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Polarization Effects of 4-1BB during CD28 Costimulation in Generating Tumor-reactive T Cells for Cancer Immunotherapy[1]

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ABSTRACT

Using murine tumor-draining lymph node (TDLN) cells, we investigated the polarization effect of 4-1BB (CD137) during CD28 costimulation in generating antitumor T cells. Costimulation of TDLN cells through the newly induced 4-1BB molecules, CD3, and CD28 using monoclonal antibodies significantly enhanced cell proliferation. The greater cell yield with 4-1BB signaling appeared to be related to the inhibition of activation-induced cell death. Activation of TDLN cells through 4-1BB in addition to CD3/CD28 signaling shifted T-cell responses toward a type 1 cytokine pattern because 4-1BB ligation plus CD3/CD28 stimulation significantly augmented type 1 cytokine (*e.g.*, IFN-γ) and granulocyte macrophage colony-stimulating factor secretion. By contrast, type 2 cytokine (*e.g.*, interleukin 10) secretion by the activated TDLN cells was significantly reduced. The *in vivo* antitumor reactivity of TDLN cells activated through 4-1BB in conjunction with CD3/CD28 pathways was examined using an adoptive immunotherapy model. The number of pulmonary metastases was significantly reduced and survival was prolonged after the transfer of anti-CD3/anti-CD28/anti-4-1BB-activated TDLN cells compared with an equivalent number of cells activated without anti-4-1BB. The antitumor effect through 4-1BB involvement during CD28 costimulation was dependent on IFN-γ production and abrogated after IFN-γ neutralization. By contrast, interleukin 10 neutralization resulted in significantly enhanced tumor regression. These results indicate that costimulation of TDLN cells through newly induced 4-1BB and CD3/CD28 signaling can significantly increase antitumor reactivity by shifting T-cell responses toward a type 1 cytokine pattern while concomitantly decreasing type 2 responses.

INTRODUCTION

Adoptive immunotherapy using tumor-reactive T cells offers an alternative approach for the treatment of cancers. Toward this end, the *ex vivo* generation of large numbers of tumor-reactive effector T cells remains a critical step for the successful clinical application of this approach. Various *ex vivo* strategies have been investigated using biological or biochemical reagents to promote T-cell proliferation and stimulate antitumor reactivity in preclinical and clinical studies. These approaches include the use of growth factors [*i.e.*, IL-2[3] (1, 2)], autologous tumor cells (3), superantigens (4, 5), pharmacological agents (6), and mAbs (7). Although effector T cells can be generated to mediate tumor regression using the above-mentioned techniques in animal models, clinical responses in adoptive immunotherapy have been confined to a small group of patients. Hence, alternative protocols need to be defined that will allow the generation of more potent tumor-reactive T cells.

Ex vivo T-cell activation using mAbs in the absence of antigen takes advantage of common signal transduction pathways that are ubiquitous to T cells. We have used this principle to expand tumor-primed T cells contained within TDLNs (5) or VPLNs. Our initial efforts involved anti-CD3 mAb (anti-CD3) to activate TDLN cells *in vitro* followed by expansion in IL-2 (8, 9). The short-term pan-T-cell activation with anti-CD3 resulted in effector cells that were capable of mediating tumor regression *in vivo* that was immunologically specific. Comparable numbers of nonactivated TDLN cells were ineffective in adoptive immunotherapy, presumably due to the low frequency of tumor-reactive T cells within the nodes. These observations have led to clinical trials in which we have evaluated anti-CD3-activated VPLN cells in advanced cancer patients. VPLN cells were induced by irradiated autologous tumor cells admixed with *Bacillus Calmette-Guérin* inoculated intradermally. These studies have demonstrated that antibody-activated T-cell therapy can result in long-term durable responses (7, 10).

During the generation of antitumor effector T cells, it is critical to identify *in vitro* correlates of effector T-cell function that are associated with *in vivo* therapeutic efficacy. In animal models, several laboratories including ours have demonstrated that cytokine elaboration to specific tumor stimulation is associated with the ability of effector T cells to mediate *in vivo* tumoricidal effects (11–14). We have reported that type 1 cytokine release (*i.e.*, IFN-γ) and GM-CSF correlate with *in vivo* tumor eradication, whereas type 2 cytokine release (*i.e.*, IL-10) was found to suppress antitumor reactivity (15). In a recent Phase II adoptive cellular trial we completed in patients with metastatic renal cell cancer, we found that the IFN-γ:IL-10 ratio of cytokine released by effector T cells in response to tumor antigen was associated with clinical responses (10). Thus, methodologies that can up-regulate type 1 while down-regulating type 2 cytokine responses of effector T cells should increase their therapeutic potential.

4-1BB (CD137) is known as a costimulatory molecule expressed by activated T cells, natural killer cells, and dendritic cells (16–18). It is a member of the tumor necrosis factor receptor superfamily. Cell signaling via 4-1BB pathways can promote T-cell proliferation and cell survival (16, 19–23). The ability of 4-1BB ligation with mAb to modulate cytokines released by antigen-stimulated T cells has been reported to be related to the differential expression of 4-1BB on CD4 and CD8 cells (24, 25). In this study, we have used an agonistic anti-4-1BB mAb as an additional stimulus to examine its modulatory effects on CD3 and CD28 activation of tumor-primed lymphoid cells.

