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Tumor-Infiltrating Programmed Death Receptor-1+ Dendritic Cells Mediate Immune Suppression in Ovarian Cancer

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Within the ovarian cancer microenvironment, there are several mechanisms that suppress the actions of antitumor immune effectors. Delineating the complex immune microenvironment is an important goal toward developing effective immune-based therapies. A dominant pathway of immune suppression in ovarian cancer involves tumor-associated and dendritic cell (DC)-associated B7-H1. The interaction of B7-H1 with PD-1 on tumor-infiltrating T cells is a widely cited theory of immune suppression involving B7-H1 in ovarian cancer. Recent studies suggest that the B7-H1 ligand, programmed death receptor-1 (PD-1), is also expressed on myeloid cells, complicating interpretations of how B7-H1 regulates DC function in the tumor. In this study, we found that ovarian cancer-infiltrating DCs progressively expressed increased levels of PD-1 over time in addition to B7-H1. These dual-positive PD-1+ B7-H1+ DCs have a classical DC phenotype (i.e., CD11c\(^+\)CD11b\(^+\)CD8\(^-\)), but are immature, suppressive, and respond poorly to danger signals. Accumulation of PD-1+B7-H1+ DCs in the tumor was associated with suppression of T cell activity and decreased infiltrating T cells in advancing tumors. T cell suppressor function of these DCs appeared to be mediated by T cell-associated PD-1. In contrast, ligation of PD-1 expressed on the tumor-associated DCs suppressed NF-\(\kappa\)B activation, release of immune regulatory cytokines, and upregulation of costimulatory molecules. PD-1 blockade in mice bearing ovarian cancer substantially reduced tumor burden and increased effector Ag-specific T cell responses. Our results reveal a novel role of tumor infiltrating PD-1+B7-H1+ DCs in mediating immune suppression in ovarian cancer. The Journal of Immunology, 2011, 186: 000–000.

O varian cancer is an immunologically active tumor that may be amenable to immune-based therapies. Over the past decade, studies have demonstrated the importance of the immune system in affecting patient outcomes, following conventional therapies such as surgery and chemotherapy. Notably, Zhang and colleagues (1) published a study that showed that infiltration of CD3 T cells was positively associated with survival. The presence of T cells was particularly beneficial for those individuals who demonstrated a complete clinical response to surgery and chemotherapy in which the 5-y survival was 74% compared with 12% for those without T cells (1). Subsequent studies have refined our understanding of intratumoral T cells, such as the study by Sato and colleagues (2) that showed that patients who had high levels of infiltrating CTLs had a median survival of 55 mo versus those with few or no CTLs who had a survival of 26 mo. The Ags to which the patients are naturally responding are now being systemically studied. For example, Goodell and colleagues (3, 4) found that ovarian cancer patients develop Ab responses to multiple proteins and that overall survival was enhanced in those patients who specifically had p53-specific Abs. Tumor Ag-specific T cell responses are also being identified. Our group has found that patients with ovarian cancer develop T cell immunity to multiple Ags overexpressed by the ovarian cancers such as folate receptor \(\alpha\) (FR\(\alpha\)) and insulin-like growth factor binding protein-2 (5, 6). Collectively, these results show that antitumor immunity is elicited against ovarian cancers and impacts favorably on the clinical course of the disease. However, the antitumor immunity is counterbalanced by an immune-suppressive microenvironment, constituted in part by lymphoid regulatory T cells (Tregs) and tolerance-inducing myeloid cells.

CD4\(^+\) Tregs are a group of different phenotypes of CD4\(^+\) T lineage (i.e., lymphoid origin) cells for which the primary function is immune regulation (7, 8). Treg-induced suppression of antitumor effector cells in the tumor microenvironment is mediated by cell surface molecules (e.g., CTLA-4) and by soluble factors (e.g., IL-10 and TGF-\(\beta\)) (7, 9–13). Increased Tregs in the ovarian cancer microenvironment portend a poor outcome (2, 14). Myeloid-derived suppressor cells (MDSCs), a more recently identified population of immune regulators, are also involved in suppressing antitumor immune responses. The tolerance-inducing MDSCs are uniquely identified by high coexpression of CD11b and Gr-1 (15). Several cancers, in humans and mice, cause the systemic accumulation of MDSCs (16–19). MDSCs may also have a central role in immune suppression of ovarian cancer in murine models (20). The predominant myeloid infiltrate in human ovarian cancer, however, appears to be CD11c\(^+\) dendritic cells (DCs), which may also
contribute to the profound immune-suppressive microenvironment (21).

