

## Anti-Programmed Death-1 Synergizes with Granulocyte Macrophage Colony-Stimulating Factor – Secreting Tumor Cell Immunotherapy Providing Therapeutic Benefit to Mice with Established Tumors

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**Abstract Purpose:** The purpose of the present study was to evaluate granulocyte macrophage colony-stimulating factor (GM-CSF) – secreting tumor cell immunotherapy, which is known to stimulate potent and long-lasting antigen-specific immune responses, in combination with PD-1 blockade, which has been shown to augment cellular immune responses.

**Experimental Design:** Survival studies were done in the B16 melanoma and CT26 colon carcinoma tumor models. Immune monitoring studies were done in the B16 model. GM-CSF – secreting tumor cell immunotherapy was administered s.c. and the anti – PD-1 antibody was administered i.p.

**Results:** The studies reported here show that combining PD-1 blockade with GM-CSF – secreting tumor cell immunotherapy prolonged the survival of tumor-bearing animals compared with animals treated with either therapy alone. Prolonged survival correlated with strong antigen-specific T-cell responses detected by tetramer staining and an *in vivo* CTL assay, higher secretion levels of proinflammatory cytokines by splenocytes, and the persistence of functional CD8<sup>+</sup> T cells in the tumor microenvironment. Furthermore, in the biweekly multiple treatment setting, repeated antigen-specific T-cell expansion was only observed following administration of the cellular immunotherapy with the PD-1 blockade and not when the cellular immunotherapy or PD-1 blockade was used as monotherapy.

**Conclusion:** The combination of PD-1 blockade with GM-CSF – secreting tumor cell immunotherapy leads to significantly improved antitumor responses by augmenting the tumor-reactive T-cell responses induced by the cellular immunotherapy. Readministration of the cellular immunotherapy with the anti – PD-1 antibody in subsequent immunotherapy cycles was required to reactivate these T-cell responses.

Induction of antitumor immune responses by granulocyte macrophage colony-stimulating factor (GM-CSF) – secreting whole cell immunotherapy has been shown to be effective in both preclinical and clinical settings (1 – 5). The tumor-specific responses generated are potent, long-lasting, and require both CD4 and CD8 T cells (1, 6, 7). One approach taken to further enhance antitumor T-cell responses induced by the cellular immunotherapy has been to combine GM-CSF – secreting tumor cell immunotherapy with antibodies targeted against distinct T-cell signaling pathways, including the negative

regulatory receptor CTLA-4 (8). In an ongoing phase 1/2 study in hormone-refractory prostate cancer patients, anti – CTLA-4 antibody seems to synergize with prostate cancer – specific, GM-CSF – secreting tumor cell immunotherapy (GVAX immunotherapy for prostate cancer), with a reported PSA decline of >50% in five of six patients at the two highest antibody dose levels (3 and 5 mg/kg; ref. 9). In other preclinical models evaluating GM-CSF – secreting tumor cell immunotherapy, cotreatment with an anti – 4-1BB antibody resulted in T-cell-mediated tumor rejection in B16 tumor – bearing animals (10), and combination with an anti – OX-40 antibody has been shown to overcome established CD8 T-cell tolerance to tumor antigens (11). These reports suggest that targeting immune checkpoint regulators during activation of tumor-specific immune responses by GM-CSF – secreting tumor cell immunotherapies may provide novel and promising combination therapies to treat patients with large tumor burden.

PD-1, an immunoinhibitory receptor belonging to the CD28 family (12, 13), is predominantly expressed on activated T cells (14). In addition, PD-1 is found on antigen-specific T cells that are chronically exposed to antigen (15 – 17). Recent studies have documented a critical role in T-cell regulation involving PD-1 and its ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC;

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### Translational Relevance

Treatment with granulocyte macrophage colony-stimulating factor (GM-CSF)-secreting tumor cells is reported to induce potent and long-lasting specific immunity. Pre-clinical studies have shown that the antitumor activity of the cellular immunotherapy can be further enhanced by combination with immunomodulatory agents. GVAX immunotherapy for prostate cancer, which is composed of two prostate tumor cell lines modified to secrete GM-CSF, has been evaluated in several phase 3 trials and in a phase 1 trial in combination with an anti-CTLA-4 antibody (ipilimumab). The identification of other effective immunomodulatory strategies for combination with GM-CSF-secreting tumor cell immunotherapies may provide other avenues to achieve antitumor immune responses in patients. The results reported here suggest that blockade of PD-1, an immune-inhibitory receptor, enhances the antitumor immune response induced by the cellular immunotherapy by potentiating tumor-reactive T cells. Thus, the combination of GM-CSF-secreting tumor cell immunotherapies and PD-1 blockade may represent a potent strategy to evaluate for the treatment of cancer.

refs. 18, 19). PD-L1 is expressed on hematopoietic cells and can be up-regulated on activation (20). PD-L1 is also found in tissues such as pancreatic islets, heart, endothelium, small intestine, and placenta (21, 22). Tumor cells that express PD-L1 use this pathway as a mechanism to evade recognition/destruction by the immune system (23–26). PD-L2 expression is restricted only to macrophages and dendritic cells and is also up-regulated on activation (20). PD-1 knockout mice develop autoimmune glomerulonephritis and arthritis (27–29), which is a milder phenotype than the one observed in CTLA-4 knockout mice, in which multiorgan autoimmunity is observed within the first few weeks of birth (30–32). Suppression of effector T-cell function by PD-1 engagement includes deletion/apoptosis (33, 34), inhibition of proliferation and production of cytokines such as interleukin (IL)-2 and IFN $\gamma$  (12), and, together with chronic antigen exposure, results in T-cell exhaustion (15). These data suggest that targeting PD-1 may augment tumor-specific T-cell responses.

The studies reported here evaluated the combination of a GM-CSF-secreting tumor cell immunotherapy with a murine anti-PD-1 antibody in the poorly immunogenic B16 melanoma and the immunogenic CT26 colon carcinoma tumor models. These two cell lines express low baseline levels of PD-1 and PD-L1 in culture and *in vivo* in solid tumors (data not shown). In the 3-day therapy setting, the combination therapy resulted in the survival of 50% and 90% of animals bearing tumors in the B16 and the CT26 tumor models, respectively. Treatment with the anti-PD-1 antibody enhanced the effectiveness of the GM-CSF-secreting tumor cell immunotherapy by significantly increasing the number, potency (as measured by augmented proinflammatory cytokine secretion), and duration of activity of tumor-specific cytolytic T cells. In addition, rapid and persistent infiltration of effector CD8<sup>+</sup> T cells was detected in the tumor microenvironment. Furthermore, multiple cycles of the therapies administered biweekly

suggested that further treatment with the cellular immunotherapy plus the anti-PD-1 antibody is required to expand the number of antigen-specific T cells initially primed by the combination therapy. Thus, anti-PD-1 antibody combined with a GM-CSF-secreting tumor cell immunotherapy significantly enhanced antitumor responses by potentiating tumor-reactive T-cell responses induced by the tumor cell immunotherapy. The combination of the cellular immunotherapy and the anti-PD-1 antibody is essential in subsequent immunotherapy cycles to expand or boost the antitumor T-cell responses.

### Materials and Methods

**Mice and cell lines.** Female BALB/c and C57BL/6 mice (Taconic) were purchased and maintained according to the Association for Assessment and Accreditation of Laboratory Animal Care guidelines. *In vivo* studies were initiated with mice between 8 and 12 weeks of age. Study designs were approved and done according to the guidelines of the Cell Genesys Care and Use Committee.

The B16F10 melanoma (termed B16) and CT26 carcinoma cells were purchased from American Type Culture Collection. The generation of the retrovirally transduced GM-CSF-secreting cell lines has been previously described (5). The GM-CSF-secreting B16 cell line was further transduced to express the MHC molecule K<sup>d</sup> to generate an immunotherapy that expresses an allogeneic component (B16.Kd.GM). B16.Kd.GM generates ~150 ng/10<sup>6</sup> cells/24 h of murine GM-CSF. GM-CSF-secreting CT26 cells (CT26.GM) generate ~80 ng/10<sup>6</sup> cells/24 h of murine GM-CSF. Cells were maintained in DMEM Medium (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 2 mmol/L L-glutamine, and 1 $\times$  penicillin/streptomycin (JRH).

To generate the OVA-expressing cell lines F10.ova and GM.ova (generates ~150 ng/10<sup>6</sup> cells/24 h of murine GM-CSF), a CD16 LAMP-1 fusion protein (35) was excised and cloned into a third-generation lentiviral transfer vector with a conditional packaging system (36). Vectors were generated by transient transfection of 293T cells as previously described (36). The transduced B16 population was stained with an OVA-specific antibody conjugated to biotin (U.S. Biological), with a streptavidin-conjugated phycoerythrin secondary antibody used for detection (BD Pharmingen). Ova-positive cells were sorted using a MoFlo (Cytomation, Inc.). The mean fluorescence intensities by flow cytometry analysis of the naive and sorted cells are 3.9 and 57.4, respectively.

