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Adoptive transfer of cytotoxic T lymphocytes induced by CD86-transfected tumor cells suppresses multi-organ metastases of C1300 neuroblastoma in mice

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Abstract In this study, we examined the therapeutic antitumor effect of cytotoxic T lymphocytes (CTL) generated against CD86-transfected mouse neuroblastoma C1300. We first generated the transfectant, CD86$^+$C1300, expressing a high level of mouse CD86 on the cell surface. While CD86$^+$C1300 cells were rejected in syngeneic A/J mice when inoculated subcutaneously, neither vaccination nor any therapeutic antitumor effect was obtained, implying that C1300 may be a poorly immunogenic tumor. However, in vitro stimulation of splenocytes from either C1300-bearing or CD86$^+$C1300-rejecting mice with CD86$^+$C1300 cells resulted in remarkable CTL activity against C1300 cells. The CTL activity induced by CD86$^+$C1300 was mediated by T cell receptor/CD3 and CD8 and was further enhanced by the addition of interleukin-2. Intravenous inoculation of C1300 cells led to multiple organ metastases including the liver, lung, kidney, ovary, lymph node and bone marrow. To examine the therapeutic effect of CTL in this metastasis model, CTL induced by parental or CD86$^+$C1300 cells were administrated into C1300-bearing mice. Adoptive transfer of CD86$^+$C1300-induced CTL resulted in marked elimination of multi-organ metastases and prolonged survival in almost all mice, 70% of which survived indefinitely. These results indicate that adoptive transfer of CTL induced by CD86-transfected tumor cells in vitro would be effective and useful for tumor immunotherapy against poorly immunogenic tumors.

Key words Adoptive immunotherapy · Cytotoxic T lymphocytes · CD86 · Metastasis · Neuroblastoma

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Introduction

Signals initiated by both the T cell receptor CD3 complex and CD28 are required for optimal activation of T cells in various immune responses [18, 22, 31]. CD28 is expressed on most thymocytes and peripheral T cells and interacts with two ligands, designated CD80 (B7/B7-1) and CD86 (B70/B7-2), which are expressed on antigen-presenting cells such as activated monocytes, dendritic cells and B cells [2, 15, 17, 19]. The CD28-mediated costimulation efficiently augments T cell proliferation, cytokine production, and generation of cytotoxic T lymphocytes (CTL) from resting T cells, indicating that CD80 and CD86 play an important role in the elimination of T cell immune responses [1, 5, 14, 21, 34].

Recently, several studies have demonstrated the genetically modified tumor cells act as antigen-presenting cells after transduction of genes encoding CD80 or CD86, often leading to rejection of transplantable tumors in mice [5, 6, 14, 21, 34, 35]. Such modified tumor cells can also elicit host immune responses against the non-transduced parental tumor cells when immunogenic tumors such as RMA, EL-4 and P815 are used [7, 35]. In contrast, several poorly immunogenic tumors, such as B16 and Ag104, have failed to induce immune responses even after in vivo immunization with CD80 and/or CD86 transfectants [7, 16]. Therefore, alternative approaches to eradicate poorly immunogenic tumors are still required.

Adoptive immunotherapy has been one strategy of therapeutic treatments against malignant tumors [25]. The in vitro expansion and subsequent adoptive transfer of tumor-specific T cells into tumor-bearing hosts have proven efficacious in a variety of experimental tumor models in mice [8, 10, 13, 30]. However, it has been generally hard to induce autologous CTL activity after secondary in vitro stimulation with poorly immunogenic tumors. In this study, we demonstrated a potent stimulator activity of CD86-transfected tumor cells for inducing tumor-specific CTL in vitro and tested the anti-metastatic effect of adoptively transferred CTL in vivo.

Materials and methods

Mice

Female A/J mice, 5–6 weeks old, were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan) and maintained in our animal facilities.

Cell lines

A murine neuroblastoma cell line, C1300, and its subclone, N18TG-2, originating from A/J mice, were kindly provided by Dr. S. Saito (Institute of DNA Medicine, Jikei University School of Medicine, Tokyo) and Dr. H. Higashida (Department of Biophysics, Kanazawa University School of Medicine, Ishikawa) respectively. A murine lung carcinoma cell line 3LL, originating from a C57BL/6 mouse, was obtained from ATCC (Rockville, Md.). These cell lines were cultured in RPMI-1640 medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% heat-inactivated fetal calf serum (FCS; Biological Industries, Haemek, Israel), 100 IU/ml penicillin G, 1 mM sodium pyruvate, 2 mM L-glutamine, 2.3 mg/ml HEPES and 2.0 mg/ml $NaHCO_3$.

