in maintaining immunological self-tolerance in physiological situations (10–18).

For introduction of multiple expression vectors into DC, we used a method for embryonic stem cell (ES cell)-mediated genetic modification of DC. Recently, we and another group established culture procedures to generate DC from mouse ES cells (2, 19). ES cell-derived DC (esDC or ES-DC) have the capacity comparable to bone marrow-derived DC to process and present protein Ags to T cells, stimulate naive T cells, and migrate to lymphoid organs in vivo (20, 21). A recent study using the method revealed the role of Notch signaling in differentiation of DC (22). For generation of genetically modified ES-DC, ES cells were transfected with expression vectors, and subsequently transfectant ES cell clones were induced to differentiate to DC, which expressed the products of introduced genes. Introduction of multiple exogenous genes by sequential transfection can readily be done with vectors bearing different selection markers (20).

In this study, we report that treatment of mice with ES-DC presenting MOG peptide in the context of MHC class II and simultaneously expressing TRAIL or PD-L1 significantly reduced the severity of EAE induced by immunization with the MOG peptide.

Materials and Methods

Mice

CBA, and C57BL/6 mice obtained from CLEA Japan or Charles River were kept under specific pathogen-free conditions. Male CBA and female C57BL/6 mice were mated to generate F_1 (CBF_1) mice, and all in vivo experiments were done using CBF_1 mice, syngeneic to TT2 ES cells. Mouse experiments met with approval by Animal Research Committee of Kumamoto University.

Peptides, protein, cell lines, and cytokines

The mouse MOG p35–55 (MEVGWYRSPFSRVVHLYRNGK), mouse myelin proteolipid protein (PLP) p190–209 (SKTSASIGSLCADARM YGVL), and mouse myelin basic protein (MBP) p35–47 (TGILDSI GRFFSG), were synthesized using the F-moc method on an automatic peptide synthesizer (PSSM8; Shimadzu) and purified using HPLC (23–25). Bovine MBP was purchased from Sigma-Aldrich. The ES cell line, TT2, derived from CBF_1 blastocysts, and the M-CSF-defective bone marrow-derived stromal cell line, OP9, were maintained, as described (2). L929, a fibroblast cell line originating from a C3H mouse was purchased from Japan Health Science Foundation (Osaka, Japan). Recombinant mouse GM-CSF was kindly provided by Kirin Brewery and was purchased from PeproTech.

Plasmid construction

Mouse *TRAIL* cDNA was prepared by RT-PCR amplification from total RNA of mouse spleen with PCR primers 5′-AACCCTCTAGACCGC CGCCACCATGCCTTCCTCAGGGGCCCTGAA-3′ and 5′-AAAGGGA TATCTTTACTGGTCATTTAGTT-3′. The design of these primers results in cloning of *TRAIL* cDNA downstream of the Kozak sequence (20). The PCR products were subcloned into a pGEM-T-Easy vector (Promega), and cDNA inserts were confirmed by sequencing analysis. cDNA for mouse *PD-L1* was kindly provided by Drs. T. Okazaki and T. Honjo (Department of Medical Chemistry, Kyoto University, Kyoto, Japan) (7). The cDNA fragments for *TRAIL* and *PD-L1* were cloned into pCAG-INeo, a mammalian expression vector driven by a CAG promoter and containing the internal ribosomal entry site (IRES)-neomycin resistance gene cassette, to generate pCAG-TRAIL-INeo or pCAG-PDL1-INeo. To generate a MOG peptide presenting vector, double-stranded oligo DNA encoding the MOG p35–55 epitope, 5′-CCGGTGATGGAAGTTGGTTGGTATCGTT CTCCATTCTCTCGTGTTGTTCATCTTTATCGTAACGGTAAG CTGCCCATGGGAGCT-3′, was inserted into the previously reported human Ii-based epitope-presenting vector, pCI30 (2). The coding region of this construct was transferred to pCAG-IPuro, an expression vector containing the CAG promoter and IRES-puromycin *N*-acetyltransferase gene cassette, to generate pCAG-MOG-IPuro. pCI-PCC is a pigeon cytochrome *c* (PCC) epitope-presenting vector derived from pCI30 (2).

Transfection of ES cells and differentiation of DC from ES cells

Transfection of ES cells and induction of differentiation of ES cells into DC were done as described (2, 20), with some minor modification as follows. The differentiating cells were transferred from OP9 to bacteriological petri dishes without feeder cells on day 10, and cultured in RPMI 1640 medium supplemented with 12% FCS, GM-CSF (500 U/ml), and 2-ME. The floating or loosely adherent cells were recovered from dishes by pipetting on days 17–19 and used for experiments.

RT-PCR to detect transgene products

Total cellular RNA was extracted using a SV Total RNA Isolation kit (Promega). All RNA samples were treated with RNase-free DNase I before reverse transcription to eliminate any contaminating genomic DNA. RT-PCR was done as described (20). The relative quantity of cDNA in each sample was first normalized by PCR for GAPDH. The primer sequences were as follows: *hCD74* (Ii), 5′-CTGACTGACCGCGTTACTCCCACA-3′ and 5′-TTCAGGGGGTCAGCATTCTGGAGC-3′; *TRAIL*, 5′-CTGACTGAC CGCGTTACTCCCACA-3′ and 5′-GAAATGGTGTCCTGAAAGGTTC-3′; *PD-L1*, 5′-CTGACTGACCGCGTTACTCCCACA-3′ and 5′-GCTTGTAG TCCGCACCACCGTAG-3′; and *GAPDH*, 5′-GGAAAGCTGTG GCGTGATG-3′ and 5′-CTGTTGCTGTAGCCGTATTC-3′. The sense-strand primer used for detection of transgene-derived mRNA was corresponding to the 5′ untranslated region included in the vector DNA. PCR products were visualized by ethidium bromide staining after separation over a 2% agarose gel. In one experiment, the level of expression of mRNA for *TGF-β* was detected by RT-PCR. The primer sequences were 5′-ACCATGCCAACTTCTGTCTG-3′ and 5′-CGGGTTGTGTTGGT TGTAGA-3′.

Flow-cytometric analysis

Staining of cells and analysis on a flow cytometer (FACScan; BD Biosciences) was done as described (2). Abs and reagent used for staining were as follows: anti-I-A^b (clone 3JP; mouse IgG2a), R-PE-conjugated-anti-mouse CD11c (clone N148; hamster IgG; Chemicon), R-PE-conjugated anti-mouse CD86 (clone RMMP-2; rat IgG2a; Caltag), FITC-conjugated anti-human CD74 (clone M-B741; mouse IgG2a; BD Pharmingen), FITC-conjugated goat anti-mouse Ig (BD Pharmingen), mouse IgG2a control (clone G155-178; BD Pharmingen), FITC-conjugated mouse IgG2a control (clone G155-178; BD Pharmingen), R-PE-conjugated hamster IgG control (Immunotech), R-PE-conjugated rat IgG2a control (clone LO-DNP-16; Caltag), biotinylated anti-mouse TRAIL (clone N2B2; rat IgG2a; eBioscience), anti-mouse PD-L1 (clone MIH5; rat IgG2a; eBioscience), rat IgG2a (Caltag), biotinylated rat IgG2a (eBioscience), FITC-conjugated anti-rat Ig (BD Pharmingen), and PE conjugated-streptavidin (Molecular Probes; Invitrogen Life Technologies). In some experiments, the DC fraction was gated by forward and side scatters. For detection of apoptosis of splenic $CD4^+$ T cell, Annexin V^{FITC} apoptosis detection kits (BioVision) were used. In brief, spleen cells isolated from mice treated with ES-DC were incubated with FITC-conjugated annexin V and R-PE-conjugated anti-mouse CD4 mAb (clone L3T4; BD Pharmingen), and subsequently analyzed by flow cytometry.

Cytotoxicity assay and proliferation assay of T cells stimulated with anti-CD3 mAb

Standard ^{51}Cr release assay was done as described (4). For proliferation assay of T cells stimulated with anti-CD3 mAb, splenic mononuclear cells were prepared from unprimed CBF_1 mice, and T cells were purified using nylon wool columns. X-ray-irradiated (35 Gy) ES-DC (2×10^4) and the T cells (1×10^5) were seeded into wells of 96-well flat-bottom culture plates precoated with anti-CD3 mAb (145-2C11; eBioscience) and cultured for 4 days. [^{3}H]Thymidine (6.7 Ci/mmol) was added to the culture (1 μCi/well) in the last 16 h. At the end of culture, cells were harvested onto glass fiber filters (Wallac), and the incorporation of [^{3}H]thymidine was measured using scintillation counting. For blocking experiments, anti-TRAIL (clone N2B2) or anti-PD-L1 (clone MIH5) blocking mAb (5 μg/ml) was added to the culture.

Analysis of presentation of MOG epitope by genetically modified ES-DC

MOG epitope-reactive T cells were prepared from inguinal lymph nodes of mice immunized according to protocol for EAE induction described below, using nylon wool columns. X-ray-irradiated (35 Gy) ES-DC as stimulator cells (2×10^4) were cocultured with the MOG-reactive T cells (1.5–2×10^5) in wells of 96-well culture plates for 3 days. Proliferation of T cells in

the last 12 h of the culture was quantified based on [^{3}H]thymidine uptake, as described above.

Induction of EAE and treatment with ES-DC

For EAE induction by synthetic peptides or purified protein, 6- to 8-wk-old female CBF_1 mice were immunized by giving a s.c. injection at the base of the tail with a 0.2-ml IFA/PBS solution containing 600 μg of MOG p35–55 peptide and 400 μg of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories) on day 0. In addition, 500 ng of purified *Bordetella pertussis* toxin (Calbiochem) were injected i.p. on days 0 and 2. For EAE induction by ES-DC presenting MOG peptide, ES-DC were injected at the base of the tail of mice (5×10^5 cells/mouse) at day 0, and the mice were given i.p. 500 ng of *B. pertussis* toxin in 0.2 ml of PBS on days 0 and 2. For prevention of EAE, mice were injected i.p. with ES-DC (1×10^6 cells/mouse/injection) on days −8, −5, and −2 (preimmunization treatment), or on days 5, 9, and 13 (postimmunization treatment). The mice were observed over a period of 42 days for clinical signs, and scores were assigned based on the following scale: 0, normal; 1, weakness of the tail and/or paralysis of the distal half of the tail; 2, loss of tail tonicity and abnormal gait; 3, partial hindlimb paralysis; 4, complete hindlimb paralysis; 5, forelimb paralysis or moribundity; 6, death.

Immunohistochemical analysis

Freshly excised spinal cords were immediately frozen and embedded in Tissue-Tek OCT compound (Sakura Finetechnical). Immunohistochemical staining of CD4, CD8, and Mac-1 was done, as described (20), but with some modification. In brief, serial 7-μm sections were made using cryostat and underwent immunochemical staining with mAbs specific to CD4 (clone L3T4; BD Pharmingen), CD8 (clone Ly-2; BD Pharmingen), or Mac-1 (clone M1/70; eBioscience), and N-Histofine Simple Stain Mouse MAX PO (Nichirei). Frozen sections of spleen were subjected to TUNEL staining by using ApopTag Fluorescein In Situ Apoptosis Detection kits (Serologicals). In brief, sections were incubated with digoxigenin-conjugated nucleotides and TdT, and subsequently with peroxidase-conjugated anti-digoxigenin Ab. The staining signals were developed using diaminobenzidene.

Analysis of T cell response to MOG or keyhole limpet hemocyanin (KLH)

Immunization of mice and restimulation of draining lymph node cells in vitro were done as described (26), but with some modification. In brief, ES-DC-treated and control mice were immunized at the base of the tail with MOG peptide, according to protocol for EAE induction, or 50 μg of KLH protein (Sigma-Aldrich) emulsified in CFA. After indicated days, inguinal lymph node cells and spleen cells were isolated and cultured (5×10^5 cells/well) in the presence of MOG peptide (0, 8, 2.5, or 80 μg/ml) or KLH (16, 50, or 160 μg/ml) in 10% horse serum/RPMI 1640/2-ME or 2% mouse serum/DMEM/2-ME/insulin-transferrin-selenium-X (Invitrogen Life Technologies), and the proliferative response was quantified based on [^{3}H]thymidine uptake, as described above. In addition, when mice were immunized with ES-DC expressing MOG peptide for EAE induction, spleen cells were isolated at day 14, and cultured (5×10^5 cells/well) in the presence of MOG peptide in 10% horse serum/RPMI 1640/2-ME, and the proliferative response was quantified based on [^{3}H]thymidine uptake, as described above. To analyze production of cytokines of spleen cells isolated from mice treated with ES-DC, isolated spleen cells were stimulated with 10 μM MOG peptide or irrelevant OVA peptide in vitro. After 72 or 96 h, cell supernatants were harvested and measured for cytokine content using ELISA kits (eBioscience) for IL-4, IL-10, and IFN-γ.

Statistical analysis

Two-tailed Student's *t* test was used to determine the statistical significance of differences. A value of $p < 0.05$ was considered significant.

Results

Induction of EAE in CBF_1 mice

To date, we found no study that EAE had been induced in CBF_1 mice. Therefore, before the study on therapeutic intervention, it was necessary to set up an experimental condition under which we could reproducibly induce EAE in CBF_1 mice. We compared several induction protocols using protein or peptide Ag of MOG, MBP, and PLP. As a result, we found that, when mice were s.c. injected at the base of the tail with a 0.2-ml IFA/PBS solution containing 600 μg of MOG p35–55 and 400 μg of *M. tuberculosis* accompanying an i.p. injection of 500 ng of purified *B. pertussis* toxin on days 0 and 2, EAE is reproducibly induced in CBF_1 mice with an average peak clinical score of 3.3 (Table I). We decided to use this protocol in the following experiments. In addition, inoculation of MBP p35–47, MBP whole protein, or PLP p190–209 together with *M. tuberculosis* and *B. pertussis* toxin also induced EAE in CBF_1 mice with a peak clinical score ranging between 2 and 3 (Table I).

