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Programmed Death Receptor-1/Programmed Death Receptor Ligand-1 Blockade after Transient Lymphodepletion To Treat Myeloma

Tyce J. Kearl,* Weiqing Jing,† Jill A. Gershan,† and Bryon D. Johnson*‡

Early phase clinical trials targeting the programmed death receptor-1/ligand-1 (PD-1/PD-L1) pathway to overcome tumor-mediated immunosuppression have reported promising results for a variety of cancers. This pathway appears to play an important role in the failure of immune reactivity to malignant plasma cells in multiple myeloma patients, as the tumor cells express relatively high levels of PD-L1, and T cells show increased PD-1 expression. In the current study, we demonstrate that PD-1/PD-L1 blockade with a PD-L1–specific Ab elicits rejection of a murine myeloma when combined with lymphodepleting irradiation. This particular combined approach by itself has not previously been shown to be efficacious in other tumor models. The antitumor effect of lymphodepletion/anti–PD-L1 therapy was most robust when tumor Ag–experienced T cells were present either through cell transfer or survival after nonmyeloablative irradiation. In vivo depletion of CD4 or CD8 T cells completely eliminated antitumor efficacy of the lymphodepletion/anti–PD-L1 therapy, indicating that both T cell subsets are necessary for tumor rejection. Elimination of myeloma by T cells occurs relatively quickly as tumor cells in the bone marrow were nearly nondetectable by 5 d after the first anti–PD-L1 treatment, suggesting that antitymoma reactivity is primarily mediated by preactivated T cells, rather than newly generated myeloma-reactive T cells. Anti–PD-L1 plus lymphodepletion failed to improve survival in two solid tumor models, but demonstrated significant efficacy in two hematologic malignancy models. In summary, our results support the clinical testing of lymphodepletion and PD-1/PD-L1 blockade as a novel approach for improving the survival of patients with multiple myeloma. The Journal of Immunology, 2013, 190: 5620–5628.

Multiple myeloma (MM) is an incurable B cell cancer arising from the monoclonal proliferation of malignant plasma cells. MM cells accumulate in the bone marrow (BM), secrete Ab, and cause progressive osteolytic bone disease and end-organ damage. Despite advances in treatment options, nearly all patients relapse and succumb to MM. Complicating the clinical management of relapsed MM are treatment-related toxicities and the frequent occurrence of drug-resistant tumor. Alternative treatment modalities to control or eradicate MM after relapse are an area of active research. Tumor immunotherapy, in particular, has exciting potential in MM as seen by clinical responses elicited by vaccination with cell-derived proteins (1).

Similar to other hematologic malignancies, MM establishes an immunosuppressive microenvironment that must be overcome for immunotherapy to be successful (2, 3). In studies that used a murine model of MM, 5T33, our laboratory recently showed that the programmed death receptor-1 (PD-1)/PD ligand-1 (PD-L1) pathway contributes to tumor-mediated suppression in vivo (4). PD-1 is a member of the Ig superfamily and is upregulated on activated T cells, B cells, NK cells, NKT cells, activated macrophages, and dendritic cells (DCs) (5). PD-L1 has two known ligands: PD-L1 (or B7-H1) and PD-L2 (or B7-DC), each with distinct and cell and tissue expression patterns. PD-L2 expression is restricted to APCs and some tumors (6, 7), whereas PD-L1 is expressed on T and B cells, APCs, various parenchymal cells, and on a wide variety of hematologic and solid tumor cancers, where its expression is generally a poor prognostic indicator (8–11). PD-L1 is rarely expressed on B cell malignancies (12), with MM the notable exception (4, 13). Although reports have shown that PD-L1 and PD-L2 can costimulate T cells in some conditions (14, 15), it is unknown if this effect is mediated through PD-1 or another receptor (16). The major effect of PD-1 ligation is inhibitory (17, 18), and PD-L expression by cancer cells impairs T cell–mediated antitumor immunity by inhibiting TCR signaling (19). Interestingly, PD-L1 also mediates T cell suppression through interactions with CD80 (16). Because PD-L1 binds two receptors, anti–PD-L1 blockade inhibits two inhibitory pathways on T cells. Anti–PD-1 blockade, in contrast, inhibits two ligands but only one pathway. It is unknown whether blocking PD-1 or PD-L1 would result in better antitumor immunity as the relative contributions of PD-L1/PD-1 and PD-L1/CD80 inhibition are unclear. Ab-based immunotherapies designed to block the immune-inhibitory effects of the PD-1/PD-L pathway have shown remarkable promise in recently reported clinical studies (20, 21).

In the J558L murine model of MM, PD-L1 blockade monotherapy delayed tumor growth but did not result in cure (22). Our laboratory previously showed that the 5T33 murine MM highly expresses PD-L1 and that T cells from 5T33-bearing mice have increased PD-1 expression and an exhausted phenotype (4). In that...
study, a multifaceted immunotherapy approach consisting of a tumor cell–based vaccine administered after hematopoietic stem cell (HSC) and T cell transfer was unsuccessful at treating established ST33 myeloma. However, the addition of a PD-L1–specific blocking Ab significantly improved immunotherapy efficacy and completely eliminated disease in ~40% of treated animals.