Using TDLN cells obtained from an animal model, we were able to demonstrate the polarizing effect of 4-1BB ligation during anti-CD3/anti-CD28 activation of tumor-reactive T cells *in vitro*. Activation of TDLN cells through 4-1BB in addition to CD3/CD28 signaling shifted T-cell responses toward a type 1 cytokine pattern. 4-1BB mAb resulted in greater proliferation of cells and enhanced IFN-γ and GM-CSF release in response to tumor antigen without a concomitant increase in IL-10 secretion. As a result, effector cells generated through 4-1BB and CD28 costimulation mediated potent *in vivo* tumor regression in the animal model. Our observations suggest that

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[3] The abbreviations used are: IL, interleukin; TDLN, tumor-draining lymph nodes; VPLN, vaccine-primed lymph node; CM, complete medium; MCA, methylcholanthrene-induced; PI, propidium iodide; AICD, activation-induced cell death; GM-CSF, granulocyte macrophage colony-stimulating factor; mAb, monoclonal antibody; FACS, fluorescence-activated cell-sorting; ATCC, American Type Culture Collection; ANN, annexin V; TCR, T Cell Receptor.

using 4-1BB as a polarizing signal during CD28 costimulation of tumor-primed lymphocytes may be a useful approach to generate effector T cells for clinical therapy.

MATERIALS AND METHODS

Mice. Female C57BL/6 (referred to henceforth as B6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). They were maintained in specific pathogen-free conditions and used for experiments at 8 weeks of age or older. Recognized principles of laboratory animal care (NIH Publication 85-23, revised in 1985) were followed, and the University of Michigan Laboratory of Animal Medicine approved all animal protocols.

Murine Tumor Cells. MCA 205 and MCA 207 murine tumors are MCA fibrosarcomas that are syngeneic to B6 mice. These tumors have been previously characterized to be weakly immunogenic with distinct tumor-specific transplantation/rejection antigens (26). Using these two tumors, we have previously reported the ability to generate tumor-reactive T cells in TDLNs that mediate the specific regression of pulmonary metastases in criss-cross experiments (27). Tumors were maintained *in vivo* by serial s.c. transplantation in B6 mice and used within the eighth transplantation generation. Tumor cell suspensions were prepared from solid tumors by enzymatic digestion in 50 ml of HBSS (Life Technologies, Inc., Grand Island, NY) containing 40 mg of collagenase, 4 mg of DNase I, and 100 units of hyaluronidase (Sigma Chemical Co., St. Louis, MO) for 3 h at room temperature. Tumor cells were washed in HBSS three times for administration into mice or resuspended in CM for *in vitro* assays. CM consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM fresh L-glutamine, 100 mg/ml streptomycin, 100 units/ml penicillin, 50 μg/ml gentamicin, 0.5 μg/ml Fungizone (all from Life Technologies, Inc.), and 0.05 mM 2-mercaptoethanol (Sigma Chemical Co.).

TDLN Preparation. To prepare TDLNs, B6 mice were inoculated with 1×10^6 tumor cells in 0.1 ml of PBS s.c. in the lower flank. Nine days later, inguinal TDLNs were removed aseptically. Multiple TDLNs were pooled from groups of mice. Lymphoid cell suspensions were prepared by mechanical dissociation with 25-gauge needles and pressed with the blunt end of a 10-ml plastic syringe in HBSS. The resultant cell suspension was filtered through nylon mesh and washed in HBSS.

FACS Analysis. TDLN cell surface expression of 4-1BB molecules was assessed by indirect immunofluorescence assays using anti-4-1BB hybridoma (kindly provided by Dr. Lieping Chen, Mayo Clinic) supernatant (rat IgG2a) and FITC-labeled mouse antirat IgG2a mAb (BD PharMingen, San Diego, CA). Control staining was performed by FITC-conjugated antibody alone. Analysis of stained cells was performed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Antibody-activated MCA 205 TDLN cells were analyzed for apoptotic and necrotic cells using a standard FACS assay (R&D Systems, Inc., Minneapolis, MN), which detects the binding of ANN-fluorescein to apoptotic cells and the binding of PI to necrotic cells.

T-cell Activation and Expansion. TDLN cells were activated with 1.0 μg/ml anti-CD3 mAb ascites plus 0.5 μg/ml anti-CD28 mAb (BD PharMingen) immobilized in 24-well plates (4×10^6 cells/2 ml/well) with or without soluble anti-4-1BB mAb (rat IgG1; BD PharMingen) at 2.5 μg/ml for 2 days. For antibody immobilization, each well of a 24-well cell culture plate (Costar, Cambridge, MA) was coated with 1 ml of anti-CD3 plus anti-CD28 at 4°C overnight or at room temperature for 5–6 h. Anti-CD3 mAb was produced as ascites by inoculating DBA/2 mice with 145-2C11 hybridoma cells (ATCC, Manassas, VA). The 145-2C11 hybridoma cells produce hamster IgG mAb against the CD3ϵ chain of the murine TCR/CD3 complex. The ascites were partially purified by 50% ammonium sulfate precipitation, and the IgG content was determined by ELISA. Secondary cross-linking antibody (antirat IgG1; 10 μg/ml) was used, together with anti-4-1BB mAb. After antibody activation, the cells were harvested and counted. The cells were then expanded in CM containing human recombinant IL-2 (Chiron Therapeutics, Emeryville, CA) starting at a concentration of 3×10^5 cells/ml in 6-well culture plates (Costar) for 5 days. The concentration of IL-2 was 60 IU/ml. During the 5-day cell expansion period, additional IL-2-containing media were added, and cells were split as they grew to keep the cell density below 3×10^6 cells/ml. At the end of the cell expansion, cells were harvested and counted to determine the fold of expansion and used for adoptive immunotherapy and cytokine secretion assessment.

Assessment of Cytokine Release. To measure cytokines released at the end of TDLN activation and expansion, culture supernatants were collected at the end of cell expansion, and cytokine release was assessed using commercially available ELISA kits (BD PharMingen).

