

## **Costimulation by CD48 and B7-1 Induces Immunity against Poorly Immunogenic Tumors**

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### **Summary**

Genetic modification of many types of mouse tumors to express the B7-1 or B7-2 molecules, natural ligands for the T cell–costimulatory molecule CD28, increases their immunogenicity. However, even after transfection with the B7-1 and/or B7-2 genes, poorly immunogenic tumors fail to elicit an efficient immune response. We report here that two such tumors, the Ag104A sarcoma and the K1735-M2 melanoma, become immunogenic after transfection of the genes encoding murine B7-1 together with CD48, which is the natural ligand for CD2. Tumor-specific CD8<sup>+</sup> cytotoxic T lymphocytes were readily generated and were effective for adoptive immunotherapy of metastasis induced by wild-type Ag104A sarcoma cells. A similar approach may be useful for developing therapy for other poorly immunogenic tumors, including those in humans.

**T**CR confers specific recognition of peptide antigens in the context of MHC molecules (1). Efficient activation of T cells, however, requires costimulatory signals (2). Several T cell surface proteins and their respective ligands on APC are involved in both increasing the strength of adhesion between the interacting cells and costimulating the T cells to respond appropriately (2, 3). Among these molecules, the CD28 and CD2 proteins on T cells play such dual roles in T cell function.

Interaction between CD2 and its natural ligand CD48 (4), the structural and functional homologue of the human LFA-3 molecule, is required for the generation of a T cell–mediated immune response. Prevention of this interaction by injecting mice with either anti-CD2 or anti-CD48 antibodies induces sustained unresponsiveness of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells to antigen stimulation (5). Administration of a combination of anti-CD2 and anti-CD48 antibodies can induce long term acceptance of allogeneic heart grafts (6). Although the role of the CD2 signaling pathway in the induction of antitumor immunity remains largely unknown, costimulation through the B7–CD28 pathway plays an important role in eliciting antitumor immunity (7). Many B7–negative tumors can be manipulated to become more immunogenic by transfer of genes encoding the B7-1 or B7-2 molecules, whereas several poorly immunogenic tumors have failed to induce immune response even after transfer of B7-1 (8) and/or B7-2 (9).

We speculated that lack of immunogenicity can be caused by a deficiency to present tumor antigens at the level required for triggering a TCR signal. If that is the case, strengthening the adhesion and signaling between TCR and MHC-presented antigens on poorly immuno-

genic tumors may improve the generation of antitumor T cell response. This possibility was tested with two such tumors, mouse sarcoma Ag104A (10) and melanoma K1735-M2 (11).

### **Materials and Methods**

**Mice and Cell Lines.** Female C3H/HeN (C3H) mice, 4–6 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME). Ag104A, a spontaneous fibrosarcoma of C3H (H-2<sup>b</sup>) origin, and Ag104B, a second spontaneous tumor in the same mouse (10), were gifts from Dr. Hans Schreiber (University of Chicago, Chicago, IL). The B7-1 transfectant of Ag104A cells (renamed here B7<sup>+</sup>Ag104A) was described (8). The C3H-derived K1735-M2 melanoma, its B7-1 transfectant (reference 12; renamed here B7<sup>+</sup>K1735-M2), and p97-transfectant 2A (11), have been described previously, as were the UV4102Pro and UV6132APro fibrosarcoma lines (10). The control Ag104A (Mock.Ag104A) and K1735-M2 (Mock.K1735-M2) lines, which were transduced with retrovirus without insert, have been described (8). The EL4 thymoma is of C57Bl/6 (H-2<sup>b</sup>) origin. YAC-1 is an NK-sensitive lymphoma. The latter two lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). ConA-stimulated lymphoblasts were prepared as described (12).

