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PD-1/PD-L1 Blockade after Transient Lymphodepletion to Treat Myeloma

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Abstract

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 antibody 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 anti-tumor effect of lymphodepletion/anti-PD-L1 therapy was most robust when tumor antigen-experienced T cells were present either through cell transfer or survival after non-myeloablative irradiation. *In vivo* depletion of CD4 or CD8 T cells completely eliminated anti-tumor 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 non-detectable by five days after the first anti-PD-L1 treatment, suggesting that anti-myeloma reactivity is primarily mediated by pre-activated 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.

INTRODUCTION

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 antibody, 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

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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 utilized a murine model of MM, 5T33, our lab recently showed that the programmed death-1 (PD-1)/PD-ligand-1 (PD-L1) pathway contributes to tumor-mediated suppression *in vivo* (4). PD-1 is a member of the immunoglobulin superfamily and is upregulated on activated T cells, B cells, NK and NKT cells, activated macrophages, and dendritic cells (5). PD-1 has two known ligands: PD-L1 (or B7-H1) and PD-L2 (or B7-DC); each with distinct cell and tissue expression patterns. PD-L2 expression is restricted to APCs and some tumors (6, 7), while 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 co-stimulate 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 anti-tumor 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, on the other hand, inhibits two ligands but only one pathway. It is unknown whether blocking PD-1 or PD-L1 would result in better anti-tumor immunity as the relative contributions of PD-L1:PD-1 and PD-L1:CD80 inhibition are unclear. Antibody-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 lab 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 5T33 myeloma. However, the addition of a PD-L1-specific blocking antibody significantly improved immunotherapy efficacy and completely eliminated disease in approximately 40% of treated animals.

In the current study, we sought to further explore the use of PD-L1/PD-1 blockade in anti-myeloma 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 mice were given sublethal, non-myeloablative total body irradiation and anti-PD-L1 mAb. These results were repeated in a larger series of experiments, and we found that the anti-myeloma response in this setting required both CD4 and CD8 T cells. In addition, the immune response was most efficacious when tumor antigen-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 AAALAC-accredited facility. C57BL/KaLwRij (KaLwRij), KaLwRij x C57BL/6.SJL (F1) mice, and B6.129S6-*Rag2*^{tm1Fwa}Tg(TcraTcrb)1100Mjb (OT-1) were bred in house. Balb/cJ and C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All animal work was reviewed and approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Tumor cells

The 5T33 murine MM cell line was derived from myeloma that spontaneously arose in a C57Bl/KaLwRij mouse (23, 24). 5T33GFP, a 5T33 line that stably expresses emerald green fluorescent protein (GFP), was created using the EmGFP control vector from the BLOCK-iT HiPerform Lentiviral Pol II miR RNAi Expression System with EmGFP (Invitrogen, Carlsbad, CA). No differences were noted in the tumorigenicity or survival of mice inoculated with either 5T33 or 5T33GFP. For experiments, 5T33 or 5T33GFP cells were thawed from a large frozen stock and cultured in RPMI 1640 + 10% fetal bovine serum for no longer than 2 weeks 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: 2×10^6 5T33 or 5T33GFP cells intravenously (*i.v.)*; 1×10^5 B16F10 or AGN2a cells *s.c.*; 1×10^6 A20, EL4 or C1498 cells *i.v.* 5T33 and 5T33GFP-bearing mice were considered moribund and euthanized when they developed paraparesis or paraplegia. Occasionally, 5T33-inoculated mice developed tumor masses or lesions and were euthanized when the size of the mass or lesion exceeded 250 mm^2; other symptoms of advanced 5T33 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.

Antibodies and flow cytometry

Fluorochrome-labeled monoclonal antibodies 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 (FJK-16s), H-2Kb (AF6-88.5.5.3), I-Ab (AF6-120.2), NK1.1 (PK136), PD-1 (J43), and PD-L1 (MIH5). Isotype control antibodies included Armenian hamster IgG and rat IgG2a kappa. Anti-rat IgG2b (G15-337) and antibodies specific to CD44 (IM7) and CD45.1 (A20) were obtained from BD Biosciences (Franklin Lakes, NJ). For cell surface and viability staining, cells were resuspended in PBS + 0.5% BSA (PBS/BSA) and stained with antibody and 7-aminoactinomycin D (7AAD) (Calbiochem, San Diego, CA) for 10 minutes on ice. Unbound antibody 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 7AAD to discriminate viable from non-viable cells. After staining, cells were either analyzed within 4 hours or resuspended and fixed in PBS/BSA + 1% paraformaldehyde for later analysis. Fluorescence-minus-one (FMO) 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 (TreeStar Inc., 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) antibodies were obtained from Bio X Cell (West Lebanon, NH). Mice were treated with 200 μg of anti-PD-L1 or control IgG, and/or 250 μg of anti-CD4, anti-CD8 or anti-NK1.1, in 200 μl PBS intraperitoneally (*i.p.)* at the indicated time points.