Recently, it was reported that the immune regulatory molecule B7-H1 (programmed death receptor-1 [PD-1] ligand), found on the surface of ovarian cancer cells, is associated with poor overall survival (22). Its ligand, PD-1, is found expressed on various adaptive immune effectors, notably CD4 and CD8 T cells, where it negatively regulates cell activation in cancers including ovarian (23–26). PD-1 expression has recently been identified on DCs, where it suppresses innate immunity against infectious disease (27). Although the immune activating and suppressive properties of ovarian cancer-associated DCs are known to be regulated by B7-H1, the molecular interactions, particularly those associated with PD-1, remain elusive (28). Thus, in the current study, the role of B7-H1 and PD-1 in regulating ovarian cancer-associated DCs, the major myeloid cell type in ovarian cancer (21) was investigated, using the murine ID8 model of peritoneal ovarian cancer. The study reveals that the tumor-associated CD11c+ DCs use both B7-H1 and PD-1 to regulate their phenotype in the tumor microenvironment.

Materials and Methods

Animals

Four to 12-wk-old female C57BL/6J (B6J) mice from in-house or The Jackson Laboratory were used for experimentation. Animal care and use was in accordance with institutional guidelines.

Cell lines

Leukocytes were cultured in RPMI 1640 supplemented with 10% FBS, 25 mM HEPES, 1.5 g/l sodium bicarbonate, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, and 50 mM 2-ME. ID8 tumor cells were derived from immortalized ovarian epithelial cells generated by repeated passage in culture (29) and were grown in DMEM supplemented with 10% FBS, 1% penicillin, 25 mM HEPES, 1.5 g/l sodium bicarbonate, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, and 2 mM l-glutamine.

Tumor implantation

ID8 tumor cells were injected i.p. at a volume of 5 × 106 cells/500 μl saline. Mice were harvested for tumor and ascites between 40 and 70 d postimplantation.

Leukocyte culture

Naive mouse leukocytes were obtained from B10D2J mice spleens by grinding the spleen through a 70-μm nylon cell strainer. The splenocytes were centrifuged at 3000 rpm for 10 min. The cells were resuspended in 4 ml ACK buffer to lyse RBCs. The cells were then washed and resuspended in T cell media. Leukocytes from ascites of tumor-bearing mice were isolated by discontinuous Ficoll gradient as described previously (30). The leukocytes were collected at the top of the 75% layer and the tumor cells at the top of the 100% layer. Tumor-infiltrating leukocytes (TILs) were harvested by mincing the tumor through a 70-μm nylon cell strainer. The TILs were then separated from tumor cells by centrifugation of the cell suspension on a discontinuous Ficoll gradient consisting of a lower 100% layer and an upper 75% layer as described above.

Fractionation of tumor tissue and purification of TILs

From single-cell suspensions of tumors and ascites, lymphocytes were magnetically isolated using an Automacs sorting machine (Miltenyi Biotec) based on the cells of interest. Leukocytes were enriched using CD11c microbeads, CD90 (th1, th2) beads, and/or CD4 and CD8 isolation kits obtained from Miltenyi Biotec (Auburn, CA). The manufacturer’s protocol was followed for all magnetic separations.

IFN-γ ELISPOT assay

ELISPOT assay was done as described previously (31). T cells isolated from tumors, spleen, and ascites were left unstimulated or stimulated with splenocytes pulsed with peptide or ID8 tumor lysates and incubated at 37°C for 24 h. To detect Ag-specific CD8 T cell responses, the H2-Kb peptide FR161 (aa 161–169, SGSHNCPV) derived from murine F8Rα, an Ag expressed on ovarian cancers, was used.

Multiplexed microsphere cytokine immunoassay

Multiplex assay was done as previously described (31). Supernatants were removed from wells containing 2.5–5.0 × 105 unstimulated or stimulated DCs from tumors, ascites, naive C57BL/6J mouse bone marrow, or naive B6/6 spleens. Cytokines and chemokines were measured using multiplex microspheres as per the manufacturer’s direction (Bio-Rad, San Diego, CA).

