Arming Tumor-Reactive T Cells with Costimulator B7-1 Enhances Therapeutic Efficacy of the T Cells

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Abstract

T cells ectopically expressing costimulators are pathogenic and contribute to autoimmunity against self-antigens. Given that tumor antigens are often self-antigen or mutated self-antigens, we hypothesize that neoexpressing a costimulator on tumor-reactive T cells may likewise enhance their reactivity to tumor. To test this hypothesis, we have expressed B7-1 on OT-1 CD8+ T-cell receptor transgenic T cells via protein transfer (or protein "painting"). Naïve OT-1 T cells, after being painted with B7-1, can self-costimulate themselves, elicit enhanced proliferative and CTL responses to E.G7-ovalbumin tumor cells (expressing a cognate antigen), and become resistant to CD4+CD25+ regulatory T-cell-mediated suppression. Importantly, these T cells, when coimplanted with E.G7-ovalbumin tumor cells into a syngeneic host, are three to nine times more potent than are control T cells (mock painted with human IgG) in inhibiting tumor growth. Further, on transfer into mice bearing established E.G7-ovalbumin tumors, B7-1-painted ex vivo–amplified OT-1 T cells induced complete tumor regression in 65% of treated mice, whereas the control T cells did so in only 28% of treated mice. Finally, on transfer into mice bearing less immunogenic 4T1 breast tumors, B7-1-painted tumor-reactive CD8+ T cells improved the survival of treated mice to a greater extent than did the control T cells. Hence, this study establishes that arming tumor-reactive T cells with a costimulator can enhance their antitumor efficacy. (Cancer Res 2006; 66(13): 6793-9)

Introduction

The idea of adoptive T-cell therapy for cancer is simple: autologous tumor-reactive T cells can be enriched from tumor-infiltrating lymphocytes or draining lymph nodes of a patient, activated and amplified ex vivo, and reininfused back to the patient to eradicate tumors (1). In practice, however, the efficacy of such therapy is often limited by the weak reactivity of the T cells to tumors (2). Moreover, on adoptive transfer, the T cells can be functionally compromised by the host immune mechanisms that are nonsupportive (i.e., the lack of antigenic, costimulatory, and/or proinflammatory signals; refs. 3, 4) and even suppressive [i.e., presence of suppressive cytokines and/or regulatory T (Treg) cells; refs. 5, 6]. Thus, additional strategies are needed to improve the efficacy of these T cells.

Costimulation is one of the key mechanisms by which T cells are regulated. Costimulation is required for T-cell activation; in the absence of costimulation, antigenic stimulation of T cells results in T-cell anergy (7). In addition, costimulation augments T-cell effector responses, including cytotoxicity, to weak antigens, such as self-antigens and tumor antigens (8–10). Further, there is evidence that strong costimulation, such as signaling via the CD28 axis (11–13), can enhance the resistance of T cells to suppression mediated by CD4+CD25+ Treg cells.

Normally, costimulators are expressed on activated antigen-presenting cells (APC), including dendritic cells. T cells that ectopically express a high level of costimulators, such as B7-1 (14, 15), CD40 (16), or LIGHT (17), are pathogenic and contribute to autoimmune diseases. Elevated autoimmune propensity, however, can be desirable for tumor-reactive T cells (18). This is because most tumor antigens are self-antigens or mutated self-antigens (19); to attack tumors effectively, tumor-reactive T cells must likewise be able to breach the barrier against autoimmunity.

Based on these notions, we hypothesize that arming tumor-reactive T cells with costimulator(s) may enhance their antitumor efficacy. By colocalizing a costimulator with its cognate receptor on same T cells, we enable the T cells to "self-costimulate" themselves in an autocrine/paracrine-like mode and bypass the costimulator-mediated mechanism(s) that limit T-cell activation and effector function, thereby eliciting enhanced antitumor responses.

