Modifying Dendritic Cells via Protein Transfer for Antitumor Therapeutics
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Abstract

Purpose: The modification of therapeutic dendritic cells (DC) with various immunostimulatory molecules represents a useful means for improving the antitumor efficacy of DC transfer–based immunotherapy. We have evaluated the feasibility of modifying therapeutic DCs with multiple immunostimulatory molecules using a time-efficient, protein transfer (or protein “painting”)–based method.

Experimental Design: Bone marrow–derived DCs were painted with either control protein human IgG (hIgG) or three immunostimulatory molecules, SLC, 4-1BBL, and TRANCE (the triad protein). Painted DCs were injected intratumorally into mice bearing established tumors. Subsequently, the capacities of painted DCs to migrate to the draining lymph nodes, recruit the host T cells, promote Th1 cytokine responses, and elicit therapeutic antitumor responses were evaluated.

Results: The triad protein transfer yields a uniform population of DCs that coexpress all three of the proteins. Compared with the hIgG-painted DCs, the triad protein–painted DCs migrate more efficiently to the draining lymph nodes and show enhanced capabilities to induce T cell infiltration of tumors and to promote Th1 cytokine responses in vivo. Furthermore, in both the EG.7 and TRAMP-C2 tumor models, compared with the DCs painted with hIgG or only one of the three proteins, the triad protein–painted DCs, upon adoptive transfer, elicit stronger therapeutic responses against established tumors. Importantly, the antitumor responses of the triad protein–painted DCs are mediated by systemic antitumor immunity.

Conclusions: This study establishes, for the first time, the feasibility of optimizing DC transfer–based immunotherapy via combinatorial protein transfer of therapeutic DCs with an array of immunostimulatory molecules.

Because of their abilities to capture and process antigens, up-regulate the expression of costimulatory and MHC molecules upon maturation, migrate to secondary lymphoid organs, and prime naive T cells, dendritic cells (DC) are the most potent antigen-presenting cells (APC; ref. 1) and are thus considered a favorable type of therapeutic cell for cancer immunotherapy. Nonetheless, DC transfer–based immunotherapy has shown limited efficacy in patients. This highlights the need to continually enhance the immunogenicity of DCs in order to enhance the antitumor efficacy of DC transfer–based immunotherapy (2).

One way to enhance the immunogenicity of DCs is to improve the presentation of tumor-associated antigens by DCs. On this front, a variety of strategies have been developed, such as pulsing DCs with tumor-associated antigens in the form of mRNA, cDNA, peptide, recombinant proteins, tumor lysates, and tumor cell–DC hybrids (3, 4). In addition, DCs may be loaded with tumor-associated antigens chimerized with other more immunogenic molecules (5, 6). For example, the targeting signal of the lysosomal-associated membrane protein 1 has been fused to idiotypes from B-cell lymphoma, to promote the MHC II–mediated presentation of the antigens by DCs (6).

Alternatively, the immunogenicity of DCs may be enhanced by modifying DCs with immunostimulatory molecules such as cytokines, chemokines, and costimulatory molecules. To achieve this goal, investigators have primarily relied on gene transfection to neoexpress one, or at most, a few immunostimulatory molecules in the transfected DCs (7–9). However, gene transfer requires a lengthy period for selecting a relatively homogeneous population of transfectants, which can limit the use of ex vivo–expanded DCs as they quickly differentiate and lose viability in culture. This emphasizes the necessity to develop alternative, more time-efficient strategies for modifying DCs, prior to infusion, with immunostimulatory molecules, in order to optimize DC transfer–based immunotherapy.

Previously, we developed a protein transfer–based strategy for coexpressing multiple proteins on cells. In this method, protein A (a commercially available, soluble Staphylococcal protein), after being chemically derivatized with palmitate, is
first incorporated onto cell membranes; in turn, this membrane-anchored palmitated-protein A (PPA) serves as a “trap” for secondarily added Fcγ-derivatized costimulator (10). Moreover, we modified the method to deliver multiple costimulators intratumorally and generate antitumor vaccines directly in situ (11).