Antibodies

Monoclonal antibodies (mAb) reactive with mouse MHC class I H-2K^k (36-7-5, mouse IgG2a), MHC class II I-A^k (11-5.2, mouse IgG2b), CD86 (GL1, rat IgG2a), CD4 (GK1.5, rat IgG2b), CD8 (53-6.7, rat IgG2a) and CD3 (145-2C11, hamster IgG) were obtained from PharMingen (San DiegoÇalif.). mAb reactive with mouse MHC class I H-2D^d (34-5-8, mouse IgG2a) and H-2L^d (30-5-7, mouse IgG2a) were kindly provided by Dr. N. Shinohara (Mitsubishi Kasei Institute, Life Science, Tokyo) and Dr. K. Udaka (Department of Biophysics, Kyoto University, Kyoto) respectively. Fluorescein-isothiocyanate (FITC)-conjugated anti-(rat IgG) and anti-(mouse IgG) antibodies were purchased from Caltag Lab. (San Francisco, Calif.).

Generation of mouse CD86 transfectants

A murine CD86 cDNA clone was isolated by cross-hybridization with the human CD86 cDNA as described previously [27]. The neuroblastoma cell line C1300 was transfected with murine CD86 cDNA in BCMGS-hyg expression vector by using Lipofectin (Gibco BRL, Gaithersburg, Md.) according to the manufacturer's instruction. A stable transfectant expressing mouse CD86 was selected by 0.75 mg/ml hygromycin B (Wako Pure Chemical Industries, Osaka) and isolated by limiting dilution.

Immunofluorescence

To examine the expression of various cell-surface antigens on C1300 and the transfectants, cells were incubated with 1 μg indicated mAb for 30 min at 4 °C, followed by FITC-labeled anti-(rat IgG) or anti-(mouse IgG). After washing twice with phosphate-buffered saline (PBS), the cells were analyzed on FACScan (Becton Dickinson, San Jose, Calif.).

Generation of cytotoxic T lymphocytes (CTL)

For the sensitization in vivo, A/J mice were given s.c. inoculations of C1300 or CD86$^+$C1300 cells (3×10^6/mouse). Between 1 and 2 weeks after the inoculation, splenocytes (2.5×10^6/ml) from the C1300-bearing or CD86$^+$C1300-rejecting mice were cocultured with C1300 or CD86$^+$C1300 cells (2.5×10^5/ml) pretreated with mitomycin C (MMC; Kyowa Hakko Kogyo Co. Ltd., Tokyo) in upright 25-cm^2 flasks at 37 °C in 5% CO_2/air. Five days later, 50 U/ml recombinant human interleukin-2 (rhIL-2; Shionogi Pharmaceutical Co., Osaka, Japan) was added and cultured for 2 more days in some experiments. The cells were collected and cytotoxic activities were measured by 6-h ^{51}Cr-release assay as described previously [20]. To examine the inhibitory effect of mAb on CTL activity, the indicated mAb were added to the cytotoxic assay at a final concentration of 10 μg/ml.

Growth of subcutaneously implanted tumor cells

C1300 and CD86$^+$C1300 cells were harvested from the culture flask with 0.05% EDTA in PBS, washed three times, resuspended in PBS and inoculated subcutaneously (s.c.) into A/J mice (3×10^6 cells/mouse). Tumor growth was measured three times per week with a digital caliper and recorded as the longest surface length (mm; *a*) and width (*b*). Tumor volume (*V*; mm^3) was calculated according to the following formula: $V = ab^2/2$. Each group consisted of 6 mice.

Multi-organ metastases of C1300

C1300 cells were prepared as described above and inoculated intravenously (i.v.) into A/J mice (1×10^5 cells/mouse). After 27 days, the mice were sacrificed and organs, including the lung, liver, kidney, ovary and mesenteric lymph node, were excised and fixed in 20% formalin/PBS. Tumor nodules in these organs were observed visually. Tumor cells in bone marrow cell suspensions were also observed under the microscope.

Adoptive transfer of CTL into C1300-bearing mice

A/J mice were first inoculated i.v. with C1300 (1×10^5 cells/mouse) on day 0, followed by i.v. administration of CTL (1×10^7 cells/mouse) on day 2 and day 9. After 27 days, the mice were sacrificed and observed for tumor metastases as described above. Another set of mice were monitored daily for survival until 4 months. Each group consisted of 6–15 mice.

