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*J Immunol* 2012; 189:1708-1716; Prepublished online 13 July 2012;
doi: 10.4049/jimmunol.1201271

http://www.jimmunol.org/content/189/4/1708

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**Supplementary Material**

http://www.jimmunol.org/content/suppl/2012/07/13/jimmunol.120127.1.DC1

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Differential Requirement for CD70 and CD80/CD86 in Dendritic Cell-Mediated Activation of Tumor-Tolerized CD8 T Cells

S. Peter Bak,* Mike Stein Barnkob,* Ailin Bai,*† Eileen M. Higham,*† K. Dane Wittrup,*§ and Jianzhu Chen*‡

A major obstacle to efficacious T cell-based cancer immunotherapy is the tolerizing-tumor microenvironment that rapidly inactivates tumor-infiltrating lymphocytes. In an autochthonous model of prostate cancer, we have previously shown that intratumoral injection of Ag-loaded dendritic cells (DCs) delays T cell tolerance induction as well as refunctionalizes already tolerized T cells in the tumor tissue. In this study, we have defined molecular interactions that mediate the effects of DCs. We show that pretreating Ag-loaded DCs with anti-CD70 Ab abolishes the ability of DCs to delay tumor-mediated T cell tolerance induction, whereas interfering with 4-1BBL, CD80, CD86, or both CD80 and CD86 had no significant effect. In contrast, CD80−/− or CD80−/−CD86−/− DCs failed to reactivate already tolerized T cells in the tumor tissue, whereas interfering with CD70 and 4-1BBL had no effect. Furthermore, despite a high level of programmed death 1 expression by tumor-infiltrating T cells and programmed death ligand 1 expression in the prostate, disrupting programmed death 1/programmed death ligand 1 interaction did not enhance T cell function in this model. These findings reveal dynamic requirements for costimulatory signals to overcome tumor-induced tolerance and have significant implications for developing more effective cancer immunotherapies. The Journal of Immunology, 2012, 189: 1708–1716.

A major focus of cancer immunotherapy has been stimulating patients’ CD8+ cytolytic T cells to kill tumor cells. In one treatment modality, tumor-infiltrating leukocytes (TILs) are isolated from the patient, activated ex vivo, and infused back into the same patient. Such adoptive cell therapy (ACT) has shown clinical benefit in treating melanoma (1). In another treatment modality, dendritic cell (DC)-based vaccines are used to stimulate the patients’ endogenous anti-tumor immune response, and recently has been approved for treating prostate cancer (2). Despite these successes, a major hurdle to widespread use of these and other treatments using CD8+ T cells is the tolerizing environment within the tumor tissue (1), which rapidly inactivates TILs and renders the therapies ineffective.

Abbreviations used in this article: ACT, adoptive cell therapy; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; PD-1, programmed death 1; PD-L1, programmed death ligand 1; pMHC, peptide MHC; TIL, tumor-infiltrating lymphocyte; TRAMP, Transgenic Adenocarcinoma of the Mouse Prostate; TRP-SIY, TRAMP mice expressing SIY; WSN-SIY, WSN influenza A virus strain expressing SIY.

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Received for publication May 8, 2012. Accepted for publication June 17, 2012.

This work is supported in part by a Postdoctoral Fellowship (12109-PF-11-025-01-LIB) from the American Cancer Society (to S.P.B.), the Margaret A. Cunningham Immune Mechanisms in Cancer Research Fellowship (to S.P.B.) from the John D. Proctor Foundation, and a Prostate Cancer Research Program grant from the United States Army Medical Research and Materiel Command (to J.C.).

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The online version of this article contains supplemental material. Abbreviations used in this article: ACT, adoptive cell therapy; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; PD-1, programmed death 1; PD-L1, programmed death ligand 1; pMHC, peptide MHC; TIL, tumor-infiltrating lymphocyte; TRAMP, Transgenic Adenocarcinoma of the Mouse Prostate; TRP-SIY, TRAMP mice expressing SIY; WSN-SIY, WSN influenza A virus strain expressing SIY.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1201271
As in human patients, effector T cells infiltrate the prostate tumor tissue and rapidly become inactivated (tolerized). The tolerized 2C T cells persist in the prostate tumor tissue (12), expressing high levels of PD-1, analogous to TILs in patients. Importantly, we have found that Ag-loaded bone marrow-derived DCs (BMDCs), when injected intraprostatically, delay the rapid tolerance induction of effector 2C T cells as they initially infiltrate the tumor tissue (13). In addition, when Ag-loaded BMDCs are injected after initial tolerance induction, they refunctionalize the persisting tolerized 2C T cells in the tumor tissue. These previous studies set the stage for defining molecular interactions that are required for prostate tumor-mediated T cell tolerance induction and DC-mediated delay and reactivation of tolerized T cells in the prostate tumor microenvironment.

