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Enhanced Antitumor Responses Elicited by Combinatorial Protein Transfer of Chemotactic and Costimulatory Molecules[1]

Shanrong Liu,*† Deborah R. Breiter,‡ Guoxing Zheng,[2]* and Aoshuang Chen[2]*

Thus far, immunotherapies based on one or a few immunostimulatory molecules have shown limited antitumor efficacy. This highlights the need to use multiple immunostimulatory molecules, to target different immune cells, including immunosuppressive cells, simultaneously. Consequently, in this study, we delivered intratumorally via protein transfer four molecules, including the chemotactic molecules secondary lymphoid tissue chemokine and Fas ligand and the costimulatory molecules 4-1BBL and TNF-related activation-induced cytokine. Secondary lymphoid tissue chemokine and Fas ligand together can attract an array of immune cells and induce apoptosis in $CD4^+CD25^+$ regulatory T cells (Treg), whereas 4-1BBL and TRANCE together can stimulate T cells and dendritic cells (DCs). We show that the transfer of all four molecules increases tumor-infiltrating neutrophils, DCs, and $CD4^+$ and $CD8^+$ T cells and decreases intratumoral Treg. We show that the treatment favors the generation of a Th1 cytokine milieu at the tumor site, which is attributed not only to an increase in IL-12-producting DCs and IFN-γ-producing $CD8^+$ T cells, but also to a decrease in IL-10-producing Treg. Importantly, in the L5178Y lymphoma model, we show that compared with transfer of the chemotactic molecules alone or the costimulatory molecules alone, transfer of all four molecules demonstrates stronger antitumor responses against established tumors. Furthermore, we show that the antitumor responses elicited by transfer of all four molecules are mediated by long-term, systemic antitumor immunity. Hence, this study demonstrates for the first time that combinatorial use of chemotactic and costimulatory molecules provides a useful strategy for enhancing antitumor responses. ***The Journal of Immunology,*** **2007, 178: 3301–3306.**

A major goal of cancer immunotherapy, such as adoptive transfer, Ab therapy, and adjuvant-based therapy, is to harness the immune system to enhance immune responses against tumor cells (1). To achieve this goal, one approach is to use immunostimulatory molecules to increase the functional capacity of immune cells. For example, costimulators and cytokines have been used to promote the activation, expansion, effector function, and/or survival of immune cells; whereas chemokines have been used to promote the migration and recruitment of the cells (2). An alternative approach to enhancing antitumor responses is to mitigate negative checkpoint signals that limit immune responses. For example, coinhibitory signals, such as those via the CTLA-4 (3) and PD1 axes (4), and immunosuppressive cells, such as the $CD4^+CD25^+$ regulatory T cells (Treg)[3] (5, 6), have been targeted to "release the brakes" on antitumor immunity.

Each of these strategies, however, has shown limited efficacy when used alone. Thus, it is conceivable that a treatment modality combining various strategies may lead to better outcome. One approach would be to apply multiple immunostimulatory molecules and target different immune cells simultaneously. To date, to enhance antitumor responses, many investigators have exploited gene transfection as a primary means to neo-express one or a few immunostimulatory molecules in transfected tumor cells or immune cells (2, 7, 8). Nonetheless, from the clinical standpoint, gene transfer has limitations, including toxicity, limited control of the levels of protein expression, and difficulty in expressing more than one protein at a time. Protein transfer (or protein "painting"), which enables simultaneous delivery of well-defined amounts of multiple proteins to a cell, provides a valuable alternative means to gene transfer (9, 10).

Previously, we developed a simple, straightforward protein transfer method. 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 Fcγ1-derivatized immunostimulatory proteins (11). Moreover, we showed that multiple costimulator-Fc fusion proteins, after being preconjugated with PPA in vitro, can be directly injected into a tumor, thereby generating cancer vaccines in situ (12).