To measure cytokines released in response to tumor stimulation, 1×10^6 activated and expanded TDLN cells were cocultured with 0.25×10^6 irradiated MCA 205 cells in 2-ml volumes/well for 24 h at 37°C. The culture supernatants were then collected and analyzed for cytokine production using ELISAs. The tumor stimulator cells were irradiated to 6000 cGy by a ^{137}Cs source. MCA 207 tumor cells were used for coculture as specificity controls.

Adoptive Immunotherapy. B6 mice were inoculated i.v. via tail vein with 2×10^5 tumor cells to establish pulmonary metastases. Three days after tumor inoculation, mice were infused i.v. with activated TDLN cells. Commencing on the day of the cell transfer, i.p. injections of IL-2 (42,000 IU) were administered in 0.5 ml of PBS and continued twice daily for 8 doses, unless otherwise indicated. At least five mice were used in each experimental group. On day 14–16, all mice were randomized and sacrificed for enumeration of pulmonary metastatic nodules. The metastases appeared as discrete white nodules on the black surface of lungs insufflated with a 15% solution of India ink (8). Metastatic foci too numerous to count were assigned an arbitrary value of >250. In some experiments, therapeutic efficacy was evaluated by the survival of the treated animals at various doses of cells transferred.

***In Vivo* Neutralization of Cytokines.** Inhibition of IFN-γ, GM-CSF, and IL-10 was performed with the i.v. administration of the neutralizing mAbs to these cytokines. Neutralizing mAbs were made from hybridoma lines R46-A2 (anti-IFN-γ, from ATCC), JES5-2A5.1.1 (anti-IL-10, from ATCC), and MP1-22E9 (anti-GM-CSF, from Dr. Keith Bishop, University of Michigan). Ascites were generated using these hybridomas by the Hybridoma Core at the University of Michigan. The efficacy of neutralizing activity was measured by ELISA performed at the Immune Monitoring Core of the University of Michigan Comprehensive Cancer Center. A 1:100 dilution of ascites could neutralize at least 300 ng/ml IFN-γ, 600 ng/ml GM-CSF, and 10,240 ng/ml IL-10. In the adoptive transfer model, 0.2 ml of ascites was administrated via caudal vein daily for 4 days after cell transfer. Control groups were given rat IgG (BD PharMingen) i.v. at the same dose and intervals as the neutralizing antibodies.

Statistical Analysis. The significance of differences in numbers of metastatic nodules between experimental groups was determined using the nonparametric Wilcoxon's rank-sum test. Two-sided *P*s of <0.05 were considered statistically significant between two groups. Student's *t* test was used to analyze cell expansion and cytokine release data.

RESULTS

Antibody Activation Up-Regulates 4-1BB Expression on TDLN Cells. The expression of 4-1BB on lymph node cells draining murine MCA 205 tumors was assessed. Freshly harvested TDLN cells did not

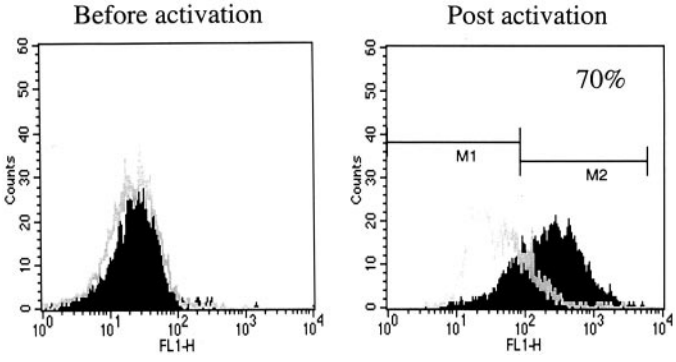

Fig. 1. 4-1BB up-regulation on TDLN cells. Murine MCA 205 TDLN cells were stained for 4-1BB expression by indirect immunofluorescence assays before and after anti-CD3/anti-CD28 activation. Data are representative of three independent experiments. Control staining was performed by FITC-conjugated secondary antibody alone (*open frame*).

Table 1 *Expansion of murine MCA 205 TDLN cells in IL-2 after antibody activation*

	Before activation	After anti-CD3/CD28			Anti-CD3/CD28/4-1BB		
Experiment	No. of cells	No. of cells	Expansion	CD3/CD4/CD8 (%)	No. of cells	Expansion	CD3/CD4/CD8 (%)
1	4×10^6	43×10^6	10.8	97/24/69	54×10^6	13.5	97/26/70
2	20×10^6	100×10^6	5.0	95/19/64	108×10^6	5.4	94/22/74
3	20×10^6	245×10^6	12.2	n.d.[a]	274×10^6	13.7	n.d.
4	20×10^6	109×10^6	5.4	95/13/78	169×10^6	8.4	98/12/78
5	40×10^6	172×10^6	4.3	n.d.	225×10^6	5.6	n.d.
6	40×10^6	153×10^6	3.8	99/25/78	172×10^6	4.3	99/20/81
		Mean ± SE[b]	6.9 ± 1.4			8.5 ± 1.7	

[a] n.d., not done.
[b] $P = 0.017$ comparing fold expansion by anti-CD3/anti-CD28 *versus* by anti-CD3/CD28/4-1BB.