Results

Generation and characterization of CD86-transfected C1300 cells

Several recent studies have shown that the introduction of costimulatory molecules, such as B7-1 (CD80) and B7-2 (CD86), was effective for the activation of tumor-reactive T cells [5–7, 14, 21, 34, 35]. To generate tumor-specific CTL, we introduced the murine CD86 (mCD86) gene into a neuroblastoma cell line, C1300, originating from an A/J mouse. The mCD86 cDNA was subcloned into the mammalian expression vector BCMGS-hyg and transfected into C1300 using lipofectin. Stable transfectants were selected by hygromycin B and cloned by limiting dilution. To verify the expression of mCD86 on the transfectants, we performed immunofluorescence using a mAb reactive with mCD86. As shown in Fig. 1A, a stable transfectant expressing a high level of mouse CD86, CD86$^+$C1300, was established. In contrast, no detectable level of CD86 or CD80 (not shown) was expressed on the parental C1300 cells. As shown in Fig. 1B, both C1300 and CD86$^+$C1300 cells expressed equivalent levels of MHC class I molecules (H-2D^d and H-2K^k), but they lacked any detectable MHC class II molecules (I-A^k).

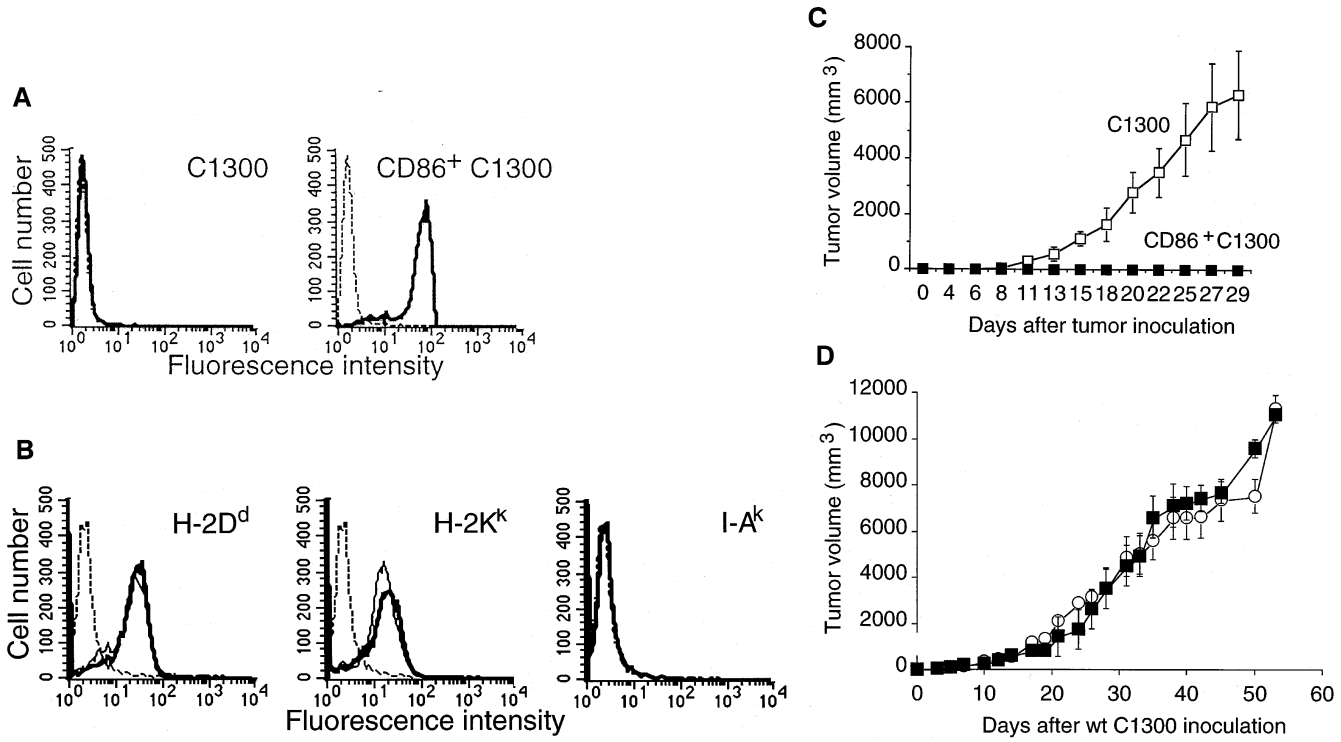

Fig. 1A–D Characterization of CD86-transfected C1300 cells. **A** Expression of murine CD86 molecule on wild type and transfected tumor lines. Wild type C1300 cells and CD86 transfectants were stained with anti-CD86 mAb (——) followed by fluorescein-isothiocyanate(FITC)-labeled goat anti-(rat IgG) antibody. - - - - - Background staining with control mouse IgG2a. **B** Cell-surface expression of MHC class I (H-2D^d, K^k) and class II (I-A^k) on C1300 (——) and CD86$^+$C1300 (——) cells. Cells were stained with mAb to H-2D^d, K^k, or I-A^k followed by FITC-labeled goat anti-(mouse IgG) antibody. - - - - - Background staining with control mouse IgG. **C** Tumorigenicity of C1300 and CD86$^+$C1300 in syngeneic mice. A/J mice were s.c. inoculated with C1300 (□) or CD86$^+$C1300 (■) (3×10^6/mouse). The results are expressed as mean ± SD tumor volume from 6 mice in each group. Similar results were obtained in two independent experiments. **D** Vaccinating effect of CD86$^+$C1300. A/J mice were first inoculated s.c. with CD86$^+$C1300 cells (3×10^6/mouse). Ten days after the inoculation of CD86$^+$C1300, these mice (■) were s.c. challenged with wild-type C1300 cells (3×10^6/mouse). Naive A/J mice (○) were also s.c. inoculated with the same number of wild-type C1300 as a control group. The results are expressed as mean ± SD tumor volume from 6 mice in each group. Similar results were obtained in two independent experiments