**Cloning and Transfection of Murine CD48.** A DNA fragment encoding the entire open reading frame of murine CD48 (13) was amplified by reverse transcription–coupled PCR of total RNA prepared from EL4 cells. The sense primer (5′-GCTAAA-GCTTCTCGAACCGCCACCATGTGCTTCATAAAACAGG-GAT3′) consisted of an oligonucleotide corresponding to nucleotides 51–72 of murine CD48 cDNA plus restriction sites for HindIII and XhoI. The antisense primer (5′-CGTAAAGCTT-CTCGGAGTCTAGAGTTCCTTGTCTCAGGTTAACAG-3′) cor-

responded to nucleotides 762–773 of murine CD48 cDNA plus sites for HindIII, XhoI, and XbaI. The PCR product was directly cloned into the vector pRC/CMV (Invitrogen, San Diego, CA). The structure of the resulting construct pRC/CMV-mCD48 is shown in Fig. 1 A. The cell lines were transfected with this vector by electroporation, and the transfectants were selected as described previously (11).

**Antibodies, Immunostaining, and Flow Cytometry Analysis.** Purified and FITC- or PE-conjugated mAbs to MHC-K<sup>k</sup>, D<sup>k</sup>, I-A<sup>k</sup>, I-E<sup>k</sup>, B7-1 (CD80), B7-2 (CD86), the intracellular adhesion molecule (ICAM)-1 and ICAM-2, the vascular cell adhesion molecule (VCAM)-1, the heat stable antigen (HSA), CD48, CD40, and the CD40 ligand (CD40L) were purchased from PharMingen (San Diego, CA). The mAbs 116-13.1 (anti-CD8) and GK1.5 (anti-CD4) were purified from culture supernatant of the respective hybridomas (ATCC), and mAb 10.2 (anti-human CD5) (ATCC) was used as a control. Rabbit polyclonal antibody, anti-Asialo GM1, was purchased from Wako Bioproducts (Richmond, VA). For immunofluorescence staining, single cell suspensions from either Ag104A or K1735-M2 were incubated on ice for 30 min with FITC-conjugated mAb or PE-conjugated mAb. After being washed twice with medium, they were analyzed by FACS<sup>®</sup>.

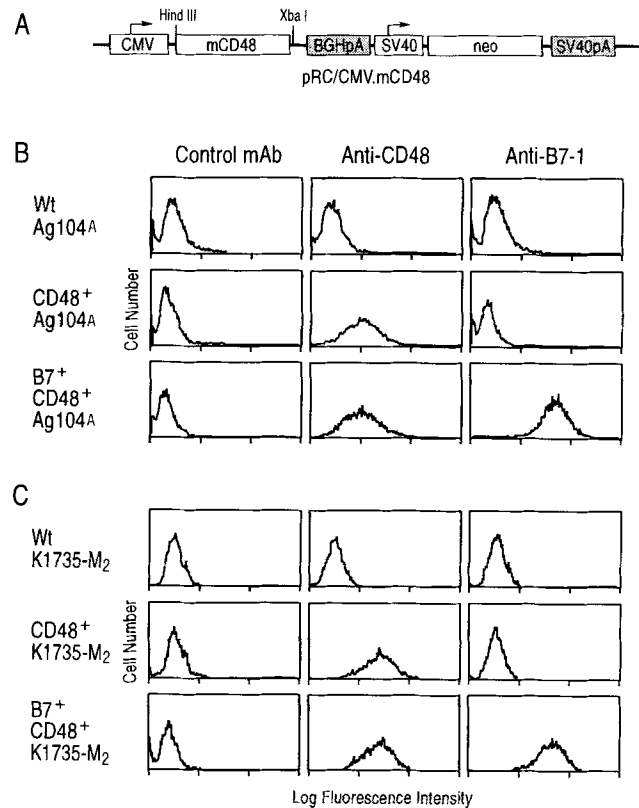
**Immunization Studies.** Mice, in groups of 5 or 10, were injected intraperitoneally with  $5 \times 10^6$  and subcutaneously with  $5 \times 10^6$   $\gamma$ -irradiated (2,500 rad) tumor cells. 14 d later, they were challenged subcutaneously with the respective wild-type tumor cells at  $10^6$ /mouse for wt Ag104A and at  $2 \times 10^6$ /mouse for wt K1735-M2, and tumor size was measured at regular intervals as described previously (11).

