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Superior Protective and Therapeutic Effects of IL-12 and IL-18 Gene-Transduced Dendritic Neuroblastoma Fusion Cells on Liver Metastasis of Murine Neuroblastoma

Hisae Inuma, Kota Okinaga, Ryoji Fukushima, Tsuyoshi Inaba, Kota Iwasaki, Akira Okinaga, Ichiro Takahashi, and Michio Kaneko

Fusion vaccine of dendritic cells (DCs) and tumor cells has the advantage of inducing an immune response against multiple tumor Ags, including unknown tumor Ags. Using the liver metastasis model of C1300 neuroblastoma cells, we assessed the protective and therapeutic effects of fusion cells transduced with the IL-12 gene and/or the IL-18 gene. Improving the fusion method by combining polyethylene glycol and electroporation increased loading efficiency. In the A/J mice vaccinated with fusion cells modified with the LacZ gene (fusion/LacZ), IFN-γ production and CTL activity increased significantly compared with that of DCs/LacZ, C1300/LacZ, or a mixture of the two (mixture/LacZ). With the transduction of IL-12 and IL-18 genes into the fusion cells (fusion/IL-12/IL-18), the level of IFN-γ increased more than five times that of other fusion groups. In addition, NK cell activity and CTL activity increased significantly compared with that of mixture/LacZ, fusion/LacZ, DC/LacZ, or C1300/LacZ. In the protective and therapeutic studies of fusion cell vaccine, mice vaccinated with fusion/LacZ, fusion/IL-12, fusion/IL-18, or fusion/IL-12/IL-18 showed a significant decrease in liver metastasis and a significant increase in survival compared with mice given a mixture/LacZ, DCs/LacZ, or C1300/LacZ. In particular, the mice receiving fusion/IL-12/IL-18 vaccine showed a complete protective effect and the highest therapeutic effects. The present study investigates the improved loading efficiency of fusion cells and suggests that the introduction of IL-12 and IL-18 genes can induce extremely strong protective and therapeutic effects on liver metastasis of neuroblastoma. The Journal of Immunology, 2006, 176: 3461–3469.

Neuroblastoma, a common malignancy in children, is derived from sympathetic nerve lineage cells and is characterized by aggressive local growth, followed by metastasis to the regional lymph nodes, liver, bones, and bone marrow (1). The prognosis for patients with neuroblastoma relates to its dissemination through the body and has not greatly improved despite multimodal treatments (2). At the time of diagnosis, two-thirds of children with this tumor present with extensive local or distant metastatic disease. The overall survival rate of patients is reported only 25% (3, 4). In the absence of an effective conventional therapy for neuroblastoma, alternative biologically based strategies should be investigated. Furthermore, a distinctive scenario exists in patients <1 yr old with stage IV-S disease in which the disease is broadly disseminated but lacks amplification of the N-myc oncogene. Of these patients, 25% have spontaneous remissions, possibly mediated by the immune system (5). These findings prompted us to investigate immunotherapy for patients with neuroblastoma.

Dendritic cells (DCs), the most potent of APCs, induce a primary antitumor immune response via direct cell-cell interactions and/or cytokine production (6, 7). This antitumor immune response of DCs has resulted in the development of DC-based tumor vaccines, which are used clinically as a form of immunotherapy (8, 9). Despite the focus on DCs, this approach has not yet resulted in any significant therapeutic benefit in neuroblastoma. DCs have been loaded with tumor-derived material in several ways, such as pulsing of synthetic peptides, tumor cell lysate, and transducing tumor-derived RNA (8–11). In many tumor cells tumor-specific Ags remain unidentified. In neuroblastoma, a few tumor-associated Ags, such as survivin, NY-ESO, and MYC-N, have been reported in humans (12–14). Most clinical tumor samples have shown heterogeneity of tumor Ag and tumor cells with immunogenicity-evading immunological surveillance. Recently, an interesting new vaccine therapy that uses a fusion of DCs and tumor cells was reported (15–21). Fusion vaccine has the advantage of inducing an immune response against multiple tumor Ags, including unknown ones. With its use, the heterogeneity of tumor cells can be overcome. When making fusion vaccines, whole tumor cells are loaded with DCs, using polyethylene glycol (PEG) or electroporation. However, the loading efficiencies of these methods are insufficient and must be improved to increase the therapeutic effect of fusion vaccine (17–21).