Genetic modification of ES-DC to express MOG peptide along with TRAIL or PD-L1

At the first step in the generation of ES-DC presenting MOG peptide and simultaneously expressing TRAIL or PD-L1, TT2 ES cells were transfected with an expression vector for TRAIL (pCAG-TRAIL-INeo) or PD-L1 (pCAG-PDL1-INeo), as shown in Fig. 1*A*. Then, ES cell clones introduced with either of the expression vectors and parental TT2 ES cells were transfected with the MOG peptide expression vector, pCAG-MOG-IPuro (Fig. 1*B*). In this vector, a cDNA for human Ii was mutated to contain an oligo DNA encoding MOG p35–55 epitope in the CLIP region (1, 2, 27, 28). Resultant single- or double-transfectant ES cell clones were subjected to differentiation to ES-DC. ES-DC expressing MOG peptide, MOG peptide plus TRAIL, and MOG peptide plus PD-L1 were designated as ES-DC-MOG, ES-DC-TRAIL/MOG, and ES-DC-PDL1/MOG, respectively. The expression of mutant human Ii

Table I. *EAE induction in CBF_1 mice*[a]

Expt.	Ag	Ag Dose (μg)	Disease Incidence	Day of Onset	Mean Peak Clinical Score
1	MOG p35–55	200 × 2[b]	1/2	9.0 ± 0	1.5 ± 0
2		400	2/2	11.0 ± 0	4.0 ± 0
3		600	44/44	10.2 ± 1.3	3.3 ± 0.5
4		800	2/2	8.0 ± 0	3.0 ± 0
5	MBP p35–47	200 × 2[b]	0/2		
6		600	8/8	5.5 ± 1.3	3.0 ± 0
7	MBP protein	200 × 2[b]	0/2		
8		600	6/6	9.7 ± 1.8	3.0 ± 0
9	PLP p190–209	200 × 2[b]	0/2		
10		600	2/2	5.0 ± 0	2.0 ± 0

[a] Data are combined from a total of 21 experiments. EAE was induced by S.C. injection at the tail base of a 0.2-ml IFA/PBS solution containing 400 μg of *M. tuberculosis* and indicated peptide or MBP protein once (on day 0) or [b] twice (on days 0 and 7), together with i.p. injections of 500 ng of purified *B. pertussis* toxin on days 0 and 2.

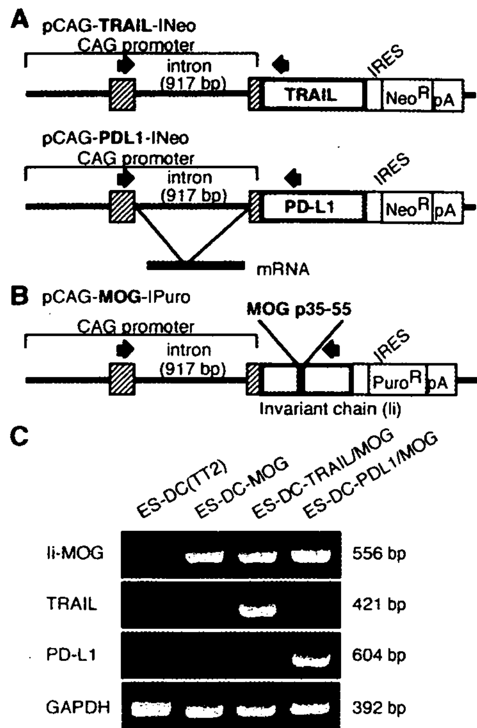

FIGURE 1. Genetic modification of ES-DC to express TRAIL, PD-L1, and Ii-MOG. *A*, The structures of pCAG-TRAIL-INeo and pCAG-PDL1-INeo, the expression vectors for TRAIL and PD-L1, and PCR primers for RT-PCR to detect transgene products are shown. Primer pairs (arrows) were designed to span the intron (917 bp) in the CAG promoter sequence to distinguish PCR products of mRNA origin (421 and 604 bp, respectively) from genome-integrated vector DNA origin. Hatched boxes indicate 5′-untranslated region of the rabbit β-actin gene included in the CAG promoter. The vectors are driven by CAG promoter (pCAG), and cDNA for TRAIL or PD-L1 are followed by the IRES-neomycin-resistance gene (Neo^R)-polyadenylation signal sequence (pA). *B*, The structure of pCAG-MOG-IPuro, the expression vector for mutant human Ii bearing MOG peptide at the CLIP region, are shown as in *A*. Primer pairs (arrows) were designed to generate PCR product of 556 bp originating from transgene-derived mRNA for CAG-MOG. *C*, RT-PCR analysis detected expression of transgene-derived mutant human *Ii* containing the MOG peptide (*Ii-MOG*), *TRAIL*, *PD-L1*, and *GAPDH* (control) mRNA in transfectant ES-DC.

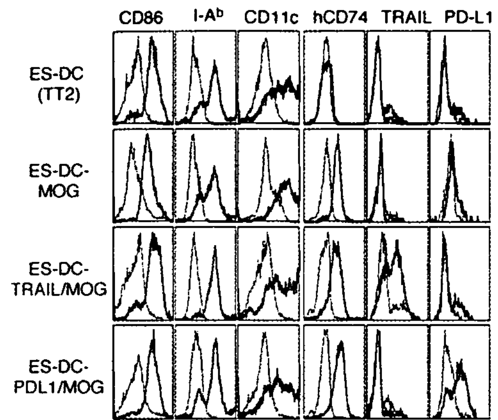

FIGURE 2. Surface phenotype of genetically modified ES-DC. Expression of cell surface CD86, I-A^b, CD11c, TRAIL, and PD-L1 on transfectant ES-DCs was analyzed by flow-cytometric analysis. Expression of mutant human Ii (hCD74) bearing MOG peptide was examined using intracellular staining. Staining patterns with specific Abs (thick line) and isotype-matched control (thin line) are shown.

containing the MOG peptide, TRAIL, and PD-L1 in ES-DC was confirmed by RT-PCR (Fig. 1*C*) and flow-cytometric analysis (Fig. 2). The mutant human Ii containing the MOG peptide was detected by intracellular staining with anti-human CD74 (Ii) mAb (Fig. 2).

ES-DC of similar morphology were generated from any of the transfectant ES cells. As shown in Fig. 2, no significant difference was observed in the level of surface expression of CD86, I-A^b, or CD11c among ES-DC derived from parental TT2 ES cells, ES-DC-MOG, ES-DC-TRAIL/MOG, and ES-DC-PDL1/MOG. Thus, forced expression of TRAIL, PD-L1, or mutant human Ii has little influence on the differentiation of ES-DC.

Functional expression of transgene-derived TRAIL and PD-L1 in ES-DC

The functional activity of TRAIL expressed in ES-DC was analyzed according to the cytotoxicity against TRAIL-sensitive L929 cells. As shown in Fig. 3*A*, ES-DC-TRAIL showed manifest killing activity against L929. In contrast, neither ES-DC (TT2) (parental TT2-derived) nor ES-DC-OVA (OVA-transfected TT2-derived ES-DC) did so. In addition, ES-DC-TRAIL inhibited the proliferation of splenic T cells stimulated with plate-coated anti-CD3 mAb (Fig. 3*B*). PD-L1 expressed on ES-DC also inhibited proliferation of splenic T cells stimulated with anti-CD3 mAb. Inhibition of anti-CD3-induced proliferation of T cells by the TRAIL and PD-L1 was abrogated by addition with anti-TRAIL and anti-PD-L1 blocking mAb, respectively (Fig. 3*B*), but not by isotype-matched control mAb (data not shown). These results indicate that transgene-derived TRAIL and PD-L1 expressed in ES-DC functioned to suppress response of T cells stimulated via TCR/CD3 complexes.

Stimulation of MOG-reactive T cells by ES-DC genetically engineered to express MOG peptide

Presentation of MOG peptide in the context of MHC class II molecules by ES-DC-MOG was investigated in vitro. MOG peptide-reactive T cells were prepared from inguinal lymph nodes of mice, which developed EAE by immunization with MOG p35–55, CFA, and *B. pertussis* toxin. Proliferative response of the MOG-reactive T cells upon coculture with transfectant ES-DC was analyzed. As shown in Fig. 4*A*, ES-DC-MOG stimulated the MOG-reactive T cells to induce proliferation. In contrast, ES-DC carrying Ii-based PCC peptide expression vector (ES-DC-PCC) (2), as a control, did not do so. No proliferative response was observed when naive splenic T cells isolated from syngeneic mice were cocultured with ES-DC-MOG under the same condition (data not shown). These results indicate that the epitope-presenting vector introduced into ES-DC functioned to present the MOG peptide in the context of MHC class II molecules to stimulate MOG-specific $CD4^+$ T cells.

It has been reported that transfer of bone marrow-derived DC preloaded with MOG peptide caused development of EAE in naive mice (29, 30). We presumed that, if ES-DC-MOG could encounter with MOG-specific T cells and stimulate the T cells with MOG peptide in vivo, EAE would be developed. We injected ES-DC-MOG or ES-DC-PCC, as a control, at the base of the tail of naive mice and also gave i.p. 500 ng of *B. pertussis* toxin on the same day and 2 days later. In the results, EAE was developed in the mice transferred with ES-DC-MOG but not those transferred with ES-DC-PCC (Fig. 4*B*).

We examined whether MOG-specific T cells were activated in vivo by injection with ES-DC-MOG. Fourteen days after the injection of ES-DC and *B. pertussis* toxin, spleen cells were isolated

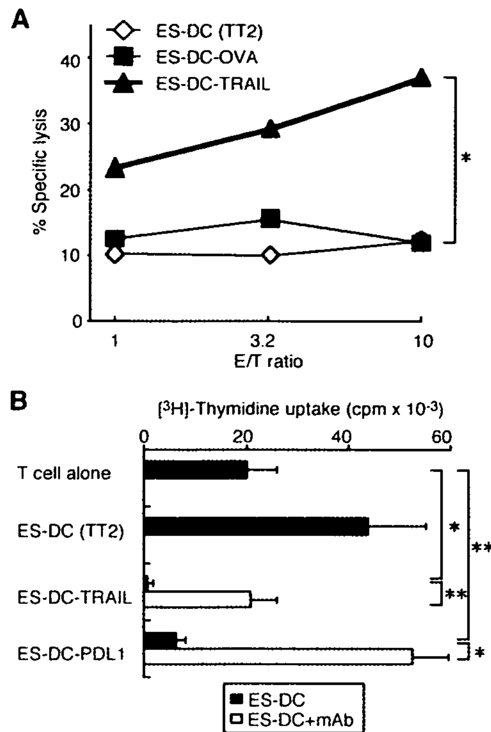

FIGURE 3. Expression of functional TRAIL or PD-L1 in ES-DC transfectants. *A*, The activity of TRAIL expressed in ES-DC was analyzed based on cytotoxicity against L929 cell. ^{51}Cr-labeled target cells (5×10^3 L929 cells) were incubated with ES-DC (TT2), ES-DC-OVA, or ES-DC-TRAIL as effector cells at the indicated E:T ratio for 12 h, and after the incubation, cytolysis of target cells was quantified by measuring radioactivity in the supernatants. Results are expressed as mean specific lysis of triplicate assays, and SDs of triplicates were <4%. *B*, Irradiated ES-DC (TT2), ES-DC-TRAIL, and ES-DC-PDL1 (2×10^4/well) were cocultured with 1×10^5 syngeneic CBF_1 splenic T cells in the presence (□) or absence (■) of blocking Ab (anti-TRAIL mAb or anti-PD-L1 mAb, 5 μg/ml) for 4 days in 96-well flat-bottom culture plates precoated with anti-CD3 mAb. Proliferation of T cells was quantified by measuring [^{3}H]thymidine incorporation. The asterisks indicate that the differences in responses are statistically significant between two values indicated by lines (*, $p < 0.01$; **, $p < 0.05$). The data are each representative of three independent and reproducible experiments with similar results.

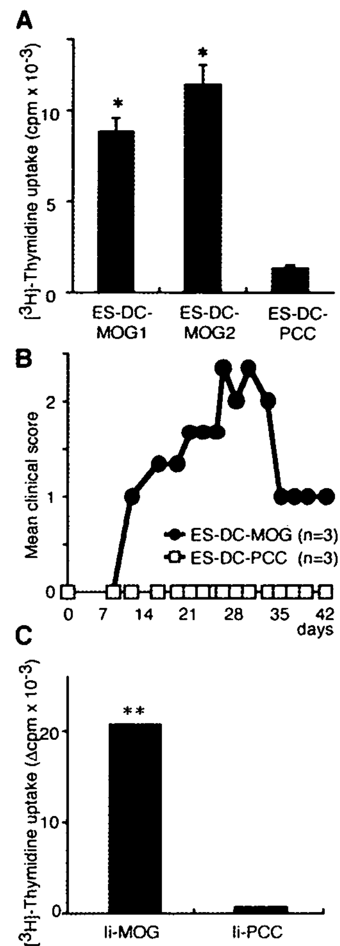

FIGURE 4. Presentation of MOG epitope by ES-DC introduced with Ii-based MOG epitope-presenting vector. *A*, T cells (1.5×10^5) isolated from inguinal lymph nodes of CBF_1 mice immunized according to the protocol for EAE induction were cocultured with one of two independent clones (2×10^4) of ES-DC-MOG or a clone of ES-DC-PCC, presenting PCC epitope, for 3 days. Proliferative response of T cells was quantified by [^{3}H]thymidine uptake in the last 12 h of the culture. *B*, CBF_1 mice (three mice per group) were injected s.c. with ES-DC-MOG or ES-DC-PCC (5×10^5) on day 0, together with i.p. injection of 500 ng of purified *B. pertussis* toxin on days 0 and 2, and the severity of induced EAE was evaluated. The disease incidence, mean day of onset ± SD, and mean peak clinical score ± SD of mice injected with ES-DC-MOG were 100%, 11.3 ± 1.7, and 2.7 ± 0.4, respectively. *C*, Spleen cells were isolated on day 14 from mice treated as in *B*, and whole spleen cells (5×10^5/well) were cultured in the presence of 1 μg/ml MOG peptide for 3 days. Proliferative response was quantified as in *A*. Data were indicated as Δcpm (value in the presence of peptide − value in the absence of peptide ($<46 \times 10^3$ cpm)), and SDs of triplicates were <9% of mean value. The asterisks indicate that the differences in responses are statistically significant compared with ES-DC-PCC (*, $p < 0.01$; **, $p < 0.05$). The data are each representative of three independent and reproducible experiments with similar results.

from the mice and cultured in the presence of MOG peptide. As shown in Fig. 4*C*, the spleen cells isolated from mice injected with ES-DC-MOG showed proliferative response to MOG peptide. In contrast, those isolated from mice injected with ES-DC-PCC did not do so. These results indicate that in vivo transferred ES-DC-MOG together with adjuvant effect of *B. pertussis* toxin stimulated MOG-specific T cells to develop EAE.

Protection from MOG-induced EAE by treatment with ES-DC expressing MOG peptide along with TRAIL or PD-L1

We examined whether TRAIL and PD-L1 expressed by ES-DC together with MOG peptide had an effect to down-modulate MOG-specific T cell responses in vitro. MOG-reactive T cells prepared as described above were cocultured with ES-DC-MOG, ES-DC-TRAIL/MOG, or ES-DC-PDL1/MOG. As shown in Fig. 5, proliferative response of the MOG-reactive T cells cocultured with ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG was significantly lower than those cocultured with ES-DC-MOG, even though the three types of ES-DC expressed an almost equal level of MOG-Ii (Fig. 2). These results indicate down-modulation of the response of MOG-reactive T cells in vitro by TRAIL and PD-L1 coexpressed together with MOG peptide on ES-DC.

