A Soluble Form of CD80 Enhances Antitumor Immunity by Neutralizing Programmed Death Ligand-1 and Simultaneously Providing Costimulation

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Abstract

Tumor cells use various methods of immunosuppression to overcome antitumor immunity. One such method is that of programmed death ligand-1 (PD-L1 or B7-H1), which upon binding its receptor PD-1 on T cells triggers apoptotic death of the activated T cells. Overexpression of the costimulatory molecule CD80 on PD-L1− tumor cells, or inclusion of a soluble form of CD80 (CD80-Fc), maintains the activation of PD-1−-activated T cells. Using T cells from CD28-deficient mice and antibodies to block CD28 on human T cells, we now report that a soluble form of CD80 mediates this effect by simultaneously neutralizing PD-1–PD-L1-mediated immunosuppression and by providing CD80–CD28 costimulation, and is more effective than antibodies to PD-L1 or PD-1 in maintaining IFNγ production by PD-1+ activated T cells. Therefore, soluble CD80 may be a more effective therapeutic than these checkpoint antibodies for facilitating the development and maintenance of antitumor immunity because it has the dual functions of preventing PD-L1–mediated immunosuppression and simultaneously delivering the second signal for T-cell activation.

Introduction

Programmed death ligand-1 (PD-L1), also known as B7 homolog 1 (B7-H1) or CD274, is expressed by many human and mouse tumor cells either constitutively or in response to exposure to IFNγ (1, 2). Expression of PD-L1 results in the suppression of antitumor immunity through multiple mechanisms. PD-L1 renders tumor cells resistant to CTL- and FasL-mediated lysis (3). It also induces apoptosis of activated T cells by signaling through its receptor PD-1. PD-L1 also reverses signals through T cell–expressed CD80 to anergize T cells, and its expression promotes the induction and expansion of regulatory T cells (Treg; refs. 1, 4–7). Some T and B cells, dendritic cells, Tregs, macrophages, and myeloid-derived suppressor cells may also express PD-L1 (8–10), and thus contribute to the inhibition of antitumor immunity.

Human and mouse tumor cells modified to express CD80 as an integral membrane protein prevent PD-L1 from binding to its receptor PD-1 (11, 12). As a result, PD-1+ T cells remain activated. Treatment with a fusion protein consisting of the extracellular domains of CD80 fused to the Fc region of human immunoglobulin G1 (IgG1; CD80-Fc) similarly maintains the viability of activated PD-1+ T cells (12).

In addition to overcoming suppression by PD-L1, membrane-bound CD80 or CD80-Fc has the potential to costimulate T-cell activation via T cell–expressed CD28 (13). We now report that CD80-Fc maintains the activation of PD-1+ T cells by simultaneously preventing PD-1–PD-L1 suppression and by providing costimulation through CD28, and is more effective than antibodies to PD-L1 or PD-1 for maintaining IFNγ production by activated T cells. These findings suggest the potential of CD80-Fc as a therapeutic agent to overcome immunosuppression and sustain antitumor immunity.

Materials and Methods

Cell lines and transfections

Human cutaneous melanoma cell line C8161 was kindly provided by Dr. Elisabeth Seftor (Children’s Memorial Research Center, Northwestern University, Evanston, IL) in 2011 and was cultured as described previously (11). Because C8161 cells were not obtained from a cell bank, they cannot be authenticated; however, the line has maintained a unique profile by short tandem repeat (STR) analysis and is routinely tested for Mycoplasma infection. C8161/CD80 transfectants were generated and maintained as described previously (11). Cell lines and procedures with human materials were approved by the University of Maryland Baltimore County (UMBC; Baltimore, MD) Institutional Review Board.
Mice

Breeding stock for C57BL/6 and CD28-deficient C57BL/6 (CD28−/−) mice was obtained from The Jackson Laboratory. Mice were bred and maintained in the UMBC animal facility. All animal procedures were approved by the UMBC Institutional Animal Care and Use Committee.

