Soluble CD80 Protein Delays Tumor Growth and Promotes Tumor-Infiltrating Lymphocytes

Abstract

Tumor cells use various immune-suppressive strategies to overcome antitumor immunity. One such method is mediated by programmed death ligand-1 (PD-L1), an inhibitory molecule of the B7 family that triggers apoptotic death or anergy upon binding programmed death-1 (PD-1) on T cells. Our previous in vitro cellular studies with human and mouse PD-L1- tumor cells demonstrated that a soluble form of the costimulatory molecule CD80 prevented PD-L1-mediated immune suppression and restored T-cell activation by binding PD-L1 and blocking interaction with PD-1. We now report that in vivo treatment of established syngeneic PD-L1+ CT26 colon carcinoma and B16F10 melanoma tumors with CD80-Fc delays tumor growth and promotes tumor-infiltrating T cells. Studies with PD-L1−/− and CD28−/− mice demonstrate that soluble CD80 acts in vivo by simultaneously neutralizing PD-1 suppression and activating through CD28. We also report that soluble CD80 mediates its effects by activating transcription factors EGR1-4, NF-kB, and MAPK, downstream signaling components of the CD28 and T-cell receptor pathways. Soluble CD80 binds to CTLA-4 on activated human peripheral blood mononuclear cells. However, increasing quantities of CTLA-4 antagonist antibodies do not increase T-cell activation. These results indicate that soluble CD80 does not suppress T-cell function through CTLA-4 and suggest that CTLA-4 acts as a decoy receptor for CD80, rather than functioning as a suppressive signaling receptor. Collectively, these studies demonstrate that soluble CD80 has therapeutic efficacy in vivo in mouse tumor systems and that its effects are due to its ability to inhibit PD-1-mediated suppression while concurrently activating T cells through CD28. Cancer Immunol Res; 6(1); 59–68. ©2017 AACR.

Introduction

Tumor cells use various methods of immune suppression to overcome antitumor immunity. One such method is mediated by programmed death ligand-1 (PD-L1), an inhibitory molecule of the B7 family that triggers apoptotic death or anergy upon binding programmed death-1 (PD-1) on T cells. Activated human and mouse T cells upregulate surface PD-1 to limit their effector functions in peripheral tissue as a mechanism to terminate an immune response and minimize damage of healthy surrounding tissue (1). Human and mouse T cells, B cells, dendritic cells, macrophages, vascular endothelial cells, pancreatic islet cells, and tumor cells may constitutively express PD-L1, and PD-L1 is upregulated in response to IFNγ (reviewed in ref. 2).

PD-L1 triggers immune suppression through numerous mechanisms including tolerization of T cells through the PD-1 pathway and reverse signaling through CD80, and by promotion of T regulatory cells (3–6). PD-L1 expression on many human and mouse tumors, including non–small cell lung carcinoma, melanoma, renal carcinoma, and Hodgkin lymphoma, has made PD-L1 a major target for tumor immunotherapies (7–11). Therefore, many antagonist monoclonal antibodies (mAbs) to both PD-L1 and PD-L1 have been developed and tested as monotherapies in clinical trials (7, 12, 13). Immune checkpoint antagonist mAbs to CTLA-4, PD-1, and PD-L1 have proven to be successful treatments in late-stage cancers and cancers once thought to be nonimmunogenic. Several of these therapies are now FDA approved for cancer patients (7, 14–19).

Nevertheless, 60% or more of patients do not respond to these therapies, which only utilize one function: blocking a ligand/receptor interaction. In both mouse and human systems, combination of checkpoint blockade antibodies with other treatments such as radiotherapy, stimulatory cytokines, vaccines, CpG ODN, or other checkpoint blockade antibodies has resulted in higher response rates (15, 20–24). We have previously described a soluble form of CD80 (CD80-Fc) as a potential immunotherapeutic (25–28) based on its binding affinity for PD-L1 (29, 30), and proposed that soluble CD80 could simultaneously act as an antagonist and agonist by (i) binding to PD-L1 and blocking PD-1 signaling and (ii) costimulating T cells through CD28. Data showed that CD80-Fc maintains the activation of human and mouse T cells in vitro in the presence of suppressive human and mouse PD-L1+ tumor cells, respectively. These studies demonstrate that soluble CD80 prevents PD-L1/PD-1 binding and suggest that CD80 also activates T cells through a CD28-dependent mechanism (26). Although these in vitro results confirmed soluble CD80 as a potential immunotherapeutic, animal studies are needed to determine if the soluble form of CD80 has
therapeutic efficacy in vivo. Likewise, additional studies are needed to clarify the mechanistic action of soluble CD80 and to determine if it acts by signaling through CD28. Given the known suppressive function of CD80 through CTLA-4, studies are also needed to ascertain if CD80-Fc minimizes T-cell function by suppressing through CTLA-4.

