Tumor Cells Expressing Anti-CD137 scFv Induce a Tumor-Destructive Environment

Yi Yang, Shilin Yang, Zhengmao Ye, Jade Jaffar, Yifeng Zhou, Erin Cutter, Andre Lieber, Ingegerd Hellström, and Karl Erik Hellström

Department of Pathology, University of Washington, Seattle, Washington

Abstract

For immunotherapy to become more effective, there is a need to maximize the antitumor response at the tumor site as well as to eliminate tumor cell variants that lack a given tumor antigen or the ability to present it. We have previously shown that wild-type (WT) cells from the K1735 melanoma (K1735-WT) are rejected following vaccination with cells (K1735-1D8) transfected to express scFv from the anti-CD137 monoclonal antibody 1D8, and that $CD4^+$ T cells and natural killer (NK) cells are needed for this rejection. We now show that tumors harvested 4 to 10 days after mice had been transplanted with K1735-1D8 cells or a mixture of K1735-1D8 and K1735-WT cells contained more NK cells and that they had an increased percentage of $CD4^+$ T lymphocytes producing IFNγ or tumor necrosis factor-α. We further show that the percentage of NK cells was higher in B16-1D8 melanomas expressing anti-CD137 scFv than in the WT tumors and that the percentage of $FoxP3^+$ cells was lower. Admixture of 10% K1735-1D8 cells prevented the progressive growth of transplanted K1735-WT cells in syngeneic mice and also of cells from the antigenically different sarcoma Ag104. Inhibition of WT tumor cells by tumor cells transfected to express anti-CD137 scFv was shown also with the TC1 carcinoma and B16 melanoma. Furthermore, injection of an adenovirus vector, Ad-1D8, which encodes anti-CD137 scFv into established B16 melanomas, significantly prolonged the survival of tumor-bearing mice and could induce regression. Our data suggest that targeting of anti-CD137 scFv to tumors should be explored for therapy for some human cancers. [Cancer Res 2007;67(5):2339–44]

Introduction

Although tumor vaccines have shown therapeutic efficacy in some models, their clinical efficacy has been modest. Various inhibitory mechanisms at the site of a growing tumor, including such mediated by regulatory T (T_{Reg}) cells, are likely to contribute to the inefficiency of tumor vaccination (1, 2), as do the frequently occurring neoplastic cell variants that lack or cannot present the antigens selected as immunologic targets (3). There is a need for therapeutic approaches to circumvent those problems.

We previously showed that cells from the K1735 melanoma, which have low immunogenicity and which express little MHC class I and lack class II, after transfection to express anti-CD137 scFv, are rejected by syngeneic mice, unless these are treated with antibodies to remove $CD4^+$ T cells, and that antibodies to natural killer (NK) cells delay the rejection (4). According to immunohistology, tumor rejection was accompanied by an increased number of infiltrating $CD4^+$ and $CD8^+$ T lymphocytes. Based on these findings, we hypothesized that the $CD4^+$ tumor-infiltrating lymphocytes (TIL) were of the T helper 1 (Th1) type, that the frequency of NK cells was increased in tumors formed by K1735 cells expressing anti-CD137 scFv, and that this can cause the destruction also of neighboring, wild-type (WT) tumor cells. Data presented here support this hypothesis and indicate that targeting protocols causing the expression, by at least some of the cells within a tumor, of an antibody-like signal that engages CD137 can be therapeutically effective.

Materials and Methods

Mice and tumor lines. Female C3H/HeN mice, 6 to 8 weeks old, were purchased (Charles River Laboratories, Wilmington, MA). The M-2 clone of the K1735 melanoma of C3H/HeN origin (5), referred to K1735 WT, has been applied in our previous experiments (4). Ag104 (6) is a spontaneous fibrosarcoma of C3H/HeN origin, which did not cross-react with K1735 when tested in assays measuring T-cell proliferation or tumor rejection (4). TC1 is a C57BL carcinoma line that expresses human papillomavirus-16 E6 and E7 proteins (7). B16, clone F1 (8), is a melanoma of C57BL origin. K1735-1D8 was obtained by transfecting K1735-WT cells with a gene encoding anti-CD137 scFv from hybridoma 1D8, and the TC1-1D8 and B16-1D8 lines were similarly constructed (see below). Each experiment used five mice per group. All mice were transplanted s.c. on the right side of the back, except in the experiment presented in Fig. 1*D*, where five mice were transplanted s.c. on both sides of the back, providing 10 tumor "sites" per group.

In experiments with the TC1 carcinoma and B16 melanoma, transfected and WT (as control) cells were sterilized by incubation with mitomycin C (MMC; Sigma, St. Louis, MO) before they were admixed with WT cells from the respective tumor. Cultured tumor cells were washed with PBS and then incubated with 50 μg of MMC per 10^7 cells for 1 h at 37°C, after which they were washed four times with PBS before they were added to live tumor cells. Controls were included to show that the MMC-treated cells did not form tumors.

