A Novel Approach to Cancer Immunotherapy: Tumor Cells Decorated with CD80 Generate Effective Antitumor Immunity


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ABSTRACT

Malignant cells often elude the immune system by lacking costimulatory signals required for the generation of effective antitumor immunity. Immunization with tumor cells genetically modified to express costimulatory molecules is a highly promising approach to cancer immunotherapy. However, genetic modification of tumor cells is not only labor/time intensive but is also less efficient and bears safety concerns. To override these complications, we have recently developed a novel technology that allows for efficient and durable display of exogenous proteins on the surface of a cell within 2 h. This technology involves modification of the cell membrane with a biotin derivative and decoration of biotinylated cells with proteins chimeric with core streptavidin. A chimeric molecule composed of the extracellular domains of the human CD80 costimulatory molecule and core streptavidin (CD80-SA) was efficiently displayed on the cell surface, where it persisted with a 1/2t of > 10 days in vivo. Tumors from patients with advanced stage gynecologic cancers decorated with CD80-SA elicited potent ex vivo tumor-specific proliferative and cytotoxic responses in autologous lymphocytes. Immunization with tumor cells decorated with CD80-SA completely prevented tumor growth in an aggressive model of mouse lymphoma. This technology may serve as a fast, efficient, and safe alternative to gene transfer approaches for engineering tumor cells for use in immunotherapy and research.

INTRODUCTION

The T-cell-mediated immune response plays a critical role against tumors (1–3). An effective T-cell response is initiated by two distinct signals: signals 1 and 2 (1–3). Signal 1 is antigen specific and transduced by T-cell receptor interaction with the peptide/MHC on the surface of APCs. Signal 2 is delivered by a series of costimulatory molecules in an antigen-nonspecific fashion (4). The combined effects of these two signals lead to T-cell activation, proliferation, and differentiation into effector cells. In the absence of signal 2, T-cell activation is abortive and cannot proceed to the stage of efficient effector function (5, 6). Tumor cells have developed multiple mechanisms, ranging from the lack of expression of tumor antigens to active suppression via anti-inflammatory cytokines or secretion of death-inducing molecules such as FasL to evade the immune system (1). However, the most frequently used strategy is the lack of costimulation (1, 3, 7–9). The most critical molecules involved in costimulation are CD80/CD86 and CD40L, which interact with their counter receptors CD28/CTLA-4 and CD40, respectively (10, 11).

Genetic modification of tumor cells through conventional gene transfer approaches to express costimulatory molecules has been shown to serve as an effective approach for immunotherapy against cancer not only in animal models but also in limited clinical trials (1, 3, 7–9, 12–15).

The most common gene transfer approaches include the use of viral and nonviral vectors to introduce DNA into the cell (12, 16–19). Both approaches are associated with low gene transfer efficiency and low transgene expression levels (17–21). Furthermore, these approaches are time/labor intensive and require the introduction of a foreign vector, which may pose safety issues, particularly in immunodeficient patients (17, 20–22). Difficulties associated with the resection of sufficient tumor mass combined with the refractory nature of certain tumor cells to genetic manipulation may require the establishment of tumor cell lines, which makes this approach impractical for patients with aggressive tumors. This raises the question whether a process that allows for the durable and rapid display of exogenous proteins bearing immunological function on the surface of tumor cells will prove to be an efficient and safe alternative to gene transfer approaches for cancer immunotherapy.

We used the extremely high affinity of biotin for SA as the basis of a novel technology designated as ProtEx to functionally display proteins on the cell surface. The SA-biotin interaction has a Kd of \( \sim 10^{-15} \) M, and as such, it is rapid and durable. Once it has occurred, this noncovalent interaction can generally be disrupted only under denaturing conditions (23–25). We reasoned that this interaction could be exploited for the durable cell-surface display of exogenous proteins chimeric with SA and used as an alternative to gene transfer approaches for a variety of applications, ranging from basic science studies to therapeutics. Furthermore, SA exists as stable tetramers and oligomers under physiological conditions (23, 25), as such, may not only allow the durable display of chimeric proteins but may also enhance signal transduction by facilitating aggregation of the proteins on the cell surface (26).