We tested whether or not development of EAE would be prevented by pretreatment of mice with genetically modified ES-DC. Mice were i.p. injected with ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG at days −8, −5, and −2 (1×10^6 cells/mouse/injection), and sequentially immunized with MOG peptide plus adjuvants at days 0 and 2 according to the protocol described in Fig. 6*A*. As shown in Fig. 6*B* and Table II, EAE was almost

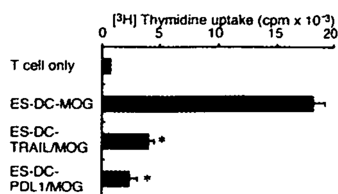

FIGURE 5. Decreased proliferative response to MOG peptide of MOG-reactive T cells cocultured with ES-DC expressing MOG plus TRAIL or MOG plus PD-L1. T cells (2×10^5) isolated from inguinal lymph nodes of CBF_1 mice immunized according to the protocol for EAE induction were cocultured with irradiated ES-DC-MOG, TRAIL/MOG, or PDL1/MOG (2×10^4) for 3 days, as in Fig. 4*A*. The asterisks indicate that the differences in responses are statistically significant ($p < 0.01$) compared with ES-DC-MOG. The data are each representative of three independent and reproducible experiments with similar results.

completely prevented by pretreatment with either of these genetically modified ES-DC. In contrast, pretreatment with ES-DC-MOG, ES-DC-TRAIL/OVA (as irrelevant Ag), or ES-DC-PDL1/OVA had no effect (Fig. 6*C* and Table II). Thus, the prevention depended on both the presentation of the MOG peptide and the expression of TRAIL or PD-L1 by ES-DC. If 2×10^6 of ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG was given as a one-injection administration, EAE was similarly prevented (data not shown). However, if 5×10^5 of ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG was used for one injection, the disease severity was not reduced (data not shown). Thus, $\sim 1 \times 10^6$ of genetically modified ES-DC as one-injection dose is apparently necessary for the prevention of EAE under this experimental condition.

We asked whether TRAIL or PD-L1 should be coexpressed by the same ES-DC as one presenting MOG peptide for their capacity to protect mice from EAE. As shown in Fig. 6*D* and Table II, coinjection of ES-DC-MOG together with ES-DC-TRAIL or ES-DC-PDL1 did not reduce the severity of EAE. Thus, coexpression of TRAIL or PD-L1 with MOG peptide by ES-DC is necessary for the protection from EAE. These results emphasize the advantage of the technology of ES cell-mediated genetic modification of DC, by which one can generate clonal transfectant DC carrying multiple expression vectors.

Next, we tested whether or not treatment with ES-DC after immunization with MOG would achieve some preventive effect on EAE. As shown in Fig. 7*A*, mice were immunized according to the protocol for EAE induction and, after that, injected with ES-DC on days 5, 9, and 13 (1×10^6 cells/mouse/injection). Even in this postimmunization treatment, injection of ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG reduced severity of the disease, but ES-DC-MOG did not do so (Fig. 7*B* and Table II).

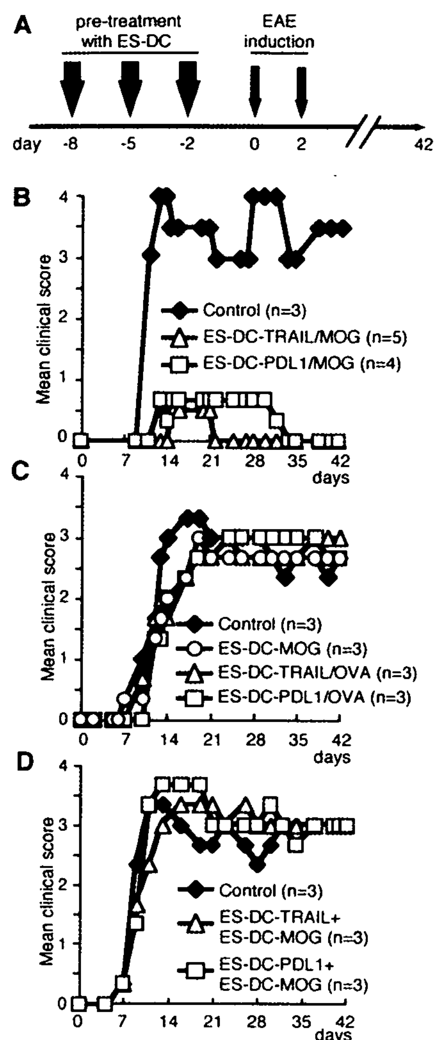

FIGURE 6. Prevention of MOG-induced EAE by pretreatment of mice with ES-DC expressing MOG plus TRAIL or MOG plus PD-L1. *A*, The schedule for pretreatment and induction of EAE is shown. CBF_1 mice (three to five mice per group) were i.p. injected with ES-DC (1×10^6 cells/injection/mouse) on days −8, −5, and −2. EAE was induced by s.c. injection of MOG peptide plus *M. tuberculosis* H37Ra emulsified in IFA on day 0, and i.p. injection of *B. pertussis* toxin on days 0 and 2. *B–D*, Disease severity of mice treated with ES-DC-TRAIL/MOG, ES-DC-PD-L1/MOG, or RPMI 1640 medium (control) (*B*), ES-DC-MOG, ES-DC-TRAIL/OVA, ES-DC-PDL1/OVA, or RPMI 1640 medium (control) (*C*), coinjection with ES-DC-MOG plus ES-DC-TRAIL, ES-DC-MOG plus ES-DC-PDL1, or RPMI 1640 medium (control) (*D*) is shown. The data are each representative of at least two independent and reproducible experiments, and data of all experiments are summarized in Table II.

Decreased T cell response to MOG in mice treated with ES-DC-TRAIL/MOG or -PD-L1/MOG

We examined whether treatment with ES-DC-TRAIL/MOG or -PDL1/MOG would reduce the activation of MOG-specific T cells. Forty-two days after the immunization according to the protocol for EAE induction (Fig. 6*A*), we isolated inguinal lymph node cells and analyzed their proliferative response upon restimulation in vitro with MOG peptide. As shown in Fig. 8*A*, the magnitude of proliferation of lymph node cells isolated from mice treated with ES-DC-TRAIL/MOG or -PDL1/MOG was not increased in response to MOG peptide. In contrast, that of lymph node cells from ES-DC-MOG-treated or untreated mice was increased with statistical significance. In the presence of 25 μg/ml MOG peptide, stimulation index (count in the presence of MOG peptide/count in the absence of Ag) for that of untreated, ES-DC-MOG, -TRAIL/MOG, and -PDL1/MOG-treated mice were 2.8, 2.4, 1.3, and 1.0, respectively. These results suggest that treatment with ES-DC-TRAIL/MOG or -PDL1/MOG inhibited the activation of MOG-specific T cells or reduced their number in mice immunized with MOG peptide and adjuvants.

Next, we examined whether or not treatment with ES-DC would affect immune responses to an irrelevant exogenous Ag. We treated mice with ES-DC-MOG, -TRAIL/MOG, -PDL1/MOG, or RPMI 1640 medium (control) using the same schedule described above, and subsequently immunized the mice with KLH/CFA. Eleven days after the immunization, we isolated inguinal lymph

Table II. *Suppression of EAE induction in CBF$_1$ mice treated with ES-DC*[a]

Treatment (ES-DC)	Disease Incidence	Day of Onset	Mean Peak Clinical Score
No Treatment (control)	26/26	10.5 ± 1.1	3.3 ± 0.4
Pre[b]- TRAIL/MOG	**3/10**	18.3 ± 2.4	**0.3 ± 0.4**
Pre- PDL1/MOG	**5/10**	13.4 ± 2.1	**0.8 ± 0.8**
Pre- MOG	8/8	10.5 ± 1.3	3.0 ± 0.3
Pre- TRAIL/OVA	6/6	10.2 ± 2.9	3.0 ± 0
Pre- PDL1/OVA	6/6	11.3 ± 0.9	3.0 ± 0
Pre- TRAIL + MOG	6/6	10.2 ± 1.2	3.2 ± 0.6
Pre- PDL1 + MOG	6/6	10.2 ± 0.6	3.3 ± 0.7
Post[c]- TRAIL/MOG	**3/6**	18.7 ± 4.4	**0.5 ± 0.5**
Post- PDL1/MOG	**3/6**	13.7 ± 1.1	**1.0 ± 1.0**
Post- MOG	6/6	10.8 ± 1.0	3.2 ± 0.3

[a] Data are combined from a total of 10 separate experiments including those shown in Figs. 6 and 7. EAE was induced by s.c. injection at the tail base of a 0.2-ml IFA/PBS solution containing 400 μg of *M. tuberculosis* and 600 μg of MOG peptide once (on day 0), together with i.p. injections of 500 ng of purified *B. pertussis* toxin on days 0 and 2. For prevention of EAE, mice were injected i.p. with ES-DC (1×10^6 cells/mouse/injection) [b]on days −8, −5, and −2 (preimmunization treatment), or [c]on days 5, 9, and 13 (postimmunization treatment). The incidence and the clinical score reduced by ES-DC treatment are indicated in boldface.

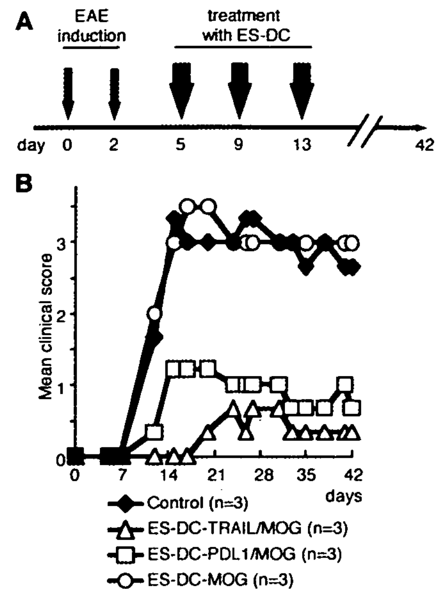

FIGURE 7. Inhibition of MOG-induced EAE by treatment with ES-DC expressing MOG plus TRAIL or MOG plus PD-L1 after immunization with MOG. *A*, The schedule for induction of EAE and treatment is shown. CBF$_1$ mice (three mice per group) were immunized on days 0 and 2 according to the EAE induction schedule described above, and subsequently i.p. injected with ES-DC (1×10^6 cells/injection/mouse) on days 5, 9, and 13. *B*, Disease severity of mice treated with ES-DC-TRAIL/MOG, ES-DC-PD-L1/MOG, ES-DC-MOG, or RPMI 1640 medium (control) is shown. The data are each representative of two independent and reproducible experiments, and data of all experiments are summarized in Table II.

node cells and analyzed their proliferative response upon restimulation with KLH in vitro. As a result, lymph node cells of ES-DC-treated and control mice showed the same magnitude of proliferative response (Fig. 8*B*), thereby indicating that the treatment with such genetically modified ES-DC did not affect the immune response to irrelevant Ags.

We immunohistochemically analyzed spinal cord, the target organ of the disease, of mice subjected to EAE induction with or without treatment with ES-DC. Massive infiltration of $CD4^+$ T cells, $CD8^+$ T cells, and Mac-1^+ macrophages was observed in spinal cords of untreated control mice (Fig. 9). In contrast, T cells and macrophages hardly infiltrated into the spinal cord of mice treated with ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG. The results of histological analysis are in parallel with the severity of EAE and activation state of MOG-specific T cells of each mouse.

Increased number of apoptotic cells in splenic $CD4^+$ T cells by treatment with ES-DC-TRAIL/MOG

With regard to the mechanism of prevention of EAE by transfectant ES-DC, we analyzed the apoptosis of $CD4^+$ T cell in spleens of mice treated with ES-DC by staining with annexin V and subsequent flow-cytometric analysis. In the results, we observed that transfer of ES-DC-TRAIL/MOG caused an increase of apoptosis of $CD4^+$ T cells in recipient mice (17.3 ± 2.5%), compared with transfer of ES-DC-MOG (12.0 ± 0.4%), ES-DC-PDL1/MOG (12.2 ± 0.5%), or RPMI 1640 medium control (10.2 ± 0.8%). In the experiments, three mice were used for each group. Increased numbers of apoptotic cells in spleen of mice transferred with ES-DC-TRAIL/MOG were also observed in histological analysis with TUNEL staining (Fig. 10). The capacity of ES-DC-TRAIL/MOG to cause apoptosis of T cells may play some role in the protection from EAE.

Discussion

DC are the most potent APC responsible for priming of naive T cells in initiation of the immune response. Recent studies revealed that DC are also involved in the maintenance of immunological self-tolerance, promoting T cells with regulatory functions, or inducing anergy of T cells. In vivo transfer of Ag-loaded DC with a tolerogenic character is regarded as a promising therapeutic means to negatively manipulate immune response in an Ag-specific manner. Various culture procedures used to generate DC with a tolerogenic character have been reported (31–36). Mouse bone marrow-derived DC generated in the presence of IL-10 and/or TGF-β or in the low dose of GM-CSF showed immature phenotypes, a low-level expression of cell surface MHC and costimulatory molecules, and induced T cell anergy in vitro and tolerance to specific Ags or allogeneic transplanted organs in vivo. In humans, monocyte-derived immature DC loaded with antigenic peptides and transferred in vivo have been shown to cause the Ag-specific immune suppression (37).

Genetic modification may be a more steady and reliable way to manipulate the character of DC. Generation of tolerogenic DC by forced expression of Fas ligand, indoleamine 2,3-dioxygemase, IL-10, or CTLA4Ig by gene transfer has been also reported (38–41). In a recent study, type II collagen-loaded bone marrow-derived DC genetically engineered to express TRAIL by using an adenovirus vector ameliorated type II collagen-induced arthritis (42).

Regarding methods for gene transfer to DC, electroporation, lipofection, and virus vector-mediated transfection have been reported (38–43). However, considering clinical applications, presently established methods have several drawbacks, i.e., efficiency of gene transfer, stability of gene expression, limitation of the size and number of genes to be introduced, potential risk accompanying the use of virus vectors, and the immunogenicity of the virus vectors. For the purpose of Ag-specific negative regulation of immune responses, the antigenicity of vector systems may lead to problems. Importantly, to efficiently down-modulate T cell responses in an Ag-specific manner, it is desirable to introduce multiple expression vectors to generate stable transfectant DC, which continuously present transgene-derived Ag and simultaneously express immunosuppressive molecules.

