Microenvironment and Immunology

Therapeutic PD-1 Pathway Blockade Augments with Other Modalities of Immunotherapy T-Cell Function to Prevent Immune Decline in Ovarian Cancer

Jaikumar Duraiswamy1, Gordon J. Freeman2, and George Coukos1,3

Abstract

The tumor microenvironment mediates induction of the immunosuppressive programmed cell death-1 (PD-1) pathway, and targeted interventions against this pathway can help restore antitumor immunity. To gain insight into these responses, we studied the interaction between PD-1 expressed on T cells and its ligands (PD-1:PD-L1, PD-1:PD-L2, and PD-L1:B7.1), expressed on other cells in the tumor microenvironment, using a syngeneic orthotopic mouse model of epithelial ovarian cancer (ID8). Exhaustion of tumor-infiltrating lymphocytes (TIL) correlated with expression of PD-1 ligands by tumor cells and tumor-derived myeloid cells, including tumor-associated macrophages (TAM), dendritic cells, and myeloid-derived suppressor cells (MDSC). When combined with GVAX or FVAX vaccination (consisting of irradiated ID8 cells expressing granuloocyte macrophage colony—stimulating factor or FL/T3 ligand) and costimulation by agonistic α-4-1BB or TLR 9 ligand, antibody-mediated blockade of PD-1 or PD-L1 triggered rejection of ID8 tumors in 75% of tumor-bearing mice. This therapeutic effect was associated with increased proliferation and function of tumor antigen-specific effector CD8+ T cells, inhibition of suppressive regulatory T cells (Treg) and MDSC, upregulation of effector T-cell signaling molecules, and generation of T memory precursor cells. Overall, PD-1/PD-L1 blockade enhanced the amplitude of tumor immunity by reprogramming suppressive and stimulatory signals that yielded more powerful cancer control.

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Introduction

At the time of diagnosis, more than 75% of patients with ovarian cancer present with advanced stage III or IV disease (1, 2). Despite appropriate surgery and receiving highly effective first-line chemotherapy, approximately 70% of patients with advanced-stage disease who achieve remission eventually relapse (1, 2). Thus, there is an immediate need for therapeutic targets for treating ovarian cancer (3). Our group and others have reported that tumor-infiltrating lymphocytes (TIL) with antitumor potential exist in patients with cancer (4–7). Studies in a primary coculture system showed that TILs from many patients with ovarian cancer secrete low to intermediate levels of IFN-γ and have limited proliferation in response to cognate peptides (unpublished observation). The programmed cell death-1 (PD-1) is an inhibitory surface receptor expressed by T cells, B cells, natural killer T cells, monocytes, and dendritic cells (DC), but not by resting T cells. PD-1 binds two ligands, programmed cell death ligand 1 (PD-L1) and PD-L2 called B7-H1 and B7-DC, respectively (8, 9). Tumors can use the PD-1 inhibitory pathway to silence the immune system (8). The expression of PD-L1 in tumors is inversely correlated with survival of patients (10, 11). This indicates that, although antitumor immunity is elicited against ovarian cancer, it is counterbalanced by immunosuppressive factors.

In ovarian tumors, myeloid cells are one of the major determinants of immune suppression. These include tumor-associated macrophages (TAM), immature/tolerogenic dendritic cells, and myeloid-derived suppressor cells (MDSC; refs. 12–21). In addition, CD4+CD25+Foxp3+ regulatory T cells (Treg) play a critical role in the control of antitumor immune responses, relying on PD-1, PD-L1, or CTLA-4 associated antigen 4 (CTLA-4) to carry out these functions (22–27). Most studies describe mechanisms for the accumulation of these immunosuppressive myeloid cells or Tregs. In this study, we show a cross-regulation among these cell types using the ID8 syngeneic mouse model of epithelial ovarian cancer. We provide evidence that T-cell dysfunction can be reversed by targeting the PD-1 pathway simultaneously in all these cell types. We found that expansion of ovarian antigen-specific CD8+ TILs was dependent on the amount of PD-L1 signaling by tumor cells, tumor-derived myeloid cells, and Tregs. Furthermore, combining PD-1 blockade with a single dose of GVAX or FVAX...
vaccination resulted in enhanced clonal expansion of antigen-specific CD8+ T cells and tumor control. Finally, we observed a further boost of CD8+ T-cell function when PD-L1 blockade was combined with both vaccination and α-4-1BB costimulation. Overall, our study shows that α-PD-L1 blockade therapy greatly synergizes with other immunotherapy modalities.