Experiments were also done in which mice that had ~3 × 3-mm tumors growing s.c. on the right side of the back were injected i.p. with 2 mg of cyclophosphamide (9), followed 4 and 5 days later by i.t. injection of 10^9 plaque-forming units (pfu) of an adenovirus vector, Ad-1D8 (see below), which was injected in 50-μL PBS, whereas controls were injected with 50-μL PBS into the tumors. For comparison, another group of mice received 5×10^8 pfu of a similarly constructed vector encoding CD80 and the same amount of a vector encoding anti-CD3 scFv.

Tumor growth was measured after s.c. transplantation of tumor cells on one or both (see Results) sides of the back of syngeneic mice by measuring the two largest perpendicular diameters with calipers and reported as average tumor area (in square millimeters).

Note: Current address for S. Yang: Department of Medicine, Vanderbilt University Medical Center, Nashville, TN. Current address for Z. Ye: Department of Microbiology and Immunology, The University of North Carolina, Chapel Hill, NC.

Initial studies were done when Y. Yang, S. Yang, Z. Ye, I. Hellström, and K.E. Hellström were working at Pacific Northwest Research Institute, Seattle, WA 98122.

Requests for reprints: Karl Erik Hellström, Department of Pathology, University of Washington, Box 35 99 39, Harborview Medical Center, 325 Ninth Avenue, Seattle, WA 98109-2499. Phone: 206-341-4714; E-mail: hells@u.washington.edu.

doi:10.1158/0008-5472.CAN-06-3593

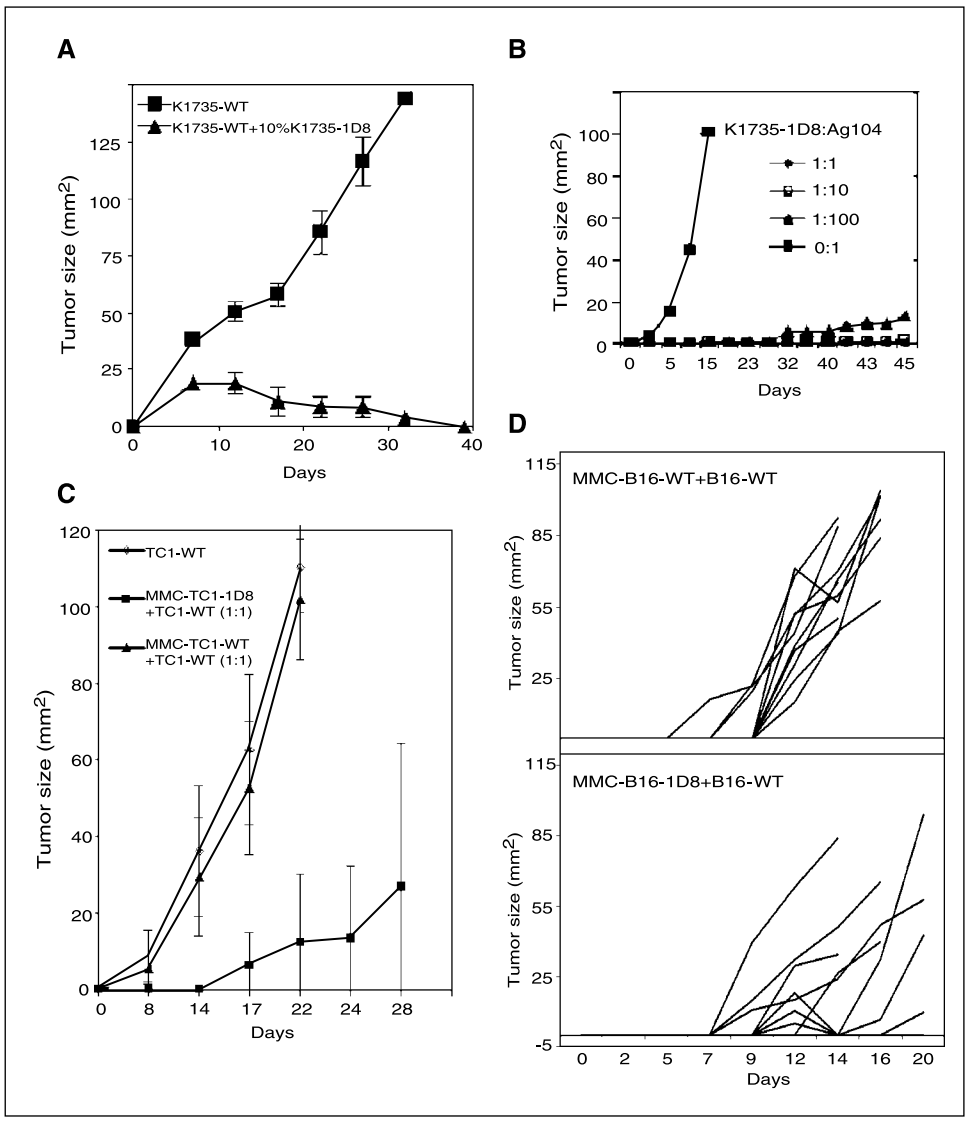

Figure 1. *A,* tumor formation in mice (five per group) transplanted s.c. with 2×10^6 K1735-WT cells admixed with 2×10^5 K1735-1D8 cells or 2×10^6 K1735-WT cells. *B,* prevention of tumor formation by 2×10^6 s.c. Ag104 cells that were mixed with K1735-1D8 cells at ratios of 1:1, 10:1, or 100:1 and transplanted s.c. to C3H/HeN mice. *C,* the outgrowth of s.c. transplanted 250,000 TC1-WT cells is inhibited after admixture of an equal number of MMC-treated TC1-1D8 cells but not after admixture of MMC-treated TC1-WT cells. *D,* the outgrowth of 10^6 B16 melanoma cells is inhibited after admixture of 2×10^6 MMC-treated B16-1D8 cells, as compared with an equal dose of MMC-treated B16-WT cells.

