

Insoluble Fraction of Tumor Cell Homogenate Is a Useful Material for Eliciting Cytotoxic T Lymphocytes: A Unique Method for Protein Solubilization

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Abstract. *Background/Aim:* Dendritic cell (DC)-based cancer immunotherapy using tumor homogenate has been evaluated. In all previously reported cases, DCs have been pulsed with a soluble fraction (lysate) of the tumor homogenate. The aim of this study was the evaluation of DCs pulsed with solubilized insoluble fraction of tumor cells. *Materials and Methods:* Solubilized recombinant murine TRP-2 and solubilized-insoluble fraction of B16 melanoma was prepared by a novel method using nucleotides. Bone marrow-derived DCs were electroloaded with the solubilized proteins. *Results:* Cytotoxic T lymphocytes were elicited in the splenocytes of C57BL/6 mice immunized with electroloaded DCs with solubilized rTRP-2. CD8⁺ T-cells derived from immunized mice with electroloaded DCs using the solubilized insoluble fraction of B16 melanoma had specific killing activity. The effects were augmented when DCs were electroloaded with both the soluble and insoluble fractions of B16 homogenate. *Conclusion:* Insoluble fraction of tumor cells is a useful material for cancer immunotherapy.

Dendritic cells (DCs) are antigen-presenting cells and powerful tools for manipulating the immune system (1). Since the method for differentiation of DCs from monocytes in peripheral blood mononuclear cells was established (2), clinical studies have examined the feasibility of manipulating DCs with tumor antigens for immunotherapy protocols. Many protocols for producing DC vaccines for cancer immunotherapy have been developed (3). The most common method involves simply co-incubating DCs with an autologous tumor lysate as the antigen; this process, known as pulsing, takes advantage of the ability of DCs to take up

extracellular material through macropinocytosis (4). The expected antitumor effect is minimal, despite some immunological responses reported in clinical studies (5, 6). Rosenberg *et al.* reviewed results of many cancer vaccine studies, including peptide vaccines, viral vaccines, and DC vaccines (7). They reported a 4.0% response rate for peptide vaccines, 7.1% for DC vaccines, and no response from viral vaccines. Based on that report, the current authors believe that the DC processing method must be improved for effective induction of antitumor effector cells.

A possible reason for the low antitumor effect of the DC vaccine may be the pulsing method because the presentation capacity via the major histocompatibility complex class I (MHC class I) pathway was low. The most common strategy for antigen presentation of MHC class I appears to be the use of a restricted peptide derived from a defined antigen. Although this technique is important for proof-of-concept studies, the use of a peptide has limitations: restriction to a given HLA type, limited number of well-characterized tumor-associated antigens (TAAs), relatively rapid turnover of exogenous peptide-MHC complexes resulting in comparatively low antigen presentation at the time DCs appear in the draining lymph node after injection, and induction of a restricted repertoire of T-cell clones, thus limiting the ability of the immune system to control tumor antigen variation (3). For efficient MHC class I-restricted presentation from cytoplasmic proteins of DCs, the ubiquitin-dependent proteolytic pathway in the cytosol is indispensable (8). Some studies have shown that tumor antigen cDNA or mRNA is expressed in DCs (9, 10). These methods are appropriate when large amounts of antigenic protein exist in the cytosol, but the safety of these methods has not been established as they are restricted by the guidelines for gene therapy. Therefore, this study focused on an electroloading method using tumor lysate (11) for efficient MHC class I presentation because the current authors believe that the method would be safer than most gene therapies. It has been reported that vaccination with DCs electroloaded with tumor lysate resulted in higher naïve T-cell activation *in vitro* than co-incubation, elicited specific

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tumor cell killing *in vitro*, and reduced tumor metastases in a mouse model (11). Regardless of DCs being subjected to electroloading, the only antigenic protein that was able to be used was tumor lysate, *i.e.*, the soluble fraction of tumor tissue homogenates. Therefore, if the insoluble fraction of tumor tissue homogenates was able to be subjected to electroloading, DCs would be able to present peptides from insoluble TAA proteins. This study hypothesized that this strategy may be a potential treatment for cancer patients. To accomplish this, it is necessary to solubilize insoluble proteins in a physiological solution, and then sterilize the solubilized protein by filtration.

Many methods for refolding of recombinant proteins have been developed, especially in the case of *Escherichia coli* expression systems (12-14). However, these methods are suitable only for specific target proteins and another device is necessary to accomplish solubilization of a mixture of proteins. A material was sought for solubilization from biogenic substances because these substances would show a low toxicity to DCs. The peptide bond frame forms hydrogen bonds with different parts of the frame and nucleotides form hydrogen bonds between the bases. The urea molecule then breaks the hydrogen bond between the nucleotide bases. When refolding of denatured protein is desired, it is usually temporarily dissolved in urea or guanidine hydrochloride solution. When the protein is continuously dialyzed against a physiological buffer, in many cases it reverts to an insoluble form. This phenomenon led the authors to believe that materials able to form hydrogen bonds must play a key role in solubilization. Therefore, it was hypothesized that monomeric nucleotides may interact with proteins and render them soluble in a physiological solution.

In this report, it is shown that insoluble recombinant murine tyrosinase-related protein-2 (rTRP-2) is recovered in phosphate-buffered saline (PBS) in the soluble form by addition of nucleotides. Solubilized rTRP-2 was incorporated into DCs by electroloading, and DCs were able to elicit TRP-2 tetramer-positive cytotoxic T lymphocytes (CTL). Furthermore, to test the assumption, the antitumor response of DCs incorporated with the solubilized insoluble fraction of B16 melanoma by a CD8⁺ cell-based killing assay was examined.

Materials and Methods

Mice and cell lines. All mouse studies were performed with the approval of the Medinet Animal Care and Use Committee and in accordance with the institution's animal ethics guidelines (Medinet Medical Institute, Tokyo, Japan). Seven-week-old male C57BL/6 mice were obtained from SLC Co., Ltd. (Shizuoka, Japan) and maintained in a specific pathogen-free condition in the host laboratory. They were used at eight to eleven weeks of age.

Mouse B16 melanoma (JCRB0202), human MeWo (JCRB0066), and G-361 (IFO50009) melanoma cell lines were obtained from

Health Science Research Resources Bank (Osaka, Japan). MDA-MB-231 was a generous gift from Dr. T. Takahashi (First Department of Internal Medicine, Sapporo University School of Medicine, Japan). These cell lines were maintained by culture in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Tissue Culture Biologicals, Tulare, CA, USA). Malme-3M human melanoma cell line was obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in Iscove's MEM (Invitrogen) supplemented with 20% FBS.

SDS-PAGE and western blotting. Cultured B-16, Malme-3M, MeWo, or G-361 cells were washed with PBS 3 times and recovered with a cell scraper as a suspension in PBS when the cells reached sub-confluence. Cells were precipitated by centrifugation at 1,000 \times g for 5 min at 4°C and stored at -80°C for further use. Cells were re-suspended in PBS (400 μ l/100 mg), and subjected to 6 cycles of freezing (-80°C) and thawing (30°C). Recovered soluble and insoluble fractions after centrifugation at 14,100 \times g for 1 h at 4°C were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins after separation by electrophoresis were stained with Coomassie Brilliant Blue (CBB) R25 (Wako Pure Chemical Industries, Osaka, Japan).