IL-12 is a 70-kDa (p70) heterodimer protein in which the 40-kDa (p40) and 35-kDa (p35) subunits are connected by one S-S bond (22, 23). IL-12, a potent proinflammatory cytokine, is produced primarily by professional APCs, such as DC and macrophages, and exerts pleiotropic effects on immune effector cells.
(24). IL-12 induces Th1 differentiation from naïve Th0 cells, stimulates IFN-γ production, promotes proliferation of T and NK cells, and enhances CTLs, NK cells, and lymphokine-activated T cells (25, 26). IL-18, an 18.3-kDa glycoprotein, was initially identified as a cytokine that facilitates the production of IFN-γ induced by endotoxin (27). IL-18 plays an essential role in inducing a Th1 response in vivo. It stimulates T cell proliferation, augments CTL activation, and enhances NK cell cytolytic activation, mediated primarily via the FasL-Fas mechanism (28, 29). Interestingly, IL-12 with IL-18 was shown to induce the highest level of IFN-γ in vitro and in vivo (30, 31). Cumulative evidence has shown the importance of IFN-γ in the induction of native and acquired immunity.

The aim of this study is to clarify the role of fusion vaccine for treating neuroblastoma with liver metastasis, and to evaluate the antitumor effect of the transduction of both IL-12 and IL-18 genes to fusion vaccine.

Materials and Methods

Mice and cell lines

Pathogen-free A/J (H-2b) female mice, 8- to 10-wk-old, were purchased from Japan SLC. During the experiments, they were kept in pathogen-free animal facilities at a controlled temperature and humidity, according to the guidelines of the university. Murine neuroblastoma C1300 originating in A/J mice was maintained in RPMI 1640 (Nissui Pharmaceutical) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. MC38 adenocarcinoma cells (H-2d) were provided by Dr. J. Primus (Vanderbilt University Medical Center, Nashville, TN), and YAC-1 lymphoma was purchased from the DNA Bank, BioResource Center, RIKEN.

Generation of bone marrow-derived DCs and phenotype of cell surface

DCs were prepared from bone marrow as described previously (32). To analyze the surface phenotype, DCs were stained with PE or FITC-conjugated mAb, including H-2Kb, I-Aa, CD11c, CD80, CD83, and CD86 (BD Pharmingen), and expression of the surface markers was examined by a FACScan cytometer and CellQuest software (BD Biosciences).

Recombinant adenoviral vectors

A mature IL-18 cDNA molecule was isolated by PCR, using full-length IL-18 cDNA expression vector plasmid (provided by Dr. M. Okamura of RIKEN, Japan). More details of the adenoviral vector and its characterizations are described in the previous publication (33). Then, mature IL-18 cDNA expression vector (pAXCAmIL-18) was constructed using the same cosmid vector. To isolate the recombinant adenovirus vector, pAXCAmIL-18 was cloned into the cosmid vector pAXCAwt using an adenovirus expression vector kit (Takara Biomedicals) to generate the pAXCAmIL-18 constructs.

Animal studies

Experimental groups for the immunization of each cell were as follows: 2) DCs transduced with the LacZ gene (DC/LacZ); 3) C1300 tumor cells transduced with the LacZ gene (C1300/LacZ); 4) C1300 tumor cells transduced with the IL-12 gene (C1300/IL-12); 5) C1300 tumor cells transduced with the IL-18 gene (C1300/IL-18); 6) C1300 tumor cells transduced with the IL-12 and IL-18 gene (C1300/IL-12/IL-18); 7) a mixture of DCs/LacZ and C1300/LacZ (mixture/LacZ); 8) a mixture of DCs/IL-12 and C1300/IL-18 (mixture/IL-12/IL-18); 9) fusion of DC/LacZ and C1300/LacZ (fusion/LacZ); 10) fusion of IL-12 gene-transduced DCs and LacZ gene-transduced C1300 tumor cells (fusion/IL-12); 11) fusion of LacZ gene-transduced DCs and IL-18 gene-transduced C1300 tumor cells (fusion/IL-18); 12) fusion of IL-12 gene-transduced DCs and IL-18 gene-transduced C1300 tumor cells (fusion/IL-12/IL-18). To examine the therapeutic effect, each vaccine (5 × 10⁶ cells/mouse) was administered i.p. to the inguinal region on days 7 and 14, before the i.v. administration of the inguinal region on days 3 and 10 after i.v. inoculation of the C1300 tumor cells. At 21 days after the tumor injection, the livers were collected and the metastases enumerated. Survival time and rates were observed until 90 days after tumor inoculation.

Cytokine production and cytotoxicity assay

The spleens were collected 2 wk after the administration of each vaccine, and cytokine production and cytotoxicity assay (NK and CTL) were examined. Splenic NK cells were isolated from spleen cells with the NK cell isolation kit (Miltenyi Biotec) for NK assay, and T cells were separated using a T Cell Immunomunocells Kit (Cedarlane Laboratories) for cytokine production and CTL assay. To characterize the effector cells, CD4- and CD8-positive cells were eliminated by AutoMACS separation system (Miltenyi Biotec). Splenic T cells were restimulated in vitro with irradiated (50 Gy) fusion cells at 37°C under 5% CO₂ for 48 h, and levels of IFN-γ, IL-4, and IL-10 were detected using an ELISA Kit (BD Pharmingen) and CTL assay.

