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MethA Fibrosarcoma Cells Expressing Membrane-Bound Forms of IL-2 Enhance Antitumor Immunity

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Abstract Tumor cells genetically engineered to secrete cytokines are effective in tumor therapy, but various unexpected side effects are observed, which may result from the bulk activation of various bystander cells. In this study, we tested tumor vaccines expressing various membrane-bound forms of IL-2 (mbIL-2) on MethA fibrosarcoma cells to focus antitumor immune responses to CTL. Chimeric forms of IL-2 with whole CD4, deletion forms of CD4, and TNF were expressed on the tumor cell surface, respectively. Tumor clones expressing mbIL-2 or secretory form of IL-2 were able to support the cell growth of CTLL-2, an IL-2-dependent T cell line, and the proliferation of spleen cells from 2C TCR transgenic mice that are responsive to the p2Ca/L^d MHC class I complex. Expression of mbIL-2 on tumor cells reduced the tumorigenicity of tumor cells, and the mice that once rejected the live IL-2/TNF tumor clone acquired systemic immunity against wild-type MethA cells. The IL-2/TNF clone was inferior to other clones in tumor formation, and superior in the stimulation of the CD8+ T cell population *in vitro*. These results suggest that the IL-2/TNF clone is the best tumor vaccine, and may stimulate CD8+ T cells by direct priming. Expression of IL-2/TNF on tumor cells may serve as an effective gene therapy method to ameliorate the side effects encountered in the recombinant cytokine therapy and the conventional cytokine gene therapy using the secretory form of IL-2.

Key words: IL-2, membrane-bound form, antitumor immunity, tumor vaccine, direct priming, CTL

Various strategies employing cytokines to enhance host immunity have been studied intensively for cancer therapy. With the successful clinical results using recombinant cytokines [2, 28], genetic modification of tumor cells with cytokine genes has drawn lots of attention. Tumor cells transduced with cytokine genes sometimes became immunogenic, regressed spontaneously *in vivo*, and eventually induced antitumor immunity [5, 12, 13, 35, 41].

As much as functions of cytokines are various and redundant, the primary targets of the cytokine gene transfers into tumor cells are diverse. The principle of the cytokine gene therapy is based on the findings that tumor bearers are often immunocompromised. T lymphocytes from the tumor bearers are defective in signaling for T cell activation [34, 38]. The IL-2 gene transduction into tumor cells compensated the defect of Th function [50]. Malfunction of the immune system in tumor bearers is also implicated with disabled dendritic cells [16]. In order to enhance dendritic cell functions, tumor cells were transduced with GM-CSF, and efficient differentiation and activation of dendritic cells were acquired, with therapeutic effect [12]. The enhanced function of dendritic cells resulted in efficient presentation of tumor-associated antigens to T cells through cross presentation [19]. However, direct priming of T cells by nonprofessional antigen-presenting cells is also functional like in fibroblasts [26]. Tumor cells do not directly activate T lymphocytes, as they usually do not express either MHC class II molecules or costimulatory molecules such as B7. Enhanced immunogenicity and successful tumor therapy were archived by gene transfer of MHC class II or costimulatory molecules into tumor cells [4, 19, 31, 32, 43]. The approaches, transducing MHC class I/II and costimulatory molecules, indicate that the modified tumor cells may directly prime T cells as antigen-presenting cells.

Recombinant IL-2 is usually applied systemically, and therapeutic effects generally are often accompanied by severe toxic side effects [8, 21, 30, 48]. The transfer of the IL-2 gene into tumor cells has the advantage that IL-2 secreted by the tumor cell itself can induce local immune

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responses at the tumor growing site [15, 17, 18, 20]. However, tumor cells engineered to produce cytokines have unexpected side effects [12, 29, 42] that may be caused by activation of bulk bystander cells, or differentiation and expansion of T cells without evident specificity for the tumor antigen [11, 37]. The multifunctional redundancy of cytokine itself and the multicellular expression of cytokine receptors may cause these. To avoid such toxicity and side effect by the tumor vaccine secreting cytokines, oriented targeting of immune responses is indispensable.