For Western blot analysis, proteins separated by electrophoresis were transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Tokyo, Japan) using the semi-dry blotting system (Trans-Blot SD Cell; Bio-Rad Laboratories). After blocking the membranes with SuperBlock Blocking Buffer in TBS (Thermo Scientific, IL, USA), proteins were incubated with anti-murine TRP-2 rabbit IgG (H-150; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-gp100 goat IgG (K-18; Santa Cruz Biotechnology), or anti-human melanoma antigen recognized by T-cell 1 (MART-1) monoclonal antibody (SPRING, CA, USA) for 1 h at 37°C. The membranes were washed four times with 0.1% Tween 20 containing PBS for 5 min, and then reacted with biotinylated anti-rabbit Ig, biotinylated anti-goat/sheep Ig, or biotinylated anti-mouse Ig at room temperature for 45 min. All secondary antibodies were obtained from GE Healthcare Japan (Hino, Japan). After the same washing procedure, membranes were incubated with streptavidin-alkaline phosphatase conjugate (GE Healthcare) at room temperature for 30 min, washed repeatedly, and reacted with BCIP/NBT solution (Wako) as the substrate.

Construction of rTRP-2 expression plasmids. Recombinant DNA procedures were performed with the approval of the Biosafety Committee of Medinet Medical Institute (Tokyo, Japan) and in accordance with institution's guidelines. Murine TRP-2 cDNA was isolated using reverse transcription-polymerase chain reaction (RT-PCR) from the total RNA fraction of B16 cells using primers (F: 5'-TATCATATGGAGGGCAGGGGGCAGTG-3' and R: 5'-ATTGGATCCTCATTATGAGAGAGAGTTGTGGACCAAC-3'). The RT-PCR procedure has been described previously (15). PCR was performed with LA Taq (TAKARA BIO, Shiga, Japan) after denaturation at 95°C for 1 min and 30 amplification cycles were performed. A single cycle involved incubation at 95°C for 30 s, 57°C for 30 s, and 72°C for 1.5 min. The amplified DNA fragment of 1.3 kbp was digested with *Nde* I and *Bam* H I and inserted in *Nde* I – *Bam* H I sites of the pET-19b vector (Novagen: MERCK, Tokyo, Japan). The nucleotide sequence was confirmed by sequencing the construct (pET19b/mTRP2). For the chimera type with an ubiquitin

fragment, cDNA of ubiquitin was amplified by PCR from MDA-MB-231 cDNA with primers (F: 5'-AGACTTAAGACCATGCAGATCTTCGTGAAGACTCTGACTGG-3' and R: 5'-TCTGGTACCGGCACCTCTGAGACGGAGTACCAGGTGC-3'). The R-primer changed the C-terminal amino acid from glycine (G76) to alanine (A76). The PCR product was digested with *Afl* II and *Kpn* I and cloned in pTracer-SV40 (Invitrogen); the construct (pTracer-Ub) was confirmed by sequencing. Second PCR was performed with pTracer-Ub as a template and primers (F: 5'-AGACA TATGCAGATCTTCGTGAAGACTCTGACTGG-3' and R: 5'-TCTCATATGGGCACCTCTGAGACGGAGTACCAGGTGC-3'). The PCR product was digested with *Nde* I, and the DNA fragment of ubiquitin was inserted in the *Nde* I site of pET19b/mTRP-2. A clone (pET19b/Ub-TRP-2) was screened from the sequencing result for confirmation of the direction and nucleotide sequence. All endonucleases were obtained from TAKARA BIO.

Expression and purification of rTRP-2 proteins. The Rosetta-gami2(DE3)pLysS *E. coli* (Novagen) was transformed with pET19b/mTRP-2 or pET19b/Ub-TRP-2; some colonies were then cultured with 50 µg/ml of ampicillin (Wako), 35 µg/ml of chloramphenicol (Wako), and 1 mM of isopropyl-β-D(-)-thiogalactopyranoside (IPTG) (Wako). All *E. coli* colonies showed large amount of recombinant protein in SDS-PAGE and Western blotting analyses with anti-murine TRP-2 antibody. Each clone was stocked as the master stock, which was then cultured overnight with antibiotic-containing Luria Bertani (LB; 200 ml) media with addition of 1 mM IPTG mixed with 500 ml of LB. *E. coli* were harvested after 6 h of culture with IPTG and washed with PBS.

The harvested cells were suspended in EDTA-free Complete™ (protease inhibitor cocktail; Roche Diagnostics, Indianapolis IN, USA) containing PBS and lysed by sonication. The pellet and supernatant were recovered after 1 h of centrifugation (10,000 ×g at 4°C). Both fractions were analyzed by SDS-PAGE, thus confirming that the recombinant proteins were in the precipitate. Precipitate were suspended in 4 M urea-PBS (pH 7.4) and centrifuged as described previously. The precipitate was recovered and re-suspended in 5 mM dithiothreitol (DTT, Wako) containing 8 M urea-PBS (pH 8.5) to dissolve the recombinant proteins. Recombinant proteins were purified by Ni-charged chelating chromatography using the HisTrap HP column (GE Healthcare) and gel filtration chromatography (GFC) using the Superose 6 column (GE Healthcare). Both chromatographies were performed using the AKTA explorer system (GE Healthcare). Elution of recombinant proteins in chelating chromatography was performed using an imidazole (Wako) gradient (40 to 500 mM) in 5 mM DTT containing 8 M urea-PBS (pH 8.5) and that for GFC was performed using 5 mM DTT containing 6 M urea-PBS (pH 8.0). After purification, reduced materials (rUb-TRP-2 or rTRP-2) were subjected to solubilization experiments.

Solubilization of insoluble proteins. The oxidized form of glutathione (GSSG, Wako) was added (final concentration, 2.5 mM) to 6 M urea solution of rUb-TRP-2 or rTRP-2, and then the nucleotide (final concentration, ~12.5 mM) was added to the solution. After 1 h of incubation at room temperature, 50% glycerol (Wako) in the amount of 1/5 of the existing volume was added, and the sample was dialyzed against 10% glycerol, 150 mM NaCl containing 20 mM Tris-HCl (pH 8.4) or 10% glycerol-PBS (pH 8.0) containing 10 µM of the corresponding nucleotide (dialysis buffer).

The nucleotide used with the dialysis buffer was the same as that added to the sample tube. The recovered rUb-TRP-2 or rTRP-2 in supernatant or precipitate was analyzed by SDS-PAGE after centrifugation. The precipitate was then dissolved in the same volume of 2× SDS sample-reagent. The dNTP mixture was obtained from GE Healthcare and the other nucleotides were obtained from Sigma. The percentage of recovered rUb-TRP-2 or rTRP-2 in the supernatant (*i.e.*, the efficacy of solubilization) was calculated using Quantity One software (1st version) of Gel-Doc 2000 (Bio-Rad) after CBB staining.