Built upon these previous studies, we here have coexpressed three molecules on the therapeutic DCs, i.e., the chemokine SLC, T cell costimulator 4-1BBL, and DC costimulator TRANCE (the triad protein) and evaluated the antitumor efficacy of the modified DCs. Our study establishes, for the first time, the feasibility of optimizing DC transfer–based immunotherapy via combinatorial protein transfer of therapeutic DCs with an array of immunostimulatory molecules.

**Materials and Methods**

**Mice and cell lines.** Female and male C57BL/6 mice (6-8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were maintained in a pathogen-free facility and used in accordance with the institutional guidelines for animal care. The EG.7 and L5178Y-R tumor cell lines were purchased from the American Type Culture Collection (Manassas, VA) and maintained according to the supplier’s recommendation. The TRAMP-C2 cell line was maintained as described (12).

**Antibodies.** FITC rat anti-mouse CD4 monoclonal antibody (mAb), APC rat anti-mouse CD11c mAb, rat anti-mouse CD40 mAb, PE goat anti-rat IgG, and FITC human IgG (hIgG) were purchased from BD Biosciences (San Diego, CA). Rat anti-mouse CD16/CD32 mAb (2.4G2), PE rat anti-mouse CD62L mAb (ME-14), APC rat anti-mouse CD80 mAb (53-6.7), FITC rat anti-mouse MHC II mAb, PE rat anti-mouse CD83 mAb (M-M1-17), PE rat anti-mouse 4-1BBL mAb (TKS-1), FITC rat anti-mouse CD80 mAb (1G10), PE or FITC rat anti-mouse CD86 mAb (GL1), PE rat anti-human TRANCE mAb, and various isotype controls were all purchased from eBioscience (San Diego, CA).

**Generation of murine bone marrow–derived DCs.** Bone marrow cell cultures were prepared from C57BL/6 mice (6-8 weeks old) as we described previously (13). Briefly, on day 0, 4 × 10^6 bone marrow cells were seeded per 100 mm dish in 4 mL of R10 medium. R10 medium is RPMI 1640 supplemented with 10% FCS, 2 mmol/L of l-glutamine, 50 µmol/L of 2-ME, 10 mmol/L of HEPES, 50 µg/mL of gentamicin, and spent medium from J558L cells secreting recombinant mouse granulocyte macrophage colony-stimulating factor (diluted at 1:30). On days 3 and 6, cells were fed with 4 mL of R10 medium. On day 7, bone marrow–derived DCs (BMDCs) were enriched by positive selection using magnetic microbeads conjugated with anti-mouse CD11c mAb (Miltenyi Biotec, Auburn, CA), following the manufacturer’s protocol; a purity of ~95% was usually achieved as assessed by immunostaining and flow cytometry.

**Recombinant Fc fusion proteins.** The production of human TRANCE Fc fusion protein (TRANCE-Fc) and murine 4-1BBL Fc fusion protein (4-1BBL-Fc) has been described previously (11, 13). The strategy for assembling chimeric expression cassettes encoding the murine SLC Fc fusion protein (SLC-Fc) mirrors that for human B7-1-Fc, which we have previously reported (10). Briefly, the COOH terminus of the coding sequence for the mature chain of murine SLC (S24-G133; Swiss-Prot accession number, P84444) was linked in-frame to a coding sequence for the Fcγ_2 domain of hIgG, within our expression construct pSLC-Fc/EE14. The fusion protein was produced by Chinese hamster ovary cell transfecants and purified by protein A-agarose chromatography, as we described previously (10). The functionality of recombinant SLC-Fc was verified by its chemotactic activity toward purified splenic CD4^+ T cells, as assessed by standard transwell assays.