Loading of tumor Ags

Three fusion methods of PEG (Sigma-Aldrich) treatment, electroporation, and a combination of PEG and electroporation were examined. DCs and irradiated (100 Gy) C1300 neuroblastoma were mixed at a ratio of 2:1 (DC: tumor cells) and centrifuged at 1500 rpm for 5 min. For fusion by PEG, 50% PEG solution was added to the cell pellet and treated for 1 min, then diluted PEG was added and incubated at 37°C, 5% CO₂. For electrofusion, a mixture of DCs and tumor cells were resuspended in hybrid medium (0.25 M glucose with 0.1 mM Ca²⁺ and 0.1 mM Mg²⁺) and dielectrophoretically aligned to form cell-cell conjugates by alternating current (100 V/cm, 20 s). Then, a fusion pulse was applied, and the cell break down the membrane and to form hybrid cells (1.2 kV/cm, 30 μs). Finally, an alternating current of postfusion was applied to solidify the conformation of the hybrid cells (100 V/cm, 30 μs) (Nepa Gene). To improve the loading efficiency, we examined a two-step fusion procedure combining PEG treatment and electroporation. In the first step, the mixture of DCs and irradiated tumor cells was treated with 50% PEG as described previously, and the cells were incubated at 37°C, 5% CO₂. In the second step, nonadherent cells, which are mostly fused cells, were collected and refused by electroporation. PEG fusion cells and electroporation cells were gathered and prepared to examine loading efficiency. To determine the loading efficiency, DCs and tumor cells were restimulated with the fluorescent dyes DiO (green fluorescence; Molecular Probes) and DiI (red fluorescence; Molecular Probes), respectively, and analyzed with a FACScan flow cytometer and CellQuest software (BD Biosciences). The form of the loading cell was confirmed by an inverted system fluorescence microscope (Olympus).

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The percentage of cytotoxicity was determined by calculating the percentage of specific 51Cr release according to the following formula: ([experimental release − spontaneous release]/maximum release − spontaneous release]) × 100. All determinations were made in triplicate.

In vivo depletion of T cell subsets and NK cells
Mice received injections i.v. with rat azide-free anti-mouse CD4 mAb (YTS191.1; Serotec), rat anti-mouse CD8 mAb (YTS169.4; Serotec), rabbit anti-mouse Asialo GM1 polyclonal Ab (Wako Chemical), or normal rat IgG (Sigma–Aldrich) at 1 mg/mouse 1 day before fusion/IL-12/IL-18 vaccine inoculation and once every 5 days thereafter for an additional 20 days. Depletion of the corresponding NK, CD4, or CD8 T cells was >80%, as confirmed by FACS analysis of spleen cells from treated mice.

Statistical analysis
Values were presented as means ± SD. Multiple groups were evaluated by ANOVA and the posthoc Scheffe multirange test. Survival estimates were determined using the Kaplan-Meier method, and data were compared by the log-rank test. A probability of <0.05 was considered significant.

Results
Comparing the loading efficiency and antitumor effect after improving the fusion method
The loading efficiency of electroporation, PEG, and PEG followed by electroporation (the two-step method) was compared using the DCs and C1300 tumor cells, which were labeled with DiO and DiI intracellular fluorescent dyes, respectively (Fig. 1). DCs loaded by whole tumor cells were characterized by the emission of both colors in the upper right of the dot plot analysis. Double-positive cells increased from 12.9 ± 3.2% using electroporation or 34.1 ± 2.6% using PEG treatment to 51.6 ± 2.1% using the two-step method (Fig. 1A). This suggests that the loading efficiency of the two-step fusion method increased 1.5 times and is 4 times higher than with PEG treatment and electroporation, respectively. In the two-step method, the loading efficiency of gene-transduced fusion cells was 45.2 ± 2.3% in fusion/LacZ, 48.1 ± 2.2% in fusion/IL-12, 49.7 ± 4.1% in fusion/IL-12/IL-18, and 50.7 ± 3.5% in fusion/IL-12/IL-18, and there were no significant differences among them (data not shown).

The samples of FACS analysis were observed under a fluorescent microscope (Fig. 1, A and B). In fluorescent micrographs of fusion cells, a unity of cell membranes of multinuclear cells was recognized. Using this method, cell viability was >80%, which was achieved by the trypan blue dye exclusion test. In contrast, simple repetition of PEG treatment or electroporation caused a significant decrease in cell viability (<50%; data not shown).