In this study, we have evaluated the role of PD-1/PD-L1 interaction in T cell tolerance induction in prostate tumor tissue and the role of CD80, CD86, 4-1BBL, and CD70 in DC-mediated delay of T cell tolerance induction and refunctionalization of tolerized T cells in prostate tumor tissue. Our results show that despite the high levels of PD-1 expression by prostate-infiltrating T cells and PD-L1 expression in the prostate, blocking PD-1/PD-L1 interaction has no effect on T cell tolerance induction in prostate tumor tissue. Although CD70 is required for DC-mediated delay of T cell tolerance induction, CD80 and CD86 are necessary for refunctionalizing the tolerized T cells in prostate tumor tissue. These findings show that different costimulatory signals are required to overcome tumor-tolerizing signals during the initial tolerance induction and reactivation of previously tolerized T cells. They also suggest approaches to overcoming the tolerizing tumor environment, to achieve more efficacious cancer immunotherapy by ACT and DC vaccination.

Materials and Methods

Mice, adoptive transfer, and influenza infection

TRP-SIY mice were generated as previously described (11). 2C TCR transgenic mice were maintained on C57BL/6 and RAG1–/– backgrounds (2C/RAG mice). CD86 and CD80/CD86 knockout mice on the C57BL/6 background were from The Jackson Laboratory (Bar Harbor, ME). CD80, PD-L1 knockout, and TRAMP mice were bred in house. Where indicated, mice were retro-orbitally injected with 1.5 × 10⁶ naive 2C cells from 2C/RAG mice and were intranasally infected with 100 PFU WSN influenza A virus strain expressing SIY (WSN-SIY) influenza A virus. All experiments were approved by the Committee on Animal Care at Massachusetts Institute of Technology.

Generation of BMDCs and intraprostatic injection

Bone marrow was collected from the femurs of C57BL/6 mice or indicated strains. Cells were resuspended at 2 × 10⁶ cells per milliliter in RPMI 1640 plus 10% FCS, 50 μM 2-ME, 4 mM L-glutamine, and 100 U/ml-100 μg/ml penicillin-streptomycin supplemented 1:30 with supernatant from 15 cells secreting GM-CSF. Cells were washed and media changed on days 2 and 5, 1 μM CFSE was added and media were harvested on day 6, and cells were harvested on day 7. Where indicated, BMDCs were loaded with 1 μg/ml SIY peptide for 1 h at 37°C and/or were labeled for 10 min at 37°C with 5 μM CFSE (Invitrogen) in PBS plus 0.1% BSA on the day of harvest. For blocking experiments, 1 × 10⁶ cell/ml were incubated in 10 μg/ml F(ab′)₂ fragments of indicated Abs for 2.5 h on ice, as previously described (14). BMDCs were rinsed and resuspended at 1 × 10⁶ cells per 40 μl PBS for intraprostatic injection (1 × 10⁶ cells per mouse).

Cells and flow cytometry

Single-cell suspensions were prepared from lymphoid tissues by grinding them between frosted glass slides and filtering the suspensions through 70-μm nylon mesh. Prostate tissues were digested with 2 μg/ml collagenase A (Roche) and 170 U/ml DNase I (Sigma-Aldrich, St. Louis, MO) in RPMI 1640 plus 10% FCS for 1 h at 37°C. Digested samples were then ground between frosted glass slides and filtered through 70-μm nylon mesh. Intracellular IFN-γ staining was performed with a BD Cytofix/Cytoperm Kit (BD Biosciences). Recovered cells were restimulated with 1 μg/ml SIY peptide for 4 h at 37°C in the presence of BD GolgiPlug containing breveldin A. Surface Ags were stained, dead cells were identified using a LIVE/DEAD Fixable Red Dead Cell Stain Kit (Invitrogen), and all cells were fixed and permeabilized. Samples were then stained with a PE-conjugated anti–IFN-γ Ab (BD Pharmingen). Gates were set using a panel of fluorescein minus one controls. Statistical analysis was conducted using a Student t test and data were considered significant if p < 0.05.