**Flow cytometry.** Cell samples were first blocked with rat anti-mouse CD16/32 (1 µg/10^6 cells) and subsequently stained with mAb(s) according to the manufacturer’s instructions. Flow cytometry was done with a FACS Calibur (BD Biosciences). Live cells were identified and gated by the FSC/SSC scatter profiles and, if necessary, by staining with 7-amino-actinomycin D (7-AAD; Invitrogen, Carlsbad, CA). Data were analyzed using the CellQuestPro software (BD Biosciences).

**Protein transfer of BMDCs.** Procedures for generating PPA (14) and using it for Fc protein transfer (10, 11) have been described previously. Briefly, conjugates of PPA and each of the Fc fusion proteins or the control protein hIgG were first generated by combining the components at a 1:2 ratio (w/w) in DMEM and incubating the mixtures on ice for 30 min. Purified BMDCs were washed and resuspended in DMEM (10^7/mL), and protein transfer of BMDCs was accomplished by incubating 10^7 cells with 30 µg each of the preformed PPA/Fc fusion protein (or hIgG) conjugates at 37°C for 30 min in 1 mL of DMEM. To detect cell surface–associated 4-1BBL-Fc or TRANCE-Fc, cells were immunostained with PE-labeled rat anti-mouse 4-1BBL mAb (TKS-1) or rat anti-human TRANCE, respectively. Of note, rat and goat antibodies were chosen in this study because neither of them binds to protein A. Because neither rat nor goat antibodies were available for the detection of murine SLC, a biotinylated derivative of SLC-Fc was used in experiments aimed at monitoring protein transfer efficiencies. SLC-Fc was biotinylated with EZ-Link Sulfo-NHS-Biotin (Pierce Biotechnology, Rockford, IL), as per the manufacturer’s protocol, and was then detected with PE-streptavidin (BD Biosciences). Cells were then analyzed by flow cytometry.

**Carboxyfluorescein diacetate succinimidyl ester labeling.** Cells were thoroughly washed with and resuspended (at 10^7 cells/mL) in PBS containing Ca^2+ and Mg^2+. Carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) was added to cells at 1 µmol/L. The reaction was incubated for 6 min at room temperature in the dark and terminated by the addition of half the volume of FCS.

**Quantitation of DCs and T cells infiltrating tumors or the tumor-draining lymph nodes.** EG.7 tumors were established by intradermally injecting 1 × 10^6 tumor cells (in 50 µL of DMEM) into the back flanks of 6- to 8-week-old female C57BL/6 mice (day 0). On day 7, 1.25 × 10^5 syngeneic BMDCs, painted with either the control hIgG or Fc derivatives of SLC, 4-1BBL, and TRANCE (the triad protein), were injected intratumorally. At different time points after the injection, tumors and draining lymph nodes (DLN) were excised, cut into small pieces, and digested for 45 min at 37°C with RPMI 1640/10% FCS/200 µg/mL type IV collagenase (Sigma-Aldrich Corp., St. Louis, MO). The tissues were mechanically disrupted, and erythrocytes were depleted. The cell suspensions were each passed through a cell strainer (40 µm). DCs in each suspension were quantified by immunostaining with APC anti-CD11c; to further quantify mature/activated DCs, cells were triple-immunostained with APC anti-CD11c, FITC anti-CD80, and PE anti-CD83. CD4^+ and CD8^+ T cells in each suspension were quantified by immunostaining with FITC anti-CD4 or APC anti-CD8, respectively; to further quantify activated T cells, cells were double-stained with PE anti-CD62L and FITC anti-CD4 or APC anti-CD8. Cells were then analyzed by flow cytometry. DCs and T cells were each identified by gating on the FSC/SSC scatter profiles. For each sample, 10^6 total cell events were analyzed.