In the current study, we sought to further explore the use of PD-L1/ PD-1 blockade in antitymoma immunotherapy. We hypothesized that immune effector cells undergo robust proliferation in the radiation-induced lymphopenic environment and that anti–PD-L1 mAb treatment during the expansion phase overcomes PD-L1/PD-1–mediated tumor immunosuppression leading to successful tumor eradication. A pilot study showed increased survival when myeloma-bearing (MB) mice were given sublethal, nonmyeloablative total body irradiation and anti–PD-L1 mAb. These results were repeated in a larger series of experiments, and we found that the antitymoma response in this setting required both CD4 and CD8 T cells. In addition, the immune response was most efficacious when tumor Ag–experienced T cells were present during the lymphopenia–induced homeostatic proliferation phase. These results indicate that lymphodepletion and PD-L1/PD-1 blockade could be a relatively simplistic therapeutic approach to treating myeloma.

Materials and Methods

Mice

All mice were housed in the Medical College of Wisconsin Biomedical Resource Center, an American Association for the Accreditation of Laboratory Animal Care–accredited facility. C57BL/KaLwRij (KaLwRij), KaLwRij × C57BL/6.SJL (F1) mice, and B6.129S6-Rag2tm1Flj (Tg[TcraTcrb]1100Mjb) (OT-1) were bred in-house. BALB/cJ and C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal work was reviewed and approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Tumor cells

The ST33 murine MM cell line was derived from myeloma that spontaneously arose in a C57BL/KaLwRij mouse (23, 24). ST33GFP, a ST33 cell line that stably expresses emerald GFP, was created using the EmGFP control vector from the BLOCK-iT HiPerform Lentiviral Pol II mRNA Interference System with EmGFP (Invitrogen, Carlsbad, CA). No differences were noted in the tumorigenicity or survival of mice inoculated with either ST33 or ST33GFP. For experiments, ST33 or ST33GFP cells were thawed from a large frozen stock and cultured in RPMI 1640 plus 10% FBS for no longer than 2 wk prior to inoculation of mice. AGN2a murine neuroblastoma is an aggressive Neuro-2a variant, derived in our laboratory as previously described (25). B16F10 murine melanoma cells were provided by Dr. Samuel Hwang at the Medical College of Wisconsin. Mice were inoculated with tumor as follows: 2 × 10^5 ST33 or ST33GFP cells i.v., 1 × 10^6 B16F10 or AGN2a cells s.c., or 1 × 10^6 A20, EL4, or C1498 cells i.v. ST33 and ST33GFP-bearing mice were considered moribund and euthanized when they developed paraparesis or paraplegia. Occasionally, ST33-inoculated mice developed tumor masses or lesions and were euthanized once the size of the mass or lesion exceeded 250 mm^2; other symptoms of advanced ST33 included splenomegaly, hepatomegaly, or neurologic impairment. B16 and AGN2a tumors were monitored by caliper measurements, and mice were considered moribund and euthanized with tumor masses >250 mm^2. A body score was used to determine when mice bearing EL4, A20, or C1498 were moribund.

Abs and flow cytometry

Fluorochrome-labeled Abs to the following cell surface or intracellular proteins were obtained from eBioscience (San Diego, CA): CD11c (N418), CD19 (1D3), CD3 (145–2C11), CD4 (GK1.5), CD62L (MEL-14), CD8 (53-6.7), Foxp3 (FK3–6c), H-2Kb (AF6-88.5.5.3), I-Ab (AF6-120.2), NK1.1 (PK136), PD-1 (J43), and PD-L1 (MIH5). Isotype control Abs included Armenian hamster IgG1 and rat IgG2a Anti-rat IgG2b (G15-337) and Abs specific to CD44 (RM7) and CD45.1 (A20) were obtained from BD Biosciences (Franklin Lakes, NJ). For cell surface and viability staining, cells were resuspended in PBS plus 0.5% BSA (PBS/BSA) and stained with Ab and 7-aminocoumarin D (7-AAD; Calbiochem, San Diego, CA) for 10 min on ice. Unbound Ab was washed off by centrifugation, and cells were resuspended in PBS/BSA. Intracellular staining for Foxp3 was performed using eBioscience’s Foxp3 Transcription Factor Staining Buffer Set according to the included instructions. For apoptosis analysis, cells were resuspended in Annexin V binding buffer (eBioscience) and stained with a combination of Annexin V (eBioscience) and propidium iodide (BD Biosciences) or 7-AAD to discriminate viable from nonviable cells. After staining, cells were either analyzed within 4 h or resuspended and fixed in PBS/BSA plus 1% paraformaldehyde for later analysis. Fluorescence-minus-one and/or isotype controls were used as negative controls for flow cytometric analysis. Flow cytometry was performed on an LSRII flow cytometer (BD Biosciences), and the data were analyzed using FlowJo software version 6.4.7 (Tree Star, Ashland, OR).