Flow cytometry

Cell-surface molecule staining and flow cytometry were done essentially as previously described (32). Flow cytometric analysis, a similar number of events, usually 20,000–100,000, were collected for all groups. Abs against CD11c, CD80, CD86, CD86, C545, CTLA-4, CD19, B7-H1, and PD-1 were from eBioscience (San Diego, CA). Abs against CD3, CD4, and MHC class II were from eBioscience and BD Pharmingen. Appropriate isotype-matched nonspecific Abs were used as controls. Stains were done on whole populations and enriched populations.

Immunofluorescent staining

CD11c+ cells were purified from ascites of ID8 tumor-bearing mice as described above. Cells were plated into chamber slides and then incubated at 37°C for 3 h to allow for adherence. Media was removed, and the cells were washed twice with PBS containing 0.5% BSA and 2 mM EDTA. Cells were then incubated for 2 h with purified anti–PD-1, anti–B7-H1, or appropriate isotype control (eBioscience) in the same media followed by washing twice to remove excess Ab and further incubation for 1 h with Alexa Fluor-conjugated anti-IgG and fluorescein-conjugated anti-IgG. Cells were washed to remove excess Ab. The chambers were removed, and the slide itself was treated with two drops of Prolong Gold anti-fade reagent (Invitrogen). A coverslip was placed on the slide and allowed to dry overnight. The cells were visualized using a confocal microscope.

In vivo PD-1 blockade

Mice were inoculated i.p. with 5 × 106 ID8 cells. Twenty to 25 d after tumor implantation, mice received 200 μg hamster IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) or 200 μg G4 clone PD-1–blocking Ab i.p. as described by Hirano and colleagues (33). The mice were treated eight times over 3 wk. After the final treatment, tumor and ascites were harvested. Tumors were weighed and processed to single-cell suspension as described above. CD4+ and CD8+ cells were isolated using negative selection beads from Miltenyi Biotec. These cells were used in ELISpot assay, as described above.

In vitro suppression assays

In vitro T cell proliferation was examined using a triated thymidine incorporation assay in 96-well plates as previously described (32). Allogeneic stimulators (S) were derived from BALB/c mice spleens and irradiated to 3000 rad before use in the MLR. To create an MLR, S cells were mixed with splenocytes derived from the B6/J mouse or B7-H1 knockout mice (B7-H1–/–) on a B6/J background called effector (E) cells. The ratio of S to E cells was 1:2–1:8. CD11c cells, derived from the ID8 tumor-bearing animals, were titrated in to the MLR reactions based on a ratio of DC to E cells. The serial dilutions of CD11c cells began from 1:1, 1:2, and 1:4. Proliferation of E cells was measured by adding 1 μCi/200 μl [3H]thymidine. Following 16 h of culture, cells were harvested on a filter harvester (PerkinElmer, Boston, MA). The filter membrane was dried and scintillation fluid added. The amount of radioactivity was measured on a Top Count NXT scintillation counter (PerkinElmer). Data are expressed as the mean percentage of control uptake or as a stimulation index calculated as the ratio of the mean value of the experimental wells over the mean value of the control wells. In experiments determining reversal, 10 μg/ml PD-1–blocking Abs were used per well. To determine if reactions with DCs are mediated by direct contact, a transwell was added to the MLR plate with a 3-μm membrane pore (Cardinal Health) to which either CD11c cells from ascites or naive spleen were added to separate them from the MLR. A hamster IgG isotype was used as a control.

Determination of phosphorylated NF-κB p65 in CD11c+ DCs

Phosphorylated p65 was evaluated using the PathScan phospho-p65 ELISA per the manufacturer’s directions (Cell Signaling Technology, Danvers,
MA) using purified ascite-derived CD11c+ DCs. B7-H1 Ig (number 1019-B7) was obtained from R&D Systems (Minneapolis, MN).

Statistical analyses
Statistical analyses were performed using GraphPad Prism version 4.00 (GraphPad, San Diego, CA; http://www.graphpad.com) for Windows (Microsoft). The Student t test, Mann–Whitney U test, or two-way ANOVA test was performed to determine statistically significant differences. A p value < 0.05 was considered significant.