Immunohistochemistry

Prostate glands were excised from mice, mixed frozen in OCT compound (Tissue-Tek; Sakura, Torrance, CA), and cryosectioned at 10 μM by the Koch Institute Histology Core Facility. Sections were fixed with acetone and stained with Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Visualization of PD-L1 staining was carried out with 3,3'-diaminobenzidine substrate (Vector Laboratories) and counterstained with eosin (Sigma-Aldrich). Images were acquisitioned using a Zeiss Axiosplan II microscope, with Zeiss 25× (0.8) lenses and a QImaging MicroPublisher 5.0 color camera. Images were captured using OpenLab 5.5 (Improvision; PerkinElmer) and analyzed using ImageJ (National Institutes of Health).

Abs and F(ab′)₂ production

Fluorescently labeled Anti-Thy-1.1 (Clone OX-7), CD27 (LG.3A10), CD11c (N418), CD28 (37.51), PD1 (RMP1-30), 41BB (17B5), OX40 (OX-86), CD30 (CD30.1), and LAG-3 (C9B7W) were from BioLegend; anti–CD68 (P03.1), CD70 (FR70), 4-1BBL (TKS-1), anti–PD-L1 (MIH5), and Tim3 (SB.2C12) from eBioscience; anti–CD80 (16-10A1), CD44 (IM7), and rat IgG2b isotype (clone A95-1) from BD Pharmingen; and biotinylated anti-rat IgG (BA-4001) from Vector Laboratories. 2C TCR was stained using a biotin-conjugated clonotypic 1B2 Ab detected with streptavidin-APC (BioLegend). Stained cells were analyzed using a FACScalibur or LSR II (BD Biosciences) instrument, and the data were processed using FlowJo software (Tree Star).

In vivo cytotoxicity assay

In vivo cytotoxicity was performed as previously described (13). Briefly, C57BL/6 (Thy1.1+) splenocytes were activated on anti-CD3–coated plates in the presence of 50 U/ml IL-2 for 3 d. Half of the activated splenocytes were then labeled with 20 μM CFSE (CFSEHL) and pulsed with SIY peptide, and half were labeled with 1 μM (CFSEL). CFSEL and CFSEHL cells were mixed 1:1 and retroorbitally injected into mice (20 × 10⁶ cells per mouse). Approximately 24 h later, the ratio of DC CFSEL to CFSEL cells in various tissues was evaluated by flow cytometry.

Results

PD-1 is expressed on prostate resident T cells and PD-L1 on the prostate stroma

To define the pathways that regulate tolerance of 2C T cells in the TRP-SIY system, we assessed the expression of inhibitory receptors on the surface of 2C T cells. PD-1, Tim3, and LAG-3, individually or in combination, negatively regulate T cell responses in tumor tissues (15, 16). T cells recovered 13 days post transfer, (or ~6 d after initial infiltration into the prostate) expressed high levels of PD-1 (Fig. 1A). Expression of PD-1 was maintained on prostate resident 2C T cells beyond 36 d post transfer into TRP-SIY mice (Fig. 1A). In contrast to PD-1, prostate resident 2C T cells did not express Tim3 or LAG-3 either 13 or 36 d post transfer (Fig. 1B, 1C). PD-L1 expression was detected on both C57BL/6-SIY and TRP-SIY prostate cells by flow cytometry (Fig. 1D) and confirmed by histological staining of prostate tissue sections (Fig. 1E).

Modulating the PD-1/PD-L1 axis does not improve T cell responses in TRP-SIY mice

The expression of PD-1 on 2C T cells and PD-L1 on prostate cells provides the basis for a possible role of PD-1/PD-L1 interaction in...
tolerance induction in the TRP-SIY system. To test this idea, we generated TRP-SIY mice on a PD-L1−/− background. Following adoptive transfer of 2C T cells and intranasal infection with WSN-SIY influenza virus, effector 2C T cells infiltrated the prostate tumor tissue of TRP-SIY PD-L1−/− mice but rapidly lost IFN-γ expression, comparable to 2C T cells in prostates of TRP-SIY mice heterozygous for PD-L1 (Fig. 2A). Thus, PD-L1 is not required for T cell tolerance induction in the prostate tumor tissue of TRP-SIY mice.