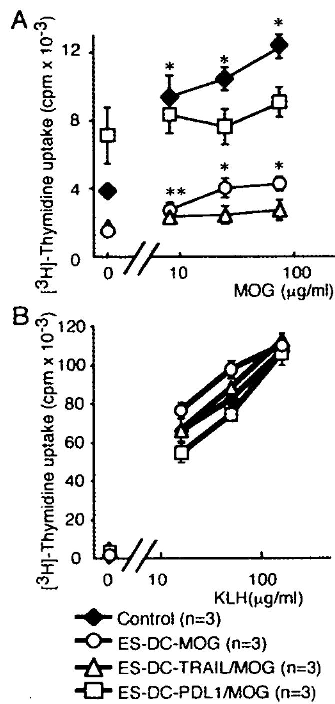

FIGURE 8. Inhibition of activation of MOG-reactive T cells and no effect of activation of KLH-specific T cell by treatment of mice with ES-DC expressing MOG plus TRAIL or PD-L1. *A*, Inguinal lymph node cells (3×10^5) were isolated from CBF_1 mice (three mice per group) of various treatment groups at over day 42, and were stimulated ex vivo with irradiated and MOG peptide-pulsed syngeneic spleen cells for 3 days. Proliferative response of T cells was quantified by [^{3}H]thymidine uptake in the last 12 h of the culture. The asterisks indicate that the differences in responses are statistically significant compared with count in the absence of Ag (*, $p < 0.01$; **, $p < 0.05$). The data are each representative of two independent and reproducible experiments with similar results. *B*, CBF_1 mice (three mice per group) were i.p. injected with ES-DC (1×10^6 cells/injection/mouse) on days −8, −5, and −2, and immunized with KLH/CFA on day 0. On day 11, inguinal lymph node cells were isolated and restimulated with the indicated concentration of KLH in vitro. Proliferation of T cells was quantified as described above.

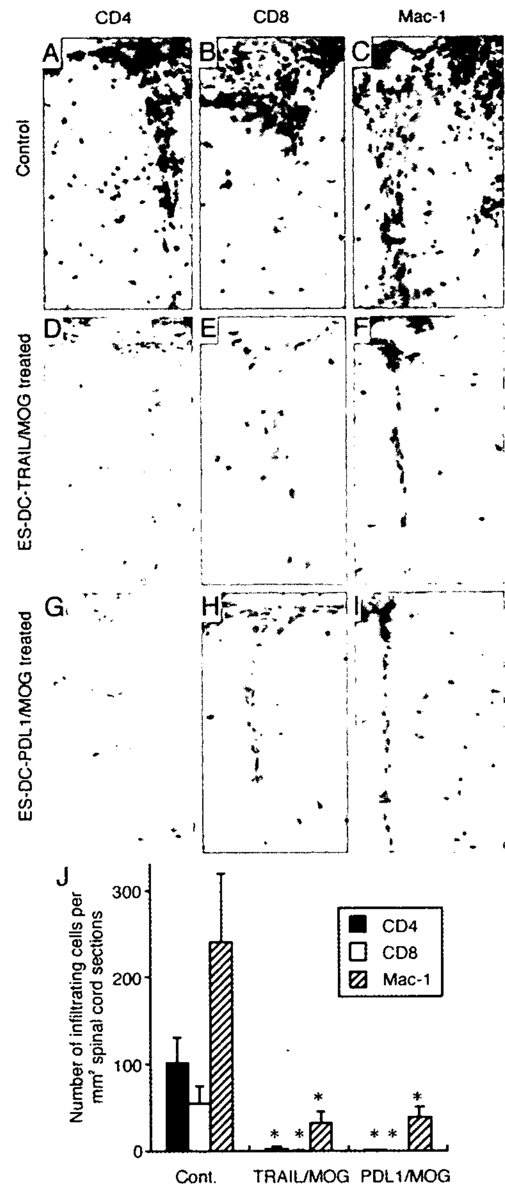

FIGURE 9. Inhibition of infiltration of $CD4^+$ T cells, $CD8^+$ T cells, and Mac-1^+ macrophages into spinal cord by treatment of mice with ES-DC expressing MOG plus TRAIL or PD-L1. Mice were pretreated with ES-DC-TRAIL/MOG, PDL1/MOG, or untreated and subsequently immunized according to the protocol for EAE induction as shown in Fig. 6*A*. The cervical, thoracic, and lumbar spinal cord was isolated at day 11 and subjected to immunohistochemical analysis. CD4 (*A*, *D*, and *G*), CD8 (*B*, *E*, and *H*), and Mac-1 (*C*, *F*, and *I*) staining are shown in representative untreated control (*A–C*), ES-DC-TRAIL/MOG-treated (*D–F*), and ES-DC-PDL1/MOG-treated (*G–I*) mice. *J*, The positive cells were microscopically counted in three sections of spinal cord. Results are expressed as mean ± SD of $CD4^+$, $CD8^+$, Mac-1^+ cells per 1 mm^2 tissue area of samples obtained from five mice. The asterisks indicate that the decreases in number of infiltrated cells are statistically significant ($p < 0.01$) compared with control.

Efficient genetic modification of mouse DC can be done by gene transfer to ES cells and subsequent differentiation of transfectant ES cells to ES-DC. By sequential transfection of ES cells using multiple expression vectors, transfectant ES-DC expressing multiple transgene products can readily be generated. In a recent study, we demonstrated that this methodology worked very effectively for induction of antitumor immunity, showing highly efficient stimulation of Ag-specific T cells by in vivo transfer of ES-DC expressing T cell-attracting chemokines along with Ag (20).

The present study demonstrates the usefulness of the genetically modified DC generated by this method for the treatment of subjects with autoimmune disease. We generated ES-DC presenting the MOG epitope in the context of MHC class II molecule and simultaneously expressing immunosuppressive molecule, TRAIL or PD-L1. By pre- or posttreatment of mice with such ES-DC, we succeeded in preventing an autoimmune disease model, EAE induced by immunization with MOG peptide (Figs. 6 and 7; Table II). Down-modulation of immune response by treatment with genetically modified ES-DC did not affect the immune response to irrelevant exogenous Ag, KLH (Fig. 8*B*). Thus, we achieved the prevention of EAE without decrease in the immune response to an irrelevant Ag.

As for the function of TRAIL, induction of apoptosis has been reported by several groups (3, 4, 42, 44). We also observed an increase in apoptosis of $CD4^+$ T cells in spleens of mice treated with ES-DC-TRAIL/MOG compared with ES-DC-MOG, PDL1/MOG or RPMI 1640 medium (control), as shown in Fig. 10. The result is consistent with a recent report by Liu et al. (42). They introduced the *TRAIL* gene into bone marrow-derived DC by adenovirus vector and injected the *TRAIL* transfectant DC into mice for prevention of collagen-induced arthritis, and also observed an increased number of apoptotic T cells in the injected mice. The

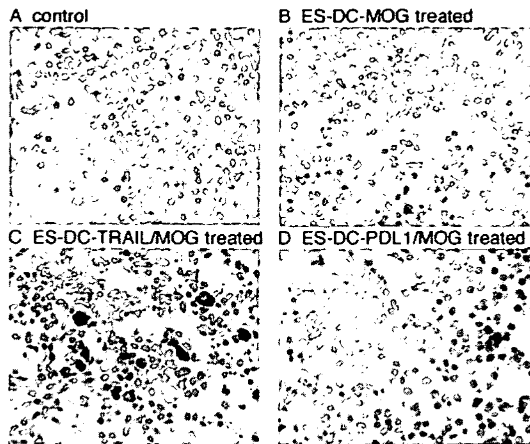

FIGURE 10. Induction of apoptosis of spleen cells by treatment of mice with ES-DC expressing TRAIL along with MOG peptide. Mice were treated with the indicated ES-DC and immunized with MOG peptide, following the schedule described in Fig. 6*A*. On day 11, spleens were isolated from the mice, and apoptotic cells were detected by in situ TUNEL staining. Original magnification, ×200. Sections of the mice untreated (*A*), treated with ES-DC-MOG (*B*), ES-DC-TRAIL/MOG (*C*), and ES-DC-PDL1/MOG (*D*) are shown. Similar results were observed for three mice used in each experimental group, and representative results are shown.

potential for ES-DC-TRAIL/MOG to cause apoptosis of T cells may have played some role in the protection from EAE, at least in part, in our experiments. In addition, our preliminary experiments suggest that ES-DC-TRAIL/MOG induced T cells with protective effects against EAE. In the experiments, we isolated splenic $CD4^+$ T cells from ES-DC-TRAIL/MOG-treated mice and adoptively transferred them to naive mice. The severity of subsequently induced EAE in the recipient mice was significantly reduced by this treatment (data not shown). At present, it may be possible that both induction of apoptosis of MOG-reactive pathogenic T cells and promotion of T cells with some regulatory function contributed to prevention of EAE by ES-DC-TRAIL/MOG. However, to clarify the precise mechanism or character of the T cell with regulatory function, further investigations are necessary.

In contrast, in case of treatment with ES-DC-PDL1/MOG, neither apoptosis of T cells nor induction of transferable disease-preventing T cells was observed (data not shown). We presume induction of anergy of MOG-reactive T cells to be likely as the mechanism of disease-preventive effect of treatment with ES-DC-PDL1/MOG, based on previous literature regarding the function of PD-L1 (7, 14, 45–47).

To determine whether the profile of cytokine production was altered by treatment with ES-DC, we did ELISA to quantify IL-10, IL-4, and IFN-γ produced by spleen cells of ES-DC-treated mice upon stimulation with MOG peptide in vitro. We observed no significant change in the amount of these cytokines produced by spleen cells from ES-DC-TRAIL/MOG-treated or ES-DC-PDL1/MOG-treated mice, compared with those from ES-DC-MOG-treated mice (data not shown). The level of expression of mRNA for TGF-β detected by RT-PCR was also unchanged compared with control (data not shown). Thus, involvement of IL-10-producing Tr-1 cells or Th2 cells in protection from EAE by treatment with ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG is unlikely, although one cannot totally rule out the possibility.

The capacity of the ES cells to differentiate to ES-DC was never impaired even after culture for at least over 4 mo. Inactivation of transcription of introduced genes due to gene silencing in ES cells can be prevented using vectors bearing the IRES-drug resistance gene or by targeted gene introduction with an exchangeable gene-trap system (2). Thus, genetically manipulated ES cells can be used as an infinite source for DC with genetically modified properties.

Recently, we established methods for generation of DC from nonhuman primate ES cells and also for genetic modification of them (S. Senju, H. Suemori, H. Matsuyoshi, S. Hirata, Y. Uemura, Y.-Z. Chen, D. Fukuma, M. Furuya, N. Nakatsuji, and Y. Nishimura, manuscript in preparation). We hope to apply this method to human ES cells to generate genetically modified human ES-DC, although some modification might be necessary. In the future, Ag-specific immune modulation therapy by in vivo transfer of human ES-DC expressing antigenic protein along with immune-regulating molecules may well be realized, based on evidence in the current study in the mouse system. Possible applications of this technology are treatment of subjects with autoimmune and allergic diseases and also for induction of tolerance to transplanted organs, especially those generated from ES cells. Thus, the methods established in the present study may have implications as a broad medical technology.

Acknowledgments

We thank Dr. S. Aizawa (RIKEN Center for Developmental Biology, Kobe, Japan) for TT2, Drs. N. Takakura (Kanazawa University, Kanazawa, Japan) and T. Suda (Keio University, Tokyo, Japan) for OP9, Dr. H. Niwa (RIKEN Center for Developmental Biology, Kobe, Japan) for pCAG-IPuro, Drs. T. Okazaki and T. Honjo (Kyoto University, Kyoto, Japan) for a cDNA clone for PD-L1, Tatsuko Kubo (Department of Molecular Pathology, Kumamoto University) for technical assistance, and Kirin Brewery Co., Ltd., for rGM-CSF. M. Ohara (Fukuoka, Japan) provided helpful comments on the manuscript.

References

1. Fujii, S., S. Senju, Y. Z. Chen, M. Ando, S. Matsushita, and Y. Nishimura. 1998. The CLIP-substituted invariant chain efficiently targets an antigenic peptide to HLA class II pathway in L cells. *Hum. Immunol. 59:607.*
2. Senju, S., S. Hirata, H. Matsuyoshi, M. Masuda, Y. Uemura, K. Araki, K. Yamamura, and Y. Nishimura. 2003. Generation and genetic modification of dendritic cells derived from mouse embryonic stem cells. *Blood 101:3501.*
3. Wiley, S. R., K. Schooley, P. J. Smolak, W. S. Din, C. P. Huang, J. K. Nicholl, G. R. Sutherland, T. D. Smith, C. Rauch, C. A. Smith, and R. G. Goodwin. 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity 3:673.*
4. Kayagaki, N., N. Yamaguchi, M. Nakayama, K. Takeda, H. Akiba, H. Tsutsui, H. Okamura, K. Nakanishi, K. Okumura, and H. Yagita. 1999. Expression and function of TNF-related apoptosis-inducing ligand on murine activated NK cells. *J. Immunol. 163:1906.*
5. Lamhamedi-Cherradi, S. E., S. J. Zheng, K. A. Maguschak, J. Peschon, and Y. H. Chen. 2003. Defective thymocyte apoptosis and accelerated autoimmune diseases in $TRAIL^{-/-}$ mice. *Nat. Immunol. 4:255.*
6. Dong, H., G. Zhu, K. Tamada, and L. Chen. 1999. B7–H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat. Med. 5:1365.*
7. Freeman, G. J., A. J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L. J. Fitz, N. Malenkovich, T. Okazaki, M. C. Byrne, et al. 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J. Exp. Med. 192:1027.*
8. Nishimura, H., M. Nose, H. Hiai, N. Minato, and T. Honjo. 1999. Development of lupus-like autoimmune diseases by disruption of the *PD-1* gene encoding an ITIM motif-carrying immunoreceptor. *Immunity 11:141.*
9. Nishimura, H., T. Okazaki, Y. Tanaka, K. Nakatani, M. Hara, A. Matsumori, S. Sasayama, A. Mizoguchi, H. Hiai, N. Minato, and T. Honjo. 2001. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science 291:319.*
10. Song, K., Y. Chen, R. Goke, A. Wilmen, C. Seidel, A. Goke, and B. Hilliard. 2000. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an inhibitor of autoimmune inflammation and cell cycle progression. *J. Exp. Med. 191:1095.*
11. Hilliard, B., A. Wilmen, C. Seidel, T. S. Liu, R. Goke, and Y. Chen. 2001. Roles of TNF-related apoptosis-inducing ligand in experimental autoimmune encephalomyelitis. *J. Immunol. 166:1314.*
12. Lunemann, J. D., S. Waiczies, S. Ehrlich, U. Wendling, B. Seeger, T. Kamradt, and F. Zipp. 2002. Death ligand TRAIL induces no apoptosis but inhibits activation of human (auto)antigen-specific T cells. *J. Immunol. 168:4881.*
13. Kayagaki, N., N. Yamaguchi, M. Abe, S. Hirose, T. Shirai, K. Okumura, and H. Yagita. 2002. Suppression of antibody production by TNF-related apoptosis-inducing ligand (TRAIL). *Cell. Immunol. 219:82.*