The ProtEx technology involves the generation of core SA fused to functional domains of proteins of interest, where these recombinant proteins are then attached to cells that have been biotinylated without the loss of protein or cell function. We have recently demonstrated that ProtEx can be used to efficiently display a chimeric molecule consisting of rat FasL chimeric with core SA on the surface of splenocytes (27). Immunomodulation with FasL-displaying splenocytes were effective in inducing apoptosis in alloreactive lymphocytes, leading to the prevention of islet allograft rejection in mice. We herein tested whether this technology can be used to convert tumor cells into APCs by displaying costimulatory molecules on the surface and if such cells can be used for tumor vaccination. A chimeric protein composed of the extracellular portion of the human CD80 costimulatory molecule and core SA was efficiently displayed on the surface of various primary and established cell lines and that the protein persisted on the cell surface for weeks both in vitro and in vivo. Tumor cells decorated with the chimeric protein served as APCs for the generation of effective antitumor immune responses ex vivo and the...
prevention of tumor growth in an aggressive model of mouse lymphoma.

MATERIALS AND METHODS

Animals and Cells. BALB/c (H-2b) male mice were purchased from Jackson laboratory (Bar Harbor, ME). PVO.1U and PVO.R8 rats were obtained from our colony at the University of Louisville. The animals were housed under specific pathogen-free conditions and cared and maintained in accordance with institutional and NIH guidelines. A20 is a B-cell lymphoma line derived from BALB/c mice and expresses MHC class I and II with low or undetectable level of CD80. A20 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 12 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol at 37°C in a humidified 5% CO2 incubator. OVCAR-3, HEC-1-A, and ME180 cell lines are derived from human ovarian cancer, endometrial cancer, and cervical cancer, respectively. These cells express HLA class I of different haplotypes but lack the expression of class II HLA and CD80 molecules. OVCAR-3, HEC-1-A, and ME180 cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 12 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate at 37°C in a humidified 5% CO2 incubator. All of the cell lines were purchased from American Type Culture Collection (Manassas, VA).

Antibodies and Other Reagents. FITC-labeled anthuman class I (clone G46-2.6), PE-labeled class II (clone TU36), PE-labeled CD80 (clone L307.4), APC-labeled anthuman CD4 (clone RPA-T4) and PE-labeled CD8 (clone RPA-T8), rabbit antirat SA serum, and horseradish peroxidase-conjugated goat antirabbit antibodies were purchased from BD-PharMingen (San Diego, CA). CFSE was purchased from Molecular Probes (Eugene, OR). Ni-NTA columns were purchased from Qiagen (Valencia, CA).

Cloning and Expression of CD80-SA. The gene encoding core SA was cloned from Streptomyces avidinii as template in PCR with specific primers (c and d; Fig. 1A). The extracellular portion of human CD80 was cloned using cDNA generated from total RNA isolated from human macrophages activated with lipopolysaccharide as template and CD80-specific primers (a and b; Fig. 1A) in PCR. These genes were then subcloned into the Drosophila pMT/BiP/V5-HisA expression vector (Invitrogen, San Diego, CA) in frame with the BiP secretion signal using standard molecular methods. Drosophila S2 cells were transfected with 20 μg of the recombinant vector using the Calcium Phosphate Transfection kit according to the manufacturer’s instructions. Stable transfectants were established by cotransfection with 1 μg of pcOHygro vector and maintenance in the presence of 300 μg/ml hygromycin. The expression of CD80-SA was induced with 600 μM copper sulfate. Supernatant was collected 1–4 days after induction and either used immediately or purified using Ni-NTA columns (Qiagen).

Characterization of CD80-SA by Western Blot. Culture supernatants were fractionated by PAGE under native and denaturing conditions. For Western blot analysis, the fractionated proteins from the gel were transferred onto polyvinylidene difluoride membranes using a dry-blot apparatus (Bio-Rad, Hercules, CA). Membranes were incubated with blocking buffer (5% dry milk and 0.5% Tween 20 in PBS), followed by incubation with antibody to goat anti-SA (Pierce, Rockford, IL) at 1:1000 dilution in the blocking buffer for 1 h. Membranes were then washed three times with washing buffer (0.2% Tween 20 in PBS) and incubated 1 h with antibody against horseradish peroxidase-conjugated goat antirabbit antibodies purchased from BD-PharMingen (San Diego, CA). Finally, membranes were washed several times and incubated in a chemiluminescent substrate according to the manufacturer’s instructions (Molecular Dynamics, San Diego, CA).