Although PD-L1 expression was not directly responsible for tolerance induction, the expression of PD-1 on 2C T cells could affect their subsequent function. Therefore, we tested whether the PD-1/PD-L1 interaction between 2C T cells and BMDCs affects the BMDC-mediated activation of 2C T cells. 2C T cells were transferred into TRP-SIY mice and activated by intranasal WSN-SIY infection, and CD44+ effector 2C T cells were recovered from the prostate an additional 6 d later and analyzed for ability to express IFN-γ. Intraprostatic injection of SIY-loaded wild-type or PD-L1−/− BMDCs stimulated similar percentages of 2C T cells to express IFN-γ when compared with PBS control (Fig. 2C, 2D). Furthermore, 30 d after initial T cell transfer and infection, when infiltrating 2C T cells were already tolerated, SIY-loaded BMDCs from wild-type or PD-L1−/− mice were injected intraprostatically to assess whether they reactivated the tolerated T cell in situ to a similar extent (Fig. 2B). Intraprostatic injection of SIY-loaded wild-type or PD-L1−/− BMDCs reactivated similar percentages of 2C T cells to express IFN-γ as compared with PBS control (Fig. 2C, 2E). These results were further confirmed using blocking Abs to PD-L1 (Supplemental Fig. 1). To determine whether PD-L1−/− BMDCs affect the number of 2C T cells within the prostate, we analyzed the numbers of 2C T cells recovered from the prostates after BMDC injection 7 or 30 d post transfer (Fig. 2F, 2G). Injection of PD-L1−/− BMDCs did not increase the number of 2C T cells as compared with PBS or wild-type BMDCs at either time point. Taken together, these data suggest that despite expression of PD-1 on 2C T cells and PD-L1 in the prostate tissue, disruption of PD-1/PD-L1 interaction does not enhance DC-mediated delay of tolerance induction or refunctionalization of already tolerated T cells in prostate tumor tissue.

**BMDCs act directly on 2C T cells in the TRP-SIY prostate**

We assessed the mechanisms by which BMDCs activate 2C T cells within the prostate tissue. To exclude the possibility that injected BMDCs nonspecifically activate 2C T cells in the prostate, we measured IFN-γ expression by endogenous SIY−non-specific CD8+ T cells. Injection of BMDCs either 7 or 30 d after Thy1.1+ 2C T cell transfer did not stimulate IFN-γ production by endogenous Thy1.SIY+ CD8+ T cells (Fig. 3A). To determine whether injection of activated BMDCs affects endogenous prostate-resident DCs, we assessed costimulatory receptor expression by the endogenous CD11c+ population following injection of either PBS or BMDCs. Compared with PBS injection, injection of activated BMDCs into the prostate of TRP-SIY did not alter the expression of CD80, CD86, CD70, or 4-IBBL on endogenous CD11c+ cells from the prostate (Fig. 3B). Furthermore, injection of SIY-loaded BMDCs did not stimulate IL-2 production by 2C T cells either 7 or 30 d after 2C T cell transfer as compared with PBS (Fig. 3C).

In our experimental system, 2C T cells are activated in the periphery by WSN-SIY infection, and CD44+ effector 2C T cells traffic to the prostate (Fig. 3D). We therefore confirmed that the activity of injected BMDCs is dependent on the direct presentation of SIY peptide to 2C T cells. We compared the effect of injecting PBS, SIY peptide alone, or activated BMDCs that had not been pulsed with the SIY peptide on IFN-γ expression by 2C T cells in the prostate. Injection of SIY peptide alone did not significantly stimulate IFN-γ expression by 2C T cells above control PBS injection (Fig. 3E), suggesting that presentation of SIY by endogenous DCs is not a major factor in reactivation of 2C T cells. Injection of activated BMDCs without being loaded with the SIY peptide in vitro stimulated a higher fraction of 2C T cells to produce IFN-γ (Fig. 3E) than did PBS or SIY peptide alone. However, the effect was less than that with SIY-loaded BMDCs (compare with Fig. 2). The observed effect is not likely due to a nonspecific effect of injected BMDCs but is likely due to uptake and presentation of SIY peptide in the prostate of TRP-SIY mice, where the SIY transgene is robustly expressed (11). Taken together, these results suggest that BMDCs activate 2C T cells in the prostate tumor partly by directly engaging 2C T cells through pMHC/TCR interaction.