14. Nishimura, H., and T. Honjo. 2001. PD-1: an inhibitory immunoreceptor involved in peripheral tolerance. *Trends Immunol. 22:265.*
15. Okazaki, T., Y. Iwai, and T. Honjo. 2002. New regulatory co-receptors: inducible co-stimulator and PD-1. *Curr. Opin. Immunol. 14:779.*
16. Salama, A. D., T. Chitnis, J. Imitola, M. J. Ansari, H. Akiba, F. Tushima, M. Azuma, H. Yagita, M. H. Sayegh, and S. J. Khoury. 2003. Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *J. Exp. Med. 198:71.*
17. Liang, S. C., Y. E. Latchman, J. E. Buhlmann, M. F. Tomczak, B. H. Horwitz, G. J. Freeman, and A. H. Sharpe. 2003. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur. J. Immunol. 33:2706.*
18. Ansari, M. J., A. D. Salama, T. Chitnis, R. N. Smith, H. Yagita, H. Akiba, T. Yamazaki, M. Azuma, H. Iwai, S. J. Khoury, et al. 2003. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J. Exp. Med. 198:63.*
19. Fairchild, P. J., F. A. Brook, R. L. Gardner, L. Graca, V. Strong, Y. Tone, M. Tone, K. F. Nolan, and H. Waldmann. 2000. Directed differentiation of dendritic cells from mouse embryonic stem cells. *Curr. Biol. 10:1515.*
20. Matsuyoshi, H., S. Senju, S. Hirata, Y. Yoshitake, Y. Uemura, and Y. Nishimura. 2004. Enhanced priming of antigen-specific CTLs in vivo by embryonic stem cell-derived dendritic cells expressing chemokine along with antigenic protein: application to antitumor vaccination. *J. Immunol. 172:776.*
21. Fairchild, P. J., K. F. Nolan, S. Cartland, L. Graca, and H. Waldmann. 2003. Stable lines of genetically modified dendritic cells from mouse embryonic stem cells. *Transplantation 76:606.*
22. Cheng, P., Y. Nefedova, L. Miele, B. A. Osborne, and D. Gabrilovich. 2003. Notch signaling is necessary but not sufficient for differentiation of dendritic cells. *Blood 102:3980.*
23. Mendel, I., N. Kerlero de Rosbo, and A. Ben-Nun. 1995. A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2^b mice: fine specificity and T cell receptor $V\beta$ expression of encephalitogenic T cells. *Eur. J. Immunol. 25:1951.*
24. Greer, J. M., R. A. Sobel, A. Sette, S. Southwood, M. B. Lees, and V. K. Kuchroo. 1996. Immunogenic and encephalitogenic epitope clusters of myelin proteolipid protein. *J. Immunol. 156:371.*
25. Zamvil, S. S., D. J. Mitchell, M. B. Powell, K. Sakai, J. B. Rothbard, and L. Steinman. 1988. Multiple discrete encephalitogenic epitopes of the autoantigen myelin basic protein include a determinant for I-E class II-restricted T cells. *J. Exp. Med. 168:1181.*
26. Senju, S., K. Iyama, H. Kudo, S. Aizawa, and Y. Nishimura. 2000. Immunocytochemical analyses and targeted gene disruption of GTPBP1. *Mol. Cell. Biol. 20:6195.*
27. Fujii, S., Y. Uemura, L. K. Iwai, M. Ando, S. Senju, and Y. Nishimura. 2001. Establishment of an expression cloning system for $CD4^+$ T cell epitopes. *Biochem. Biophys. Res. Commun. 284:1140.*
28. Uemura, Y., S. Senju, K. Maenaka, L. K. Iwai, S. Fujii, H. Tabata, H. Tsukamoto, S. Hirata, Y. Z. Chen, and Y. Nishimura. 2003. Systematic analysis of the combinatorial nature of epitopes recognized by TCR leads to identification of mimicry epitopes for glutamic acid decarboxylase 65-specific TCRs. *J. Immunol. 170:947.*
29. Weir, C. R., K. Nicolson, and B. T. Backstrom. 2002. Experimental autoimmune encephalomyelitis induction in naive mice by dendritic cells presenting a self-peptide. *Immunol. Cell Biol. 80:14.*
30. Legge, K. L., R. K. Gregg, R. Maldonado-Lopez, L. Li, J. C. Caprio, M. Moser, and H. Zaghouani. 2002. On the role of dendritic cells in peripheral T cell tolerance and modulation of autoimmunity. *J. Exp. Med. 196:217.*
31. Bonham, C. A., L. Lu, R. A. Banas, P. Fontes, A. S. Rao, T. E. Starzl, A. Zeevi, and A. W. Thomson. 1996. TGF-β1 pretreatment impairs the allostimulatory function of human bone marrow-derived antigen-presenting cells for both naive and primed T cells. *Transpl. Immunol. 4:186.*
32. Lutz, M. B., R. M. Suri, M. Niimi, A. L. Ogilvie, N. A. Kukutsch, S. Rossner, G. Schuler, and J. M. Austyn. 2000. Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. *Eur. J. Immunol. 30:1813.*
33. Steinbrink, K., M. Wolfl, H. Jonuleit, J. Knop, and A. H. Enk. 1997. Induction of tolerance by IL-10-treated dendritic cells. *J. Immunol. 159:4772.*
34. Menges, M., S. Rossner, C. Voigtlander, H. Schindler, N. A. Kukutsch, C. Bogdan, K. Erb, G. Schuler, and M. B. Lutz. 2002. Repetitive injections of dendritic cells matured with tumor necrosis factor-α induce antigen-specific protection of mice from autoimmunity. *J. Exp. Med. 195:15.*
35. Sato, K., N. Yamashita, M. Baba, and T. Matsuyama. 2003. Regulatory dendritic cells protect mice from murine acute graft-versus-host disease and leukemia relapse. *Immunity 18:367.*
36. Sato, K., N. Yamashita, M. Baba, and T. Matsuyama. 2003. Modified myeloid dendritic cells act as regulatory dendritic cells to induce anergic and regulatory T cells. *Blood 101:3581.*
37. Dhodapkar, M. V., R. M. Steinman, J. Krasovsky, C. Munz, and N. Bhardwaj. 2001. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J. Exp. Med. 193:233.*
38. Takayama, T., Y. Nishioka, L. Lu, M. T. Lotze, H. Tahara, and A. W. Thomson. 1998. Retroviral delivery of viral interleukin-10 into myeloid dendritic cells markedly inhibits their allostimulatory activity and promotes the induction of T-cell hyporesponsiveness. *Transplantation 66:1567.*
39. Lu, L., A. Gambotto, W. C. Lee, S. Qian, C. A. Bonham, P. D. Robbins, and A. W. Thomson. 1999. Adenoviral delivery of CTLA4Ig into myeloid dendritic cells promotes their in vitro tolerogenicity and survival in allogeneic recipients. *Gene Ther. 6:554.*
40. Min, W. P., R. Gorczynski, X. Y. Huang, M. Kushida, P. Kim, M. Obataki, J. Lei, R. M. Suri, and M. S. Cattral. 2000. Dendritic cells genetically engineered to express Fas ligand induce donor-specific hyporesponsiveness and prolong allograft survival. *J. Immunol. 164:161.*
41. Terness, P., T. M. Bauer, L. Rose, C. Dufter, A. Watzlik, H. Simon, and G. Opelz. 2002. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J. Exp. Med. 196:447.*
42. Liu, Z., X. Xu, H. C. Hsu, A. Tousson, P. A. Yang, Q. Wu, C. Liu, S. Yu, H. G. Zhang, and J. D. Mountz. 2003. CII-DC-AdTRAIL cell gene therapy inhibits infiltration of CII-reactive T cells and CII-induced arthritis. *J. Clin. Invest. 112:1332.*
43. Morita, Y., J. Yang, R. Gupta, K. Shimizu, E. A. Shelden, J. Endres, J. J. Mule, K. T. McDonagh, and D. A. Fox. 2001. Dendritic cells genetically engineered to express IL-4 inhibit murine collagen-induced arthritis. *J. Clin. Invest. 107:1275.*
44. Giovarelli, M., P. Musiani, G. Garotta, R. Ebner, E. Di Carlo, Y. Kim, P. Cappello, L. Rigamonti, P. Bernabei, F. Novelli, et al. 1999. A "stealth effect": adenocarcinoma cells engineered to express TRAIL elude tumor-specific and allogeneic T cell reactions. *J. Immunol. 163:4886.*
45. Brown, J. A., D. M. Dorfman, F. R. Ma, E. L. Sullivan, O. Munoz, C. R. Wood, E. A. Greenfield, and G. J. Freeman. 2003. Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. *J. Immunol. 170:1257.*
46. Carter, L., L. A. Fouser, J. Jussif, L. Fitz, B. Deng, C. R. Wood, M. Collins, T. Honjo, G. J. Freeman, and B. M. Carreno. 2002. PD-1:PD-L inhibitory pathway affects both $CD4^+$ and $CD8^+$ T cells and is overcome by IL-2. *Eur. J. Immunol. 32:634.*
47. Selenko-Gebauer, N., O. Majdic, A. Szekeres, G. Hofler, E. Guthann, U. Korthauer, G. Zlabinger, P. Steinberger, W. F. Pickl, H. Stockinger, et al. 2003. B7-H1 (programmed death-1 ligand) on dendritic cells is involved in the induction and maintenance of T cell anergy. *J. Immunol. 170:3637.*

ELSEVIER

Available online at www.sciencedirect.com

SCIENCE @ DIRECT®

Biochemical and Biophysical Research Communications 335 (2005) 5–13

BBRC

www.elsevier.com/locate/ybbrc

Cancer prevention with semi-allogeneic ES cell-derived dendritic cells ☆

Daiki Fukuma [a,b], Hidetake Matsuyoshi [a], Shinya Hirata [a], Akari Kurisaki [a], Yutaka Motomura [a,c], Yoshihiro Yoshitake [a,b], Masanori Shinohara [b], Yasuharu Nishimura [a], Satoru Senju [a,*]

[a] *Department of Immunogenetics, Kumamoto University, Graduate School of Medical Sciences, Kumamoto, Japan*
[b] *Department of Oral and Maxillofacial Surgery, Kumamoto University, Graduate School of Medical Sciences, Kumamoto, Japan*
[c] *Department of Gastroenterological Surgery, Kumamoto University, Graduate School of Medical Sciences, Kumamoto, Japan*

Received 31 May 2005
Available online 28 June 2005

Abstract

Dendritic cells (DC) genetically modified to present tumor-associated antigen are a promising means for anti-cancer immunotherapy. By introducing expression vectors into ES cells and subsequently inducing differentiation to DC (ES-DC), we can generate transfectant DC expressing the transgenes. In the future clinical application of this technology, the unavailability of human ES cells genetically identical to the patients will be a problem. However, in most cases, semi-allogeneic ES cells sharing some of HLA alleles with recipients are expected to be available. In the present study, we observed that model tumor antigen (OVA)-expressing mouse ES-DC transferred into semi-allogeneic mice potently primed OVA-reactive CTL and elicited a significant protection against challenge with OVA-expressing tumor. Genetic modification of ES-DC to overexpress SPI-6, the specific inhibitor of granzyme B, further enhanced their capacity to prime antigen-specific CTL in semi-allogeneic recipient mice. These results suggest the potential of ES-DC as a novel means for anti-cancer immunotherapy.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Anti-cancer immunotherapy; Embryonic stem cells; Dendritic cells; Serine proteinase inhibitor

Activation of CTL reactive to tumor-associated antigens is crucial for anti-tumor immunotherapy. Dendritic cells (DC) are potent immune-stimulators, and the adoptive transfer of antigen-loaded DC has proven to be an efficient method for priming T cells specific to the antigen. DC-based methods are now regarded as a promising approach for anti-cancer immunotherapy. For loading tumor antigens to DC for anti-cancer immunotherapy, gene-based antigen-expression by DC is considered to be superior to loading antigen as peptide, protein, or tumor cell lysate [1]. For efficient gene transfer to DC, the use of virus-based vectors is required because DC are relatively reluctant to genetic modification. Clinical trials using DC genetically modified with virus vectors, for example, monocyte-derived DC introduced with adenovirus vectors encoding for tumor antigens, are now in progress. Considering the broader medical applications of this method, the drawbacks of genetic modification with virus vectors include the potential risk accompanying the use of virus vectors and legal restrictions related to it. Thus, the development of safer and more efficient means would be desirable.

We recently established a novel method for the genetic modification of DC [2]. In the method, we generated DC from mouse embryonic stem (ES) cells

☆ *Abbreviations:* ES cell, embryonic stem cell; ES-DC, embryonic stem cell-derived dendritic cell; BM-DC, bone marrow-derived dendritic cell; SPI-6, serine proteinase inhibitor 6; PI-9, proteinase inhibitor 9; neo-R, neomycin resistant; IRES, internal ribosomal entry site.

* Corresponding author. Fax: +81 96 373 5314.
E-mail address: senjusat@gpo.kumamoto-u.ac.jp (S. Senju).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.06.096

by in vitro differentiation. The capacity of ES cell-derived DC (ES-DC) to simulate T cells was comparable to that of DC generated in vitro from BM cells (BM-DC). We can readily generate genetically modified ES-DC by introducing expression vectors into ES cells and the subsequent induction of their differentiation into ES-DC. The transfection of ES cells can be done with electroporation using plasmid vectors, and the use of virus-based vectors is not necessary. Once a proper ES cell transfectant clone is established, it then serves as an infinite source for genetically modified DC.

In the future clinical application of this technology, we will face the problem of histoincompatibility between patients to be treated and the ES cells as source of DC. In general, ES cells genetically identical to patients will not be available. However, ES cells sharing some of HLA class I alleles with the patients are expected to be available in most cases. By adoptive transfer of ES-DC derived from such semi-allogeneic ES cells, we will be able to stimulate tumor antigen-specific CTL restricted to the shared HLA molecules. The obstacle to performing anti-cancer immunotherapy by this strategy would be the presence of allogeneic antigen-reactive T cells, which mainly recognize the HLA molecules expressed by ES-DC but not by the recipients. It is anticipated that such allo-reactive T cells, mainly $CD8^+$ CTL, may attack the transferred APC and rapidly eliminate them, based on a previous report [3]. Mouse BM-DC bearing antigens adoptively transferred are rapidly eliminated, if CTL recognizing the antigens already exist in the recipient mice [4]. Thus, the crucial point is whether the transferred ES-DC can activate tumor antigen-specific CTL restricted to the shared MHC class I molecules before they are eliminated by allo-reactive CTL.

In the present study, we addressed this issue using a mouse system. We adoptively transferred OVA-expressing ES-DC to semi-allogeneic mice and examined whether or not they could activate OVA-specific CTL and elicit protective immunity against tumor cells expressing OVA. We introduced an expression vector for SPI-6, a molecule specifically inhibiting the apoptotic effect of granzyme B, to OVA-transfectant ES cells, and generated double transfectant ES-DC expressing OVA and simultaneously overexpressing SPI-6. We then evaluated this strategy for improving the efficiency of ES-DC to prime antigen-specific CTL, by making ES-DC resistant to attack by CTL.

Materials and methods

Mice. CBA, BALB/c, and C57BL/6 mice were purchased from Clea Animal (Tokyo, Japan) or Charles River (Hamamatsu, Japan) and kept under specific pathogen-free conditions. Male CBA and female C57BL/6 mice were mated to produce (CBA × C57BL/6) F1 mice, and male BALB/c and female C57BL/6 mice were mated to produce (BALB/c × C57BL/6) F1 mice. The animal experiments in this study were approved by Animal Experiment Committee of Kumamoto University (permission number A16-074).