For solubilization of the insoluble fraction of cell homogenates, precipitate was dissolved in 8 M urea-PBS and the efficacy of solubilization was estimated by protein assays: the soluble fraction by the BCA method (BCA protein assay kit; Thermo Scientific) and the insoluble fraction by the Bradford method (16). The concentration of residual urea was confirmed after dialyses using the Quantichrom Urea Assay Kit (BioAssay Systems, Hayward, CA, USA). The average value was 50 µg/ml, which was lower than that in peripheral blood (220 µg/ml).

Generation and culture of murine DCs. Normal C57BL/6 male mice were anesthetized by Nembutal injection (Dainippon Pharmaceutical, Osaka, Japan). Blood was collected from mouse hearts and serum obtained from the blood was used for culturing DCs. DCs were generated as described by Weiss *et al.* (11) and Balkow *et al.* (17), with some modifications. In brief, bone marrow cells collected from murine tibias and femurs after blood sampling were washed twice with AIM-V media (BioWhittaker, Walkersville, MD, USA) and passed through a nylon mesh. Cell suspension of AIM-V supplemented with 0.2% FBS, 2 mM L-glutamine (Invitrogen), 30 ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech, London, UK), and 10 ng/ml recombinant murine interleukin (IL)-4 (PeproTech) was seeded in 75 cm² or 225 cm² tissue culture flasks (Sumilon, Tokyo, Japan) at a cell density of lower than 2.5×10⁶ cells/ml. On day three, the same volume of fresh medium was added to the culture flasks. On days five and seven, fresh media was exchanged for half of the media volume. On day nine, the suspended cells were harvested and transferred to T75 flasks (Ultra Low Attachment Surface; Corning, NY, USA) or 6-well plates (Ultra Low Attachment Surface; Corning). The maturation medium (AIM-V supplemented with 0.2% FBS, 2 mM L-glutamine, 30 ng/ml recombinant murine GM-CSF, and 1 µg/ml lipopolysaccharide) was added to reach a cell density of 5×10⁵ cells/ml. The suspended cells were harvested on day ten, and a small aliquot of the cells was analyzed for surface markers by flow cytometry (FCM). Mature DCs with 0.2% normal C57BL/6 mouse serum (replacing FBS in the maturation medium) were used for antigen presentation experiments on day ten.

Electroporation and antigen roading of DCs. Harvested mature DCs were washed with X-VIVO 20 medium (Lonza, Basel, Switzerland) and adjusted to a density of 5×10⁷ cells/ml with the medium. Then, 400 µl of DCs were mixed with 4 µl of 50 mg/ml fluorescein isothiocyanate (FITC)-dextran (Sigma) and transferred to a cuvette (EC-002S, NEPA GENE, Chiba, Japan). Electroporation (Ep) was performed using the CUY21Pro-Vitro model (NEPA GENE) set to a poring pulse of 170 V (P on: 15 ms, P off: 50 ms) and a driving pulse of 20 V (P on: 50 ms, P off: 50 ms, ten cycles). After Ep, DCs were transferred to a 24-well plate

with AIM-V supplemented with 0.2% mouse serum, 2 mM L-glutamine, 30 ng/ml recombinant murine GM-CSF, and 10 ng/ml of murine interferon gamma (IFN- γ ; PeproTech), and incubated for 1 h at 37°C. For the solubilized protein and cell lysate, a final protein concentration of 2.0 mg/ml (the volume was less than 20%) was added to DCs and incubated for 16 h after Ep. An antigenic peptide (TRP-2: SVYDFFVWL) synthesized by Invitrogen was used for peptide pulsing. DCs were cultured with the maturation medium, added to the peptide at a final concentration of 1.0 μ g/ml, and further cultured for 6 h.

Immunization with DCs. Antigen-pulsed DCs were washed with 0.2% mouse serum containing PBS, and 1×10^6 viable DCs/mouse (for tetramer assay) or 5×10^5 viable DCs/mouse (for killing assay) were injected in the abdominal cavities of normal C57BL/6 male mice (performed in duplicate for tetramer assay and in triplicate for killing assay). Immunization was performed once per week (three times in all). Splenocytes were recovered from the immunized mice one week after the last immunization. Red blood cells in the splenocytes were lysed with 0.83% ammonium chloride and 20 mM Tris (pH 7.4). Splenocytes were then washed with 0.5% FBS containing PBS (for FCM analysis) or complete RPMI-1640 medium supplemented with 10% FBS (for cytotoxic assay).

FCM. Bone marrow-derived DCs were analyzed routinely for surface makers using monoclonal antibodies to CD11b (FITC-anti-CD11b, clone M1/70), CD11c (PE-anti-CD11c, clone N418), CD80 (FITC-anti-mouse CD80, clone 16-10A1), and CD86 (Biotin anti-mouse CD86, clone GL-1). The isotype controls were FITC-rat IgG2b (clone RTK4530), PE Armenian Hamster IgG (clone HTK888), FITC Armenian Hamster IgG (clone HTK888), and Biotin rat IgG2a (clone RTK2758), respectively. Streptavidin-PE/Cy5 was also used for CD86 analyses. All antibodies to analyze DC phenotype were purchased from Biolegend (San Diego, CA, USA). For the tetramer assay, H-2K^b TRP-2 tetramer -SVYDFFVWL-PE (MBL, Aichi, Japan) was used to elicit peptide-specific CTL and H-2K^b OVA tetramer -SIINFEKL-PE (MBL) was used as a negative control. Anti-CD8a-FITC (clone 53-6.7) and isotype control rat IgG2a (clone R35-95) were obtained from BD Bioscience. For B16 phenotype analyses, monoclonal antibodies to H-2K^b (clone AF6-88.5, PE), H-2D^b (clone KH95, FITC) and isotype controls, mouse IgG2a (clone G155-178, PE) and mouse IgG2b (clone MPC-11, FITC), were obtained from BD Biosciences. The cells were analyzed by FCM using an Epics XL (Beckman Coulter).

In vitro cytotoxicity assay. CD8a⁺ T-cells were isolated from splenocytes of immunized mice by negative selection using the CD8a⁺ T-Cell Isolation kit and MS MACS Columns (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD8a⁺ T-cells were cultured for six days with DCs using 10 U/ml of recombinant human IL-2 (Proleukin; Chiron) and complete RPMI-1640 medium. B16 cells were cultured for 24 h with 5.0 ng/ml of IFN- γ to express MHC and were then labeled with 5 μ g/ml of calcein-AM (Dojindo, Kumamoto, Japan). The labeled target cells were cultured for 3 h with the effector CD8a⁺ T-cells. Results were normalized by subtracting spontaneous calcein-AM fluorescence from tumor cells and presented as a percentage of maximal cell-killing (using 2% Triton X-100 to obtain maximal release). Cytotoxicity (%) was calculated using Terascan VP (Minerva Tech K.K., Tokyo, Japan) software.

Statistics. Student's paired *t*-test (JMP 6) was used to determine statistical significance between experimental groups. Results were considered to be significant for $p < 0.05$.