Hematopoietic stem cells and T cell enrichment

Femurs and tibias from either KaLwRij or (KaLwRij × C57BL/6.SJL) F1 mice were harvested, and bone marrow cells were processed into single cell suspensions. T cells for adoptive cell transfer (ACT) were enriched from splenocytes of either myeloma-bearing (eight days post-inoculation with 2×10^6 tumor cells *i.v.*) or naïve mice. Spleens were processed into single cell suspensions by pressing through wire mesh screens. Red blood cells were lysed by brief exposure to a hypoosmotic 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, myeloma-bearing 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 $2.5\text{-}10\times10^6$ bone marrow cells plus/minus $5\text{-}6\times10^6$ 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 bone marrow 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 Student's T-test or another test as noted in the figure legends. P-values of <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 5T33 MM and that PD-1 expression on splenic T cells is related to 5T33 burden (4). However, we did not know if PD-1 were 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 myeloma-bearing mice. As expected, T cells from myeloma-bearing mice had increased PD-1 expression in both the spleen and bone marrow as compared to naïve mice (Fig. 1A). Since PD-1 expression on T cells is known to be related to cell activation, we hypothesized that PD-1^+ T cells in the bone marrow and spleen of myeloma-bearing mice represent tumor-specific T cells as has been shown with PD-1^+ tumor-infiltrating lymphocytes (TILs) from melanoma patients (27). 5T33 tumor antigens

have not been identified, preventing us from directly addressing PD-1 expression levels on tumor antigen-specific T cells. Instead, we used an indirect approach where CD8 T cells from OT-1 mice served as a source of tumor non-specific 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 one week later. T cells were analyzed when the mice became moribund, approximately 4-5 weeks after tumor challenge. In myeloma-bearing (MB) mice, while host CD8 T cells upregulated PD-1 expression in both the spleen and bone marrow, OT-1 CD8 T cells did not (Fig. 1B, C). In naïve 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 non-specific T cells.

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

Based on the results from Figure 1, we hypothesized that PD-1^{+} T cells in myeloma-bearing mice represent functionally exhausted tumor-specific T cells capable of mediating anti-tumor immune responses upon blockade of the PD-1/PD-L1 pathway. Since PD-L1/PD-1 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 antibody treatment. Mice with established myeloma were treated with 500 cGy total body irradiation one week after tumor cell inoculation, followed by a series of six anti-PD-L1 or control IgG injections as illustrated in Figure 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 from myeloma progression. Subsequent experiments confirmed that irradiation was necessary for the anti-myeloma 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 post-tumor cell inoculation (or at the equivalent time post-irradiation for naïve mice), CD8 T cells isolated from spleen and bone marrow, and IFN-γ ELISPOT assays performed to determine frequencies of 5T33-specific T cells in these tissues. Myeloma-bearing mice treated with anti-PD-L1 had significantly greater frequencies of tumor-reactive CD8 T cells in both the spleen and bone marrow 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-1 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-naïve mice treated with PD-L1.

Experiments were performed to assess the timing of the anti-myeloma response after total body irradiation and anti-PD-L1 treatment. For these experiments, mice were irradiated and treated as illustrated in Figure 2A, and cohorts were euthanized at days 18 or 32 after 5T33GFP inoculation to assess tumor burden. Myeloma cells could not be distinguished from background until day 18 in the bone marrow, as shown in representative flow cytometric histograms (Fig. 3A; control IgG). By day 32 after tumor inoculation, tumor cells could be detected in both bone marrow and spleen. Unexpectedly, total body irradiation and anti-PD-L1 treatment resulted in the elimination of tumor cells in the bone marrow by day 18, which was only 5 days 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 Figure 4A. At days 0, 7, 12, 18, and 32 after myeloma inoculation, mice from each group were euthanized, spleen and bone marrow 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 bone marrow 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 bone marrow of anti-PD-L1 mice. This difference is likely due to different tumor burdens present in the two groups of mice since MM infiltration in bone marrow 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 (CD4 and CD8 T cells, B cells, NK cells, and DC) generally followed the same pattern of decreased counts immediately post-irradiation followed by gradual reconstitution. Although the different immune cell types had different sensitivities to irradiation, anti-PD-L1 treatment had no impact on cell reconstitution, with the exception of NK cells. No treatment-related differences were seen in the percentages of cells undergoing apoptosis or in naïve/effector memory/central memory T cell frequencies (based on CD62L and CD44 immunophenotyping) (data not shown).

We also examined the spleens of both myeloma-bearing and 5T33-naïve 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 myeloma-bearing mice, but not in 5T33-naïve mice. In myeloma-bearing mice, anti-PD-L1 treatment did not result in increased PD-1 expression on T cells whereas in 5T33-naïve 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).

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

While both activated T and NK cells are known to upregulate PD-1, we postulated that re-activation of ‘exhausted’ T cells was responsible for the anti-myeloma effect induced by sublethal total body irradiation and anti-PD-L1. To determine which lymphocytes are involved in the anti-myeloma response, *in vivo*-depleting antibodies targeting CD4 T cells, CD8 T cells, or NK cells were administered to myeloma-bearing mice according to the schedule shown in Figure 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. Since the 5T33 myeloma only expresses MHC class I proteins, these results suggest that CD8 effector T cells require the ongoing presence of CD4 T cell ‘help’ in order to mount an effective immune response.

Myeloma-bearing hosts contain tumor-reactive T cells in their lymphoid tissues as early as 8 days after tumor inoculation

Our laboratory previously showed that treatment of myeloma-bearing mice with a combination of lethal total body irradiation, HSC transplantation (bone marrow plus added splenocytes), and PD-L1 blockade completely eliminated myeloma in up to 40% of animals, but co-treatment with a tumor vaccine was required to observe this effect (4). In those experiments, naïve splenocytes were added to the bone marrow graft to provide a source of T cells, since murine bone marrow is relatively T cell-deficient. Unlike lethal total body