Materials and Methods

Mice and tumor lines
All experiments were carried out using protocols approved by the University of Pennsylvania Laboratory Animal Resources policies. A mouse ovarian epithelial papillary serous adenocarcinoma cell line (ID8) was obtained from Dr. K.F. Roby (University of Kansas Medical Center, Kansas City, KS; ref. 28). Development of ID8 cells expressing murine granulocyte macrophage colony—stimulating factor (GM-CSF; ID8-GVAX) or Flt3-ligand (ID8-FVAX) was based on methods described previously (29).

Blocking and agonistic antibodies
Rat anti-mouse PD-1 (29F.1A12, at IgG2a, κ), PD-L1 (10F.9G2, rat IgG2b, κ), PD-L2 [3.2, mouse immunoglobulin G1 (IgG1)], and isotype control antibodies were used (7). In addition, the rat anti-mouse 4-1BB antibody from BioXcell was used.

Tumor experiments
C57BL6 mice (n = 12) were given an intraperitoneal injection containing 5 × 10^6 ID8 cells. Two hundred micrograms of rat α-mouse-PD-1, -PD-L1, and -PD-L2 antibodies as well as 100 μg of α-CTLA-4 (clone 9D9) or isotype control antibodies were administered intraperitoneally, 4 weeks after tumor inoculation five times on alternate days. For costimulation, 200 μg of α-4-1BB antibody from BioXcell was given once together
with the first aforementioned dose of blocking antibodies. In vaccination experiments, $10^6$ irradiated (150 Gy) gene-modified ID8-GVAX or ID8-FVAX cells were given by intraperitoneally once, 3 weeks after tumor inoculation. Mice were monitored by their weight gain twice a week. Mice weighing more than 35 g as a result of tumor growth and/or ascites were euthanized.

Materials and Methods are detailed in the Supplementary Data.

Results

Lack of T-cell infiltration in ovarian tumors is acquired

We have previously reported that, at the steady state, established ID8 tumors are poorly infiltrated by T cells (30) and are, thus, considered intrinsically nonimmunogenic, mimicking many human ovarian cancers lacking TILs (6). In this orthotopic model of ovarian cancer, gross metastatic intraperitoneal nodules appear at approximately 28 days, and tumor and ascites rapidly accumulate, leading to death at 55 to 60 days (Fig. 1A). We investigated the changes in the frequency of TILs in ovarian tumors, ascites, and spleens of mice from early (~28 days) to late times of tumor growth (~52 days). Interestingly, CD3$^+$ (CD8$^+$ and CD4$^+$) T cells infiltrating during the early tumors were almost completely absent in advanced tumors (Fig. 1B).

Interestingly, a high percentage of T cells in advanced tumors were Tregs (31), whereas there were no detectable CD8$^+$ T cells left and the depletion of T cells was restricted to

Figure 2. Expression of PD-L1, PD-L2, and PD-1 in ID8 mice. Mice were inoculated intraperitoneally with $5 \times 10^6$ ID8 tumor cells ($n = 12$), and their tumor, ascites, spleen, liver, lung, and blood were harvested from half of the mice at early and the other half at advanced tumor stages. Tumor cells (EpCAM$^+$), macrophages (CD45$^+$CD11B$^+$F4/80$^+$), dendritic cells (CD45$^+$CD11c$^+$), and MDSCs (CD45$^+$CD11B$^+$Gr-1$^+$) were isolated from the tumor, ascites, and spleen. Histograms show PD-L1 (A) and PD-L2 (B) expression by ID-8 tumor cells as well as macrophages, dendritic cells, and MDSCs from tumor, ascites, and spleen of ID-8 tumor-bearing mice. C, PD-1 expression on CD8$^+$ T cells from tumor, ascites, spleen, blood, liver, and lung of ID-8 tumor-bearing mice. Results are from one of the three experiments.
tumors. Parallel to the observed disappearance of CD3+ TILs and the increase in Tregs, we found a significant increase in MDSCs (CD11b+ Gr-1+; P = 0.024; Fig. 1B; Supplementary Fig. S1) and TAMs (CD11b+ F4/80+, P = 0.048) in tumors, but no significant changes in CD11c+ dendritic cells (P = 0.10; data not shown). Thus, lack of immunogenicity in established ID8 tumors seems to be acquired rather than intrinsic.