**Generation and Assay of CTL Activity.** C3H mice were immunized with irradiated (2,500 rad) Ag104A transfectants as described above. 2–3 wk later, single-cell suspensions were prepared from the spleen and cocultured for 5 d with  $\gamma$ -irradiated (10,000 rad) wt Ag104A cells in a 24-well plate (Costar Corp., Cambridge, MA). For the generation of T cell lines, spleen cell cultures were restimulated every 7–10 d with  $\gamma$ -irradiated wt or CD48<sup>+</sup>Ag104A cells in the presence of irradiated (3,000 rad) splenocytes from C3H mice and human recombinant IL-2 (Cetus Corp., Emeryville, CA) at 5 U/ml (11). CTL clones were generated by limiting dilution. Cytolytic activity was examined in a standard 4-h <sup>51</sup>Cr release assay at different E/T ratios as indicated in the figures. In T cell subset depletion experiments, effector cells at  $\sim 10^6$ /ml were incubated for 30 min on ice with 30  $\mu$ g/ml of the indicated mAb and were subsequently treated with 1:100 diluted rabbit complement (Cedarlane Laboratories, Hornby, Canada) and washed three times before use.

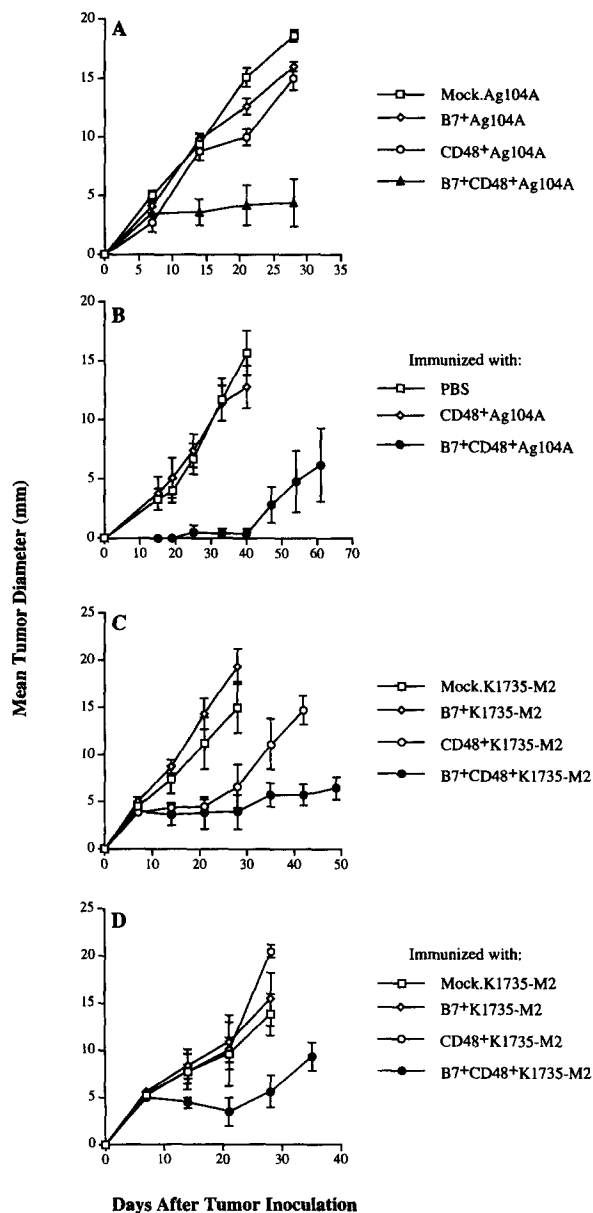
**Adoptive Immunotherapy of Micrometastasis Induced by wt Ag104A Sarcoma.** C3H mice, in groups of 5 or 10, were injected intravenously with Ag104A tumor cells at  $10^6$ /mouse. 3 d later they were injected intravenously with  $5 \times 10^6$  cells from the YL1 CTL line (see text) or from a C3H-derived anti-H-2<sup>d</sup> CTL line, which was used as a control, and they were given daily intraperitoneal injections of  $10^4$  U of human recombinant IL-2 in PBS for 3 d. Other groups of mice were treated with the same dosage of IL-2 or PBS. 3 wk after tumor injection, the mice were killed, the lungs were removed and fixed in Bouin's solution, and metastatic nodules were counted under a dissecting microscope; 200 metastatic nodules was the upper limit that could be accurately counted. In some experiments, the mice were monitored daily for survival. When a moribund mouse was found, it was killed, and a gross pathology examination was performed to identify metastases.