**CD80 and CD86 are required to reactivate tolerated T cells in prostate tumor tissue**

Next, we determined cell surface molecules on BMDCs that are required for reactivating 2C T cells, in addition to SIY/MHC. Upregulated upon maturation of DCs, CD80 and CD86 provide an important stimulus in the context of the TCR/peptide MHC-1 engagement (3). Specific to prostate cancer, provision of antagonistic CD80 or CD86 Abs abrogates the immunosuppressive environment and reduces tumor growth (17). BMDCs exhibit robust
expression of CD80 and CD86 before intraprostate injection (Fig. 4A). Therefore, we compared the effect of intraprostatic injection of SIY-loaded wild-type DCs and DCs deficient in CD80, CD86, or both in TRP-SIY mice. Experiments were carried out as in Fig. 2B, except SIY-loaded BMDCs from CD80−/− or CD86−/− mice were injected 7 d after initial T cell transfer and analyzed after 6 d (F) or 7 d (G) later. Graphs are from three independent experiments, with at least four mice per group. NS, Compared with PBS control.
CD86 is required for DC-mediated delay of tolerance induction in prostate tumor tissue.

In addition to their importance in T cell primary responses, CD80 and CD86 are vital for simulating productive secondary responses (18, 19). To determine the requirement for CD80 and/or CD86 in DC-mediated refunctionalization of persisting tolerized T cells in the prostate tumor tissue, we injected SIY-loaded DCs from wild-type, CD80<sup>−/−</sup>, CD86<sup>−/−</sup>, or CD80<sup>−/−</sup>CD86<sup>−/−</sup> mice 30 d after initial 2C cell transfer and infection. At 6–7 d later, Thy1.1<sup>−</sup>CD8<sup>+</sup> T cells were harvested from prostate tissue and analyzed for IFN-γ expression. IFN-γ versus CD8 plots were gated on live CD8<sup>+</sup> Thy1.1<sup>+</sup> cells. The numbers indicate percentage of IFN-γ<sup>+</sup> cells. (B) TRP-SIY mice were injected with PBS or WT-DCs in the prostate. At 6–7 d later, prostate tissues were dissociated, and single-cell suspensions were stained with CD11c and CD80, CD86, CD70, 4-1BBL (black line), or isotype control (filled gray line). Histograms are gated on live CD11c<sup>+</sup> cells. (C) TRP-SIY mice were injected with PBS- or SIY-loaded WT BMDCs on either day 7 or day 30 after initial 2C cell transfer and infection. At 6–7 d later, Thy1.1<sup>+</sup> 2C T cells were harvested from prostate tissue, stimulated with SIY peptide, and analyzed for IL-2 expression. IFN-γ versus Thy1.1 flow cytometry plots were gated on live 2C TCR<sup>+</sup> Thy1.1<sup>+</sup> cells. The numbers indicate percentage of IL-2<sup>+</sup> cells. (D) 2C T cells recovered from the prostates of TRP-SIY were assayed for CD44 expression 13 d post transfer and infection with WSN-SIY virus. Histograms are gated on Thy1.1<sup>−</sup>2C TCR<sup>+</sup> T cells with either CD44 (black line) or isotype (filled gray line) and representative of three independent experiments. (E) TRP-SIY mice were injected with PBS, SIY peptide, or LPS-activated wild-type (WT) BMDCs not pulsed with SIY peptide 7 d after initial 2C cell transfer and infection. At 6 d later, Thy1.1<sup>−</sup> 2C T cells were harvested from prostate tissue, stimulated with SIY peptide, and analyzed for IFN-γ expression. IFN-γ versus Thy1.1 flow cytometry plots were gated on live 2C TCR<sup>+</sup> Thy1.1<sup>+</sup> cells. The numbers indicate percentage of IFNγ<sup>+</sup> cells.
CD70, but not 4-1BBL, is required for BMDC-mediated delay in T cell tolerance