**PD-1 ligands are present in the microenvironment of ovarian tumors**

Previous immunohistochemical studies have reported upregulation of PD-L1 in human ovarian cancers (10). Here, we observed high levels of PD-L1 and moderate levels of PD-L2 on ID8 tumor cells, as well as macrophages, dendritic cells, and MDSCs derived from the same tumors (Fig. 2A and B; Supplementary Fig. S2A). Similarly, myeloid cells derived from ascites showed moderate expression of high levels of PD-L1 and PD-L2; however, spleens showed low-level expression of only PD-L1, but not PD-L2. Next, we found that PD-1 was expressed by matched CD8+ T cells in tumor at both early and later stages (Fig. 2C; Supplementary Fig. 2B), but PD-1 expression was not detected in other organs except PD-1dim CD8+ T cells in the liver at early stages of tumor. Thus, T cells specifically upregulate PD-1 in the tumor microenvironment where they are likely to receive inhibitory signals.

**PD-1 or PD-L1, but not PD-L2, blockade therapy causes regression of ovarian tumors**

We next tested whether the blockade of PD-1 could reverse the decline of T-cell immunity and promote tumor rejection in mice. In addition, we sought to compare the relative contribution of PD-L1 versus PD-L2. We inoculated C57BL/6 mice intraperitoneally with ID8 tumor cells and then administered α-PD-1, α-PD-L1, or α-PD-L2 antibodies starting on day 28 (Fig. 3A, left). Treatment with α-PD-1 or α-PD-L1 antibodies resulted in tumor rejection in 25% (3 of 12) of the mice, as indicated by normalized mouse weights after treatment (weight gain is due to ascites and is an accurate surrogate of tumor growth in this model), and long-term survival. On the contrary, α-PD-L2 antibody did not reject tumors. To test whether PD-1-mediated TIL suppression was active at even earlier stages of tumor development, we treated mice with α-PD-L1, starting on day 21. PD-L1 blockade resulted in tumor rejection in 60% (7 of 12) of the mice (Fig. 3A, right). Thus, the PD-1 pathway is highly relevant, and is active very early in the process of establishment of ovarian tumors.

**PD-1 or PD-L1, but not PD-L2, blockade therapy prevents immune decline in the tumor microenvironment**

Next, we tested whether PD-1 blockade was associated with reversal of immune decline in tumors and enhanced TIL infiltration. We selected the more advanced treatment schedule (starting on day 28), as earlier. A week after completion of the treatment (~day 45), residual peritoneal tumor deposits were resected and analyzed. The frequency of CD8+ and CD4+ T cells (as well as total CD45+ leukocytes) was markedly increased following the administration of α-PD-1 and α-PD-L1. Interestingly, despite the lack of tumor response, there was an increase in CD8+ and total CD45+ cells following α-PD-L2 (Fig. 4A). Importantly, PD-1 and PD-L1 blockade significantly reduced Tregs and, thereby, increased the CD8+ to Treg and CD4+ to Treg ratios within the tumor (Fig. 4B). In contrast, no such decrease in Tregs was seen after α-PD-L2 treatment and, thus, there was no significant change in the CD8+ or CD4+ Treg ratios.

