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Cutting Edge: Delay and Reversal of T Cell Tolerance by Intratumoral Injection of Antigen-Loaded Dendritic Cells in an Autochthonous Tumor Model

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Abstract

The tumor environment exerts a powerful suppressive influence on infiltrating tumor-reactive T cells. It induces tolerance of adoptively transferred effector T cells as they enter tumors and maintains the tolerance of persisting tumor-infiltrating T cells. In an autochthonous prostate cancer model, in which tumor-reactive CD8 T cells are trackable, we demonstrate that both depletion of endogenous dendritic cells (DCs) and intratumoral injection of Ag-loaded mature DCs delayed the tolerization of tumor-infiltrating effector CD8 T cells. Intratumoral injection of Ag-loaded DCs also reactivated tolerized CD8 T cells in the tumor tissue. The observed effects lasted as long as the injected DCs persisted. These findings are consistent with a critical role of DCs in modulating T cell reactivity in the tumor environment. They also suggest new potential strategies to extend the functionality of transferred effector T cells and to restore function to tolerized tumor-infiltrating T cells for cancer immunotherapy.

Tumor-infiltrating lymphocytes (TILs) are found in many solid tumors in patients. Although a significant fraction of TILs recognize tumor-derived Ags, they are usually nonfunctional (tolerized), as they lack cytolytic activity and/or effector cytokine production (1). A major objective of cancer immunotherapy is to reactivate TILs so that they can kill cancer cells. One such approach has been adoptive cell transfer (ACT), in which TILs are isolated from patients, expanded *ex vivo*, and reintroduced into patients. ACT has been effective in treating metastatic melanoma, achieving response rates of over 50% when combined with patient-preconditioning strategies (2,3). However, a limit to the effectiveness of ACT is that activated T cells can lose their function through tumor-induced tolerance, so additional strategies are needed to help maintain the function of activated TILs in the tumor environment.

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Another potential approach to induce antitumor T cell responses is through intratumoral injection of dendritic cells (DCs). DCs are specialized APCs that capture, process, and present Ags and subsequently direct T cell function (4). Although functional mature DCs can initiate robust antitumor T cell responses, DCs acted on by tumor-derived factors are generally suppressive (5). It is anticipated that functional DCs injected into tumors could overcome the effects of resident suppressive DCs. Indeed, intratumoral administration of either Ag-loaded or plain DCs has induced antitumor responses in transplanted tumor models in mice and rats (6,7). In humans, intratumoral injection of autologous DCs induced tumor regression in 6 out of 10 patients with melanoma and breast cancer (8,9). Similarly, intratumoral and intradermal administration of tumor lysate-pulsed autologous DCs promoted longer survival in patients with glioma than intradermal administration alone (9).

Despite the successes of inducing antitumor immunity and tumor regression, it has not been directly demonstrated whether intratumoral injection of DCs can reactivate persisting TILs in situ. In previous studies with intratumoral DC administration, antitumor immunity has generally been evaluated through gross measurements of tumor regression, with minimal analysis of the function of tumor-reactive T cells in the tumor tissue. In studies that analyzed antitumor T cell immunity, CD8 and/or CD4 T cells from the spleens, lymph nodes, or peripheral blood of tumor-bearing animals were assayed for Ag-specific cytolytic activity and/or effector cytokine production. However, the function of tumor-reactive T cells in the periphery can be significantly different from that in the tumor tissue itself (10). Furthermore, most previous studies used transplanted tumors, which may not capture the essential properties of TILs found in spontaneous primary tumors. Thus, although intratumoral DC administration has been shown to enhance antitumor immunity, it is not clear whether TILs can be directly reactivated by functional DCs within autochthonous tumors or whether intratumoral injection of DCs can sustain the function of effector T cells in the tumor tissue during ACT.

In an autochthonous prostate cancer model, we show in this study that intratumoral injection of ex vivo-matured, Ag-loaded DCs can both delay the tolerization of tumor-infiltrating effector T cells and reactivate persisting tolerized T cells in the tumor tissue. These findings provide new strategies to augment ACT as well as to reactivate tolerized TILs in situ for cancer immunotherapy.