Results
CD11c+ myeloid cells derived from the ovarian cancer microenvironment demonstrate an immune-suppressive phenotype
Tumor-associated CD11c+ DCs often have an immature suppressor phenotype. To assess whether ovarian cancer-associated DCs in the murine ID8 model have an activating or inhibitory effect, CD11c+ cells were purified from ascite-derived DCs (A-DCs) of mice and were tested for suppression in in vitro assays (Fig. 1). As shown in Fig. 1A, magnetic isolation results in enrichment of CD11c+ high adherent DCs with mixed morphology and size. As shown in Fig. 1A, A-DCs dose-dependently suppressed T cell proliferation responses (p < 0.003). In contrast, DCs purified from naive B6 spleen failed to mediate any suppression at a 1:1 E/DC ratio. Both tumor-derived DCs (T-DCs) and A-DCs were characterized by an immune regulatory (34–37) rather than a proinflammatory cytokine signature, namely higher spontaneous release of IL-6, IL-10, and G-CSF in the absence of IL-12 p40 (Fig. 1B, 1C). Both T-DCs and A-DCs had blunted or nonexistent IL-12p40 production responses to TLR stimulation as assessed by response to LPS and CpG as compared with bone marrow-derived DCs (BMDCs) (Fig. 1C). The IL-12p40 responses in T-DCs and A-DCs were comparable to normal spleen immature DCs (NSDCs). In contrast, both T-DCs and A-DCs had blunted IL-10 production responses to CpG as compared with NSDCs (Fig. 1C). In fact, the T-DCs demonstrated reduced production following TLR stimulation. There were negligible levels of IL-2 and IFN-γ secretion by these different DCs, indicating no contamination from lymphocytes (data not shown). Collectively, these data indicate that DCs within the ovarian cancer microenvironment have an immature, suppressive, and altered cytokine production phenotype.

Tumor-associated DCs have a nonactivated CD11c+CD8− GR-1lo/intCD11b+ phenotype
Although the majority of ovarian cancer-associated DCs expressed both MHC class I and II, the levels were qualitatively lower than the levels observed in splenic DCs (Fig. 2). CD86 was not expressed by either the A-DCs or T-DCs commensurate with an immature phenotype. Whereas ICAM-1 (CD54) was present at moderate levels, the inhibitory molecule, surface CTLA-4, was expressed at low levels on a subpopulation of the T-DCs and A-DCs. Both A-DCs and T-DCs were CD11b+Gr-1int/low and CD11c+CD8−, which, together with their suppressor phenotype, classifies them as immune regulatory DCs of the classical DC lineage but apparently distinct from typical GR-1hiCD11b+CD11c− MDSCs (38). In addition, tumor-associated DCs expressed the DC-specific CD209a marker (Fig. 2B). Staining for other T cell markers (CD3, CD4), the B cells marker CD19, and NK cell marker CD49 demonstrated minimal contamination of the gate with other infiltrating leukocytes. Additionally, conventional MDSCs (GR-1hiCD11b+CD11c−) were not detected in the tumor but were detected in spleens of tumor-bearing animals (not shown).
Ovarian cancer-associated DCs coexpress PD-1 and B7-H1 and accumulate in the ovarian cancer microenvironment

PD-1 is inducibly expressed on CD4^+ T cells and CD8^+ T cells upon activation (24). The interaction of PD-1 expressed on T cells with B7-H1 present on the same cell type or different cell type such as APCs results in inactivation of effector T cells (23). Given the inhibitory nature of CD11c^+ DCs observed in Fig. 1, it was hypothesized that they expressed PD-1 and/or B7-H1. Consistent with prior work, it was found that a fraction of T cells infiltrating into tumor expressed PD-1 along with B7-H1 (not shown). A key finding, however, was the coexpression of PD-1 and B7-H1 on the CD11c^+ DCs derived from both tumor and ascites (Fig. 3A,3B). PD-1 expression on BMDCs and NSDCs was minimal (<5%, data not shown).

To determine if PD-1 expression was dynamic on the DCs over the course of the disease, time points were chosen at middle to late disease in which tumor was processed and analyzed using flow cytometry. Tumors are first visible in this model at about day 40. As shown in Fig. 3C, CD11c^+ infiltration increased over the course of disease and constituted ~11% of total leukocytes harvested from the tumor. The numbers of PD-1^+ DCs among the total leukocytes also increased as expression increased on the tumor-associated DCs over the course of the disease (Fig. 3C). On day 42, post-tumor challenge, ~65% of the CD11c^+ cells were PD-1^+, which increased to 92% by day 61 (Fig. 3C).