**Anti-PD-1 antibody.** Anti-mouse PD-1 antibody, 4H2, was generated by immunization of rats with mouse PD-1-immunoglobulin fusion protein. Binding of the antibody to mouse PD-1 was shown by ELISA to PD-1-immunoglobulin fusion and by flow cytometry with transfected Chinese hamster ovary cells expressing mouse PD-1. The antibody was selected for its ability to inhibit the interaction between mouse PD-1 and its ligand PD-L1 or PD-L2. The variable (V) region sequences of this antibody were determined and VH and VK sequences were grafted onto the murine IgG1 Fc region and the murine C $\kappa$  constant region, respectively, to generate an antibody with reduced binding to mouse Fc receptors. Chinese hamster ovary cell lines that express the chimeric antibody were selected and used for production of the antibody. The chimeric 4H2 (mIgG1) was used in these studies. Stock solutions of anti-PD-1 were kept at 4°C.

**Antitumor efficacy studies.** In the B16 model, mice ( $n = 10$  per group) were inoculated with  $2 \times 10^5$  live B16 cells by s.c. injection at a dorsal site and immunized with  $1 \times 10^6$  irradiated allogeneic GM-CSF-secreting B16 cells (B16.Kd.GM) by s.c. injection at a ventral site on day 3, 7, or 11. This model was designed to result in ~20% long-term survival (70 d) when animals received B16.Kd.GM on day 3 after tumor inoculation, but no therapeutic benefit when immunotherapy was

administered later, thereby providing a model suitable for the evaluation of agents that may have synergistic and/or additive effects when combined with GM-CSF-secreting tumor cell immunotherapy. Delaying the initiation of therapy beyond day 3 allows the assessment of the effectiveness of combination therapies in animals with larger tumor burden. In the CT26 model, mice ( $n = 10$  per group) were inoculated with  $5 \times 10^5$  live CT26 cells by s.c. injection at a dorsal site and immunized with  $1 \times 10^6$  irradiated CT26.GM cells by s.c. injection at a ventral site on day 3. In the combination treatment groups of both models, the anti-PD-1 antibody was administered i.p. on the day of (200  $\mu\text{g}$  per injection) and the day after (100  $\mu\text{g}$  per injection) immunotherapy, respectively. Animals were monitored and scored for the formation of palpable tumors twice weekly and sacrificed if tumors became necrotic or exceeded the predetermined size of 1,500  $\text{mm}^3$ .

**In vivo proliferation.** In the single treatment cycle setting,  $1 \times 10^6$  OT-1 transgenic T cells were adoptively transferred into tumor-bearing C57BL/6 mice that had been inoculated the day before with  $2 \times 10^5$  F10.ova cells. Three days later, mice ( $n = 5$  per time point per group) were immunized with  $1 \times 10^6$  irradiated GM-CSF-secreting ovalbumin-expressing cells (GM.ova) as immunotherapy alone or immunotherapy was followed by i.p. injections of 200 and 100  $\mu\text{g}$  of anti-PD-1 antibody on days 3 and 4, respectively, as combination therapy. At indicated time points, draining lymph nodes were harvested from selected mice and evaluated for antigen-specific T cells by costaining with anti-CD8 antibody and SIINFEKL tetramer.

In multiple cycles of treatment setting,  $1 \times 10^6$  OT-1 transgenic T cells were adoptively transferred into tumor-bearing C57BL/6 mice that had been inoculated the day before with  $2 \times 10^5$  F10.ova cells. Three days later, mice ( $n = 5$  per time point per group) were immunized with  $1 \times 10^6$  irradiated GM.ova cells as immunotherapy alone or immunotherapy was followed by i.p. injections of 200 and 100  $\mu\text{g}$  of anti-PD-1 antibody on days 3 and 4, respectively, as combination therapy. Subsequently, animals received either the combination therapy on a biweekly schedule or the combination therapy on a monthly schedule followed by anti-PD-1 antibody alone in between the combination therapy schedule. At indicated time points, spleen cells were harvested from selected mice and evaluated for antigen-specific T cells by costaining with anti-CD8 antibody and SIINFEKL tetramer.

**In vivo CTL assay.** Mice were inoculated with F10.ova cells on day 0 and immunized with  $1 \times 10^6$  irradiated GM.ova cells on day 3 as immunotherapy alone or immunotherapy was followed by i.p. treatment with 200 and 100  $\mu\text{g}$  of anti-PD-1 antibody on the day of and the day after immunotherapy, respectively, as combination therapy. At indicated time points, mice ( $n = 5$  per group) were injected with syngeneic splenocytes labeled for 15 min at  $37^\circ\text{C}$  with 5  $\mu\text{mol/L}$  carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes; CFSE<sup>hi</sup> cells) and 0.5  $\mu\text{mol/L}$  CFSE (CFSE<sup>lo</sup> cells). CFSE<sup>hi</sup> cells were pulsed with SIINFEKL peptide at 1  $\mu\text{g/mL}$  for 1 h at  $37^\circ\text{C}$ . Unpulsed CFSE<sup>lo</sup> cells served as an internal control. Splenocytes were analyzed 18 h after injection for the detection and quantification of CFSE-labeled cells by flow cytometry. Naïve animals with no CTL activity display two equal CFSE<sup>hi</sup> and CFSE<sup>lo</sup> peaks at 1:1 ratio, whereas experimental animals that contain lytic T cells specific for the peptides that have been loaded onto the splenocytes display a reduction in the CFSE<sup>hi</sup> population. The formula to calculate percent specific killing is as follows: % lysis =  $[(\text{naïve } m_2/m_1)/(\text{experimental } M_2/M_1)] \times 100$ , where  $m_1$  is CFSE<sup>hi</sup> and  $m_2$  is CFSE<sup>lo</sup>.

**ELISPOT assay.** Antigen-specific responses were enumerated by an IFN $\gamma$  ELISPOT assay (R&D Biosystems) according to the manufacturer's instructions. Briefly, 96-well filtration ELISPOT plates (Millipore) were coated with 4  $\mu\text{g/mL}$  of capture antibody in 100  $\mu\text{L}$  of reagent diluent for 2 h at  $37^\circ\text{C}$ . Plates were washed twice with wash buffer (PBS with 0.5% Tween 20) and blocked for 2 h at room temperature in blocking buffer containing PBS supplemented with 1% bovine serum albumin and 5% sucrose. Erythrocyte-depleted splenocytes ( $5 \times 10^5$ ;  $n = 5$  per group) were plated and incubated for 48 h at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  with 1  $\mu\text{mol/L}$  of the K<sup>b</sup> binding peptide derived from Trp2 (Genmed

Synthesis) or irradiated B16 cells. ELISPOT plates were developed according to the manufacturer's specifications. Wells containing medium and spleen cells only served as negative control. Spots were enumerated by an automated plate scanning service from Cellular Technology Ltd.

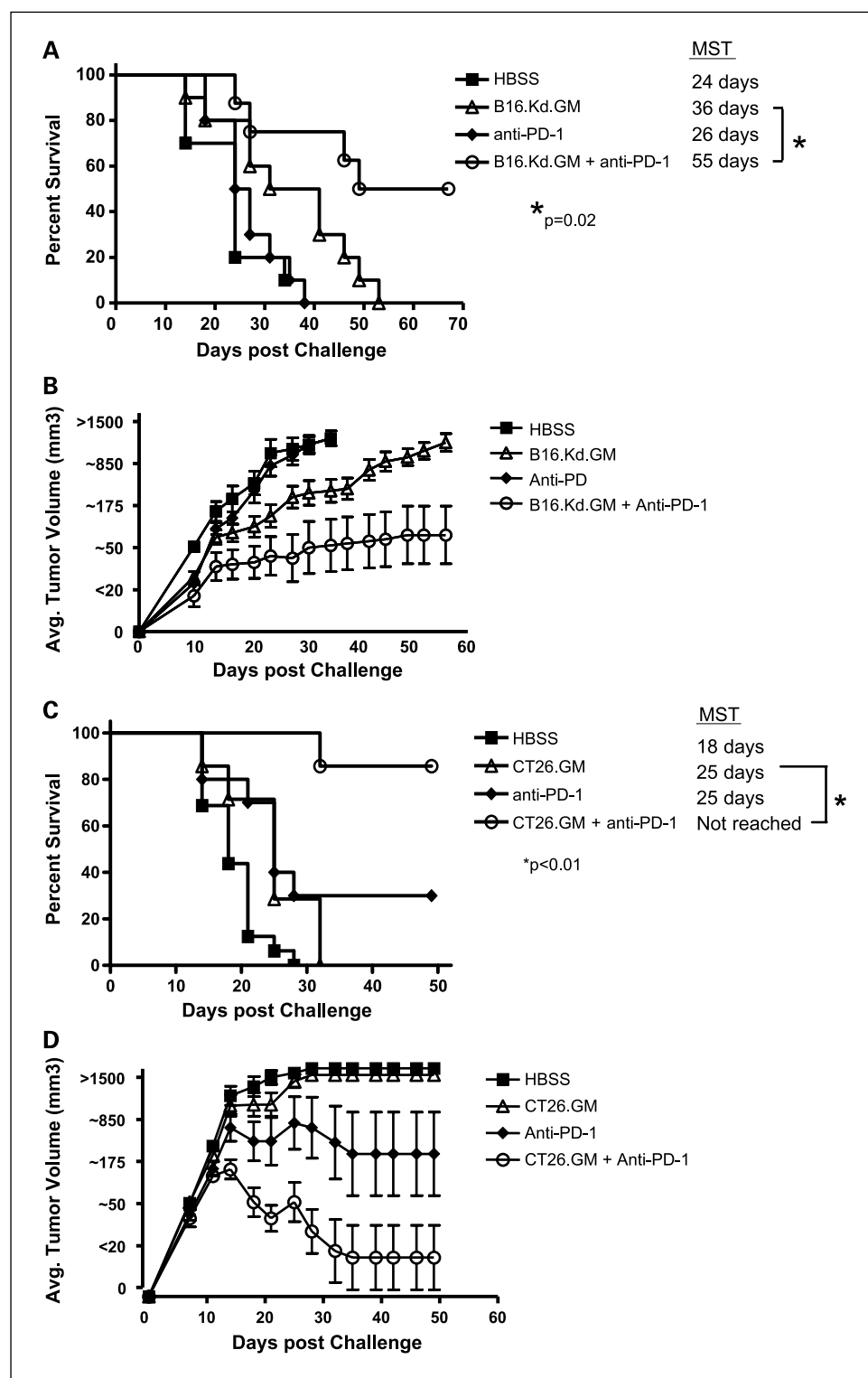
**Cytokine evaluation.** Irradiated B16 cells were cocultured with splenocytes for 2 d. Cytokine secretion analyses by flow cytometry of cocultured supernatant were done using Th1/Th2 and inflammatory cytometric bead array kits (BD Pharmingen) according to the manufacturer's instructions and analyzed by BD CBA system (BD Pharmingen).