Materials and Methods

Mice, adoptive transfer, and influenza infection

Transgenic adenocarcinoma of the mouse prostate (TRAMP) mice expressing SIYRYYGL (TRP-SIY) were generated as previously reported (11). 2C TCR transgenic mice were maintained on a RAG1^{-/-} background (2C/RAG). CD11c-DTR/GFP mice were on a C57BL/6 background. Where indicated, mice were retro-orbitally injected with 1.5×10^6 naive 2C cells from 2C TCR transgenic mice maintained on a RAG1^{-/-} background and intranasally infected with 100 PFU influenza A virus of the WSN strain expressing SIYRYYGL (WSN-SIY) (12). Experiments were performed in compliance with the institutional guidelines on animal care.

Generation of bone marrow chimeras and depletion of DCs

Approximately 2-mo-old TRP-SIY mice were lethally irradiated (2×500 rad) and reconstituted through retro-orbital injection of 12×10^6 CD11c-DTR/GFP bone marrow cells. DCs were depleted through i.p. injection of 20 ng/g diphtheria toxin (Sigma-Aldrich, St. Louis, MO) in PBS.

Generation of bone marrow-derived DCs and intraprostatic injection

Bone marrow was collected from femurs of C57BL/6 mice. After RBC lysis, cells were resuspended at 2×10^5 cells/ml in RPMI 1640 plus 10% FCS, 50 μ M 2-ME, 4 mM L-Glu, and 100 U/ml penicillin, and 100 μ g/ml streptomycin supplemented with GM-CSF-containing supernatant from J5 cells. Half of the media was replaced on days 3 and 5, 1 μ g/ml LPS was added on day 6, and cells were harvested on day 7. Where indicated, bone marrow-derived DCs were loaded with 1 μ g/ml SIY peptide for 1 h at 37°C and/or labeled for 10 min at 37°C with 5 μ M CFSE (Invitrogen, Carlsbad, CA). Mice were injected with 1×10^6 bone marrow-derived DCs either in the prostate (40 μ l) or s.c. (250 μ l). For intraprostatic injection, each lobe was injected five times with a total volume of 10 μ l.

Abs and flow cytometry

Abs to CD8, B220, CD11c, and Thy1.1 were from Biolegend (San Diego, CA), Thy1.2 and plasmacytoid DC Ag-1 (PDCA-1) were from eBioscience (San Diego, CA), and Gr-1 was from BD Biosciences (San Jose, CA). 2C TCR was stained using a clonotypic Ab. For intracellular IFN- γ staining, cells were restimulated with 1 μ g/ml SIY peptide for 4 h at 37°C in the presence of BD GolgiPlug (BD Biosciences). Cells were analyzed using an FACSCalibur (BD Biosciences), and data were processed using FlowJo (Tree Star, Ashland, OR).

In vivo cytotoxicity assay

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 splenocytes were then labeled with CFSE and pulsed with SIY peptide. CFSE⁺ and CFSE⁻ cells were mixed 1:1 and retro-orbitally injected (20×10^6 cells/mouse). Twenty-four hours later, the ratio of CFSE⁺ to CFSE⁻ cells in various tissues was evaluated by flow cytometry.

Results and Discussion

DCs tolerize tumor-infiltrating effector T cells

We have previously reported the construction of TRP-SIY transgenic mice that express the nominal CD8 T cell epitope SIY in the prostate tissue on the TRAMP background (11). TRP-SIY mice develop SIY-expressing prostate cancer that can be recognized by CD8 T cells displaying the 2C TCR. To study the response of CD8 T cells to SIY-expressing prostate tumors, TRP-SIY mice were adoptively transferred with naive 2C T cells and challenged intranasally with WSN-SIY. 2C cells were robustly activated and infiltrated prostate tumors, but rapidly lost their effector functions (11).

To evaluate whether DCs in the tumor could be tolerogenic, we determined if the DCs exhibit a suppressive phenotype. CD11c^{low} cells from the spleens and prostates of normal C57BL/6 (B6) mice and tumor-bearing TRAMP and TRP-SIY mice were compared for expression of PDCA-1, Gr-1, and B220, markers associated with immunosuppressive plasmacytoid DCs (13,14). Although no significant differences were detected in the phenotype of DCs in the spleens, the percentage of CD11c^{low} cells that expressed PDCA-1, Gr-1, and B220 was significantly increased in the prostates of TRAMP and TRP-SIY mice relative to that in B6 mice (Fig. 1A). Thus, a greater fraction of DCs in the prostate tumor tissue exhibit a suppressive phenotype.