In vivo lymphocyte depletion

Purified anti–PD-L1 (10F.9G2), control rat IgG2b (LTF2), anti-CD4 (GK1.5), anti-CD8 (2.43), and anti-NK1.1 (PK136) Abs were obtained from BioXCell (West Lebanon, NH). Mice were treated with 200 μg anti–PD-L1 or control IgG and/or 250 μg anti-CD4, anti-CD8, or anti-NK1.1 in 200 μl PBS i.p. at the indicated time points.

Hematopoietic stem cells and T cell enrichment

Femurs and tibias from either KaLwRij or (KaLwRij × C57Bl6/SJL) F1 mice were harvested, and BM cells were processed into single-cell suspensions. T cells for adoptive cell transfer (ACT) were enriched from splenocytes of either MB (8-48 h postinoculation with 2 × 10^5 tumor cells i.v.) or naive mice. Spleens were processed into single-cell suspensions by passing through wire mesh screens. RBCs were lysed by brief exposure to a hypo-osmotic solution. Total T cells were negatively enriched using an AutoMACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany) and the murine Pan T Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer’s directions. T cell purity was typically >85% based on flow cytometric analysis (data not shown).

Irradiation, HSC transplantation, and adoptive T cell transfer

In general, MB recipient mice were given total body irradiation as a single sublethal (500 cGy) or lethal (1100 cGy) dose. Radiation was administered by a Shepherd Mark I Cesium Irradiator in accordance with established guidelines. Twenty-four hours after total body irradiation, some mice received HSC transplantation and ACT as a single i.v. injection of 2.5–10 × 10^6 BM cells ± 5 to 6 × 10^6 enriched T cells.

IFN-γ ELISPOT

To assess for the presence of tumor-reactive IFN-γ–secreting CD8 T cells, T cells harvested from spleens and BM were purified by immunomagnetic sorting as described above. ELISPOT assays were done using the mouse IFN-γ ELISPOT Kit (BD Biosciences) as described previously (26).

Statistics

Survival curves were compared using the log-rank (Mantel Cox) test. Other experiments were compared using the Student t test or another test as noted in the figure legends. The p values <0.05 were considered significant. Statistical analysis was done using Prism version 5.0a software (GraphPad Software, La Jolla, CA).

Results

PD-1 expression is increased on tumor-specific T cells

Our previous work demonstrated that the PD-1/PD-L1 pathway is important in suppressing immune responses to ST33 MM and that PD-1 expression on splenic T cells is related to ST33 burden (4). However, we did not know if PD-1 was upregulated globally on T cells or only on myeloma-specific cells. To examine this, we compared expression of PD-1 on T cells from separate locations in moribund MB mice. As expected, T cells from MB mice had increased PD-1 expression in both the spleen and BM as compared with naive mice (Fig. 1A). Because PD-1 expression on T cells is known to be related to cell activation, we hypothesized that PD-1–1 T cells in the BM and spleen of MB mice represent tumor-specific T cells as has been shown with PD-1+ tumor-infiltrating lymphocytes (TILs) from melanoma patients (27). ST33 tumor Ags have not been identified, preventing us from directly addressing PD-1 expression levels on tumor Ag–specific T cells. Instead, we used an indirect approach in...
Splenocytes and femoral BM cells were analyzed by flow cytometry to determine PD-1 expression levels on host (CD45.1+) versus OT-1 (CD45.1) T cells. CD8 T cells were gated as CD3 +CD8+7-AAD−. These results demonstrated a potent synergism between non-specific T cells. OT-1 T cells express a transgenic TCR specific to the epitope SIINFEKL, which is not found in 5T33 cells. We hypothesized that if PD-1 expression is increased on all T cells in the tumor microenvironment, then transferred OT-1 T cells will upregulate PD-1 equivalently to host T cells. CD45.1−OT-1 T cells were adoptively transferred into sublethally irradiated host F1 (CD45.1+) mice, and the mice were challenged with 5T33 MM 1 wk later. T cells were analyzed when the mice became moribund, ∼4 to 5 wk after tumor challenge. In MB mice, whereas host CD8 T cells upregulated PD-1 expression in both the spleen and BM, OT-1 CD8 T cells did not (Fig. 1B, 1C). In naive mice, no differences were seen in the expression of PD-1 by either host or donor T cells. These results suggest that 5T33 myeloma contributes to the upregulation of PD-1 on tumor-specific T cells but not on tumor nonspecific T cells.