Decoration of Cells with CD80-SA. Various cell types (splenocytes, primary tumor cells, and established tumor cell lines) were biotinylated in 5 μM freshly prepared EZ-Link Sulfo-NHS-LC-Biotin (Pierce) in PBS for 30 min at room temperature. Cells were then washed extensively and incubated for 30 min with 50–100 ng of CD80-SA/106 cells in PBS. The presence of CD80-SA on the cell surface was assessed using PE-labeled anthuman CD80 mAb (clone L307.4).

MLTRs. Blood and tumor samples for this study were collected from subjects with informed consent under guidelines and procedures approved by the institutional review board of the University of Louisville. PBLs from healthy volunteers or cancer patients were isolated by centrifugation on Ficoll-Paque Plus followed by plastic adherence for 2 h. PBLs were resuspended in complete human MLTR medium (RPMI 1640 supplemented with 10 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, 274 μM l-arginine, and 5% pooled human serum) and used as responders against irradiated (10,000 cGy) tumor cell lines of various antigenic disparities. After 3–5 days of incubation, cultures were pulsed with 1 μCi/well [3H]thymidine (NEN Life
and higher order structures under native PAGE conditions (Fig. 1A) using S2 cells. The chimeric CD80-SA molecule formed tetramers on the surface of several different was effectively displayed (Fig. 2A).

For mouse experiments, splenocytes from animals inoculated with tumors were harvested 30–58 days after tumor challenge. These cells were co-incubated with irradiated A20 cells in the presence of IL-2 for 5 days under culture conditions similar to those used for human cells. Effector cells were harvested and tested for cytotoxic activity against tumor targets at different E:T ratios.

Immunotherapy. BALB/c mice were challenged s.c. into the right flank with a lethal dose (1 × 10^6) of viable A20 cells immediately followed by immunization with a dose of 1 × 10^7 irradiated (5000 cGy) A20 cells decorated with CD80-SA at the same site. Controls included: unmodified irradiated cells; cells decorated with a nonfunctional human CD40L chimeric with SA; and biotinylated cells treated with S2 culture supernatant. Animals were palpated for the presence of tumors, and once detected, tumors were measured every other day using calipers. Average tumor size was measured in two perpendicular diameters, and animals were euthanized when the tumor size reached ~30 mm in diameter or upon any sign of ill health.

RESULTS

Generation and Characterization of the CD80-SA Protein. A chimeric molecule containing the extracellular domains of the human CD80 costimulatory molecule and the biotin-binding, tetramer-forming domains of SA were generated using gene-specific primers in PCR (Fig. 1A). The extracellular portion of CD80 was cloned NH2-terminal to SA for proper folding because CD80 is a type 1 protein (29). The CD80-SA gene was then subcloned in frame with the secretion signal of Drosophila in a metal-inducible expression vector using S2 cells. The chimeric CD80-SA molecule formed tetramers and higher order structures under native PAGE conditions (Fig. 1B). However, dissociation of chimeric CD80-SA into monomers occurred when the sample was heated at 100°C but not 60°C (Fig. 1C). These structural features of CD80-SA are consistent with those of native SA (23–25). The chimeric protein effectively bound to purified primary human T cells or rat T-cell lines expressing the counter receptors CD28/CTLA-4 (Fig. 1D). Taken together, these results clearly demonstrate that CD80-SA has the structural features of native SA and CD80 molecules with respect to forming tetramers/oligomers and binding to its counter receptors CD28/CTLA-4 on T cells.