To test this hypothesis, in this study, we have neoexpressed the costimulator B7-1 on tumor-reactive CD8+ T cells and evaluated the functional capacities of the T cells so engineered both in vitro and in vivo. The study establishes that arming tumor-reactive T cells with a costimulator enhances their antitumor efficacy.

Materials and Methods

Mice and tumor cell lines. BALB/c and C57BL/6 mice (3- to 5-week old) were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-1 T-cell receptor (TCR) transgenic mice (20) in the C57BL/6 background were bred at the animal facility of the University of Illinois College of Medicine at Rockford (Rockford, IL) using hemizygous males and wild-type females. To screen for transgenic offspring, 1 ml blood was collected from the tail, immunostained with FITC-labeled anti-Vα2 and allophycocyanin-labeled anti-CD8 monoclonal antibodies (mAb), and analyzed by flow cytometry. Mice with nearly all of their CD8+ T cells stained positive for Vα2 were selected. The animals were maintained in a pathogen-free facility and used in accordance with the institutional guidelines for animal care. E.G7-ovalbumin, EL4, 4T1, and L5178Y tumor cell lines were purchased from the American Type Culture Collection (Manassas, VA) and maintained as per supplier’s recommendation.

Antibodies. FITC-labeled rat anti-mouse Vα2 mAb (B20.1) was from Caltag Laboratories (Burlingame, CA). Rat anti-mouse CD3 mAb (TK3) was purchased from Serotec Ltd. (Oxford, United Kingdom). Rat anti-mouse CD16/32 mAb (clone 93), FITC-labeled rat anti-mouse B7-1 mAb (1G10), FITC-labeled rat anti-mouse B7-2 mAb (GL1), PE-labeled or nonlabeled...
hamster anti-mouse CD28 mAb (37,51), allophycocyanin-labeled rat anti-mouse CD8 mAb, PE-labeled rat anti-mouse CD62L mAb (MEL-14), and various isotype controls were from eBioscience (San Diego, CA). Anti-mouse CD28 Fab was generated from the corresponding mAb (37,51) using the ImmunoPure Fab preparation kit (Pierce Biotechnology, Rockford, IL). Control Fab was purchased from the Jackson ImmunoResearch Laboratories (West Grove, PA).

**T-cell isolation.** All T cells were purified by magnetically activated cell sorting using various isolation kits (all from Miltenyi Biotec, Auburn, CA). OT-1 T cells were purified from bulk splenocytes via negative selection using a mouse CD8+ T-cell kit. CD4+CD25+ T cells were isolated from lymph nodes by the use of a CD4+ T-cell kit (negative selection) followed by the use of a CD25+ cell kit (positive selection). T-cell purity >97% was achieved as determined by flow cytometry.

**Flow cytometry.** Cell samples were first blocked with rat anti-mouse CD16/32 (1 µg per 106 cells) and subsequently stained with mAb(s) as per manufacturers’ instructions. Flow cytometry was done with a FACS Calibur (BD Biosciences, San Jose, CA). Live cells were identified and gated by forward and side scatter and, if necessary, by staining with 7-amino-actinomycin D (7-AAD; Invitrogen, Carlsbad, CA). Data were analyzed using the CellQuest Pro software (BD Biosciences).

**Protein transfer.** T cells were suspended to 2 × 107/mL in DMEM/0.1% bovine serum albumin (BSA)/50 µM/L j-mercaptoethanol (transfer buffer). Palmitated protein A was generated as we described previously (21) and added to the cells at 30°C for 15 minutes and then at 42°C for 5 minutes. Cells were washed and resuspended at 4 × 106/mL in transfer buffer, and Fcγ-derivatized mouse B7-1 (B7-1-Fc; ref. 22) or human IgG (IgG; Sigma-Aldrich Corp., St. Louis, MO) was added to cells at 30 µg/mL. Cells were incubated at 4°C for 15 minutes. Unbound proteins were removed by washing.