Based on these previous results, in the present study, we aim to enhance antitumor responses by applying intratumorally, via protein transfer, four immunostimulatory molecules that together may affect a selected spectrum of immune cells. Specifically, we combine two chemotactic molecules, namely, secondary lymphoid tissue chemokine (SLC) and Fas ligand (FasL), with two costimulatory molecules, namely, 4-1BBL and TNF-related activation-induced cytokine (TRANCE). SLC attracts naive T cells and dendritic cells (DCs) (13), whereas FasL

*Department of Biomedical Sciences, University of Illinois College of Medicine at Rockford, IL 61107; †Department of Histology and Embryology, Second Military Medical University, Shanghai, People's Republic of China; and ‡Department of Biochemistry and Chemistry, Rockford College, Rockford, IL 61107

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[2] Address correspondence and reprint requests to Drs. Aoshuang Chen or Guoxing Zheng, Department of Biomedical Sciences, University of Illinois College of Medicine at Rockford, 1601 Parkview Avenue, Rockford, IL 61107. E-mail addresses: aoshuang@uic.edu and guoxingz@uic.edu

[3] Abbreviations used in this paper: Treg, regulatory T cell; PPA, palmitated protein A; SLC, secondary lymphoid tissue chemokine; FasL, Fas ligand; TRANCE, TNF-related activation-induced cytokine; DC, dendritic cell; Foxp3, Foxhead box P3; 7-AAD, 7-aminoactinomycin D.

has been shown to mediate proinflammatory, antitumor responses via attracting neutrophils (14–16). 4-1BBL is a potent T cell costimulator (17), whereas TRANCE is a potent DC costimulator that functions as a "DC survival factor" (18, 19). Of note, in light of recent findings that FasL effectively induce apoptosis in Treg (20), and that Treg increase or preferentially accumulate within tumors (21–23), the inclusion of FasL may also enhance antitumor responses via depleting intratumoral Treg. Our results show that combined use of the four molecules enhances the recruitment of various immune cells, decreases Treg, and increases Th1 cytokine responses at the tumor site. More importantly, we show that the therapy using the four molecules demonstrates stronger therapeutic efficacy than control therapy using either the chemotactic or the costimulatory molecules.

Materials and Methods

Mice and cell lines

Female DBA/2J mice (6–8 wk of age) were purchased from The Jackson Laboratory and maintained and used in accordance with the institutional guidelines for animal care. L5178Y and EG.7 tumor cell lines were purchased from American Type Culture Collection and maintained as per the supplier's recommendation.

Antibodies

Anti-mouse CD16/CD32 (2.4G2), anti-mouse CD8-allophycocyanin (53-6.7), anti-mouse Gr-1-PE (RB6-8C5), anti-mouse CD4-FITC (GK1.5), anti-mouse CD25-PE (7D4), anti-mouse Foxp3-PE (JFK-16S), anti-human FasL (NOK-1), anti-mouse CD11c-allophycocyanin, anti-rat IgG-PE, human IgG-FITC, and various isotype controls were purchased from *e*Bioscience.

Recombinant Fc fusion proteins

The production of human TRANCE Fc fusion protein (TRANCE-Fc) (12, 24) and murine 4-1BBL Fc fusion protein (4-1BBL-Fc) (12) has been described previously. The strategy for assembling chimeric expression cassettes encoding the murine SLC Fc fusion protein (SLC-Fc) mirrors that for human B7-1-Fc we previously reported (11). Briefly, the C 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 Fcγ1 domain of human IgG1 within our expression construct pSLC-Fc/EE14. The fusion protein was produced by a Chinese hamster ovary cell transfectant and purified by protein A-agarose chromatography as we described previously (11). The functionality of recombinant SLC-Fc was verified by its chemotactic activity toward purified splenic $CD4^+$ T cells as assessed by the standard Transwell assay.

Intratumoral protein transfer

Procedures for generating PPA (25) and using it for Fc protein transfer (11, 12) have been described previously. Briefly, conjugates of PPA and each of the Fc fusion proteins 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. Mixtures of the conjugates were injected intratumorally (in 50 μl of DMEM).

Flow cytometry

Cells were first incubated with anti-mouse CD16/32 (1 μg/10^6 cells) and subsequently stained with mAb(s) as per the manufacturer's instructions. Flow cytometry was performed with a FACSCalibur (BD Biosciences). Live cells were identified and gated by forward scatter/side scatter profiles and, if necessary, by staining with 7-aminoactinomycin D (7-AAD; Invitrogen Life Technologies). Data were analyzed using the CellQuest Pro software (BD Biosciences).