**Therapy using Fc protein–transferred BMDCs.** For DC therapy in the EG.7 tumor model, tumors were established (five mice for each treatment group), as described above (day 0). On days 7, 10, and 13, 1.25 × 10^5 syngeneic BMDCs (in 50 µL DMEM/0.1% bovine serum albumin), painted with either hIgG or Fc fusion protein(s), were injected intratumorally. Cured mice were rechallenged with 1 × 10^6 EG.7 tumor cells (i.p. injection), 2 to 3 months after complete tumor regression. For DC therapy in the TRAMP-C2 tumor model, tumors were established (six mice for each treatment group) by injecting (s.c.) 0.5 × 10^6 TRAMP-C2 cells (in 50 µL of DMEM) into 6- to 8-week-old male C57BL/6 mice (day 0). On days 7, 14, and 21, 1 × 10^6 syngeneic BMDCs (in 50 µL DMEM/0.1% BSA), painted with hIgG or the triad protein, were injected intratumorally. Cured mice were rechallenged with 1 × 10^6 TRAMP-C2 tumor cells (i.p. injection), 3 months after
complete tumor regression. All of the mice were monitored daily, and tumor sizes were measured once or twice per week with a caliper. Mice were euthanized when they became moribund or when their tumors exceeded 400 mm² in size.

**Cytokine determination from tumor nodules, DLNs, and spleens.** For the study in the EG.7 tumor model, the cytokine profiles in tumors and DLNs were determined in mice treated with DCs painted with hIgG or the triad protein. On days 1, 3, 5, and 7 post-DC injection, tumors and DLNs were excised, cut into small pieces, digested, and homogenized. The homogenates were assayed for the production of interleukin 2 (IL-2), tumor necrosis factor α (TNF-α), IFN-γ, IL-4, and IL-5 by flow cytometry using a Cytometric Bead Array kit for Th1/Th2 cytokines and normalized against concentrations of total proteins. For the study in the TRAMP-C2 tumor model, the cytokine profiles after secondary stimulation with the tumor cells were determined. Bulk splenocytes were obtained from the mice cured by the triad protein–painted DCs, 2 to 4 weeks after the rechallenge. Splenocytes were cocultured with mitomycin C–treated, CFSE-labeled EG.7 tumor cells in a 24-well plate at 1:10 ratio (2 × 10⁶ total cells/mL) for 5 days. Subsequently, viable cells were harvested and used as effectors in CTL assays, as we described (15). Briefly, effector splenocytes were mixed with mitomycin C–treated, CFSE-labeled EG.7 or TRAMP-C2 (specific target) or L5178Y-R (nonspecific) tumor cells at different effector/target ratios. After 12 h, the cultures were stained with 7-AAD and analyzed by flow cytometry. Lysed target cells were identified as the CFSE−/7-AAD− population. 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 the percentage of 7-AAD− target cells in the absence of effector cells (spontaneous lysis).

**Statistical analysis.** Student’s t-test was used for pairwise comparison. The difference is deemed statistically significant if P < 0.05.

**Results**

**DCs can be efficiently modified by protein transfer.** We first determined whether DCs can be modified efficiently with multiple immunostimulatory molecules via the protein transfer method we previously described (10, 11). To this end, murine BMDCs were incubated with a mixture of the FcγI, derivatives of SLC, 4-1BBL, and TRANCE, each preconjugated with PPA. These three proteins (the triad protein) were chosen for their documented immunostimulatory activities. SLC is a chemokine capable of modulating DC–T cell interactions by attracting and colocalizing both cell types (16). TRANCE (RANK ligand) is a known DC maturation and survival factor (17, 18). 4-1BBL is known for its ability to costimulate T cell responses, especially CD8+ T cell responses (19). Of note, the expression of the triad protein was not meant for deciphering the role of each of the three proteins in enhancing the efficacy of DCs, but rather for a robust demonstration of combinatorial protein transfer of therapeutic DCs.