Using CD80-Fc, we have now tested the ability of soluble CD80 to control progression of PD-L1+ tumors in mice, and we report that in vivo treatment of established CT26 and B16F10 tumors with CD80-Fc delays tumor growth and promotes the influx of T cells into solid tumors. We clarified the mechanism by which soluble CD80 mediates its effects and report that it stimulates downstream signaling components of the CD28 and T-cell receptor pathways. We also demonstrated that CD80-Fc binds to CTLA-4. However, the binding did not affect T-cell function, suggesting that CTLA-4 serves as a competitive inhibitor for CD80 and does not in itself have suppressive activity.

Materials and Methods

Cell lines and mice
Human melanoma cell line C8161 and mouse C57BL/6-derived melanoma B16F10 and BALB/c-derived colon carcinoma CT26 cells were obtained and cultured as previously described (25, 26, 31). Jurkat cells were obtained from and cultured as recommended by the ATCC. Cell lines were authenticated by the original suppliers and were routinely mycoplasma tested in our laboratory. Cell lines were typically maintained in culture for 2 weeks prior to use. C57BL/6 CD28-deficient, C57BL/6 PD-1-deficient, and breeding stocks of BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory. Mice were bred (BALB/c and C57BL/6) and maintained (all strains) in the UMBC animal facility, and all animal procedures were approved by the UMBC Institutional Animal Care and Use Committee.

Tumor inoculation and tumor growth
Female 6-week-old BALB/c or C57BL/6 mice were inoculated subcutaneously (s.c.) in the flank with $5 \times 10^5$ CT26 colon carcinoma or $1 \times 10^6$ B16F10 melanoma cells, respectively. For most in vivo experiments, day 0 corresponds to the start of therapy. Intratumoral (i.t.) injections of mouse CD80-Fc ($20 \mu g$ in 50 $\mu L$ per mouse; R&D Systems), rat IgG2a clone 2A3 ($20 \mu g$ in 50 $\mu L$ per mouse; BioXCell), or Cpg ODN 1555 ($100 \mu g$ in 50 $\mu L$ per mouse) were started on day 0 and administered twice a week for 3 weeks. Tumor diameters at the start of therapy are stated in the figure captions. For some experiments, mice were inoculated with B16F10 cells on both flanks, and tumors on only one flank were treated. Intraportal (i.p.) injections of mouse CD80-Fc ($200 \mu g$ per mouse; R&D Systems), rat IgG2a clone 2A3 ($200 \mu g$ per mouse; BioXCell), or anti-mouse PD-L1 clone 10F.9G2 ($200 \mu g$ per mouse; BioXCell) were administered on days 3, 6, 9, and 22 following tumor inoculation. Mice were sacrificed when they became moribund, and their tumors were cryopreserved and analyzed by immunohistochemistry (IHC). Tumors were measured with a vernier caliper every 2 to 3 days in two perpendicular diameters. Tumor volume $= 4/3\pi r^3$, where $r$ = (diameter 1 + diameter 2)/2.

Reagents, antibodies, and flow cytometry
Soluble CD80 (CD80-Fc) and TROY-Fc were from R&D Systems (Biotechne). Human antibodies CD3-APC (clone OKT3) and CD28-FITC (clone CD28.2) were from BioLegend. Cells ($2 \times 10^5$) were stained for cell-surface expression, subjected to flow cytometry as described, and analyzed using a Beckman Coulter Cyan ADP flow cytometer and Summit V4.3 software (25, 26).