Ovarian cancer-associated DCs block T cell proliferation through PD-1 and in a contact-dependent manner

Ovarian cancer-associated DCs are immune regulatory, express PD-1, and accumulate in the cancer microenvironment. Predicted from these observations would be the presence of tolerized T cells in the tumor, possibly T cell depletion, and a role for the B7-H1/PD-1 pathway. As shown in Fig. 4A, T cells isolated from the tumor at day 50 failed to respond to bryostatin and ionomycin, whereas normal naive spleen-derived T cells responded robustly over the course of 10 d, which is evident from the increase in the cell number. Similar results were observed using anti-CD3– and anti-CD28–coated beads. The observation that the numbers of CD3^+CD4^+ Th or Tregs and CD3^+CD8^+ CTL or regulatory CD8 T cells increased with the advancing tumor until day 47 but then eventually nearly completely disappeared during the same time course consistent with a developing immune-suppressive microenvironment (Fig. 4B,4C). To ascertain whether PD-1 was involved in immune suppression mediated by the ovarian cancer-associated DCs, blocking anti–PD-1 Abs were used in in vitro suppression assays. As shown in Fig. 4D, a complete reversal in suppression was observed when blocking PD-1 Ab was added. Conversely, in the wells to which no PD-1 Ab or isotype control Ab was added, suppression of effector T cell proliferation was...
The role of PD-1+ DCs in inhibiting the proliferation of T cells was confirmed by using anti–PD-1 Ab with E and S cells (Fig. 4E). Whether suppression induced by DCs depends on direct cell contact or not was examined using transwell chambers. As shown in Fig. 4F, the majority of the DC-induced suppression of E cells was reversed with the transwell filter. There was some suppression when the DCs were separated by the transwell, which may be due to the production of immune regulatory cytokines. Thus, it was questioned, using T cells from B7-H1−/− mice, whether the inhibitory effects of PD-1+ DCs were mediated through the interaction of PD-1 on the DCs and B7-H1 on the effector T cells. The results revealed that B7-H1 on the DCs was mediating the suppressive actions (Fig. 4G). Indeed, flow cytometry revealed coexpression of PD-1 and B7-H1 on the tumor-associated DCs (Fig. 4H).

**FIGURE 4.** Ovarian cancer-associated DCs block T cell proliferation through PD-1 and in a contact-dependent manner. A, Numbers of TILs derived from tumor (TIL) (left panel) and splenocytes (SP) (right panel) cultured in the presence/absence of bryostatin/ ionomycin (BI) and anti-CD3/CD28 beads. Each data point is the mean (± SEM) of three to four determinations, and the experiment is representative of three or four experiments. B and C, The mean (± SEM) proportions of helper/regulatory and CTL/regulatory (i.e., CD3+CD4+ T cells and CD3+CD8+ T cells, respectively) on days 42, 47, and 61. T cells are expressed as a percent of total leukocytes. Shown are the means (± SEM) of two to three replicates. Panels are representative of three independent experiments. D, Tritiated thymidine incorporation into E cells in an MLR assay in the absence or presence of A-DCs. Some wells also contained either anti–PD-1 Ab (αPD1) or an isotype control Ab (iso). E, Tritiated thymidine incorporation into E cells in the wells containing either anti–PD-1 Ab (αPD1) or an isotype control Ab (iso). F, Thymidine incorporation into E cells in the presence or absence of A-DCs or NSDCs. The DCs were either in direct contact (C) with the E cells or were separated by a transwell membrane (T). G, Thymidine incorporation into E cells derived from normal B6 mice or B7-H1−/− in the presence or absence of A-DCs and anti–PD-1 Abs. In all panels, each bar is the mean and SEM of three replicates. The experiment is representative of three similar experiments. H, Dot plots of T-DCs or A-DCs, respectively, at day 47 following tumor challenge, stained for B7-H1 on the x-axis and PD-1 on the y-axis. Inset numbers are the percentages for the respective quadrants. Cells were gated on CD11c.

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**Treatment of ovarian cancer-bearing mice with anti–PD-1 Abs enhances T cell immunity in the tumor and reduces tumor burden**

The in vivo importance of PD-1 in the control of ovarian tumors was examined in a treatment model in which mice were challenged with tumor, i.p., followed by treatment with i.p. injections of anti–PD-1 or irrelevant Abs. As shown in Fig. 5A, mice treated with PD-1–blocking Ab have a significantly reduced tumor size as compared with mice treated with appropriate isotype Ab. Tumors in mice treated with anti–PD-1 Abs also had significantly higher levels of IFN-γ–producing CD4 T cells (Fig. 5B). The immune
response observed in PD-1–treated mice was Ag-specific as discerned by evaluation of response of the tumor localized CD8 T cells to an H2d peptide, FRα p161–169 (Fig. 5C). Treatment of mice with anti–PD-1 Ab also resulted in a significant elevation of splenic IFN-γ–producing CD4 T cells (Fig. 5D). Overall, the results are consistent with a regulatory role of PD-1 in suppressing the generation of local and systemic T cell activation.