Subsequently, we analyzed the coexpression of all three proteins on the painted DCs by flow cytometry. Significantly, the triad protein transfer yielded a nearly uniform population (98-100%) of DCs that coexpress all three of the proteins (Fig. 1A). As a control protein, FITC-conjugated hIgG was also efficiently painted on DCs (data not shown). Next, we determined the duration of the proteins painted on DC surfaces in vivo, using SLC as a representative. To this end, CFSE-labeled DCs, painted with hIgG or biotinylated SLC-Fc, were injected intratumorally into mice bearing established EG.7 tumors. Over a period of 60 h postinjection, the surface expression of SLC-Fc on injected DCs was assessed by flow cytometry. After 36 h, a significant level of SLC was still detected on the surfaces of painted DCs (Fig. 1B). Given that a few hours to 1 day is sufficient for DCs to prime naive T cells (20) and that DCs themselves are short-lived in vivo (21), the painted proteins can persist over a significant portion of the life span of the injected DCs. Thus, these results establish the feasibility of protein transfer of DCs.

**The triad protein–painted DCs show improved ability to migrate to the tumor-draining lymph nodes (DLNs).** To begin to determine the effect of protein painting on the function of DCs, we first examined the migration of intratumorally injected DCs to the DLNs. To that end, CFSE-labeled DCs, painted with hIgG or the triad protein, were injected intratumorally. After 3 days, injected DCs (CD11c+/CFSE−) present in the DLNs were quantified. Consistent with the previous results from others (22–24), only a small portion (<4%) of the injected DCs reached the DLNs. Nonetheless, when compared with the mice injected with the hIgG-painted DCs, mice injected with the triad protein–painted DCs had significantly more injected DCs in the DLNs (Fig. 2A). The results suggest that the triad protein painting improves the ability of the painted DCs to migrate to the DLNs.

Of note, a majority (>90%) of the triad protein–painted DCs were initially (i.e., prior to injection) of an immature phenotype, as indicated by their low expression of the maturation
The triad protein–painted DCs show enhanced activity to induce infiltration/activation of T cells in vivo. In parallel to the influx of these mature DCs into the DLNs, the triad protein–painted DCs also caused an increase in the percentages of activated CD4+ and CD8+ T cells in the DLNs (Fig. 3A). Of note, we did not observe a significant difference in the absolute numbers of CD4+ and CD8+ T cells between mice treated with the hlgG-painted DCs and mice treated with the triad protein–painted DCs. This is not surprising given that lymph nodes are largely constituted by lymphocytes. Nonetheless, 3 to 5 days postinjection, mice treated with the triad protein–painted DCs had significantly higher percentages of activated CD4+ and CD8+ T cells in the DLNs than those treated with the hlgG-painted DCs. The results point to the association of the improved migration to the DLNs of the triad protein–painted DCs with the increased priming/activation of T cells in the DLNs.

In addition, we determined whether the triad protein painting could increase the ability of DCs to induce T cell infiltration of tumors, which signifies increased immune responses against the tumors. Of note, the majority of the tumor-infiltrating CD4+ and CD8+ T cells showed activated phenotypes (CD62Llow), regardless of whether DCs were painted with hlgG (data not shown) or the triad protein (Fig. 3B). This implies that under our experimental conditions, tumor-infiltrating T cells might have been activated before infiltration or promptly become activated after infiltration. Nonetheless, when compared with tumors injected with the hlgG-painted DCs, tumors injected with the triad protein–painted DCs showed significant increase in the absolute number of T cells, especially CD8+ T cells (Fig. 3C). This difference was particularly evident on day 5, with the CD8+ T cells being increased by ~4-fold. In summary, the results that the triad-painted DCs increase the absolute quantities of tumor-infiltrating T cells, and that the majority of tumor-infiltrating T cells are of activated phenotypes, point to the that the triad-painted DCs cause an overall increase in activated T cells in the tumor microenvironment, which is indicative of enhanced antitumor responses.