CD80 and CD86 do not affect the ability of BMDCs to delay 2C T cell tolerance, indicating other costimulatory molecules may potentiate these effects. TNF costimulatory molecules, such as 4-1BB and CD70, promote T cell activation during initial priming and may be important for 2C T cell function during initial infection within the prostate tissue (8). For example, engagement of 4-1BB on CD8+ T cells by 4-1BB enhances T cell cytotoxic function (20, 21). Similarly, CD70 functions to maintain T cell survival and proliferation in the periphery (22, 23). Activated BMDCs express higher levels of CD70 and 4-1BBL relative to the expression on endogenous prostate DCs (Fig. 5A, compared with Fig. 3B). We used anti-CD70 and anti-4-1BB Abs previously shown to block CD70/CD27 and 4-1BBL/4-1BB interactions in vivo, to define the contribution of CD70 and 4-1BB in DC-mediated delay of tolerance induction (14, 24). To exclude possible FcR-mediated effect on the DCs, we generated an F(ab’)_2 fragment of each Ab lacking the Fc portion of the molecule but still retaining binding activity, as assessed by the ability to compete with fluorescently labeled full-length Ab for cell surface binding (Fig. 5B). As a control, we used F(ab’)_2 of the Ab mixture against FcRs CD16 and CD32 (25). SIY-loaded BMDCs were incubated with each Ab fragment and then injected into prostate tumor tissue of TRP-SIY mice 7 d after 2C T cell transfer and WSN-SIY infection. FcR-blocking Abs did not diminish the ability of BMDCs to stimulate prolonged IFN-γ expression by infiltrating 2C T cells (Fig. 5C). Blocking the interaction between 4-1BB on 2C T cells and 4-1BB on BMDCs did not impair the ability of DCs to extend IFN-γ expression by infiltrating 2C T cells. However, blockade of CD70 on BMDCs significantly reduced their ability to stimulate prolonged IFN-γ expression by infiltrating 2C T cells in the prostate (Fig. 5C). The observed difference was not due to differential retention of anti-CD70–treated BMDCs within prostate tissue, compared with control–treated BMDCs (Fig. 5E), consistent with previous experiments with untreated BMDCs (13). These results show that CD70/CD27 interaction is required for DC-mediated delay of 2C T cell tolerance induction in prostate tumor tissue.

As 4-1BBL and CD70 have been shown to enhance recall responses and reactivate previously tolerized T cells (26, 27), we examined the effect of 4-1BBL and CD70 on reactivation of already tolerized 2C T cells. We injected Ab-treated, SIY-loaded BMDCs into the prostate of TRP-SIY mice 30 d after 2C T cell transfer and WSN-SIY infection. Blockade of either CD70 or 4-1BBL did not significantly reduce the fractions of persisting 2C T cells that were induced to express IFN-γ (Fig. 5D), indicating that CD70 and 4-1BBL are not required for reactivation of tolerized 2C T cells in prostate tumor tissue.

**CD27 is downregulated on prostate resident T cells**

The costimulatory molecules necessary to delay tolerance induction and reactivate already tolerized 2C T cells are distinct. BMDCs expressed similar levels of costimulatory molecules, whether they were used to delay tolerance induction or reactivate already tolerized T cells. The functional differences between these two time points may be a result of phenotypic changes on prostate resident 2C T cells. Thus, we assessed 2C T cell expression of CD28, CD27, and 4-1BB before transfer and 13, 23, and 36 d following transfer and infection in TRP-SIY mice. CD28 expression on 2C T cells from prostate remained similar at the three different time points and was similar to that of naive 2C T cells (Fig. 6A–C). The expression of 4-1BB was low on 2C T cells recovered from TRP-SIY prostate throughout the 36 d following transfer (Fig. 6D), indicating that CD27 and 4-1BBL are not required for reactivation of tolerized 2C T cells in prostate tumor tissue.
**Discussion**