## Results

FACS<sup>®</sup> analysis with specific mAbs revealed that the Ag104A line expresses a high level of H-2K<sup>k</sup>, D<sup>k</sup>, and VCAM-1, whereas it does not express MHC class II or several potential costimulatory molecules, such as B7-1, B7-2, CD48, ICAM-1, ICAM-2, HSA, CD40, and CD40L. The K1735-M2 line expresses very low levels of MHC class I and II molecules and has none of the accessory molecules tested for (data not shown). A recombinant plasmid, pRC/CMV.mCD48, containing the murine CD48 cDNA, was constructed (Fig. 1). Four tumor lines, wt Ag104A, B7<sup>+</sup>Ag104A (12), wt K1735-M2, and B7<sup>+</sup>K1735-M2 (8, 11), were transfected with this vector, and clones obtained from each line were screened by FACS<sup>®</sup> analysis for the expression of CD48. Three to five clones from each tumor line with stable cell surface expression of CD48, B7-1, or both were picked. After in vivo tumorigenicity tests (Fig. 2), one clone from each line was selected for further exper-



**Figure 1.** Expression of CD48 and B7-1 molecules on tumor cells. (A) Structure of the pRC/CMV.mCD48 construct. The murine CD48 open reading frame is driven by the CMV promoter. The vector contains the neomycin resistant gene (*neo*) for drug selection. (B) Cell surface expression of murine CD48 and B7-1 on transfected Ag104A lines. The wt Ag104A, CD48<sup>+</sup>Ag104A, and B7<sup>+</sup>CD48<sup>+</sup>Ag104A cells were stained with FITC-conjugated anti-CD48 mAb, anti-B7-1 mAb, or the control mAb and subjected to FACS<sup>®</sup> analysis as described in Materials and Methods. A total of 5,000 cells were analyzed for each sample. (C) Cell surface expression of murine CD48 and B7-1 on transfected K1735-M2 cells. The wt K1735-M2, CD48<sup>+</sup>K1735-M2, and B7<sup>+</sup>CD48<sup>+</sup>K1735-M2 cells were stained and analyzed as described in B.



**Figure 2.** Tumorigenicity and immunogenicity of the transfectants derived from the Ag104A and K1735-M2 cells in C3H mice. C3H mice, in groups of 10, were injected subcutaneously with the transfectants from Ag104A at  $10^6$ /mouse (A) or from K1735-M2 at  $2 \times 10^6$ /mouse (C). Tumor size was assessed at regular intervals. The experiments were terminated when some tumors reached 20–25 mm in diameter, severe ulceration or bleeding had developed, or the mice had died. For immunization, C3H mice, in groups of 5 or 10, were injected subcutaneously and intraperitoneally with PBS or with irradiated (2,500 rad) transfectants from Ag104A cells (B) or from K1735-M2 cells (D) at  $5 \times 10^6$ . 2–3 wk later, they were challenged subcutaneously at a distant site with wt Ag104A cells (B) or with wt K1735-M2 cells (D). The results are expressed as mean diameters (in millimeters) of tumors from all mice in the group. Error bars represent SD of the means. Similar results were obtained in a repeat experiment with each cell line.

iments. The CD48 and B7-1 expression in several representative clones from both Ag104A and K1735-M2 cells is shown in Fig. 1.

The tumorigenicity of the transfectants was tested by subcutaneous injection of C3H mice with cells from each clone at a minimal tumorigenic dose (8). The wt Ag104A tumor grew progressively in all mice, as did tumors induced by CD48<sup>+</sup>Ag104A and B7<sup>+</sup>Ag104A cells. In contrast, transfectants expressing both CD48 and B7-1 (B7<sup>+</sup>CD48<sup>+</sup>Ag104A) grew more slowly or not at all in the mice (Fig. 2 A). 6 out of 10 mice inoculated with B7<sup>+</sup>CD48<sup>+</sup>Ag104A remained tumor-free for >2 mo. Similar results were obtained using K1735-M2 transfectants (Fig. 2 C).