The triad protein–painted DCs promote the release of Th1 cytokines. Next, we determined the effect of the triad protein painting of DCs on the release of Th1 cytokines in vivo. It is generally accepted that a Th1 cytokine milieu favors the generation of cell-mediated antitumor response. Especially, IFN-γ and TNF-α have been shown to play a role in tumor regression mediated by CD8+ T cells (25). To that end, we injected the triad protein–painted DCs intratumorally, and analyzed the production of IFN-γ and TNF-α and the change in the ratio of Th1 to Th2 cytokines within the tumors and DLNs. On days 3 and 5 postinjection, the mice treated with the triad protein–painted DCs had higher levels of IFN-γ and TNF-α at both the tumor site and the DLNs than those treated with the hlgG-painted DCs (Fig. 4). On the other hand, the levels of Th2 cytokines (such as IL-4 and IL-5) were low in treated tumors, regardless of whether DCs were painted with the triad protein or hlgG (data not shown). Thus, the triad protein painting leads to an increase in the ratio of Th1 to Th2 cytokines and, thus, stronger Th1 responses in vivo.

The triad protein–painted DCs elicit enhanced responses against established EG.7 lymphoma, which are mediated by persistent, systemic antitumor immunity. Having determined...
the effects of the triad protein painting on DC function in vivo, we next assessed the therapeutic antitumor efficacy of the triad protein–painted DCs against established EG.7 tumors. As shown in Fig. 5A and B, the triad protein–painted DCs elicited strong antitumor responses, resulting in complete tumor regression in 60% to 100% of treated mice, whereas untreated mice or mice treated with the hlgG-painted DCs all died from tumors. Of note, DCs painted with only one of the three proteins showed partially enhanced antitumor efficacy and delayed the growth of tumors; particularly, TRANCE– or 4-1BBL–painted DCs yielded complete tumor regression in ~20% of treated mice. These results point to an additive or synergistic effect as a result of the triad protein combination, as we initially anticipated.

Having documented the local tumor regression in a substantial percentage of mice treated with the triad protein–painted DCs, we next determined the existence of systemic antitumor immunity. To this end, cured mice were rechallenged with the EG.7 tumor cells injected at sites (i.p.) distant from the original tumor, 2 to 3 months after the local tumor regression. As shown in Fig. 5C, all of the mice cured by the triad protein–painted DCs were resistant to the rechallenge, whereas naïve mice inoculated with the same tumor cells all died of tumor. Moreover, 4 to 6 weeks after tumor rechallenge, we recovered bulk splenocytes from the mice cured by the triad protein–painted DCs and looked for specific CTL activity. The splenocytes, upon restimulation with the EG.7 tumor cells, showed strong CTL activity against the EG.7 tumor cells. Of note, the freshly isolated splenocytes did not elicit detectable CTL response (data not shown). Significantly, the CTL activity was EG.7 tumor cell–specific, as the splenocytes showed significantly lower CTL activity to the control LS178Y-R tumor cells (Fig. 5D). Furthermore, spleen cells from naïve mice failed to develop any CTL activity against EG.7 tumor cells during the in vitro restimulation phase, indicating that the CTL response is dependent on an immunizing effect resulting from previous treatment of the tumor. Together, these results point to a persistent, systemic antitumor immunity that is invoked by intratumoral injection of the triad protein–painted DCs.

**The triad protein–painted DCs elicit stronger antitumor responses against established TRAMP-C2 prostate tumors.** Finally, we wanted to evaluate the therapeutic efficacy of the triad protein–painted DCs in a clinically relevant tumor model.