Several cytokines, including IL-1 [27], TNFα [25], LTα [6], and IFN-γ [1] are expressed as both membrane-associated forms and soluble forms. Interestingly, the genetically engineered membrane-bound form of TNFα or LTα on tumor cells induced antitumor immunity with lower toxic side effect [6, 33]. Such approach using a membrane-bound form of cytokine has been expanded to GM-CSF [14, 40, 49], Flt3 ligand [9], IL-4 [7, 24], IL-12 [10], and IL-2 [7]. Furthermore, anchoring of IL-2 via the diphtheria toxin T domain on tumor cells induced successful anticancer immunity [36]. Although the intended target cells are different depending on applied cytokines, those approaches share a common rationale of direct priming of tumor-associated antigens. The genetically modified tumor cells expressing a membrane-bound form of cytokines may exert their effect on restricted cells, which are able to contact physically with the engineered tumor cells.

In the present study, we modified MethA cells to express membrane-bound forms of IL-2 (mbIL-2), and studied their tumorigenicity and induction of systemic antitumor immunity. We envisaged that if the tumor cells displayed the proper tumor-associated antigen/MHC complex together with IL-2 as a membrane-bound form, then tumor antigen-specific CTL precursors might be selectively activated and side effects of bystander cell activation would be minimized.

Materials and Methods

Animals, Cell Lines, and Antibodies

Female BALB/c and C57BL/6 mice between 6 and 8 weeks of age were purchased from the Korea Research Institute of Chemical Technology (Daejeon, Korea). The methylcholanthrene-induced fibrosarcoma MethA (BALB/c originated) was cultured in RPMI-1640 medium (Sigma, St. Louis, MO, U.S.A.) supplemented with 5% fetal bovine serum (FBS) (Sigma, St. Louis, MO, U.S.A.), 50 U/ml penicillin, 50 μg/ml streptomycin, and occasionally *in vivo* passage. An IL-2-dependent cytotoxic cell line, CTLL-2, was obtained from Dr. Charles Janeway, Jr. (Yale University, CT, U.S.A.), and maintained in RPMI-1640 medium supplemented with 5% FBS and IL-2 (10 U/ml). IL-2 was provided in the form of culture supernatant from the EL-4 cells transfected with IL-2 cDNA expressing vector. The 2C CTL clone was maintained in RPMI-1640 medium supplemented with 10% FBS, antibiotics and 10% rat Concanavalin A stimulated supernatant. At every 2 weeks,

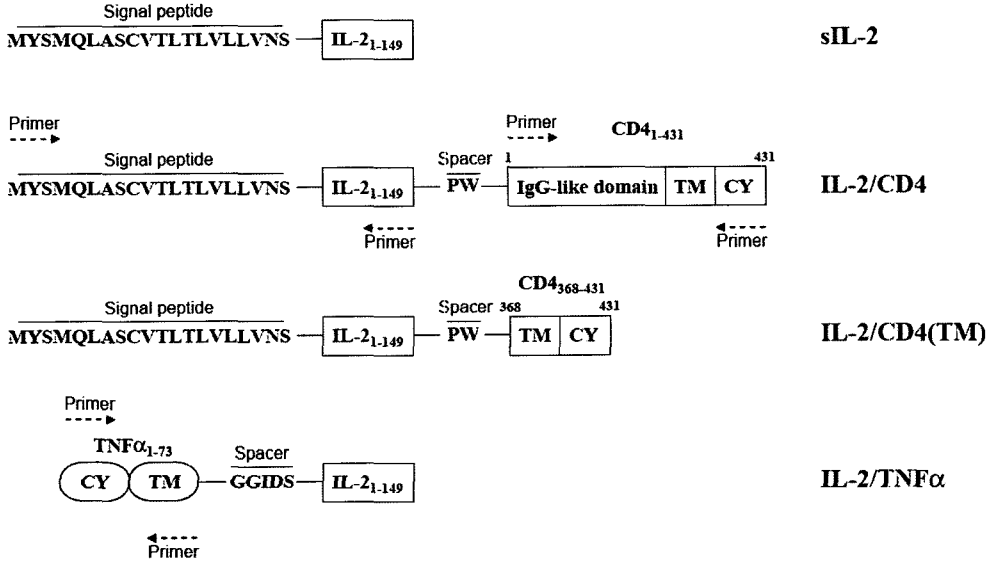

Fig. 1. Construction of the SIL-2, IL-2/CD4, IL-2/CD4(TM), and IL-2/TNF chimeric cDNA.
The IL-2/CD4 chimeric gene encoded a protein comprising the signal peptide (from −20 to −1) and coding sequence (from 1 to 149) of IL-2, two amino acids spacer (PW), and whole amino acids of CD4 (from 1 to 431) except signal peptide. The IL-2/CD4(TM) chimeric gene encoded the same as the above IL-2/CD4, truncated extracelluar four Ig-like domains. The IL-2/TNF chimeric gene encoded a protein comprising the cytoplasmic domain (from −75 to −45), the transmembrane domain (from −44 to −24), and the 19 extracellular amino acids (from −23 to −5) of TNF, and five amino acids spacer (GGIDS), and IL-2 (from 1 to 149) without signal peptide.