**Tumor-infiltrating lymphocyte characterization.** At designated time points, tumors were removed from mice ( $n = 5$  per group) and digested in 1 mg/mL collagenase (Sigma) and 0.1 mg/mL DNase (Sigma) for 1 h at  $37^\circ\text{C}$ . Dissociated cells were filtered through a 0.3- $\mu\text{m}$  filter and leukocytes were positively selected using anti-CD45 MACs beads according to the manufacturer's instructions (Milteny Biotec). Enriched leukocytes were directly stained with conjugated antibodies (BD Pharmingen) for phenotype characterization by flow cytometry.

**Statistical analysis and data presentation.** Multiparameter statistics for the Kaplan-Meier survival curves were done by a log-rank test using Prism Software (GraphPad, Inc.). Relative differences between groups were also done using a Student's  $t$  test and GraphPad Prism Software.

## Results

**Anti-PD-1 antibody in combination with a GM-CSF-secreting tumor cell immunotherapy improves survival of tumor-bearing animals.** Allogeneic tumor cell lines are currently used in clinical studies to evaluate the potency of GM-CSF-secreting tumor cell immunotherapies in cancer patients. In these studies, GM-CSF-secreting B16 cells were modified to express the MHC molecule K<sup>d</sup> (B16.Kd.GM) to generate an immunotherapy that expresses an allogeneic component in the K<sup>b</sup> genetic background of C57BL/6 animals in a therapeutic model of established s.c. B16 melanoma. Mice were inoculated with a lethal dose of live B16 tumor cells followed by a single administration of B16.Kd.GM immunotherapy 3 days after tumor inoculation. Anti-PD-1 antibody was administered either as monotherapy or to B16.Kd.GM immunotherapy-treated animals as combination therapy on days 3 and 4 at 200 and 100  $\mu\text{g}$ , respectively. HBSS-injected control animals succumbed to tumor burden rapidly and had a mean survival time (MST) of 24 days, and treatment with anti-PD-1 antibody alone did not provide a significant improvement in tumor growth rate or survival compared with control animals, with a MST of 26 days. Animals treated with B16.Kd.GM monotherapy showed a delay in tumor growth and prolonged survival with a MST of 36 days ( $P < 0.05$ , HBSS versus B16.Kd.GM), which was further significantly extended to 55 days in animals treated with the combination therapy, with 50% long-term (>70 days) survival ( $P = 0.02$ , compared with B16.Kd.GM monotherapy; Fig. 1A and B). To evaluate the potency of this combination therapy in a second model, the *in vivo* study was repeated in the autologous immunogenic CT26 colon carcinoma tumor model in BALB/c animals. In this model, anti-PD-1 antibody and CT26.GM monotherapies both prolonged the survival of tumor-bearing animals to 25 days compared with HBSS-injected control animals with a MST of 18 days (Fig. 1C and D). Consistent with the findings in the B16 model, CT26 tumor-bearing animals treated with the combination therapy showed a significant survival advantage over animals treated with either monotherapy, with 90% of animals surviving



**Fig. 1.** Combination with anti – PD-1 antibody enhances the potency of GM-CSF – secreting tumor cell immunotherapy *in vivo*. C57BL/6 mice ( $n = 10$  per group) were inoculated s.c. with  $2 \times 10^5$  B16 tumor cells on day 0. A single treatment with  $1 \times 10^6$  irradiated allogeneic GM-CSF – secreting B16 cells (B16.Kd.GM) was given on day 3 as GMKd immunotherapy alone (A). BALB/c mice ( $n = 10$  per group) were inoculated s.c. with  $5 \times 10^5$  CT26 tumor cells on day 0. A single treatment with  $1 \times 10^6$  irradiated GM-CSF – secreting CT26 cells (CT26.GM) was given on day 3 as CT26.GM immunotherapy alone (C). Anti – PD-1 antibody was given by i.p. injections on days 3 and 4 at 200 and 100  $\mu\text{g}$ , respectively, as antibody monotherapy or to cellular immunotherapy – treated animals as combination therapy (A). Mice were assessed for the formation of tumors twice weekly (B and D) and sacrificed when tumors became necrotic or exceeded 1,500  $\text{mm}^3$ . A Kaplan-Meier survival curve was used for the evaluation.

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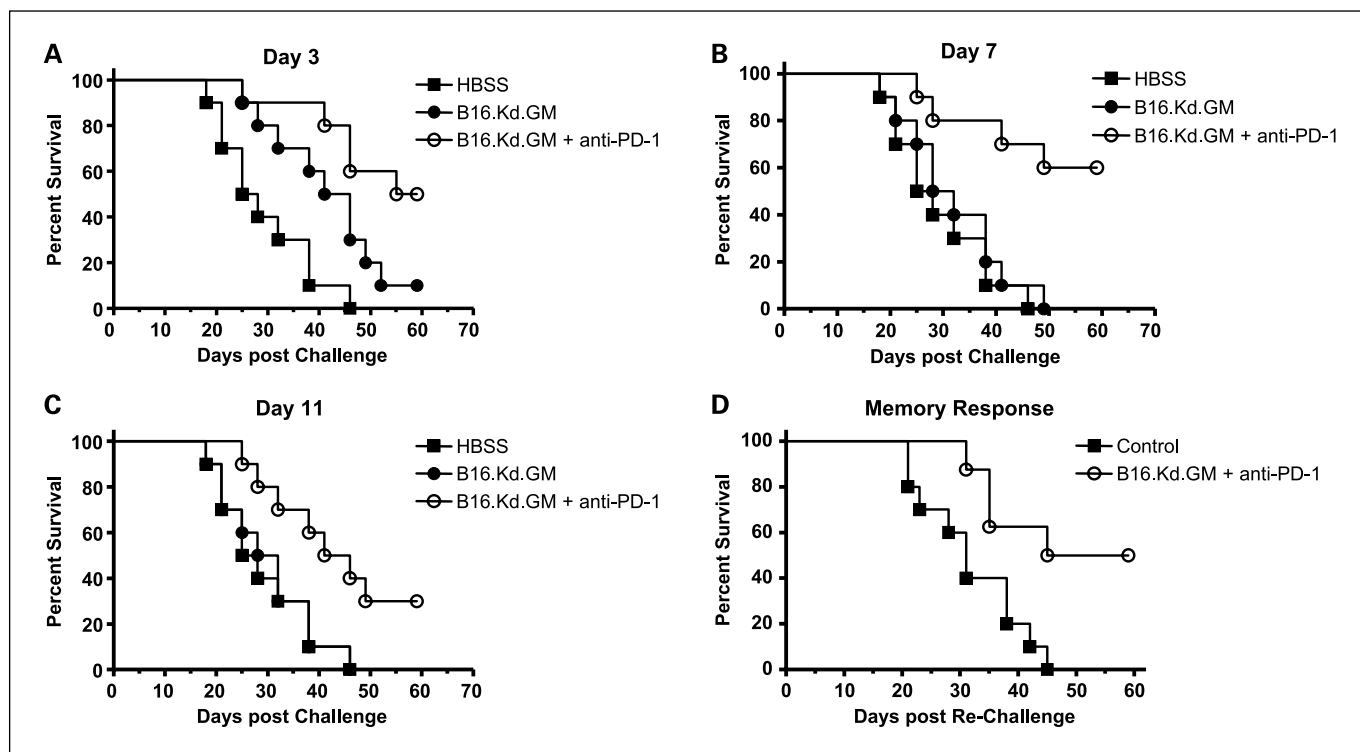
long-term ( $P < 0.01$ , compared with CT26.GM alone). Thus, anti-PD-1 antibody in combination with GM-CSF-secreting tumor cell immunotherapy significantly prolonged the survival of animals bearing either a nonimmunogenic or an immunogenic tumor when compared with either monotherapy.