To examine tumorigenicity of the CD86 transfectants in vivo, parental C1300 and CD86$^+$C1300 (3×10^6 cells/mouse) were s.c. inoculated into syngeneic A/J mice. As shown in Fig. 1C, parental C1300 cells formed progressively growing tumor in all mice, but the CD86$^+$C1300 cells completely regressed after a transient growth in all recipients. We next examined the efficacy of CD86$^+$C1300 cells as a tumor vaccine in syngeneic mice. After the primary rejection of s.c. inoculated CD86$^+$C1300 cells, the mice were challenged s.c. with the same number of parental C1300 cells. Unexpectedly, as shown in Fig. 1D, parental C1300 cells were not rejected in these recipients. This indicated that a protective immunity against C1300 cells was not elicited by the primary rejection of CD86$^+$C1300 cells. Immunization with irradiated C1300 or CD86$^+$C1300 cells also did not elicit a protective immunity against C1300 cells (data not shown). Moreover, no therapeutic effect was observed when the CD86$^+$C1300 cells were s.c. inoculated into C1300-bearing mice (data not shown). These results suggested that C1300 is poorly immunogenic in syngeneic hosts and, therefore, another therapeutic strategy was required to eradicate the established tumors.

Induction of C1300-specific CTL by stimulation with CD86$^+$C1300 cells in vitro

To examine the presence of CTL precursors and to generate tumor-specific CTL in vitro, we next performed mixed lymphocyte/tumor cultures (MLTC) by using splenocytes from naive, C1300-bearing, or CD86$^+$C1300-rejecting A/J mice. As shown in Fig. 2A, in vitro stimulation with the CD86$^+$C1300 cells could induce strong cytotoxic activity against C1300 cells in splenocytes from either C1300-bearing or CD86$^+$C1300-rejecting mice, but not in those from naive mice. In contrast, no cytotoxic activity was observed in these splenocytes when stimulated with C1300 cells. CTL induced by CD86$^+$C1300 also lysed a subclone of C1300, N18TG-2, but not an irrelevant allogeneic tumor 3LL (Fig. 2B). Furthermore, the cytotoxic activity was inhibited by mAb to CD3, CD8, MHC class I, and LFA-1, but not by those to CD4 or MHC class II (Fig. 2C), suggesting that the induced effector cells were typical CD8$^+$ CTL whose TCR/CD3 might recognize some tumor antigen of C1300 presented by MHC class I.