irradiation, the sublethal irradiation used in earlier figures provides non-myeloablative lymphodepletion in which some endogenous lymphocytes survive irradiation and contribute to the reconstitution of the immune compartment. We speculated that some endogenous tumor antigen-experienced T cells survive the sublethal irradiation and are responsible for the anti-tumor 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 myeloma-bearing mice (8 days after 5T33 inoculation) could contribute to the anti-myeloma 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) seven days after 5T33 MM inoculation. Mice were rescued one day later by transfer of syngeneic bone marrow supplemented with 5-6×10^6 T cells from either myeloma-bearing (8 days after 5T33 inoculation) or naïve 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 Figure 6A. The effect of T cell transfer from myeloma-bearing donors was surprisingly impressive in anti-PD-L1-treated mice. Whereas less than 25% of anti-PD-L1-treated mice given naïve T cells survived long-term, 100% of anti-PD-L1-treated mice given T cells from myeloma-bearing mice survived to day 100 (Fig. 6B). In mice treated with control IgG, T cells from myeloma-bearing donors did statistically improve survival, but there were no long-term (100 day) survivors as seen in anti-PD-L1 treated mice. Together, the improved survival seen after transfer for tumor-experienced T cells indicates that tumor antigen-reactive T cells are present in the lymphoid tissues of myeloma-bearing mice within 8 days after tumor inoculation.

Sublethal irradiation and anti-PD-L1 mAb treatment is 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 5T33 (Fig. 7A). PD-L1 expression was further increased after 48 hours 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 5T33 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 antibody treatment results in elimination of established murine 5T33 MM. The anti-myeloma effect is T cell-dependent and requires both CD4 and CD8 T cell subsets. This combined therapy is potently synergistic as non-myeloablative 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, while PD-L1 blockade prevents re-inactivation of the T cells via the PD-1/PD-L1 pathway. This is the first report to document the efficacy of this combined treatment for myeloma.

Anti-PD-L1 antibody therapy after irradiation-induced lymphopenia has been shown to improve anti-tumor responses in other studies, but efficacy was only seen in the presence of concurrent vaccine administration. Hallett et al. demonstrated improved survival in myeloma-bearing mice treated with 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 (4). 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 anti-tumor responses. To address this possibility, future experiments are planned to determine the cells responsible for anti-tumor responses in the different settings (sublethal versus lethal irradiation). Pilon-Thomas et al. investigated the addition of PD-L1 blocking antibody to a peptide-pulsed DC vaccine in the B16 and M05 murine melanoma models (29). 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 Figure 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-Ll. B16 is known to be a poorly immunogenic tumor and it expresses lower levels of PD-L1 than 5T33. This may explain why anti-PD-L1 monotherapy fails and why vaccination and transfer of tumor-specific or tumor antigen-primed T cells are needed to achieve efficacious anti-tumor responses in the B16 model.

A lymphopenic environment can be caused by lethal (myeloablative) or sublethal (non-myeloablative) irradiation or by chemotherapy with drugs including cyclophosphamide, fludarabine, and melphalan (30-35). After lymphodepletion, lymphocytes undergo spontaneous, antigen-independent expansion called homeostatic proliferation that restores the pre-lymphodepletion 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 two months 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 lymphodepleting 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 antigen (gp100)-specific autologous 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 non-myeloablative conditioning and demonstrated the importance of modulating the host environment through lymphodepletion. When rapidly-expanded TILs containing polyclonal CD4 and CD8 T cells were used for ACT in combination with non-myeloablative conditioning, objective responses reached 50 percent (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 gamma 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 (ex. 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 anti-tumor effects of CD8 T cells used for ACT (52), and effective therapy by ACT positively corresponds with levels of common gamma chain cytokines in lymphodepleted hosts (43).

Recently, Schietinger et al. showed that tolerized CD8 transgenic T cells transferred into sublethally irradiated (500 cGy) hosts were reactivated during homeostatic proliferation and responded to antigenic-challenge (53). This response occurred whether or not the cognate antigen was present in the host. However, transferred T cells were re-tolerized after homeostatic proliferation ceased and the host's T cell compartment was reestablished. The authors attribute this finding to epigenetic changes in the tolerant T cells that are overcome during homeostatic proliferation, but re-assert themselves in the post-homeostatic proliferative environment, even in the absence of cognate antigen. We found in lethally irradiated mice that ACT with T cells from myeloma-bearing 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 re-challenged with tumor 100 days after initial inoculation, and the animals uniformly rejected the secondary challenge without additional anti-PD-L1 treatment (data not shown). We hypothesize that tumor antigen-experienced T cells from the myeloma-bearing mice are able to break tolerance during homeostatic expansion and that re-tolerization is prevented by blockade of the PD-1/PD-L1 pathway, which could possibly alter pro-tolerization epigenetic changes. This hypothesis will be tested in future studies.

Our results demonstrate that the combination of lymphopenia induced by non-myeloablative irradiation and PD-L1/PD-1 blockade is sufficient for the generation of an effective anti-myeloma immune response. Since the intensive high-dose therapy used prior to autologous HSCT is associated with increased side effects and mortality, reduced-intensity conditioning is attractive, particularly since 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 red blood cell 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 percent after addition of 200 and 1200 cGy total body irradiation, respectively, to the preparative regimen (51).

Bracci et al. showed that *ex vivo* depletion of CD4 T cells, but not CD8 T cells, NK cells, or macrophages from transferred splenocytes abrogated the enhanced anti-tumor response seen with lymphodepletion plus ACT in a murine melanoma model (44). Similarly, we found that *in vivo* depletion of CD4 cells eliminated the response to 5T33 MM 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 since we hypothesize that the anti-myeloma effect is due to the re-activation 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 anti-myeloma response. If our hypothesis is correct, the data suggests that CD4 T cells provide necessary ‘help’ to facilitate the re-activation 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 T helper cells (51, 55). Schmidmaier et al. showed that helper T cell numbers in autologous HSC transplants were positively correlated with event free survival in MM (56). Several studies have shown that CD4 T cells are required for the development of anti-tumor 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 blockade, could result in synergistic anti-tumor immunity, similar to the synergism observed here in our study.