Based on the encouraging results obtained with the combination therapy in these two tumor models, the

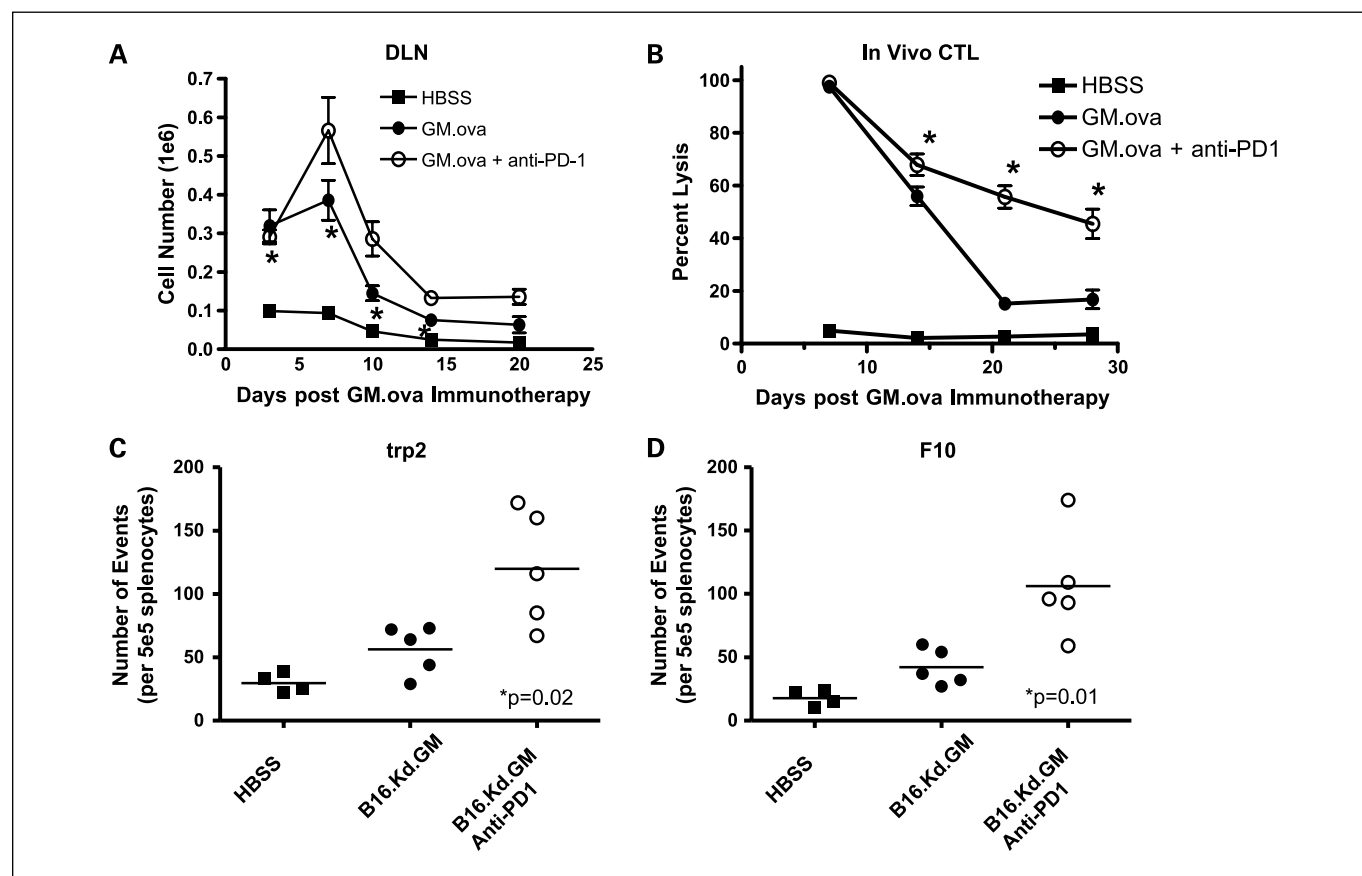
combination therapy was further evaluated in the B16 model, with treatment starting at later time points to assess the overall potency in animals with well-established tumors. Animals were inoculated with a lethal dose of live B16 tumor cells and followed by a single administration of B16.Kd.GM immunotherapy on day 3, 7, or 11 after tumor inoculation. Animals in the combination therapy group also received 200

and 100  $\mu\text{g}$  of anti-PD-1 antibody on the day of and 1 day after immunotherapy. In this study, HBSS-injected control animals had a MST of 26 days and animals treated with B16.Kd.GM immunotherapy on day 3 had a MST of 44 days with 10% long-term survival ( $P = 0.01$ , HBSS versus B16.Kd.GM). MST was further prolonged to 57 days in animals that were treated with the combination therapy on day 3 with 50% long-term survival ( $P < 0.05$ , B16.Kd.GM + anti-PD-1 versus B16.Kd.GM; Fig. 2A). MSTs were 30 days when B16.Kd.GM immunotherapy was delayed to either day 7 or day 11 after tumor challenge and all animals succumbed to tumor burden by day 45 to day 50. Again, enhanced antitumor activity was observed in animals that received the combination therapy initiated either on day 7 or day 11, with 60% (MST not reached) and 30% (MST, 42 days) of animals surviving long-term, respectively [ $P < 0.005$  (day 7 initiation of therapy) or  $P = 0.01$  (day 11 initiation of therapy)], long-term survival of B16.Kd.GM + anti-PD-1 versus B16.Kd.GM; Fig. 2B and C]. Furthermore, on day 90, rechallenge of surviving mice in both immunotherapy alone and combination therapy groups with 2.5 times the initial dose of live tumor cells showed the presence of a potent B16-specific memory response, suggesting that the induction of memory responses is B16.Kd.GM dependent (Fig. 2D). Thus, the combination of GM-CSF-secreting tumor cell immunotherapy and anti-PD-1 antibody is potent enough to significantly delay tumor growth of well-established primary tumors and to maintain memory responses for protection against tumor reoccurrence.

**In vivo PD-1 blockade augments antigen-specific T-cell responses induced by cellular immunotherapy in tumor-bearing animals.** To evaluate the *in vivo* kinetics of T-cell activation, B16 tumor-bearing animals were treated with either the cellular immunotherapy alone or in combination with anti-PD-1 antibody. T-cell activation was monitored using tetramers specific for ovalbumin (SIINFEKL tetramer), a surrogate antigen expressed by the immunotherapy cells in this particular experiment. On day 0, T-cell receptor transgenic T cells specific for ovalbumin (OT-1 T cells;  $1 \times 10^6$ ) were adoptively transferred into B16 tumor-bearing animals challenged on day -1 with  $2 \times 10^5$  live tumor cells. On day 3, animals were immunized with  $1 \times 10^6$  irradiated GM-CSF-secreting B16 cells expressing ovalbumin (GM.ova) as monotherapy or GM.ova immunotherapy was followed by 200 and 100  $\mu\text{g}$  of anti-PD-1 antibody on days 3 and 4, respectively, as combination therapy. At indicated time points, draining lymph nodes were harvested from selected animals and evaluated for the presence of ovalbumin-specific CD8<sup>+</sup> T cells by costaining cells with anti-CD8 antibody and SIINFEKL tetramer. As shown in Fig. 3A and Supplementary Fig. S1, HBSS-injected control animals showed low numbers of tetramer-positive CD8 T cells at all time points evaluated. In contrast, animals that received GM.ova monotherapy or in combination with anti-PD-1 antibody showed a significant increase in the number of tetramer-positive CD8 T cells compared with HBSS control animals ( $P < 0.05$ , HBSS versus GM.ova or combination). Both treatment groups displayed a similar T-cell kinetics, with an initial expansion phase within 1 week posttreatment with



**Fig. 2.** GM-CSF – secreting tumor cell immunotherapy in combination with anti-PD-1 antibody enhances the survival of tumor-bearing animals in the delayed treatment setting. C57BL/6 mice ( $n = 10$  per group) were inoculated s.c. with  $2 \times 10^5$  B16 tumor cells on day 0. A single treatment with  $1 \times 10^6$  irradiated allogeneic GM-CSF – secreting B16 cells (B16.Kd.GM) was given on day 3 (A), day 7 (B), or day 11 (C) as immunotherapy alone or GMKd treatment was followed by i.p. injections of 200 and 100  $\mu\text{g}$  of anti-PD-1 antibody on the day of and 1 d after immunotherapy, respectively, as combination therapy. Mice were assessed for the formation of tumors twice weekly and sacrificed when tumors became necrotic or exceeded  $1500 \text{ mm}^3$ . On day 90, tumor-free animals from A, B, and C treatment schedules were rechallenged with  $5 \times 10^5$  (2.5-fold of initial dose) B16 tumor cells and monitored for survival (D). A Kaplan-Meier survival curve was used for the evaluation.