For intracellular staining, cells were fixed with 2% paraformaldehyde, permeabilized with 0.2% saponin, and subsequently stained with the following fluorescent antibodies. For assessment of phosphorylated signal transduction factors, $2 \times 10^5$ cells/mL (RPMI, 10% FBS, 1% glutamax, $5 \times 10^{-5}$ M $\beta$$\text{-mercaptoethanol}$, and 1% penicillin–streptomycin) were stimulated with agonist anti-human CD80 (2 $\mu g$/mL; clone 28.2; BioLegend or BD), CD80-Fc (10 $\mu g$/mL), TROY-Fc (10 $\mu g$/mL), or mouse IgG1 isotype (2 $\mu g$/mL; BD) for 30 minutes at 37°C, transferred to ice, fixed and permeabilized, and then stained with rabbit mAbs for phospho-p44 MAPK + p42 MAPK pYly204 (clone B.742.5; ThermoScientific), pNF-kBp65 (S536, clone 9H1; Cell Signaling Technologies), or rabbit IgG isotype control (ThermoFisher Scientific, catalog #02-6102), followed by F(ab’)2 goat-anti-rabbit IgG-FA488 mAb (ThermoFisher, catalog #A-11070).

In vitro PBMC and T-cell activation
Cryopreserved peripheral blood mononuclear cells (PBMC) from healthy donors were provided by Dr. Dean Mann (UMB Medical School). Tumor cells (50 Gy irradiated; $3 \times 10^5$) and PBMCs ($6 \times 10^4$) were cocultured in triplicate in 96-well plates as described (26) with the following modifications. In some experiments, human CD80-Fc, human TROY-Fc, human IgG1 (R&D Systems), antagonist anti-CTLA-4 mAb 13D10 (BioLegend), pilumumab (Bristol-Meyers Squibb), or CTLA-4–Fc was added at 10 $\mu g$/mL or the specified concentration. IFNγ in culture supernatants was quantified by ELISA according to the manufacturer's protocol (R&D Systems) as previously described (26). Percent T-cell activation $= [(\text{IFN}\gamma_{\text{Experimental}})/\text{(IFN}\gamma_{\text{Control}})] \times 100$. PBMCs ($1 \times 10^4$) were stimulated for 72 hours with PHA (5 $\mu g$/mL) and then treated for 30 minutes with human CD80-Fc or human TROY-Fc (10 $\mu g$/mL) or agonist CD28 antibody (2 $\mu g$/mL; clone 28.2; BioLegend).

Cryopreservation and immunohistochemistry
Tumors at the indicated times were cryopreserved in OCT by freezing in a slurry of dry ice plus 2-methylbutane. Frozen tumors were stored at −80°C until they were sectioned, fixed in cold acetone, dried, and stained with H&E or hematoxylin and/or stained with anti-CD3 mAb (5 $\mu g$/mL; BioLegend). A dozen random fields were analyzed per sample using a Zeiss Universal microscope and 10× and 20× objectives.

Western blots
A total of $2 \times 10^5$ Jurkat cells were left untreated or incubated at 37°C for 30 minutes with 20 $\mu g$/mL CD80-Fc or anti-CD28 mAb. Cells were harvested, resuspended in M-PER Mammalian Protein Extraction Reagent with Halt Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific), and lysed in M tubes (Millipore Biotech) using the gentleMACS dissociator program Protein_01 according to the manufacturer’s recommendation. Protein concentration was assayed via Bradford Assay, protein was electrophoresed on 15% SDS-PAGE gels in SDS running buffer (BioRad) at 100 volts for 90 minutes, and transferred overnight in transfer buffer (BioRad) at 30 volts to PVDF membranes (GE Healthcare).
Membranes were blocked with 5% milk in TBST. pNF-κB and pMAPK were detected with anti-p-κB (clone 93H1; 1:1,000 dilution in 10 mL 2.5% milk/TBST) or anti-pMAPK (clone D13.14.4E; 1:2,000 dilution in 10 mL 2.5% milk/TBST) (both from Cell Signaling Technology) followed by goat anti-rabbit-HRP (Biolegend; 1:10,000 dilution in 10 mL 2.5% milk/TBST). Beta-actin was detected with anti-β-actin (clone AC-15; Sigma-Aldrich; 1:10,000 dilution in 10 mL 2.5% milk/TBST) followed by sheep anti-mouse-HRP (GE Healthcare Life Sciences). Protein was visualized using an HRP detection kit (HyGLO-HRP Detection kit, Denville Scientific, Inc.).