In addition, we noticed PD-L1 surface expression on Tregs (Supplementary Fig. S1). To understand whether PD-1–PD-L1 interactions contribute directly to the inhibitory function of tumor Tregs, CD4+ CD25+ Tregs isolated from ID8 tumors were incubated with responder spleen CD8+ T cells stimulated...
Figure 4. PD-1 or PD-L1 blockade increases immune activation of TILs in ID8 tumor. One week following completion of blockade treatment, the TILs were harvested from regressing tumors and stained with various markers. Percentages of CD8<sup>+</sup> and CD4<sup>+</sup> TIL (CD45<sup>+</sup>) infiltration of total leucocytes (A) and the ratio of CD8<sup>+</sup> T cells to Tregs (B) are shown in treated versus untreated groups. C, blocking PD-1–PD-L1 interaction reduced Treg-mediated suppression of CD8<sup>+</sup> T cells in vitro. CFSE-labeled CD8<sup>+</sup> T cells were cocultured with syngeneic, αCD3-loaded dendritic cells with or without Tregs and α-PD-L1 or αCTLA-4 as indicated. CD8<sup>+</sup> T cells and stimulator APCs were obtained from naive B6 mice. Tregs were obtained from ID8–tumor-bearing mice. Treg-mediated suppression of proliferation of naïve CD8<sup>+</sup> T cells was noticed. Results from one of three experiments are shown. D, the ratio of CD8<sup>+</sup> T cells to MDSCs are shown. E, CD8<sup>+</sup> TILs from α-PD-L1–treated mice were stained with arginase-1 (8C9 clone from SantaCruz Biotechnology) and analyzed by flow cytometry. The CD11b<sup>+</sup> arginase-1<sup>+</sup> MDSCs within CD45<sup>+</sup> TILs are shown. F, tumor-derived MDSCs were plated at 1 × 10<sup>6</sup>/well in 24-well plates and stimulated with equal amount of tumor supernatants (from ID8 cells). Following stimulation, cells were added with α-PD-L1 and then arginase I was analyzed after 24 hours following washing with PBS and lysis buffer. G, percentage of Ki67<sup>+</sup> and Granzyme B<sup>+</sup> as well as mean fluorescence intensity of pT-bet, pEomes, pS6, and pAkt expression by CD8<sup>+</sup> TILs are shown. The results are the sum of three independent experiments with 8 to 10 mice per group. Bars represent mean ± SEM. *, P ≤ 0.05; **, P ≤ 0.01.
by immobilized α-CD3 antibody, in the presence or absence of PD-L1 blocking antibody. T-cell proliferation was assessed by carboxy-fluorescein diacetate succinimidyl ester (CFSE) dilution. Although Tregs suppressed responder cell proliferation, α-PD-L1 attenuated the ability of Tregs to suppress CD8+ T-cell proliferation (Fig. 4C; \( P < 0.05 \); Supplementary Fig. S3).

Thus, PD-L1 blockade not only reduced the relative frequency of Tregs in ID8 tumors, but also attenuated the suppressive function of tumor-derived Tregs.

In addition, MDSCs were decreased specifically by PD-L1 blockade, which thereby increased the ratio of T cells to MDSCs within the tumor (Fig. 4D). MDSCs suppress effector T cells through the production of arginase-I (32, 33). Interestingly, we detected a significantly lower number of arginase-I–positive MDSCs in ID8 tumors after treatment with α-PD-L1 (Fig. 4E). To assess whether arginase-I is involved in the mechanisms through which PD-L1+ MDSCs suppress TILs in ID8 tumors, CD11b+ Gr1+ isolated from ID8 tumors were further cultured with ID8 tumor cell supernatants in the presence or absence of α-PD-L1 antibody. Arginase catalyzes the conversion of arginine to ornithine and urea in the urea cycle. Arginase activity was evaluated by measuring the urea concentration in the media. We found that PD-L1 blockade decreased the level and activity of arginase-I (Fig. 4F; \( P = \ldots \))

Figure 5. Synergistic effect of PD-1 blockade, GVAX or FVAX vaccination and α-4-1BB costimulation on ovarian ID8 tumor rejection. A, 3 weeks after ID8 tumor inoculation, the mice were administered 2 × 106 irradiated (150 Gy) GVAX (top) or FVAX (bottom) intraperitoneally once. A week later, mice were injected five times intraperitoneally with α-PD-1 (200 µg), α-PD-L1 (200 µg), or α-PD-L2 (200 µg) on alternate days or a single dose 200 µg of α-4-1BB either alone or in combination as indicated. Results are from one of three experiments.

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0.0003). Thus, PD-L1 blockade not only reduced the relative frequency of CD11b+ Gr1+ cells, but also reduced their suppressive phenotype by reducing arginase-I activity, which seems to be dependent, in part, on PD-1–PD-L1 interactions.