Results

Solubility of proteins including TAA in melanoma cells. Many TAA have been identified until date; however, not all are soluble proteins in the natural state. To resolve any doubt, the solubility of some melanoma-associated antigens was analyzed. Native mouse TRP-2 and gp100 antigens in B16 melanoma or human MART-1 antigens in Malme-3M, MeWo, or G-361 melanomas were detected by Western blotting. These cells were harvested and prepared as PBS suspensions. The cells in PBS were homogenized by freeze/thaw cycles. After centrifugation, supernatant contained the soluble proteins. Insoluble proteins were present in the precipitate fraction. The amount of total protein in these fraction derived from human melanoma are shown in Table I. Approximately 59%-47% protein was present in the insoluble fraction. Whether the TAA were present in the soluble or insoluble fraction was analyzed. Native TRP-2 was recovered from the soluble fraction and gp100 from the insoluble fraction of the B16 homogenate (Figure 1A). Most of the MART-1 protein was recovered from the insoluble fraction of the homogenate of all human cell lines (Figure 1B). Only a small amount of soluble MART-1 was detected in the soluble fraction of MeWo and G-361 homogenates. From these results, it was concluded that a large amount of TAA had previously been discarded with the insoluble fraction when tumor lysate was used to prepare pulsed DCs. A procedure was devised to avoid this problem, which is described in the next subsections.

The effect of nucleotides on solubilization of insoluble proteins. Native TRP-2 protein was recovered in the soluble fraction of melanoma (Figure 1A), but rTRP-2 and rUb-TRP-2 were insoluble in an *E. coli* expression system, forming an inclusion body. Thus, the above proteins were used as models for insoluble proteins. The rTRP-2 and rUb-TRP-2 proteins were partially purified by Ni²⁺ affinity chromatography and GFC. Urea solution of partially purified rTRP-2 or rUb-TRP-2 was used as the starting material for solubilization studies. Neither of these proteins were detected in the soluble fraction after dialyzation against PBS (pH 7.4) or Tris-buffered saline (pH 7.4) (data not shown). Therefore, solubilization was attempted by adding GSSG before dialysis against PBS with or without 10% glycerol. No soluble protein was detected in either case (the result for rUb-TRP-2 is shown in Figure 2-1, Figure 2-2). Next, arginine, dNTP, or both were added to the reaction tubes. Although soluble proteins were not detected after addition of arginine (Figure 2-3), solubilized proteins (51%) were detected after dNTP addition (Figure 2-4). Arginine repressed the effect of dNTP effect on protein

Table I. Protein amount in soluble (*sup.*) or insoluble (*ppt.*) fractions after freezing and thawing using human melanoma lines.

Cell line	Malme-3M	MeWo	G-361
Wet weight (mg)	37	52	57
Sup. (μg)	611±0	853±21	671±16
Ppt. (μg)	720±36	768±30	964±44

Cells were harvested in phosphate-buffered saline (PBS) from confluent culture in 75 cm² flasks using cell-scrappers. Wet weight of cell pack was measured and cells were resuspended in PBS (400 μl/100 mg). Cells were subjected to six successive cycles of freeze and thaw. After centrifugation, protein assays were performed. Protein amounts in the soluble fractions were estimated by the BCA method. Protein amounts in the insoluble fractions were estimated by the Bradford method after solubilization with same volume of 8 M urea-PBS solution. Data are representative of two independent experiments±SE.

Table II. The effects of GMP or UMP on solubilization of the MeWo-insoluble fraction.

Nucleotide	GMP	UMP	None
Soluble protein (μg)	83.3±2.97 *	109±8.62 *	65.0±11.2
Recovery (%)	45.8	59.9	35.9

The 8 M urea-PBS solution of the MeWo-insoluble fraction was divided between three tubes. Each tube contained 182 μg protein estimated by Bradford method. The oxidized form of glutathione at a final concentration of 10 mM was added to all tubes and 10 mM of GMP, UMP or none were added respectively. After dialysis against 10% glycerol-PBS (pH 7.4) containing 10 μM of each nucleotide or none, the protein amount of each supernatant was assayed by BCA method. Data are representative of two independent experiments±SD (n=4). **p*<0.02.

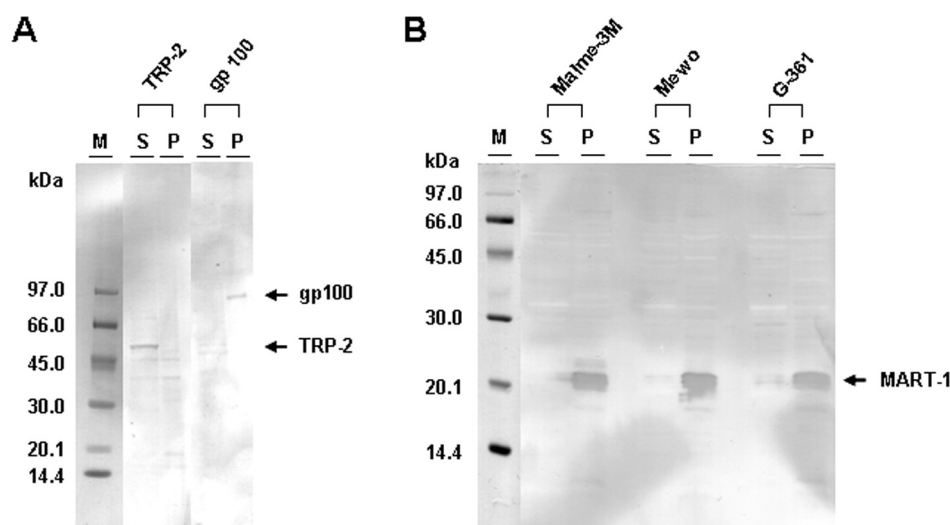


Figure 1. Solubility of melanoma-associated antigen in melanoma cells were analyzed by Western blotting. Melanoma cells from (A) mouse B16, or (B) human Malme-3M, MeWo, or G-361 were harvested from culture flasks. The insoluble fractions (precipitation: P) after freezing and thawing were solubilized in 2× SDS-PAGE sample reagent in the same volume as the soluble fractions (supernatant: S). The same volumes of both fractions in 1× SDS-PAGE sample reagent were applied to 10.5% (A) or 13.5% (B) acrylamide gel. Various protein transferred membranes were subjected to immunoblotting using (A) anti-TRP-2 antibody, or anti-gp100 antibody, or (B) anti-human MART-1 antibody. Data are representative of two independent experiments. M: Molecular weight marker. Transverse arrows show migration points of gp100, TRP-2, and MART-1, respectively.

solubilization (Figure 2-5). From this experiment, it was concluded that addition of deoxyribonucleotides might be an effective method to solubilize insoluble proteins.