**PD-1 regulates cytokine production and NF-κB activation by tumor-associated DCs and maintains their immature phenotype**

Although PD-1 is well known to suppress activation of T cells, its role in regulating the activity of DCs is relatively unknown. Given that PD-1+B7-H1+ DCs likely do not use their PD-1 to block T cell proliferation (Fig. 4G), the role of PD-1 on tumor-associated DCs was questioned by incubating purified CD11c+ cells with blocking anti–PD-1 Abs. In mice treated with irrelevant or anti–PD-1 Abs, IFN-γ+ CD4 T cells in B and D were detected without stimulation. Values (mean ± SEM) in B-D are calculated from eight, three, and five replicates, respectively, from three independent experiments.

**Discussion**

The PD-1/B7-H1 pathway is an important inhibitory pathway in the tumor microenvironment (23, 24). PD-1 expression on tumor-infiltrating CD8 T cells and increased expression of B7-H1 on tumor cells correlates with poor prognosis in patients with different types of cancers, such as breast, ovarian, pancreatic, gastric, kidney, and bladder cancers (39–44). In ovarian cancer, the PD-1/B7-H1 pathway seems to be a dominant immune-suppression mechanism. For example, Hamanishi and colleagues (22) found that 5-y survival in ovarian cancer was significantly worse for those with high-level B7-H1 expression compared with those with low-level expression (i.e., 80.2 versus 52.6 y, respectively; p = 0.016). The model infers that PD-1 on E cells such as CD8 T cells interacts with B7-H1 expressed in the tumor microenvironment and inhibits antitumor activity of T cells (45). In contrast to this paradigm, in this study, we observed that PD-1 along with B7-H1 is expressed on suppressive ovarian tumor-infiltrating CD11c+CD8α+Gr-1lo/−CD11b+ DCs as well as tumor-infiltrating T cells. Expression of PD-1 was dynamic and increased on DCs with advancing disease. It was observed that ligation of PD-1 on the CD11c+ DCs resulted in negative signals that constitutively blocked release of cytokines. Also, PD-1 expression on the CD11c+ DCs regulated release of cytokines, predominantly immune regulatory, a unique finding that could have implications for therapies that aim to activate intratumoral DCs with adjuvants (46).

It could be argued that the myeloid cells identified in the current study are MDSCs because of coexpression of CD11b with low to intermediate levels of Gr-1. MDSCs are a heterogeneous population of suppressor cells that have been identified in most cancer patients and experimental tumor-bearing mice (47). Mouse studies show that MDSCs accumulate not only in the tumor but also in the spleen, blood, and bone marrow (47). The problem with classifying the cells identified in the current study as MDSCs is the expression of CD11c, which is thought to be highly restricted to DCs and is a marker not typically found on tumor-infiltrating MDSCs (48). Furthermore, flow cytometry studies revealed expression of another DC-specific marker, CD209a, the mouse homolog of human DC-specific ICAM-3-grabbing nonintegrin. CD209a is a 238-aa type II transmembrane lectin that is almost exclusively expressed on CD8-negative DCs (49). Despite that, however, Gr-1high MDSCs have been observed in the ascites of ID8-bearing mice by Liu and colleagues (50). Furthermore, these tumor-associated MDSCs express PD-1, which regulates their arginase-dependent immune-suppressive activity (50). In contrast, we were unable to detect Gr-1highCD11b+ MDSCs in the tumor but could detect them in the spleens of tumor-bearing mice (K.L. Knutson, unpublished observations). Our findings that CD11c+ DCs are the predominant suppressor cells in the tumor microenvironment are consistent with recent studies from Conejo-Garcia and colleagues (51) using a similar ID8 model system. However, there are also key differences between the current study and the published studies of Conejo-Garcia. For example, they have found that the predominant suppressive DC population infiltrating tumors is CD8α+CD80− DCs (21). One likely reason for the differences observed is the use of ID8 cells transfected with Vegf-A, which could alter patterns of infiltration.