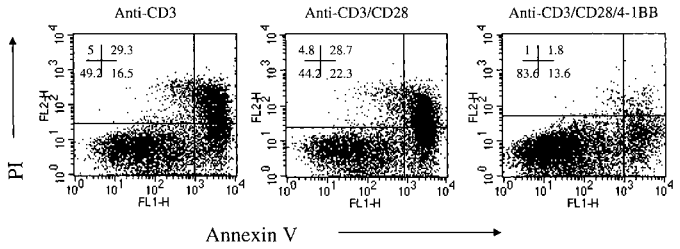

Fig. 2. 4-1BB signaling enhances cell proliferation by preventing apoptosis and necrosis of antibody-activated TDLN cells. TDLN cells were activated with anti-CD3 alone, anti-CD3/anti-CD28, or anti-CD3/anti-CD28/anti-4-1BB, followed by cell expansion in IL-2. After culture, TDLN cells were analyzed by FACS for ANN and PI staining. The *bottom left quadrant* (ANN−/PI−) represents viable cells, the *bottom right quadrant* (ANN+/PI−) depicts cells in early apoptosis, and the *top right quadrant* (ANN+/PI+) shows late apoptotic and necrotic cells.

express detectable 4-1BB by FACS analysis (Fig. 1). However, anti-CD3/anti-CD28 antibody activation up-regulated 4-1BB expression on these TDLN cells. Notably, as shown in Fig. 1, 2 days after anti-CD3/anti-CD28 activation, 4-1BB expression on murine TDLN cells was increased from negligible to approximately 70%.

Costimulation of 4-1BB and CD28 Enhances the Proliferation of Lymph Node Cells by Preventing AICD. To study the functional significance of 4-1BB expression on the cell surfaces of lymph node cells after antibody activation, we assessed their proliferation to IL-2 in culture.

Based on the observation that 4-1BB molecules could be up-regulated effectively on TDLN cells within a short period of time (Fig. 1), an antimouse 4-1BB mAb was used at the same time anti-CD3/anti-CD28 mAbs were used to activate TDLN cells. Table 1 summarizes the expansion of MCA 205 TDLN cells in IL-2 after antibody activation using anti-CD3/anti-CD28 with or without the anti-4-1BB mAb in six independent experiments. In all six experiments, cell expansion in the presence of anti-4-1BB mAb was higher than that in the absence of it. The mean fold expansion was 6.9 and 8.5, respectively, when TDLN cells were activated using anti-CD3/anti-CD28 in the absence or presence of 4-1BB stimulus. By FACS analysis, there was no difference in the CD4:CD8 ratio in the absence or presence of 4-1BB costimulation (Table 1).

FACS analysis of the activated TDLNs was performed to evaluate the increased cell yield when anti-4-1BB was used. Assessment of IL-2 receptor expression after anti-CD3/anti-CD28 *versus* anti-CD3/anti-CD28/anti-41BB activation of TDLN cells revealed no significant differences (data not shown). We then examined the degree of apoptosis and necrosis of TDLN cells after activation and expansion in IL-2. At the end of cell expansion after anti-CD3 or anti-CD3/anti-CD28 activation, the viable (ANN/PI−) cells comprised 44–50% of the whole population (Fig. 2). In contrast, when TDLN cells were activated in the presence of anti-4-1BB in addition to anti-CD3/anti-CD28, more than 80% of the cells were viable, and less than 5% underwent necrosis. A significantly larger proportion of anti-CD3/anti-CD28-activated TDLN cells underwent early apoptosis as compared with anti-CD3/anti-CD28/anti-4-1BB-activated cells (22.3 *versus* 13.6%, respectively). Hence, the greater cell yield with 4-1BB signaling appears to be related to protection against AICD. These studies indicated the important role of 4-1BB costimulation with CD28 in promoting the proliferation of immune cells isolated from tumor-bearing hosts and suggest the usefulness of anti-4-1BB mAb in generating large numbers of effector T cells.

Polarization Effects of 4-1BB during CD28 Costimulation in Up-Regulating Type 1 *versus* Type 2 Cytokine Secretion of Tumor-reactive T Cells. We measured cytokines released by the TDLN cells activated through CD3 and CD28 in the presence of anti-4-1BB mAb. Culture supernatants were collected at the end of cell expansion after antibody activation, and cytokines released into

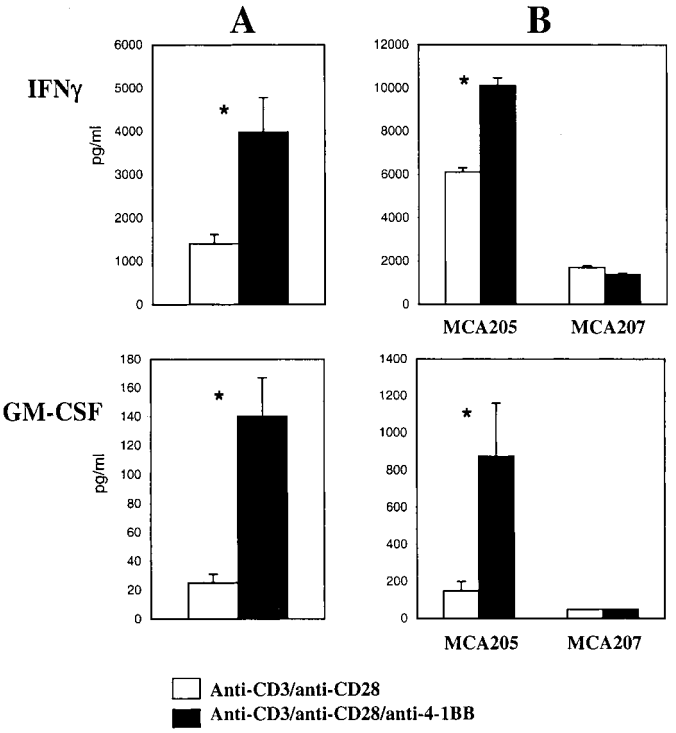

Fig. 3. 4-1BB and CD28 costimulation of TDLN cells preferentially up-regulates IFN-γ and GM-CSF secretion. MCA 205 TDLN cells were activated with anti-CD3 plus anti-CD28 in the absence (□) or presence (■) of anti-4-1BB mAb, followed by cell expansion in IL-2. *A*, cytokine secretion at the end of cell expansion by activated TDLN cells without encountering tumors. *B*, cytokine secretion of activated/expanded TDLN cells in response to tumor stimulation. TDLN cells (1×10^6) collected at the end of activation and expansion were cocultured with irradiated MCA 205 cells or MCA 207 cells (specificity control). After 24 h, supernatants were collected for cytokine analysis. *, $P < 0.05$ comparing TDLN cells activated by anti-CD3/anti-CD28 with or without anti-4-1BB mAb.