**Carboxyfluorescein diacetate succinimidyl ester labeling.** Cells were thoroughly washed with and suspended (at 2.5 × 105/mL) in PBS containing Ca2+ and Mg2+. Carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) was added to cells at 1 µM/L. The reaction was allowed to last for 5 minutes at room temperature and terminated with the addition of 0.5 volume of FCS.

**In vitro proliferation assay.** Tumor cells (serving as APC) were suspended at 2 × 105/mL in DMEM/2% FCS and mitotically inactivated with mitomycin C (Sigma-Aldrich) added to 100 µg/mL. Cells were treated at 37°C for 1 hour and then thoroughly washed before use. CFSE-labeled OT-1 T cells were seeded in triplicate wells (5 × 106 per well) in RPMI 1640/10% FCS/15 mM/L HEPES/50 µM/L j-mercaptoethanol (R10 medium) in a U-bottomed 96-well plate together with mitomycin C-treated EG7-ovalbumin or EL4 tumor cells (5 × 104 per well). After 48 or 72 hours, cells were harvested and stained with allophycocyanin-labeled anti-CD8 mAb (to allow us to gate on the OT-1 T cells) and analyzed by flow cytometry.

**In vivo proliferation assay.** CFSE-labeled OT-1 T cells were painted with B7-1 or hIgG, mixed with E.G7-ovalbumin tumor cells at 1:1 ratio, and injected (0.5 × 105 to 1 × 106 total cells per injection) into the footpads of the hind legs of syngeneic C57BL/6 mice (two – three mice per group for each experiment). The cell mixture containing the B7-1-painted T cells was injected into the right footpads (test); that containing the hIgG-painted T cells, into the contralateral left footpads (control). The recipient mice were maintained in the dark for 4 days and then sacrificed. Cell suspensions were prepared from pooled popliteal lymph nodes draining the same injection site, stained with allophycocyanin-labeled anti-CD8 mAb and 7-AAD, and analyzed by flow cytometry (gated on the CD8+ population).

**Detection of cytokines.** OT-1 T cells were cocultured with tumor cells for 48 hours, and the conditioned media were analyzed by flow cytometry using a cytometric bead array kit for Th1/Th2 cytokines (BD Biosciences) as per manufacturer’s protocol.

**CTL assay.** Effector T cells were seeded in triplicate wells of a U-bottomed 96-well plate together with CFSE-labeled, mitomycin C–inactivated tumor cells (target) at different E:T ratios. At 12 hours, the cultures were mixed with an equal volume of PBS containing 2 µg/mL 7-AAD. Cells were analyzed by flow cytometry. CFSE-labeled cells (target) were gated. Raw data were converted into specific lysis by the formula: specific lysis = (x – c)/(100 – c), where x is the percentage of 7-AAD+ target cells in the presence of effector cells and c is that in the absence of effector cells (spontaneous lysis).

**Analysis of antitumor titer in vivo.** The antitumor activity of OT-1 T cells was titered by the Winn assay (23). Briefly, naïve OT-1 T cells painted with B7-1 or hIgG were serially diluted and mixed with 1 × 106 EG7-ovalbumin tumor cells to reach various OT-1 to EG7-ovalbumin cell ratios. Each mixture of a defined OT-1 to EG7-ovalbumin ratio was tested in a group of C57BL/6 mice (four mice per group); mixtures were each injected i.d. into mice. As a negative control group, mice were injected with the tumor cells alone. Growth of tumors in all groups was measured weekly.