qRT-PCR

CD3+ T cells from the blood of healthy human donors were isolated via negative selection using a Miltenyi Human T-cell Isolation Kit according to the manufacturer’s protocol and were >85% CD3+ T cells. 1×10^7 cells were incubated for 72 hours with 5 μg/mL PHA followed by a 2-hour treatment with LEAF-purified mouse IgG1K isotype control antibody (2 μg/mL; Biolegend), agonist CD28 mAb clone 2D8.2 (2 μg/mL), soluble TROY-Fc (10 μg/mL; R&D Systems), or CD80-Fc (10 μg/mL; R&D Systems). RNA was isolated from T cells using the Maxwell RSC kit (QiaGen, Promega) according to the manufacturer’s protocol and quantified using a BioTek Synergy 2 microplate reader. cDNA was created from each RNA sample using the Maxima cDNA kit (Thermo Scientific) per the manufacturer’s protocol. Specific transcripts were amplified using the KapaStart SYBR Green qPCR ReadyMix iQ (Sigma Life Science), following the manufacturer’s protocol, and detected using a CFX96 Real-Time PCR Detection System (Bio-Rad). Each sample was analyzed in triplicate using the 2-ΔΔCt method, and each primer set was tested by a melting curve. Relative expression of each experimental transcript was normalized to β-actin. Primers used were: EGR1 forward: ACCGCAAGGCTTCTTTCATGCA; EGR1 reverse: GTGGGTGGTCATCATCTCAC; EGR2 forward: ACCGCTTCTTGCAGACCTC; EGR2 reverse: AATGGTGTATGCATCCTGCC; EGR3 forward: GGTAGCATGACAGTGTGC; EGR3 reverse: ACCAGATGGCATTACATCTCTCTGT; EGR4 forward: TACGGAGTGTTCGCGACCC; EGR4 reverse: TCAAGAAGTCGCTGCTCC; β-actin forward: CTTGCGCTTTGCGATCC; β-actin reverse: AATCCCTTCTGACCATGCC.

Statistical analysis

Statistical analysis of tumor growth rates was conducted using the compare growth curves function of the Statmod software package (http://bioinf.wehi.edu.au/software/compareCurves/). Statistical analysis of Kaplan–Meier graphs was conducted using the log-rank test function of the Statmod software package (http://bioinf.wehi.edu.au/software/russell/logrank/). Student t test was utilized to determine statistical significance between two sets of data using Microsoft Excel Version 2010. One-way ANOVA with Friedman’s multiple comparisons test was performed using GraphPad Prism, v7.00. P values <0.05 were considered statistically significant.

Results

CD80-Fc slows tumor growth and extends survival of tumor-bearing mice

We previously reported that in vitro CD80-Fc potentially acts simultaneously as an antagonist and agonist by binding to PD-L1 to block PD-1 signaling and by costimulating T cells through CD28, respectively (25–27). To ascertain if CD80-Fc is therapeutic in vivo, BALB/c and C57BL/6 mice were injected with CT26 colon carcinoma cells and B16F10 melanoma cells, respectively. Tumors were allowed to grow to approximately 4.5 mm in diameter. On day 0, when tumors were established, the mice were given intratumoral (i.t.) injections of CD80-Fc or control IgG antibody twice a week for 3 weeks and monitored for tumor progression. Day 0 on the graphs is the day therapy was started. CD80-Fc treatment slowed tumor growth in both CT26 (P = 0.029) and B16F10 (P = 0.045) tumor-bearing mice in comparison with control IgG treatment (Fig. 1A and B).