mitomycin C (MMC)-treated BALB/c splenic feeder cells were supplemented. mAb to IL-2 (S4.B6.34, rat IgG1) was kindly provided by Dr. Charles Janeway, Jr., and mAb to MHC class I specific to L^d (28-14-8S) was obtained from Dr. Peter Cresswell (Yale University, CT, U.S.A.). Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-CD8 mAb, FITC- or PE-conjugated anti-CD4 mAb, FITC-goat anti-mouse IgG, and FITC-goat anti-rat IgG antibodies were purchased from Sigma (St. Louis, MO, U.S.A.).

Plasmid Construction

The cDNA of mouse IL-2 was purchased from American Type Culture Collection (ATCC) (Rockville, MD, U.S.A.). The cDNA of mouse CD4 was obtained from Dr. Soon-Cheol Hong (Medical College of Ohio, OH, U.S.A.) and the cDNA of mouse TNFα was obtained from Dr. Sang-Young Chun (Chonnam National University, Korea). To construct IL-2/CD4 chimeric cDNA, primers specific for IL-2 (forward: 5'CGC GAA TTC ATG TAC AGC ATG CAG CTC GCA3', reverse: 5'GCG CCA TGG TTG AGG GCT TGT TGA GAT GAT3') and CD4 (forward: 5'GCG CCA TGG AAG ACG CTG GTG CTG GGG AAG3', reverse: 5'CGC GAA TTC TCA GAT GAG ATT ATG GCT CTT3') were used to amplify the respective cDNA fragments by polymerase chain reaction (PCR) (Fig. 1). To construct IL-2/CD4(TM) chimeric cDNA, cDNA encoding the extracellular region of CD4 (immunoglobulin-like domain), that is, the NcoI/BamHI fragment (approximately 1 Kbp), was deleted from the IL-2/CD4 expression vector. To generate IL-2/TNF chimeric cDNA, the PCR-amplified and subcloned IL-2 cDNA into pBluescipt II was digested with HincII/BamHI and the cohesive ends were filled in. The HincII/BamHI fragment encoded only the mature form of IL-2 (excluding IL-2 leader sequences). To amplify partial TNFα cDNA encoding transmembrane and cytoplasmic regions, the following primers were used; forward, 5'GC GGA TCC ATG AGC ACA GAA3', and reverse, 5'C GCG AAT TCC TCC GGC CAT AGA ACT3'. The amplified TNFα fragment was digested with BamHI/EcoRI and the cohesive ends were filled. Finally, the blunt ended IL-2 cDNA fragment (approximately 800 bp) and the blunt ended TNFα cDNA fragment (240 bp) were ligated, and subcloned into the BamHI cloning site of the pNeoSRαII expression vector by the blunt ligation procedure. All the PCRs were performed with a capillary PCR machine (FTC-2000, Daehan Medical Co., Seoul, Korea) and the structure of the chimeric DNAs was confirmed by DNA sequence analysis.

Transfection and Drug Selection

For electroporation, MethA cells were harvested and washed twice in HBSS (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose, 20 mM HEPES, pH 7.0; Gibco-BRL, Grand Island, NY, U.S.A.). The cells (2×10^7 cells) were resuspended in HBSS and mixed with 20 μg plasmid DNA. After incubation on ice for 10 min, they were electroporated at 320 V, 960 μF (Bio-Rad, Hercules, CA, U.S.A.) and transferred to normal medium. After 48 h, cells were plated in 96-well plates in (1 g/l) G418-containing medium. The drug-resistant colonies were usually visible after 2–3 weeks.