**Adoptive immunotherapy.** For immunotherapy in the EG7-ovalbumin model, OT-1 T cells were prepared. naïve OT-1 T cells were preactivated for 2 days with anti-CD3 (plate bound at 6 µg/mL) and interleukin-2 (IL-2; 100 IU/mL) and amplified for 4 days with IL-2; subsequently, the T cells were painted with hIgG or B7-1 as described earlier. For treatment, EG7-ovalbumin tumors were established by injecting i.d. 1 × 106 EG7-ovalbumin tumor cells into the right flank of C57BL/6 mice (day −5). Before treatment, the mice were divided into two groups (five mice per group) and individually ear marked within each group. On days 0 (tumors were ∼25 mm2 in size) and 1, each group of mice was injected intratumorally (1 × 106 T cells per injection) with the hIgG-painted or B7-1-painted OT-1 T cells. Tumor sizes were measured weekly after the treatment. Cured mice were rechallenged with 1 × 105 EG7-ovalbumin tumor cells injected i.p. to 3 months after the complete tumor regression.

**For immunotherapy in the 4T1 tumor model, 4T1 tumor-reactive T cells were prepared.** To that end, BALB/c mice were immunized twice with mitomycin C-treated 4T1 tumor cells. The immunized mice were then challenged with live 4T1 tumor cells on both sides of the flank; the mice that showed partial resistance to this challenge were used as the donors for 4T1 tumor-reactive CD8+ T cells. The CD8+ T cells isolated from tumor-draining lymph nodes in the donor mice were preactivated for 2 days with anti-CD3 (plate bound at 6 µg/mL) and IL-2 (100 IU/mL), amplified for 4 days with IL-2, and painted with hIgG or B7-1. For treatment, tumors were established in BALB/c mice by injecting i.d. 3 × 106 4T1 tumor cells on the right flank of BALB/c mice (day −4). On day −1, the mice were given an ip. injection of cyclophosphamide (2.5 mg in 100 µL water) to slow down tumor growth (24). Before the T-cell therapy, mice were divided into three groups (five mice per group). On day 0, each group of mice was injected intratumorally (3 × 106 cells per injection) with medium (DMEM/0.1% BSA) or the 4T1 tumor-reactive CD8+ T cells painted with either IgG or B7-1 (described above). Tumor sizes were measured weekly. Mice were euthanized when they became moribund or when their tumors exceeded 400 mm2 in size.

**Results**

**Expressing B7-1 on primary T cells via protein transfer.** OT-1 TCR transgenic CD8+ T cells, expressing a TCR specific for the MHC I–restricted ovalbumin peptide antigen (ovalbumin257-264), are a well-established system for studying antigen-specific CD8+ responses. naïve OT-1 T cells express a high level of costimulatory receptor CD28 but not its ligand B7-1 or B7-2 (data not shown). To neoeexpress costimulator B7-1 on OT-1 T cells, we used the protein transfer (or protein painting) method we developed previously (21).

In that method, protein A, after being chemically derivatized with palmitate in a simple reaction, is first incorporated into cell membranes; in turn, this membrane-anchored palmitated protein A serves as a “trap” for secondarily added Fc fusion proteins. With its simplicity in expressing defined amounts of proteins and in coexpressing more than one protein at a time (21, 22, 25), the method provides an alternative means to gene transfer for expressing proteins on cells, especially on those primary cells that are resistant to gene transfer. Here, as shown in Fig. 1A, naïve primary OT-1 T cells, after being painted with palmitated protein A
were mixed (1:1) with E.G7-ovalbumin (OVA) and murine B7-1 Fc fusion protein (B7-1-Fc), were double positive for both CD28 and B7-1.