**Fig. 3.** Anti-PD-1 antibody augments antitumor T-cell responses induced by GM-CSF-secreting tumor cell immunotherapy. *A*, on day 0,  $1 \times 10^6$  ovalbumin-specific, transgenic T cells (OT-1 cells) were adoptively transferred into tumor-bearing C57BL/6 mice that had been inoculated on day -1 with  $2 \times 10^5$  F10.ova. On day 3, mice were immunized with  $1 \times 10^6$  irradiated GM-CSF-secreting B16 cells expressing ovalbumin (GM.ova) as immunotherapy alone or GM.ova immunotherapy was followed by 200 and 100  $\mu$ g of anti-PD-1 antibody on days 3 and 4, respectively, as combination therapy. At the indicated time points, draining lymph nodes (DLN) were harvested from selected mice ( $n = 5$  per group) and evaluated for ovalbumin-specific T cells by tetramer staining. *B*, following the same study design as in *A*, with the exception of the adoptive transfer, at indicated time points, mice ( $n = 5$  per group) were injected with CSFE-labeled nonpulsed and ovalbumin-specific peptide (SIINFEKL)-pulsed syngeneic splenocytes. Eighteen hours later, splenocytes were harvested and evaluated for cytolytic activity by measuring the ratio of CSFE-labeled cells. *C*, following the same study design as in *A*, with the exception of the adoptive transfer and the use of non-ovalbumin-expressing B16 cells, on day 17, an ELISPOT was done to evaluate the number of IFN $\gamma$ -secreting cells per  $5 \times 10^5$  splenocytes ( $n = 5$  per group) when stimulated with irradiated B16 cells or B16-specific K<sup>b</sup> peptide, Trp2 (*D*).

GM.ova immunotherapy that was followed by a rapid contraction phase. The response was greater with a trend toward statistical significance at all evaluated time points in the combination therapy-treated animals compared with animals that had received cellular immunotherapy alone. Tetramer-positive CD8 T cells were costained with Annexin V, and the data confirmed that the increased number of antigen-specific T cells was not a result of apoptosis prevention by the anti-PD-1 therapy but rather due to increased expansion of these cells (data not shown). These data suggest that blockade of PD-1 receptor during immune activation by GM-CSF-secreting tumor cell immunotherapy leads to an increased expansion of antigen-specific T cells potentially by preventing the inhibitory signal triggered by the interaction of PD-1 with PD-L1 and/or PD-L2.

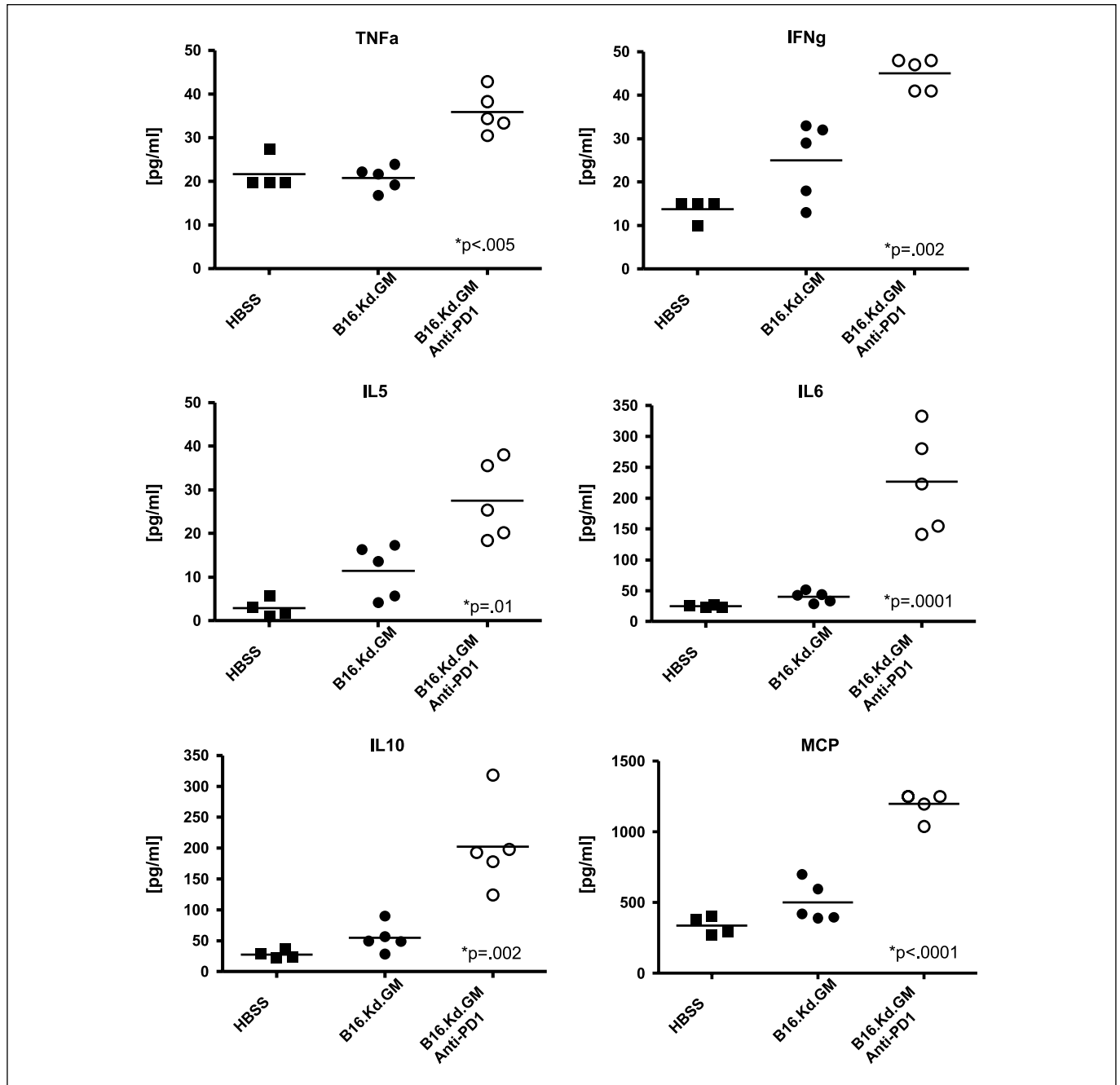
To evaluate the potency and functionality of the induced T cells, an *in vivo* CTL assay was done. The same experimental setup was followed as used in the tetramer staining assay with the exception that the adoptive transfer of OT-1 transgenic T cells was omitted to permit the evaluation of the response of naïve T cells to the surrogate antigen expressed by the cellular immunotherapy. At indicated time points, animals were

injected with syngeneic CSFE-labeled nonpulsed splenocytes and splenocytes pulsed with SIINFEKL peptide. Cytolytic activity was determined 18 hours later by measuring the difference in the ratio of CSFE-labeled cells in splenocytes loaded with SIINFEKL peptide and unloaded splenocytes. HBSS control animals displayed low levels of antigen-specific cytolytic T-cell activity, whereas animals treated with either GM.ova monotherapy or the combination therapy exhibited close to maximal cytolytic activity at the peak of the response on day 7 following treatment (Fig. 3B). The antigen-specific cytolytic activity of animals treated with GM.ova monotherapy gradually declined to background level by day 21, whereas animals treated with the combination therapy showed a significantly higher and more persistent T-cell response at all other time points, declining to ~50% of the peak response on day 28 (last time point evaluated;  $P < 0.05$ , GM.ova versus combination). These data suggested that the antigen-specific CD8 T cells induced at higher numbers in animals treated with the combination of GM-CSF-secreting tumor cell immunotherapy and anti-PD-1 are functional *in vivo*, exhibiting potent lytic activity.