In conclusion, our data show that lymphodepletion and PD-L1 blockade synergize to eradicate MM. While 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) is encouraging as it demonstrates that the anti-tumor 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 lymphodepletive-conditioning and PD-1/PD-L pathway blockade.

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Abbreviations used in this article

PD-L1/2	programmed death ligand-1
PD-1	programmed death-1
MM	multiple myeloma
MB	myeloma-bearing
7AAD	7-aminoactinomycin D
ACT	adoptive cell transfer
HSC	hematopoietic stem cell
NK	natural killer cell
DC	dendritic cell
TIL	tumor infiltrating lymphocyte

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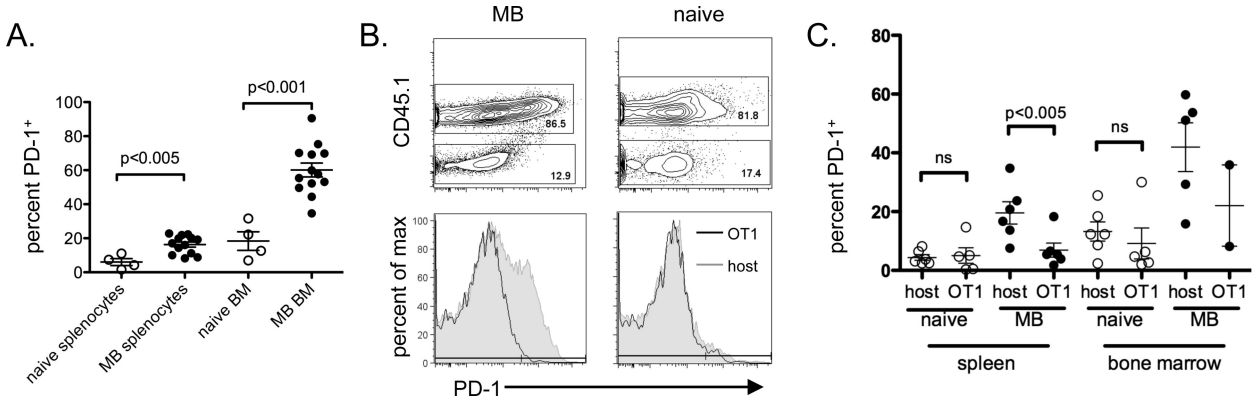

Figure 1. Increased PD-1 expression on CD8 T cells in the spleens and bone marrow of myeloma-bearing mice occurs preferentially on T cells with the potential for tumor antigen reactivity

A. KaLwRij mice were inoculated with 2×10^6 5T33 cells *i.v*. Myeloma-bearing (MB) mice became moribund and were euthanized between days 28-40 after inoculation. Splenocytes and femoral bone marrows were harvested and the CD8 T cells analyzed by flow cytometry for PD-1 expression. At the same time points, naïve (non-myeloma-bearing) mice were analyzed as controls. CD8 T cells were gated as $CD3^+CD8^+7AAD^-$. PD-1 percentage was based on isotype controls. Data are combined from 4 independent experiments; n=13 (MB) and 4 (naive) mice. **B. and C**. (KaLwRij × B6.SJL) F1 mice were sublethally irradiated (500 cGy) and given 1.5×10^6 purified OT-1 CD8 splenocytes *i.v*. Some mice were inoculated 1-2 weeks later with 2×10^6 5T33 cells *i.v*. Myeloma-bearing (MB) mice were euthanized when they became moribund, between days 28-35 after inoculation; naive (non-myeloma-bearing) control mice were euthanized at the same time points. Splenocytes and femoral bone marrow 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 $CD3^+CD8^+7AAD^-$. **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 naïve mice. **C**. Percentages of $PD\text{-}1^+$ host and OT-1 CD8 T cells in individual mice are shown. Each group contains of 2-6 mice from 2 independent experiments, and the mean +/− SEM for each group is shown. OT-1 cells could only be found in the bone marrow of 2/5 MB mice and 5/6 naive mice.