Proliferation Assay

Each transfectant was assayed for IL-2 biological activity by measuring proliferation of the IL-2-dependent cell line, CTLL-2 cells, and spleen cells from 2C TCR transgenic mice [22]. TCRs on T cells from 2C transgenic mice recognize p2Ca/L-d MHC class I on BALB/c cells. The p2Ca peptide is a natural endogenous antigenic peptide, which exists on cells of BALB/c mice [44, 45]. Briefly, mbIL-2 tumor cells were inactivated with MMC (50 μg/ml) for 2 h and co-cultures were set up with CTLL-2 cells or spleen cells from 2C transgenic mice (1×10^4 cells) and an equal number of transfectant in a final volume of 200 μl, using flat-bottomed 96-well plates. Cultures were incubated for 48 h at 37°C, pulsed for the final 12 h with 0.5 μCi of ^{3}H-thymidine (Amersham, Piscataway, NJ, U.S.A.) per well, and samples were collected using a PHD cell harvester (Skatron; Sterling, VA, U.S.A.). ^{3}H-thymidine incorporation was determined by a liquid scintillation counter and expressed as total counts per minute (cpm).

Flow Cytometry Analysis

The transfected clones were first incubated for 30 min at 4°C, with primary antibody in staining buffer (1× PBS containing 0.02% sodium azide and 0.5% FBS). Cells were washed and incubated for an additional 30 min at 4°C, with FITC-goat anti-mouse IgG antibody or FITC-goat anti-rat IgG antibody (Sigma, St. Louis, MO, U.S.A.). The stained cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.).

Stimulation of Whole Spleen Cells and Enriched Splenic T Cells by IL-2 Transfectants

Single-cell suspensions of spleen cells from normal mice or MethA-immunized BALB/c mice were prepared and red blood cells were removed by using a hypotonic solution [23]. MethA tumor cells (1×10^6 cells) were incubated with MMC (50 μg/ml) for 2 h at 37°C, washed with PBS, and injected intraperitoneally into the mice. After a week, the spleen was removed and the cell suspension was prepared and passed through nylon wool column. Approximately more than 80% were T cells after T cell enrichment in terms of CD3, CD4, or CD8 expression. To set up mixed cell cultures, one million responder cells were mixed with one-tenth of transfectants inactivated by MMC, as stimulators in 2-ml volume in 24-well plates. After 5 days co-culture, the cells were harvested, washed with staining buffer, and

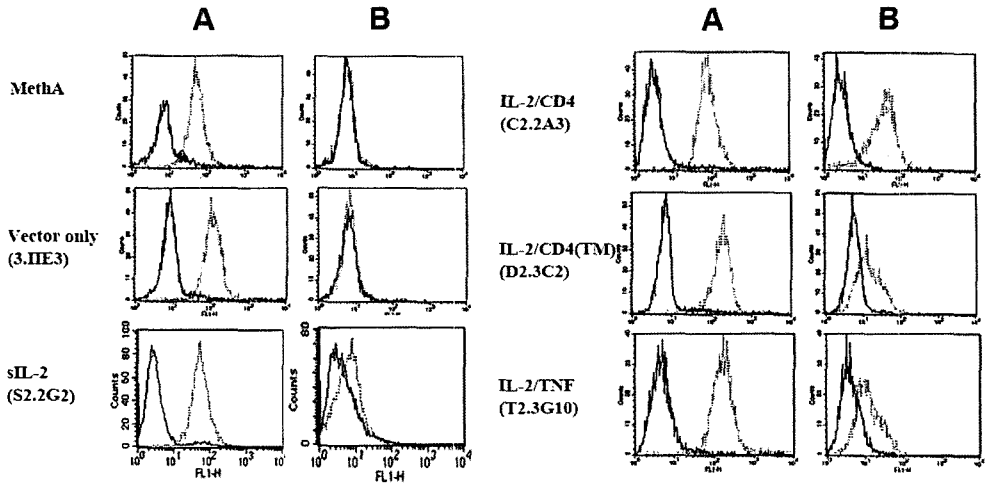

Fig. 2. Surface expression of IL-2 on transfected MethA cells.
Left panel (**A**) stained with the anti-L^d MHC class I mAb, 28-14-8S antibody, and the right panel (**B**) stained with the anti-IL-2 mAb, S4.B6.34 antibody. Solid lines: control secondary FITC-goat anti-mouse (left) and anti-rat (right) IgG antibodies. The identity of the cell clone in each histogram is as follows: MethA cell; vector clone (3.IIE3); sIL-2 clone (S2.2G2); IL-2/CD4 clone (C2.2A3); IL-2/CD4(TM) clone (D2.3C2); IL-2/TNF clone (T2.3G10).

followed by FITC-conjugated anti-CD8 mAb and PE-conjugated anti-CD4 mAb. The stained cells were analyzed by a FACSCalibur flow cytometer (Beckton Dickinson, San Jose, CA, U.S.A.), and nonviable cells were excluded by propidium iodide (Sigma, St. Louis, MO, U.S.A.) staining.