CD80-SA Is Durably Displayed on the Cell Surface in Vitro and in Vivo. The use of ProtEx as an alternative to conventional gene transfer approaches for immunotherapy depends upon not only efficient cell surface display of exogenous proteins but also their persistence on the surface for periods of time necessary for the elicitation of the anticipated response. To test whether our technology met these requirements, rat and mouse splenocytes were biotinylated with various concentrations of a biotin derivative, Sulfo-NHS-LC-Biotin, under physiological conditions and analyzed for cell surface biotin using flow cytometry. There was dose-dependent display of biotin on the cell surface, reaching a plateau at 5 μM (data not shown). CD80-SA was effectively displayed (Fig. 2A) on the surface of several different biotinylated primary (splenocytes), as well as established human cancer cell lines (OVCAR-3, HEC-1A, and ME180) where it persisted with a t1/2 of >12 days for cancer cell lines and >10 days for splenocytes in culture (Fig. 2B). Importantly, modification of the cells with biotin and decoration with CD80-SA were not toxic to the cells because a tumor cell line displaying CD80-SA on the surface showed similar kinetics of proliferation in culture as compared with the unmodified cells (data not shown). To assess the cell surface persistence of the chimeric protein in vivo, rat splenocytes were labeled with the intracellular dye CFSE, decorated with CD80-SA, and injected i.v. into syngeneic hosts. Spleens were harvested at various times after

Fig. 2. Persistence of CD80-SA on the surface of rodent splenocytes in vitro and in vivo. Rat splenocytes were biotinylated and decorated with CD80-SA. The cell surface presence of CD80-SA was determined using the PE-labeled antihuman CD80 mAb in flow cytometry (A). Biotinylated cells incubated with S2 control supernatant served as negative controls. Splenocytes and three human cell lines (OVCAR-3, HEC-1A, and ME180) decorated with CD80-SA were cultured in vitro for various times, and the cells were analyzed for the presence of CD80-SA molecule on the surface using flow cytometry (B). Splenocytes labeled with CFSE and decorated with CD80-SA were injected i.v. into syngeneic animals. Splenocytes harvested at various times after injection were analyzed for the presence of CD80-SA on the surface of CFSE-positive cells using flow cytometry (C). Results shown are representative of three independent experiments with a minimum of 6 animals/data point.
injection and analyzed for the presence of CD80-SA. The turnover kinetics of CD80-SA from the cell surface in vivo were similar to those observed for in vitro, with a t_{1/2} of >10 days. As shown in Fig. 2C, >65% of CFSE-positive cells maintained the chimeric protein on the surface 10 days after injection. Taken together, these data clearly demonstrate that ProtEx can be used as a platform technology to display proteins on the cell surface in vivo for weeks, if not months, without detectable toxicity to the cell.

**Decoration of Tumor Cells with CD80-SA Converts Them into APCs with Potent T-Cell Activation Function.** The combination of cell membrane modification for rapid display of exogenous proteins combined with extended persistence of these proteins on the cell surface in vivo suggests that ProtEx can serve as a practical and powerful immunotherapeutic approach for the treatment of cancers. To test this prediction, three different human tumor cell lines (OVCAR-3, ovarian cancer; HEC-1-A, endometrial cancer; and ME180, cervical cancer) were decorated with CD80-SA molecule and used for the activation of lymphocytes from healthy individuals (30). All these tumor cell lines expressed HLA class I but lacked the expression of class II and CD80 molecules (data not shown). CD80-SA-decorated tumor cells resulted in effective display of this chimeric molecule on the surface (Fig. 3A). Tumor cells displaying the chimeric molecule generated potent proliferative responses in PBLs from healthy volunteers (Fig. 3B). The proliferative response was specific to CD80-SA because unmanipulated tumor cells or biotinylated cells treated with culture supernatant of S2 cells transfected with a nonfunctional gene, denoted control S2, had no or a minimal proliferative response. The proliferative response was primarily associated with CD80+ T cells but not CD4+ T cells (Fig. 3C), which is consistent with the lack of class II expression on tumor cells (Fig. 3A). This observation is also consistent with previous published studies demonstrating that tumor cells genetically modified to express CD80 primarily generate a CD80+ T-cell response (9, 12, 31). This proliferative response was attributable to costimulatory signals transduced by CD80-SA because a recombinant core SA protein did not generate a response (data not shown). CD80-SA-decorated tumor cells not only induced the proliferation of CD80+ T cells but also generated an effector response. T cells primed with CD80-SA-decorated tumor cells, but not with unmanipulated cells, effectively lysed tumor targets in an antigen-specific manner (Fig. 3D). These results indicate that the CD80-SA molecule delivers effective costimulatory signals that lead to not only CD80+ T-cell proliferation but also differentiation into killer cells.