the culture media were assessed using ELISA. As shown in Fig. 3*A*, costimulation of TDLN cells through 4-1BB and CD3/CD28 signaling pathways significantly enhanced type 1 (IFN-γ) cytokine and GM-CSF secretion compared with CD3 ligation plus CD28 costimulation ($P < 0.05$). This release profile was seen in three of three experiments. We then measured the cytokines released by activated TDLN cells in response to tumor antigen. After coculturing activated MCA 205 TDLN cells with irradiated MCA 205, or with MCA 207 tumor cells as a specificity control, the culture supernatants were collected and analyzed for cytokines. As shown in Fig. 3*B*, costimulation of TDLN cells through newly up-regulated 4-1BB plus CD3/CD28 preferentially elevated IFN-γ and GM-CSF secretion of the activated TDLN cells in response to specific tumor stimulation ($P < 0.05$). These results were observed in five of six experiments.

Similar studies were performed to look at the release of the type 2 cytokine, IL-10. As shown in Fig. 4*A*, 4-1BB ligation during CD3/CD28 costimulation significantly decreased IL-10 levels produced by activated TDLN cells in eight of eight experiments performed ($P = 0.003$). On the other hand, in 10 of 12 experiments (Fig. 4*B*), IL-10 secretion remained unchanged or was slightly decreased after the coculture of activated TDLN cells with MCA 205 tumor cells ($P = 0.20$).

These results demonstrate the polarizing effects of 4-1BB during costimulation of TDLN cells *in vitro*. In the context of antigen reactivity, 4-1BB preferentially promotes type 1 (IFN-γ) and GM-CSF cytokine release of committed effector cells, but not type 2 (IL-10) responses.

Effector Cells Generated through 4-1BB and CD28 Costimulation Mediate Potent *in Vivo* Tumor Regression. The *in vivo* antitumor reactivity of TDLN cells activated through CD3/CD28/4-1BB pathways was examined in the adoptive immunotherapy model. Mice with 3-day established MCA 205 pulmonary metastases were treated by the adoptive transfer of TDLN cells activated with anti-CD3/anti-CD28 mAbs with or without anti-4-1BB mAb. Varying numbers of activated TDLN cells were transferred. Approximately 14–16 days after treatment, all mice were euthanized for enumeration of pulmonary metastatic nodules. As indicated in Fig. 5, the number of metastases was significantly reduced after the transfer of low doses of anti-CD3/anti-CD28/anti-4-1BB-activated TDLN cells (2.5×10^6 or 1×10^6) compared with an equivalent number of anti-CD3/anti-CD28 activated cells ($P < 0.05$). These findings were reproduced in a second independent experiment (data not shown). In other experiments, therapeutic efficacy was evaluated by the survival of tumor-bearing mice treated with anti-CD3/anti-CD28/anti-4-1BB-activated TDLNs. The treatment was performed by transferring 3×10^6 or 10×10^6 of anti-CD3/anti-CD28/anti-4-1BB-activated TDLN cells and compared with equal numbers of anti-CD3/anti-CD28-activated cells. As shown in Fig. 6, survival of mice with established pulmonary metastases was prolonged after adoptive immunotherapy using anti-CD3/anti-CD28/anti-4-1BB-activated TDLN cells compared with an equal number of anti-CD3/anti-CD28-activated cells. These findings were reproduced in a second independent experiment (data not shown). These studies therefore revealed that, on a per cell basis, anti-CD3/anti-CD28/anti-4-1BB-activated TDLN cells were more po-

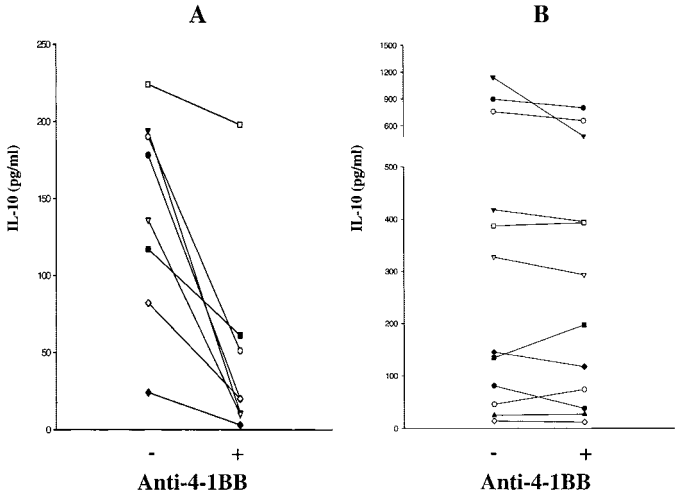

Fig. 4. Regulation of IL-10 production by TDLN cells after 4-1BB costimulation. MCA 205 TDLN cells were activated using anti-CD3/anti-CD28 with (+) or without (−) anti-4-1BB mAb followed by cell expansion as described in the Fig. 3 legend. After activation, >95% of the cells were $CD3^+$ T cells. Individual experiments are depicted by connecting lines. *A*, IL-10 secretion at the end of cell activation and expansion ($n = 8$; $P = 0.003$ comparing with or without anti-4-1BB). *B*, IL-10 secretion of activated/expanded TDLN cells after coculturing with MCA 205 tumor cells ($n = 12$; $P = 0.20$ comparing with or without anti-4-1BB). The IL-10 secretion was immunologically specific because negligible IL-10 was measured in response to MCA 207 tumor cells (data not shown).