To further expand the tumor-specific CTL for adoptive immunotherapy, we examined the effect of exogenous IL-2

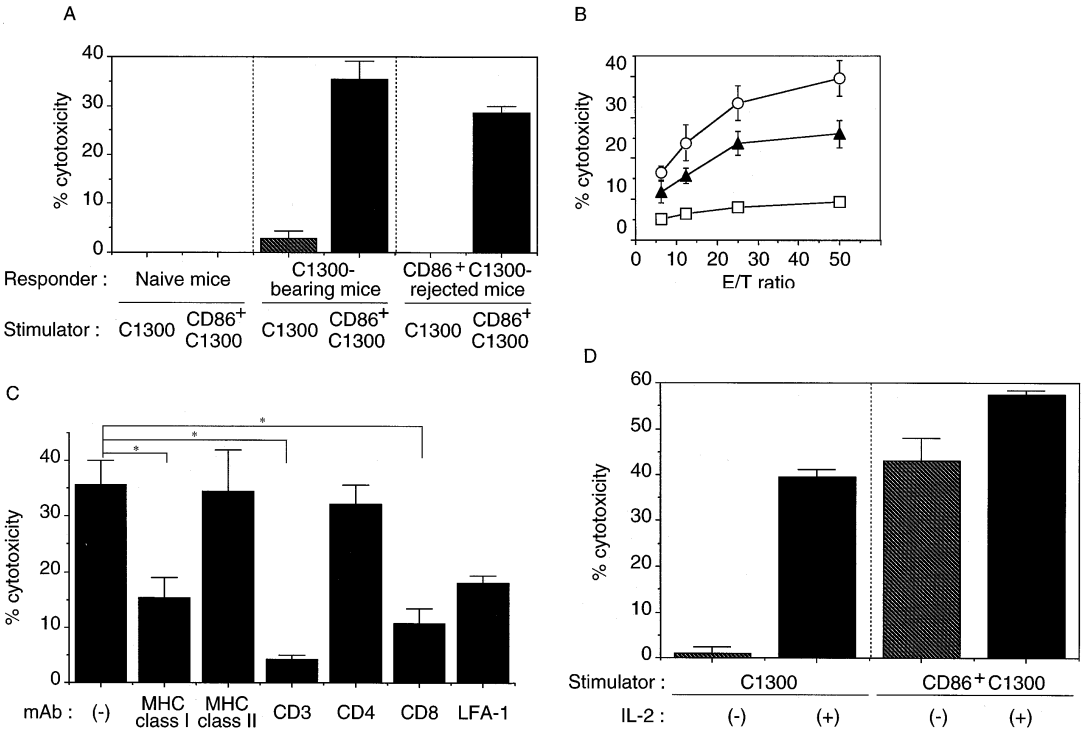

Fig. 2A–D Characterization of cyotoxic T lymphocytes (CTL) induced by CD86-transfected C1300 cells. **A** CTL activity after mixed lymphocyte/tumor cell culture. A/J mice were s.c. inoculated with C1300 or CD86$^+$C1300 cells (3×10^6/mouse). Seven days later, spleen cells (responder) were prepared from naive, C1300-bearing, or CD86$^+$C1300-rejecting mice and cocultured with mitomycin-C(MMC)-treated C1300 or CD86$^+$C1300 cells (stimulator) for 5 days. Cytotoxic activity against wild-type C1300 cells was tested by 6-h ^{51}Cr-release assay at an E/T ratio of 50. Data are indicated as means ± SD of triplicate wells. Similar results were obtained in three independent experiments. **B** Specificity of CTL induced by CD86$^+$C1300 cells. Splenocytes from CD86$^+$C1300-rejecting mice were cultured with MMC-treated CD86$^+$C1300 cells for 5 days and then used as effector cells. Cytotoxic activity against C1300 (○), N18TG-2 (▲), and 3LL (□) target cells was tested by 6-h ^{51}Cr-release assay at the indicated E/T ratios. Data represent means ± SD of triplicate wells. Similar results were obtained in two independent experiments. **C** Inhibitory effect of monoclonal antibodies (mAb) on CTL activity. CTL were generated as described in B and cytotoxic activity against C1300 target cells was tested in the presence or absence of mAb to the indicated molecules (10 μg/ml each) at an E/T ratio of 50. Data represent means ± SD of triplicate wells. * $P<0.01$. Similar results were obtained in two independent experiments. **D** Enhancement of cytotoxic activity by interleukin-2 (*IL-2*). Splenocytes from CD86$^+$C1300-rejecting mice were cultured with MMC-treated wild-type C1300 or CD86$^+$C1300 cells for 5 days followed by an additional 2-day culture in the presence or absence of 50 U/ml IL-2. Cytotoxic activity against C1300 target cells was tested by 6-h ^{51}Cr-release assay at an E/T ratio of 50. Data represent means ± SD of triplicate wells. Similar results were obtained in two independent experiments