**Primary Tumor Cells Decorated with CD80-SA Generate Potent Antitumor Responses in Autologous Lymphocytes.** Although a potent stimulation was observed in T cells from healthy individuals, there remained the question whether primary tumors decorated with CD80-SA would elicit a response in autologous T cells from cancer patients with a high tumor burden or who had been subjected to chemotherapy. Primary tumor cells were isolated from three patients...
with gynecologic malignancies; one was a 56-year-old female with advanced stage, high-grade adenocarcinoma of the ovary without previous treatment (patient A; PA), the second was a 48-year-old female with advanced stage, high-grade adenocarcinoma of undetermined origin with a previous history (10 year) of treated cervical cancer (patient B; PB), and the third was a 75-year-old female with stage IIIC adenocarcinoma of ovarian origin (patient C; PC). Tumor specimens were obtained after pathologic assessment, and single-cell suspensions were prepared by mechanical dissociation. The isolated tumor cells were decorated with CD80-SA and coincubated with autologous PBLs harvested at the time of surgery. There was 20-fold more T-cell proliferation in cultures where peripheral lymphocytes from subject PA were coincubated with autologous tumors displaying CD80-SA than in cultures from the same subject with unmanipulated or S2-treated tumors cells (Fig. 4A). All three patients generated vigorous cytolytic T-cell responses ranging from 50 to 60% specific killing against tumor cells after a 5-day in vitro stimulation with autologous tumors decorated with CD80-SA (Fig. 4B; data for patients PB and PC are not shown). The cytolytic response was antigen-specific because there was minimal killing (~10%) of an allogeneic cell line. Stimulation with unmodified tumor cells or biotinylated tumor cells treated with S2 supernatant or core SA generated minimal cytolytic response. These results clearly demonstrate that tumor antigen-specific cytolytic cells can readily be elicited in patients with treatment history and large tumor burdens in a short period of time using autologous tumor cells decorated with CD80-SA as stimulators, additionally illustrating the potency of this approach.

**Immunization with Tumor Cells Decorated with CD80-SA Results in Protective Immunity in a Mouse Model of Cancer.** To assess the immunotherapeutic effectiveness of ProtEx in vivo, a mouse B-cell lymphoma line, A20, was used as a model system (7). This cell line expresses MHC class I, class II, and costimulatory molecule CD86 with low to undetectable level of the endogenous CD80 molecule (13). BALB/c test animals were challenged s.c. into the right flank with a lethal dose of A20 cells (1 x 10^6) followed by 1 x 10^5-irradiated cells decorated with CD80-SA, and tumor growth was followed for 8 weeks. All test animals that were immunized with A20 cells displaying CD80-SA were free of tumor growth within the observation period (Fig. 5A). In marked contrast, all of the mice immunized with irradiated unmanipulated or biotinylated A20 cells developed tumors within 18–20 days (Fig. 5, A and B). The tumor inhibition effect was specific to CD80-SA because all of the mice immunized with A20 cells decorated with a nonfunctional costimulatory molecule, human CD40L chimeric with core SA (SA-CD40L), developed tumors (Fig. 5, A and B). This tumor inhibition response was correlated with the generation of an antitumor cytolytic response. Animals immunized with CD80-SA-decorated tumor cells generated a potent cytolytic response to A20 cells (>50% specific killing; Fig. 5C). In marked contrast, the cytolytic response in tumor-bearing animals immunized with nonfunctional SA-CD40L-decorated tumor cells was ~10%, which was similar to the response generated by immunization with biotinylated cells treated with S2 control supernatant or by unmodified cells (Fig. 5C). Cytotoxic T cells generated in animals vaccinated with CD80-SA-decorated tumor cells were independent of costimulation for their effector function (Fig. 5D). These observations are consistent with previously published studies demonstrating that tumor cells genetically modified to express CD80 primarily generate a CD8+ T-cell response (3, 12, 31). Moreover, when tumor-free mice were rechallenged with live tumor cells 55 days after the initial immunotherapy, the majority of them (8 of 11) did not develop a tumor within the observation period of 110 days, demonstrating long-term immunological memory. These results show that tumor cells modified to display exogenous CD80-SA on their surface can effectively be used as an immunotherapeutic approach for the treatment of cancer.