To determine whether CD11c⁺ DCs can tolerize effector 2C cells in the tumor tissue, we generated bone marrow chimeric (BMT) mice by lethally irradiating TRP-SIY mice and reconstituting them with bone marrow cells from CD11c-DTR/GFP (15) donors. Eight weeks postreconstitution, the BMT mice were injected with diphtheria toxin, and over 90%

of the CD11c⁺ cells were depleted from the spleens and prostates within 48 h (Supplemental Fig. 1). Additional BMT mice were injected with naive 2C cells and infected intranasally with WSN-SIY virus. Six days postinfection (dpi), half of the mice were treated with diphtheria toxin. Four days after that (10 dpi), 2C cells from spleens, prostate draining lymph nodes (PDLNs), and prostates were assayed for their ability to produce IFN- γ (Fig. 1B). In the spleens and PDLN, the percentage of 2C cells that expressed IFN- γ was similar in all BMT mice (Fig. 1C and data not shown). In contrast, in the prostates, the percentage of IFN- γ ⁺ 2C cells was significantly higher in BMT mice with DC depletion than those without DC depletion (average of 47% versus 35%; $p < 0.05$). These results suggest that CD11c⁺ DCs are one of the major factors contributing to the tolerization of effector 2C cells in the prostate tumor tissue of TRP-SIY mice.

Intraprostatic injection of Ag-loaded DCs 7 dpi delays tolerization of tumor-infiltrating effector T cells

We wanted to evaluate whether infusion of functional DCs could overcome the influence of suppressive DCs in TRP-SIY mice. To determine if intraprostatic injection of SIY-loaded DCs could delay the tolerization of tumor-infiltrating effector 2C cells, we injected SIY-loaded DCs into the prostate tissue 7 d after WSN-SIY infection. As effector 2C cells do not infiltrate the prostate until ~7 dpi (11), the delayed injection would maximize the potential interactions between SIY-loaded DCs and effector 2C cells in the tumor tissue. TRP-SIY mice were injected retro-orbitally with naive 2C cells and infected intranasally with WSN-SIY. At 7 dpi, either PBS or ex vivo-matured SIY-loaded DCs were injected into the prostates. At 11, 13, 15, and 20 dpi, 2C cells from the prostate tumor tissues were assayed for IFN- γ expression (Fig. 2A). Following injection of PBS, only 25–30% of 2C cells in the prostates expressed IFN- γ at all time points, indicating that the tumor-infiltrating effector 2C cells had been tolerized (Fig. 2B). In contrast, following injection of SIY-loaded DCs, 50–60% of 2C cells in the prostates expressed IFN- γ 11 and 13 dpi, and over 45% of 2C cells still expressed IFN- γ 15 dpi. By 20 dpi, however, the percentage of 2C cells that expressed IFN- γ had fallen to the same level as in PBS-injected mice. Thus, intraprostatic injection of SIY-loaded DCs 7 dpi could delay the tolerization of tumor-infiltrating effector 2C cells by at least 8 d.

As controls, we evaluated whether intraprostatic injection of either SIY peptide or ex vivo-matured plain DCs (not loaded with SIY) 7 dpi could delay tolerance induction. Eleven and 13 dpi, similarly low percentages of 2C cells in the prostates expressed IFN- γ when TRP-SIY mice were injected with either PBS or SIY peptide (Fig. 2B, 2C). At 11 dpi, a higher percentage (35%) of 2C cells expressed IFN- γ in the prostates when plain DCs were injected, although the effect was not as dramatic as when the DCs were loaded with SIY. This effect may be due to the plain DCs acquiring SIY in the tumor tissue and thereby helping maintain the function of infiltrating 2C cells. By 13 dpi, IFN- γ expression by 2C cells in the prostate was comparable with injection of either PBS or plain DCs.

Why is the effect of SIY-loaded DCs transient? To answer this question, we evaluated DC persistence following intraprostatic injection of CFSE-labeled DCs. At 3 d postinjection, a significant population of CFSE⁺ DCs was detected in the prostate tissue, and a small fraction had drained to the PDLN, but no CFSE⁺ DCs were detected in the spleens or peripheral lymph nodes (Fig. 2D and data not shown). At 8 dpi, however, only a small number of CFSE⁺ DCs were detected in the prostate tissue and PDLNs. Thus, the timing for loss of IFN- γ expression by 2C cells correlates with the depletion of injected DCs from the tumor tissue. Consistently, intraprostatic injection of SIY-loaded DCs on day 0 could not overcome tolerance induction in the tumor tissue, as too few injected DCs likely persisted by the time the effector 2C cells infiltrated the tumor tissue 7 dpi (Supplemental Fig. 2).