C3H mice, in groups of 5 or 10, were immunized by the double transfectants from either Ag104A or K1735-M2 and then challenged with wt tumor cells to examine whether they had developed any protective tumor immunity. All mice immunized with B7<sup>+</sup>CD48<sup>+</sup>Ag104A cells were resistant to the wt tumor challenge, whereas mice immunized with CD48<sup>+</sup>Ag104A or given PBS as a control displayed no protective immunity (Fig. 2 B). However, the protective immunity induced by B7<sup>+</sup>CD48<sup>+</sup>Ag104A was not long lasting, since 60% of the mice developed progressive tumors at the injection site that were detected 55 d after tumor challenge. Similar results were obtained after immunization of mice with B7<sup>+</sup>CD48<sup>+</sup>K1735-M2 cells: although all mice immunized with mock-, CD48-, or B7-1-transfected K1735-M2 cells displayed no protective immunity, fewer tumors grew out in mice immunized with B7<sup>+</sup>CD48<sup>+</sup>K1735-M2 cells (Fig. 2 D). 40% of the mice were resistant to outgrowth of cells from wt K1735-M2 tumor during the initial 4 wk, although only 20% of them were tumor free 5 wk after challenge. We conclude that growth of Ag104A and K1735-M2 was inhibited and that protective immunity could be induced against them, but only when both CD48 and B7-1 were expressed in the immunizing tumor cells.

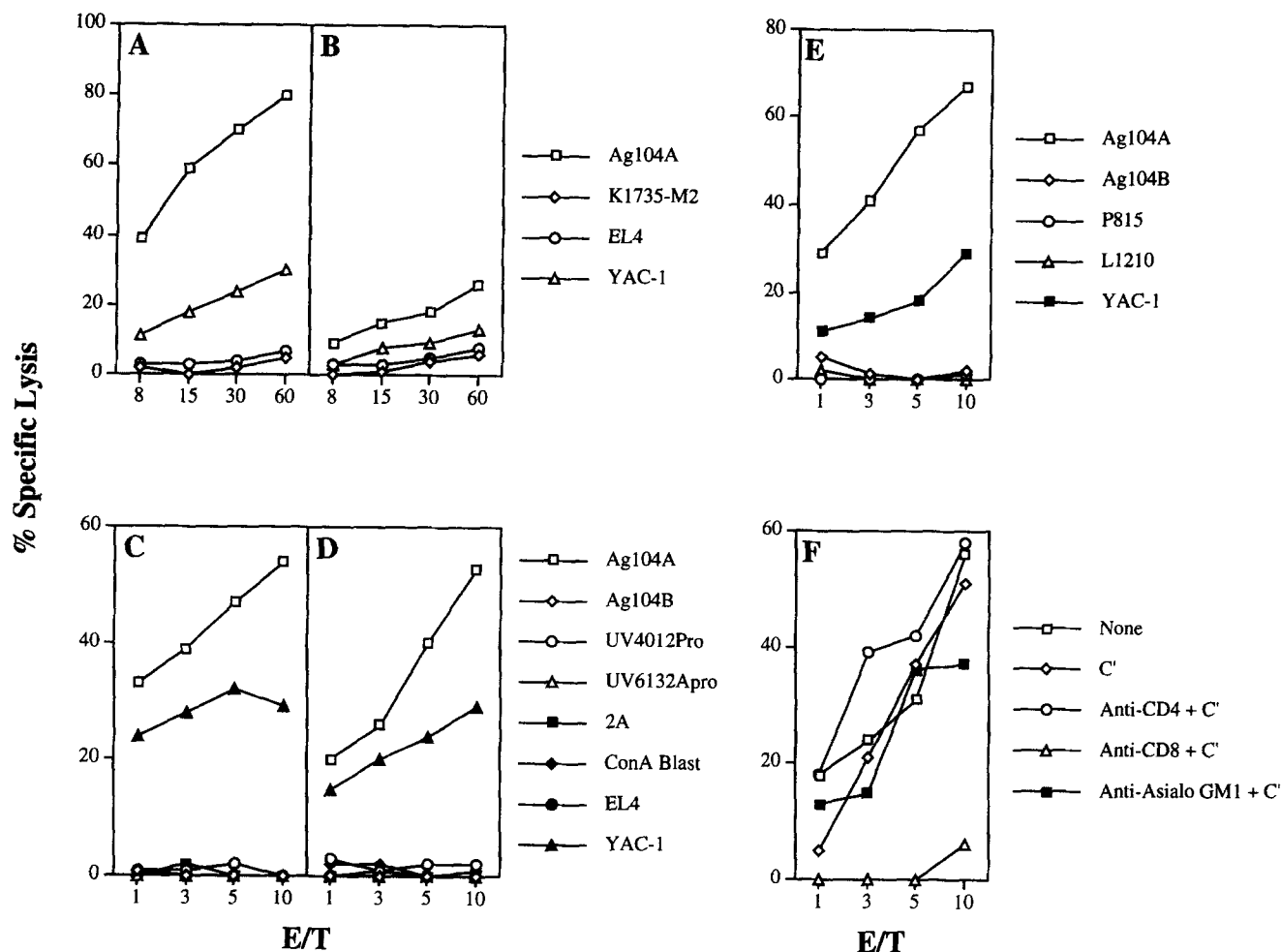
Splenocytes from mice immunized with the double transfectants were harvested and further stimulated *in vitro* by cocultivation with irradiated wt Ag104A cells for 5 d. Subsequently, they were tested for CTL activity in a standard <sup>51</sup>Cr release assay. As indicated in Fig. 3, bulk-cultured spleen cells from mice immunized with B7<sup>+</sup>CD48<sup>+</sup>Ag104A revealed a high level of CTL activity against wt Ag104A cells (Fig. 3 A). In contrast, bulk-cultured spleen cells from the mice immunized with CD48<sup>+</sup>Ag104A (Fig. 3 B) or B7<sup>+</sup>Ag104A (12) did not show any significant CTL activity to wt Ag104A targets. The bulk-cultured CTL from mice immunized with B7<sup>+</sup>CD48<sup>+</sup>Ag104A also lysed the NK-sensitive YAC-1 cell line, albeit at a lower level.