**B7-1 painting augments OT-1 T-cell proliferation in response to tumor cells.** We hypothesized that expressing a costimulator on tumor-reactive T cells may enable the T cells to self-costimulate themselves in an autocrine/paracrine-like mode, thereby increasing their reactivity to tumor cells. To establish “proof of principle,” we first determined whether B7-1 painting can augment the proliferative response of OT-1 T cells to EG.7-ovalbumin tumor cells. EG.7-ovalbumin cells are transfected EL4 cell line expressing a cognate ovalbumin antigen, and they lack B7-1 and other known costimulators (26); thus, they are a well-established tumor model for antigen-specific T cells. As shown in Fig. 1B, B7-1-painted OT-1 T cells proliferated vigorously in response to EG.7-ovalbumin tumor cells in coculture, with nearly half of the population reaching their third cell division within 48 hours, whereas hlgG-painted control T cells, cultured under the same conditions, proliferated to a less extent, lagging by approximately one cell division. The effect of B7-1 painting was blocked by anti-CD28 Fab but not by control Fab, which indicates that the B7-1 painted on the T cells functions via signaling CD28 and, thus, enables the T cells to self-costimulate themselves. B7-1 painting did not alter the specificity of OT-1 T cells to the antigen, as B7-1-painted OT-1 T cells did not proliferate at all in response to EL4 tumor cells. The difference in proliferation between hlgG-painted and B7-1-painted T cells became more marked when the coculture was prolonged to 72 hours, but the CFSE patterns started to degrade (data not shown). Anti-CD28 Fab blocked the effect of B7-1 at 72 hours as well (data not shown). Together, these results show that B7-1 painting specifically enhances the proliferation of OT-1 T cells in response to EG.7-ovalbumin tumor cells in vitro. Of note, the painting process itself does not affect T-cell responses, as we detected no functional difference between nonpainted and hlgG-painted OT-1 T cells using in vitro proliferation, cytokine production, or cytotoxicity assays (data not shown).

To verify our finding, we determined the effect of B7-1 in vivo. CFSE-labeled OT-1 T cells were mixed with EG.7-ovalbumin tumor cells and adoptively transferred into syngeneic C57BL/6 mice via footpad injection. Subsequently, the proliferation of the donor OT-1 T cells in the draining lymph nodes (popliteal) was analyzed by flow cytometry. The majority of the B7-1-painted OT-1 T cells divided two to three times following adoptive transfer, whereas the majority of hlgG-painted control T cells divided only once or stayed undivided (Fig. 1C).

In summary, these data indicate that the expression of B7-1 on OT-1 T cells can augment the proliferation of the T cells in response to tumor cells both in vitro and in vivo.

**B7-1 painting enhances antitumor effector function of OT-1 T cells in vitro.** Having documented the effect of B7-1 painting on the proliferation of OT-1 T cells in response to tumor cells, we wanted to substantiate our finding and determine whether B7-1 expression also has an effect on effector responses of the T cells, particularly Th1 cytokines and CTL responses, against the tumor cells. After stimulation by EG.7-ovalbumin tumor cells, B7-1-painted OT-1 T cells produced higher levels of Th1 cytokines, especially IL-2 and IFN-γ, than did hlgG-painted control T cells (Fig. 2A). Further, B7-1-painted OT-1 T cells, on a per cell basis, displayed stronger CTL activity against EG.7-ovalbumin tumor cells than did hlgG-painted T cells (Fig. 2B and C). Together, these results show that the expression of B7-1 on OT-1 T cells can enhance antitumor effector responses of the T cells in vitro.
B7-1 painting renders OT-1 T cells resistant to CD4⁺CD25⁺ Treg cell-mediated suppression. There is growing evidence that, in patients bearing progressively growing tumors, antitumor T cells are functionally suppressed by the Treg cells (5, 6). On the other hand, previous studies have also indicated that T cells are resistant to suppression when strongly costimulated (11–13). Thus, we determined whether B7-1-painted OT-1 T cells, with endowed ability to self-costimulate themselves (Fig. 1), are resistant to suppression mediated by CD4⁺CD25⁺ Treg cells isolated from lymph nodes draining progressively growing E.G7-ovalbumin tumors.

In the absence of the Treg cells, the B7-1-painted T cells again proliferated in response to E.G7-ovalbumin tumor cells more vigorously than did the hIgG-painted control T cells; as expected, neither the B7-1-painted cells nor the control T cells proliferated in response to EL4 tumor cells (Fig. 3). Significantly, Treg cells inhibited the proliferation of hIgG-painted T cells in a dose-dependent manner (Fig. 3A-C) but had only minimal effect on the proliferation of B7-1-painted T cells under the same conditions (Fig. 3D-F). These results show that B7-1 painting renders OT-1 T cells resistant, at least partially, to suppression mediated by CD4⁺CD25⁺ Treg cells.