Mice with established tumors of >5-mm mean diameter were checked daily, and, to prevent suffering, mice were euthanized when their tumors reached an average area of 100 mm^2. The time for transplanted tumors to reach that size is referred to as survival. Evidence of "biological activity" was recorded as CR, complete regression of an established tumor; PR, >50% decrease of an established tumor; and stabilization, no increase of tumor size for a minimum of 1 week.

The animal facilities are Association for Assessment and Accreditation of Laboratory Animal Care certified and our protocols were approved by the respective Institutional Animal Care and Use Committees (at Pacific Northwest Research Institute for the initial studies and at University of Washington for the subsequent work).

Vectors and transfection of tumor cells. As previously reported (4), K1735-1D8 cells were engineered by transfecting a retrovirus vector expressing the variable region genes from the anti-CD137 rat hybridoma 1D8 (10), using published techniques (11), and the same approach was taken to engineer TC1 and B16 cells, establishing the TC1-1D8 and B16-1D8 lines. The cells transfected with the *1D8* gene express surface-bound anti-CD137 scFv from hybridoma 1D8 whereas the WT cells do not. We previously showed that K1735-WT cells transfected to express the product of a similarly constructed control vector encoding antihuman CD28 scFv do not induce a tumor-destructive immune response (4).

To produce an adenovirus vector, Ad-1D8, the *1D8* gene (3) was cloned into pAd.RSV (8) as *Hin*dIII/blunted *Xba*I fragment under the control of the Rous sarcoma virus (RSV) promoter. Recombinant Ad-1D8 virus was produced in 293 cells by recombination with pBHG10 (Microbix, Toronto, Canada) and virus was banded in CsCl gradients, dialyzed, and stored in aliquots as described elsewhere (12, 13). Titers were determined by plaque titration on 293 cells and the contamination with the WT virus was examined by real-time PCR. Only vector preparations that contained less than one WT viral genome in 10^6 genomes were used in further studies. Lack of endotoxin contamination was shown with a *Limulus* amoebocyte lysate entotoxin detection kit (BioWhittaker, Walkerville, MD). A similar approach was applied to construct vectors encoding mouse CD80 (14) and antimouse CD3 (15), which were tested in parallel with an Ad-1D8 vector for comparison. I.t. adenoviral injections were done with 50 μL of adenovirus vectors in PBS.

Analysis of tissues by flow cytometry. The following labeled rat anti-mouse monoclonal antibodies (mAb) were purchased from BD PharMingen (San Diego, CA): CD3-phycoerythrin (PE), CD4-FITC, CD8-FITC, and NK1.1-PE. Rat anti-mouse IFNγ-PE and tumor necrosis factor α (TNFα)-PE were purchased from Beckman Coulter (Miami, FL). A FoxP3-FITC mAb was bought from eBiosciences (San Diego, CA). For detection of intracellular cytokines, cells were first stained with anti-CD4-FITC followed by staining for intracellular IFNγ and TNFα, using a kit purchased from BD

PharMingen. For detection of FoxP3, cells were first fixed with IntraPrep Permeabilization reagent (Beckman Coulter, Fullerton, CA) according to the manufacturer's protocol.

Mice were euthanized when their tumors had a mean diameter of 4 to 6 mm following transplantation of 2×10^6 to 4×10^6 cells from either K1735-WT or K1735-1D8, which was 4 to 10 days after transplantation before the latter tumors started to regress (4). We also analyzed lymphoid cells from mice transplanted s.c. with a mixture of 10% K1735-1D8 plus 90% K1735-WT cells.

Spleen and lymph node cell suspensions were mechanically prepared, and the spleen cells were incubated with RBC lysing buffer (Sigma) for 5 min at room temperature. Tumors were cut into 1-mm^2 pieces and digested for 3 h at room temperature in 1 mmol/L HEPES Hanks buffer containing 5 units/mL collagenase, 0.1% hyaluronidase, and 0.01% DNase, using enzymes purchased from Invitrogen (Carlsbad, CA). The suspensions were filtered, the cells collected by centrifugation, and erythrocytes lysed. After washing with PBS, cells were added to a mouse lymphocyte isolation buffer (Cedarlane, Ontario, Canada) and centrifuged at $200 \times g$ for 20 min, after which the lymphocyte band was collected and the resulting TILs were washed with PBS. After counting, the TILs were stained and analyzed.