In an attempt to obtain tumor-reactive CTL lines for adoptive immunotherapy, bulk-cultured spleen cells were repeatedly stimulated *in vitro* with irradiated Ag104A cells, and they were then tested for CTL activity. A representative CTL line, YL1 (Fig. 3 C), and a clone, c18 (Fig. 3 D), had high cytolytic activity against wt Ag104A cells, whereas there was no lysis of a panel of other targets, including the

syngeneic tumor lines Ag104B, UV4102Pro, UV6132APro, 2A, syngeneic ConA lymphoblasts, and several allogenic tumor lines, including EL4 (H-2<sup>b</sup>) (Fig. 3, C and D), P815 (H-2<sup>b</sup>), and L1210 (H-2<sup>b</sup>) (Fig. 3 E). However, both YL1 and c18 lysed YAC-1 at a significant level. The YL1 line has been passaged in vitro over a 10-mo period and retains its cytolytic activity against wt Ag104A. Incubation of the YL1 line with an anti-CD8 mAb, but not with an anti-CD4 mAb or with an anti-Asialo GM1 antiserum, in the presence of complement, eliminated the cytolytic activity (Fig. 3 F), whereas the same treatment with anti-Asialo GM1 antiserum abrogated NK activity of spleen cells from mice injected with polyI:C (14) (data not shown). We conclude that CD8<sup>+</sup>, MHC class I-restricted CTL that are specific for Ag104A can be generated from mice immunized with B7<sup>+</sup>CD48<sup>+</sup>Ag104A cells and that they can be maintained in vitro for >10 mo.