Quantitation of tumor-infiltrating DCs and T cells

L5178Y tumors were established by injecting intradermally 0.5×10^6 tumor cells (in 50 μl of DMEM) into the flank of DBA/2J mice (day 0). From day 4 on, protein transfers were performed intratumorally as indicated in Table I. On day 8, tumors were each 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). Tissues were mechanically disrupted, and erythrocytes were depleted. DCs were quantified by immunostaining with anti-CD11c-allophycocyanin; neutrophils, anti-Gr-1-PE; $CD4^+$ T cells, anti-CD4-FITC; $CD8^+$ T cells, anti-CD8-allophycocyanin; and Treg, anti-CD4-FITC, and anti-CD25-PE or anti-Foxp3-PE. Cells were then analyzed by flow cytometry. For each tumor sample, numbers of various immune cells in 1×10^6 total tumor cells were each determined.

Table I. *Protein transfers of chemotactic and/or costimulatory molecules*

Group	Days 4 and 5	Days 6 and 7
PPA	PPA	PPA
FasL/SLC	FasL/SLC	PPA
4-1BBL/TRANCE	4-1BBL/TRANCE	PPA
FasL/SLC → 4-1BBL/TRANCE	FasL/SLC	4-1BBL/TRANCE

Determination of cytokines

To quantify cytokines in tumor nodules, after protein transfer, tumors were excised, digested, and homogenized. IL-12 and IL-10 in homogenized tumor tissues were each quantified by ELISA using commercial kits (Pierce) and normalized against total proteins and IFN-γ by flow cytometry using a cytometric bead array kit (BD Biosciences). To quantify intracellular cytokines produced by immune cells within tumors, single cells prepared from treated tumors were cultured (5×10^6 cells/ml) for 24 h with 5 μg/ml Con A (Sigma-Aldrich) and 3 μM monensin (eBioscience). Cells were single- or double-stained with anti-CD11c-PE-Cy5, anti-CD4-FITC, and anti-CD25-PE-Cy5, or anti-CD4-FITC and anti-CD8-PE-Cy5. Cells were fixed in 4% paraformaldehyde/Dulbecco's PBS and permeabilized using a commercial kit (BD Biosciences). Cells were then stained with anti-IL-12-PE, anti-IFN-γ-allophycocyanin, or anti-IL-10-allophycocyanin and analyzed by flow cytometry.

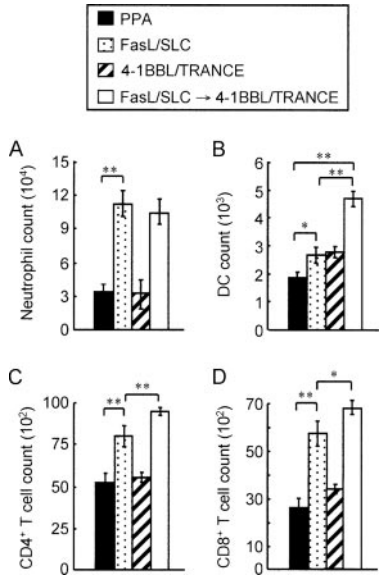

FIGURE 1. Intratumoral transfer of chemotactic and/or costimulatory molecules leads to increase in intratumoral infiltration of immune cells. L5178Y tumors were established in DBA/2 mice (day 0). Protein transfers were performed intratumorally as indicated in Table I. On day 8, single-cell suspensions were each prepared from excised tumors. Tumor-infiltrating neutrophils (*A*), DCs (*B*), $CD4^+$ T cells (*C*), and $CD8^+$ T cells (*D*) were quantified by immunostaining for Gr-1, CD11c, CD4, and CD8, respectively, and flow cytometry. The number of specified cells in 1×10^6 total cells is shown. Each bar represents the mean ± SD of six tumor samples from three independent experiments. The difference in the number of tumor-infiltrating cells between different groups is statistically significant (**, $p < 0.01$; *, $p < 0.05$).