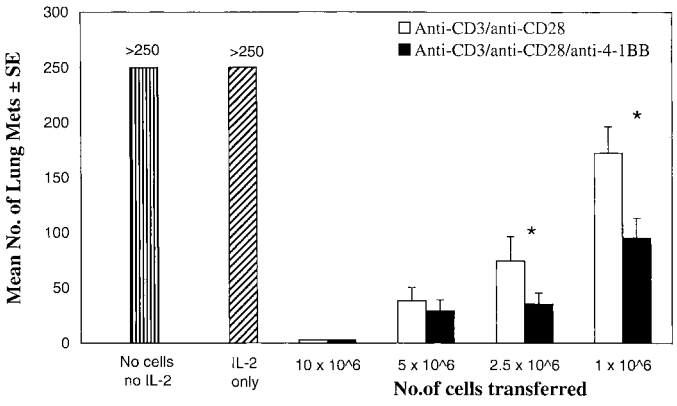

Fig. 5. Effector cells generated with 4-1BB ligation in addition to anti-CD3/anti-CD28 activation mediate effective tumor regression. B6 mice bearing 3-day established MCA 205 pulmonary metastases were treated by the adoptive transfer of anti-CD3/anti-CD28/anti-4-1BB-activated MCA 205 TDLN cells. Treatment was compared with an equal number of MCA 205 TDLN cells activated with anti-CD3/anti-CD28 without 4-1BB ligation. *, $P < 0.05$ comparing equal number of transferred cells generated with or without anti-4-1BB mAb.

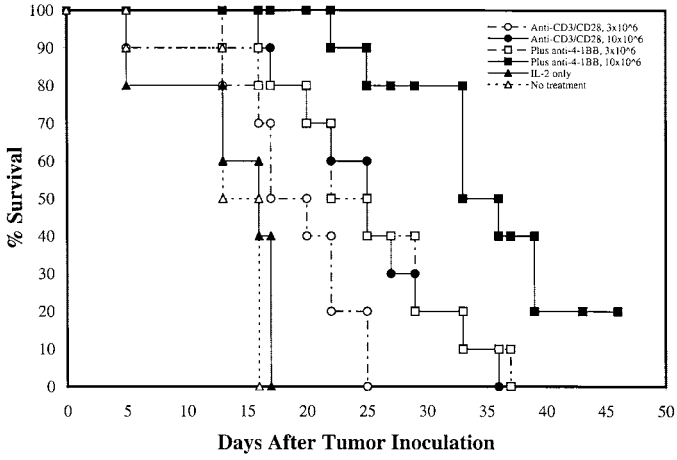

Fig. 6. Survival of mice with established pulmonary metastases is prolonged after adoptive immunotherapy using anti-CD3/anti-CD28/anti-4-1BB-activated TDLN cells. B6 mice bearing 3-day established MCA 205 pulmonary metastases were treated by the adoptive transfer of 3×10^6 or 10×10^6 anti-CD3/anti-CD28/anti-4-1BB-activated MCA 205 TDLN cells in conjunction with the administration of exogenous IL-2. Therapeutic efficacy was compared with equal number of anti-CD3/anti-CD28-activated MCA 205 TDLN cells.

tent than anti-CD3/anti-CD28-activated TDLN cells in therapeutic efficacy.

Effects of *in Vivo* Neutralization of Cytokines after Adoptive Immunotherapy. The augmented *in vivo* antitumor activity mediated by anti-CD3/anti-CD28/anti-4-1BB-activated TDLN cells was associated with the observed high levels of IFN-γ and GM-CSF secretion, which suggested that the effects of 4-1BB during CD28 costimulation in up-regulating type 1 *versus* type 2 cytokine secretion of tumor-reactive T cells may be an important mechanism. To prove this, we used neutralizing mAb to different cytokines after T-cell transfer. B6 mice bearing 3-day established MCA 205 pulmonary metastases were treated by the adoptive transfer of anti-CD3/anti-CD28/anti-4-1BB-activated MCA 205 TDLN cells as in the previous experiments. All mice received 5×10^6 cells unless otherwise indicated. Inhibition of IFN-γ, GM-CSF, and IL-10 was performed with the i.v. administration of the neutralizing antibodies to these cytokines. As shown in Table 2, cytokine neutralization confirmed that therapeutic efficacy mediated by anti-CD3/anti-CD28/anti-4-1-BB-activated TDLN cells involved the release of IFN-γ and GM-CSF. Whereas the *in vivo* neutralization of GM-CSF after the adoptive transfer of anti-CD3/anti-CD28/anti-4-1BB-activated TDLN cells only partially inhibited the antitumor response, the neutralization of IFN-γ or both cytokines almost completely abrogated the therapeutic efficacy. On the other hand, the administration of anti-IL-10 mAb significantly enhanced the tumor regression mediated by the transferred T cells, indicating the suppressive effect of IL-10 on the antitumor response.