B7-1 painting increases antitumor titer of naïve OT-1 T cells in vivo. Having determined the effects of B7-1 painting on proliferation, effector function, and resistance to Treg cells, we next determined whether B7-1 expression can increase the antitumor titer of OT-1 T cells in vivo using the classic Winn assay (23). This assay measures the combined outcome of both tumor incidence and tumor growth rate as a function of T-cell dose. To that end, 1 × 10⁶ E.G7-ovalbumin tumor cells were premixed with hIgG-painted or B7-1-painted naïve OT-1 T cells in defined ratios and inoculated into syngeneic C57BL/6 mice. Subsequently, the antitumor activity of the T cells was titered by determining the minimal number of the T cells required for blocking tumor growth. To that end, 1 × 10⁶ E.G7-ovalbumin tumor cells were premixed with hIgG-painted or B7-1-painted naïve OT-1 T cells in defined ratios and inoculated into syngeneic C57BL/6 mice. Subsequently, the antitumor activity of the T cells was titered by determining the minimal number of the T cells required for blocking tumor growth. To that end, 1 × 10⁶ E.G7-ovalbumin tumor cells were premixed with hIgG-painted or B7-1-painted naïve OT-1 T cells in defined ratios and inoculated into syngeneic C57BL/6 mice. Subsequently, the antitumor activity of the T cells was titered by determining the minimal number of the T cells required for blocking tumor growth. To that end, 1 × 10⁶ E.G7-ovalbumin tumor cells were premixed with hIgG-painted or B7-1-painted naïve OT-1 T cells in defined ratios and inoculated into syngeneic C57BL/6 mice. Subsequently, the antitumor activity of the T cells was titered by determining the minimal number of the T cells required for blocking tumor growth. To that end, 1 × 10⁶ E.G7-ovalbumin tumor cells were premixed with hIgG-painted or B7-1-painted naïve OT-1 T cells in defined ratios and inoculated into syngeneic C57BL/6 mice. Subsequently, the antitumor activity of the T cells was titered by determining the minimal number of the T cells required for blocking tumor growth.
minimal tumor growth was observed. Thus, we estimated that B7-1 painting increased the antitumor titer of OT-1 T cells by between 3- and 9-fold.

B7-1 painting increases the therapeutic efficacy of ex vivo–amplified OT-1 T cells against established tumors. Typically, adoptive T-cell therapy involves infusion of ex vivo–amplified/activated T cells back to the host to eradicate established tumors. Thus, we further tested whether B7-1 painting can increase the efficacy of preactivated OT-1 T cells against established EG.7-ovalbumin tumors, defined as having 100% probability to kill the host if without treatment. To that end, OT-1 T cells were first activated and amplified in culture with anti-CD3 and IL-2. Such ex vivo–amplified T cells were readily painted with B7-1 (Fig. 5A).

After intratumoral transfer into tumor-bearing mice at a predetermined suboptimal dose (1 × 10⁶ cells per tumor), whereas hlgG-painted control T cells cured only 20% of the treated animals, the B7-1-painted T cells cured 60% of the treated animals (Fig. 5B). On average (over three experiments), hlgG-painted T cells cured 28% of the treated mice whereas the cure rate for B7-1-painted T cells was 65%. As a negative control, nontreated animals all died of the tumor (data not shown).