Single-cell suspensions were washed in PBS, resuspended in FACS buffer (PBS + 2% fetal calf serum), and aliquoted into Falcon plates at 5×10^4 per well in 100-μL FACS buffer. After staining, the cells were collected, washed twice in 100-μL FACS buffer, resuspended in a mixture of 100-μL FACS buffer and 500-μL PBS, and analyzed within 30 min using Coulter Epics FACS and the Coulter Epics × 1.2 software program. The percentages of PE- or FITC-positive cells in the gated cell populations were compared for each group tested.

Results

Cellular composition and lymphokine production in tumors, spleens, and lymph nodes. The frequency of lymphoid cells expressing CD4, CD8, or NK1.1 in K1735 melanomas and lymphoid tissues was studied by flow cytometry. We found, confirming a published study (4), that tumors harvested 4 to 10 days after transplantation of K1735-1D8 cells (i.e., as they started to regress) contained three to four times more $CD4^+$ and $CD8^+$ T cells than the K1735-WT tumors, and the same was true for tumors formed by transplantation of an equal mixture of K1735-WT and K1735-1D8 cells (data not shown). As shown in Table 1, we also found a 4- to 5-fold increase in the frequency of NK cells in tumors formed by K1735-1D8 cells or a mixture of 90% K1735-WT and 10% K1735-1D8 cells. This experiment was repeated with similar results. Another experiment was done in which we instead analyzed tumors formed after transplantation of 2×10^6 WT cells from the B16 melanoma or B16-1D8 cells that express anti-CD137 scFv. When mice were euthanized 10 days after transplantation, the number of NK cells was increased in B16-1D8 tumors by a factor of 2.8 as compared with B16-WT tumors ($P < 0.01$).

Table 2 shows that the frequency of $CD4^+$ T cells containing IFNγ or TNFα, a characteristic of Th1 type cells, was higher in spleens, lymph nodes, and tumors from mice transplanted with either K1735-1D8 cells or a mixture of K1735-1D8 and K1735-WT cells than in the corresponding tissues from the group transplanted with WT cells alone.

Table 3 presents an experiment showing that the percentage of T cells expressing FoxP3, a marker of T_{Reg} cells (2, 16), was significantly lower in mice bearing B16-1D8 melanoma (expressing anti-CD137 scFv) than in mice with B16-WT tumors, and that the percentage of $CD4^+$ and $CD3^+$ cells was higher. This experiment was repeated once with similar results.

Bystander effect against WT antigen-positive and antigen-negative tumor cells. A pilot experiment was done in which 2×10^6 K1735-WT cells were mixed at three different ratios with K1735-1D8 cells (1:1; 10:1, and 100:1) and transplanted s.c. to two syngeneic mice for each mixture, whereas another two mice were given 2×10^6 K1735-WT cells alone. Mice transplanted with WT cells alone developed progressively growing tumors. In contrast, tumors regressed in the mice given a mixture of WT and 1D8-transfected cells except in one of the two mice transplanted with 100 WT cells per 1D8 cell (data not shown). This experiment was repeated (Fig. 1*A*) in which five mice were transplanted s.c. with 2×10^6 K1735-WT cells that had been admixed with 2×10^5 K1735-1D8 cells and another five mice were injected with 2×10^6 K1735 WT cells alone. All mice initially displayed tumors, but the tumors regressed in the mice receiving one K1735-1D8 cell per 10 K1735-WT cells, whereas mice transplanted with K1735-WT cells alone had to be sacrificed after tumors reached end-point size. This experiment was repeated with similar results.

Experiments were then done based on our hypothesis that the increased number of NK cells and CD4 cells producing IFNγ and TNFα in the presence of K1735-1D8 cells will inhibit the outgrowth also of cells from a different tumor. Fig. 1*B* depicts an experiment (five mice per group) to test this hypothesis. We mixed WT cells from the antigenically unrelated, syngeneic sarcoma Ag104 with (untreated) K1735-1D8 cells at various ratios and transplanted mixtures composed of 2×10^6 Ag104 cells to each mouse. Mice that received Ag104 cells alone developed progressively growing tumors. In contrast, mixtures of 1 K1735-1D8 cell per 1, 10, or 100 Ag104 cells did not form tumors, except in two of five mice receiving 1 K1735-1D8 cell per 100 Ag104 cells, which developed tumors after a long latency period. The experiment was repeated with similar results.

To investigate whether these findings have general validity, we used two additional models, the TC1 carcinoma and the B16 melanoma. Figure 1*C* shows that addition of an equal amount of MMC-sterilized TC1-1D8 cells, as compared with MMC-treated TC1-WT cells, significantly ($P < 0.001$ on days 17 and 22 after tumor transplantation) delayed tumor formation by TC1-WT cells; there was no difference in tumor growth in mice that received live TC1-WT cells alone or admixed with MMC-treated TC1-WT cells. There was also some, but not statistically significant, delay of tumor outgrowth when 50,000 MMC-treated TC1-1D8 cells were admixed to 250,000 TC1-WT cells.