Studies have shown that intratumoral injection of DCs can help maintain T cell function in lymphoid tissues (16). Our results further demonstrate that intratumorally injected DCs can also sustain the function of effector T cells within the tumor tissue. Therefore, strategically timed intratumoral injection of mature DCs, especially if Ag-loaded, could augment the therapeutic efficacy of ACT.

Intraprostatic injection of Ag-loaded DCs 30 dpi reactivates persisting tolerized T cells in the tumor tissue

We have previously shown that following WSN-SIY challenge, 2C cells persist long-term in the prostates of TRP-SIY mice, but do not respond to Ag challenge (11). Accordingly, we evaluated whether intraprostatic injection of SIY-loaded DCs could reactivate tolerized 2C cells that persist in the prostate tumor tissue. TRP-SIY mice were injected with naive 2C cells and infected with WSN-SIY. At 30 dpi, PBS or SIY-loaded DCs were injected into the prostate tissue. At 35, 37, and 40 dpi, 2C cells in the prostates were assayed for effector cytokine expression or cytolytic activity (Fig. 3A).

At 35 dpi, an average of 2.4×10^4 2C cells were recovered per prostate when PBS was injected, and only 20% of the cells could be stimulated to produce IFN- γ (Fig. 3B). When SIY-loaded DCs were injected, an average of 8.1×10^4 2C cells were recovered per prostate, and 44% of the cells could be stimulated to produce IFN- γ . Associated with the higher percentage of cells expressing IFN- γ , 2C cells in TRP-SIY prostates also acquired the ability to kill Ag-displaying target cells in the tumor tissue. In the prostates of TRP-SIY mice injected with SIY-loaded DCs, over 60% of SIY-pulsed, CFSE-labeled target cells were lysed within 24 h (Fig. 3C), whereas in the prostates of mice injected with PBS, the lysis of target cells was minimal. Consistently, histologic analysis of prostate tissue samples revealed a dramatic increase in leukocyte infiltration in TRP-SIY mice injected with SIY-loaded DCs (Fig. 3D). As a control, s.c. injection of SIY-loaded DCs did not stimulate persisting 2C cells to express IFN- γ (Fig. 3E). Even at 37 dpi, over 50% of 2C cells still expressed IFN- γ in the prostates of TRP-SIY mice injected with SIY-loaded DCs (Fig. 3B). By 40 dpi, however, the percentage of IFN- γ^+ 2C cells was no longer increased (Fig. 3B), consistent with the limited survival of the injected DCs (Fig. 2D). Together, these results demonstrate that intraprostatic injection of ex vivo-matured, SIY-loaded DCs can reactivate persisting tolerized T cells in the prostate tumor tissue for at least 7 d.