Anti–PD-L1 mAb treatment synergizes with lymphodepletive irradiation to facilitate a T cell–mediated antmyeloma response

Based on the results from Fig. 1, we hypothesized that PD-1+ T cells in MB mice represent functionally exhausted tumor-specific T cells capable of mediating antitumor immune responses upon blockade of the PD-1/PD-L1 pathway. Because PD-L1/PD-L1 blockade alone is not able to facilitate the elimination of established myeloma (4), we tested our hypothesis by using a combined approach of non-myeloablative total body irradiation and anti–PD-L1 Ab treatment.

Mice with established myeloma were treated with 500 cGy total body irradiation 1 wk after tumor cell inoculation, followed by a series of six anti–PD-L1 or control IgG injections as illustrated in Fig. 2A. Remarkably, sublethal total body irradiation and anti–PD-L1 treatment induced the rejection of myeloma in approximately two-thirds of mice (Fig. 2B). Mice treated with control IgG all died of myeloma progression. Subsequent experiments confirmed that irradiation was necessary for the antitymoma response, as mice given anti–PD-L1 treatment alone nearly all succumbed to myeloma progression (Fig. 2C). These results demonstrated a potent synergism between non-myeloablative total body irradiation and PD-L1 blockade.

Some of the 500 cGy–treated mice were euthanized at day 17 posttumor cell inoculation (or at the equivalent time postirradiation for naive mice), CD8 T cells isolated from spleen and BM and IFN-γ ELISPOT assays performed to determine frequencies of 5T33–specific T cells in these tissues. MB mice treated with anti–PD-L1 had significantly greater frequencies of tumor-reactive CD8 T cells in both the spleen and BM than IgG-treated control mice (Fig. 2D). This effect was augmented when anti–PD-L1 was added in vitro to the assay, indicating that PD-L1/PD-L1 blockade affects tumor cell/CTL interactions directly. The increase in 5T33–reactive CD8 T cells was tumor specific and not due to a generalized increase of T cell reactivity after anti–PD-L1 therapy, as few 5T33–reactive T cells were found in 5T33–naive mice treated with PD-L1.

Experiments were performed to assess the timing of the antitymoma response after total body irradiation and anti–PD-L1 treatment. For these experiments, mice were irradiated and treated as illustrated in Fig. 2A, and cohorts were euthanized at days 18 or 32 after 5T33 GFP inoculation to assess tumor burden. Myeloma cells could not be distinguished from background until day 18 in the BM, as shown in representative flow cytometric histograms (Fig. 3A, control IgG). By day 32 after tumor inoculation, tumor cells could be detected in both BM and spleen. Unexpectedly, total body irradiation and anti–PD-L1 treatment resulted in the elimination of tumor cells in the BM by day 18, which was only 5 d after the first anti–PD-L1 treatment (Fig. 3B).

To determine the extent of lymphodepletion achieved by 500 cGy total body irradiation, groups of mice were treated as shown in Fig. 4A. At days 0, 7, 12, 18, and 32 after myeloma inoculation, mice from each group were euthanized, spleen and BM cellularity assessed, and the splenocytes and marrow cells were phenotyped by flow cytometry. After irradiation, absolute cell counts in the spleen dropped dramatically and then largely recovered by day 32 (Fig. 4B). Absolute cell counts in the BM were less affected by irradiation. Little difference was seen between anti–PD-L1 and control IgG-treated mice, except for an increased cellularity at day 32 in the BM of anti–PD-L1 mice. This difference is likely due to different tumor burdens present in the two groups of mice because MM infiltration in BM displaces normal cells and decreases cellularity. Absolute numbers (Fig. 4C, solid lines) and percentages (Fig. 4C, dashed lines) of immune cell populations in the spleen

FIGURE 1. Increased PD-1 expression on CD8 T cells in the spleens and BM of MB mice occurs preferentially on T cells with the potential for tumor Ag reactivity. (A) KaLwRij mice were inoculated with 2 × 106 5T33 cells i.v. MB mice became moribund and were euthanized between days 28 and 40 after inoculation. Splenocytes and femoral BMs were harvested and the CD8 T cells analyzed by flow cytometry for PD-1 expression. At the same time points, naive (non-MB) mice were analyzed as controls. CD8 T cells were gated as CD3−CD8+7-AAD−. PD-1 percentage was based on isotype controls. Data are combined from four independent experiments; n = 13 (MB) and 4 (naive) mice. (B and C) (KaLwRij × B6.SJL) F1 mice were sublethally irradiated (500 cGy) and given 1.5 × 106 purified OT-1 CD8 splenocytes i.v. Some mice were inoculated 1 to 2 wk later with 2 × 106 5T33 cells i.v. MB mice were euthanized when they became moribund, between days 28 and 35 after inoculation; naive (non-MB) control mice were euthanized at the same time points. Splenocytes and femoral BM cells were analyzed by flow cytometry to determine PD-1 expression levels on host (CD45.1+) versus OT-1 (CD45.1−) CD8 T cells. CD8 T cells were gated as CD3−CD8+7-AAD−. (B) Representative flow cytometry dot/contour plots and histograms depicting PD-1 expression on host and transferred OT-1 CD8 T cells from MB and naive mice. (C) Percentages of PD-1+ host and OT-1 CD8 T cells in individual mice are shown. Each group contains two to six mice from two independent experiments, and the mean ± SEM for each group is shown. OT-1 T cells could only be found in the BM of two out of five MB mice and five out of six naive mice.