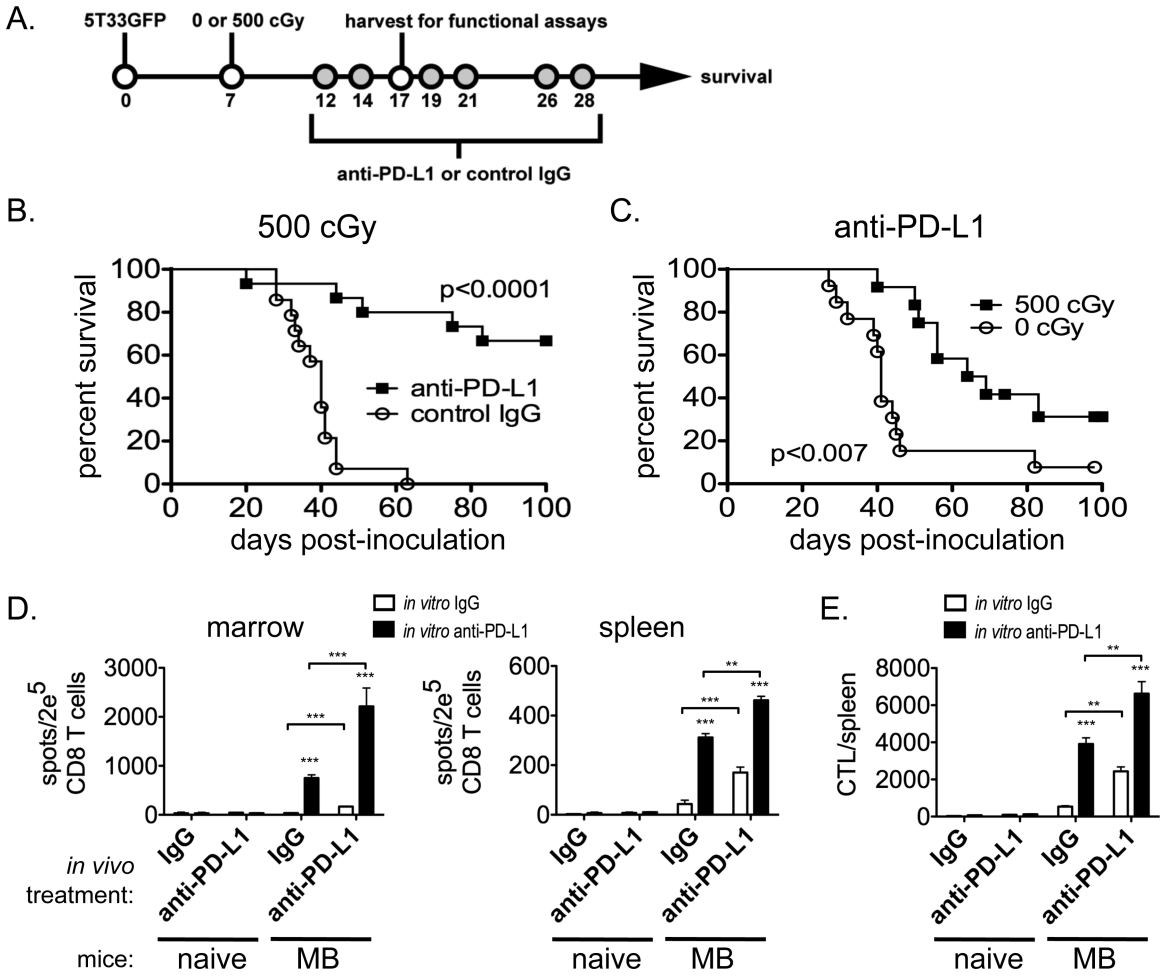

Figure 2. Sublethal-irradiation and anti-PD-L1 administration facilitate the rejection of myeloma

A. Experimental design: Myeloma-bearing KaLwRij (**B**) or (KaLwRij × B6.SJL) F1 (**C**) mice received either 500 cGy or no irradiation (0 cGy) seven days after tumor cell inoculation. Treatment with anti-PD-L1 or control IgG (200 μg *i.p.*) was initiated 5 days later, and specifically given 12, 14, 19, 21, 26 and 28 days after tumor inoculation. Some mice were euthanized at day 17 for use in IFN-γ ELISPOT assays (**D** & **E**). **B** & **C**. Survival curves from showing the combined data from 3 (**B**) or 4 (**C**) independent experiments; n=12-15 mice per experimental group. **D** & **E**. CD8 T cells were isolated from spleens and BM 17 days after tumor inoculation or 10 days after irradiation in naïve mice treated with anti-PD-L1 or control IgG. 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 2 independent experiments in which the CD8 T cells for each group were pooled from 5 individual mice. ***p<0.001; **p<0.01.