Next, we compared the ability for CTL production of these fusion cells generated by the different protocols (Fig. 1C). The cytotoxicity of splenic lymphocytes from mice vaccinated with two-step fusion cells was significantly higher than that of PEG treatment or electroporation alone. These results show the usefulness as cancer vaccines of fusion cells generated by the two-step method.

Cell surface phenotypes of DCs, tumor cells, and fusion cells
The cell surface markers of each vaccine were analyzed by FACS (Fig. 2). C1300 tumor cells transduced with the LacZ gene (C1300/LacZ) showed only the expression of MHC class I Ags (H-2K) but not MHC class II (I-A), CD11c, or costimulatory molecules (CD80, CD83, CD86). DCs transduced with the LacZ gene (DCs/LacZ) expressed high levels of MHC class II Ags and CD11c, CD80, and CD86, and a low level of CD83. Almost the same levels of DC-derived markers were identified on the surface of the fusion cells transduced with the LacZ gene (fusion/LacZ) or the IL-18 gene (fusion/IL-18). In contrast, transduction with the IL-12 genes enhanced the expression of CD83 molecule on the surface of the fusion cells, and a high level of CD83 was shown in fusion/IL-12 and fusion/IL-12/IL-18.

Cytokine production by gene-transduced DCs and tumor cells
At a MOI 100, the gene transduction efficiency for IL-12 in DCs was 61%, and that for IL-18 in C1300 tumor cells was 68% (data not shown). Fig. 3A shows the IL-12 protein levels in the medium of each group. Significant levels of p70 IL-12 proteins were produced in DCs or fusion cells following transduction with the IL-12 gene. The p70 IL-12 levels of the DCs/LacZ, fusion/LacZ, DCs/
IL-12, fusion/IL-12, and fusion/IL-12/IL-18 were 166 ± 51 pg/ml, 185 ± 65 pg/ml, 3210 ± 702 pg/ml, 2820 ± 568 pg/ml, and 2672 ± 468 pg/ml, respectively. There were no significant differences in IL-12 levels between the DCs/IL-12, fusion/IL-12, and fusion/IL-12/IL-18. Fig. 3B shows the IL-18 protein levels in the medium of each group. Significant levels of IL-18 proteins were produced in C1300 cells or fusion cells by transduction with the IL-18 gene. The IL-18 levels of C1300/LacZ, fusion/LacZ, C1300/IL-18, fusion/IL-18, and fusion/IL-12/IL-18 cells were 58 ± 25 pg/ml, 78 ± 35 pg/ml, 1280 ± 308 pg/ml, 1036 ± 202 pg/ml, and 1006 ± 282 pg/ml, respectively. No significant differences in IL-18 levels were found between the C1300/IL-18, fusion/IL-18, and fusion/IL-12/IL-18.

Cytokine production by splenic T cells

Production of IFN-γ by splenic T cells collected from mice immunized with each vaccine and cell surface phenotypes of effector cells in IFN-γ production were examined (Fig. 4). IFN-γ levels in the culture supernatant of splenic T cells were significantly higher in the mice vaccinated with fusion/LacZ, fusion/IL-12, fusion/IL-18, and fusion/IL-12/IL-18 than with the mixture of DC/LacZ and C1300/LacZ (mixture/LacZ), DCs/LacZ, C1300/LacZ, or PBS (Fig. 4A). The fusion/IL-12/IL-18-vaccinated group showed the highest levels of IFN-γ compared with that of fusion/LacZ, fusion/IL-12, and fusion/IL-18. In contrast, IL-4 and IL-10 levels from splenic T cells were very low in all groups, and there were no significant differences between these groups (data not shown). To characterize the T cells that produce the IFN-γ, CD8+ T cells and/or CD4+ T cells were depleted by negative selection of MACS (Fig. 4B). In the mice vaccinated with fusion/LacZ, fusion/IL-12, fusion/IL-18, or fusion/IL-12/IL-18, production of IFN-γ was significantly inhibited by depletion of splenic T cells expressing not only CD4+ but also CD8+ T cells. These results suggest that a fusion vaccine itself has the ability to induce a Th1 immune response, and cotransduction of the IL-12 and IL-18 genes causes a strong shift to a Th1 response by markedly increasing production of IFN-γ by CD4+ and CD8+ T cells.