Having shown local tumor regression in a substantial portion of mice treated by B7-1-painted OT-1 T cells, we next looked for evidence of systemic antitumor immunity. To that end, cured mice were rechallenged with a lethal dose of EG.7-ovalbumin tumor cells injected i.p. 2 to 3 months after complete tumor regression. As shown in Fig. 5C, all of the animals cured by B7-1-painted OT-1 T cells resisted rechallenge. The results point to a systemic antitumor immunity that is evoked by intratumoral transfer of B7-1-painted OT-1 T cells. Such systemic immunity, however, was not B7-1 specific because animals cured with hlgG-painted control T cells were able to reject systemic tumor rechallenge as well (data not shown). Moreover, the systemic antitumor immunity in cured animals was also largely directed toward the original pseudotumor antigen (hen ovalbumin). As such, when the EL4 parental tumor cells, which do not express the ovalbumin pseudotumor antigen, were inoculated into the same mice that had rejected the rechallenge by EG.7-ovalbumin tumor cells (Fig. 5C), the cells grew into tumors albeit at a considerably slower rate than that in naive mice (data not shown).

In summary, these data show that B7-1 painting improves the therapeutic antitumor efficacy of ex vivo–amplified/activated OT-1 T cells against established tumors.

B7-1 painting increases antitumor efficacy of 4T1 tumor-reactive CD8⁺ T cells. Thus far, we have shown in the previous figures the effects of B7-1 painting on antitumor efficacy of OT-1 TCR transgenic CD8⁺ T cells, a well-defined immunologic system for studying antigen-specific responses. Next, we wanted to further substantiate our findings in T cells that are of high heterogeneity and with relatively poor reactivity to tumors, a scenario resembling more closely to clinical settings. To that end, we determined the effects of B7-1 painting on CD8⁺ T cells harvested from mice bearing the murine mammary carcinoma 4T1, a model for human stage IV breast cancer (27). Compared with the EG.7-ovalbumin tumors, the 4T1 tumors are less immunogenic and more metastatic.

First, to generate 4T1 tumor-reactive CD8⁺ T cells, syngeneic BALB/c mice were immunized twice with mitotically inactivated
4T1 tumor cells and subsequently inoculated with live 4T1 tumor cells on both sides of the flank. Although the prior immunization failed to prevent tumor occurrence completely, retarded tumor growth was noted in about half of the immunized animals (data not shown). Hence, the tumor-draining lymph nodes from the mice that showed retarded tumor growth were analyzed by flow cytometry. The total count of CD8$^+$ T cells was $\sim 22\%$ (Fig. 6A), which was similar to that of corresponding lymph nodes from naïve mice (data not shown). However, $\sim 18\%$ (4% divided by 22%) of these CD8$^+$ T cells were CD62L$^{\text{low}}$ (suggestive of their previous encounter with antigens; Fig. 6A) compared with 11% of CD8$^+$ T cells in corresponding lymph nodes from naïve mice (data not shown).

Therefore, total CD8$^+$ T cells from the tumor-draining lymph nodes were used as the source of antitumor T cells. After ex vivo amplification in the presence of IL-2, these T cells retained their effector function, as shown by their specific CTL activity against the 4T1 tumor cells but not control L5178Y tumor cells (Fig. 6B). Again, these CD8$^+$ T cells were readily painted with B7-1 (Fig. 6C).

Next, we determined whether B7-1 painting can increase the antitumor efficacy of these T cells on intratumoral transfer into mice bearing 4-day established 4T1 tumors. The treatment did not cure any of the animals. However, as depicted in Fig. 6D, a single injection of $3 \times 10^6$ B7-1-painted T cells significantly prolonged the survival of the treated mice, whereas the injection of the same number of hIgG-painted T cells was less effective. These results show that B7-1 painting improves the therapeutic effect of natural, heterogeneous T cells originated from the tumor-draining lymph nodes, thus further solidify our findings made in the OT-1 T cells.