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**Fig. 3.** Injection of the triad protein–painted DCs leads to enhanced T cell infiltration of the DLNs and tumors. A, BMDCs painted with hlgG or the triad protein were injected into EG.7 tumors. At the indicated time points postinjection, single cell suspensions were each prepared from the DLNs and quantified for activated CD4+ and CD8+ T cells by immunostaining and flow cytometry. Points, mean of six DLNs from three experiments; bars, SD. The difference in percentages of activated T cells (identified as CD62L+ populations) between mice injected with the hlgG-painted DCs and those with the triad protein–painted DCs is statistically significant (**P < 0.01; ***P < 0.001). B, 3 d postinjection, single cell suspensions were prepared from tumors treated with the triad protein–painted DCs. Cells were immunostained for CD62L and CD4 or CD8, and analyzed by flow cytometry. The majority of CD4+ and CD8+ T cells were of activated phenotypes and identified as CD62L+ populations. C, at indicated time points post-DC injection, single cell suspensions were prepared from excised tumors. Tumor-infiltrating CD4+ T cells and CD8+ T cells were quantified by staining for CD4 and CD8, respectively, and analyzing by flow cytometry. Lymphocytes were identified by gating on the FSC/SSC scatter profiles. The number of specified cells in 1 × 10^6 total cell events are shown. Columns, mean of six tumor samples from three experiments; bars, SD. The difference in the number of tumor-infiltrating T cells between mice injected with the hlgG-painted DCs and those with the triad protein–painted DCs is statistically significant (**P < 0.01; Similar results were obtained in three experiments. [Image reference: http://www.aacrjournals.org/clinicanimmunology]
For that purpose, we chose the TRAMP-C2 prostate tumor model (12, 26). Again, the triad protein–painted DCs showed strong antitumor efficacy against established TRAMP-C2 tumors. As shown in Fig. 6A, the hIgG-painted DCs caused complete tumor regression in ~33% of the treated mice, whereas the triad protein–painted DCs did so in ~83% of the treated mice. Importantly, all the mice cured by the triad protein–painted DCs rejected rechallenge with the TRAMP-C2 tumor cells injected at sites (i.p.) distant from the original tumor, 2 to 3 months after tumor regression (Fig. 6B). Furthermore, the bulk splenocytes isolated from the rechallenged mice, upon restimulation by the TRAMP-C2 tumor cells, produced high levels of Th1 cytokines IL-2, IFN-γ, and TNF-α, upon in vitro restimulation with the TRAMP-C2 tumor cells (Fig. 6C). The splenocytes also showed strong CTL activity against the TRAMP-C2 tumor cells (Fig. 6D). Of note, freshly isolated splenocytes did not show significant CTL and cytokine responses (data not shown). Therefore, consistent with the observations made in the EG.7 lymphoma model, the results from the TRAMP-C2 tumor model also point to enhanced therapeutic responses elicited by the triad protein–painted DCs that are mediated by a persistent, systemic antitumor immunity.

Discussion

In this study, we have shown that compared with DCs painted with hIgG, DCs painted with three immunostimulatory molecules (SLC, 4-1BBL, and TRANCE) show enhanced capacities to migrate to the DLNs, induce T cell infiltration of tumors, and promote Th1 cytokine responses in vivo. More importantly, we have shown in two different tumor models that such DCs can elicit stronger therapeutic responses against established tumors. Thus, our study establishes, for the first time, the feasibility of improving the immunogenicity of therapeutic DCs, immediately prior to adoptive transfer, via simple and time-efficient protein transfer.

In choosing immunostimulatory proteins for DC modification, we have considered some of the limitations associated with ex vivo–expanded DCs. First, the majority of such DCs are unable to migrate from the injection site to the regional lymph nodes (27, 28), where the encounters between the DCs and T cells occur; this directly reduces the ability of the DCs to prime T cells. We used TRANCE, also called DC survival factor, to bypass this problem, because we and others have shown that TRANCE can promote DC migration to regional lymph nodes, likely via enhancing the longevity of DCs and/or counteracting FasL-induced DC apoptosis (13, 17). Second, to improve the interaction between intratumorally injected DCs and endogenous T cells in the DLNs, we also painted the DCs with SLC, a CC chemokine capable of attracting and colocalizing DCs and naïve T cells via the CCR7 receptor on both DCs and naïve T cells (16). Last, even though ex vivo–expanded DCs naturally express T cell costimulators required for priming T cells, it is unclear whether the levels of the expression are sufficient to prime, rather than tolerize, antitumor T cells. Given that antitumor T cells often have low affinities for tumor antigens (29, 30), it is conceivable that these T cells may require strong costimulation. We intended to bypass this problem by painting DCs with 4-1BBL, a potent costimulator that plays an essential role in T cell–mediated immunity.