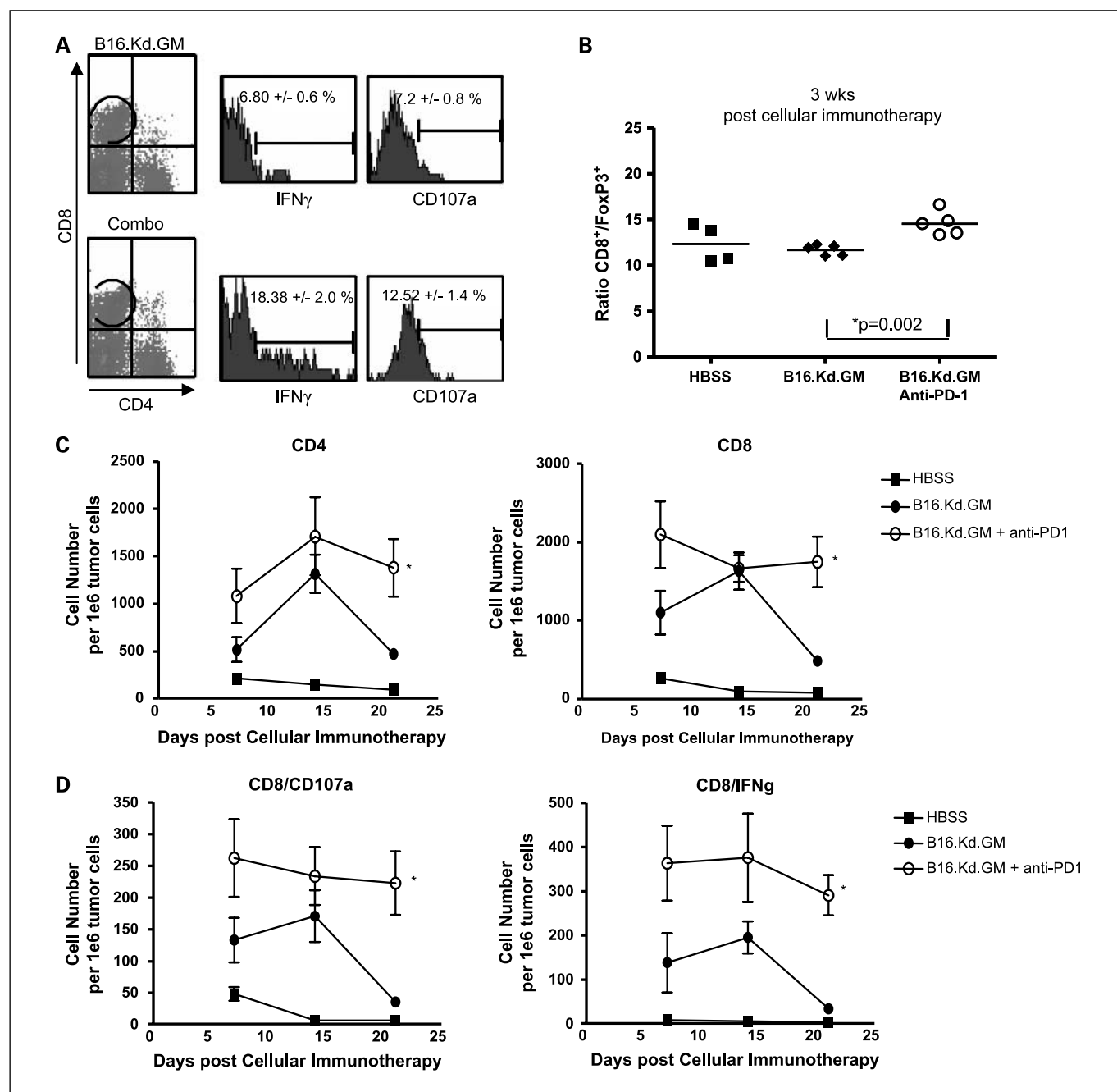
An ELISPOT assay measuring IFN $\gamma$  secretion by splenocytes after restimulation with either the B16-specific Trp2 peptide or

irradiated B16 whole cells further confirmed the functionality of these CD8 T cells in both treatment groups. For the ELISPOT assay, ovalbumin was omitted as a surrogate marker in the immunotherapy cells because B16 peptide-specific responses can be detected in this assay in animals receiving GM-CSF-secreting tumor cell immunotherapy (10, 37, 38). Tumor-bearing animals were either immunized with  $1 \times 10^6$  irradiated B16.Kd.GM cells as monotherapy or anti-PD-1 antibody was added as described in the *in vivo* CTL assay as combination

therapy. On day 17, to specifically monitor the tumor responses, splenocytes were harvested and restimulated either with the  $K^b$ -specific peptide for Trp2 or with irradiated B16 whole cells that did not express the allogeneic component  $K^d$ . HBSS-injected control animals had low numbers of  $IFN\gamma$ -secreting splenocytes following either of the two stimuli (Fig. 3C and D) as was expected in this poorly immunogenic tumor model. In contrast, splenocytes from animals injected with B16.Kd.GM monotherapy had increased numbers of



**Fig. 4.** Anti-PD-1 antibody enhances proinflammatory cytokine secretion in animals treated with GM-CSF-secreting tumor cell immunotherapy. On day 0, mice were inoculated s.c. with live B16 tumor cells. On day 3, mice were immunized with  $1 \times 10^6$  irradiated B16.Kd.GM cells as immunotherapy alone or B16.Kd.GM immunotherapy was followed by 200 and 100  $\mu$ g of anti-PD-1 antibody on days 3 and 4, respectively, as combination therapy. On day 17, splenocytes from selected mice ( $n = 5$  per group) were cultured with irradiated B16 cells for 48 h in 96-well plates. Supernatants were evaluated for the presence of tumor necrosis factor- $\alpha$  (*TNF $\alpha$* ),  $IFN\gamma$ , IL-5, IL-6, IL-10, and MCP-1.



**Fig. 5.** Addition of anti-PD-1 antibody enhances the number of tumor-infiltrating T cells in animals treated with GM-CSF-secreting tumor cell immunotherapy. On day 0, mice were inoculated s.c. with live tumor cells. On day 3, mice were immunized with  $1 \times 10^6$  irradiated B16.Kd.GM cells as immunotherapy alone or B16.Kd.GM immunotherapy was followed by 200 and 100  $\mu$ g of anti-PD-1 antibody on days 3 and 4, respectively, as combination therapy. At indicated time points, tumors from mice ( $n = 5$  per group) were excised and digested, and single-cell suspensions evaluated by flow cytometry. Shown are the percentages of IFN $\gamma$ - and CD107a-expressing cells in the CD8 tumor-infiltrating lymphocyte subpopulation in the immunotherapy alone and the combination group (A). Also shown is the ratio of CD8<sup>+</sup>/FoxP3<sup>+</sup> cells in the tumor at 3 wk after cellular immunotherapy (B). Also included is the absolute number of CD4<sup>+</sup> and CD8<sup>+</sup> T cells per  $1 \times 10^6$  tumor cells (C) and the absolute number of CD8<sup>+</sup> and IFN $\gamma$ <sup>+</sup> T cells and CD8<sup>+</sup> and CD107a<sup>+</sup> T cells per  $1 \times 10^6$  tumor cells (D).

IFN $\gamma$ -secreting cells compared with HBSS control animals when stimulated with either the Trp2 peptide ( $P = 0.04$ ) or B16 cells ( $P = 0.02$ ). A further significant increase in the number of IFN $\gamma$ -secreting cells was observed in splenocytes from animals treated with the combination therapy compared with those from animals treated with cellular immunotherapy alone ( $P = 0.02$  for Trp2 peptide or  $P = 0.01$  for B16 cells). These data suggested that the addition of a blocking anti-PD-1 antibody to cellular

immunotherapy *in vivo* increases T-cell responses specific for antigens derived from the cellular immunotherapy potentially by preventing a T-cell inhibitory signal during T-cell priming.