Next, the solubilizing ability of each nucleotide was examined separately because the above finding was based on the addition of dNTP mixture. dATP, dTTP, dCTP, and dGTP were added at a final concentration of 10 mM, separately to rTRP-2 urea solutions and an experiment similar to that performed previously with the dNTP mixture was performed. The solubilizing ability of dTTP, dCTP, and dGTP was equal

to that of the dNTP mixture, whereas that of dATP was inferior (Figure 3A). To find the appropriate concentration for solubilizing insoluble proteins and explore whether other nucleotides have the same capacity, 2, 10, and 50 mM of GTP or dGTP were added in turn and a similar experiment was performed. Addition of 2–10 mM GTP or dGTP solubilized rTRP-2, but addition of 50 mM GTP or dGTP was ineffectual (Figure 3B). Similarly, the effect of 0.5–10 mM GMP, GTP, UMP, or UTP was examined. It was found that 0.5–10 mM GMP, 0.5–2 mM GTP, 0.5–10 mM UMP, and

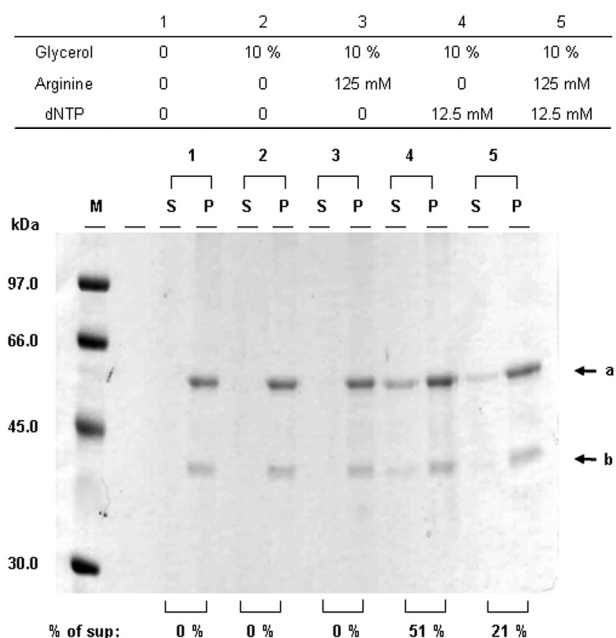


Figure 2. The effect of dNTP mixture on solubilization of rUb-TRP-2 was analyzed by SDS-PAGE. Reduced rUb-TRP-2 was divided between 5 tubes and 2.5 mM of GSSG was added to all tubes. Glycerol, arginine or dNTP was added before dialyzation as shown in the figure. Recovered soluble (S) and insoluble (P) fractions after dialyzation were analyzed by SDS-PAGE as described in the Materials and Methods. Data are representative of 2 independent experiments. Arrow a is the migration point of rUb-TRP-2 and arrow b is the point of truncated rUb-TRP-2. Percentage of sup was calculated after CBB R25 staining as follows, $\% = S(v) \times 100 / S(v) + P(v)$, where $v = \text{area} \times \text{concentration}$ of (a) + area \times concentration of (b). The other symbols are same as those in Figure 1.

0.5-2 mM UTP showed the solubilizing effect (Figures 3C and 3D). From these experiments, it was concluded that various nucleotides, including deoxyribonucleotide 5'-triphosphates (with the exception of dATP), GTP, GMP, UTP, and UMP have the ability to suppress aggregation of protein, despite differences in the appropriate concentration.

GMP and UMP were selected for further analyses because these nucleotides were more cost-effective compared with the other nucleotides and they have an acceptable capacity for solubilization. Instead of rTRP-2, the MeWo insoluble fraction was used. The recovery of solubilized proteins with GMP addition was 45.8% and that with UMP addition was 59.9% after performing the solubilizing procedure. These values were significantly higher than those in the nucleotide free condition (35.9%) (Table II). Furthermore, it was examined whether it was possible to solubilize the MART-1 protein in the insoluble fraction of MeWo (refer to Figure 1B) by this method. The result of Western blotting with anti-MART-1 antibody is shown in Figure 4. The MART-1 protein was clearly detected in both the soluble and insoluble fraction

after solubilization with GMP or UMP. From the results derived from the experiments that have been performed in this study, we concluded that various TAAs were able to be recovered as solubilized protein from an insoluble fraction of tumor cell homogenate using this method.

DC preparation using solubilized rTRP-2 protein and eliciting CTL. Weiss *et al.* have previously reported efficient responses in a murine renal tumor model by electroloading DCs with tumor lysate (11). It was expected that solubilization of insoluble proteins would be applicable to antigen presentation of DCs using the Weiss *et al.* procedure. The AIM-V culture medium supplemented with 0.2% FBS was used to generate DCs from bone marrow cells because it has been suggested that FBS influences CD4⁺ T-cells and may induce tolerance in immunized individuals (18), and mature DCs cultured with serum-free AIM-V showed low viability after Ep in our preliminary experiments (data not shown). Figure 5A shows data typical of the DC phenotype from this preparation. In almost all cases, more than 80% of gated cells in a left panel expressed CD11c (CD11c⁺ CD11b⁺ plus CD11c⁺ CD11b⁻) and more than 85% of gated cells in a left panel expressed CD80 or CD86 (CD80⁺ CD86⁺ plus CD80⁺ CD86⁻ plus CD80⁻ CD86⁺). To confirm the transduction efficacy of DCs by Ep in this system, the ratio of FITC-dextran-incorporated cells was analyzed by FCM. Figure 5B shows one of the results. The ratio of FITC-positive DCs in case of co-culture with FITC-dextran was 2.50% and that in case of Ep was 86.2%. Cell viabilities of both cases were greater than 90% or 80% estimated by trypan blue uptake, respectively. It can therefore be concluded from these results that the electroporated DCs may retain their activity in the bodies of mice.

Before performing the immunization experiment using DCs electroloaded with proteins, a preliminary experiment was performed using TRP-2 peptide. An equivalent level of TRP-2 tetramer-positive cells (CTL) was observed in splenocytes of mice immunized with intradermal or intraperitoneal (*i.p.*) injection, and the deviation of results from *i.p.*-injected mice was small (data not shown). Thus, *i.p.* was selected as the injection route. To examine the capacity of electroloaded DCs with solubilized protein, solubilized rTRP-2 was used as a model protein, which had been obtained by the addition of UMP or GMP as described previously in this report. DCs for immunizations were prepared using the same Ep protocol as that for FITC-dextran. After overnight incubation with 0.2% normal mouse serum, GM-CSF, and IFN- γ -containing medium, DCs were harvested, washed, and injected *i.p.* in normal C57BL/6 mice. CTL induction *in vivo* was analyzed after the third immunization using TRP-2 tetramer. Electroloading DCs with solubilized TRP-2 obtained by addition of UMP and/or GMP induced a level of CTL equivalent to that obtained