In this study, we show that both depleting endogenous DCs and intratumorally injecting ex vivo-matured, Ag-loaded DCs can delay the tolerization of tumor-infiltrating effector CD8 T cells. The latter can also refunctionalize persisting tolerized CD8 T cells. These complementary results provide strong support for a critical role of DCs in modulating T cell reactivity in the tumor environment. To our knowledge, this is the first time that intratumoral DC administration has been directly demonstrated to both delay and reverse T cell tolerance in the tumor tissue, thereby overcoming the tolerizing influence of endogenous DCs. Our study overcomes important limitations of previous studies that evaluated the effect of intratumoral DC administration. In contrast to transplanted tumor models, TRP-SIY mice develop autochthonous prostate cancer that more closely resembles primary tumors in humans and is thus more suitable for studying both TILs and effector T cells introduced by ACT. Furthermore, we directly analyzed tumor-infiltrating effector T cells and persisting tolerized T cells in the tumor tissue, in addition to the peripheral lymphoid tissues, and demonstrated that the function of tumor-reactive T cells can differ dramatically between those environments. Our findings suggest possible approaches to extend the functionality of transferred effector T cells during ACT and directly refunctionalize persisting tolerized TILs for cancer immunotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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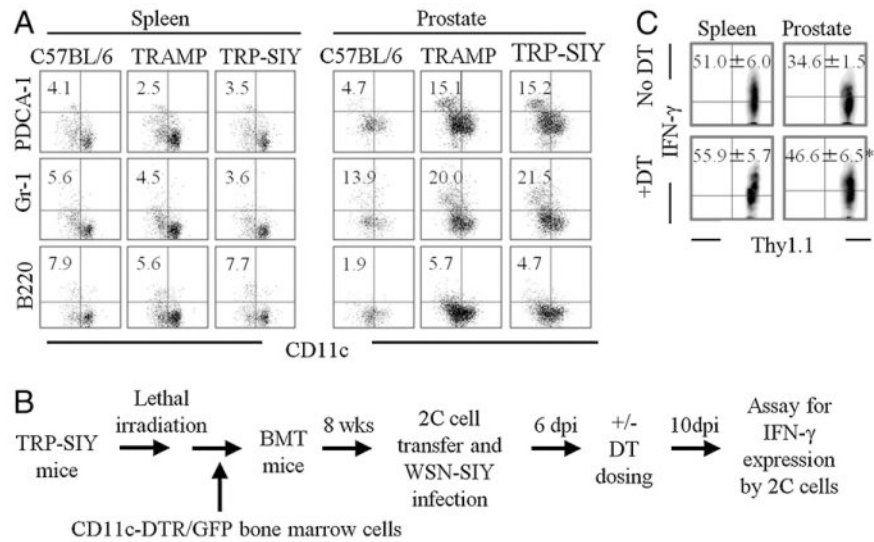
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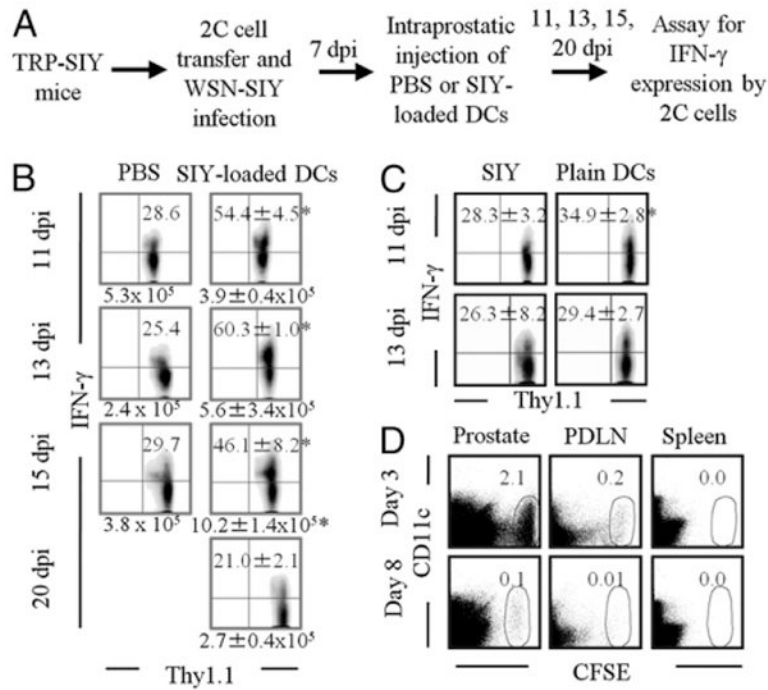
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Abbreviations used in this paper

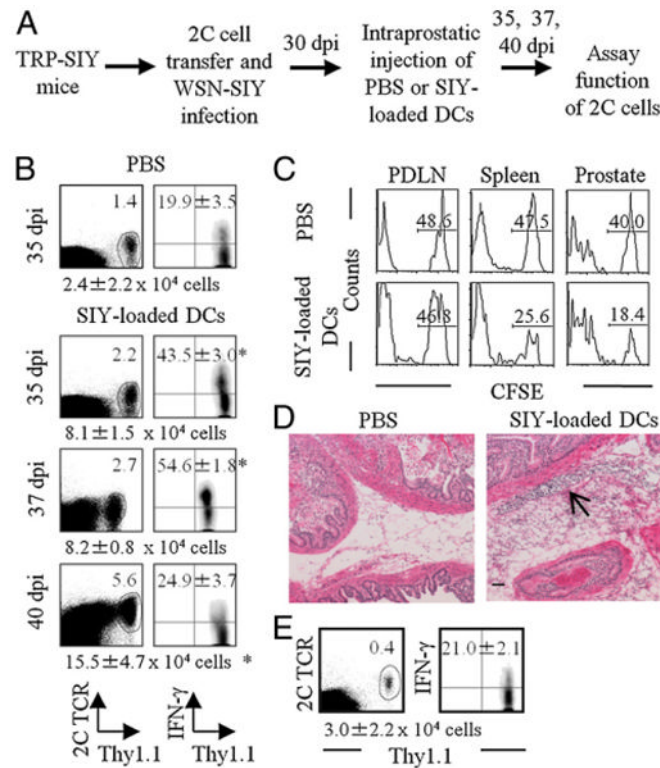
ACT	adoptive cell transfer
BMT	bone marrow chimeric
DC	dendritic cell
dpi	days postinfection
PDCA-1	plasmacytoid dendritic cell Ag-1
PDLN	prostate draining lymph node
SIY	SIYRYYYGL
TIL	tumor-infiltrating lymphocyte
TRAMP	transgenic adenocarcinoma of the mouse prostate
TRP-SIY	TRAMP mice expressing SIY
WSN-SIY	influenza A virus of the WSN strain expressing SIY