Cytotoxicity of splenic NK cells and splenic T cells

Productivity of cytotoxic splenic NK cells against NK-sensitive YAC-1 cells and C1300 cells was examined (Fig. 5A). Splenic NK cells of fusion/LacZ-vaccinated mice showed a significant increase of cytotoxicity against YAC-1 cells compared with mice vaccinated with DC/LacZ, C1300/LacZ, and PBS. Furthermore, NK activities were increased by transduction of IL-12 and/or IL-18 genes. Splenic NK cells of the mice vaccinated with fusion IL-12/IL-18, fusion/IL-12, fusion/IL-18, or nonfused mixture/IL-12/IL-18 showed a significant increase of cytotoxicity against YAC-1 cells and C1300 cells compared with that of mixture/LacZ, DC/LacZ, C1300/LacZ, or PBS-administered mice. The group vaccinated with fusion/IL-12/IL-18 showed the highest NK activity in
all groups. The NK activity against YAC-1 cells was higher than that of C1300 cells. These results suggest that the fusion vaccine has the ability to induce NK activity, and that transduction of IL-12 and IL-18 genes greatly increase the NK activities.

Next, we examined the CTL activities of splenic T cells against parental C1300 tumor cells and allogeneic MC38 tumor cells (Fig. 5B). In the mice transduced with the LacZ gene, CTL activity against C1300 tumor cells was significantly higher in the mice vaccinated with fusion cells compared with those vaccinated with mixture/LacZ, DCs/LacZ, C1300/LacZ, and PBS. The CTL activities of splenic T cells in mice vaccinated with fusion/IL-12, fusion/IL-18, and fusion/IL-12/IL-18 were higher than that of mice vaccinated with fusion/LacZ, and mice vaccinated with fusion/IL-12/IL-18 showed the highest level of activity in these groups. In contrast, mice vaccinated with nonfused mixture/IL-12/IL-18 or mixture/LacZ did not show a significant level of CTL activity. These results suggest that the formation of fusion cells is important for CTL induction. Cytotoxic activity against allogeneic MC38 adenocarcinoma was not detected in any group.

We then determined the surface phenotype and the location of MHC restriction of the cytotoxic effector cells induced by each fusion vaccine (Fig. 5C). The cytotoxic activity in all fusion groups (fusion/LacZ, fusion/IL-12, fusion/IL-18, and fusion/IL-12/IL-18) was significantly inhibited by the depletion of CD8$^+$ T cells but not by CD4$^+$ T cells. In the blocking assay of MHC Ags, CTL activities of all fusion groups were significantly decreased by treatment in anti-H-2K$^b$ mAb. However, treatment with anti-I-A$^k$ mAb did not affect cytotoxicity. These results indicate that fusion cells could induce MHC class I-restricted CD8$^+$ CTLs.

**Protective effects of fusion vaccine**

The protective effect induced by each vaccine was assessed by the number of liver metastases at 21 days and the survival rates 90 days after tumor inoculation (Table I and Fig. 6A). Each vaccine was administered on days 7 and 14, before the i.v. inoculation of C1300 tumor cells. In mice vaccinated with fusion/LacZ, a significant decrease in the number of liver metastases was observed compared with that of mice vaccinated with mixture/LacZ, DCs/LacZ, C1300/LacZ, or PBS. Transduction with IL-12 or IL-12 and IL-18 gene into fusion cells led to a significantly lower liver metastasis number compared with mice vaccinated with C1300/IL-12, C1300/IL-18, mixture/LacZ, DCs/LacZ, C1300/LacZ, or PBS. In particular, fusion/IL-12/IL-18 vaccine showed a dramatic decrease of liver metastasis, and all mice were tumor-free.

In a comparison of survival rates, the mice vaccinated with fusion/LacZ showed a significant increase compared with mice vaccinated with mixture/LacZ, C1300/LacZ, DC/LacZ, or PBS (Fig. 6A). Transduction with the IL-12 and/or IL-18 gene into fusion cells led to a higher survival rate compared with that of fusion/LacZ. In particular, fusion/IL-12/IL-18 vaccine showed a complete protection, and all mice remained tumor-free for at least 90 days. In contrast, mice vaccinated with mixture/IL-12/IL-18, C1300/IL-12/IL-18, C1300/IL-18, and C1300/IL-18 showed partial protection, and all mice remained tumor-free for at least 90 days. These results demonstrate that the fusion cells transduced with the IL-12 and IL-18 genes lead to a dramatic protective effect and that conformation of fusion cells is important for strong tumor protection.

**Therapeutic effects of fusion vaccine**

To assess the therapeutic effects, each vaccine was administered after tumor inoculation, and the number of liver metastasis and survival rates were examined (Table II and Fig. 6B). In the study of liver metastasis, the mice vaccinated with fusion/LacZ, fusion/IL-18, C1300/IL-12/IL-18, or mixture/IL-12/IL-18 demonstrated a significant decrease in liver metastasis number compared with that
of mice vaccinated with mixture/LacZ, DC/LacZ, C1300/LacZ, or PBS. Fusion/IL-12 showed a significant decrease in the number of liver metastases compared with C1300/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ, or PBS. In contrast, transduction of the IL-12 and IL-18 genes into fusion cells showed the lowest number of liver metastases in all groups, and this level differed significantly from that of C1300/IL-12, C1300/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ, or PBS.