Because multiple routes of drug delivery are now being used clinically, CD80-Fc therapy has also been administered intraperitoneally (i.p.). BALB/c mice were inoculated s.c. with 5×10^5 CT26 cells and either left untreated or i.p. treated with 200 μg CD80-Fc, anti-PD-L1 mAb, or isotype mAb (rat IgG2a) on days 3, 6, 9, and 22 following tumor inoculation. By day 42 after tumor inoculation, 100% of the CD80-Fc–treated mice were still alive, whereas only 65% of the PD-L1 mAb-treated, 50% of the untreated, and none of the isotype mAb-treated mice survived. To determine if CD80-Fc promotes an influx of tumor-infiltrating lymphocytes (TILs), tumors were harvested at the time of euthanasia, according to IACUC guidelines (day 42 for all groups except isotype-treated mice, which were euthanized on day 39), and frozen sections were stained for CD3+ T cells. CD80-Fc therapy resulted in increased CD3+ TILs compared with the IgG control, untreated, and PD-L1 mAb-treated mice (Fig. 1C).

Treatment of both mice and cancer patients with CD80-Fc in combination of immune monotherapies has, in some cases, produced greater reduction in tumor growth than either monotherapy alone (reviewed in refs. 32 and 33). To determine if CD80-Fc has the potential to amplify the effect of another immune-based therapeutic, we combined i.t. CD80-Fc therapy with CpG ODN 1555 therapy. CpG was selected because it facilitates both adaptive and innate immunity by enhancing the activation of dendritic cells through the engagement of TLR9, and it reprograms myeloid-derived suppressor cells (MDSC) to become nonsuppressive macrophages, thus, eliminating another potent immune suppressive mechanism in the tumor microenvironment (34). However, CpG induces PD-L1 expression on human B cells (35), a drawback that should be neutralized by soluble CD80. BALB/c mice were inoculated on day 1 with 5×10^5 CT26 tumor cells. On day 14, when tumors were an average of 6.5±1.1 mm in diameter, mice were started on twice-weekly i.t. injections of either control IgG, CpG, or combination CD80-Fc and CpG. Monotherapy with CpG alone slowed primary tumor growth. However, combination therapy of CpG plus CD80-Fc was more effective and significantly delayed tumor progression (Fig. 2). Collectively, the in vivo studies demonstrate that CD80-Fc delays tumor progression of established tumors, enhances the therapeutic efficacy of other immune monotherapies, and promotes TILs.

CD80-Fc activates the CD28 pathway and downstream targets of the TCR

CD80 is an established costimulatory molecule and second signal for T-cell activation (36). Our previous studies using wild-type and CD28-deficient T cells demonstrated that CD80-Fc binds to CD28, suggesting that in addition to preventing PD-1 suppression, CD80-Fc costimulates through CD28. If CD80-Fc uses the CD28 pathway, then CD80-Fc binding to CD28
should activate EGR1-4, downstream components of the CD28 signaling pathway (37). To test this hypothesis, T cells were purified from PBMCs of healthy human donors, treated with PHA for 72 hours, and then treated with either a CD28 agonist antibody, CD80-Fc, or the irrelevant recombinant protein TROY-Fc. RNA was then isolated and screened by qRT-PCR. In CD80-Fc-treated cells, mRNAs were upregulated approximately 3-fold for EGR1, EGR2, EGR3, and EGR4 in comparison with the TROY-Fc–treated T cells (P = 0.032, 0.041, 0.029, 0.035, respectively; Fig. 3A). T cells treated with the agonist CD28 antibody had upregulation of EGR1, EGR2, EGR3, and EGR4 similar to that of CD80-Fc–treated cells (Fig. 3A). EGR1–4 were similarly activated when human Jurkat T lymphoma cells were used (Supplementary Fig. S1).
MAPK and NF-κB are established downstream signal transducers of the TCR and CD28 pathways (38-40). To confirm that CD80-Fc activates T cells through these pathways, CD3⁺ CD28⁺ Jurkat cells were stimulated for 30 minutes at 37°C with agonist anti-CD28 mAb, TROY-Fc, or CD80-Fc. The resulting cells were then fixed and permeabilized and stained with mAbs to CD3, pMAPK, and pNF-κB. CD3⁺ cells were gated and analyzed (Fig. 3B). To confirm the flow cytometry findings, Jurkat cells were analyzed by Western blotting for pMAPK and pNF-κB following stimulation with agonist anti-CD28 mAb or CD80-Fc (Fig. 3C). CD3⁺ cells treated with either agonist CD28 or CD80-Fc contained elevated phosphorylated MAPK and NF-κB (Fig. 3B and C). These results demonstrate that CD80-Fc activates transcription factors downstream of CD28 and the TCR.