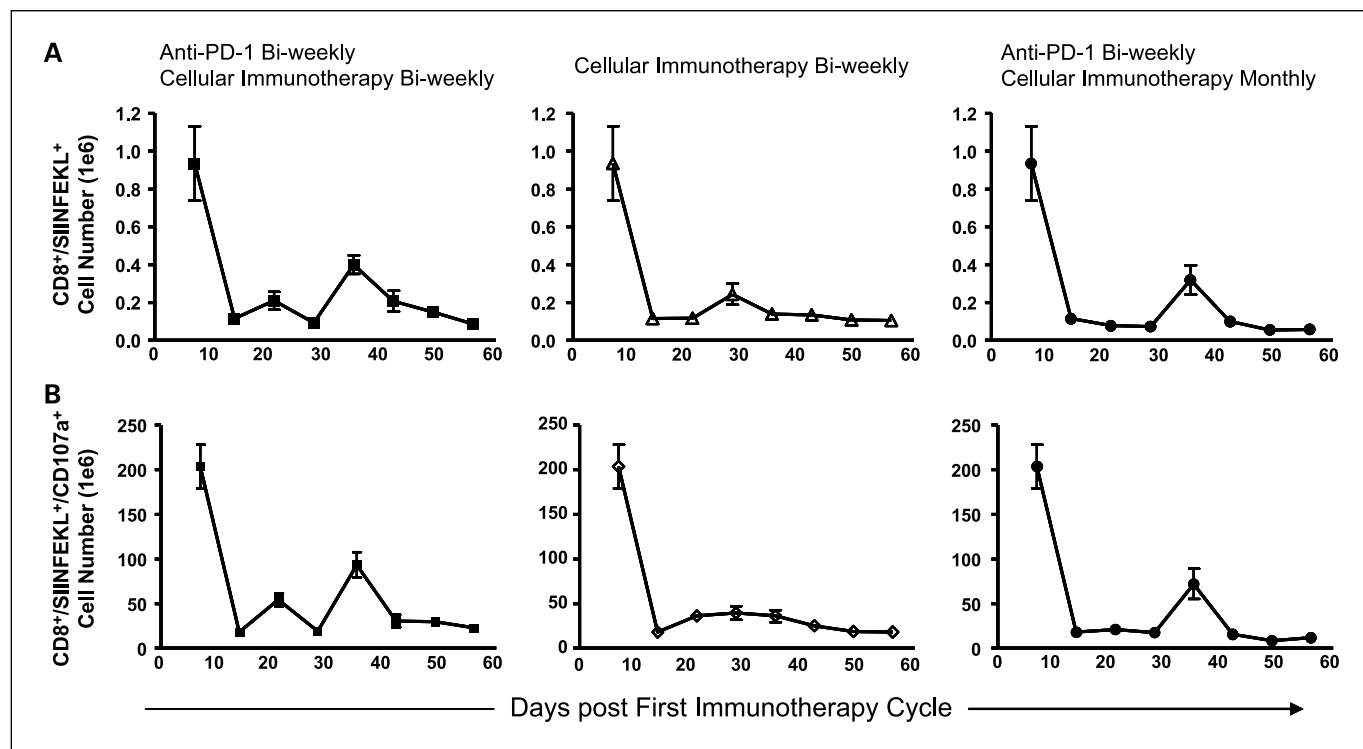
**PD-1 receptor blockade enhances proinflammatory cytokine production by splenocytes in B16.Kd.GM-treated tumor-bearing animals.** Cytokines are secreted proteins that mediate and regulate immunity, inflammation, and hematopoiesis. They are produced *de novo* in response to various immune stimuli, and



different cytokines affect distinct immune pathways. Th1 cytokines stimulate cellular immunity and inflammation, and Th2 cytokines stimulate antibody production by B cells. To examine the cytokine profile of splenocytes from animals treated with B16.Kd.GM monotherapy or in combination with the anti-PD-1 antibody, supernatants from splenocytes stimulated with irradiated B16 whole cells were evaluated. The same experimental protocol was followed as described for the IFN $\gamma$  ELISPOT assay. On day 10 (7 days after immunotherapy treatment),  $5 \times 10^5$  splenocytes were isolated and cocultured with irradiated B16 cells. Two days later, supernatants were collected and analyzed using Th1/Th2 and inflammation cytokine cytometric bead array kits. Secretion levels of IL-2, IL-4, and IL-12 were nondetectable in all experimental groups (data not shown). Secretion levels of tumor necrosis factor- $\alpha$  and IL-6 were comparable between splenocytes from B16.Kd.GM-treated and HBSS-injected animals, but both cytokines were significantly increased in animals treated with the combination therapy (Fig. 4). The levels of IFN $\gamma$ , IL-5, IL-10, and MCP-1 (Fig. 4) were increased in splenocyte supernatants from B16.Kd.GM-treated animals compared with supernatants from HBSS control animals and were significantly increased in cultures from animals treated with combination therapy compared with the B16.Kd.GM group. These data suggest that a more potent tumor-specific T-cell response occurs in animals treated with the combination therapy compared with animals that received cellular immunotherapy alone,

suggesting once more that anti-PD-1 strongly augments B16.Kd.GM-induced immune responses.

**High numbers of effector CD8 T cells infiltrate into tumors of animals treated with B16.Kd.GM immunotherapy and anti-PD-1 antibody.** High numbers of tumor-infiltrating lymphocytes within tumors have been shown to correlate with overall therapeutic benefit (39, 40). To examine the extent of infiltrating lymphocytes in tumors of B16.Kd.GM-treated animals compared with animals treated with the combination therapy, the same experimental protocol was followed as described for the IFN $\gamma$  ELISPOT assay. At indicated time points, tumors were removed, collagenase digested, and evaluated for the presence of CD4 and CD8 T cells by flow cytometry. CD4/IFN $\gamma$  costaining was used to identify effector CD4 T cells and CD8/IFN $\gamma$ , or CD8/CD107a costaining was used to identify effector CD8 T cells within tumors. IFN- $\gamma$  secreted by T cells has previously been shown to correlate with immunoregulatory and antitumor properties (41), whereas expression of CD107a is associated with cytolytic activity of T cells (42, 43). Animals receiving the combination therapy showed an increase in the percentage of activated CD8 effector T cells within tumors compared with animals that received B16.Kd.GM monotherapy (Fig. 5A and B). Furthermore, HBSS control animals had minimal CD4 and CD8 T-cell infiltration into tumors, correlating with tumor progression of these animals (Fig. 5C and D; Supplementary Fig. S2). Comparatively, animals treated with B16.Kd.GM monotherapy displayed



**Fig. 6.** Combination of GM.ova immunotherapy with anti-PD-1 antibody is required in each boost cycle for the expansion of antigen-specific T cells. On day 0, ovalbumin-specific, transgenic T cells (OT-1 cells;  $1 \times 10^6$ ) were adoptively transferred into tumor-bearing C57BL/6 mice that had been inoculated on day -1 with live  $2 \times 10^5$  F10.ova cells. On day 3, mice were immunized with  $1 \times 10^6$  irradiated GM-CSF-secreting B16 cells expressing ovalbumin (GM.ova) as immunotherapy alone or GM.ova immunotherapy was followed by 200 and 100  $\mu$ g of anti-PD-1 antibody on days 3 and 4, respectively (the first therapy cycle). For subsequent therapy cycles, one group of animals received biweekly GM.ova and anti-PD-1 antibody and another group received biweekly anti-PD-1 antibody with GM.ova administered with every other anti-PD-1 treatment (monthly). Shown are the absolute numbers of ovalbumin-specific CD8 T cells in the spleen by tetramer staining (A) and ovalbumin-specific CD8 T cells costained with the activation marker CD107a (B).

gradual infiltration of T cells into the tumor environment with peak infiltration seen on day 14, which correlated with the delay in tumor growth observed in this group ( $P < 0.05$ , HBSS versus B16.Kd.GM for all time points). Animals treated with the combination therapy exhibited a rapid and persistent tumor infiltration of functional CD4 and CD8 T cells ( $P < 0.05$ , B16.Kd.GM versus combination therapy on day 21) that correlated with its increased antitumor efficacy.

A favorable intratumoral ratio of effector T cells to regulatory T cells after immunotherapy has been described to correlate with overall antitumor activity (44) and was therefore assessed in tumors of animals treated with either B16.Kd.GM monotherapy or the combination therapy. At the early time points (weeks 1 and 2 after immunotherapy), the intratumoral ratio of effector T cells to regulatory T cells was comparable between the B16.Kd.GM monotherapy and the combination therapy treatment groups (data not shown). In contrast, 3 weeks after immunotherapy, the ratio of effector T cells to regulatory T cells was significantly increased in animals that received the combination therapy when compared with B16.Kd.GM immunotherapy-treated animals (Fig. 5B). The correlation between the improved ratio of effector T cells to regulatory T cells observed in animals treated with the combination therapy and the better control of tumor growth in this group again suggests that the presence of regulatory T cells in tumors blunts CD8 T-cell activity and that cancer immunotherapies that shift this ratio in favor of the effector T cells are more effective at providing antitumor responses. In summary, these data showed a strict correlation between the potency of the antitumor T-cell responses measured in the periphery, the number of effector T cells infiltrating into the tumors, and the overall control of disease progression in animals treated with either B16.Kd.GM monotherapy or in combination with an anti-PD-1 antibody.