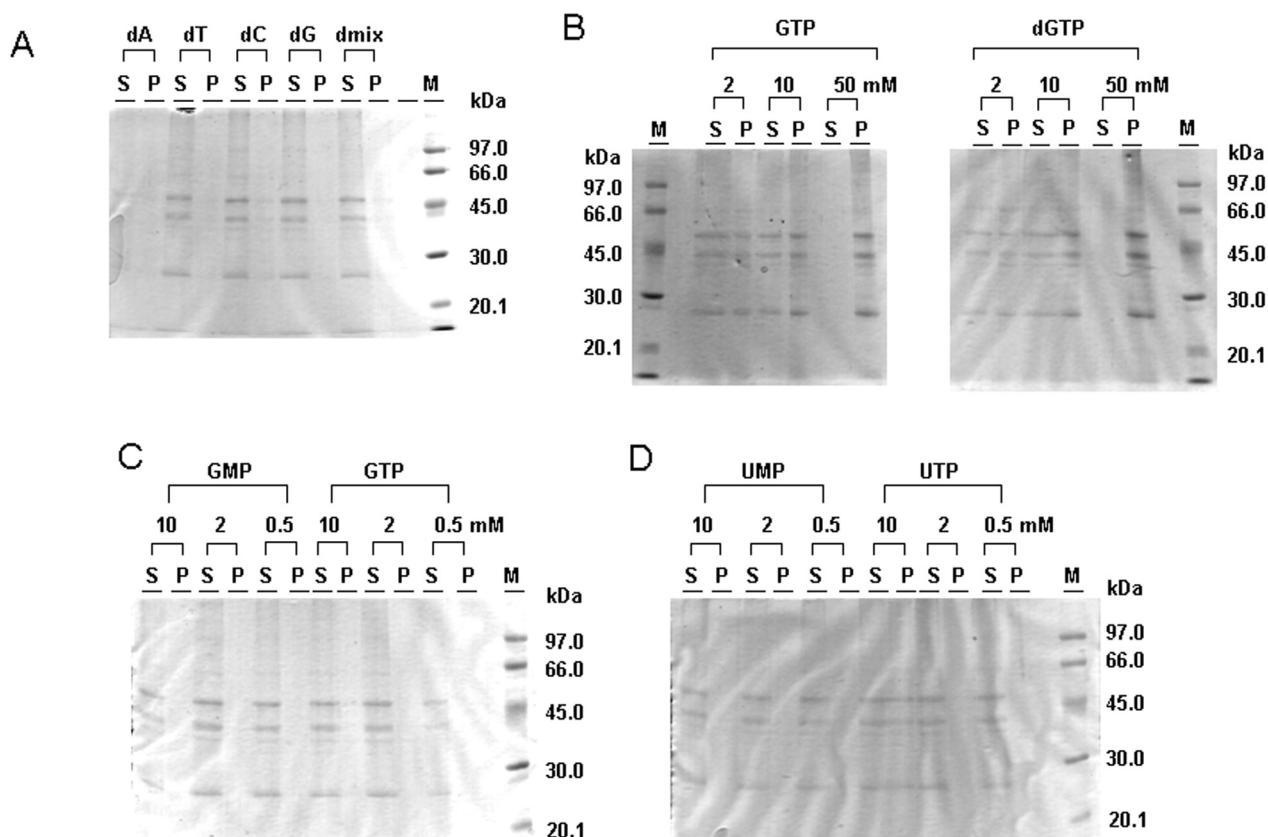


Figure 3. The effects of various nucleotides on solubilization of rTRP-2 were analyzed by SDS-PAGE. Reduced rTRP-2 was divided between 5 tubes and 2.5 mM of GSSG was added to all tubes. (A) 10 mM dATP (dA), dTTP (dT), dCTP (dC), dGTP (dG), or dNTP mixture (dmix) was added to all tubes. The ensuing procedure and symbols are the same as those in Figure 2. (B) GTP, dGTP, (C) GMP, GTP, or (D) UMP, UTP was added at concentrations shown in the figure when the reaction with GSSG was started as in (A). The ensuing procedure and symbols are same as (A). Data are representative of two independent experiments.

from peptide-pulsed DCs (Figure 6). This result suggests that the presented number of H-2K^b restricted peptide from the transduced protein is equal to that from pulsed peptide.

Killing effect of CD8⁺ T-cells in splenocytes derived from mice immunized with tumor cell insoluble fraction-incorporated DCs. The efficacious antigen-presentation was demonstrated by MHC class I of electroloaded DCs incorporated with solubilized rTRP-2 at the former stage. It was next hypothesized that many TAAs may exist in the insoluble fraction of tumor cell homogenates and they would be efficacious for antigen presentation by DCs. To explore this, a B16-targeted killing assay was performed. C57BL/6 mice were immunized with DCs electroloaded with a B16-solubilized insoluble fraction, prepared by the solubilizing procedure with UMP. DCs electroloaded with an equivalent amount of B16 cell lysate and TRP-2 peptide-pulsed DCs were used as the controls for immunization. Furthermore, mice were immunized with DCs electroloaded with both the

solubilized (previously insoluble) fraction and B16 lysate for future clinical study protocols. After three immunizations, splenocytes were recovered from the immunized mice and CD8⁺ cells were isolated from them. CD8⁺ cells were cultured with the corresponding DCs *in vitro* for six days before the killing assay.

For the killing assay using B16 cells, cells were pretreated with IFN- γ to express MHC class I molecules because expression levels did not stabilize without treatment. One result of MHC class I (H-2K^b and/or H-2D^b) expression level of B16 cells after pretreatment is shown in Figure 7A. The proportion of H-2K^b and H-2D^b-positive cells was 79% and that of double-negative cells was only 16%. It was concluded that the expression level was sufficient for a cytotoxic assay based on MHC class I-T cell receptor binding. Recovered CD8⁺ cells from *in vitro* culture were co-cultured with calcein-AM-labeled B16 cells at various tumor/effecter ratios, and the killing effects were assessed. The comparative result in electroloaded DCs is shown in

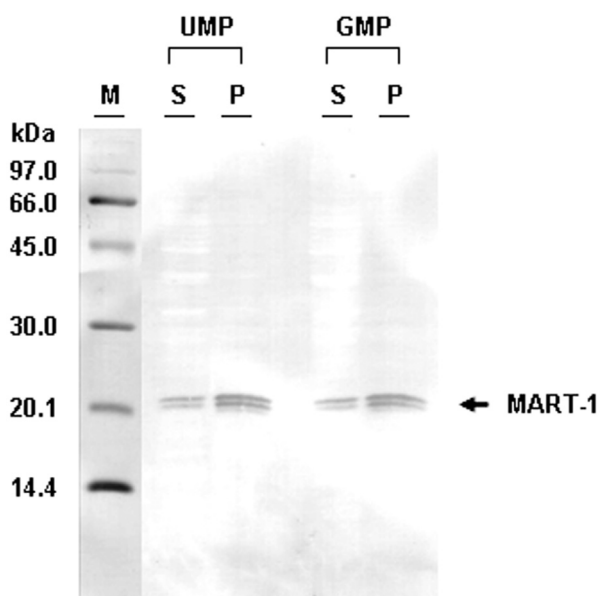


Figure 4. Human MART-1 protein was detected in the supernatant after solubilization of MeWo insoluble-fraction. The insoluble fraction of the MeWo cell homogenate was solubilized by addition of GMP or UMP. The same volumes of both supernatant and precipitate fractions after solubilization were analyzed SDS-PAGE. Other conditions (including Western blotting) were identical to those described in Figure 1.

Figure 7B. The killing activity of CD8⁺ cells derived from mice immunized with DCs electroloaded with B16-solubilized precipitate (Ep-P) and with B16 lysate (Ep-L) tended to be stronger than that of CD8⁺ cells derived from mice immunized with peptide-pulsed DCs (peptide) at E/T 40. As expected, CD8⁺ cells derived from mice immunized with DCs electroloaded with both fractions (Ep- L+P) showed the highest killing activity (E/T=10, 40). The effect was significant in comparison with the effects of CD8⁺ cells from Ep-P, Ep-L, or peptide-pulsed groups.

From these results, it can be concluded that the insoluble fraction of a tumor cell homogenate was useful for antigen presentation of DCs and that the CTL-inducing effect was augmented by combination with the lysate.