CD80-Fc prevents CTLA-4 suppression by saturating CTLA-4

Because the inhibitory receptor CTLA-4 is an established receptor for CD80, it was unexpected that blocking CD80-Fc in the presence of CD80 did not increase T-cell activation. Early studies suggest that CTLA-4/CD80 interactions mediate T-cell suppression through CTLA-4 signaling (41, 42). However, downstream signaling molecules have not been identified for CTLA-4, and studies speculate that CTLA-4 limits T-cell function because it is a decoy receptor for CD80 (reviewed in ref. 43). If CTLA-4 is a decoy receptor, then CTLA-4 should bind CD80-Fc, preventing CD80-Fc from binding to PD-L1 and CD28. To test this hypothesis, we first determined the minimum dose of CD80-Fc that maintains T-cell activation in the presence of PD-L1⁺ tumor cells (Supplementary Fig. S2). We then cocultured PHA-activated human PBMCs for 3 days with PD-L1⁺ human C8161 melanoma cells in the presence of 2 μg/mL CD80-Fc. Increasing quantities of CTLA-4–Fc or irrelevant control recombinant protein TROY-Fc were included in the cultures to determine if CTLA-4 competed with CD80-Fc and suppressed T-cell production of IFNγ. Increasing concentrations of CTLA-4–Fc, but not irrelevant TROY-Fc, prevented CD80-Fc from restoring T-cell activation in the presence of PD-L1⁺ tumor cells (Fig. 5), consistent with the concept that CTLA-4 suppresses T-cell function by binding CD80 and preventing activation of T cells through CD28.

In vitro effect of CD80-Fc requires neutralization of PD-1 and CD28 engagement

In vitro studies indicate that CD80-Fc functions by simultaneously blocking PD-1/PD-L1 suppression while activating through CD28. To determine if these functions occur in vivo, C57BL/6 wild-type, CD28⁻/⁻, and PD-1⁻/⁻ mice were inoculated s.c. with B16F10 tumor cells and treated with either CD80-Fc or PBS starting when tumors were ~5 mm in diameter. We reasoned
that if CD28 activation was essential for a therapeutic effect, then CD80-Fc therapy would not benefit CD28−/− mice. However, if neutralizing PD-1 suppression while activating through CD28 was essential, then the therapeutic effect of CD80-Fc would be the same in PD-1+/− and PD-1−/− mice. To control for the impact of genetic background on tumor progression, the mice were also injected in their contralateral flank on day 0 with tumor cells, and these tumors were not treated. Treated tumors progressed significantly more rapidly in PBS-treated wild-type and CD80-Fc–treated CD28−/− mice as compared with CD80-Fc–treated wild-type and CD80-Fc–treated PD-1−/− mice (Fig. 6A), demonstrating that the CD80-Fc therapeutic effect involves both PD-1 and CD28. Progression of the contralateral untreated tumors in the CD28−/− and PD-1−/− mice was not significantly different from progression of the injected tumors, further demonstrating that CD80-Fc had no effect unless CD28 was present and the PD-1 pathway was inhibited (Fig. 6B).

Discussion

This present study demonstrates that CD80-Fc enhances T-cell activation in the tumor site, extends survival time either as a monotherapy or in conjunction with another immunotherapy, and that the effects occur in mice with relatively large, established tumors. These in vivo results complement earlier in vitro studies showing that soluble CD80 activates tumor-reactive T cells and is a potential therapeutic for facilitating antitumor immunity (25–27). The present study also reveals that soluble
CD80 functions by activating the CD28 and TCR signal transduction pathways. A concern of our earlier studies was that soluble CD80 might limit T cell–mediated antitumor activity by interacting with CTLA-4. However, the current studies demonstrate that CTLA-4 does not affect the therapeutic effect of soluble CD80.

Studies using a biochemical reconstitution system and intact cells have demonstrated that PD-L1 signaling through PD-1 results in the dephosphorylation of CD28 by PD-1–recruited Shp2 phosphatase (44). Therefore, PD-1 suppresses T-cell function by inhibiting costimulation through CD28. Our initial hypothesis that CD80-Fc acts by inhibiting PD-1 signaling while concurrently activating through CD28 is, therefore, not only supported by the studies presented here, but is also consistent with this identified mechanism explaining PD-1–mediated suppression.