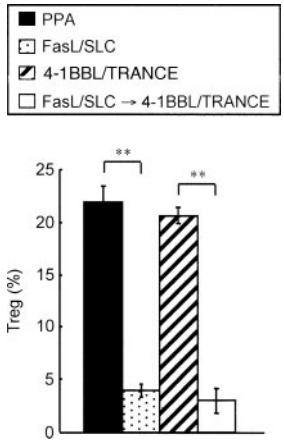

FIGURE 2. Intratumoral transfer of FasL leads to a decrease in intratumoral Treg. Protein transfers were performed, and single-cell suspensions were prepared as described in Fig. 1. Intratumoral Treg were quantified by immunostaining for CD4, CD25, and Foxp3 and flow cytometry. The percentage of Treg (in total $CD4^+$ T cells) is shown. Each bar represents the mean ± SD of six tumor samples from three independent experiments. The difference in the number of intratumoral Treg between different groups is statistically significant (**, $p < 0.01$).

Therapy experiments in the L5178Y lymphoma model

L5178Y tumors were established in DBA/2J mice as described above (day 0). From day 4 on, protein transfers were performed (once daily) intratumorally as indicated in Table I. Subsequently, tumor size was measured twice weekly. Mice were euthanized when they became moribund or when the tumors exceeded 400 mm^2. Cured mice were each rechallenged (i.p.) with 5×10^5 L5178Y tumor cells at least 8 wk after the initial tumor inoculation.

CTL assay

Bulk splenocytes from cured mice, prepared 4–6 wk after tumor rechallenge, were restimulated with mitomycin C-treated tumor cells at a 10:1 ratio for 5 days. Viable cells were used as effectors in the CTL assay as we described elsewhere (26). Briefly, effector cells were cultured with CFSE-labeled, mitomycin C-treated tumor target cells. After 12 h, the cultures were stained with 7-AAD and analyzed for dead target cells ($CFSE^+$/$7\text{-}AAD^+$) by flow cytometry. Specific lysis was calculated as we described previously (26).

Results

Intratumoral protein transfer promotes the infiltration of neutrophils, DCs, and $CD4^+$ and $CD8^+$ T cells at the tumor site

To enhance antitumor responses, tumors were protein transferred first with the chemotactic molecules SLC and FasL and, subsequently, with the costimulators 4-1BBL and TRANCE. The rationale here was to first modulate the tumor microenvironment by increasing the infiltration of neutrophils, DCs, and T cells and subsequently to increase the functional capacity of tumor-infiltrating immune cells, DCs and T cells in particular. As controls, tumors were also protein transferred only with the chemotactic molecules or the costimulators. The protein transfer schemes are summarized in Table I. Of note, we also tested various other schemes, such as cotransferring all four proteins at the same time, or transferring FasL first and then SLC/4-1BBL/TRANCE (data not shown). The schemes in Table I yielded most consistent results and were, thus, used throughout the present study.

Given that tumors, inevitably, varied in size at the time of harvest for assessment of infiltration by immune cells, we quantified immune cells in 1×10^6 total tumor cells to normalize the results from different tumor samples. As expected, transfer of proteins including FasL increased infiltration of neutrophils (Fig. 1*A*). Mice treated with the costimulators (4-1BBL/TRANCE) showed no increase in tumor-infiltrating neutrophils, when compared with mice treated with PPA (a control); whereas mice treated with all four proteins or with the chemotactic molecules (SLC/FasL) showed a significant increase (~3-fold) in neutrophil infiltration. In parallel, mice treated with all four proteins showed a significant increase in the infiltration of DCs (Fig. 1*B*); in comparison, mice treated with either SLC/FasL or 4-1BBL/TRANCE only showed a slight increase. These results suggest a synergistic/additive effect between the chemotactic and the costimulatory molecules, presumably attributed to the functional complement between SLC and TRANCE (to recruit and prolong the survival of DCs, respectively). Furthermore, transfer of either all four proteins or SLC/FasL, but not 4-1BBL/TRANCE, caused a significant increase in the infiltration of $CD4^+$ (Fig. 1*C*) and $CD8^+$ (Fig. 1*D*) T cells, which was presumably due to the chemotactic activity of SLC toward T cells. The relatively long half-life of T cells (compared with DCs) may explain their increase in the absence of T cell costimulators (such as 4-1BBL) in mice treated with only SLC/FasL.

Collectively, these results indicate that although the use of the chemotactic molecules is sufficient to increase the infiltration of neutrophils and T cells, combined use of the chemotactic and costimulatory molecules is necessary to significantly increase and sustain the accumulation of DCs within tumors.