Antibodies, reagents, and flow cytometry

Mouse CD3-Pacific Blue (clone 17A2), mouse CD28-PE-Cy7 (clone E18), mouse PD-1-APC (clone RMP1–30), mouse PD-L1-PE (clone 10F.9G2), mouse CD152-APC (clone UC10-4B9), and low endotoxin, azide-free human CD80 (clone 2D10) monoclonal antibodies (mAb) were from BioLegend. Mouse CD69-FITC (clone H1.2F3) and functional grade mouse IgG1 (clone P3.6.2.8.1) were from BD Biosciences and eBioscience, respectively. Anti-PD-L1 (clone 5H1) was provided by Dr. Eugene Kwon (Mayo Clinic, Rochester, MN). Anti-human IgG1-Alexa Fluor 488 and anti-mouse IgG-Alexa Fluor 647 polyclonal antibodies were from Invitrogen. Cells were stained for cell surface expression and subjected to flow cytometry as described previously (14, 15), and analyzed using a Beckman Coulter CyAn ADP flow cytometer and Summit V4.3. For internal staining, cells were fixed with 2% paraformaldehyde and permeabilized before staining.

Human PBMC activation

Cryopreserved peripheral blood mononuclear cells (PBMC) were obtained from healthy human donors as described previously (16). PBMCs (6 × 10^5) and tumor cells (50 Gy-irradiated; 3 × 10^5) were cocultured in 96-well plates as described previously (11). Human CD80-Fc, human TROY-Fc (TNF receptor superfamily member) recombinant fusion proteins (R&D Systems), and antibodies to human PD-L1 (clone 29E.2A3; BioLegend) and human PD-1 (clone P1D1.3.1.3; Miltenyi Biotec) were added to some wells at 10 μg/mL. Mouse IgG1 (clone P3.6.2.8.1), and antibodies to human CD80 (clone 2D10) were from eBioscience and BioLegend, respectively. Human IFNγ production was measured by ELISA on day 3 as described previously (16).

CD80-Fc and PD-1-Fc binding

C8161/CD80 cells were incubated in the presence of 10 or 100 μg/mL of anti-human CD80 (BioLegend) or mouse IgG1 control followed by incubation with recombinant human CD28-Fc, staining with anti-human IgG-Alexa Fluor 488, and analysis by flow cytometry. C, CD80 blockade diminishes, but does not eliminate the ability of CD80 to maintain T-cell activation. PHA-activated PBMCs from healthy human donors were cocultured with human C8161 or C8161/CD80 cells. Ten, 50, or 100 μg/mL of CD80 blocking mAb or IgG1 control were included in some wells. IFNγ production was measured by ELISA. Statistical evaluation was performed by the Mann–Whitney test. D, human PBMCs from healthy donors were undepleted, CD8-depleted, CD4-depleted, or CD8- and CD4-depleted, and subsequently incubated with PHA for 72 hours. IFNγ production was measured by ELISA. Data are from three, two, two, and two independent experiments for A, B, C, and D, respectively. Slightly modified versions of this experiment using five different tumor cell lines were done with PBMCs from six different healthy donors.
IgG1 (eBioscience) mAbs, followed by incubation with a fusion protein consisting of the extracellular region of CD28 fused to the Fc region of human IgG1 (CD28-Fc; R&D Systems), and detected by anti-human IgG-Alexa Fluor 488 (Invitrogen). C8161 and C8161/CD80 cells were incubated in the presence of human PD-1-Fc, followed by anti-human IgG-Alexa Fluor 488 (Invitrogen) as described previously (11).

**Mouse splenocyte activation** Splenocytes from C57BL/6 and CD28−/− mice were depleted of red blood cells and cultured at 37°C, 5% CO2 in 96-well plates...
at 1 × 10^5 cells per 200 μL per well with 20 ng/mL phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich) plus 1 μg/well ionomycin (EMD Millipore) in the presence or absence of mouse TROY-Fc or CD80-Fc (R&D Systems) at 1, 5, and 10 μg/mL. IFNγ production was measured on day 2 by ELISA according to the manufacturer’s directions (BioLegend).

**Statistical analysis**

SD, one-way ANOVA, and Tukey honestly significant difference (HSD) test were performed using Excel version 2010. The Mann–Whitney test was performed using [http://faculty.vassar.edu/lowry/VassarStats.html](http://faculty.vassar.edu/lowry/VassarStats.html).