FIGURE 5. NK and cytotoxic T cell activities of splenic NK cells or T cells from mice immunized with each vaccine. A, NK activity. On day 14 after vaccination by DC/LacZ, C1300/LacZ, mixture/LacZ, mixture/IL-12/IL-18, fusion/LacZ, fusion/IL-12, fusion/IL-18, or fusion/IL-12/IL-18, NK cells were isolated from the spleen and cocultured with irradiated DC/C1300 fusion cells, and cytotoxicity was measured against YAC-1 and C1300 tumor cells. *, p < 0.05 (fusion/IL-12/IL-18, fusion/IL-12, fusion/IL-18, mixture/IL-12/IL-18 vs mixture/LacZ, DC/LacZ, C1300/LacZ, PBS). †, p < 0.05 (fusion/LacZ vs DC/LacZ, C1300/LacZ, PBS). Mean ± SD (n = 5). B, CTL activity. On day 14 after vaccination by each vaccine, T cells were separated from spleen cells and cocultured with irradiated DC/C1300 fusion cells, and cytotoxicity was measured against parental C1300 tumor cells and MC38. *, p < 0.05 (fusion/IL-12/IL-18, fusion/IL-12, fusion/IL-18 vs mixture/IL-12/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ, PBS). †, p < 0.05 (fusion/LacZ vs mixture/LacZ, DC/LacZ, C1300/LacZ, PBS). Mean ± SD (n = 5). C, Phenotype and MHC restriction of CTL induced by fusion vaccines. On day 14 after immunization of fusion vaccines (fusion/LacZ, fusion/IL-12, fusion/IL-18, or fusion/IL-12/IL-18), splenic T cells were collected and cocultured with irradiated DCs/C1300 fusion cells, and phenotype and MHC restriction were examined. To examine the phenotype of CTL, CD8+ and/or CD4+ cells were depleted by MACS, and CTL activity was compared with that of undeleted T cells. The ratio of E:T cells was 25:1. *, p < 0.01 (CD8 deple vs CD4 deple, untreated). Mean ± SD (n = 5). To examine the MHC restriction of effector cells, C1300 target cells were pretreated with anti-H-2Kk mAb, anti-I-Ak mAb or medium, and cytotoxicities were measured. The ratio of E:T cells was 25:1. *, p < 0.01 (anti-H-2Kk vs anti-I-Ak, medium). Mean ± SD (n = 5).
In the study of survival rates, all of the mice vaccinated with fusion/LacZ died within 57 days of tumor inoculation (Fig. 6B). The mice vaccinated with fusion/IL-12, fusion/IL-18, or mixture/IL-12/IL-18 had increased survival rates; however, their levels were <20%. In contrast, mice vaccinated with fusion/IL-12/IL-18 showed a significant increase of survival rates compared with that of all other groups, and 60% of mice remained tumor-free for at least 90 days. The survival rates of mice vaccinated with mixture/IL-12/IL-18 decreased significantly compared with that of mice vaccinated with fusion/IL-12/IL-18, suggesting the significance of conformation of fusion cell for strong therapeutic effect. These results demonstrate that the therapeutic effect of the fusion cells transduced with the IL-12 and IL-18 genes is superior to that of any other group.

Next, we investigated the participation of immune cell subsets in the generation of the therapeutic effects of the fusion/IL-12/IL-18 vaccine (Fig. 7). The NK, CD8 T cells, or CD4 T cells of mice were depleted by the administration of anti-asialoGM1, anti-CD4, or anti-CD8 mAb. The depleted NK cells, CD8 T cells, or CD4 T cells showed a significant increase of liver metastasis compared with normal IgG-injected mice. These results suggest that NK, CD8 T cells, and CD4 T cells are necessary and are associated with the therapeutic effects of the fusion vaccine transduced with the IL-12 and IL-18 genes.

**Discussion**

In the present study, we demonstrated the protective and therapeutic effect of DC/tumor fusion cells on neuroblastoma with liver metastasis. The transduction of both the IL-12 and the IL-18 genes to fusion cells induced the highest levels of IFN-γ, NK cell activity, and CTL activity. Furthermore, the fusion vaccine transduced with IL-12 and IL-18 genes showed complete protective and highly significant therapeutic effects on liver metastasis of neuroblastoma in mice.