Some of the 500 cGy–treated mice served as a source of tumor nonspecific T cells. OT-1 T cells express a transgenic TCR specific for the epitope SIINFEKL, which is not found in 5T33 cells. We hypothesized that if PD-1 expression is increased on all T cells in the tumor microenvironment, then transferred OT-1 T cells will upregulate PD-1 equivalently to host T cells. CD45.1−OT-1 T cells were adoptively transferred into sublethally irradiated host F1 (CD45.1+) mice, and the mice were challenged with 5T33 MM 1 wk later. T cells were analyzed when the mice became moribund, ∼4 to 5 wk after tumor challenge. In MB mice, whereas host CD8 T cells upregulated PD-1 expression in both the spleen and BM, OT-1 CD8 T cells did not (Fig. 1B, 1C). In naive mice, no differences were seen in the expression of PD-1 by either host or donor T cells. These results suggest that 5T33 myeloma contributes to the upregulation of PD-1 on tumor-specific T cells but not on tumor nonspecific T cells.
were euthanized at day 17 for use in IFN-γ ELISPOT assays. (B and C) Survival curves showing the combined data from three (B) or four (C) independent experiments; n = 12–15 mice per experimental group. (D and E) CD8 T cells were isolated from spleens and BM 17 d after tumor inoculation or 10 d after irradiation (0 cGy) 7 d after tumor cell inoculation. The CD8 T cells were assayed in IFN-γ ELISPOT assays with tumor cell stimulators to determine tumor-reactive IFN-γ-secreting cell frequencies. Anti–PD-L1 or control IgG (10 μg/ml) was added to the assays in vitro. The graphs are representative of two independent experiments in which the CD8 T cells for each group were pooled from five individual mice. *p < 0.01, ***p < 0.001.

Figure 2. Sublethal irradiation and anti–PD-L1 administration facilitate the rejection of myeloma. (A) Experimental design. MB KaLwRij (B) or (KaLwRij × B6.SJL) F1 (C) mice received either 500 cGy or no irradiation (0 cGy) 7 d after tumor cell inoculation. Treatment with anti–PD-L1 or control IgG (200 μg i.p.) was initiated 5 d later and specifically given 12, 14, 19, 21, 26, and 28 d after tumor inoculation. Some mice were euthanized at day 17 for use in IFN-γ ELISPOT assays. (B and C) Survival curves showing the combined data from three (B) or four (C) independent experiments; n = 12–15 mice per experimental group. (D and E) CD8 T cells were isolated from spleens and BM 17 d after tumor inoculation or 10 d after irradiation (0 cGy) 7 d after tumor cell inoculation. The CD8 T cells were assayed in IFN-γ ELISPOT assays with tumor cell stimulators to determine tumor-reactive IFN-γ-secreting cell frequencies. Anti–PD-L1 or control IgG (10 μg/ml) was added to the assays in vitro. The graphs are representative of two independent experiments in which the CD8 T cells for each group were pooled from five individual mice. *p < 0.01, ***p < 0.001.

MB hosts contain tumor-reactive T cells in their lymphoid tissues as early as 8 d after tumor inoculation

Our laboratory previously showed that treatment of MB mice with a combination of lethal total body irradiation, HSC transplantation (BM plus added splenocytes), and PD-L1 blockade completely eliminated myeloma in up to 40% of animals, but cotreatment with a tumor vaccine was required to observe this effect (4). In those experiments, naive splenocytes were added to the BM graft to provide a source of T cells, because murine BM is relatively T cell deficient. Unlike lethal total body irradiation, the sublethal irradiation used in earlier figures provides nonmyeloablative lymphodepletion in which some endogenous lymphocytes survive irradiation and contribute to the reconstitution of the immune compartment. We speculated that some endogenous tumor Ag–experienced T cells survive the sublethal irradiation and are responsible for the antitumor response after anti–PD-L1 treatment, as suggested by the absence of tumor at day 18 in sublethal irradiation/anti–PD-L1–treated mice. As a way to begin addressing this possibility, we asked whether transfer of T cells from the spleens of MB mice (8 d after ST33 inoculation) could contribute to the antilymphoma response in lethally irradiated (1100 cGy) recipients.