A key finding in the current study is that when PD-1 is blocked on the DC, there was a large increase in release of immune regulatory cytokines, such as IL-10, IL-6, and G-CSF. The accumulation of high levels of immune regulatory cytokines may explain why PD-1 blockade in vivo had only a partial antitumor effect. This conclusion, however, may be simplistic, because blockade of PD-1 completely reversed the suppressive activity of the DCs in vitro.

**FIGURE 5.** PD-1 regulates ovarian cancer immunity in vivo. A, Tumor weight in milligrams in tumor-bearing mice treated with an irrelevant or PD-1–specific Ab. Tumor burden was measured on day 40 following tumor challenge. Bars are the mean of 25 mice, representative of five separate experiments. B–D, Shown are the mean (SEM) numbers of IFN-γ–producing T cells in the tumor (total IFN-γ+ CD4 T cells and FR161-specific CD8 T cells) and the spleen (total IFN-γ+ CD4 T cells), respectively, in mice treated with irrelevant or anti–PD-1 Abs. IFN-γ+ CD4 T cells in B and D were detected without stimulation. Values (mean ± SEM) in B–D are calculated from eight, three, and five replicates, respectively, from three independent experiments.

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Despite that, however, in vitro assays using MHC mismatch are not likely to reflect self-Ag–specific T cell activation in vivo, as the mismatch assays may be less sensitive to immune-suppressive cytokines. To confirm the role of PD-1 in immune suppression mediated by ovarian tumor-associated DCs in in vivo studies, a mouse model selectively depleted for PD-1 on DCs (i.e., PD-1 DC knockout mice) is required, and unfortunately, these mice are not yet available. Furthermore, the immune microenvironment greatly influences the types of local immune suppression observed. For example, Yang and colleagues (20) showed that suppression of viral Ag-specific T cell cytokine secretion responses by murine ovarian cancer (ID8)-derived MDSCs are mediated through a CD80-dependent activation of CD4+CD25+ Tregs. Nonetheless, multiple redundant mechanisms of immune suppression and tolerance induction in the peritoneal microenvironment may explain the propensity for ovarian cancer to thrive in this environment with minimal spread to other remote regions of the body, such as the lungs, liver, and brain. In fact, multiple cytokine responses observed upon blockade of PD-1 on tumor-derived DCs using anti–PD-1 Ab confirms the fact that multiple mechanisms rather than one single parameter are being affected by interaction of PD-1 on DCs with B7-H1 on DCs. This complicates the clear analysis of the role of PD-1 on tumor-derived DCs in this study. Thus, future studies addressing the functional role of PD-1 on these ID8 tumor-derived DCs are warranted.
monocytogenes. Even though the signaling mechanism of inhibitory function of PD-1 on DCs remains to be determined, prior work has shown that PD-1 ligation in lymphocytes results in phosphorylation of PD-1’s immunoreceptor tyrosine-based switch motif followed by recruitment of Src homology region 2 domain-containing phosphatase-2, which subsequently suppresses tyrosine phosphorylation-based signaling cascades (53–55). Future studies could be directed at determining if similar Src homology region 2 domain-containing phosphatase-1 activation occurs and, importantly, whether such inhibitory signaling regulates maturation of DCs into the activating phenotype. In fact, some of the data obtained in this study suggest that blockade of PD-1 on these DCs results in the maturation of these cells that otherwise have an immature phenotype.

Previous reports suggest that along with Tregs, MDSCs, and soluble immunosuppressive factors such as IL-10 and TGF-β, DCs (both plasmacytoid DCs and myeloid DCs) in the ovarian tumor microenvironment also play a role in inducing immune suppression or promoting tumor formation that is mediated by factors such as IL-8, TNF-α, and IDO produced by these inhibitory DCs (56, 57). In this study, we show that DCs in the ovarian tumor microenvironment express both PD-1 and B7-H1 and use this well-known B7-H1/PD-1 inhibitory pathway in mediating immune suppression. In conclusion, our studies reveal that ovarian cancer-derived immune-suppressive DCs not only use the B7-H1/PD-1 pathway to block adaptive immune responses, but are also themselves regulated by this pathway. Additionally, the results underscore the importance of understanding that mechanisms of immune suppression are dynamic in the ovarian cancer microenvironment, suggesting that interventions in early disease may not be suitable for advanced disease. Thus, the immune-suppressing microenvironment in ovarian cancer is a complex network and may require multiple therapeutic approaches to block and reverse this suppression to favor tumor eradication.

Disclosures

The authors have no financial conflicts of interest.

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