**DISCUSSION**

Immunotherapy with tumor cells modified via gene transfer approaches to express immunostimulatory molecules has shown great promise in experimental and clinical settings (12). There are, however, a series of difficulties that remain to be overcome before the full potential of gene transfer-based immunotherapy is realized clinically. These difficulties include the refractory nature of some tumor cells to conventional gene transfer approaches and the associated low transfer efficiency and transgene expression level (18). Conventional gene transfer techniques are also elaborate, time consuming, and have biosafety concerns arising from the introduction of foreign genetic material into the patient (22). Therefore, a method that allows for the rapid and durable display of exogenous proteins on the cell surface within a short period of time may have important therapeutic implications, particularly in the area of cancer immunotherapy, and hence may offer a viable alternative to gene transfer approaches.

Herein, we report the use of a novel technology, ProtEx that permits the long-term display of exogenous proteins on the cell surface for cancer immunotherapy. This method involves biotinylation of cells and decoration with immunostimulatory proteins chimeric with core SA under physiological conditions without major toxicity to the manipulated cells. The procedure is efficient because 100% of the targeted cells display the chimeric protein on the surface and practical.

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**Fig. 4.** Primary tumor cells decorated with CD80-SA elicit potent tumor-specific response in autologous lymphocytes. Tumors from adenocarcinoma patients (PA, adenocarcinoma of the ovary) without treatment was resected, processed into a single-cell suspension, and decorated with CD80-SA. Cells were then irradiated (10,000 cGy) and used as stimulators for autologous PBLs harvested at the time of surgery in a 4-day MLTR culture (A). Undecorated tumor cells (none) and biotinylated cells treated with S2 culture supernatants (S2) served as negative controls. Tumor cells decorated with CD80-SA prime autologous PBLs for the generation of antitumor cytotoxic response (B). Lymphocytes from patient PA were stimulated with CD80-SA-decorated autologous primary tumor cells in the presence of IL-2 for 5 days (）。Effector cells were harvested and tested at various E:T ratios against autologous (）and third-party (）tumor cells in cytotoxic assays. Lymphocytes primed with unmodified (）or biotinylated cells decorated with S2 supernatant or CSA (）served as controls with minimal killing activity against autologous tumors. The killing response was antigen specific because there was minimal lysis of third-party OVCAR3 cells (）。Data are presented as percentages of specific killing for patient PA.
Two to 6 animals/group were tested, with similar results.

ified A20 cells were used for stimulation and as targets.

and comparable with those shown in (C).
The cytolytic responses generated were antigen specific
were used to test the antigen specificity of the response.

(C) or CD80-SA-decorated (B).

used (B). Immunization with CD80-SA-decorated tumor cells resulted in potent antitumor cytotoxicity (C).

Splenocytes were harvested from animals with and without tumors 55 days after tumor challenge and tested against tumor cells after a 5-day restimulation with irradiated A20 tumor cells in culture.

Data are presented as percentages of specific killing (C). The effector function of T cells does not depend upon CD80-SA-mediated costimulation (D). Splenocytes from vaccinated animals without tumor were stimulated with A20 decorated with CD80-SA for 5 days and used as effectors against A20 (□) or A20 decorated with CD80-SA (●). Unmodified (□) or CD80-SA-decorated (△) EL-4 lymphoma cells were used to test the antigen specificity of the response.

The powerful immunostimulatory efficacy of our approach may be attributable to the high levels of CD80-SA on the cell surface and/or aggregation into tetramers or higher order structures, leading to effective delivery of costimulatory signals. This notion is further consistent with our observation that immunotherapy with one dose of tumor cells decorated with CD80-SA is sufficient to eradicate the tumor in all of the mice challenged with a lethal dose of tumor cells.