Discussion

Nucleotides are components of DNA or RNA and raw materials of biosynthesis; therefore, we believed that nucleotides would not influence the viability of cultured cells when they were transduced into the cells. This was confirmed by preparation of electroloaded DCs. Nucleotides have the ability to solubilize insoluble proteins and since the solubilized proteins are applied for various purposes, it is very important that no toxic material is present. The role of nucleotides in solubilization is not interpretable, but we are of the opinion that nucleotides interact

with the structure of peptide bonds through hydrogen bonding. The interaction of a nucleotide with a protein is not very strong because the elution time of a solubilized protein with a nucleotide (dNTP) did not shift compared with that of a solubilized protein without nucleotide on a gel-filtration chromatography analysis at room temperature (the data was obtained using RNase A as a model protein and is not shown). We believe the mechanism is not same as that of ATP binding (19), GDP binding (20), or DNA binding to specific proteins (21), and must be different from the cases of refolding, based on a study of chaperonin with ATP (22). This study is a direction for future investigation, and one example of an application for this method was shown. The recovery of solubilized rTRP-2 without nucleotide (10% glycerol was present) was higher than that of rUb-TRP-2 in the current experiment (Figures 2 and 3). This result may be influenced by the ratio of conjugated glutathione/molecular weight of rUb-TRP-2, which is lower than that of rTRP-2, because cysteine residue is absent from the Ub-fragment sequence.

On the basis of the tetramer assay, this study demonstrated the efficacious induction of TRP-2 epitope-specific CTLs using DCs electroloaded with solubilized rTRP-2 protein. The effect was equivalent to that of peptide-pulsed DCs. This result suggests that antigenic proteins introduced into the cytoplasm would have efficient presentation with MHC class I, even if the protein is solubilized. Waeckerle-Men *et al.* have demonstrated that vaccination using multi-epitope-pulsed DCs showed efficient cellular antitumor response using human materials (23). Therefore, multi-epitope presentation from antigenic proteins will be an important technique for cancer treatment. Although percentages of CTLs based on only a single epitope were shown, it can be hypothesized that other antigen-specific epitopes must be presented by MHC class I and class II when rTRP-2 protein is used as the antigen along with tumor-insoluble fraction. If nothing contradicts this hypothesis, the antitumor effect of DCs would be stronger than that of peptide-pulsed DCs. This hypothesis was supported both by the theory of Palucka *et al.* (3) and the result of the killing assay performed in this study. The antitumor effect derived from DC vaccination with insoluble antigens should be obtained as an additional effect with DCs electroloaded with tumor lysate, as shown by Weiss *et al.* (11). We believe that the method developed in the present study and that of Weiss *et al.* will be important because these methods will make it possible to use antigenic proteins having a point mutation in individual patient. It is very difficult to achieve this by gene transduction or peptide pulsing. The use of plural antigenic proteins in tumor tissues for antigen presentation of DCs for antitumor vaccination shows promise. A report by Shojaeian *et al.* (24) supports the idea that co-pulsing of two antigenic proteins to DCs results in higher frequency of antigen-specific responding cells and significantly more IFN- γ production in a mouse model.

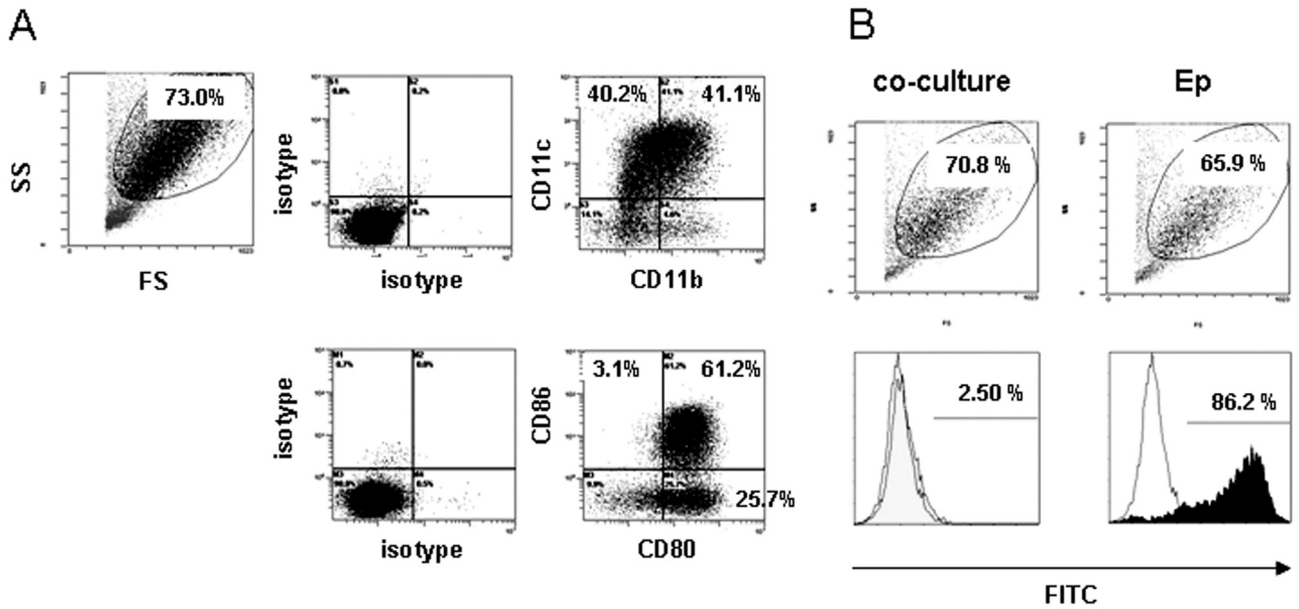


Figure 5. Phenotype and efficient loading of bone marrow-derived DCs. (A) Phenotype of DCs harvested at day 10 of culture. DCs were stained with antibodies against CD11b, CD11c, CD80, and CD86 and analyzed by FCM. Representative data are from many experiments. (B) DCs were electroloaded with FITC-dextran and results of FCM analysis 1 h after Ep are shown. Intensity of FITC-positive DCs (gated in upper panels) are shown in the lower panels. Comparison between co-culture (left side, gray histogram) and Ep (right side, black histogram) with FITC-dextran is shown. The white histogram shows co-cultured DCs without FITC-dextran.