Table 1. Increased percentage of TIL with NK markers in melanomas composed of at least 10% of K1735-1D8 cells and transplanted 4 d before euthanasia

Group	Mean*
K1735-WT TIL	4.4 ± 1.8
K1735-1D8 TIL	16.2 ± 4.2 ($P < 0.04$)
10% K1735-1D8 + 90% K1735-WT TIL	22.5 ± 5.4 ($P < 0.02$)

NOTE: Each mouse received a total of 4×10^6 tumor cells. $n = 5$ per group.
*P values according to two-tailed Student's t test, as compared with the WT group. When the data from the K1735-1D8 group are combined with those from the 10% K1735-1D8 + 90% K1735-WT group, the difference in comparison with the ST group is significant at the $P < 0.003$ level. The absolute number of TILs in the three groups was approximately equal.

Table 2. Increased ratios of the number of $CD4^+$ cells in spleens, lymph nodes, and tumors that contain IFNγ or TNFα in groups transplanted with K1735-1D8 cells or a mixture of 10% K1735-1D8 and 90% K1735-WT cells, as compared with the group transplanted with K1735-WT cells

Group	IFNγ ratio, *P**	TNFα ratio, *P**
K1735-1D8 spleen	2.4 ± 0.3, <0.01	3.5 ± 1.9, <0.1
K1735-1D8 lymph nodes	2.3 ± 0.3, <0.05	2.0 ± 0.4, <0.1
K1735-1D8 TIL	2.5 ± 0.4, <0.05	5.6 ± 0.4, <0.001
10% K1735-1D8 + 90% K1735-WT spleen	2.7 ± 0.2, <0.01	4.4 ± 1.9, <0.05
10% K1735-1D8 + 90% K1735-WT lymph nodes	4.4 ± 0.7, <0.01	3.7 ± 0.3, <0.01
10% K1735-1D8 + 90% K1735-WT TIL	5.2 ± 0.9, <0.05	7.7 ± 2.9, <0.1

NOTE: The ratios, given as mean ± SE, were calculated by dividing the number of $CD4^+$ cells containing the respective lymphokine with the corresponding number in the group transplanted with K1735-WT cells. n = 3 to 5 per group.
*Statistical significance is calculated for each entry relative to the WT group according to two-tailed Student's t test. When the ratios obtained with TIL from the K1735-1D8 group are combined with those from the 10%K1735-1D8 + 90% K1735-WT group, the differences in relation to the WT group are significant at the $P < 0.01$ level for IFNγ and at the $P < 0.01$ level for TNFα.

Evidence for a "bystander effect" against WT cells was seen also in the B16 model, where addition of 2 MMC-treated B16-1D8 cells per WT cell inhibited growth ($P < 0.001$ for the difference in tumor areas on day 14 after tumor transplantation) of the WT cells. The data are presented for individual tumors in Fig. 1*D*. Five mice per group were transplanted s.c. on both sides of the back, providing 10 tumor sites per group. There were three complete, albeit short-lived, tumor regressions in the group with the admixed B16-1D8 cells. The experiment was repeated with similar results.

Therapeutic vaccination of mice with B16 melanoma by i.t. injection of an adenovirus vector encoding anti-CD137 scFv. Based on the above findings, experiments were done with the B16 melanoma, which was chosen in spite of the fact that B16 is less sensitive to the bystander effect than K1735, Ag104, or TC1. Mice were transplanted with 2×10^5 B16 cells on one side of the back and, when they had ~3 × 3 mm tumors, they were, based on previous findings in other vaccination experiments (9), injected with 2 mg of cyclophosphamide to decrease the effect of T_{Reg} cells, followed 4 and 5 days later with i.t. injection of an adenovirus vector, Ad-1D8, which encodes anti-CD137 scFv. We also included a group in which tumors were injected with a mixture of two similarly prepared adenovirus constructs, one encoding CD80 and one encoding anti-CD3 scFv. As shown in Table 4, survival, defined as the time until mice had to be euthanized because their tumors reached 100 mm^2 surface area, was significantly prolonged in mice whose tumors had been injected with the Ad-1D8 vector. In contrast, s.c. transplantation of 2×10^6 MMC-treated B16-1D8 cells had no therapeutic effect with the mice surviving 17.0 ± 3.1 days as compared with 17.2 ± 1.8 days for mice transplanted with the same dose of MMC-treated B16-WT cells. A combination of adenovirus vectors encoding CD80 and anti-CD3 scFv did not significantly prolong survival; these negative data, using similarly constructed vectors, are important in view of the fact that a "dummy" adenovirus control was not available at the time. There was evidence of biological activity, including two CR, in the group vaccinated with the Ad-1D8 vector but not in the other groups, except for temporary stabilization of tumor growth in one of ten mice given the Ad-CD80 + Ad-anti-CD3 scFv vectors. The experiment was repeated with similar results.