**Results**

**CD80-mediated costimulation is not exclusively responsible for maintaining activated T cells in the presence of PD-L1+ human tumor cells**

Coexpression of membrane-bound CD80 by PD-L1+ tumor cells prevents the binding of PD-1 to tumor cell–expressed PD-L1 (Fig. 1A), suggesting that CD80 may sustain T-cell activation by preventing PD-1–PD-L1–mediated immunosuppression. In addition to binding PD-L1, CD80 also functions as a costimulatory molecule by binding to T cell–expressed CD28 (13). We have shown previously that PD-L1+ human and mouse tumor cells modified to express CD80 or treated with a soluble recombinant CD80 (CD80-Fc) facilitate the activation of T cells (11), an effect that could be due to either blocking of PD-1–PD-L1 interactions and/or CD80–CD28 costimulation. To test the latter possibility, PD-L1+ human melanoma C8161 cells transfected with CD80 (C8161/CD80) were incubated with 10 or 100 μg/mL anti-CD80 blocking mAb or IgG1 isotype control followed by human CD28-Fc. Binding of CD28-Fc was detected by staining with fluorescently labeled goat anti-human IgG and analysis by flow cytometry (Fig. 1B). Pretreatment with 10 or 100 μg/mL anti-CD80 mAb decreased CD28-Fc binding by 73% and 94%, respectively, whereas pretreatment with 10 or 100 μg/mL of control IgG mAb decreased the binding by no more than 4%. Because treatment with 100 μg/mL of anti-CD80 antibodies inhibited the binding of CD28-Fc, it is reasonable to expect that CD80 antibodies–treated PD-L1+ cells cannot bind to CD28 and costimulate.

To determine whether CD80–CD28 costimulation was exclusively responsible for maintaining T-cell activation in the presence of PD-L1+ tumor cells, phytohemagglutinin (PHA)-activated human PBMCs were cocultured with PD-L1+ C8161 or with C8161/CD80 cells in the presence or absence of increasing concentrations of either anti-CD80 blocking mAb or IgG1 isotype control antibody (Fig. 1C). T-cell activation was measured by the production of IFNγ. If CD80 only costimulates, then cultures of C8161/CD80 cells with anti-CD80 mAbs will display suppression similar to cultures containing CD80– C8161 cells. C8161 cells suppressed the activation, whereas C8161/CD80 cells increased IFNγ production. Inclusion of anti-CD80 mAb, but not IgG1 isotype control, at 10, 50, or 100 μg/mL, decreased the activation to levels similar to those of activated PBMCs alone, but more than that of activated PBMCs plus C8161 cells. IFNγ was produced by CD4+ and CD8+ T cells as depletion of these cells eliminated IFNγ production (Fig. 1D). Therefore, anti-CD80 mAbs partially decreased T-cell activation by C8161/CD80 cells, suggesting that in the absence of costimulation, CD80 maintains T-cell activation by preventing PD-1–PD-L1–mediated suppression. This latter conclusion is supported by our finding that CD80+ PD-L1+ tumor cells do not bind to PD-1 (Fig. 1A).

**CD80-Fc overcomes suppression by PD-L1+ PBMCs, including PD-L1–expressing activated CD4+ and CD8+ T cells**

In addition to tumor cells, activated CD4+ and CD8+ T cells as well as non-CD3+ PBMCs express PD-1 and PD-L1 (Fig. 2A),...
and may contribute to suppression. To test this possibility, human PBMCs were activated for 72 hours with PHA in the presence of anti-PD-L1 mAb (clone 29E.2A3), anti-PD-1 mAb (PD1.3.1.3), or an isotype control (Fig. 2B). IFNγ production was measured by ELISA. Antibodies to both PD-L1 and PD-1 increased the activation relative to the untreated or IgG controls, confirming that T cell–expressed PD-L1 suppresses activation. We have shown previously that a soluble form of CD80 (CD80-Fc) is equally effective as membrane-bound CD80 in maintaining the activation of both mouse and human T cells (12), and therefore included CD80-Fc and a negative control recombinant protein (TROY-Fc) in this experiment. CD80-Fc enhanced activation more efficiently than treatment with either antibody (Fig. 2B). Experiments with four other anti-PD-L1 mAbs (MIH1, 5H1, MIH3, and 130021) and four other anti-PD-1 mAbs (EH12.1, EH12.2H7, J116, and MIH4) gave similar results. Therefore, expression of PD-L1 by activated T cells and other PBMCs also reduces T-cell activation, and T-cell production of IFNγ is more effectively enhanced by CD80-Fc than by antibodies to PD-L1 or PD-1.