Up-Regulated IFN-γ Secretion Is Not Due to a Nonspecific Reactivity of the Secondary Cross-linking Antibody. In earlier studies with tumor-primed lymphoid cells, we found that anti-4-1BB mAb, when used in a soluble form with a secondary cross-linking antibody, was more effective in promoting cell expansion and cytokine production compared with an immobilized antibody (data not shown). Those early studies led us to the current protocol in this report, *i.e.*, immobilized anti-CD3/anti-CD28 mAbs plus soluble anti-4-1BB and its cross-linking antibody as described in "Materials and Methods." The antimouse 4-1BB mAb we used was a rat IgG1, and the secondary cross-linking antibody was therefore antirat IgG1. To eliminate the possibility that the effect of anti-4-1BB in shifting T-cell responses toward a type 1 cytokine pattern was due to the nonspecific reactivity of the secondary cross-linking antibody, we performed a control experiment. In Fig. 7, all groups of MCA 205 TDLN cells were activated with immobilized anti-CD3/anti-CD28. In one group (*striped bar*), soluble antimouse 4-1BB and its cross-linking antibody, antirat IgG1, were added. In another group (Fig. 7, ■), only the secondary cross-linking antibody was used without the anti-4-1BB antibody. IFN-γ release was analyzed after the coculture of these antibody-activated TDLN cells with MCA 205 tumor cells. The elevated production of IFN-γ in response to tumor took place only when both primary anti-4-1BB and secondary cross-linking antibody were used in addition to anti-CD3/anti-CD28 during the T-cell activation. This experiment confirms the involvement of the 4-1BB signaling pathway during its costimulation with CD28 in up-regulating type 1 cytokine secretion.

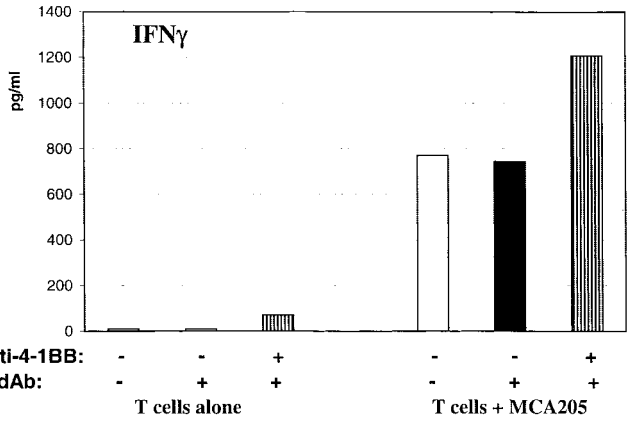

Fig. 7. Polarization effects of 4-1BB during CD28 costimulation in up-regulating IFN-γ secretion are not due to the nonspecific reactivity of the secondary cross-linking antibody. MCA 205 TDLN cells were activated with either anti-CD3/anti-CD28 alone (□) or anti-CD3/anti-CD28 plus soluble anti-4-1BB and its cross-linking antibody, anti-rat IgG1 (*striped bar*). In addition, another group was activated with anti-CD3/anti-CD28 plus the cross-linking antibody only (■). All groups of activated/expanded cells were then subject to expansion in IL-2-containing medium. The activated TDLN cells were subsequently cocultured with MCA 205 tumor cells for IFN-γ secretion analysis as described in the Fig. 3 legend.

Table 2 *In vivo neutralization of cytokines after adoptive immunotherapy of established pulmonary metastases*

Group	TDLN cells[a]	*In vivo* Ab[b]	Mean no. of lung metastases (SE) Expt. 1	Expt. 2
1	None	None	189 (32)	>250
2	+	None	94 (15)[c]	47 (12)[c]
3	+	Rat Ig	79 (21)[c]	41 (10)[c]
4	+	Anti-IFN-γ	210 (37)[d]	203 (20)[d]
5	+	Anti-GM-CSF	—	84 (16)[d]
6	+	Anti-IFNγ + anti-GM-CSF	—	210 (25)[d]
7	+	Anti-IL-10	5 (0)[e]	11 (4)[e]

[a] Mice with 3-day established lung metastases received 5×10^6 TDLN cells activated with anti-CD3/anti-CD28/anti-4-1BB. Neutralizing mAb was administered i.v. as described in "Materials and Methods."
[b] Ab, antibody; Expt., experiment.
[c] $P < 0.05$ compared with group 1.
[d] $P < 0.05$ compared with group 3.
[e] $P < 0.05$ compared with group 3.

DISCUSSION

Using murine TDLN cells, we found that costimulation through newly induced 4-1BB and CD28 can polarize T-cell differentiation toward a Th1/Tc1 phenotype by significantly enhancing IFN-γ and GM-CSF secretion, whereas IL-10 production was either reduced or remained nonmodulated. Consequently, involvement of 4-1BB signaling during CD28 costimulation resulted in effector T cells that were highly potent in mediating tumor regression in an adoptive transfer model. The augmented antitumor effect due to the use of anti-4-1BB was dependent on the enhanced IFN-γ secretion and to a lesser extent on GM-CSF production.

We recently completed a Phase II adoptive immunotherapy clinical trial in patients with metastatic renal cell cancer (10). Patients were treated with anti-CD3-activated VPLN cells plus IL-2, which resulted in a 27% response rate (complete and partial responders), and prolonged survival. Importantly, we identified that an increased IFN-γ:IL-10 cytokine release ratio of the VPLN cells in response to autologous tumor cells was associated with a greater likelihood of tumor response. Using a modified protocol from the one described in our murine model, we evaluated the application of 4-1BB and CD28 costimulation on anti-CD3-activated human VPLN cells. In preliminary studies, we found that the activation of VPLN cells by anti-CD3 and anti-CD28 required 4 days for the induction of 4-1BB expression. At that time point, anti-4-1BB was used to activate the cells followed by expansion in IL-2. In two of two human samples examined, we observed the up-regulation of a type 1 cytokine response and the simultaneous down-regulation of a type 2 response of the activated cells when exposed to autologous tumor cells (data not shown). This provides a rationale strategy in generating effector cells for adoptive immunotherapy.