Discussion

Previously reported cell depletion experiments showed that K1735-1D8 tumors, which express cell-bound anti-CD137 scFv at their surface, regress when transplanted to immunocompetent mice by a mechanism involving $CD4^+$ T cells and NK cells. In contrast, depletion of $CD8^+$ T cells did not hamper the rejection of transplanted K1735-1D8 cells, probably because K1735 cells express very low levels of MHC class I and are relatively resistant to killing by CTL (4). Previous studies also showed by immunohistology that K1735-1D8 tumors were composed of a larger number of $CD4^+$ and $CD8^+$ T cells than corresponding WT tumors of similar size and that vaccination of mice with small growing K1735-WT tumors by s.c. transplantation of K1735-1D8 cells increased the number of $CD4^+$ and $CD8^+$ T cells in the WT tumors (4). We now show, using flow cytometry, an increased infiltration of NK cells in transplanted mouse melanomas from an inoculum containing tumor cells transfected with the *1D8* gene to stably express anti-CD137 scFv at their surface and that there is an increased number of Th1 cells among the $CD4^+$ TILs. In the K1735 model, only 10% of the transfected (K1735-1D8) cells was sufficient to increase the frequency of NK cells to approximately the same level as in

Table 3. Decreased frequency of T cells expressing FoxP3 in spleens and tumors from mice transplanted with 2×10^6 B16-1D8 as compared with 2×10^6 B16-WT cells 10 d before the mice were euthanized

Group	CD3	CD8	CD4	FoxP3	FoxP3/CD3	FoxP3/CD4
B16-WT spleen	38.6	15.4	20.5	1.4	0.036	0.068
TIL	21.8	7.9	17.7	6.3	0.289	0.356
B16-1D8 spleen	49.6*	17.3	27.0*	0.9	0.018*	0.033*
TIL	36.9	9.9	27.8	2.9	0.079*	0.104

*$P < 0.05$, compared with B16-WT cells; three to five mice per group.

Table 4. Survival (defined as time to outgrowth of 100-mm^2 tumors) of C57BL mice that were transplanted with 2×10^5 B16 cells on one side of the back and injected, when they had $\sim 3 \times 3$-mm tumors, with 2 mg of cyclophosphamide given i.p.

Group	No. mice	Survival	*P*	Biological activity			
				CR	PR	Stabilization	Progression
Ad-1D8	15	24.7 ± 6.6	<0.001*	2	4	3	6 †
Ad-D3 + Ad-CD80	10	19.3 ± 6.3	NS	0	0	1	9
PBS	15	17.1 ± 2.7	Contr.	0	0	0	15

NOTE: After 4 and 5 d, the tumors were injected with Ad-1D8, an adenovirus vector that encoded anti-CD137 scFv from hybridoma 1D8, with a combination of Ad-CD80 and Ad-anti-CD3 vector, or with an equal volume of PBS (as control).
Abbreviation: NS, not significant.
*$P < 0.05$, compared with CD3/CD80.
† $P < 0.001$, compared with PBS; $P < 0.045$, compared with CD3/CD80.

tumors composed only of K1735-1D8 cells. Furthermore, there was an increased frequency of Th1 type cells among the CD4$^+$ TILs, suggesting that various lymphokines produced by those cells can facilitate tumor rejection (e.g., by activating NK cells and macrophages; refs. 17, 18). Although the accumulation of Th1 lymphocytes and NK cells in tumors must have been initiated by cell-to-cell contact because tumor cells transfected to express soluble anti-CD137 scFv are not rejected by immunocompetent mice,[1] we do not know which of the several lymphoid cell types that express CD137 (19–22) was responsible for the initial event, leading to the formation of chemokines such as IFNγ and TNFα. Our data also indicate that engagement of CD137 can negatively influence the generation of T_{Reg} cells, a matter that needs to be studied further.

We hypothesized that the ability of K1735-1D8 cells to attract NK cells, CD8$^+$ T cells, and Th1-type CD4$^+$ T cells can cause tumor destruction and showed that admixture of even a small proportion (10%) of K1735-1D8 cells prevented the outgrowth of transplanted K1735-WT cells. We next investigated whether the increased number of NK cells and CD4 lymphocytes that produce IFNγ and TNFα in the presence of K1735-1D8 cells could inhibit the outgrowth also of cells from a different syngeneic tumor, Ag104. This was found to be the case. The findings with Ag104 are of particular interest because mice immunized against K1735 reject transplanted K1735, but not Ag104, cells and develop CD4$^+$ and CD8$^+$ T cells that recognize K1735 but not Ag104 (4). They are also noteworthy in view of the lack of immunogenicity of Ag104 when tested by classic transplantation experiments (6).

To investigate whether these findings have general validity, we used two additional models, TC1 carcinoma and B16 melanoma, for *in vivo* studies. Tumor growth was inhibited in the presence of cells transfected with the *1D8* gene also in these models, although the effects seen were smaller than those observed with K1735, perhaps because the K1735-M2 clone is more immunogenic.

The bystander effect, as studied in the K1735 model, is not antigen specific and we hypothesize that Th1 type lymphokines such as TNFα and IFNγ are at least partially responsible by acting on NK cells and probably also on macrophages, as shown in other systems (17, 18), as well as by affecting the tumor cells directly and damaging the tumor vasculature. Whereas the role of CTL against K1735 tumors seems to be minor, this is not the case in many other systems, wherein lymphokines such as TNFα and IFNγ are likely to expand the CTL activity. Most likely, there may also be antigen-specific bystander effects (e.g., when WT and 1D8-transfected cells are combined from the same tumor).