Molecular interactions that underlie the ability of DCs to overcome tumor-induced T cell tolerance are largely unknown. In an autochthonous model of prostate cancer, we have previously defined two stages at which activated DCs can overcome tumor micro-environment: delaying tolerance induction of tumor-infiltrating T cells and reactivating already tolerized T cells in the tumor tissue. In this study, we have now identified molecules necessary for the effects of DCs. Our data show that CD70/CD27 interaction between T cells and BMDCs is required for DC-mediated delay in 2C T cell tolerance. Our study further refines the role of CD70 by demonstrating that CD70/CD27 interaction can sustain intratumoral T cell activity in an otherwise tolerizing environment. Previous reports of CD70 function in tumor models have been restricted to transplantable cell lines. Overexpression of CD70 by endogenous APCs enhances priming of T cells against the EL4-NP and B6F10 tumor cell lines (9, 28). Furthermore, activation of clonotypic T cells with an anti-CD27 Ab increases the rejection of another transplantable melanoma cell line (29). These studies focus on priming of anti-tumor T cells in transplantable tumor models. In contrast, we analyze the requirement of costimulatory molecules to overcome tolerization in an autochthonous prostate tumor model. Studies have shown that provision of Ag-specific CD4 T cells to TRAMP mice sustains SV40-specific CD8 T cell function through CD40L activation of endogenous DCs (30). As CD40L stimulation promotes CD70 expression on DCs (31), CD70-dependent signals from DCs may be a common pathway to activate prostate tumor-reactive T cells. Notably, CD80, CD86, and 4-1BB do not play a direct role in delaying prostate tumor-induced T cell tolerance, perhaps because they do not influence CD70 expression.

The CD80 and CD86 costimulatory molecules are best known for their role in naive T cell activation. Our results presented in this study demonstrate that CD80 is also required to reactivate tolerized T cells in prostate tumor tissue. It is notable that CD80-/- DCs are as potent as wild-type DCs in reactivating tolerized T cells. In contrast, blocking CD70 or 4-1BBL does not affect the ability of DCs to reactivate tolerized T cells, and this function is completely abolished when combined with CD86 deficiency. These results reveal distinct functions for CD80 and CD86 in reactivating tolerized T cells. CD80-/- DCs exhibit a significantly reduced ability to reactivate tolerized T cells, and this function is completely abolished when combined with CD86 deficiency. These results reveal distinct functions for CD80 and CD86 in reactivating tolerized T cells. In contrast, blocking CD70 or 4-1BB does not affect the ability of DCs to reactivate tolerized 2C T cells. This finding is perhaps explained by the lack of CD27 and low 4-1BB expression on persisting T cells in the TRP-SIY prostate. These findings highlight the spatiotemporal specificity of costimulatory molecules in augmenting tumor-specific T cell responses.

Our studies demonstrate that the ability of BMDCs to overcome T cell tolerance is based on a combination of Ag specificity and costimulation. Despite Ag expression within the TRP-SIY prostate (11), and a population of tissue resident DCs (13), 2C T cells are...
tolerized within the prostate. Indeed, we show that endogenous DCs within the prostate express levels of costimulatory molecules barely above background (Fig. 3B). Previous work has shown that depletion of prostate DCs reverses 2C T cell tolerance (13). Considering that tolerance can result from pMHC presentation by DCs lacking costimulatory signals (32), these data suggest that steady-state prostate resident DCs promote T cell tolerance, perhaps through pMHC/TCR engagement without costimulation.

However, when peptide-pulsed, LPS-activated, BMDCs are injected into the prostate, 2C T cells are reactivated in a CD70 or CD80/CD86-dependent manner. The injection of activated BMDCs may provide a costimulatory signal that is normally lacking in the TRP-SIY prostate. This notion is supported by three lines of evidence. First, we have demonstrated that 2C T cell reactivation is dependent on the interaction between costimulatory receptors and ligands (Figs. 4, 5). Second, LPS-activated BMDCs without SIY peptide activate a greater percentage of 2C T cells, compared with PBS injection, but less than SIY pulsed LPS activated DCs. This observation suggests that enough residual SIY peptide is present in the prostate to be acquired and presented by activated BMDCs. Third, the window in which 2C T cells remain activated in this model directly depends on the presence of BMDCs within the prostate (13), reinforcing the importance of the presence of costimulatory molecules.