The literature does not conclusively identify the mechanism by which CTLA-4 suppresses T cells. Several mechanisms have been proposed: (i) CTLA-4 initiates a negative signal transduction pathway; (ii) CTLA-4 increases T-cell motility that limits antigen presenting cell/T-cell interactions; or (iii) CTLA-4 is a decoy receptor that sequesters CD80 and CD86 away from CD28. There are no known signaling molecules activated by CTLA-4 (reviewed in ref. 43), so we have not explored if soluble CD80 activates a CTLA-4–negative signal transduction pathway. However, we have demonstrated that CTLA-4 can suppress T cells by competing for CD80-Fc and preventing it from binding to PD-L1 and CD28. Therefore, therapy with soluble CD80 will depend on the saturation of CTLA-4 at both the tumor site and the tumor draining lymph node, and excess soluble CD80 will be necessary to allow costimulation through CD28 and neutralization of PD-L1. Saturating quantities of soluble CD80 may have the added benefit
of preventing CTLA-4–mediated suppression and, thereby, reducing T-cell exhaustion.

Therapeutic antibodies and recombinant proteins have been delivered via multiple routes including intravenous (i.v.), i.p., and i.t. Antibody accumulation within solid tumors does not differ between the i.v. and i.p delivery methods (45). Although i.v. delivery has been the traditional route, i.t. delivery is increasingly being used. Intratumoral injection has been utilized for the delivery of bacteria, oncolytic viruses, immune-agonists, STING agonist, cytokines, activated immune cells, and antibodies (reviewed in ref. 46), and many stage I, II, and III clinical trials are solely utilizing i.t. injections of immunotherapies (www.clinicaltrials.gov). Studies using i.t. injections of anti-CTLA-4 mAbs in mice have resulted in systemic antitumor immune responses without the serious adverse side effects of a systemic injection (47, 48). Other studies have shown that some immunotherapeutic drugs only elicited a therapeutic response when delivered i.t. and not i.v. (49). Intratumoral injection makes it likely that the drug arrives at the tumor site in high concentrations where it can immediately bind to PD-L1 and costimulate tumor-specific T cells. Intratumoral injection may also be beneficial because it decreases the potential for off-target side effects.

A 2006 clinical trial of the superagonist anti-CD28 mAb TGN1412 rendered 6 healthy recipients critically ill with a systemic inflammatory response and cytokine release syndrome (“cytokine storm”; ref. 50). CD28 superagonist antibodies fully activate naïve T cells in the absence of TCR ligation and downstream phosphorylation of ZAP70 and the TcRζ. Their effects are due to their binding to the exposed C′D loop of the CD28 Ig-like domain, in contrast to conventional anti-CD28 antibodies that bind to a region close to the binding site for CD80 and CD86 (51).
Superagonist anti-CD28 mAbs can also induce T regulatory cells (52). In contrast to superagonist antibodies, soluble CD80 binds to the natural binding site of CD28 and has a binding constant five orders of magnitude lower than an antibody. Therefore, it is unlikely that soluble CD80 will have the same adverse effects as TG1412.

Our mouse studies here indicate that CD80-Fc therapy is more effective than monotherapy with PD-L1 antibody at promoting TILs. The specific PD-L1 antibody used in these experiments was selected because it is known to have therapeutic efficacy in mice. Because the binding constants for mAbs are much higher than the binding constant for CD80 to PD-L1 (10^{-6}–10^{-9} for antibodies vs. ~1.4 μmol/L for CD80; ref. 29), the difference in function is not due to higher affinity. Several mechanisms could be involved: (i) The ability to costimulate through CD28 allows soluble CD80 to deliver additional activating signals to T cells while concurrently preventing T-cell anergy through PD-1; (ii) unlike antibody to PD-L1, which only prevents suppression via PD-L1, soluble CD80 may also prevent tolerance by reverse signaling of PD-L1 to PD-L1, which only prevents suppression via PD-L1, soluble CD80 binds

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Tumors and suggest it may have the potential for the treatment of cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Writing, review, and/or revision of the manuscript: L.A. Horn, T.M. Long, S. Ostrand-Rosenberg

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Study supervision: S. Ostrand-Rosenberg

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