Intratumoral protein transfer results in a decrease in intratumoral $CD4^+CD25^+Foxp3^+$ Treg

It has recently been shown in vitro that FasL can effectively induce apoptosis in Treg (20). Thus, we determined whether transfer of proteins including FasL can lead to a decrease in intratumoral

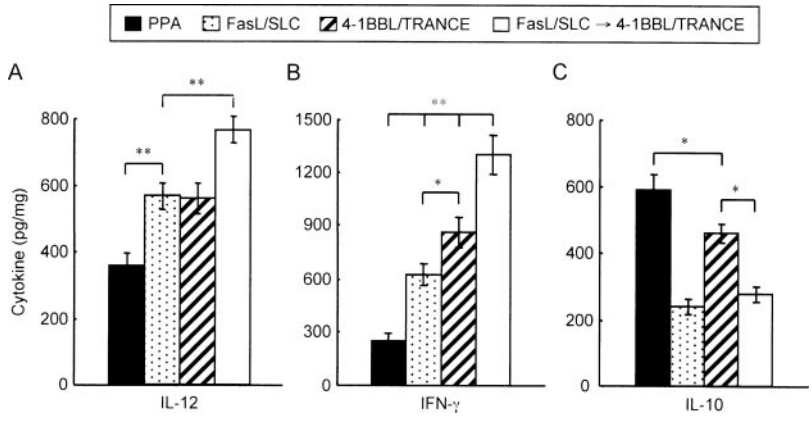

FIGURE 3. Intratumoral transfer of chemotactic and/or costimulatory molecules leads to change in cytokine milieu at the tumor site. Protein transfers were performed as described in Fig. 1. On day 8, tumors were harvested and homogenized. Homogenized tumor tissues were quantified for IL-12 (*A*), IFN-γ (*B*), and IL-10 (*C*) and normalized against total proteins. Each bar represents the mean ± SD of tumor samples from three independent experiments. The differences between different groups are statistically significant (**, $p < 0.01$; *, $p < 0.05$).

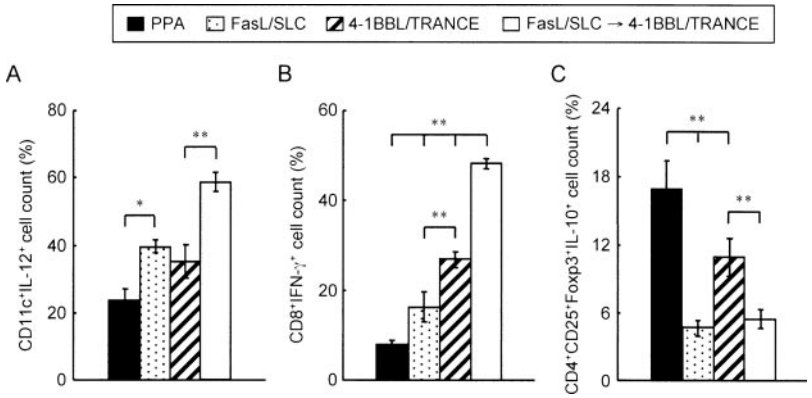

FIGURE 4. The change in cytokine milieu at the tumor site is attributed to the change in the quantity of tumor-infiltrating immune cells resulted from protein transfer. Protein transfers were performed as described in Fig. 1 legend. On day 8, single-cell suspensions were prepared from treated tumors. Cells were cultured for 24 h in the presence of Con A (5 μg/ml) and monensin (3 μM). Cells were first stained for CD11c, CD4, CD25, and/or CD8, fixed, and permeabilized. Subsequently, cells were stained for intracellular IL-12, IFN-γ, IL-10, and/or Foxp3. Samples were quantified by flow cytometry for percentage of activated DCs ($CD11c^+/IL\text{-}12^+$) in total DCs (*A*), activated $CD8^+$ ($CD8^+/IFN\text{-}\gamma$) T cells in total $CD8^+$ T cells (*B*), and IL-10-secreting Treg ($CD4^+/CD25^+/Foxp3^+/IL\text{-}10^+$) in total $CD4^+$ T cells (*C*). Each bar represents the mean $\pm$ SD of tumor samples from three independent experiments. The differences between different groups are statistically significant (**, $p < 0.01$; *, $p < 0.05$).