The earlier data indicate that PD-1 or PD-L1 blockade significantly attenuated the number and function of Tregs and/or MDSCs. We, thus, tested whether the earlier changes were associated with activation of CD8+ TILs. We detected significant upregulation of Ki67 and intracellular granzyme B, as well as increased levels of phosphorylated transcription factors, T-bet, Eomes, and S6 kinase, and Akt, supporting the theory that effective PD-1 blockade activated function and survival pathways in CD8+ TILs (Fig. 4G; Supplementary Fig. S4; refs. 34, 35).

**Vaccination synergizes with PD-1 blockade to prevent immune decline in tumors**

We theorized that vaccine administered in combination with PD-1 blockade could enhance the efficacy of PD-1 blockade in preventing immune decline in tumors. Previous studies have described a therapeutic efficacy of B16 autologous melanoma tumor vaccines expressing either GM-CSF or FMS-like tyrosine kinase 3 ligand (Flt3-ligand), when combined with the blocking CTLA-4 (29, 32, 36–38). Transduced ID8 whole tumor cells to express either GM-CSF (ID8-GVAX; Fig. 5A) or Flt3L (ID8-FVAX; Fig. 5B) and used irradiated cells as vaccine in combination with PD-1, PD-L1, or PD-L2 blockade. Mice received weekly vaccine, starting 3 weeks after tumor inoculation. A week later, mice were treated with PD-1–blocking antibodies five times on alternate days. Although GVAX alone had no effect on tumor growth or mouse survival, the addition of GVAX doubled the effect of checkpoint blockade on mouse survival. Fifty percent of mice that received GVAX plus PD-1 or PD-L1 blockade rejected their tumors, whereas antibodies alone resulted in 25% tumor rejection (Fig. 5A, left). In contrast, α-PD-L2 did not show any positive interaction with vaccination. Similar results were obtained with FVAX (Fig. 5B, left).

Interestingly, the positive interaction between vaccine and checkpoint blockade required full development of the vaccine effect, and this was achieved by increasing the time interval between vaccination and checkpoint blockade to 1 week (data not shown).

**Costimulatory signals further augment the combination of vaccine plus PD-L1 blockade**

The earlier results indicate that PD-1 or PD-L1 blockade can prevent immune decline in tumors, and that addition of vaccine further enhances this effect. Next, we hypothesized that adding a costimulatory signal, 4-1BB (CD137), a TNF receptor gene family member (33, 39–42), could further boost TILs and enhance the effects of checkpoint blockade and vaccination. Mice were vaccinated with GVAX or FVAX starting 3 weeks after tumor inoculation, and a week later, we administered five doses of α-PD-L1 on alternate days plus a single dose of α-4-1BB (200 μg) antibody with the first dose of α-PD-L1 (Fig. 5A right). Administration of the α-4-1BB antibody alone had no impact on tumor growth or survival. Furthermore, addition of α-4-1BB antibody to PD-L1 blockade had moderate impact on survival (Fig. 5A, right). Remarkably, 75% of mice receiving GVAX followed by α-PD-L1 plus α-4-1BB antibodies rejected their tumors. Similar results were obtained with FVAX (Fig. 5B, right).

In tumors, costimulatory signals could be provided by properly activated antigen-presenting cells (APC), which express the 4-1BB ligand as well as other important costimulatory ligands. Hypothesizing that similar therapeutic benefit as earlier could be obtained through T-cell costimulation by TLR-activated APCs, we used an agonistic murine TLR9 ligand, CpG 1668 (43). Mice were treated with five doses of CpG 1668 intraperitoneally (5 μg/mouse) in combination with α-PD-L1 antibody (Supplementary Fig. S5). Similar to α-4-1BB, we found significant therapeutic benefit by adding CpG. Importantly, the effect from this combination was seen only when CpG was given in combination with α-PD-L1 antibody, but not if CpG was administered earlier in combination with the vaccine, indicating that CpG did not contribute at enhancing T-cell priming. Thus, the therapeutic PD-L1 blockade has the potential to induce significant tumor rejection, which can be maximized if PD-L1 blockade is preceded by tumor vaccination, and accompanied by costimulation.