Immunotherapy has been put forward as a feasible strategy for treating neuroblastoma based upon the observation that some aggressive neuroblastomas spontaneously regress (5). Although clinical trials of DC-based immunotherapy were investigated, its effects on several carcinomas were limited by the low number of defined tumor-associated Ags and the heterogeneity of tumor cells. In contrast, a new strategy using a DC-based tumor vaccine reported that DCs loaded with whole tumor cells or tumor lysate have the advantage of inducing antitumor immunity to multiple

### Table I. **Protective effects of each vaccine on liver metastasis**

<table>
<thead>
<tr>
<th>Immunogen Tumor-Free Mice</th>
<th>No. of Liver Metastasis</th>
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<tr>
<td>PBS</td>
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*p = 0.05 (C1300/IL-12, C1300/IL-18 vs. C1300/LacZ, PBS).

*p p < 0.05 (C1300/IL-12/IL-18, mixture/IL-12/IL-18, fusion/IL-18 vs. C1300/IL-12/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).

*p = 0.05 (fusion/LacZ vs. mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).

*p = 0.05 (fusion/LacZ vs. mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).

† p * = 0.05 (fusion/IL-12 vs all groups except fusion/IL-18 and mixture/IL-12/IL-18).

‡ p = 0.05 (fusion/IL-12, fusion/IL-12/IL-18 vs C1300/IL-12, C1300/IL-18, mixture/LacZ, C1300/LacZ, DC/LacZ or PBS).

§ p = 0.05 (fusion/LacZ, C1300/IL-12/IL-18 vs fusion/IL-12/IL-18, fusion/IL-12, mixture/LacZ, C1300/LacZ, DCs/LacZ or PBS).

¶ p = 0.05 (C1300/IL-12, C1300/IL-18 vs fusion/IL-12/IL-18, fusion/IL-12, fusion/IL-18, mixture/IL-12/IL-18, C1300/LacZ or PBS).
tumor Ags, including unidentified tumor Ags. A phase I clinical trial of tumor lysate-pulsed DC treatment for patients with neuroblastoma has been conducted and a stable clinical response demonstrated (35). In contrast, our preliminary data have shown that the fusion vaccine of DCs and tumors cells induce a stronger anti-tumor immunity than that of tumor lysate (our unpublished observations). In previous experimental models using mice, Ag presentation and the antitumor effects of the fusion vaccine were reported in adenocarcinoma, plasmacytoma, hepatocellular carcinoma, and melanoma (15–23). Recently, we reported not only an increase in the antitumor effect, but also protective and therapeutic effects of an IL-2 gene-modified fusion vaccine in mice with pulmonary metastasis of fibrosarcoma (36). In clinical trials of the fusion vaccine in patients with melanoma, glioma, and renal cell carcinoma, complete remission in melanoma, a partial clinical response in glioma, and stability in renal cell carcinoma were demonstrated (37–39).

Although the chemical agent PEG and electrofusion have been used for the fusion vaccine, the fusion process of these methods is different. In PEG treatment, the lipid bilayer of the cell membrane is thought to be broken down through the dehydration action of PEG, followed by an increase in the fluidity of the cell membrane. In contrast, the fundamental step in electrofusion is reversible membrane breakdown. When short-duration, direct current electric impulses applied to the cell membrane exceed a critical threshold, that membrane will become transiently but highly permeable through the formation of micro pores. Moreover, the adjacent process of touching cells may form channels and lead to the formation of new spherical hybrid cells. The loading efficiency of PEG and electrofusion was reported to be 17.0–35.0% and 5.0–25.0%, respectively (15–21). Cell fusion is known to be influenced by the characteristics of the cell membrane, and loading efficiency differs markedly among tumor cells. To increase and stabilize loading efficiency, we improved the fusion method by combining PEG and electrofusion. By this two-step method, tumor-loading efficiency was increased 1.6 times that of PEG treatment and 4 times that of electrofusion, and allowed higher CTL activities. We expect that the combination method of PEG and electrofusion may have beneficial effects on the stable loading of various types of tumor cells.

Previous immunotherapeutic approaches to neuroblastoma have focused on the use of IL-2 to activate both T cell-dependent and -independent cytotoxic immune responses (40, 41). Recent strategies for treating neuroblastoma have incorporated advances in molecular biology to engineer a tumor cell for the induction of a more efficient immune response. Yoshida et al. (42) showed that murine neuroblastoma retrovirally transduced with the IL-2 or GM-CSF gene exhibited tumor-specific acquired immunity. Shimizu et al. (43) and Redlinger et al. (44) reported that DCs transduced with the IL-12 gene by adenovirus could induce an antitumor immune response in an established murine neuroblastoma. However, the efficacy of DC transduced with IL-18 gene has not been reported in the murine neuroblastoma model. It has been reported that IL-18 could induce high-level expression of IFN-γ by T cells, NK cells, B cells, and monocytes and play an important role in CTL activation and enhancement of NK cell cytotoxic activity (27, 28). Furthermore, IL-18 acts synergistically with IL-12, but not IL-2 or GM-CSF, in inducing IFN-γ production by T cells (30, 31). Both IL-12 and IL-18 may be required for an effective differentiation into Th1 cells. In this study, we evaluated the Th1 immune response, CTL activity, and NK activity using fusion cells modified with both IL-12 and IL-18 genes. The production of IFN-γ by CD4+ and CD8+ T cells was markedly increased by the vaccination of fusion cells transduced with IL-12 and IL-18 genes. Moreover, transduction with the IL-12 and IL-18 genes in fusion cells showed a significant increase of MHC class I-restricted CD8+ CTL activity and NK activity.