We then test whether anti-Ag104 CTL can be used for the treatment of pulmonary micrometastasis induced by

Ag104A. C3H mice were injected intravenously with wt Ag104A cells at 10<sup>6</sup>/mouse, and 4 d later the mice were treated with either YL1 CTL plus IL-2 or IL-2 alone. In contrast to the formation of lung metastases in mice treated with IL-2 alone, six out of seven mice treated by YL1 plus IL-2 were free of lung tumors at day 20. The antimetastatic effect of the CTL was specific for Ag104A tumor since the same treatment did not inhibit the growth of lung metastatic tumors induced by either Ag104B or K1735-M2 (Table 1). The effect of anti-Ag104 CTL on survival of tumor-bearing mice was also observed. Intravenous injection of mice with wt Ag104A cells at 10<sup>6</sup>/mouse killed 100% of the mice within 50 d. In contrast, 10 out of 10 mice injected with cells from the YL1 line plus IL-2 remained alive after >160 d, whereas the mice treated with either IL-2 or IL-2 combined with a control CTL line did not live any longer than the PBS-treated group (Fig. 4). Mice that died were shown to have tumor growth in the lungs, and in some cases also in the back, neck, and bones.



**Figure 3.** Cytolytic activity of CTL generated from mice immunized with B7<sup>+</sup>CD48<sup>+</sup>Ag104A. C3H mice were injected subcutaneously with either CD48<sup>+</sup>B7<sup>+</sup>Ag104A cells (A) or CD48<sup>+</sup>Ag104A cells (B) at 10<sup>6</sup>/mouse. 10 d later, tumor nodules were removed surgically. Spleen cells were prepared 14 d after injection and were cocultivated with  $\gamma$ -irradiated wt Ag104A cells for 5 d. CTL activity against <sup>51</sup>Cr-labeled target cells, as indicated, was measured as described in Materials and Methods. Cytolytic activity of YL1 (C and E) and c18 clone (D) of CTL against <sup>51</sup>Cr-labeled target cells, as indicated, was also measured. For T cell subset depletion experiments (E), cells from the YL1 line were pretreated with anti-CD4 and anti-CD8 mAbs or anti-Asialo GM1 antiserum in the presence of complement, as described in Materials and Methods before assay for their cytolytic activity.

## Discussion

We have demonstrated that coexpression of CD48 with B7-1 in two classically poorly immunogenic tumors, the Ag104A sarcoma and the K1735-M2 melanoma, made them immunogenic, as indicated by their inhibited growth in syngeneic C3H mice and by their ability to induce a certain level of protective immunity against challenge with wt tumor cells. Furthermore, tumor-specific CTL could be generated in vitro (Fig. 3) and used for adoptive immunotherapy to cure micrometastasis induced by wt Ag104 cells (Fig. 4). Our results suggest that poor immunogenicity of tumors does not always reflect an inherent lack of tumor antigens or failure of processing/presentation of such antigens.

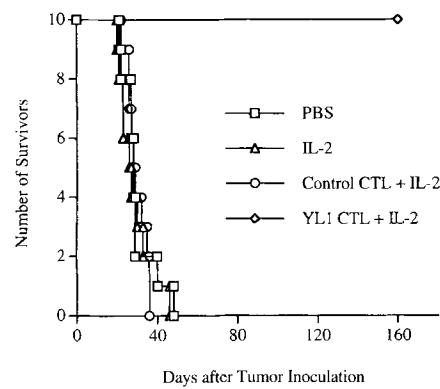
Transfection of both CD48 and B7-1 was required for induction of immunity against Ag104A and K1735-M2 since tumor cells expressing either the CD48 or the B7-1 gene alone were poorly immunogenic (Fig. 2). There is evidence that CD2 signaling can stimulate the signal transduction pathway in a manner similar to that of TCR-MHC antigen ligation (3). Another consequence of CD48-CD2 interaction is an increased cell-cell adhesion (3), and this may also engage a greater number of receptor-ligand pairs. CD48 transfection into tumor cells may, therefore, elevate the signal delivered by MHC-presented tumor antigen to the threshold critical for triggering the TCR. The expression of the E7 protein of human papilloma virus type 16, in conjunction with B7-1, makes K1735-M2 highly immunogenic (11), demonstrating that this tumor line has the machinery needed for antigen processing/presentation, despite an undetectable MHC class I expression according to FACS<sup>®</sup> analysis (Table 1). However, immunization by B7<sup>+</sup>CD48<sup>+</sup>K1735-M2 appears to be less effective in inducing systemic immunity (Fig. 2 D) than that seen with the Ag104A double transfectant (Fig. 2 B). This may be caused by a lack of other accessory molecules and/or the