Tumor Challenge

For tumorigenicity studies, syngeneic BALB/c mice were injected with 1×10^6 live cells intraperitoneally, and tumor growth was monitored every other day. After initial tumor clone injection, all mice that once rejected tumor clones were rechallenged with wild-type MethA cells intraperitoneally, and tumor growth was monitored. As control, a new group of age-matched mice (10 mice) were injected with live MethA cells at the same time. The mice were killed when they became moribund according to animal care guidelines.

Results

Expression of the Membrane-Bound Forms of IL-2 (mbIL-2) is Stable on MethA Tumor Cells

IL-2 cDNA was engineered in chimeric forms with CD4 and TNFα cDNAs, to express on tumor cell surface as membrane-bound forms (Fig. 1). Since proper interaction between IL-2 and IL-2R may require specific orientation, we used CD4 as a representative type I transmembrane protein (IL-2/CD4) and TNFα as a representative type II transmembrane protein (IL-2/TNF). To remove the effect of the extracellular domain of CD4, a chimeric protein with only the transmembrane domain and cytoplasmic domain of CD4 was also generated (IL-2/CD4(TM)). The chimeric IL-2 cDNA expression vectors were transfected into MethA fibrosarcoma cells. Tumor clones producing the secretory form of IL-2 (sIL-2) and mock vector transfectant clones were also generated for comparison with the tumor clones expressing mbIL-2. The expression of mbIL-2 on transfected MethA tumor cells was analyzed by flow cytometry using anti-IL-2 antibody (S4.B6.34) (Fig. 2). The IL-2 was expressed on tumor cell surface in all three mbIL-2 tranfectant clones. These clones also expressed similar levels of H-$2L^d$ MHC class I, compared with parental MethA cells. The transfectant clone expressing the secretory form of IL-2 also expressed similar levels of H-$2L^d$ MHC class I to wild-type MethA cells (data not shown). The expression of mbIL-2 and sIL-2 was stable for years. We chose representative clones from each group of transfectant for further study (3.IIE3, mock vector transfectant; C2.2A3, IL-2/CD4 chimeric form; D2.3C2, IL-2/CD4(TM) chimeric form; T2.3G10, IL-2/TNF chimeric form).

mbIL-2 and sIL-2 in MethA Tumor Cells Retain Biological Activity

To test whether mbIL-2 molecules on tumor cells retain their biological activity, a proliferation assay using the IL-2-dependent CTLL-2 cells and spleen cells from 2C TCR transgenic mice was set up. Most of the CD8+ T lymphocytes of the 2C TCR transgenic mouse usually express 2C TCR (20–90%), and the T lymphocytes are responsive to p2Ca peptides on L^d molecules [39, 44]. We assumed that the proliferation of spleen cells from the 2C TCR transgenic mouse would be increased in response to p2Ca/L^d molecules on MethA cells, if mbIL-2 molecules retain biological function. The responder cells, CTLL-2 and 2C spleen cells, were mixed-cultured with the X-ray inactivated wild-type MethA clones and transfectant clones for 48 h, respectively, and

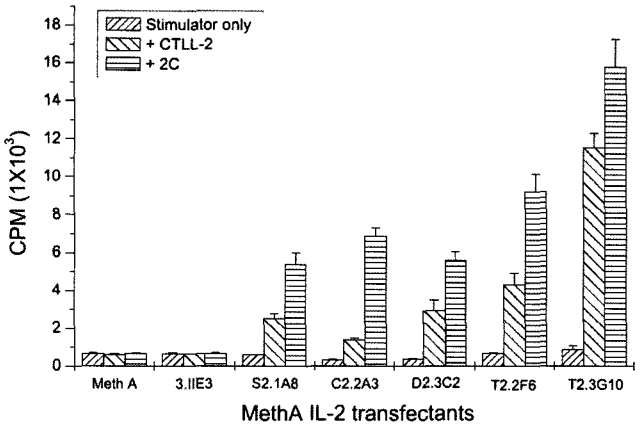

Fig. 3. Proliferation of CTLL-2 and 2C spleen cells in response to IL-2 MethA clones.
CTLL-2 and 2C spleen cells were plated at 1×10^4 per well in a flat-bottomed 96-well plate, and each MethA clone was treated with MMC for 2 h, washed four times, and plated at 1×10^4 per well in triplicate. After 48 h, all of the cell culture was harvested and proliferation was measured by incorporation of ^{3}H-thymidine for a final 12 h. 3.IIE3 (vector transfectant clone), S2.1A8 (sIL-2), C2.2A3 (IL-2/CD4), D2.3C2 (IL-2/CD4(TM)), T2.2F6 (IL-2/TNF), and T2.3G10 (IL-2/TNF) clones were used as stimulators.

