not significant.

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addition, TGFβ promotes angiogenesis and stimulates the generation of cancer-associated fibroblasts in the tumor microenvironment (35). TGFβ also inhibits antitumor immune responses, as TGFβ directly inhibits the proliferation and activities of macrophages and NK cells, $CD4^+$ helper T cells, and $CD8^+$ cytotoxic T cells, and promotes the proliferation and function of regulatory T cells and myeloid-derived suppressor cells (36). Moreover, TGFβ1 upregulates PD-1 on tumor reactive T cells and suppressed antitumor T-cell immunity (37).

Targeting the TGFβ pathway is an emerging strategy in cancer therapy. Galunisertib (LY2157299, Eli Lilly), a small-molecule inhibitor of TGFβ receptor I kinase, has an acceptable safety profile in phase I and II clinical trials (38–40). Several clinical trials with galunisertib are ongoing in hepatocellular carcinoma (NCT01246986; ref. 41) and pancreatic adenocarcinoma (NCT01373164; ref. 42). Another TGFβ kinase inhibitor, vactosertib (TEW-7197, MedPacto), is currently in clinical trials for relapsed or refractory multiple myeloma in combination with pomalidomide (NCT03143985).

Blocking immune-checkpoint receptors and TGFβ is a promising combination strategy for enhancing antitumor T-cell responses (43). TGFβ has been demonstrated to attenuate antitumor immune responses induced by PD-L1 blockade in bladder cancer patients (13). In addition, coinhibition of TGFβ and PD-1 promotes robust tumor regression in mouse tumor models (13, 14, 44). Clinical trials combining galunisertib with ICIs are currently under way in patients with non–small cell lung cancer, hepatocellular carcinoma, and pancreatic cancer (NCT02423343 and NCT02734160). Vactosertib is also expected to begin clinical trials in combination with anti–PD-L1 or anti–PD-1 for non–small cell lung cancer, gastric cancer, and colorectal cancer. In our data, blocking the TGFβ signal significantly enhanced anti–PD-1-induced functional restoration in BM $CD8^+$ T cells in multiple myeloma patients, suggesting a clinical trial testing coblockade of TGFβ and PD-1 in patients with multiple myeloma.

There are a few limitations to our study. First, the expression of myeloma antigens (NY-ESO-1 and HM1.24) in myeloma cells was not examined, although myeloma antigen–specific $CD8^+$ T cells were studied in multiple myeloma patients. HM1.24 is widely expressed in myeloma cells (45, 46). However, NY-ESO-1 is known to be expressed in < 30% of multiple myeloma patients (45–47). Compared with this low positive rate for NY-ESO-1 expression in historic data, NY-ESO-$1_{157\text{-}165}$–specific $CD8^+$ T cells were detected with a relatively high rate (20/29, 69.0%) in the present study. Therefore, investigating both myeloma antigen expression and myeloma antigen–specific $CD8^+$ T cells in same patients is necessary in the future study. Second, although the frequency of myeloma antigen–specific cells was very low in BM $CD8^+$ T cells from EMZL, MGUS, and SMM patients (Supplementary Fig. S6), $CD8^+$ T-cell proliferation in the presence of myeloma antigen peptides was higher in BMMCs from EMZL, MGUS, and SMM patients than in multiple myeloma patients (Supplementary Fig. S21). These data indicate that there might be antigen-nonspecific $CD8^+$ T-cell proliferation in our assay system, although BMMCs were *ex vivo* stimulated with myeloma antigen peptides. Antigen-nonspecific $CD8^+$ T-cell proliferation might result from bystander activation. Third, a relatively low number of MHC-I multimer$^+$$CD8^+$ T cells may hamper accurate phenotyping. Phenotypic analysis of myeloma antigen–specific $CD8^+$ T cells requires caution particularly when the frequency of MHC-I multimer$^+$$CD8^+$ T cells is low.

In summary, BM $CD8^+$ T cells and myeloma antigen–specific $CD8^+$ T cells express increased levels of PD-1 and have a terminally exhausted phenotype in multiple myeloma patients. Under TGFβ inhibition, anti–PD-1 reinvigorates BM $CD8^+$ T cells from multiple myeloma patients, but PD-1 blockade alone does not restore the function of BM $CD8^+$ T cells. Blocking both TGFβ and PD-1 can be a promising therapeutic strategy for the treatment of multiple myeloma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Kwon, C.G. Kim, H. Lee, I.-C. Song, D.-Y. Jo, J.S. Kim, I. Choi, E.-C. Shin
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