**Table 1.** Treatment of Mice with Lung Micrometastasis by Adoptive Transfer of the Anti-Ag104A CTL Line YL1

Tumor	YL1	No. lung metastases
Ag104A	+	0, 0, 4, 0, 0, 0, 0, 0*
	-	11, 8, 12, 7, 6, 5, 1
Ag104B	+	15, 16, 12, 18, 5, 10, 8
	-	2, 10, 24, 17, 11, 2, 5, 8
K1735-M2	+	>200 (in all six mice)
	-	>200 (in all five mice)

C3H mice were injected intravenously with 10<sup>6</sup> tumor cells as indicated. 4 d after tumor inoculation, mice were injected intravenously with 5 × 10<sup>6</sup> cells of YL1 CTL, followed by intraperitoneal injection with 10<sup>4</sup> U of recombinant human IL-2; injection of IL-2 was repeated once a day for 3 consecutive days. Control groups received IL-2 treatment without CTL. 20 d after tumor inoculation, mice were killed and the number of lung metastases from each individual mouse was recorded.

\*Significantly different from the number of metastases obtained with control group without injection of YL1 CTL (*P* < 0.01).



**Figure 4.** Cure of 3-d pulmonary micrometastasis of wt Ag104A cells by adoptive transfer of YL1 CTL. Mice, in groups of 10, were injected intravenously with wt Ag104A cells at 10<sup>6</sup>/mouse. 3 d later they were treated with 5 × 10<sup>6</sup> cells of YL1 CTL line by intravenous injection and followed the next day with an intraperitoneal injection of 10<sup>4</sup> U of recombinant human IL-2 once a day for 3 d. Control groups included mice injected with either PBS alone, IL-2 alone, or a combination of the control CTL line and IL-2. The mice were monitored daily for survival. Similar results were obtained with each CTL line in a repeat experiment.

very low expression of MHC class I molecules on K1735-M2 cells, whereas Ag104A cells express high levels of MHC class I and VCAM-1 molecules (Table 1). It is noteworthy that although the protective immunity was induced by CD48 and B7-1 double transfectants, many immune mice eventually developed tumors (Fig. 2, B and D). The possibilities that the tumors lose antigen or MHC class I molecules so as to escape the immune destruction are under investigation.

CD2 is expressed by 80–90% of human NK cells, and expression of CD58 and CD59 (ligands for CD2) in target cells can activate NK cells (15), as can B7-CD28 costimulation (16). This may explain the lysis of YAC-1 cells by bulk-cultured spleen cells from mice immunized with B7<sup>+</sup>CD48<sup>+</sup>Ag104A (Fig. 3 A). The CTL activity against Ag104A cells observed in our studies is, however, not likely to be mediated by NK cells, since the YL1 line and the c18 clone behave as typical MHC class I-restricted CD8<sup>+</sup> CTL selective for Ag104A cells, except for their ability to lyse YAC-1 cells (Fig. 3, C and D). The fact that the lysis on Ag104A cells was not eliminated by treatment of the YL1 line with an anti-Asialo GM1 antiserum (Fig. 3 E), combined with published evidence that antigen-specific CTL can be promiscuous by lysing a variety of tumor cells (17), supports this possibility. It is noteworthy that our CTL could be propagated in vitro by exposure to irradiated wt Ag104A cells, that is, that it did not require the presence of B7 and CD48 in the Ag104A cells. This emphasizes that the expression of CD48 and B7-1 in the tumor cells is primarily needed for the in vivo priming of the CTL.

It remains to be explored whether the poor immunogenicity of other tumors can also be reversed by the transfection of both CD48 and B7-1. If so, an approach similar to that described here may become useful for the development of therapy of human malignancies, which are normally poorly immunogenic.

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