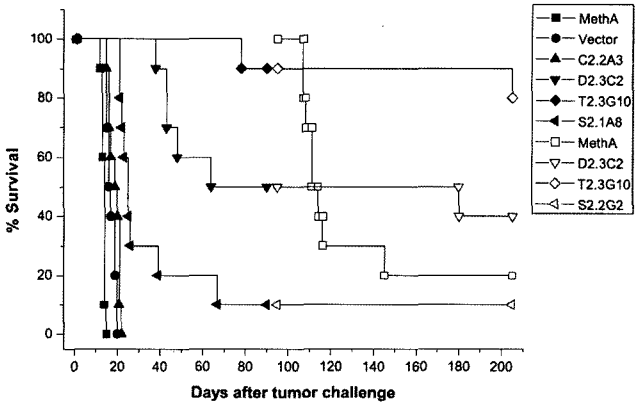

Fig. 4. Survival of mice injected with MethA clones and acquisition of protective immunity to MethA tumor cells.
Mice (10 per group) were injected with each clone (1×10^6 cells per mouse) intraperitoneally, and survival was monitored every other day. On the 95th day, the surviving mice (sIL-2, IL-2/CD4(TM), and IL-2/TNF clones) were re-challenged with wild-type MethA cells (1×10^6 cells per mouse) intraperitoneally. As the control, 10 age-matched normal mice were injected with wild-type MethA tumor cells at the same time.

pulse-labeled with ^{3}H-thymidine for the last 12 h. As shown in Fig. 3, all the mbIL-2 clones and sIL-2 clone supported the proliferation of CTLL-2 cells and spleen cells from 2C transgenic mice. These results indicate that both mbIL-2 on tumor cells and IL-2 by sIL-2 clone are biologically functional. Among them, the IL-2/TNF clone showed the strongest supporting activity compared with the other clones, even though the expression level of IL-2 on cell surface was similar. The IL-2/TNF clone also effectively stimulated spleen cells from 2C TCR transgenic mice to proliferate. The IL-2/CD4 and IL-2/CD4(TM) clones showed relatively weak biological activity compared with the IL-2/TNF clone.

IL-2/TNF Clone has Low Tumorigenicity and Induces Antitumor Immunity to MethA Tumor

To evaluate the tumorigenicity of mbIL-2 tumor clones, survival of BALB/c mice was monitored after injection with tumor clones (1×10^6 cells per group) intraperitoneally (Fig. 4). All of the mice injected with wild-type MethA cells died within 25 days, but nine out of 10 mice injected with the IL-2/TNF tumor clone survived for more than 2 months. To investigate whether surviving mice acquired antitumor immunity, the mice were re-challenged with wild-type MethA cells (1×10^6 cells per group) intraperitoneally. As a control, a group of new mice was challenged with the same number of wild-type MethA tumor cells. On In contrast to the new group of mice susceptible to the tumor cells, 8 out of 9 mice that once rejected the IL-2/TNF tumor clone and 4 out of 5 mice that rejected the IL-2/CD4 clone showed resistance to the wild-type MethA tumor cells. These experiments show that the IL-2/TNF tumor clone has low tumorigenicity and induces systemic antitumor immunity effectively against MethA tumor.

mbIL-2 Tumor Clones are Effective in Stimulation of Syngeneic Spleen Cells or Purified T Cells

BALB/c spleen cells syngeneic to MethA tumor cells were cultured with MMC-treated MethA tumor clones expressing

Table 1. Distribution of T cell subpopulations of spleen cells after mixed-cell culture with MMC-inactivated MethA transfectant clones.[a]

	CD4−/CD8+	CD4+/CD8+	CD4−/CD8−	CD4+/CD8−
Responder only	13.7	2.5	28.9	54.9
MethA	12.4	3.2	42.1	42.3
3.IIE3	12.3	2.4	50.1	35.2
S2.1A8	13.5	1.6	53.0	31.9
C2.3C11	N/D	N/D	N/D	N/D
D2.3C2	13.0	3.6	37.0	46.4
T2.3G10	18.3	2.0	35.3	44.4

[a]This experiment was performed more than three times and a representative result is presented.