**Combination therapy reprograms the tumor immune microenvironment**

We next asked what were the changes in the tumor microenvironment induced by the ineffective vaccines GVAX and FVAX. Interestingly, vaccine alone increased significantly the frequency of TILs, especially CD8+ cells (P < 0.01; Fig. 5A; Supplementary Fig. S7A). Because vaccines proved ineffective, we asked whether this was associated with an increase in immunosuppressive populations. Importantly, we noticed that both GVAX and FVAX failed to reduce the frequency of Tregs in tumors. Moreover, GVAX increased the frequency of the MDSC population in tumor leukocytes (Fig. 6C). Conversely, FVAX increased the frequency of plasmacytoid dendritic cell (pDC) levels in tumors (Supplementary Fig. S6), which have been shown to have immunosuppressive properties (44). Thus, vaccine alone increased not only TILs but also immunosuppressive cells.

Mice treated with GVAX or FVAX plus α-PD-L1 showed significantly increased infiltration of total inflammatory CD45+ cells and T cells in tumors compared with mice treated with α-PD-L1 (P < 0.01) or vaccine (P < 0.001) alone. Among
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**A**

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them, CD8$^+$ T cells were more abundant than CD4$^+$ T cells (Fig. 6A; Supplementary Fig. S7A). When PD-L1 blockade was combined with GVAX or FVAX, there was a moderate reduction in Tregs as well as MDSC levels in the tumor (Fig. 6B and 6C; Supplementary Figs. S7B and C). The addition of α-4-1BB to α-PD-L1 significantly increased TILs relative to each agent alone, both CD8$^+$ and CD4$^+$. In addition, it markedly reduced Tregs relative to each agent alone. Consequently, the combination of α-4-1BB plus α-PD-L1 resulted in a marked increase in the CD8/Treg and CD4/Treg ratios, superior than each antibody alone. The combination of α-PD-L1 and α-4-1BB with GVAX or FVAX yielded the highest frequency in TILs, both CD8$^+$ and CD4$^+$. Furthermore, the addition of vaccine did not increase Tregs when α-PD-L1 and α-4-1BB were combined with GVAX or FVAX, thus resulting in the highest CD8/Treg and CD4/Treg ratios seen in this study. In addition, the triple combination yielded the highest frequency of Ki67$^+$ CD8$^+$ T cells compared with that seen with the other combinations (Figs. 6D; Supplementary Fig. ?D). Combination treatment (either GVAX or FVAX plus α-4-1BB and α-PD-L1) also increased granzyme B expression, suggesting an enhancement in cytolytic potential of effector T cells (Fig. 6D and Supplementary Fig. 7D). Increased phosphorylation of T-bet and Eomes was induced by α-PD-L1 antibody alone, and addition of α-4-1BB to α-PD-L1 produced only moderate further increases (Figs. 6E and 7E). Thus, the PD-L1 blockade induced TIL activation, and the combination of GVAX (or FVAX) and α-4-1BB treatment resulted in a further stimulation of the proliferative and cytolytic capability of TILs. Taken together, these changes could result in a more permissive environment for immune-mediated tumor rejection.

Combination therapy results in maximal stimulation of tumor-reactive CD8$^+$ TILs

Given the significant changes in the tumor microenvironment, we next tested whether the functional capacity of tumor-reactive CD8$^+$ TILs was increased following combination therapy. Tumors from the various treatment groups were freshly dissociated enzymatically, and total leukocytes isolated from these tumors were seeded in primary mixed cocultures and stimulated with K$^+$-restricted peptides of folate receptor-α or mesothelin, two known ovarian tumor antigens expressed by ID8 cells. TIL cultures derived from mice treated with GVAX or FVAX showed minimally increased IFN-γ$^+$ and TNF-α$^+$ CD8$^+$ TIL when stimulated with the FR-α (SSGHNECPV) and mesothelin (LSIFKHKL and LIWIPALL) peptides (Fig. 7A and B; Supplementary Fig. S8). TIL cultures derived from mice treated with α-PD-L1 alone showed modestly increased IFN-γ$^+$ and TNF-α$^+$ CD8$^+$ TIL when stimulated with the peptides. TIL cultures derived from mice treated with GVAX (or FVAX) plus α-PD-L1 (not shown) or α-4-1BB showed further increased IFN-γ$^+$ and TNF-α$^+$ CD8$^+$ TIL when stimulated with the peptides. However, when mice were treated with α-PD-L1 combined with GVAX (or FVAX) and α-4-1BB, TILs exhibited the highest numbers of IFN-γ$^+$ and TNF-α$^+$ CD8$^+$ T cells ex vivo (Fig. 7A and B). Importantly, the increase in IFN-γ production by TILs specific to FR-α and mesothelin peptides seemed to be correlated with a decrease in tumor load (Figs. 3 and 5). The functional status of CD8$^+$ TILs was corroborated by testing the phosphorylation status of key transcription factors. Triple combination therapy showed significant increases in pT-bet and pS6K levels, although only modest increase in pEomes (Fig. 7C).