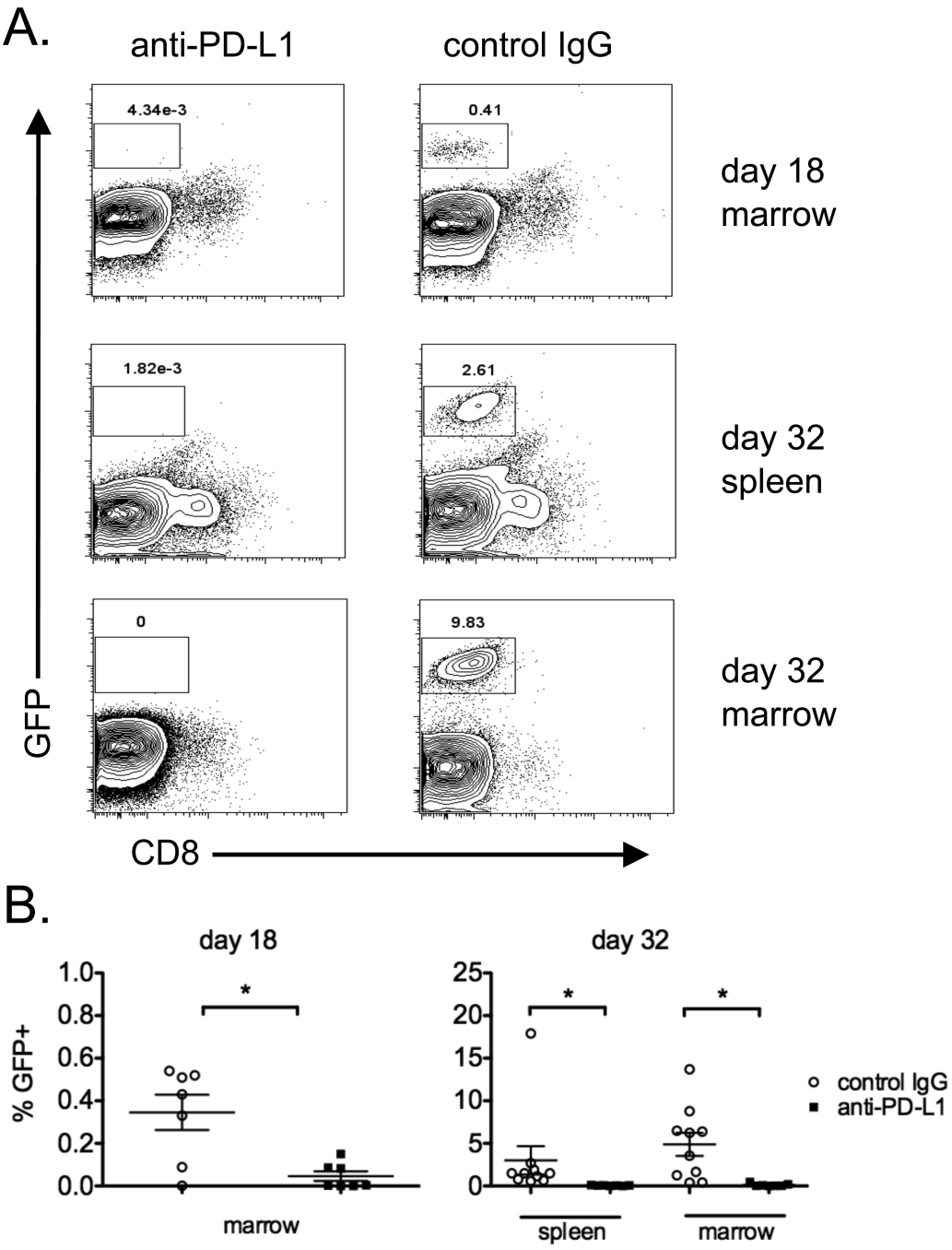

Figure 3. The anti-myeloma effect induced by sublethal irradiation and anti-PD-L1 occurs relatively quickly

Myeloma-bearing mice were treated with 500 cGy irradiation on day 8 after 5T33GFP inoculation, and anti-PD-L1 or control IgG was administered on days 13, 15, 20, 22, 27 and 29. Mice were euthanized on days 18 and 32 and spleens and BM were harvested. The tissues were analyzed by flow cytometry to detect the presence of 5T33GPF. **A**. Representative flow cytometry dot/contour plots depicting GFP expression (tumor cells) on the y-axis and CD8 on the x-axis. **B**. The percentages of GFP+ 5T33 myeloma cells in the tissues of individual experimental mice are shown. Spleens were not analyzed on day 18 due to the inability to detect myeloma at this time point. The data is representative of 2 separate

experiments; n=7-10 mice per group at each time point. Groups were compared using the Mann Whitney test.

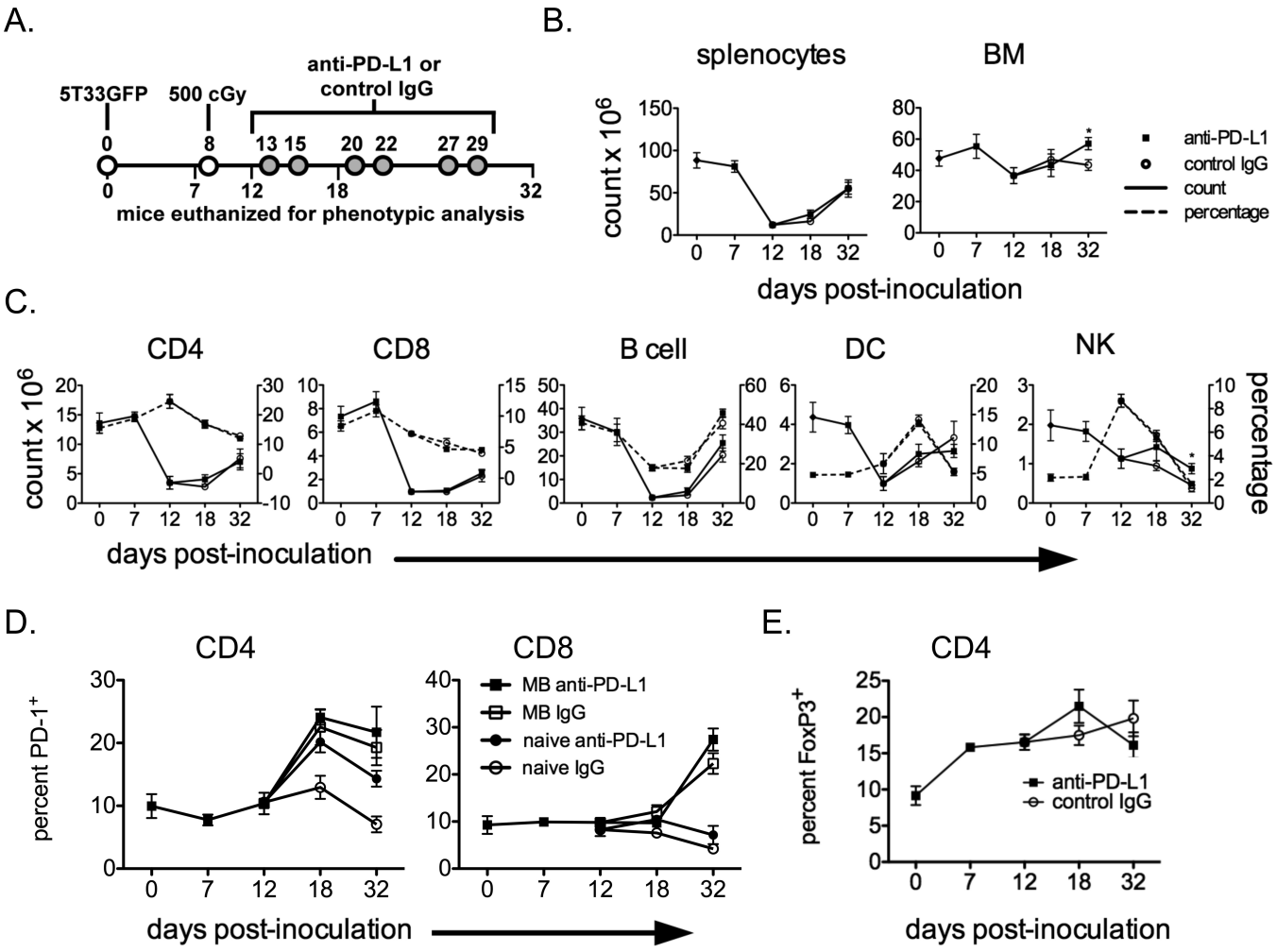

Figure 4. The time course of irradiation-induced lymphodepletion and the effect of irradiation and anti-PD-L1 treatment on PD-1 and FoxP3 expression