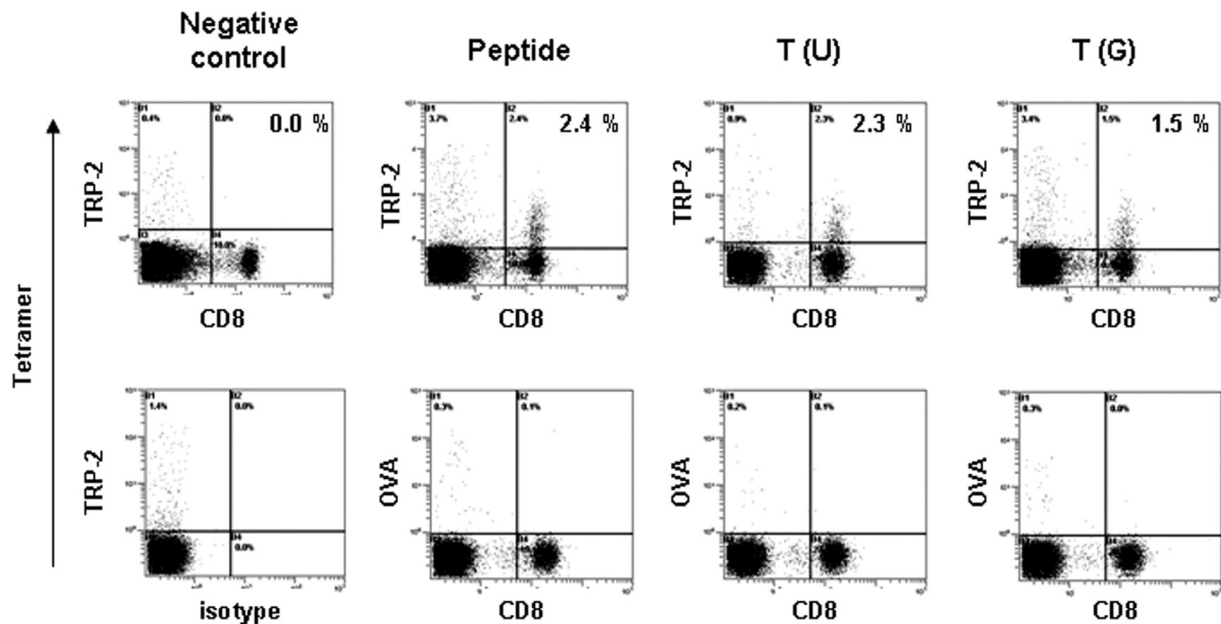


Figure 6. Murine TRP-2 epitope-specific CD8⁺ T-cell-inducing abilities of DCs were analyzed using TRP-2 tetramer. Splenocytes were recovered from C57BL/6 mice 1 week after last immunization with DCs and stained with TRP-2 tetramer (upper panels), OVA tetramer as a non-specific control (lower panels), and antibodies against CD8 or isotype control. Splenocytes of normal mouse were used as a negative control. Peptide: TRP-2 peptide-pulsed DCs; T (U): solubilized TRP-2 protein (with UMP) loaded DCs; T (G): solubilized TRP-2 protein (with GMP) loaded DCs. The percentages of CD8⁺ cells stained with TRP-2 tetramer are indicated in the upper panels. Data are representative of 2 out of 4 independent experiments.

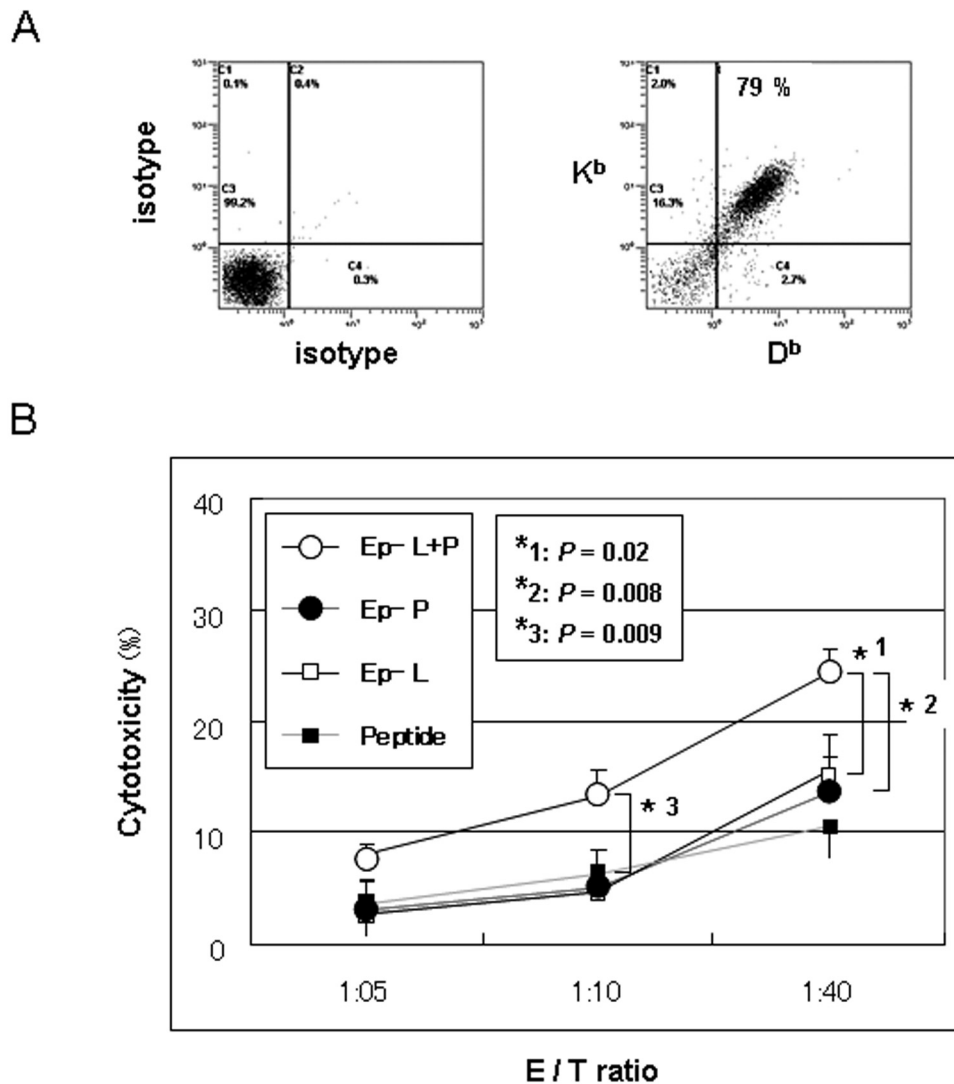


Figure 7. Cytotoxic activity of CD8⁺ splenocytes on murine TRP-2 expressing B16 melanoma. Splenocytes were obtained from C57BL/6 mice on day 6 after last immunization with dendritic cells (DCs) (n=3/group). TRP-2 peptide-pulsed DCs (peptide), DCs electroloaded with B16 soluble fraction (Ep-L), DCs electroloaded with B16 insoluble fraction after solubilization (Ep-P), DCs electroloaded with mixture of both B16 fractions (Ep-L+P) were used for immunization. CD8⁺ cells recovered from immunized mice were cultured with corresponding DCs respectively for 6 days *in vitro* (responder cells:stimulator cells=10:1). (A) Cell surface expression of H-2K^b and H-2D^b on B16 cells after cultivation with interferon-gamma was analyzed by FCM. B16 cells were labeled with calcein-AM and used as target cells in experiments shown in (B). (B) Specific killing activity of CD8⁺ cells was examined using labeled B16 cells. Cytotoxicities (%) were calculated based on the amount of calcein-AM fluorescence left in B16 cells after 3 h of incubation with effector CD8⁺ cells at various E/T ratios. Values in the figure are the mean±SD of 3 determinations. Data are representative of two independent experiments (n=3).

We expect that electroloading DCs with the insoluble fraction of tumor cells will be a powerful tool for manipulating the immune system. This evidence suggests the usefulness of the tumor insoluble fraction for DC vaccination therapy, such as helper T-cell function and/or antitumor effect using a mouse model *in vivo*, to be tested in future clinical studies.

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