**FIGURE 1.**

DCs induce tolerance of tumor-infiltrating effector T cells in the prostate tumor tissue. *A*, CD11c⁺ cells were isolated from the spleens and prostates of B6, TRAMP, and TRP-SIY mice by cell sorting and were then stained for PDCA-1, Gr-1, or B220. Dot plots shown are from five pooled mice in each group and are representative of two similar experiments. Experimental protocol (*B*) and effect of DC depletion on 2C T cell function (*C*). Cells from the spleens and prostates of BMT mice were stained for 2C TCR, Thy1.1, and intracellular IFN- γ . Density plots show Thy1.1 versus IFN- γ profiles of 2C TCR⁺ Thy1.1⁺ cells. Numbers indicate the average percentage of positive cells \pm 1 SD. Data are representative of two to three mice per group. * p < 0.05 when compared with control.

**FIGURE 2.**

Intraprostatic injection of Ag-loaded DCs 7 dpi delays tolerization of tumor-infiltrating effector T cells. Experimental protocol (A) and effect of intraprostatic injection of SIY-loaded mature DCs on IFN- γ expression by tumor-infiltrating 2C cells (B). Cells from prostates were stained for 2C TCR, Thy1.1 and intracellular IFN- γ . Density plots are as described in Fig. 1C. Numbers inside the plots indicate the percentage or the average percentage of positive cells \pm 1 SD. Numbers outside the plots indicate total number of 2C cells \pm 1 SD in the prostate. Data are from four independent experiments with two to three mice per group. C, SIY peptide and plain DC controls. Experimental protocol is the same as in A, except SIY peptide or plain DCs were injected, and prostates were analyzed 11 and 13 dpi. Representative data from two similar experiments are shown with two to three mice per group. D, Persistence of injected DCs. DCs were labeled with CFSE, loaded with SIY peptide, and injected into TRAMP prostates. Three and 8 d later, the presence of CFSE⁺ DCs in the indicated tissues was determined by flow cytometry. Dot plots show CD11c versus CFSE profiles of live cells. Numbers indicate percentage of CFSE⁺ cells. Representative data are shown from two to three mice per group. * $p < 0.05$ when compared with control.

**FIGURE 3.**

Intraprostatic injection of Ag-loaded DCs reactivates persisting tolerized T cells in the prostate tumor tissue. Experimental protocol (A) and effect of intraprostatic injection of SIY-loaded mature DCs on reactivation of persisting tolerized 2C cells (B). Cells from prostates were stained for 2C TCR, Thy1.1, and intracellular IFN- γ . Dot plots show Thy1.1 versus 2C TCR profiles of live cells, and density plots are as described in Fig. 1C. Numbers inside the plots indicate the percentage or the average percentage of positive cells \pm 1 SD in the gated region. Numbers outside the plots indicate total number of 2C cells \pm 1 SD. Data are from three independent experiments with two to three mice per group. C, In vivo CTL assay. A 1:1 mixture of SIY-pulsed, CFSE-labeled, activated T cells (Thy1.1⁺) and unlabeled, unpulsed, activated T cells (Thy1.1⁺) were retro-orbitally injected into TRP-SIY mice (Thy1.2⁺) on day 34. The following day, the proportions of CFSE⁺ versus CFSE⁻ target cells were determined by flow cytometry. CFSE histograms are shown for live Thy1.1⁺ cells. Numbers indicate percentage of CFSE⁺ cells. Representative data from two similar experiments are shown. D, H&E stains of prostate tissues from TRP-SIY mice injected intraprostatically with either PBS or SIY-loaded DCs 5 d earlier. Arrow indicates leukocyte infiltration in the prostate. Scale bar, 100 μ m. E, Effect of s.c. injection of SIY-loaded DCs. The experiment was performed as in A and B, except SIY-loaded mature DCs were injected s.c., and prostates were analyzed 35 dpi. * $p < 0.05$ when compared with control.