Most immunotherapies exploit DCs matured ex vivo, because mature DCs are generally considered more immunogenic than immature DCs (1, 31). Nonetheless, under certain conditions, DCs matured ex vivo have been shown to preferentially induce Th2 responses or tolerize the cognate T cells (32–38); in addition, fully differentiated or mature DCs have been shown to be short-lived in vivo (39), which reduces their ability to prime T cells. In an attempt to bypass this dilemma, we decided to use immature DCs and modify them with TRANCE, which can stimulate DC maturation. The rationale here is to allow the immature DC to uptake tumor antigens in situ and differentiate into mature DCs under the influence of TRANCE. Nonetheless, more experiments are needed to determine whether this is indeed the case.

Fig. 4. The triad protein–painted DCs promote the release of Th1 cytokines. BMDCs painted with hIgG or the triad protein were injected into EG.7 tumors. On days 3 and 5 postinjection, the tumors (A) and DLNs (B) were harvested and homogenized. The homogenates were quantified for TNF-α and IFN-γ by flow cytometry and normalized against concentrations of total proteins. Columns, mean of six samples from three experiments; bars, SD. The difference in cytokine release between mice injected with the hIgG-painted DCs and those with the triad protein–painted DCs is statistically significant (*, P < 0.05; **, P < 0.01).
Fig. 5. The triad protein–painted DCs elicit enhanced responses against established EG.7 lymphoma. A, EG.7 tumors were established intradermally (day 0). On days 7, 10, and 13, 1.25 × 10⁵ BMDCs painted with the indicated protein(s) were injected intratumorally. Tumor sizes were measured and graphed for individual animals (○), tumor size of a single animal. B, the percentage of survival (of total animals in each group) was graphed. Similar results were obtained from three experiments.

C, 2 to 3 mo after tumor regression, a lethal dose (1 × 10⁶) of EG.7 tumor cells were inoculated i.p. (five mice per group) into mice cured by the triad protein–painted BMDCs (○), or naive mice (●). Mice were monitored weekly for the appearance of tumor. D, bulk splenocytes, prepared from the control naive mice or mice cured by the triad protein–painted BMDCs 3 to 6 wks after the rechallenge, were restimulated with mitomycin C–treated EG.7 tumor cells. After 5 d, the splenocytes (effector) were mixed with CFSE-labeled, mitomycin C–treated EG.7 (●, specific target) or L5178Y-R (○, nonspecific target) tumor cells at indicated effector/target (E:T) ratios. After 12 h, CTL activity was assessed by 7-AAD staining and flow cytometry; dead target cells were identified as CFSE+/7-AAD+. As a negative control, splenocytes from naive mice were assayed for CTL activity against the EG.7 tumor cells (○). Similar results were obtained from three experiments.
Protein transfer is particularly well-suited for engineering DCs not only because it provides simplicity in delivering multiple proteins onto the same cell surfaces, but also because it is relatively safe. Particularly, it permits temporary modification of immune cells, as 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 effects, such as the induction of autoimmunity. Significantly, because the initial establishment of the “proof-of-principle” of protein transfer, achieved via the GPI modification (40–42), several protein transfer methods, including the method used in this study, have been developed (43, 44). These protein transfer methods and other cell-modifying methods, combined with the expanding repertoire of immunostimulatory molecules, now provide an enlarging set of options for engineering DCs and other immune cells for treating cancer and autoimmune diseases as well.

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References

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