A. Experimental design: Myeloma-bearing (KaLwRij × B6.SJL) F1 mice were euthanized on the indicated days during treatment with irradiation and anti-PD-L1 or control IgG. Splenocytes and bone marrow cells were harvested. **B**. Absolute numbers of splenocytes and bone marrow (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 $7AAD^{-}CD3^{+}$, B cells were gated as $7AAD^{-}CD3^{-}CD19^{+}$, DC were gated as $7AAD^{-}CD3^{-}CD11c^{+}$, and −NK cells were gated as $7AAD^{-}CD3^{-}NK1.1^{+}$. The effect of treatment at specific time points was compared using the Student's T test (*$p<0.05$). **D**. percentages of splenic $PD\text{-}1^{+}$ CD4 and CD8 T cells. **E**. Percentages of splenic $Foxp3^{+}$ CD4 T cells. The data are the combined results of 1-3 separate experiments; n=4-14 mice per group at each time point.

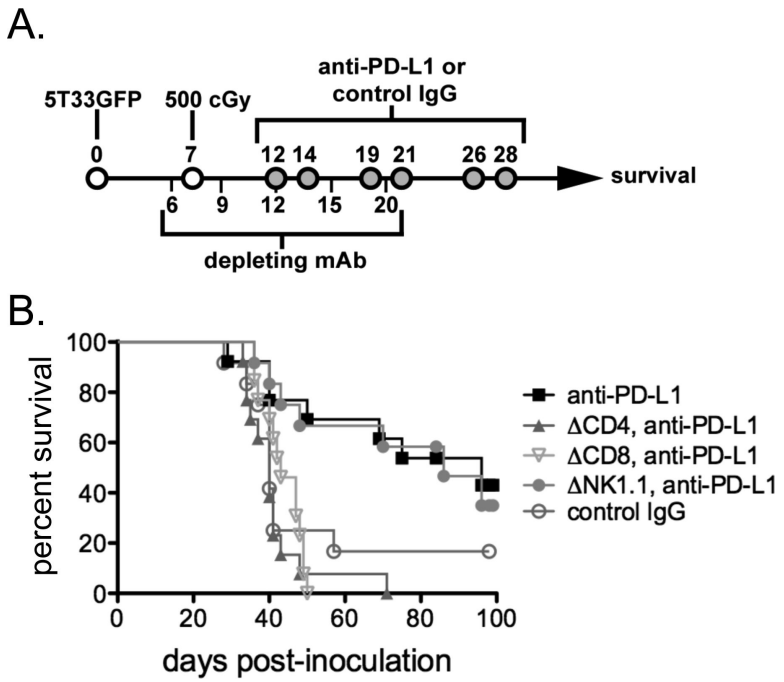

Figure 5. The anti-myeloma effect of sublethal irradiation and anti-PDL1 is dependent upon both CD4 and CD8 T cells

A. Experimental design: Myeloma-bearing KaLwRij mice received *in vivo* depleting antibodies (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-PDL1 blocking antibody as illustrated. Peripheral blood from some mice was obtained after administration of depleting antibodies 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 3 independent experiments; n=12-13 mice per experimental group.