**Discussion**

Previously, we used the protein transfer method to paint APCs and tumor cells with costimulators in an effort to improve the immunogenicity of these cells (21, 22). In the present study, we apply the same method to tumor-reactive T cells to enhance their antitumor efficacy. Our data show that neoexpression of B7-1 via protein transfer on naïve OT-1 TCR transgenic CD8$^+$ T cells enables the T cells to self-costimulate themselves. Such self-costimulating T cells produce enhanced proliferative and CTL responses to EG.7-ovalbumin tumor cells expressing a cognate antigen and become resistant to Treg cell-mediated suppression. Significantly, when cotransplanted with the EG.7-ovalbumin tumor cells into syngeneic animals, these B7-1-painted OT-1 cells show three to nine times higher antitumor titer than do hIgG-painted control T cells. Moreover, in both EG.7-ovalbumin (highly immunogenic) and 4T1 (poorly immunogenic) tumor models, B7-1 painting of ex vivo–amplified tumor-reactive CD8$^+$ T cells improves their therapeutic efficacy against established tumors. Together, these data support our hypothesis that antitumor T cells can be functionally enhanced by arming them with a costimulator.

Until now, adoptive T-cell immunotherapies have largely relied on ex vivo amplification to increase the number of therapeutic T cells. Our present study suggests that arming tumor-reactive T cells with a costimulator may further enhance the in vivo antitumor efficacy of these T cells on reinfusion into the patient. Thus, engineering self-costimulating T cells may provide an alternative means to improve the efficacy of adoptive immunotherapies.

With its simplicity in efficiently anchoring premanufactured recombinant costimulators onto cell surfaces, protein transfer is particularly suited for engineering tumor-reactive T cells and other immune cells. Moreover, from the safety standpoint, protein transfer has an advantage over gene transfer. It permits temporary modification of immune cells because the painted proteins will “wear off” over time, likely due to metabolism and/or shedding. Thus, protein transfer is unlikely to cause long-term side effect, such as the induction of autoimmune disease. Furthermore, after the initial proof of principle of protein transfer obtained via the glycosylphosphatidylinositol modification (28–30), several protein transfer methods, including the method used in this study, have been developed (reviewed in refs. 31, 32). These protein transfer methods, combined with the expanding repertoire of costimulators, now provide an enlarging set of options for engineering T cells and other immune cells for treating cancer and autoimmune diseases.

Mechanistically, the enhanced antitumor efficacy of self-costimulating T cells can be attributed to the involvement of costimulation in the priming and/or the effector stages of T-cell function. For the B7-1-painted naïve OT-1 T cells, the enhanced antitumor titer may result, at least partially, from direct priming of the T cells by the tumor cells, which may otherwise be suppressed (by the Treg cells and/or other factors) or be ineffective in the absence of B7-1. This interpretation is consistent with both the data in this study (Figs. 2 and 3) and a recent finding that priming of naïve antitumor T cells can occur within a tumor with enforced costimulation and lead to tumor regression (33). For the ex vivo–amplified T cells, however, their enhanced antitumor efficacy may
derive from the costimulation of effectors (8–10); in the present study, the need for costimulation from the tumor target or tumor-infiltrating APCs is bypassed by directly painting the effector T cells with B7-1.

Liu and Janeway (34) showed previously that antigen and B7 need to be expressed on the same accessory cells to achieve maximal costimulation. It is unclear whether self-costimulation by B7-1-painted T cells can meet such spatial requirement. A spatial separation between antigenic and costimulatory signals is inevitable if the B7-1 can only function in a paracrine mode, through T-cell–T-cell interaction. On the other hand, if the B7-1 can also function in an autocrine mode, it may satisfy the spatial requirement by aligning closely with antigen, in a manner similar to what achieved by colocalizing B7 and antigen on the same accessory cells. Only future experiments will resolve this issue.

Our finding that B7-1-painted T cells are resistant to Treg-mediated suppression apparently contradicts the previous finding by Paust et al. (35) that B7 expression on target T cells facilitates mediated suppression apparently contradicts the previous finding. This discrepancy may be due to what achieved by colocalizing B7 and antigen on the same accessory cells.

References

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Self-Costimulating Antitumor T Cells
Arming Tumor-Reactive T Cells with Costimulator B7-1 Enhances Therapeutic Efficacy of the T Cells


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