Treg, which would be conducive to antitumor responses. Significantly, transfer of all four proteins or SLC/FasL resulted in a dramatic decrease (>80%) in intratumoral Treg (Fig. 2). In comparison, such decrease was not observed in mice treated with the costimulators alone.

Intratumoral protein transfer changes cytokine milieu in the tumor microenvironment

Next, we determined whether combinatorial transfer of all four proteins promotes the Th1 cytokine response, which favors the generation of T cell-mediated antitumor response. Mice treated by all four proteins showed an increase in the Th1 cytokines IL-12 (Fig. 3*A*) and IFN-γ (Fig. 3*B*) at the tumor site; in comparison, mice treated with SLC/FasL or 4-1BBL/TRANCE also showed such an increase, although to a lesser degree. Significantly, a decrease in the Th2 cytokine IL-10 was observed in mice treated with either all four proteins or SLC/FasL (Fig. 3*C*).

Next, we determined whether the observed increase in IL-12 and IFN-γ was attributed to the observed increase in tumor-infiltrating DCs (Fig. 1*B*) and $CD8^+$ T cells (Fig. 1*D*), respectively. Mice treated with all four proteins showed a significant increase in IL-12-producing DCs (Fig. 4*A*) and IFN-γ-producing $CD8^+$ T cells (Fig. 4*B*). In comparison, mice treated with SLC/FasL or 4-1BBL/TRANCE also showed such an increase, although to a lesser degree. Of note, although transfer of 4-1BBL/TRANCE did not increase the absolute number of $CD8^+$ T cells (Fig. 1*D*), it increased the number of activated, IFN-γ-producing $CD8^+$ T cells here, presumably via enhancing 4-1BBL-mediated costimulation of the T cells.

In parallel, given that Treg have been shown to produce IL-10 and other suppressive cytokines (27, 28), we also determined whether the observed decrease in IL-10 (Fig. 3*C*) was linked to the decrease in intratumoral Treg (Fig. 2). As shown in Fig. 4*C*, mice treated with all four proteins or with SLC/FasL showed a significant decrease in IL-10-secreting Treg. The result confirms that transfer of all four proteins or SLC/FasL leads to a decrease in IL-10 at the tumor site via, primarily, the reduction of intratumoral Treg.

Collectively, these data indicate that compared with the transfer of the chemotactic molecules or the costimulatory molecules, transfer of all four molecules increases the ratio of Th1:Th2 cytokines, thereby generating a microenvironment that favors cell-mediated antitumor responses.

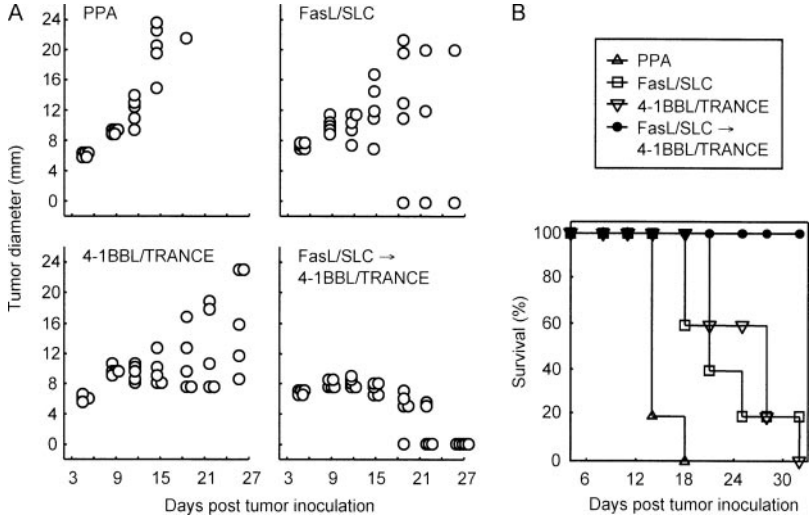

FIGURE 5. Transfer of both chemotactic and costimulatory molecules leads to stronger therapeutic efficacy than does transfer of either the chemotactic or the costimulatory molecules alone. L5178Y tumors were established (day 0). Intratumoral transfers were performed (five mice per treatment group) as indicated in Table I. *A*, After protein transfer, tumor size was measured and graphed for individual animals; each circle represents the tumor size of a single animal. The representation stops when the animal dies. *B*, Survival rate of each treatment group was graphed. Data are representative of three independent experiments.