Discussion

In this study, we show a critically important role of the PD-1 pathway in establishing an immune privilege site in the ovarian cancer microenvironment. Although tumors were infiltrated by T cells in very early stages of tumor orthotopic inoculation, a progressive reduction was noted in the frequency of CD8$^+$ cells and the CD8:Treg ratio, until very few TILs were finally found in advanced tumors. Most of these were Tregs. The role of Tregs in mediating immune suppression and tumor growth has been previously established in ovarian cancer (24, 31). A parallel increase in MDSCs and TAMs completed the picture of a well-organized tumor immune-suppressive environment. These findings are important in two ways. First, they indicate that lack of tumor immunogenicity is not intrinsic, but rather is acquired, at least in ID8 ovarian tumors. Second, we found that such progressive establishment of immune suppression can be reversed by blocking the PD-1 pathway. Thus, PD-1, besides mediating TIL exhaustion, can facilitate a profound depletion of TILs. Importantly, this phenomenon was mediated by PD-L1, as PD-L1 blockade produced similar results, but not PD-L2. PD-1 blockade restored the immune decline and led to expansion of TILs. An increase in the number of CD4$^+$ and CD8$^+$ cells was associated with increased CD8/Treg and CD4:Treg ratios. This was associated with a moderate clinical response, together with molecular evidence of functional activation of TILs. Reversal of decline was time-sensitive, as PD-1 blockade was more effective in earlier times, when more TILs were present in the tumor microenvironment. Moreover, the therapeutic effect of blocking PD-1/PD-L1 correlated with increased numbers of polyfunctional ovarian tumor-antigen (mouse folate receptor-α and mesothelin) specific CD8$^+$ T cells. In addition, our study provides further insight that tumor antigen-specific CD8$^+$ T cells upregulate the key effector and
memory precursor signaling molecules (T-bet, Eomesodermin, Akt, and S6 kinase) upon PD-1/PD-L1 blockade. This is in agreement with recent studies that described inhibition of signaling pathways by PD-1/PD-L1–mediated T-cell exhaustion (45–50).

Given this observation, we theorized that expanding the pool of tumor-reactive TILs through a whole-tumor antigen vaccine (GVAX or FVAX) could be beneficial in more advanced time points of the disease. We found that, in fact, although ineffective on its own, vaccine could double the efficacy of PD-L1 blockade. Importantly, vaccine inefficiency was not due to its inability to expand tumor-reactive TILs. Rather, increase in TILs was accompanied by a similar (compensatory) increase in Treg and other immunosuppressive populations (such as MDSCs or pDCs). Thus, failure of vaccines was due to their failure to reprogram the tumor microenvironment, which continued to take advantage of homeostatic anti-inflammatory mechanisms such as Treg, MDSCs, and/or pDCs. Importantly, PD-L1 blockade again was able to break these homeostatic mechanisms, to establish tumor rejection. In addition, we found that administration of agonistic 4-1BB stimulation and α-PD-L1 after vaccination produced maximal expansion of TILs and tumor rejection. We acknowledge that the dose of 4-1BB antibody (200 μg) used was high for human translation and that, in humans, 4-1BB agonistic antibodies have resulted in liver toxicity (51). However, we believe that our studies still provide proof of principle for adding costimulation to checkpoint blockade as a beneficial therapeutic combination. Interestingly, similar benefit was also obtained by administering CpG, a TLR9 agonist, intraperitoneally.