for CD86$^+$C1300-stimulated CTL. Splenocytes from tumor-rejecting mice were cultured with CD86$^+$C1300 cells for 5 days without IL-2 followed by the addition of 50 U/ml IL-2 for 2 days. The addition of IL-2 doubled the collected effector cell number and enhanced the cytotoxic activity (Fig. 2D). This cytotoxic activity was also blocked by mAb to CD3 and CD8 (not shown). The addition of IL-2 to the splenocytes stimulated with C1300 also enhanced the cytotoxic activity (Fig. 2D), but this was not specific for C1300 and was not inhibited by anti-CD3 or anti-CD8 mAb (not shown), suggesting that lymphokine-activated killer (LAK) cells, but not CTL, were induced by exogenous IL-2 in these conditions. These results indicated that CD86 expressed on the tumor cells plays a critical role in the induction of C1300-specific CTL in vitro.

Adoptive transfer of CTL induced by CD86 transfectants suppresses multi-organ metastases and prolongs the survival

To investigate the therapeutic effect of the C1300-specific CTL induced by CD86$^+$C1300 stimulation in vitro, a metastasis model was established by i.v. inoculation of C1300 cells into syngeneic A/J mice. After 27 days, all mice had numerous tumor nodules in multiple organs, including the lung, liver, kidney, and ovary (Fig. 3A), and the tumor cells also invaded the mesenteric lymph node and bone marrow (not shown).

We then divided the A/J mice that had been i.v. inoculated with 1×10^5 C1300 cells on day 0 into three treatment groups, which were i.v. administered PBS (group 1), 1×10^7 C1300-induced CTL (group 2), or 1×10^7 CD86$^+$C1300-induced CTL (group 3) on day 2 and day 9. The presence of metastatic nodules in various organs was examined on day 27. As shown in Fig. 3B and summarized in Table 1,

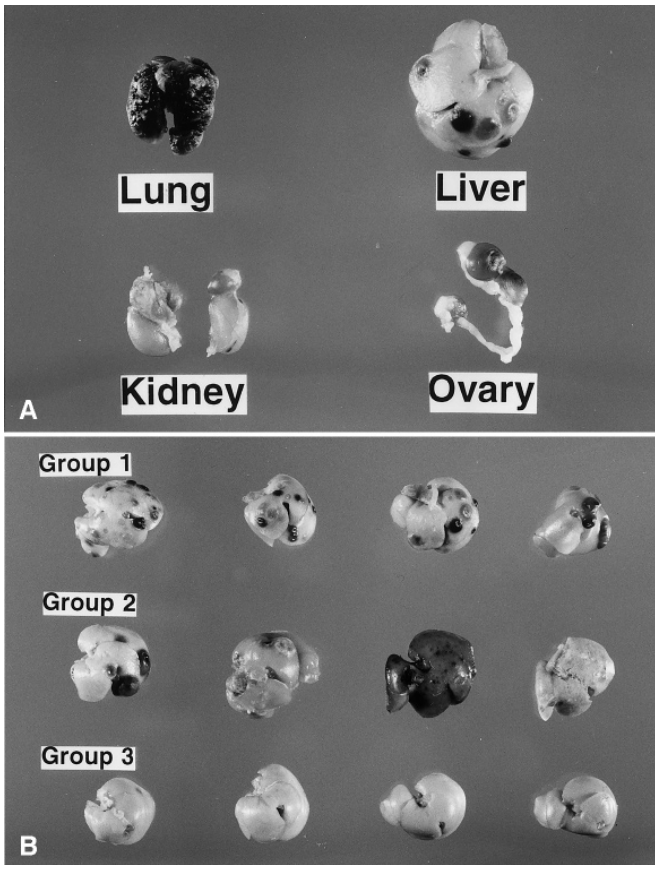

Fig. 3A, B Anti-metastatic effect of adoptively transferred CTL. **A** Metastases of C1300 cells in multiple organs. A/J mice were i.v. inoculated with wild-type C1300 cells (1×10^5/mouse) and, 27 days after the inoculation, the mice were sacrificed to examine the metastases. Multiple metastatic nodules were found in the lung, liver, adrenal gland, kidney and ovary. **B** Adoptive transfer of CD86$^+$C1300-induced CTL suppresses liver metastasis of C1300. Multiple metastatic nodules were found in the liver when treated with phosphate-buffered saline or C1300-induced CTL. Reduction of metastases is apparent when treatment was with CD86$^+$C1300-induced CTL