Our results showed that fusion cells of DCs and neuroblastoma, but not a mixture of DCs and neuroblastoma, significantly reduced liver metastasis and significantly increased survival rates compared with DCs or tumor cells alone, suggesting that the formation of fusion cells is important in inducing strong antitumor immunity. Orentas et al. (17) reported the protective effect of electrically prepared fusion cells on mice with Neuro-2a neuroblastoma cells. However, in his paper the loading efficiency by electrofusion was only 5–10%. In contrast, the loading efficiency of our two-step fusion method showed 45–52% efficiency. By improving loading efficiency, we demonstrated not only the protective effects but also the therapeutic effects of the fusion cells themselves. Furthermore, our study showed that the transduction of the fusion cells with both IL-12 and IL-18 genes produced complete protective effects and highly significant therapeutic effects on liver metastasis and survival. The relative importance of the NK cell, CD4+, and CD8+ T cell subsets for the therapeutic effect of the fusion/IL-12/IL-18 vaccine was demonstrated. To the best of our knowledge, this is the first study to demonstrate the protective and therapeutic effects

### Table II. Therapeutic effects of each vaccine on liver metastasis

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Tumor-Free Mice</th>
<th>No. of Liver Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0/10</td>
<td>48.0 ± 23.2</td>
</tr>
<tr>
<td>DC/LacZ</td>
<td>0/10</td>
<td>46.8 ± 21.6</td>
</tr>
<tr>
<td>C1300/LacZ</td>
<td>0/10</td>
<td>47.9 ± 21.4</td>
</tr>
<tr>
<td>C1300/IL-12</td>
<td>0/10</td>
<td>38.5 ± 10.0</td>
</tr>
<tr>
<td>C1300/IL-12/IL-18</td>
<td>0/10</td>
<td>30.2 ± 11.2</td>
</tr>
<tr>
<td>Mixture/LacZ</td>
<td>0/10</td>
<td>19.6 ± 5.9</td>
</tr>
<tr>
<td>Mixture/IL-12/IL-18</td>
<td>1/10</td>
<td>41.3 ± 14.5</td>
</tr>
<tr>
<td>Fusion/LacZ</td>
<td>0/10</td>
<td>41.3 ± 14.5</td>
</tr>
<tr>
<td>Fusion/IL-12</td>
<td>1/10</td>
<td>16.8 ± 9.3</td>
</tr>
<tr>
<td>Fusion/IL-12/IL-18</td>
<td>2/10</td>
<td>9.7 ± 6.2</td>
</tr>
<tr>
<td>Fusion/IL-12/IL-18</td>
<td>6/10</td>
<td>2.6 ± 3.4</td>
</tr>
</tbody>
</table>

* Mean ± SD (n = 10). The data presented were pooled from two separated experiments, and the reproducibility of results was shown.

* p < 0.05 (fusion/LacZ, fusion/IL-18, C1300/IL-12/IL-18, mixture/IL-12/IL-18 vs. mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).

/10. The data presented were pooled from two separated experiments, and the reproducibility of results was shown.

* p < 0.05 (fusion/IL-12 vs. C1300/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).

* p < 0.05 (fusion/IL-12/IL-18 vs. C1300/IL-12, C1300/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).

FIGURE 7. Participation of CD4, CD8, and NK cells in fusion/IL-12/IL-18 vaccine-induced immunity. After C1300 tumor inoculation, mice were administered fusion/IL-12/IL-18 vaccine or PBS (nonimmunized). Anti-CD4 mAb, anti-CD8 mAb, anti-asialo GM1 Ab, or normal rat IgG was injected i.v. before vaccination and once every 5 days thereafter for an additional 20 days. On day 21, the number of liver metastasis was counted. * p < 0.01 (nonimmunized, anti-asialo GM1, anti-CD8 mAb, anti-CD4 mAb vs IgG). Mean ± SD (n = 10).
of an IL-12 and IL-18 gene-modified fusion vaccine on murine neuroblastoma with liver metastasis. In conclusion, we demonstrated that improved loading efficiency may provide a basis for using a fusion vaccine and that introducing both IL-12 and IL-18 genes can induce extremely strong protective and therapeutic effects.

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Disclosures

The authors have no financial conflict of interest.

References