In this model, PD-L1 blockade was very effective, whereas PD-L2 blockade was ineffective. This effect is likely due to the high expression level of PD-L1 in ID8 tumor cells as well as tumor-derived myeloid cells. Although α-PD-L2 blockade resulted in moderately increased levels of TIL infiltration (CD45+, CD8+), proliferation (Ki67+), and signaling molecules (Eomes, Akt, and S6 kinase), Treg and MDSC levels were not reduced after α-PD-L2 blockade. In this regard, we previously noticed low-level expression of PD-L2 in other types of tumor models (23). Therefore, the lack of response to α-PD-L2 blockade may be due to low-level expression of PD-L2, lacking sufficient endogenous response upon in vivo blocking PD-L2. Although our studies provide strong evidence of a role of PD-L1 in the syngeneic ovarian model, they do not allow us to discern the relative contribution of PD-L1 expressed by tumor cells versus tumor stroma or infiltrating leukocytes. Future studies using PD-L1 knockout mice and bone marrow chimeras will be required to clarify this point. PD-L1 blockade overcame the upregulation of MDSCs and pDCs by GVAX and FVAX, respectively. Previous studies reported that FVAX was more effective in combination with blockade therapy than GVAX (34). Our study used different clones of rat anti-mouse PD-1 (RMP1-30) and rat anti-mouse PD-L1 (10F.9G2) compared with the Bioxcell antibodies α-PD-1 (RMP1-14) and α-PD-L1 (9G2) used in their study, which may have accounted for eliminating the differences previously observed between the two vaccine preparations.

Our findings have important implications in understanding the immunobiology of ovarian cancer and developing rational therapies. We have previously described the classification of ovarian cancers in tumors with and tumors without intraepithelial TILs (4). The present findings suggest that tumors lacking intraepithelial TILs may have a very active PD-L1–mediated suppression, leading to depletion of TILs. This would be in agreement with observations by Hamanishi and colleagues reporting that increased expression of PD-L1 (but not PD-L2) is associated with poor survival in patients with advanced ovarian cancer, similar to the lack of intraepithelial TILs (10). Our finding of high frequency of Tregs in tumors lacking TILs is consistent with an association of Tregs with poor outcome in human ovarian cancer (4) and with the observation that, in these tumors, we found increased expression of vascular endothelial growth factor (4), which also correlates with Treg preponderance in the tumor microenvironment (31). On the basis of the present findings, human ovarian cancer lacking intraepithelial TILs should not lack intrinsic immunogenicity, consistent with the notion that most advanced ovarian cancers express known tumor-associated antigens and harbor tons of nonsynonymous mutations, which could give rise to immunogenic epitopes. Rather, powerful immunosuppressive factors would eliminate TILs, suggesting that reversal of these factors would restore immune recognition and attack.

PD-L1 blockade could be an important step toward reversing such immunosuppressive environment, but additional maneuvers are likely required to optimize tumor immune attack.

In the present study, addition of a whole-tumor antigen vaccine significantly enhanced the ability of PD-L1 blockade. Importantly, just as in human patients with ovarian cancer treated with GVAX (37, 52), vaccine alone was ineffective in the mouse, but was quite beneficial in association with checkpoint blockade. We found that the reason whole-tumor vaccines were ineffective was because they elicited a significant activation of tumor microenvironment homeostatic mechanisms, with increased MDSCs or pDCs and no decrease in Tregs. Importantly, PD-L1 blockade abrogated this rebound increase in immunosuppressive cells and depleted Tregs, resulting in clinical benefit. Concomitant costimulation, through an agonistic antibody or CpG, further enhanced this benefit. Whole-tumor antigens have been proposed as better alternatives than molecularly defined vaccines and can be effective in inducing antitumor immune responses in patients with ovarian cancer (53). The increased availability of immunomodulatory antibodies could provide a new role for whole-tumor antigen vaccines according to our findings.

**Disclosure of Potential Conflicts of Interest**

G.J. Freeman has ownership interest (including patents) in CoStim Pharmaceuticals, Bristol-Myers Squibb, Roche, Merck, EMD Serono, Boehringer-Ingelheim, and Amplimmune and is a consultant/advisory board member of CoStim Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: J. Duraiswamy, G. Coukos

Development of methodology: J. Duraiswamy, G.J. Freeman

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Duraiswamy
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Duraiswamy
Writing, review, and/or revision of the manuscript: J. Duraiswamy, G.J. Freeman, G. Coukos
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Duraiswamy
Study supervision: J. Duraiswamy, G. Coukos

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