Table 1 Adoptive transfer of CD86$^+$C1300-induced cytotoxic T lymphocytes (*CTL*) prevents multi-organ metastases of C1300. A/J mice were i.v. inoculated with wild-type C1300 cells (1×10^5/mouse), followed by i.v. inoculation of phosphate-buffered saline (*PBS*), C1300-induced CTL (1×10^7/mouse), or CD86$^+$ C1300-induced CTL (1×10^7/mouse) on day 2 and day 9. On day 27, the mice were sacrificed to examine the metastases in multiple organs. Results are presented as the number of mice with metastatic nodules/total number of mice examined, and the percentage incidence is in parentheses. Results are a summary of three independent experiments

Treatment	Frequency of metastases						
	Lung	Liver	Adrenal gland	Kidney	Ovary	Lymph node	Bone marrow
PBS	13/15 (87%)	11/15 (73%)	8/15 (53%)	2/15 (13%)	9/15 (60%)	9/15 (60%)	10/15 (67%)
C1300-induced CTL	6/6 (100%)	4/6 (67%)	6/6 (100%)	1/6 (17%)	5/6 (83%)	4/6 (67%)	5/6 (83%)
CD86$^+$ C1300-induced CTL	0/14* (0%)	1/14* (7%)	3/14* (21%)	0/14* (0%)	0/14* (0%)	2/14* (14%)	0/14* (0%)

* Significantly different from the PBS or C1300-induced CTL group ($P < 0.01$)

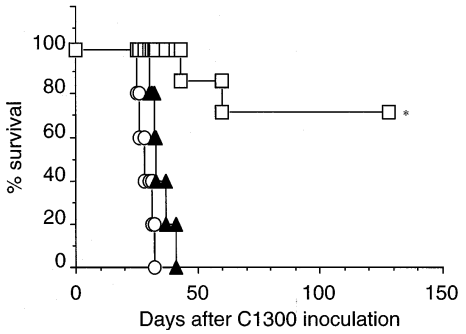

Fig. 4 Adoptive transfer of CD86$^+$C1300-induced CTL prolongs the survival of C1300-bearing mice. A/J mice were i.v. inoculated with C1300 cells (11×10^5/mouse) on day 0, and PBS (○), C1300-induced CTL (▲), or CD86$^+$C1300-induced CTL (□) (11×10^7/mouse) was administered on day 2 and day 9. Survival of tumor-bearing mice was monitored daily for 4 months. Data are presented as a Kaplan-Meier plot. Each group consisted of 6–15 mice. * $P<0.01$ as compared to the PBS or C1300-induced CTL group

the mice receiving adoptively transferred C1300-induced CTL (group 2), as well as control mice (group 1), exhibited high incidences of tumor metastases in various organs. In contrast, the mice inoculated with the CD86$^+$C1300-induced CTL (group 3) developed few metastases in almost all organs examined. Furthermore, while all mice in group 1 and group 2 died within 40 days, the mice in group 3 showed marked prolongation of survival and 10/14 survived indefinitely (Fig. 4). These results indicate that tumor-specific CTL induced by the stimulation with CD86 transfectants in vitro exert a potent therapeutic effect in vivo for eliminating metastatic tumors in various organs.

Discussion

We here demonstrated that CD86-transfected tumor cells function as antigen-presenting cells to induce tumor-specific CTL in splenocytes from tumor-bearing mice in vitro. CTL induced by stimulation with CD86$^+$C1300 could efficiently lyse the parental tumor cells, and the delayed addition of IL-2 enhanced the expansion of CTL. We also demonstrated a potent antitumor effect of such CTL when adoptively transferred into tumor-bearing mice. Only two i.v. inoculations of CTL resulted in elimination of the majority of metastases in multiple organs and markedly improved survival.

CD86, as well as CD80, acts as a counterreceptor for CD28 and can provide a costimulatory signal for T cell activation [2, 5, 21]. Several recent studies have suggested that CD80 and CD86 may play differential roles in regulating immune responses [4, 32]. It has been reported that CD86 was preferentially involved in the development of T helper 2 cells producing IL-4 in vitro and in vivo [11, 28]. Differential abilities of CD80 and CD86 to confer immu-

nogenicity on some murine tumor cells have been reported consistently by several groups [12, 23, 24]. However, it has also been reported that the transfectants expressing either CD80 or CD86 exhibited a similar ability to costimulate T cell proliferation, cytokine production, and generation of CTL [5, 21] and that CD86 could induce antitumor immunity as efficiently as CD80 [35]. We also generated C1300 transfectants expressing either CD80 or CD86. Both transfectants regressed when s.c. inoculated into syngeneic mice, and CTL activities were similarly induced when splenocytes from tumor-bearing mice were stimulated with $CD80^+$C1300 or $CD86^+$C1300 in vitro (data not shown). Therefore, the differential abilities of CD80 and CD86 to elicit antitumor immunity may depend on the nature of the tumor cells used.