In addition to stimulatory molecules, 2C T cells within the prostate express high levels of the inhibitory receptor PD-1. In human patients and mouse models of cancer, T cells express PD-1, Tim3, and LAG3 individually or in combination, depending on tumor type (16, 33, 34). We find tumor-infiltrating 2C T cells express, and maintain, uniformly high levels of PD-1 but do not express Tim3 or LAG3. Despite the expression of PD-L1 within the tumor islets of the TRP-SIY prostate, genetic deletion of PD-L1 on the TRP-SIY background did not inhibit the induction of T cell tolerance in prostate tumor tissue. This finding indicates that PD-1 is not critical for tolerance induction, but is perhaps a marker of exhausted T cells, similar to dysfunctional T cells in chronic viral infections (35). Furthermore, PD-L1−/− BMDCs were as active as wild-type DCs in reactivating tolerized 2C T cells in prostate tumor tissue. This unexpected finding is seemingly at odds with the literature outlining the increase in tumor-associated T cell function after blockade of PD-1 (5, 36). The specifics of our model system likely account for these differences. We note that in many systems restoration of tumor tolerized T cell function requires blockade of multiple inhibitory pathways to regain full function (16, 33, 37). As we found that 2C T cells upregulate PD-1 and not LAG-3 or Tim3, there may be additional, unidentified coinhibitory receptors functioning in this system. Further, ineffectiveness of PD-L1 blockade in the context of BMDC injection may be a consequence of the powerful stimulatory capacity of DCs. BMDCs may provide

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** CD27 expression is progressively lost on TRP-SY prostate resident 2C T cells. (A) Naive 2C T cells pooled from spleens and lymph nodes were assayed for expression of CD28, 4-1BB, and CD27. Histograms are gated on Thy1.1+ 2C TCR+ cells stained with isotype Ab (shaded gray) or specific Ab (black line). (B) Naive Thy1.1+ 2C T cells were retro-orbitally injected into TRP-SIY mice, along with intranasal infection with WSN-SIY virus. Mice were sacrificed 13 (bold black line), 23 (gray line), and 36 (thin black line) d post transfer, and CD28, 4-1BB, and CD27 expression on prostate resident 2C T cells was assayed by flow cytometry. Histograms are gated on live 2C TCR+ Thy1.1+ cells with isotype Ab (shaded gray) or specific Ab (black line). (C) Mean fluorescence intensity of CD28, CD27, or 4-1BB staining from Thy1.2+ 2C TCR+ T cells from naive mice or recovered from prostates at indicated times post transfer and WSN-SIY infection. Data shown are from at least two independent experiments (mean ± SD). (D) Time course of CD27 expression on 2C T cells recovered from TRAMP and C57BL/6 (B6) prostates 13 (bold black line), 23 (gray line), and 36 (thin black line) d post transfer. (E) As above, with 2C transfer/WSN-SIY infection and analysis of CD27 on 2C TCR+Thy1.1+ 2C T cells 13 (bold black line), 23 (gray line), and 36 (thin black line) d post transfer in spleens from TRP-SIY, TRAMP, and B6 mice.
the maximal stimulation of tolerant T cells regardless of PD-1/ PD-L1 engagement. This highlights the tissue (TRP-SIY prostates) and context (DC-mediated reactivation) specificity in the utility of checkpoint blockades in tumor resident T cell activity.

In summary, the differential requirement for CD70 and CD80/ CD86 in T cell function within the tumor environment suggests approaches to enhance ACT- and DC-based vaccines for cancer immunotherapy. For example, enhancing the CD27/CD70 interaction may delay tolerization of adaptively transferred T cells in the tumor microenvironment, and enhancing CD80 expression by DCs could stimulate anti-tumor response by endogenous tolerized CD8+ T cells. Defining the molecular interactions necessary to overcome tumor-induced tolerance is critical, as provision of costimulatory Fc fusion proteins, transduction of whole-cell tumor vaccines with costimulatory molecules, and viral vectors expressing costimulatory molecules are under development for the treatment of tumors (2). Our findings provide a basis for the rational design of ACT and DC vaccines targeting tumor-specific immune responses.

Acknowledgments

We thank Camille Jusino, Carol McKinley, Marisha Mikell, and the Biotechnology Core Facility at the Koch Institute for technical support; members of the Chen Lab for helpful discussions; and Dr. Arlene Sharpe (Harvard Medical School, Boston, MA) and Dr. Leiping Chen (Yale University, New Haven, CT) for CD80 and PD-L1 knockouts, respectively.

Disclosures

The authors have no financial conflicts of interest.

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