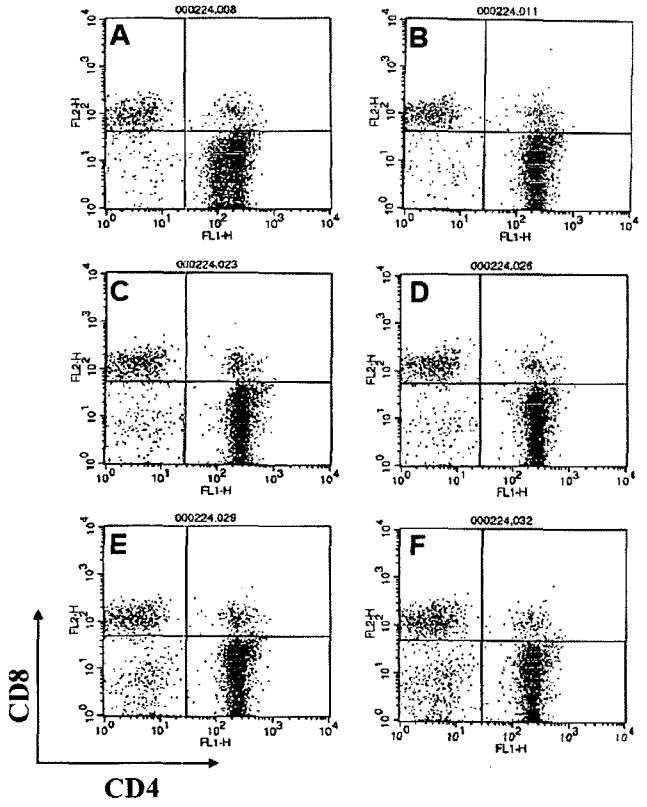

Fig. 5. Flow cytometry analysis of enriched T cells after mixed cell culture with IL-2 transfectants.

Single-cell suspension of spleen cells from three syngeneic BALB/c mice immunized with MMC-inactivated MethA (1×10^6 cells per mouse) was prepared. To enrich T cells, the cell suspension was passed through nylon a wool column. Effluent cells were about 80% T cells using CD4/CD8 double staining. One million responder cells and MMC-inactivated responder cells (1×10^5 cells) were mixed-cultured for 5 days and analyzed for CD4/8 expression. Responder only (**A**), 3.IIE3 (vector) (**B**), S2.1A8 (sIL-2) (**C**), C2.3C11 (**D**) D2.3C2 (IL-2/CD4(TM)) (**E**), and T2.3G10 (IL-2/TNF) (**F**) clones were used as stimulators.

mbIL-2 or sIL-2. After five days of *in vitro* mixed-cell culture, spleen cells were harvested and analyzed for the populations of CD4+ and CD8+ cells. In the spleen cells activated with inactivated MethA cells, the CD4+ population was 42.3% and CD8+ was 12.4% (Table 1), whereas in the group of spleen cells activated with inactivated MethA cells expressing IL-2/TNF, the CD4+ population was 44.0% and CD8+ was 18.3%. The percentage of CD8+ T cells in the group stimulated with the sIL-2 clone was somewhat less than that by IL-2/TNF. This result indicates that the IL-2/TNF clone is superior to wild-type MethA cells and other transfectant clones in stimulating CD8+ T cells to proliferate.

To minimize the effect of monocytes/macrophages in *in vitro* mixed culture, splenic T cells were enriched by panning on plastic culture ware, and through nylon wool column, from BALB/c mice previously immunized with MMC-inactivated MethA cells. After five days in mixed-cell culture with inactivated mbIL-2 tumor clones, cells were analyzed for the populations of CD4+ and CD8+ (Fig. 5 and Table 2). The IL-2/TNF clone was superior to the other clones in stimulating CD8+ T cells to proliferate in the condition that T cells are enriched as responder cells. These results suggest that the mbIL-2 clone may activate CD8+ T cells by direct priming.

Discussion

IL-2 is a growth factor that stimulates the proliferation of Th, CTL, NK cells, and lymphokine-activated killer (LAK) cells, all of which participate in the antitumor immune responses. Among these cells, systemic antitumor immunity relies mainly on Th and final effector CTL. For effective tumor-specific Th and CTL activation, recombinant IL-2 and tumor vaccine transduced with the IL-2 gene have been extensively studied. Unfortunately, systemic administration of recombinant IL-2 and cytokine gene-transduced tumor vaccine encountered unexpected toxic side effects. At present, developing a tumor vaccine that selectively activates tumor-specific CTL is one of the goals in tumor therapy.