An experimental design similar to that previously used in our laboratory was employed. Briefly, mice with established myeloma were treated with lethal irradiation (1100 cGy) 7 d after ST33 MM inoculation. Mice were rescued 1 d later by transfer of syngeneic BM supplemented with 5 to 6 × 10^6 T cells from either MB (8 d after ST33 inoculation) or naive syngeneic mice. Mice in each treatment group were then given a series of six anti–PD-L1 or control IgG treatments at the times indicated in Fig. 6A. The effect of T cell transfer from MB donors was surprisingly impressive in anti–PD-L1–treated mice. Whereas <25% of anti–PD-L1–treated mice given naive T cells survived long-term, 100% of anti–PD-L1–treated mice given T cells from MB mice survived to day 100 (Fig. 6B). In mice treated with control IgG, T cells from MB donors did statistically improve survival, but there were no long-term (100 d) survivors as seen in anti–PD-L1–treated mice. Together, the improved survival seen after transfer of tumor-experienced T cells indicates that tumor Ag–reactive T cells are required for effective antilymphoma immunity after irradiation and anti–PD-L1 treatment.

Although both activated T and NK cells are known to upregulate PD-1, we postulated that reactivation of exhausted T cells was responsible for the antilymphoma effect induced by sublethal total-body irradiation and anti–PD-L1. To determine which lymphocytes are involved in the antilymphoma response, in vivo–depleting Abs targeting CD4 T cells, CD8 T cells, or NK cells were administered to MB mice according to the schedule shown in Fig. 5A. By day 22, mice were depleted of >95% of the corresponding cell type (data not shown). Surprisingly, depletion of either CD4 or CD8 T cells completely abrogated the therapeutic efficacy of irradiation plus anti–PD-L1 (Fig. 5B); depletion of NK cells did not significantly affect therapeutic efficacy. Because the ST33 myeloma only expresses MHC class I proteins, these results suggest that CD8 effector T cells require the ongoing presence of CD4 T cell help to mount an effective immune response.

CD4 and CD8 T cells are required for effective antilymphoma immunity after irradiation and anti–PD-L1 treatment

We also examined the spleens of both MB and ST33-naive mice treated with sublethal total body irradiation and anti–PD-1 for possible effects on the percentages of PD-1+ T cells and Foxp3+ regulatory T cells over time in the spleen. The percentages of T cells expressing PD-1 were significantly increased over time in MB mice, but not in ST33-naive mice. In MB mice, anti–PD-L1 treatment did not result in increased PD-1 expression on T cells, whereas in ST33-naive mice, the percentage of CD4 T cells expressing PD-1 was increased after anti–PD-L1 treatment (Fig. 4D). The PD-1/PD-L1 pathway has been shown to be important in the generation of regulatory T cells (28). Splenic CD4+Foxp3+ T cell percentages increased after tumor inoculation but were minimally affected by anti–PD-L1 treatment (Fig. 4E).

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present in the lymphoid tissues of MB mice within 8 d after tumor inoculation.

Sublethal irradiation and anti–PD-L1 mAb treatment significantly improves the survival of mice bearing A20 B cell lymphoma or C1498 leukemia, but not mice bearing B16F10 melanoma, AGN2a neuroblastoma, or EL4 T cell lymphoma

PD-L1 is commonly expressed by tumors other than MM. The murine tumor cell lines B16F10, A20, EL4, and C1498, but not AGN2a neuroblastoma, express PD-L1 although not as highly as ST33 (Fig. 7A). PD-L1 expression was further increased after 48 h of culture in 100 ng/ml IFN-γ (data not shown). We tested whether sublethal irradiation and anti–PD-L1 treatment is able to improve the survival of mice bearing these other tumors. When lethal doses of these tumor cells were tested in a similar experimental strategy to that used for ST33 MM, sublethal irradiation and anti–PD-L1 treatment significantly improved survival in the A20 and C1498 models but was ineffective at eliminating tumors in the B16F10 and AGN2a models (Fig. 7B). Although EL4-bearing mice treated with irradiation and anti–PD-L1 survived longer, and tumor cells were eliminated in two mice, the improved survival was not statistically significant.

Discussion

We report that irradiation-induced lymphopenia combined with PD-L1–specific Ab treatment results in elimination of established murine ST33 MM. The antitumor effect is T cell dependent and requires both CD4 and CD8 T cell subsets. This combined therapy is potently synergistic as nonmyeloablative irradiation or anti–PD-L1 alone has no effect on myeloma progression. We hypothesize that the lymphopenic environment allows for the functional recovery of inactivated tumor-reactive T cells, whereas PD-L1 blockade prevents reactivation of the T cells via the PD-L1/PD-L1 pathway. This is the first report, to our knowledge, to document the efficacy of this combined treatment for myeloma.