Fig. 5. Immunization with CD80-SA-decorated tumor cells abrogates tumor growth. BALB/c mice were challenged s.c. with a dose of $1 \times 10^4$ live A20 syngeneic tumor cells (A). The animals were simultaneously immunized with a dose of $1 \times 10^5$-irradiated unmodified cells (□) or cells decorated with CD80-SA (△), nonfunctional SA-CD40L (○), or S2 supernatant (●). The animals were observed for s.c. growth of the tumor for 55 days.

Groups include none (n = 15), S2 (n = 9), CD80-SA (n = 15), and SA-CD40L (n = 7). The results are expressed as mean diameter in mm of tumors from groups of 9–18 mice each, except for SA-CD40L, where 7 animals were used (B).

Immunization with CD80-SA-decorated tumor cells resulted in potent antitumor cytotoxicity (C).

Splenocytes were harvested from animals with and without tumors 55 days after tumor challenge and tested against tumor cells after a 5-day restimulation with irradiated A20 tumor cells in culture. Data are presented as percentages of specific killing (C). The effector function of T cells does not depend upon CD80-SA-mediated costimulation (D).

The cell surface displays of exogenous proteins have previously been reported. These approaches included the display of proteins on the cell membrane via a glycosyl phosphatidylinositol moiety, a hydrophilic tail, or chemical derivatization with palmitic/immunodacetic acids (34–37). However, these approaches only resulted in short-term display of proteins ranging from 1 to 3 days in vitro and are not reported to have been tested in vivo. Their failure to cause long-term persistence may be attributable to the weak nature of chemical interactions involved in anchorage of proteins to the cell membrane in these systems.

Furthermore, some of these approaches are cumbersome (35) and others involve random integration of proteins into the cell membrane in a way that may interfere with anticipated functions (34).

Taken together, our ex vivo and in vivo data clearly demonstrate that tumor cells can be converted into potent APCs by displaying the CD80-SA protein on the cell surface. These results are consistent with a series of studies showing that tumor cells genetically manipulated to express CD80 serve as efficient APCs to generate antitumor responses (3, 7, 8, 19, 38–40).

Our results in samples from cancer patients, demonstrating that tumor cells decorated with CD80-SA generate potent antitumor responses in autologous lymphocytes from patients with large tumor burden and treatment history in a short period of time (5 days), are of great importance. Particularly, lymphocytes from patients with a large tumor burden or treatment history do not in general respond to autologous tumors ex vivo (8, 38, 39).

Stimulation with tumor cells genetically modified to express CD80 alone or in combination with other immunostimulatory molecules results in the generation of antitumor responses that reportedly can be measured only after several rounds of in vitro stimulation (8, 38, 39).

The powerful immunostimulatory efficacy of our approach may be attributable to the high levels of CD80-SA on the cell surface and/or aggregation into tetramers or higher order structures, leading to effective delivery of costimulatory signals. This notion is further consistent with our observation that immunotherapy with one dose of tumor cells decorated with CD80-SA is sufficient to eradicate the tumor in all of the mice challenged with a lethal dose of tumor cells.

This contention is consistent with recent findings that effective transduction of many key immunological signals requires cell surface aggregation of receptors and their ligands (26). However, additional experiments are needed to delineate the underlying mechanisms of immunotherapy using CD80-SA-decorated tumor cells in our model.

Ex vivo manipulation of primary tumor cells to achieve the display of exogenous immunostimulatory proteins on 100% of the targeted cells within a short period of time provides a great deal of procedural flexibility and offers great potential in the area of tumor immunotherapy. ProExs may also be used as a general immunomodulatory approach not only for up-regulating the immune response to fight cancer and infections but also for down-regulating the immune response to treat or prevent a series of immune-based disorders such as autoimmune diseases and foreign graft rejection. In support of this notion, we have recently shown that immunomodulation with allogeneic APCs displaying the extracellular domains of FasL death molecule resulted in the blockade of alloreactive immune responses, leading to the
prevention of islet allograft rejection (27). In conclusion, rapid and durable cell surface display of immunostimulatory proteins possesses the simplicity, safety, and efficacy required to make it a clinically relevant alternative that will accomplish the same end as gene transfer approaches in the treatment of a broad spectrum of immune-based disorders. This technology also provides a convenient and rapid means of displaying exogenous proteins on the cell membrane for fundamental, preclinical, and clinical research purposes.

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