Administration of an agonistic mAb is the most straightforward approach to engage CD137 (23). However, working in a transgenic model with carcinomas expressing epitopes encoded by the *neu* gene, Zhang et al. (24) found that vaccination with cells transfected to express anti-CD137 scFv was more efficacious and less toxic than administration of anti-CD137 mAb. According to published data, immunization via transfected anti-CD137 scFv is more therapeutically efficacious than immunization with cells expressing the CD137 ligand (4, 23, 25), perhaps because the ligand can induce T-cell apoptosis (26) whereas scFv induces an antibody-like response in the presence of the relevant tumor antigens.

To study whether delivery of anti-CD137 scFv to tumors could be therapeutically effective, we transplanted mice with B16 melanoma cells and, when they had small palpable tumors, injected them with 2 mg cyclophosphamide to decrease the effect of T_{Reg} cells (2, 9). I.t. injection of an adenovirus vector, Ad-1D8, which encodes anti-CD137 scFv, given 4 and 5 days later, delayed tumor growth as compared with mice whose tumors were injected with PBS or with a combination of vectors encoding CD80 and anti-CD3 scFv. There was also evidence of biological activity, including CR and PR, in some of the mice whose tumors were injected with Ad-1D8. It is noteworthy that s.c. transplantation of MMC-treated B16-1D8 cells had no therapeutic effect (i.e., immune stimulation by anti-CD137 scFv had to be targeted to the tumor site). In contrast, s.c. transplanted, MMC-treated K1735-1D8 cells were shown to be effective against K1735-WT tumors of similar size (4).

Tumor cell populations continously undergo genetic changes (27), which cause the emergence of variants that have lost tumor antigens and/or the mechanisms needed for antigen presentation. In addition, the conditions under which tumors grow can influence antigen expression. Consequently, all cells within a tumor are highly unlikely to express and present those antigens that are commonly the targets for immunotherapy. For tumor vaccination or adoptive transfer of immune T cells to be clinically successful, also those tumor cells that lack or fail to present the antigen

[1] Unpublished findings.

vaccinated against need to be destroyed. The same is true for administration of antitumor antibodies. We speculate, therefore, as have others (28), that the dramatic therapeutic effects seen in some patients given an antitumor antibody such as 17.1A (29) are secondary to the antibody-mediated induction of an active immune response, and the same may explain the decrease in large tumor masses in occasional patients given an antibody construct targeting Lewis Y (30). Our data suggest that the targeting of anti-CD137 scFv to tumors and tumor-draining lymph nodes may facilitate the generation of an immune response that involves effector cells capable of also killing antigen-negative tumor cells, as shown by our data with sarcoma Ag104, together with the generation of an antigen-specific response as indicated by previous data (4). As indicated by our data in the B16 model, the delivery of anti-CD137 scFv to the tumor site is more therapeutically effective than the s.c. administration of a tumor vaccine. A bispecific antibody (31) or a tumor-seeking, recombinant virus (32) may be a suitable vehicle for such targeting, and the therapeutic efficacy may be further increased by incorporating additional immunostimulatory agent(s) and by finding more specific drugs than cyclophosphamide to circumvent down-regulatory mechanisms. An advantage of the targeting is that it may facilitate the generation of immune responses to a variety of epitopes expressed by the given tumor.

Acknowledgments

Received 9/27/2006; revised 11/14/2006; accepted 12/13/2006.

Grant support: NIH grant RO1-112073.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. N. Kiviat for her support; Drs. J.A. Ledbetter and M. Hayden-Ledbetter for discussions when the studies were initiated; Dr. M.L. Disis and H.O. Sjogren for comments on the manuscript; Daniel Bushyhead for technical assistance; and Pacific Northwest Research Institute, where the study was initiated, for permission for continued use of their flow cytometry facility.