Anti–PD-L1 Ab therapy after irradiation-induced lymphopenia has been shown to improve antitumor responses in other studies, but efficacy was only seen in the presence of concurrent vaccine administration. Hallett et al. (4) demonstrated improved survival in MB mice treated with a tumor-based vaccine plus anti–PD-L1 after lethal total body irradiation and HSC transplantation, but they saw no response in the absence of vaccine administration. Although the current study used a similar experimental design, the transfer of bulk splenocytes in the previous study may have resulted in the transfer of regulatory cell populations that inhibited successful antitumor responses. To address this possibility, future experiments are planned to determine the cells responsible for antitumor responses in the different settings (sublethal versus lethal irradiation). Pilon-Thomas et al. (29) investigated the addition of PD-L1–blocking Ab to a peptide-pulsed DC vaccine in the B16 and M05 murine melanoma models. Without total body irradiation, DC vaccination plus anti–PD-L1 had no impact on survival or tumor growth. However, when mice received 600 cGy total-body irradiation, delayed tumor growth was seen with DC vaccination plus anti–PD-L1; delayed tumor growth was not observed when only total body irradiation and anti–PD-L1 were administered, similar to our results in the B16 model shown in Fig. 7. Overall survival was significantly improved when tumor-specific or T cells from vaccinated donors were adoptively transferred into irradiated mice treated with the DC vaccine and anti–PD-L1. B16 is known to be a poorly immunogenic tumor, and it expresses lower levels of PD-L1 than ST33. This may explain why anti–PD-L1 monotherapy fails and why vaccination and transfer of tumor-specific or tumor Ag–primed T cells are needed to achieve efficacious antitumor responses in the B16 model.

A lymphopenic environment can be caused by lethal (myeloablative) or sublethal (nonmyeloablative) irradiation or by chemotherapy with drugs including cyclophosphamide, fludarabine, and melphalan (30–35). After lymphodepletion, lymphocytes undergo spontaneous, Ag-independent expansion called homeostatic proliferation that restores the prelymphodepletion lymphocyte compartment. In addition to endogenous lymphocytes, adoptively transferred T cells undergo homeostatic proliferation when placed in a lymphopenic environment (36). Although transferred HSCs can differentiate into T cells, thymopoiesis is inversely correlated with age, and only low levels are expected to occur in most MM patients, especially in the 2 mo following HSC transplantation; reconstitution of the lymphopenic compartment early after HSC transplantation is due to expansion of lymphopenia induction-resistant T cells and/or T cells present in the transplant inoculum (37–39). Adoptive cellular therapy after lymphodepletion conditioning is known to cause regression of established tumors in murine models (40–44). Although this effect has been noted for many years (45, 46), it was not until the past decade that the observation has been effectively translated to human trials. Early clinical studies using melanoma Ag (gp100)–specific au-

![Figure 3](image_url)
tologenous clonal CD8 T cells as ACT for the treatment of melanoma did not incorporate lymphodepletive conditioning and saw no complete responses (47). Subsequent phase I/II studies added chemotherapy-based nonmyeloablative conditioning and demonstrated the importance of modulating the host environment through lymphopenia. When rapidly expanded TILs containing polyclonal CD4 and CD8 T cells were used for ACT in combination with nonmyeloablative conditioning, objective responses reached 50% (31, 48). Lymphopenia contributes to effective ACT through several different mechanisms including an increased availability of immune stimulatory cytokines and the creation of an environment conducive to the disruption of T cell tolerance.

Homeostatic proliferation of various lymphocyte subsets is driven and influenced by the common γ-chain cytokines IL-7 and IL-15 (36, 49, 50). Immune stimulatory cytokines are more available after lymphodepletion (30, 51), possibly due to the ablation of cell populations acting as cytokine sinks (e.g., NK cells) or through upregulation of cytokine production by marrow and other cells in the periphery (44). In addition to affecting proliferation, IL-15 also enhances the antitumor effects of CD8 T cells used for ACT (52), and effective therapy by ACT positively corresponds with levels of common γ-chain cytokines in lymphodepleted hosts (43).

Recently, Schietinger et al. (53) showed that tolerized CD8-transgenic T cells transferred into sublethally irradiated (500 cGy) hosts were reactivated during homeostatic proliferation and responded to antigenic challenge. This response occurred whether the cognate Ag was present in the host. However, transferred T cells were retolerized after homeostatic proliferation ceased, and the host’s T cell compartment was re-established. The authors attribute this finding to epigenetic changes in the tolerant T cells that are overcome during homeostatic proliferation, but reassert themselves in the posthomeostatic proliferative environment, even in the absence of cognate Ag. We found in lethally irradiated mice that ACT with T cells from MB mice provided superior tumor rejection, suggesting that they were able to break tolerance; however, PD-L1 blockade was required to ensure elimination of myeloma. Some surviving mice from our experiments were rechallenged with

**FIGURE 4.** The time course of irradiation-induced lymphodepletion and the effect of irradiation and anti–PD-L1 treatment on PD-1 and Foxp3 expression. (A) Experimental design: MB (KaLwRij × B6.SJL) F1 mice were euthanized on the indicated days during treatment with irradiation and anti–PD-L1 or control IgG. Splenocytes and BM cells were harvested. (B) Absolute numbers of splenocytes and BM (femurs and tibias) are shown. (C) Absolute numbers (solid lines) and percentages (dotted lines) of the indicated splenocyte immune cell populations. T cells were gated as 7-AAD–CD3+; B cells were gated as 7-AAD–CD3+CD19–, DCs were gated as 7-AAD–CD3+CD11c+, and NK cells were gated as 7-AAD–CD3–NK1.1+. The effect of treatment at specific time points was compared using Student t test. (D) Percentages of splenic PD-1+ CD4 and CD8 T cells. (E) Percentages of splenic Foxp3+ CD4 T cells. The data are the combined results of one to three separate experiments; n = 4–14 mice per group at each time point. *p < 0.05.