Table II. *Protein transfer treatment of L5178Y tumors*

Expt.	Fraction of Mice Cured per Treatment Group			
	PPA	FasL/SLC	4-1BBL/TRANCE	FasL/SLC → 4-1BBL/TRANCE
1	0/5	1/5	0/5	5/5
2	0/5	0/5	0/5	2/5
3	0/7	1/7	0/7	4/7
Total	0/17	2/17	0/17	11/17
Percentage	0	12	0	65

Intratumoral protein transfer leads to enhanced antitumor responses in the L5178Y tumor model

We next assessed the antitumor efficacy of intratumoral protein transfer. Established L5178Y tumors were treated with various protein combinations, as described in Table I, and tumor growth was assessed. As negative control, PPA-treated mice all showed progressive tumor growth and died, whereas a portion of the mice treated with SLC/FasL or 4-1BBL/TRANCE showed delayed tumor growth (Fig. 5*A*). Also, complete tumor regression was observed in a small portion of mice treated with SLC/FasL (Fig. 5). On average, transfer of SLC/FasL resulted in complete tumor regression in 12% of the treated mice, whereas transfer of 4-1BBL/TRANCE did not result in regression in treated mice (Table II). In comparison, when mice were treated with all four proteins, retardation of tumor growth and complete tumor regression were observed in a substantial portion of treated mice (Fig. 5). On average, complete tumor regression was observed in 65% of the treated mice (Table II).

Collectively, these results indicate that therapy combining the chemotactic molecules with the costimulatory molecules has stronger therapeutic efficacy than that using either the chemotactic or the costimulatory molecules alone.

Establishment of persistent, systemic immunity in mice treated with all four proteins

Having documented the local tumor regression in a substantial portion of mice treated with all four proteins, we next assessed the establishment of persistent, systemic antitumor immunity in treated mice. To this end, cured mice were rechallenged with the L5178Y tumor cells injected at a site (i.p.) distant from the original tumor at least 2 mo after the initial tumor inoculation. All of the cured mice were resistant to the rechallenge, whereas naive mice inoculated with the same tumor cells all died of tumors (Fig. 6*A*). It is especially notable that all of the cured mice subjected to i.p. tumor rechallenge >6 mo after the initial tumor inoculation were still resistant to tumor (data not shown). These rechallenge experiments point to a persistent, systemic antitumor immunity that is established in mice treated with both the chemotactic molecules and the costimulatory molecules in combination.

To further support the establishment of systemic immunity, we recovered bulk splenocytes from the cured mice, 4–6 wk after tumor rechallenge, and checked for CTL responses. Upon in vitro restimulation with tumor cells, the splenocytes showed strong CTL activity against the L5178Y tumor cells (Fig. 6*B*). The CTL activity was L5178Y tumor cell specific, as the lysis of irrelevant, control EG.7 tumor cells was at a significantly lower level. Thus, specific CTL can be recovered from a secondary lymphoid organ distal from the tumor cells at the treatment site.

Collectively, these results demonstrate a persistent, systemic antitumor immunity that is established in mice cured by therapy using both the chemotactic and costimulatory molecules.

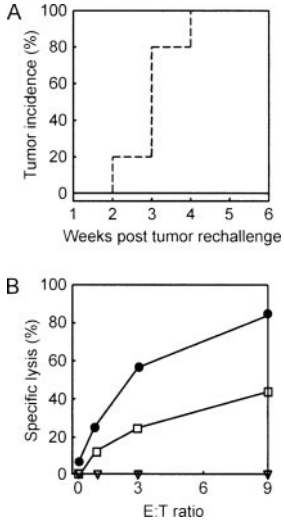

FIGURE 6. Mice cured by intratumoral transfer of both chemotactic and costimulatory molecules demonstrate persistent, systemic antitumor immunity. *A*, A lethal dose (0.5×10^6) of the L5178Y tumor cells were inoculated (i.p.) into naive mice (- - -) or mice cured by intratumoral transfer of all four proteins (—) 2–3 mo after initial tumor inoculation. Mice were monitored for the appearance of tumors weekly. *B*, Bulk splenocytes were prepared 3–6 wk after the rechallenge experiment described in *A* and restimulated in vitro with mitomycin C-treated L5178Y tumor cells (at a 10:1 ratio). Subsequently, splenocytes (effector) were incubated for 12 h with the L5178Y (●, specific target) or EG.7 (□, nonspecific target) tumor cells at indicated E:T ratios, and CTL activity was determined by 7-AAD staining and flow cytometry. As negative control, splenocytes from naive mice were assayed for CTL activity against the L5178Y tumor cells (▽). Data are representative of two independent experiments.