References

1. Hellstrom KE, Hellstrom I. Novel approaches to therapeutic cancer vaccines. Expert Rev Vaccines 2003; 2:517–32.
2. Turk MJ, Guevara-Patino JA, Rizzuto GA, Engelhorn ME, Houghton AN. Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. J Exp Med 2004;200:771–82.
3. Seliger B, Maeurer MJ, Ferrone S. Antigen-processing machinery breakdown and tumor growth. Immunol Today 2000;21:455–64.
4. Ye Z, Hellstrom I, Hayden-Ledbetter M, Dahlin A, Ledbetter JA, Hellstrom KE. Gene therapy for cancer using single-chain Fv fragments specific for 4-1BB. Nat Med 2002;8:343–8.
5. Fidler IJ, Hart IR. Biological and experimental consequences of the clonal composition of solid tumors. Cancer Res 1981;41:3266–7.
6. Ward PI, Koeppen H, Hurteau T, Schreiber H. Tumor antigens defined by cloned immunological probes are highly polymorphic and are not detected on autologous normal cells. J Exp Med 1989;170:217–32.
7. Feltkamp MCW, Smits HL, Vierboom MPM, et al. Vaccination with cytotoxic T lymphocyte epitoe-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. Eur J Immunol 1993;23:2242–9.
8. Fidler IJ, Gersten DM, Budmen MB. Characterization *in vivo* and *in vitro* of tumor cells selected for resistance to syngeneic lymphocyte-mediated cytotoxicity. Cancer Res 1976;36:3160–5.
9. Di Paolo NC, Tuve S, Ni S, Hellstrom KE, Hellstrom I, Lieber A. Effect of adenovirus-mediated heat shock protein expression and oncolysis in combination with low-dose cyclophosphamide treatment on antitumor immune responses. Cancer Res 2006;66:960–9.
10. Shuford WW, Klussman K, Tritchler DD, et al. 4-1BB costimulatory signals preferentially induce $CD8^+$ T cell proliferation and lead to the amplification *in vivo* of cytotoxic T cell responses. J Exp Med 1997; 186:47–55.
11. Winberg G, Grosmaire LS, Klussman K, et al. Surface expression of CD28 single chain Fv for costimulation by tumor cells. Immunol Rev 1996;153:209–23.
12. Carlson CA, Shayakhmetov DM, Lieber A. An adenoviral expressionsystem for AAV Rep 78 using homologous recombination. Mol Ther 2002;6:91–8.
13. Carlson CA, Steinwaerder DS, Stecher H, Shayakhmetov DM, Lieber A. Rearrangements in adenovirual genomes mediated by inverted repeats. Methods Enzymol 2002;346: 277–92.
14. Chen L, Ashe S, Brady WA, et al. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. Cell 1992;71: 1093–102.
15. Chapoval AI, Nelson H, Thibault C. Anti-CD3 × anti-tumor F(ab′)2 bifunctional antibody activates and retargets tumor-infiltrating lymphocytes. J Immunol 1995;155:1296–303.
16. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfinin $CD4^+CD25^+$ T regulatory cells. Nat Immunol 2003;4:337–42.
17. Fidler IJ, Poste G. Macrophage-mediated destruction of malignant utmor cells and new strategies for the therapy of metastatic disease. Springer Semin Immunopathol 1982;5:161–74.
18. Kahn M, Sugawara H, McGowan P, et al. $CD4^+$ T cell clones specific for the human p97 melanoma-associated antigen can eradicate pulmonary metastases from a murine tumor expressing the p97 antigen. J Immunol 1991;146:3235–41.
19. Hurtado JC, Kim SH, Pollok KE, Lee ZH, Kwon BS. Potential role of 4-1BB in T cell activation. Comparison with the costimulatory molecule CD28. J Immunol 1995; 155:3360–7.
20. Melero I, Johnston JV, Shufford WW, Mittler RS, Chen L. NK1.1 cells express 4-1BB (CDw137) costimulatory molecule and are required for tumor immunity elicited by anti-4-1BB monoclonal antibodies. Cell Immunol 1998;190:167–72.
21. Langstein J, Schwartz H. Identification of CD137 as a potent monocyte survival factor. J Leukoc Biol 1999;65: 829–33.
22. Futagawa T, Akiba H, Kodama T, et al. Expression and function of 4-1BB and 4-1BB ligand on murine dendritic cels. Int Immunol 2002;14:275–86.
23. Melero I, Shuford WW, Newby SA, et al. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. Nat Med 1997;3:682–5.
24. Zhang H, Knutson L, Hellstrom KE, Disis ML, Hellstrom I. Antitumor efficacy of CD137 ligation is maximized by the use of a CD137 single-chain Fv-expressing whole-cell tumor vaccine compared with CD137-specific monoclonal antibody infusion. Mol Cancer Ther 2006;5:149–55.
25. Melero I, Bach N, Hellstrom KE, Aruffo A, Mittler RS, Chen L. Amplification of tumor immunity by gene transfer of the co-stimulatory 4-1BB ligand: synergy with the CD28 co-stimulatory pathway. Eur J Immunol 1998; 28:1116–21.
26. Schwarz H. Biological activities of reverse signal transduction through CD137 ligand. J Leukoc Biol 2005; 77:281–6.
27. Bielas JH, Loeb KR, Rubin BP, True LD, Loeb LA. Human cancers express a mutator phenotype. Proc Natl Acad Sci U S A 2006;103:18238–42.
28. Koprowski H, Herlyn D, Lubeck M, DeFreitas E, Sears HF. Human anti-idiotype antibodies in cancer patients: is the modulation of the immune response beneficial for the patient? Proc Natl Acad Sci U S A 1984; 81:216–9.
29. Sears HF, Herlyn D, Steplewski Z, Koprowski H. Initial trial use of murine monoclonal antibodies as immunotherapeutic agents for gastointestinal adenocarcinoma. Hybridoma 1986;5:109–15.
30. Pai LH, Wittes R, Setser A, Willingham MC, Pastan I. treatment of advanced solid tumors with immunotoxin LMB-1: an antibody linked to Pseudomonas exotoxin. Nat Med 1996;2:350–3.
31. Segal DM, Weiner GJ, Weiner LM. Introduction: bispecific antibodies. J Immunol Methods 2001;248: 1–6.
32. Gaggar A, Shayakhmetov DM, Lieber A. CD46 is a cellular receptor for group B adenoviruses. Nat Med 2003;9:1408–12.

Tumor Cells Expressing Anti-CD137 scFv Induce a Tumor-Destructive Environment

Yi Yang, Shilin Yang, Zhengmao Ye, et al.

Cancer Res 2007;67:2339-2344.