**FIGURE 5.** The antimyeloma effect of sublethal irradiation and anti–PD-L1 is dependent upon both CD4 and CD8 T cells. (A) Experimental design: MB KaLwRij mice received in vivo–depleting Abs (250 μg i.p. of anti-CD4, anti-CD8, or anti-NK1.1) on days 6, 9, 12, 15, and 20 after 5T33 inoculation. The mice were irradiated (500 cGy) and treated with anti–PD-L1–blocking Ab as illustrated. Peripheral blood from some mice was obtained after administration of depleting Abs to verify depletion. In each case, >95% of the targeted cell population was depleted (data not shown). (B) The survival curves depict the combined results of three independent experiments; n = 12 to 13 mice per experimental group.
tumor 100 d after initial inoculation, and the animals uniformly rejected the secondary challenge without additional anti–PD-L1 treatment (data not shown). We hypothesize that tumor Ag-experienced T cells from the MB mice are able to break tolerance during homeostatic expansion and that retolerization is prevented by blockade of the PD-1/PD-L1 pathway, which could possibly alter protolerization epigenetic changes. This hypothesis will be tested in future studies.

Our results demonstrate that the combination of lymphopenia induced by nonmyeloablative irradiation and PD-L1/PD-1 blockade is sufficient for the generation of an effective antimyeloma immune response. Because the intensive high-dose therapy used prior to autologous HSC transplant is associated with increased side effects and mortality, reduced-intensity conditioning is attractive, particularly because most myeloma patients are elderly and more susceptible to the side effects. A randomized trial of previously untreated multiple myeloma patients directly compared high-dose and reduced-intensity conditioning regimens (54). Reduced-intensity conditioning resulted in less mucositis, a smaller duration of neutropenia and thrombocytopenia, a shorter hospital stay, fewer RBC and platelet transfusions, and a decreased need for antibiotic administration. The possibility of fewer treatment-induced complications, however, must be weighed against data showing increased responses to ACT with higher intensity preparative regimens. For example, response rates to ACT with TILs for metastatic melanoma increased to 52 and 72% after addition of 200 and 1200 cGy total body irradiation, respectively, to the preparative regimen (51).

Bracci et al. (44) showed that ex vivo depletion of CD4 T cells, but not CD8 T cells, NK cells, or macrophages from transferred splenocytes abrogated the enhanced antitumor response seen with lymphodepletion plus ACT in a murine melanoma model. Similarly, we found that in vivo depletion of CD4 cells eliminated the antimyeloma response that was induced by lymphodepletion and PD-L1 blockade, despite the fact that 5T33 cells only express MHC class I proteins (no class II). This finding was surprising because we hypothesized that the antimyeloma effect was due to the reactivation of PD-1+ T cells and not through the generation of new effectors. We therefore expected that depletion of only CD8 T cells would eliminate the antimyeloma response. If our hypothesis is correct, the data suggest that CD4 T cells provide necessary help to facilitate the reactivation of tolerized CD8 T cells. Just how this help is mediated is unclear. Much of the focus of early ACT clinical trials was on the transfer of autologous CD8 T cells only. The incomplete clinical efficacy of CD8 T cell–only ACT may reflect the need to transfer appropriate Th cells (51, 55). Schmidmaier et al. (56) showed that Th cell numbers in autologous HSC transplants were positively correlated with event-free survival in MM. Several studies have shown that CD4 T cells are required for the development of antitumor memory after ACT and HSC transplantation (26, 57, 58), further underscoring the need for CD4 T cells in ACT. Although high-intensity conditioning results in increased lymphodepletion, it is unlikely that irradiation or chemotherapy-based conditioning can completely remove immune regulatory elements. Therefore, other methods of
overcoming regulatory elements, such as PD-1/PD-L blockades, could result in synergistic anti-tumor immunity, similar to the synergism observed in our study.

In conclusion, our data show that lymphodepletion and PD-L1 blockade synergize to eradicate MM. Although this therapy was highly effective at eliminating established myeloma, it did not eliminate disease in all of the animals. However, we believe that this combined immunotherapeutic approach could serve as an exciting clinical platform for other immunotherapies, including ACT, to achieve even better outcomes. The results we obtained in other tumor models (A20 and C1498) are encouraging, as they demonstrate that the antitumor effect obtained with this combination therapy is not limited to murine myeloma and may have a role in the treatment of other hematologic malignancies. These observations provide support for the clinical testing of therapeutic strategies involving lymphodepleting conditioning and PD-1/PD-L1 pathway blockade.

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Disclosures
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