Cells. The ES cell line TT2, derived from (CBA × C57BL/6) F1 embryo, was maintained on a feeder cell layer of mouse primary embryonic fibroblasts, as previously described [5]. The OVA-transfectant ES cell clone (TT2-OVA) generated previously by introduction of OVA-expression vector, pCAG-OVA-IP, to TT2 ES cells was maintained with a sporadic selection with puromycin (2 μg/ml) [6]. MO4 was generated by the transfection of C57BL/6-derived melanoma B16 with the pAc-neo-OVA plasmid, as described [7]. The procedure for inducing the differentiation of ES cells to ES-DC has been described previously [2].

Generation of BM-DC. The generation of dendritic cells from mouse BM cells was done according to the reported procedures [8] with some minor modifications. In brief, bone marrow cells were isolated from (C57BL/6 × CBA) F1 mice and cultured in bacteriological petri dishes (3.0×10^6/7.5 ml medium/90 mm dish) in RPMI-1640 medium supplemented with 10% FCS, GM-CSF (500 U/ml), IL-4 (20 ng/ml), and 2-ME (50 μM) [4]. The culture medium was changed by half on day 3, and floating cells harvested by pipetting between 6 and 8 days of the culture were used in the assays.

Analysis of the priming of CTL in vivo. The indicated number of ES-DC or BM-DC was injected i.p. into the mice twice with a 7-day interval. In some experiments, ES-DC were heat-killed by incubation at 70 °C for 20 min before injection. The mice were sacrificed 7 days after the second injection and spleen cells were isolated. After hemolysis, the spleen cells were cultured in RPMI-1640/10% horse serum/2-ME (50 μM) containing $OVA_{257-264}$ peptide (0.1 μM) and recombinant human IL-2 (100 U/ml). Five days later, the cells were recovered and used as effector cells in a cytotoxicity assay. As target cells, EL-4 thymoma cells were labeled with sodium [^{51}Cr]chromate for 1 h and washed. Subsequently, target cells were incubated in 24-well culture plates (1×10^6 cells/well) with or without 10 μM OVA peptide for 3 h, washed, and seeded into 96-well round-bottomed culture plates (5×10^3 cells/well). The effector cells were added to the target cells according to the indicated E/T ratio and incubated for 4 h at 37 °C. At the end of the incubation, supernatants (50 μl/well) were harvested and counted on a gamma counter. The percentage of specific lysis was calculated as: 100 × [(experimental release − spontaneous release)/(maximal release − spontaneous release)]. The spontaneous release and maximal release were determined in the presence of medium alone and PBS-1% Triton X-100, respectively.

Tumor challenge experiments. The indicated number of genetically modified ES-DC was transferred i.p. into (CBA × C57BL/6) F1 or C57BL/6 mice. Such transfer was done twice with a 7-day interval and, 7 days after the second transfer, 2 or 3×10^5 MO4 cells were injected s.c. in the shaved left flank region. The tumor sizes were determined biweekly in a blinded fashion and the survival rate of mice was also monitored. The tumor index was calculated as: tumor index (in millimeters) = square root (length × width).

Western blot analysis. The cell samples were lysed in an appropriate amount of lysing buffer, 150 mM NaCl, 50 mM Tris, pH 7.4, 1% Nonidet P-40, 1 mM sodium orthovanadate (Wako, Osaka, Japan), 1 mM EDTA, plus a protease inhibitor tablet (Amersham, Arlington Heights, IL). The supernatant fluids of the lysates were separated by 10% SDS–PAGE and then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were then blocked with 5% skimmed milk and 0.2% Tween 20 in Tris-buffered saline. Subsequently, the membranes were incubated with the anti-human PI-9 (mouse mAb, Alexis Biochemicals), cross-reactive to mouse SPI-6, or the anti-β-actin (mouse mAb, SIGMA), followed by HRP-conjugated rabbit anti-mouse Ig. The signal was detected using the ECL detection kit (Amersham Bioscience).

Generation of double transfectant ES-DC expressing OVA and overexpressing SPI-6. Mouse SPI-6 cDNA was prepared by RT-PCR amplification from total RNA of mouse spleen cells with PCR primers 5′-gagactcgagcccgccgccaccatgaatactctgtctgaaggaaat-3′ and 5′-gagagcggccgcgtgtctttatggagatgagaacct-3′. The design of these primers results in the cloning of SPI-6 cDNA downstream of the Kozak sequence. The PCR products were subcloned into a pGEM-T-Easy vector (Promega, Madison, WI), and the cDNA insert was then confirmed by a sequencing analysis. The cDNA fragments were cloned into pCAG-INeo, a mammalian expression vector driven by a CAG promoter and containing the internal ribosomal entry site (IRES)-neomycin resistance gene cassette [6], to generate pCAG-SPI-INeo (Fig. 4B). The transfection of ES cells and the induction of the differentiation of ES cells into DC were done as previously described [2].

Statistical analysis. Two-tailed Student's t test was used to determine any statistical significance in the differences in the lytic activity of the spleen cell preparations and tumor growth between treatment groups. A value of $p < 0.05$ was considered to be significant. The Kaplan–Meier plot for survivals was assessed for significance using the Breslow–Gehan–Wilcoxon test. Statistical analyses were made using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

Results

Priming of antigen-specific CTL by adoptive transfer of antigen-expressing ES-DC into syngeneic and semi-allogeneic recipients

We tested whether or not OVA-transfectant ES-DC (ES-DC-OVA) derived from TT2 ES cells (H-$2^{k/b}$), which originated from a CBA (H-2^k) × C57BL/6 (H-2^b) F1 embryo, could prime OVA-specific CTL upon adoptive transfer into semi-allogeneic C57BL/6 mice. Both the TT2-derived ES-DC and C57BL/6 mice possess the H-2^b haplotype, but MHC of the H-2^k haplotype expressed in ES-DC is allogeneic to the recipient C57BL/6 mice.

ES-DC-OVA or non-transfectant ES-DC (ES-DC-TT2) were injected i.p. into syngeneic (CBA × C57BL/6) F1 mice (H-$2^{k/b}$) or semi-allogeneic C57BL/6 mice twice with a 7-day interval. The spleen cells were isolated from the mice 7 days after the second injection and cultured in vitro in the presence of a K^b-binding $OVA_{257-264}$ peptide. After 5 days, the cultured spleen cells were recovered and assayed for their capacity to kill EL-4 thymoma cells (H-2^b) pre-pulsed with the OVA peptide. The results shown in Figs. 1A and B indicate that OVA-specific, H-2^b-restricted CTL was induced in both (CBA × C57BL/6) F1 and C57BL/6 mice injected with ES-DC-OVA but not in those injected with ES-DC-TT2. Although the results suggest that ES-DC-OVA primed OVA-specific CTL before they were killed by H-2^k-reactive T cells, it was also possible that the OVA protein released from ES-DC-OVA killed by allo-reactive CTL was taken up by endogenous APC and presented to prime OVA-specific CTL. To assess this possibility, we heat-killed ES-DC-OVA before injection into C57BL/6 mice. As shown in Fig. 1C, injection of heat-killed ES-DC-OVA did not result in priming of OVA-specific CTL. These results indicate that priming of OVA-specific CTL was mostly mediated by the direct presentation of OVA epitope by ES-DC-OVA, but not by cross-presentation by endogenous host APC. Thus, antigen-expressing ES-DC injected into semi-allogeneic mice can prime CTL specific to the antigen before they are killed by allo-reactive T cells. In addition, ES-DC-OVA primed OVA-specific CTL also in (BALB/c × C57BL/6) F1 mice (H-$2^{d/b}$) (Fig. 1D).

We next examined the priming of OVA-specific CTL by semi-allogeneic ES-DC under the condition by which the allo-reactive CTL that could attack the ES-DC had been pre-activated. We injected ES-DC-TT2 without an expression of OVA into C57BL/6 mice and 7 days later injected ES-DC-OVA. Under this condition, the first injected ES-DC-TT2 activated H-2^k-reactive CTL, and the ES-DC-OVA injected 7 days later would be attacked more rapidly by the once primed H-2^k-reactive CTL than in the former condition. The spleen cells were isolated 7 days after the second injection, and the CTL activity was analyzed by the same procedure as described above. As shown in Fig. 1E, a substantial priming of K^b-restricted OVA-specific CTL was also observed under this condition. This result indicates that, even in the presence of pre-activated allo-reactive CTL, antigen-expressing ES-DC is able to prime the antigen-specific CTL.

Induction of protective immunity against OVA-expressing tumor cells by ES-DC expressing OVA in semi-allogeneic recipients

We next asked whether CTL primed by ES-DC-OVA adoptively transferred into semi-allogeneic mice could protect the recipient mice from a subsequent challenge with tumor cells expressing OVA antigen. ES-DC-OVA were i.p. transferred into (CBA × C57BL/6) F1 mice or C57BL/6 mice twice with a 7-day interval and 7 days after the second transfer, the mice were inoculated s.c. with MO4, OVA-expressing B16 melanoma cells originating from a C57BL/6 mouse. As shown in Figs. 2A and B, the transfer of ES-DC-OVA into syngeneic (CBA × C57BL/6) F1 mice elicited a significant degree of protection against the challenge with MO4 in comparison to the transfer of ES-DC-TT2, and these findings were consistent with our previous report [6]. The transfer of ES-DC-OVA provided protection also in the semi-allogeneic C57BL/6 mice (Figs. 2C and D). These results suggest that the anti-cancer cellular vaccination with ES-DC genetically engineered to express tumor antigens is effective not only in syngeneic but also in semi-allogeneic recipients.

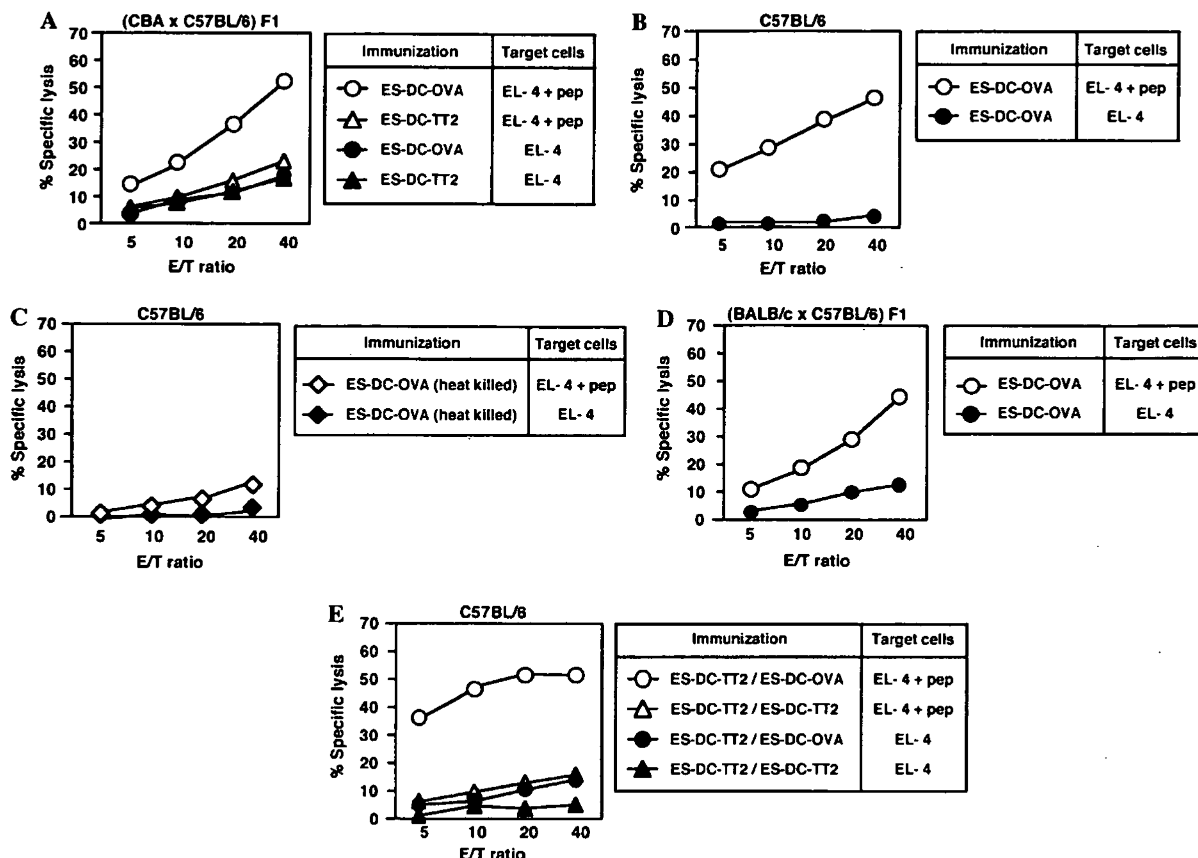

Fig. 1. Stimulation of OVA-specific CTL by ES-DC expressing OVA in syngeneic and semi-allogeneic mice. (CBA × C57BL/6) F1 (A), C57BL/6 (B, C), or (BALB/c × C57BL/6) F1 (D) mice were injected i.p. twice with ES-DC-OVA or ES-DC-TT2 (1×10^5/injection/mouse) on days −14 and −7. In (C), ES-DC-OVA were heat-killed before injection. C57BL/6 mice were injected with ES-DC-TT2 on day −14 and injected with ES-DC-OVA on day −7 (E). Spleen cells from the ES-DC-injected mice were harvested on day 0, pooled for each group (three mice per group), and cultured in the presence of $OVA_{257-264}$ peptide (0.1 μM) for 5 days. Next, the cells were harvested and assayed for their activity to kill EL-4 tumor cells either pulsed with 10 μM OVA peptide or left unpulsed. The results are expressed as the mean specific lysis of triplicate assays and SEM of triplicates were less than 2%.

Genetic modification of ES-DC to express antigen is superior to the loading of antigenic peptide to BM-DC in the priming of antigen-specific CTL in semi-allogeneic mice

The above described results were somewhat unexpected, considering the results of a previous study with peptide antigen-loaded BM-DC [4]. In that study, the presence of CTL in the recipient mice recognizing certain antigens presented by transferred DC severely diminished the priming of CTL specific to another antigen presented by the same DC. A possible reason for the substantial priming of antigen-specific CTL observed in our present experiments is that ES-DC is superior to BM-DC in priming antigen-specific CTL upon transfer to semi-allogeneic mice. Another possible reason is that, as a means for loading the antigen to DC to simulate CTL, the genetic modification of DC to produce antigenic protein is more efficient than the loading of antigenic peptide to DC.