In this study, we demonstrated that a MethA clone expressing IL-2/TNF as a type II transmembrane protein induced antitumor immunity. The induced antitumor immunity by the IL-2/TNF clone is superior to the IL-2/CD4 and IL-2/CD4(TM) clones, which are expressed as a type I transmembrane protein. The difference may result from the orientation of the chimeric IL-2 molecules on the tumor

Table 2. Distribution of T cell subpopulations of enriched T cells after mixed-cell culture with MMC-inactivated MethA transfectant clones.[a]

	CD4−/CD8+	CD4+/CD8+	CD4−/CD8−	CD4+/CD8−
MethA	9.2	3.8	2.5	84.6
3.IIE3	10.3	5.2	1.7	82.8
S2.1A8	11.7	3.9	3.4	81.0
C2.3C11	10.6	3.2	2.8	83.5
D2.3C2	13.7	4.8	6.4	75.2
T2.3G10	15.1	3.0	7.5	74.4

[a]This experiment was performed more than three times and a representative result is presented.

cell surface. In comparison with the sIL-2 clone, mbIL-2 clones were similar or stronger to the IL-2 biological activity. However, the IL-2/TNF and IL-2/CD4(TM) clones showed stronger activity in antitumor immunity induction. It should be noted that the amount of secreted IL-2 by the sIL-2 clone is not comparable with the expression level of IL-2 on the cell surface on the mbIL-2 tumor clone. The produced IL-2 amount and the half-life of soluble IL-2 and IL-2 on the tumor cell surface would be different. Furthermore, from the tumorigenicity study, the mice injected with the sIL-2 clone died in a shorter period compared with those injected with the IL-2/CD(TM) clone or IL-2/TNF. The result indicates that the survival time of the sIL-2 tumor clone *in vivo* is longer than that of the IL-2/CD4(TM) and IL-2/TNF clones.

In induction of antitumor immunity, a cross presentation mechanism of tumor-associated antigen (TAA) involving antigen-presentation cells like dendritic cells has been proposed [12]. Using a dendritic cell growth factor, GM-CSF, the *in vitro* cultured dendritic cells and GM-CSF transduced tumor cells were effective in tumor therapy [14, 40, 49]. Transfer of dendritic cells pulsed with apoptotic tumor cells also induced antitumor immunity effectively. These approaches all depend on the antigen-presenting cells, which express MHC classes I and II. However, it is not known how TAA is picked up by dendritic cells and presented to CTL through the TAA peptide/MHC class I complex. On the other hand, there are many reports suggesting direct priming of CTL by tumor cells [46, 47]. Expression of co-stimulatory molecules on tumor cells also resulted in CTL activation [3, 19, 45], suggesting direct contact of tumor cells with CTL. Co-stimulatory molecules on the tumor cell surface may act on corresponding receptors on CTL during the physical contact.

Similarly, tumor cells engineered to express a membrane-bound form of TNFα also induced antitumor effect [33]. Membrane-bound forms of GM-CSF [14 40, 49] and Flt3 ligand [9] were also functional in stimulation of antigen-presenting cells. Tumor cells engineered to express the GPI-anchored form of IL-12 induced antitumor immunity [10]. A cancer vaccine anchoring recombinant IL-2 via diphtheria toxin T domain induced tumor specific CTL activity [36]. Through this approach, they could observe a reduced toxicity of IL-2. The IL-4 on MethA tumor cells and the IL-2 on B16 melanoma cells also induced systemic antitumor immunity when they were expressed as membrane-bound forms [7, 24]. All of these reports suggest that membrane-bound form of molecules retain biological activity and are able to stimulate target cells by cell-to-cell contact. In our study, we compared the stimulatory effect of each clone by setting up mixed-cell cultures with spleen cells from BALB/c mice. The IL-2/TNF clone was superior to the other clones in supporting proliferation of CD8+ T cells (Table 1). Furthermore, the selective stimulation of CD8+ T cells were observed in the mixed-cell culture using enriched T cells (Table 2). This result suggests that the IL-2/TNF may stimulate CD8+ T cells by direct priming.

The presented data in this report suggest that the IL-2/TNF expression on tumor cells could be an effective approach for cytokine gene therapy of tumors, probably focusing on immune response to tumor-specific CTL.

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