4-1BB is an inducible T-cell surface receptor (16–18). Cross-linking of 4-1BB molecules by agonistic antibody transmits a distinct costimulatory signal for T-cell activation and differentiation (20), increases graft-*versus*-host disease (23), and accelerates the rejection of cardiac allograft and skin transplants (20). The role of tumor necrosis factor receptor-associated factor 2 and p38 mitogen-activated protein kinase was reported during 4-1BB-dependent immune response (21). The use of 4-1BB mAb as a therapeutic agent for established cancers has been explored previously by several investigators. The *in vivo* administration of anti-4-1BB was first discovered by Melero *et al.* (22) to have antitumor efficacy against weakly immunogenic s.c. tumors that was mediated by both $CD4^+$ and $CD8^+$ T cells. On the other hand, Kim *et al.* reported that poorly immunogenic s.c. and pulmonary tumors were refractory to anti-4-1BB (28). This refractoriness has been postulated by Wilcox *et al.* (29) to be due to an immune "ignorance" that can be overcome by active immunization with tumor-associated peptides in combination with anti-4-1BB administration. From these studies, it is apparent that the utility of anti-4-1BB mAb therapy involves the prior induction of antigen-primed T cells in the host. The *ex vivo* generation of antigen-specific effector T cells for adoptive immunotherapy represents an ideal application for agonistic 4-1BB mAb costimulation.

The *ex vivo* generation of tumor-reactive T cells with 4-1BB costimulation has been examined previously in adoptive transfer models. Kim *et al.* (28) observed that TDLN cells activated *in vitro* with anti-CD3/anti-4-1BB were less potent than anti-CD3-activated cells in mediating tumor regression *in vivo*. In their study, the proliferation of TDLN cells in IL-2 after anti-CD3/anti-4-1BB activation was significantly enhanced compared with anti-CD3 alone. However, this increased proliferation resulted in the expansion of non-tumor-reactive T cells. The lack of CD28 costimulation during *in vitro* activation in their study suggests that CD28 ligation is important for activating tumor-primed reactive T cells. Strome *et al.* (30) reported the use of anti-4-1BB in activating T cells for adoptive immunotherapy. Similar to our findings, they observed that the use of anti-4-1BB resulted in the generation of T cells that were more effective than those activated by anti-CD3 alone or anti-CD3/anti-CD28 in mediating antitumor reactivity *in vivo*. However, the mechanisms for these findings were not clearly defined.

Our study suggests that activation of TDLN cells through newly induced 4-1BB in addition to CD3/CD28 signaling shifted T-cell responses toward a type 1 cytokine pattern. This may represent an important mechanism accounting for the potent therapeutic efficacy of activated TDLN cells. In this report, the involvement of 4-1BB during CD3/CD28 costimulation elevated IFN-γ and GM-CSF production both by activated TDLN cells alone and by activated TDLN cells in response to specific tumor stimulation. By contrast, anti-4-1BB significantly decreased IL-10 levels produced by activated TDLN cells, even though IL-10 secretion remained unaltered after coculture of the activated TDLN cells with tumor cells. It is not clear why there are different IL-10 secretion profiles of TDLN cells after 4-1BB activation in the presence or absence of tumor cells. The mechanism for this observation remains to be defined. Regardless, in the context of tumor reactivity, 4-1BB preferentially promotes type 1 (IFN-γ) and GM-CSF cytokine release of these effector cells, but not type 2 (IL-10) responses. We also assayed for IL-4 in these culture systems and have not been able to detect measurable amounts of the cytokine.

In our studies, we observed an up-regulation of 4-1BB expression on TDLN cells after CD28 costimulation that may contribute to increased viability of tumor reactive T cells by anti-4-1BB during *in vitro* activation. Our data indicate that costimulation of TDLN cells through 4-1BB molecules and CD3/CD28 significantly enhanced cell proliferation, and the greater cell yield with 4-1BB signaling appeared to be related to the inhibition of AICD. It would be of great interest to further explore what is occurring in the adoptively transferred mice by examining the traffic and the survival of the anti-4-1BB-activated TDLN cells *in vivo*.

Although we found that 4-1BB ligation enhanced proliferation of TDLN cells, there was no preferential expansion of $CD4^+$ or $CD8^+$ cells. Kwon and colleagues have evaluated the ability of anti-4-1BB to costimulate allostimulated $CD4^+$ or $CD8^+$ cells (24). We have previously shown that both $CD4^+$ and $CD8^+$ tumor-reactive cells can be generated from TDLN after anti-CD3/anti-CD28 activation (31). T-cell subset depletion or the use of T-cell subgroup-deficient animals would confirm our prediction that both cell subsets are activated with the combination of anti-CD3/anti-CD28/anti-4-1BB, and both are involved in mediating tumor regression.

During T-cell activation, cross-linking of the membrane receptors, *i.e.*, CD3, CD28, and 4-1BB, is required for subsequent cell signaling. The cross-linking of the receptors on the cell surface can be completed by antigen, antibodies immobilized on solid surfaces (culture plate or beads), soluble antibodies with a secondary cross-linking antibody, or artificial antigen-presenting cells expressing ligands for the T-cell receptors (32). We found that the use of 4-1BB antibody in different forms (*i.e.*, plate-bound *versus* soluble) could alter its potential to polarize T-cell activation (data not shown) and that both primary and a secondary cross-linking antibody are required for the functioning of anti-4-1BB when it is used in a soluble form. Further evaluation of the utilization of CD3, CD28, and 4-1BB mAbs in different forms and combinations should enable us to optimize the condition for 4-1BB signaling in shifting T-cell differentiation toward the Th1/Tc1 phenotype during CD28 costimulation. Relevant to our clinical experience in cellular therapies (10), the findings in this study may allow for the generation of more potent effector cells.

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