To address the former possibility, we compared the efficiency of priming of OVA-specific CTL by the transfer of BM-DC and ES-DC. We isolated BM cells from (CBA × C57BL/6) F1 mice and generated BM-DC, which were genetically identical to ES-DC-TT2. BM-DC or ES-DC-TT2 were pre-pulsed with $OVA_{257-264}$ synthetic peptide (10 μM) for 2 h and injected i.p. into C57BL/6 mice. The injections were done twice with a 7-day interval, and 7 days after the second injection the spleen cells were isolated and the priming of OVA-specific CTL was analyzed by the method described above. The degree of priming of OVA-specific CTL by peptide-loaded BM-DC was very slight. $OVA_{257-264}$ peptide-loaded ES-DC-TT2 primed OVA-specific CTL more efficiently than the BM-DC did (Fig. 3A). These results indicate that ES-DC is superior to BM-DC in priming antigen-specific CTL upon loading with antigen and transfer to semi-allogeneic mice. However, the magnitude of priming of OVA-specific CTL by OVA peptide-loaded ES-DC-TT2 was lower than that primed

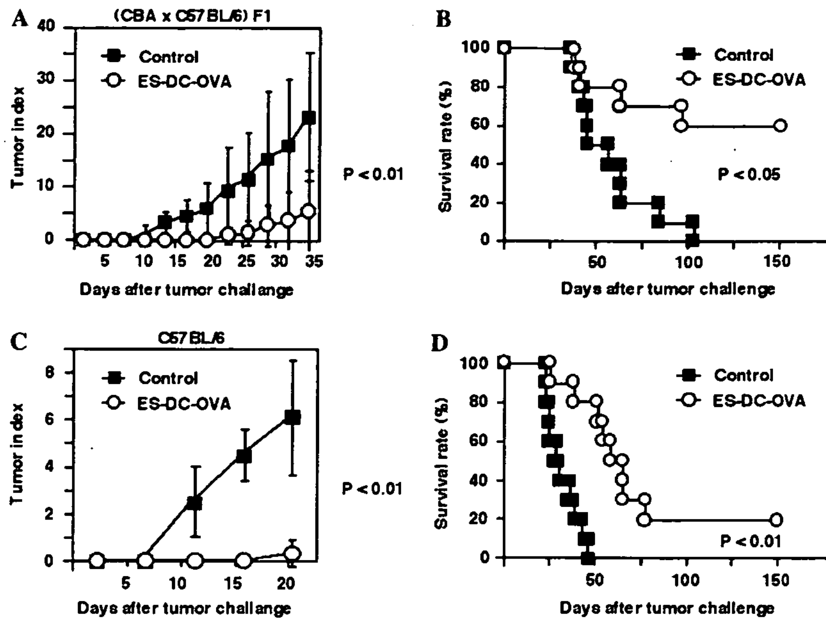

Fig. 2. Induction of protective immunity by ES-DC expressing OVA against OVA-expressing tumor cells in syngeneic and semi-allogeneic mice. (CBA × C57BL/6) F1 mice were injected i.p. twice on days −14 and −7 with ES-DC-OVA (2×10^4/injection/mouse) or medium (RPMI-1640) only as control and were challenged s.c. with MO4 tumor cells (3×10^5/mouse) on day 0 (A,B). C57BL/6 mice were injected with ES-DC-OVA (3×10^4/injection/mouse) and challenged with MO4 (2×10^5/mouse) by the same schedule (C,D). Growth of tumor (A,C) and survival of mice (B,D) were monitored. The tumor size was indicated as tumor index, square root of (length × width) in mm, ± SEM. The measurement of tumor sizes was stopped at the time point when one mouse of either of the mouse groups died (at day 35 in A and at day 20 in C). The differences in the tumor index and survival rate between ES-DC-OVA and control were significant ($P < 0.01$ in A, $P < 0.05$ in B, $P < 0.01$ in C, and $P < 0.01$ in D). For each experimental group, 10 mice were used.

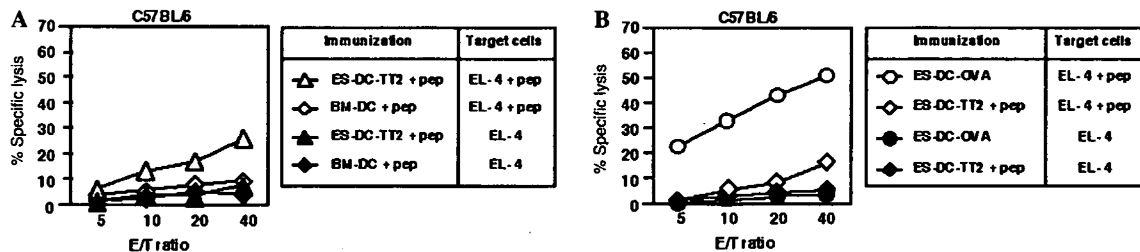

Fig. 3. Priming of OVA-specific CTL by OVA peptide-loaded DC or OVA-expressing ES-DC in semi-allogeneic mice. BM-DC or ES-DC-TT2 (non-transfectant ES-DC) were pulsed with $OVA_{257-264}$ synthetic peptide (10 μM) for 2 h and injected i.p. into C57BL/6 mice (1×10^5/injection/mouse) (A). ES-DC-OVA or OVA peptide-pulsed ES-DC-TT2 were injected i.p. into C57BL/6 mice (1×10^5/injection/mouse) (B). Injections were done twice on days −14 and −7. Spleen cells from the mice were harvested on day 0, and cytotoxic activity of OVA-specific CTL was analyzed as in Fig. 1.

by ES-DC-OVA, expressing transgene-derived OVA (Fig. 3B). The latter possibility mentioned above may thus also be true in that the genetic modification of DC to express antigen is superior to loading the antigenic peptide to DC in the priming of antigen-specific CTL.

Enhanced priming of antigen-specific CTL by ES-DC overexpressing SPI-6

As shown in Fig. 3A, ES-DC was superior to BM-DC in priming CTL in semi-allogeneic recipient mice. One possible reason for this was that ES-DC might be relatively resistant to attack by CTL and can survive for a longer period of time after transfer and thus primed OVA-specific CTL more efficiently than BM-DC did.

SPI-6 is a specific inhibitor of granzyme B, the major mediator of cytotoxic activity of CTL, and has been presumed to make DC resistant to attack by CTL during stimulation of CTL. As shown in Fig. 4A, SPI-6 was scarcely detected in BM-DC. On the other hand, ES-DC showed an evident expression of SPI-6. Thus, the substantial priming of OVA-specific CTL by ES-DC-OVA in semi-allogeneic mice may be attributed, at least in part, to the higher expression level of SPI-6. To verify the hypothesis that SPI-6 protected ES-DC from the cytotoxicity of allo-reactive CTL and resultingly enabled

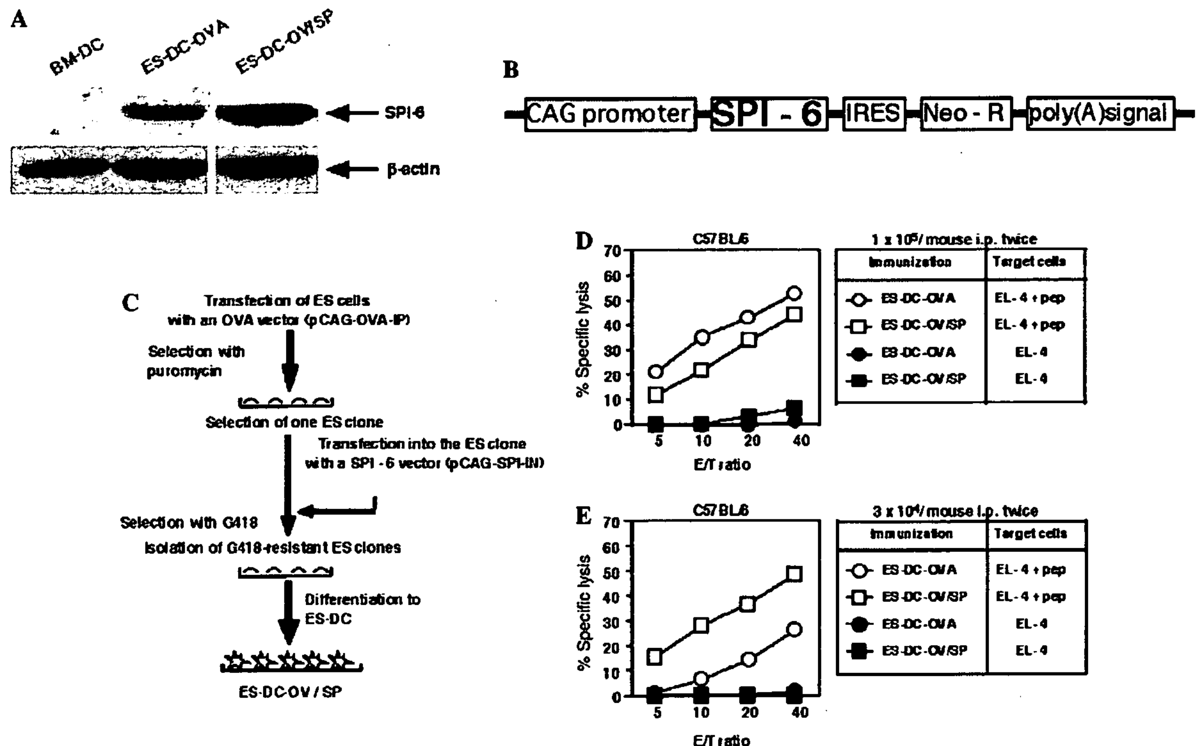

Fig. 4. Expression of SPI-6 in DC and priming of antigen-specific CTL by ES-DC expressing OVA and SPI-6 in semi-allogeneic mice. (A) The levels of expression of SPI-6 in BM-DC, ES-DC-OVA, and ES-DC-OV/SP were analyzed by a Western blotting analysis. The same samples were analyzed also for β-actin expression as control. (B) Structure of SPI-6 expression vector, pCAG-SPI-IN. (C) Schematic depiction of the generation of double transfectant ES-DC expressing OVA and overexpressing SPI-6. (D,E) ES-DC-OVA or ES-DC-OV/SP were injected i.p. to C57BL/6 mice (1×10^5/injection/mouse in D and 3×10^4/injection/mouse in E). Injections were done twice on days −14 and −7. Spleen cells were harvested from the mice on day 0, and activity of OVA-specific CTL was analyzed as shown in Fig. 1.

ES-DC to prime OVA-specific CTL more efficiently, we decided to generate double transfectant ES-DC expressing OVA and simultaneously overexpressing SPI-6. We made an expression vector for SPI-6 and introduced it into the OVA-gene transfectant ES cells (Fig. 4B). We then subjected the double transfectant ES cells to an induction culture for ES-DC, thereby generating ES-DC-OV/SP (Fig. 4C). As shown in Fig. 4A, ES-DC-OV/SP expressed a higher level of SPI-6 than ES-DC-OVA did. We compared ES-DC-OVA and ES-DC-OV/SP in their capacity to prime OVA-specific CTL in semi-allogeneic mice. The two clones of transfectant ES-DC were injected i.p. into C57BL/6 mice twice and the priming of OVA-specific CTL was analyzed. As shown in Fig. 4D, when 1×10^5 ES-DC were used for one injection, the degree of CTL-priming by ES-DC-OV/SP was similar to or somewhat lower than that primed by ES-DC-OVA. On the other hand, when lower number of ES-DC (3×10^4) were injected, ES-DC-OV/SP primed OVA-specific CTL more efficiently than ES-DC-OVA did (Fig. 4E). It is presumed that, when the lower number of ES-DC were transferred, the survival period of the injected ES-DC influenced more greatly the efficiency of priming OVA-specific CTL. Thus, the data shown in Figs. 4D and E suggest that an overexpression of SPI-6 in ES-DC improved the efficiency of priming OVA-specific CTL, and the effect was evident when a lower number of ES-DC were transferred for immunization.

Discussion

In recent years, a number of tumor-associated antigens have been identified, by the aid of genetic approaches such as expression cloning with tumor-reactive CTL, serological analysis of recombinant cDNA expression libraries (SEREX), or cDNA microarray analysis [9–13]. These antigens are potentially good targets for anti-cancer immunotherapies. To establish truly effective anti-cancer immunotherapy, development of a means for potently polarizing the immune system toward these tumor-associated antigens is essential. Anti-tumor immunotherapy with DC loaded with HLA-binding peptides derived from tumor antigens has been clinically tested in many institutions [14]. In most cases, DC are generated by the culture of monocytes obtained from peripheral blood of the patients. Apheresis, a procedure which is sometimes invasive for patients with cancer, is necessary to obtain a sufficient

number of monocytes as a source for DC. In addition, the culture to generate DC should be done separately for each patient and for each treatment, and thus the presently used method is labor-intensive and also expensive.

As a means for loading of tumor antigen to DC, genetic modification of DC to express antigenic protein has several advantages in comparison to the loading of peptide antigen to DC. The expression of genes encoding for tumor-specific antigens circumvents the need for identification of specific CTL epitopes within the protein. The expression of tumor antigens within DC provides a continuous and renewable supply of antigens for presentation, as opposed to a single pulse of peptides or tumor cell lysates. In most cases, adenovirus vector is used for the genetic modification of human monocyte-derived DC. However, there are several problems related to the use of adenovirus vectors, i.e., the efficiency of gene transfer, the stability of gene expression, and the potential risk accompanying the use of virus vectors. In addition, use of virus-based vectors outside of isolated laboratories is prohibited by law in many countries.

As we showed in both our previous and the present report, we can use ES cell transfectants as an infinite source of genetically modified DC. If the ES cell-based method can be clinically applied, then the repeated isolation of monocytes from patients is not necessary. In addition, we will be able to generate genetically engineered DC without the use of virus vectors, because the genetic modification of ES cells can be done with the introduction of plasmid DNA by electroporation. Furthermore, it is feasible to generate multiple gene-transfectant ES-DC with enhanced capacity to elicit anti-tumor immunity, by the sequential transfection with multiple expression vectors as demonstrated in our present and previous reports [6,15].

Considering clinical application, one drawback of the ES-DC method is the unavailability of human ES cells genetically identical to the patients to be treated. Based on previous studies, the stimulation of antigen-specific CTL by antigen-bearing, semi-allogeneic APC is considered to be difficult. The efficiency of priming antigen-specific CTL by adoptively transferred BM-DC presenting the antigen significantly decreased if the DC were targets of a pre-existing CTL [4]. APC transferred to MHC-incompatible mice were rapidly eliminated by allo-reactive CTL of the recipient mice [3]. However, the results of the present study revealed that adoptively transferred mouse ES-DC expressing OVA stimulated OVA-specific CTL not only in syngeneic (CBA × C57BL/6) F1 mice but also in semi-allogeneic C57BL/6 and (BALB/c × C57BL/6) F1 mice (Fig. 1). The OVA-specific CTL, and probably also the OVA-specific helper T cells, were primed by OVA-expressing ES-DC and protected the recipient C57BL/6 mice from subsequent challenge with tumor cells bearing OVA (Fig. 2). These results thus show the promise of prevention of cancer with ES-DC.