**Antigen-specific T-cell expansion requires the combination therapy in each boost cycle.** To determine the optimal schedule for administration of each component of the combination therapy to expand antigen-specific T cells effectively in a biweekly multitreatment setting, three different combination therapy treatment schedules were evaluated. To first establish an antigen-specific T-cell response, all B16 tumor-bearing animals received GM.ova immunotherapy and anti-PD-1 antibody at the first therapy cycle. In all subsequent therapy cycles, one group of animals received biweekly GM.ova immunotherapy and anti-PD-1 antibody; another group of animals received biweekly GM.ova immunotherapy alone; and yet another treatment group received biweekly anti-PD-1 antibody and monthly GM.ova immunotherapy. At indicated time points, splenocytes from selected animals were isolated and evaluated for the presence of ovalbumin-specific CD8<sup>+</sup> T cells by costaining the cells with anti-CD8 antibody and the SIINFEKL tetramer. The study showed the expansion and contraction of ovalbumin-specific CD8 T cells in the spleen after each treatment cycle (Fig. 6A) and that the number of ovalbumin-specific CD8 T cells peaked 7 days after each therapy cycle only in animals that were concurrently treated with the cellular immunotherapy and the PD-1 blockade. After the second treatment cycle, the combination therapy and the cellular monotherapy groups both showed an ~2-fold expansion in the number of tetramer-positive cells from the baseline levels reached after the first peak. This expansion was significantly weaker than the one observed after the initial

treatment cycle. In animals that received the cellular immunotherapy alone as the boost treatment, the peak of the ovalbumin-specific CD8 T-cell response was observed 14 days after the second therapy cycle whereas it was reached more quickly in the combination therapy group (7 days after the second therapy cycle). No T-cell expansion was detected after subsequent therapy cycles in the group treated with the cellular immunotherapy only. In contrast, in animals that received biweekly anti-PD-1 antibody and monthly GM.ova immunotherapy, expansion of the ovalbumin-specific CD8 T cells was only observed after the therapy cycle that consisted of the cellular immunotherapy and the PD-1 blockade and not when only the antibody was administered. The effector phenotype of these ovalbumin-specific CD8 T cells was confirmed by costaining the cells with CD107a, a marker for the lysosome membrane protein that is up-regulated on activated lytic CD8 T cells (Fig. 6B). The number of tetramer-positive cells correlated with the number of cells with an effector phenotype in all treatment groups at all evaluated time points. In addition, serum levels of autoantibodies (anti-nuclear antibodies) from the different therapy groups correlated with the expansion of antigen-specific T cells (Supplementary Fig. S3). In summary, these data suggested that readministration of the combination of cellular immunotherapy and the anti-PD-1 antibody in treatment boost cycles was required to expand antigen-specific T cells.

## Discussion

The therapeutic efficacy of immunotherapies generally correlates with the generation of strong antigen-specific T- and B-cell responses, and augmentation of such responses may increase the overall potency of immunotherapies. This study showed that PD-1 blockade in combination with GM-CSF-secreting tumor cell immunotherapy increases tumor-specific T-cell responses and that these increases correlated with an overall increase in antitumor therapeutic benefit. Other immune-modulatory antibodies, including anti-CTLA-4, anti-4-1BB, and anti-OX-40, have also been reported to enhance antitumor immune responses induced by GM-CSF-secreting tumor cell immunotherapies in preclinical tumor models (10, 11, 45). The anti-CTLA-4 antibody, which among the three is the most advanced antibody in clinical development, is currently being evaluated in combination with GM-CSF-secreting tumor cell immunotherapy in a phase 1/2 study in patients with castration-resistant prostate cancer.

PD-1 is an inhibitory receptor, and the blockade of PD-1 receptor on activated T cells during immunotherapy-driven T-cell priming resulted in the increase of tumor-specific T-cell responses *in vivo*. The detection of increased numbers of tetramer-positive T cells implies that preventing the interaction of the PD-1 receptor with its ligands during immunotherapy-mediated T-cell activation leads to sustained proliferation of activated T cells, which translates to enhanced therapeutic efficacy in tumor-bearing animals, just as reported with CTLA-4 blockade. As has been described for the anti-CTLA-4 antibody (8, 45), monotherapy with an anti-PD-1 antibody does not provide therapeutic benefit to animals bearing poorly immunogenic tumors such as the B16 melanoma but shows some activity in highly immunogenic tumor models such as the CT26 model. However, in both models, when used in combination

with GM-CSF–secreting tumor cell immunotherapy, treatment with either antibody results in enhanced antitumor immunity and improved therapeutic activity compared with animals treated with the cellular immunotherapy alone.

In addition to the rapid recruitment of high numbers of IFN $\gamma$  and CD107a-expressing effector CD8 T cells to the tumors of animals that received the combination therapy, the data also showed a significantly greater ratio of effector T cells to regulatory T cells (Total CD8<sup>+</sup>/CD4<sup>+</sup>FoxP3<sup>+</sup>) in animals that received the combination therapy compared with cellular immunotherapy alone (Fig. 5B). A favorable ratio of effector T cells to regulatory T cells within tumors has previously been shown to be essential for tumor rejection in animals treated with a GM-CSF–secreting tumor cell immunotherapy in combination with anti-CTLA-4 antibody (44). The latter data are in agreement with the data presented in this study showing that anti-PD-1 antibody augments the number of cellular immunotherapy–specific tumor-reactive T cells, induces better infiltration of these effector T cells into tumors, and changes the intratumoral balance of effector T cells to regulatory T cells in favor of the former, which correlated with enhanced overall antitumor responses.

The PD-1/PD-L pathway consists of the PD-1 receptor and two ligands, PD-L1 and PD-L2. PD-L1 is expressed on dendritic cells, macrophages, B cells, and T cells and is further up-regulated in all of these cell types on activation (20). In contrast, PD-L2 is up-regulated exclusively on dendritic cells and macrophages on activation. High PD-L1 expression has been found in most human cancers tested thus far and is believed to be one of the mechanisms by which tumors escape immune recognition by the induction of anergy and/or apoptosis of tumor-reactive effector T cells at the tumor site (33, 46). In the absence of PD-1 or if binding to PD-1 is blocked, PD-L1 can have a stimulatory effect on T cells (18, 47). Thus, the direct antitumor activity of anti-PD-1 and its effects on tumor-specific T cells observed in these studies are suggestive of and consistent with an effect of PD-1 blockade at the level of T-cell priming. The increase in the number and proliferation of antigen-specific T cells and the increased cytokine secretion by these cells, as well as the rapid onset of immune responses observed in animals treated with the combination therapy as compared with the cellular therapy alone, are evidence of a systemic effect of PD-1 blockade. These data are also consistent with observations by Probst et al. (48) in which the absence of PD-1 converted a tolerogenic dendritic cell into a priming stimulus for CD8 T cells. Importantly, the addition of PD-1 blockade to the allogeneic cellular immunotherapy did not disproportionately increase the allogeneic over the tumor-specific immune response (data not shown), both of which were increased by about 1.5- to 2-fold, which is crucial considering the repeated cycles of both therapies.

PD-1 receptor blockade in combination with the cellular immunotherapy enhanced the recruitment to and prolonged the persistence of immune effector cells at the tumor compared with the cellular immunotherapy alone, which may be a consequence of PD-L1 expression at the tumor site. Similar expression levels of PD-L1 are observed on the B16 tumors from animals that received HBSS, B16.Kd.GM monotherapy, or the combination therapy (data not shown). These data suggested that the suppressive nature of the tumor by PD-L1

should be similar between all experimental groups. Furthermore, because the expression levels of PD-1 on the tumor-infiltrating T cells could not be assessed in this study due to lack of a suitable anti-PD-1 antibody, it could not be determined whether the disruption of PD-1/PD-L1 by the anti-PD-1 antibody within the tumor microenvironment contributed to the more favorable effector T-cell to regulatory T-cell ratio in animals treated with the combination therapy.

These studies also showed that the cellular immunotherapy and the anti-PD-1 antibody both need to be administered in each boost cycle to optimally and consistently expand or boost the antigen-specific T cells. In addition, serum levels of autoantibodies (anti-nuclear antibodies) from the different therapy groups correlated with the extent of antigen-specific T-cell expansion, suggesting that therapeutic benefit and potential immune-related adverse events might correlate. Although PD-1 blockade has the ability to reverse antigen-specific T-cell suppression and exhaustion (16, 17), the presence of antigen is also critical for expanding the population of effector cells. In addition, although cellular immunotherapy has the ability to expand the T cells, these T cells could potentially be exhausted and the anti-PD-1 antibody would therefore be required to reverse the exhaustion. Thus, the antigen-specific T-cell expansion observed in animals that received the combination therapy at each immunotherapy cycle could potentially be a combined effect of the cellular immunotherapy driving the T-cell expansion and the reversal of T-cell suppression and exhaustion by the anti-PD-1 antibody. Finally, the peak of the SIINFEKL-positive T cells in the spleen after the immunotherapy boost cycles was significantly lower than the peak observed after the initial treatment. This observation could potentially be a result of the differences in homing of activated, antigen-specific T cells after multiple cycles of therapy or could be due to the treatment schedule used in this study. Thus, studies evaluating other lymphoid organs for the presence of antigen-specific T cells and evaluation of various treatment schedules (biweekly versus monthly) are necessary before moving this promising combination therapy into clinical development.

In conclusion, the data in this report suggest that blockade of the PD-1 receptor during immune activation by a GM-CSF–secreting tumor cell immunotherapy leads to an increased expansion of antigen-specific T cells by preventing the inhibitory signals triggered by the interaction of PD-1 with its ligands PD-L1 and/or PD-L2, thereby altering the intratumoral balance of effector to regulatory T cells in favor of the former, resulting in enhanced tumor rejection. In addition, each immunotherapy cycle requires the presence of the cellular immunotherapy for the expansion of effector T cells and the anti-PD-1 therapy for the reversal of T-cell exhaustion.

### Disclosure of Potential Conflicts of Interest

B. Li, M. VanRoey, and K. Jooss were employed by Cell Genesys, Inc. C. Wang, T. Chen, and A. Korman are employed by Medarex.

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