**CD80 increases T-cell activation independent of costimulation**

The experiments depicted in Figs. 1 and 2 demonstrated that CD80, either as a membrane-bound protein or as a soluble recombinant protein, contributes to maintaining T-cell activation, and that its effect is partially due to the costimulation via CD28. To test the function of CD80 in the absence of costimulation, wild-type and CD28−/− C57BL/6 mouse splenocytes were treated with PMA plus ionomycin for 48 hours, and the resulting T cells were analyzed by flow cytometry for the activation marker CD69 and for the expression of PD-1 and PD-L1. Activated splenocytes were also tested for CTLA-4 expression because CD80 can cause anergy/apoptosis by binding to T cell–expressed CTLA-4 (17). Because cell surface CTLA-4 has a short half-life, both intracellular and cell surface CTLA-4 were measured (Fig. 3 and Supplementary Fig. S1). Both wild-type and CD28−/− T cells displayed increased CD69 and PD-1 expression following treatment with PMA plus ionomycin, demonstrating that both cell populations were activated. Mouse T cells also upregulated PD-L1 after activation, indicating that activated mouse T cells, like activated human T cells, may also contribute to PD-L1–mediated immunosuppression. CTLA-4 was also expressed by activated mouse T cells.

To test whether murine T cell–expressed PD-L1 decreased T-cell activation, splenocytes from C57BL/6 wild-type and CD28−/− mice were activated with PMA plus ionomycin and cultured for 48 hours with either CD80-Fc or control TROY-Fc. T-cell activation was assessed by measuring IFNγ production (Fig. 4A). If CD80-Fc prevents suppression by blocking PD-1–PD-L1 interactions, then CD80-Fc will increase IFNγ production by CD28−/− T cells. However, if CD80-Fc only increases T-cell activation by costimulation, then there will be no effect when CD80-Fc is added to the CD28−/− T cells. Inclusion of CD80-Fc, but not TROY-Fc, increased IFNγ production by CD28−/− T cells, and the dose-dependent increase was most dramatic for the CD28−/− T cells (Fig. 4A and B). IFNγ production was increased by CD80-Fc despite the expression of CTLA-4 on activated T cells. This finding suggests...
that CD80-Fc is not inducing T-cell anergy/apoptosis via CTLA-4 or that such interactions are minimal. Collectively, these results demonstrate that, similar to the effects of CD80 on human T cells, CD80 also sustains the activation of murine T cells through a costimulation-independent mechanism.

Discussion

Previously, we have demonstrated that treatment of PD-L1+ tumor cells with a soluble form of the extracellular domains of CD80 or coexpression of membrane-bound CD80 maintains the activation of both human and mouse T cells (11, 12). In these previous studies, it was unclear if and how much of the effect of CD80 was due to its ability to costimulate through CD28 versus its ability to prevent PD-1+ T cells in the presence of PD-L1+ cells. As recent clinical trials have demonstrated, PD-L1−mediated suppression is a potent mechanism used by many tumor cells to evade antitumor immunity, and antibody blocking of PD-L1 or PD-1 significantly increases patient responses and survival times (18, 19). In the present studies, CD80-Fc was more effective than antibodies to PD-L1 or PD-1 at maintaining T-cell production of IFNγ. This enhanced activity may be because of the dual action of CD80-Fc in simultaneously blocking PD-1−PD-L1 suppression and providing costimulation and, therefore, CD80-Fc may be a more effective therapeutic than mAbs to PD-L1 and/or PD-1 for restoring and maintaining antitumor immunity in patients with cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: S.T. Haile, S. Ostrand-Rosenberg
Development of methodology: S.T. Haile
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.T. Haile, L.A. Horn, S. Ostrand-Rosenberg
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.T. Haile, L.A. Horn, S. Ostrand-Rosenberg
Writing, review, and/or revision of the manuscript: S.T. Haile, L.A. Horn, S. Ostrand-Rosenberg

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.T. Haile
Study supervision: S. Ostrand-Rosenberg

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References

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