Discussion

Although this study was not intended for systematic evaluation of synergies among various combinations of immunostimulatory molecules, we took into consideration the functional complementation among the chosen molecules. FasL and SLC were combined to recruit cells from both the innate and the adapted arms of immunity, including neutrophils, DCs, and T cells. TRANCE and 4-1BBL were combined to enhance the function of DCs and T cells, which are the key players in eliciting antitumor immunity.

Moreover, inclusion of FasL and TRANCE in the same therapy was intended to minimize the potential loss of DCs caused by FasL-induced apoptosis, because we have previously shown that TRANCE can counteract FasL-induced apoptosis in DCs (24). We show here that the use of either SLC/FasL or 4-1BBL/TRANCE leads to only a slight increase in DC infiltration (Fig. 1*B*). This is not surprising. Transfer of SLC/FasL alone may be insufficient to sustain the recruited DCs, which, in the absence of TRANCE, can undergo spontaneous or FasL-induced apoptosis. In contrast, transfer of 4-1BBL/TRANCE alone may be insufficient to increase DCs, without DC recruitment. Congruent with our rationale, the use of all four molecules leads to a significant increase in DC infiltration (Fig. 1*B*).

Originally, our decision to include FasL stemmed from its ability to recruit neutrophils and mediate proinflammatory, antitumor responses (14–16). During the course of this study, Fritzsching et al. (20) reported that compared with $CD4^+CD25^-$ naive effector T cells, Treg are highly susceptible to FasL-mediated apoptosis in vitro. Consistent with this finding, our study shows that intratumoral transfer of proteins including FasL results in a significant decrease (>80%) in intratumoral Treg (Fig. 2). Such a decrease, in turn, leads to a decrease in IL-10 (Figs. 3*C* and 4*C*), which is often

produced by immunosuppressive cells including tumor cells and Treg. Furthermore, we have recently confirmed the susceptibility of Treg to FasL-induced apoptosis in vitro and in vivo; more importantly, we have also shown that depletion of intratumoral Treg using FasL enhances the antitumor efficacy of adoptive T cell transfer, and that the effect of FasL can be diminished by adding Treg during adoptive transfer (manuscript submitted for publication). Papiernik and colleagues (29), however, observed resistance of prestimulated Treg to apoptosis induced by anti-Fas mAb (29). One explanation for this discrepancy among different groups may be that we and Fritzsching et al. (20) used FasL, which might bind and multimerize the Fas receptor differently from the anti-Fas mAb used by Papiernik and colleagues (29).

Of note, this study was designed for a robust demonstration of combinatorial in situ transfer of chemotactic and costimulatory molecules, not for elaboration of the role played by each of the molecules. The functional plurality of the molecules used here warrants further study in the future to evaluate how the functional capacities of each of the molecules, such as FasL-mediated neutrophil recruitment, FasL-induced apoptosis of Treg, and TRANCE-mediated increase in DC longevity, contribute to the overall enhanced antitumor efficacy.

With the simplicity in delivering defined amounts of immunostimulatory molecules in combination, protein transfer offers a useful alternative means to gene transfer for cancer vaccination. Moreover, from the safety standpoint, protein transfer bypasses the need to use toxic reagents, such as certain viral vectors. It also permits temporary modification of vaccine cells because the transferred proteins will "wear off" over time, likely due to metabolism and/or shedding; thus, it is unlikely to cause long-term side effects, such as the induction of autoimmunity. Significantly, since the initial establishment of the "proof-of-principle" of protein transfer, achieved via the GPI modification (30–32), several protein transfer methods, including the method used in this study, have been developed (9, 10). These methods, combined with the expanding repertoire of immunostimulatory molecules, now provide an enlarging set of options for cancer vaccination.

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Disclosures

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