Some tumor cells transfected with costimulatory molecules or cytokines often act as a cellular vaccine capable of inducing protective and therapeutic host immune responses [9]. Several studies have shown that the transduction of CD80 or CD86, increased the tumor immunogenecity and induced CTL responses to the tumors. Previous work showed that CD80-transfected tumors were rejected in syngeneic hosts and conferred protection against subsequent challenge with parental CD80-negative tumors [3, 14, 34]. It was also reported that such an antitumor immune response was similarly elicited by tumor cells expressing CD86 [35]. In this study, we also examined the vaccinating effect of $CD86^+$C1300 cells. However, as shown in Fig. 1D, we found that such a protective immunity was not elicited by the primary rejection of s.c. inoculated $CD86^+$C1300 cells, suggesting that C1300 is a poorly immunogenic tumor. A similar case has been reported with CD80-transfected B16 melanoma cells, against which the primary rejection was likely mediated by natural killer cells and the protective immunity was not elicited [36]. Consistent with this notion, C1300 but not $CD86^+$C1300 cells grew in T cell-deficient nude mice (data not shown), suggesting a major contribution of non-T cells to the primary rejection.

To overcome such an inability of $CD86^+$C1300 cells to elicit protective immunity in vivo, we next tried to raise CTL in vitro for adoptive immunotherapy. To date, several kinds of effector cells have been applied to adoptive immunotherapy against murine or human tumors. One approach has been the adoptive transfer of LAK cells that are induced from peripheral blood lymphocytes (PBL) by high doses of IL-2 and preferentially lyse a variety of tumor cells in vitro. Although LAK cells can be easily generated, they accumulate poorly in the tumor site and achieve limited success [29]. Therefore, alternative effectors, such as tumor-specific CTL, have been expected to be more efficient for adoptive immunotherapy. Several studies have indicated that tumor-infiltrating lymphocytes (TIL) were enriched for tumor-reactive T cells [26] and that adoptive transfer of CTL induced from TIL by MLTC appeared to be more effective than LAK cells [29, 33]. However, it remains generally difficult to generate tumor-specific CTL from PBL or splenocytes because of the low frequency of tumor-reactive T cells. In fact, no significant cytotoxic activity could be induced by MLTC of tumor-bearing splenocytes with C1300 cells, and only LAK cells, but not C1300-specific CTL, were induced in the presence of IL-2 (Fig. 2). Adoptive transfer of such LAK cells did not exert an antitumor effect in vivo (Figs. 3, 4).

To expand tumor-specific CTL from splenocytes containing infrequent precursor cells in vitro, additional costimulatory signals may be necessary. Previous studies showed that the CD28 costimulation plays a critical role in the expansion of both $CD4^+$ T cells and $CD8^+$ T cells, mainly by regulating IL-2 production [22, 31]. Indeed, C1300-specific CTL could be efficiently induced by MLTC with $CD86^+$C1300 cells in splenocytes from either C1300-bearing or $CD86^+$C1300-rejecting mice (Fig. 4A). The fact that C1300-specific CTL could be induced from C1300-bearing mice but not from naive mice suggests that C1300-reactive CTL precursors had been expanded but had not differentiated into effector CTL in C1300-bearing mice. Azuma et al. demonstrated a critical role of CD28 costimulation for inducing the cytotoxic effector function in $CD8^+$ T cells [1, 3]. Therefore, CD86 expressed on C1300 cells seems to act as a costimulator not only for expanding but also for directly driving cytotoxic differentiation of C1300-specific CTL in vitro.

The work described here demonstrated that tumor-specific CTL were efficiently induced by in vitro stimulation with CD86-transfected tumor cells and were effective for eliminating multiple organ metastases of poorly immunogenic C1300 neuroblastoma. We recently found that CTL activity against human lung adenocarcinomas can be also induced when PBL from the patients were cocultured with autologous tumor cells expressing a high level of CD80 (unpublished observation). Therefore, the strategy described here may be generally applicable to the generation of tumor-specific CTL for adoptive immunotherapy.

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