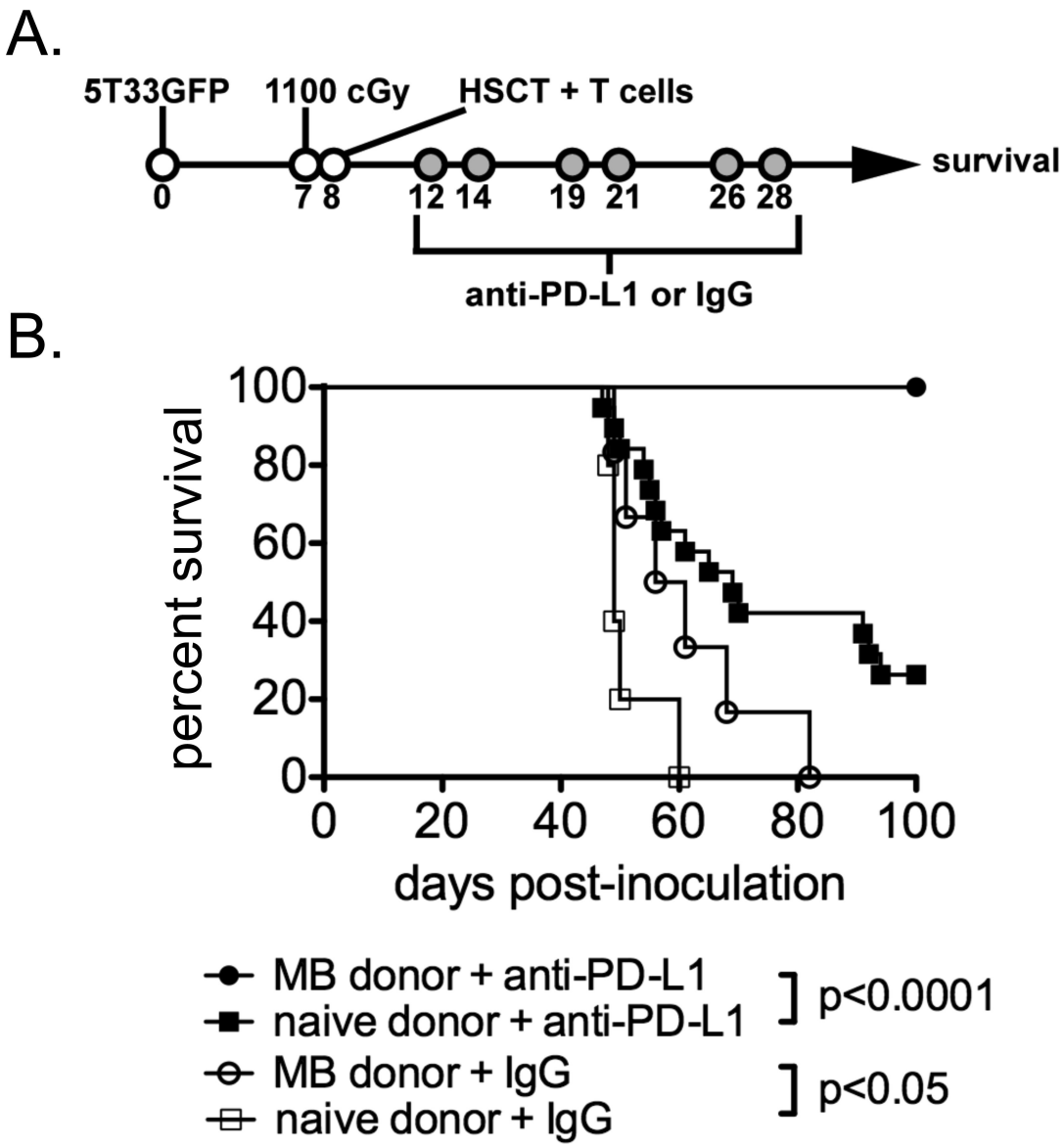

Figure 6. ACT with T cells from syngeneic myeloma-bearing donor mice provides superior anti-myeloma immunity in combination with anti-PD-L1 treatment

A. Experimental design: Myeloma-bearing (KaLwRij × B6.SJL) F1 mice were lethally-irradiated (1100 cGy) seven days after 5T33 inoculation (some mice were irradiated on day 8). One day following irradiation, the mice were intravenously injected with 5-10×10^6 bone marrow cells from naïve mice and 6×10^6 purified T cells from myeloma-bearing (MB) or naïve donors. Experimental groups were treated with control IgG or anti-PD-L1 blocking antibody as depicted. **B**. The survival curves compare experimental groups of mice given T cells from MB versus naïve donors. Data for control IgG mice are from 1 experiment; n=5-6. Data for anti-PD-L1 mice are combined from 4 independent experiments; n=19-20 mice per experimental group.

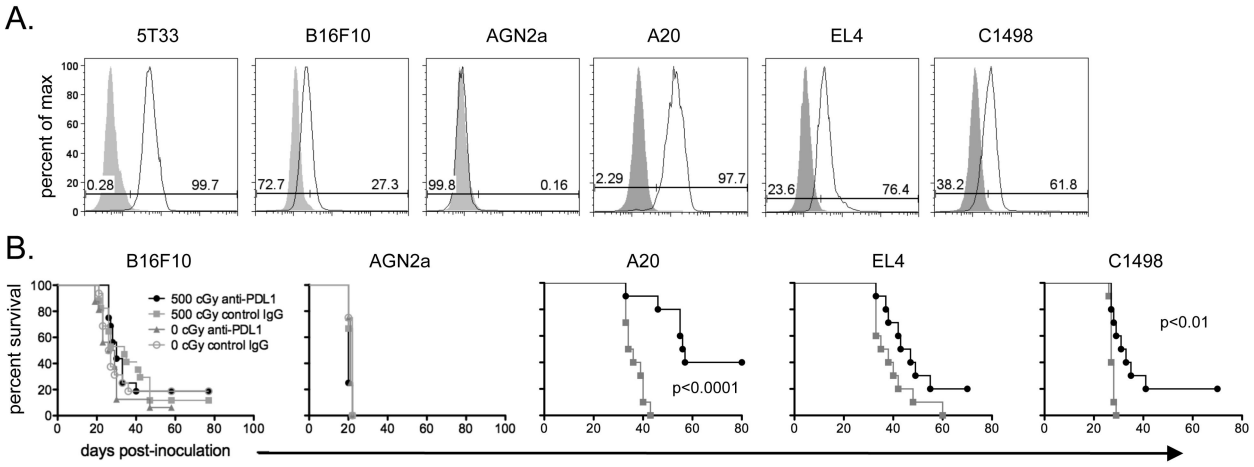

Figure 7. Sublethal irradiation and anti-PD-L1 is also effective in the treatment of other hematologic malignancies

A. PD-L1 expression on cultured 5T33, B16F10, AGN2a, A20, EL4, and C1498 cells is depicted in flow cytometry histograms. Solid curves represent isotype controls. **B**. Survival curves are shown for mice inoculated with the indicated tumors. The experimental design followed that depicted in Figure 2A. Briefly, approximately one week after inoculation mice received 0 or 500 cGy irradiation and then were treated on six days with anti-PD-L1 or control IgG. Survival curves are from 1-3 independent experiments; n=8-20 mice per experimental group.