As shown in Fig. 4A, upon loading with $OVA_{257-264}$ peptide and transfer into semi-allogeneic C57BL/6 mice, ES-DC primed $OVA_{257-264}$-specific CTL more potently than BM-DC did, thus suggesting that ES-DC was superior to BM-DC in priming antigen-specific CTL in semi-allogeneic conditions. ES-DC-OVA, ES-DC genetically engineered to express OVA, was further more potent than OVA peptide-loaded ES-DC in the priming of OVA-specific CTL (Fig. 4B). Thus, the substantial priming of antigen-specific CTL by ES-DC-OVA in semi-allogeneic mice may be partly due to the efficient CTL-priming capacity of ES-DC and also due to the method of loading of antigen, namely genetic modification.

The level of expression of SPI-6 in ES-DC was higher than that in BM-DC (Fig. 4). SPI-6, the mouse homologue of human protease inhibitor 9 (PI-9), is a specific inhibitor of granzyme B, the major mediator of cytotoxicity of CTL [16–18]. SPI-6 is expressed in CTL, DC, and mast cells and it has been hypothesized to protect these cells from granzyme B-mediated apoptosis during immune responses [19–22]. It has recently been reported that the co-administration of expression vector for SPI-6 with a DNA vaccine for tumor antigen enhanced the vaccination potency, possibly because the expression of the vector-derived SPI-6 made antigen-presenting DC resistant to cytotoxic activity of CTL [23]. Thus, an evident intrinsic expression of SPI-6 in ES-DC may be one reason for that the capacity of OVA-expressing ES-DC to stimulate CTL in semi-allogeneic recipient mice was more potent than that of BM-DC. To address this possibility, we introduced OVA-transfectant ES cells with an expression vector for SPI-6, and thus generated double transfectant ES-DC expressing OVA and overexpressing SPI-6, ES-DC-OV/SP. ES-DC-OV/SP primed OVA-specific CTL more efficiently than ES-DC-OVA did when lower number (3×10^4/injection) of cells was transferred for immunization. It is thus suggested that the overexpression of SPI-6 by genetic modification of ES-DC prolonged their survival after transfer to semi-allogeneic mice and enhanced the priming of OVA-specific CTL (Fig. 4C).

Bcl-2 and Bcl-xL are anti-apoptotic proteins which block the apoptosis induced by various apoptotic signals, and they are reported to be involved in the control of the lifespan of DC [24–26]. We also examined the level of expression of Bcl-2 and Bcl-xL in BM-DC and ES-DC. Both ES-DC and BM-DC expressed Bcl-2 and Bcl-xL, and ES-DC expressed lower level of Bcl-2 and higher level of Bcl-xL than BM-DC did (data not shown). It is possible that an overexpression of Bcl-2 or Bcl-xL by genetic modification of ES-DC may also have an ability to enhance the efficiency to prime antigen-specific CTL in vivo.

For the efficient induction of cytotoxic effector function of $CD8^+$ T cells, $CD4^+$ helper T cells are known to play a crucial role. They produce cytokines such as IL-2 and IFN-γ, which directly stimulate CTL, and make DC more potent in activation of T cells, via CD40–CD40-ligand interactions. After the adoptive transfer of semi-allogeneic ES-DC presenting tumor antigen, a large number of allo-reactive $CD4^+$ T cells of the recipients may be activated by MHC class II molecules expressed on ES-DC and provide potent help for priming of antigen-reactive CTL. Therefore, while the expression of allogeneic MHC class I by transferred ES-DC may reduce the efficiency of the induction of anti-tumor immunity, allogeneic MHC class II expressed by ES-DC may confer considerable advantages for induction of anti-tumor immunity.

In order to realize the future clinical application of ES-DC, we recently established a method to generate ES-DC from non-human primate, cynomolgus monkey, ES cells, and also for genetic modification of them (manuscript in preparation). We believe that this method should be applicable to human ES cells, although some modifications might be necessary. It is expected that human ES cells sharing some of the HLA alleles with patients are available in most cases. Although HLA genes are highly polymorphic, a few prevalent alleles exist in each locus of HLA gene for each ethnic group in general. For example, the gene frequency of HLA-A*0201, A*0206, A*2402, and A*2601 in Japanese population is 0.11, 0.10, 0.36, and 0.10, respectively [27]. This indicates that more than 90% of the Japanese people possess at least one of these four alleles in the HLA-A locus. So far, a number of human ES cells have been established, and most of the human ES cells probably have HLA alleles dominant in the ethnic group to which the donors belong. We can thus expect that human ES cell lines sharing some of the HLA alleles with patients to be treated will be available in most cases.

In the future, antigen-specific anti-tumor immunotherapy by the in vivo transfer of human ES-DC expressing tumor antigen may well be achieved. The overexpression of PI-9, the human homologue of mouse SPI-6, by genetic modification is a promising way to enhance the effect of the cellular vaccination using human ES-DC semi-allogeneic to the recipients. We believe that the present study paves the way for the future clinical application of anti-cancer immunotherapy utilizing ES-DC.

Acknowledgments

Grant support: Grants-in-Aid 12213111, 14370115, 14570421, and 14657082 from the Ministry of Education, Science, Technology, Sports, and Culture, Japan and a Research Grant for Intractable Diseases from Ministry of Health, Labour and Welfare, Japan, and grants from the Tokyo Biochemical Research Foundation and the Uehara Memorial Foundation are acknowledged.

References

[1] J.M. Kaplan, Q. Yu, S.T. Piraino, S.E. Pennington, S. Shankara, L.A. Woodworth, B.L. Roberts, Induction of antitumor immunity with dendritic cells transduced with adenovirus vector-encoding endogenous tumor-associated antigens, J. Immunol. 163 (1999) 699–707.

[2] S. Senju, S. Hirata, H. Matsuyoshi, M. Masuda, Y. Uemura, K. Araki, K. Yamamura, Y. Nishimura, Generation and genetic modification of dendritic cells derived from mouse embryonic stem cells, Blood 101 (2003) 3501–3508.

[3] V. Loyer, P. Fontaine, S. Pion, F. Hetu, D.C. Roy, C. Perreault, The in vivo fate of APCs displaying minor H antigen and/or MHC differences is regulated by CTLs specific for immunodominant class I-associated epitopes, J. Immunol. 163 (1999) 6462–6467.

[4] I.F. Hermans, D.S. Ritchie, J. Yang, J.M. Roberts, F. Ronchese, $CD8^+$ T cell-dependent elimination of dendritic cells in vivo limits the induction of antitumor immunity, J. Immunol. 164 (2000) 3095–3101.

[5] S. Senju, K. Iyama, H. Kudo, S. Aizawa, Y. Nishimura, Immunocytochemical analyses and targeted gene disruption of GTPBP1, Mol. Cell. Biol. 20 (2000) 6195–6200.

[6] H. Matsuyoshi, S. Senju, S. Hirata, Y. Yoshitake, Y. Uemura, Y. Nishimura, Enhanced priming of antigen-specific CTLs in vivo by embryonic stem cell-derived dendritic cells expressing chemokine along with antigenic protein: application to antitumor vaccination, J. Immunol. 172 (2004) 776–786.

[7] L.D. Falo Jr., M. Kovacsovics-Bankowski, K. Thompson, K.L. Rock, Targeting antigen into the phagocytic pathway in vivo induces protective tumour immunity, Nat. Med. 1 (1995) 649–653.

[8] M.B. Lutz, N. Kukutsch, A.L. Ogilvie, S. Rossner, F. Koch, N. Romani, G. Schuler, An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow, J. Immunol. Methods 223 (1999) 77–92.

[9] Y.T. Chen, M.J. Scanlan, U. Sahin, O. Tureci, A.O. Gure, S. Tsang, B. Williamson, E. Stockert, M. Pfreundschuh, L.J. Old, A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening, Proc. Natl. Acad. Sci. USA 94 (1997) 1914–1918.

[10] T. Boon, P.G. Coulie, B. Van den Eynde, Tumor antigens recognized by T cells, Immunol. Today 18 (1997) 267–268.

[11] S.A. Rosenberg, A new era for cancer immunotherapy based on the genes that encode cancer antigens, Immunity 10 (1999) 281–287.

[12] T. Nakatsura, T. Kageshita, S. Ito, K. Wakamatsu, M. Monji, Y. Ikuta, S. Senju, T. Ono, Y. Nishimura, Identification of glypican-3 as a novel tumor marker for melanoma, Clin. Cancer Res. 10 (2004) 6612–6621.

[13] Y. Yoshitake, T. Nakatsura, M. Monji, S. Senju, H. Matsuyoshi, H. Tsukamoto, S. Hosaka, H. Komori, D. Fukuma, Y. Ikuta, T. Katagiri, Y. Furukawa, H. Ito, M. Shinohara, Y. Nakamura, Y. Nishimura, Proliferation potential-related protein, an ideal esophageal cancer antigen for immunotherapy, identified using complementary DNA microarray analysis, Clin. Cancer Res. 10 (2004) 6437–6448.

[14] V. Cerundolo, I.F. Hermans, M. Salio, Dendritic cells: a journey from laboratory to clinic, Nat. Immunol. 5 (2004) 7–10.

[15] S. Hirata, S. Senju, H. Matsuyoshi, D. Fukuma, Y. Uemura, Y. Nishimura, Prevention of experimental autoimmune encephalomyelitis by transfer of embryonic stem cell-derived dendritic cells expressing myelin oligodendrocyte glycoprotein peptide along with TRAIL or programmed death-1 ligand, J. Immunol. 174 (2005) 1888–1897.

[16] C.H. Bird, V.R. Sutton, J. Sun, C.E. Hirst, A. Novak, S. Kumar, J.A. Trapani, P.I. Bird, Selective regulation of apoptosis: the cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway, Mol. Cell. Biol. 18 (1998) 6387–6398.

[17] J. Sun, L. Ooms, C.H. Bird, V.R. Sutton, J.A. Trapani, P.I. Bird, A new family of 10 murine ovalbumin serpins includes two homologs of proteinase inhibitor 8 and two homologs of the granzyme B inhibitor (proteinase inhibitor 9), J. Biol. Chem. 272 (1997) 15434–15441.

[18] J.P. Medema, J. de Jong, L.T. Peltenburg, E.M. Verdegaal, A. Gorter, S.A. Bres, K.L. Franken, M. Hahne, J.P. Albar, C.J. Melief, R. Offringa, Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors, Proc. Natl. Acad. Sci. USA 98 (2001) 11515–11520.

[19] B.A. Bladergroen, M.C. Strik, A.M. Wolbink, D. Wouters, R. Broekhuizen, J.A. Kummer, C.E. Hack, The granzyme B inhibitor proteinase inhibitor 9 (PI9) is expressed by human mast cells, Eur. J. Immunol. 35 (2005) 1175–1183.

[20] J.P. Medema, D.H. Schuurhuis, D. Rea, J. van Tongeren, J. de Jong, S.A. Bres, S. Laban, R.E. Toes, M. Toebes, T.N. Schumacher, B.A. Bladergroen, F. Ossendorp, J.A. Kummer, C.J. Melief, R. Offringa, Expression of the serpin serine protease inhibitor 6 protects dendritic cells from cytotoxic T lymphocyte-induced apoptosis: differential modulation by T helper type 1 and type 2 cells, J. Exp. Med. 194 (2001) 657–667.

[21] B.A. Bladergroen, M.C. Strik, N. Bovenschen, O. van Berkum, G.L. Scheffer, C.J. Meijer, C.E. Hack, J.A. Kummer, The granzyme B inhibitor, protease inhibitor 9, is mainly expressed by dendritic cells and at immune-privileged sites, J. Immunol. 166 (2001) 3218–3225.

[22] C.E. Hirst, M.S. Buzza, C.H. Bird, H.S. Warren, P.U. Cameron, M. Zhang, P.G. Ashton-Rickardt, P.I. Bird, The intracellular granzyme B inhibitor, proteinase inhibitor 9, is up-regulated during accessory cell maturation and effector cell degranulation, and its overexpression enhances CTL potency, J. Immunol. 170 (2003) 805–815.

[23] T.W. Kim, C.F. Hung, D.A. Boyd, L. He, C.T. Lin, D. Kaiserman, P.I. Bird, T.C. Wu, Enhancement of DNA vaccine potency by coadministration of a tumor antigen gene and DNA encoding serine protease inhibitor-6, Cancer Res. 64 (2004) 400–405.

[24] A. Nopora, T. Brocker, Bcl-2 controls dendritic cell longevity in vivo, J. Immunol. 169 (2005) 3006–3014.

[25] W.S. Hou, L. Van Parijs, A Bcl-2-dependent molecular timer regulates the lifespan and immunogenicity of dendritic cells, Nat. Immunol. 5 (2004) 583–589.

[26] H. Hon, E.B. Rucker 3rd., L. Hennighausen, J. Jacob, bcl-xL is critical for dendritic cell survival in vivo, J. Immunol. 173 (2004) 4425–4432.

[27] H. Tanaka, T. Akaza, T. Juji, Report of the Japanese central bone marrow data center, Clin. Transpl. 1 (1996) 139–144.

Therapeutic effect of α-galactosylceramide-loaded dendritic cells genetically engineered to express SLC/CCL21 along with tumor antigen against peritoneally disseminated tumor cells

Hidetake Matsuyoshi,[1,2] Shinya Hirata,[1] Yoshihiro Yoshitake,[1] Yutaka Motomura,[1] Daiki Fukuma,[1] Akari Kurisaki,[1] Tetsuya Nakatsura,[1] Yasuharu Nishimura[1] and Satoru Senju[1,3]

[1]Department of Immunogenetics and [2]Department of Otolaryngology-Head and Neck Surgery, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan

(Received August 5, 2005/Revised September 23, 2005/Accepted September 29, 2005/Online publication December 8, 2005)

The close cooperation of both innate and acquired immunity is essential for the induction of truly effective antitumor immunity. We tested a strategy to enhance the cross-talk between NKT cells and conventional antigen-specific T cells with the use of αGalCer-loaded dendritic cells genetically engineered to express antigen plus chemokine, attracting both conventional T cells and NKT cells. DC genetically engineered to express a model antigen, OVA, along with SLC/CCL21 or monokine induced by IFN-γ/CXCL9, had been generated using a method based on *in vitro* differentiation of DC from mouse ES cells. The ES-DC were loaded with α-GalCer and transferred to mice bearing MO4, an OVA-expressing melanoma, and their capacity to evoke antitumor immunity was evaluated. *In vivo* transfer of either OVA-expressing ES-DC, stimulating OVA-reactive T cells, or α-GalCer-loaded non-transfectant ES-DC, stimulating NKT cells, elicited a significant but limited degree of protection against the i.p. disseminated MO4. A more potent antitumor effect was observed when α-GalCer was loaded to ES-DC expressing OVA before *in vivo* transfer, and the effect was abrogated by the administration of anti-CD8, anti-NK1.1 or anti-asialo GM1 antibody. α-GalCer-loaded double transfectant ES-DC expressing SLC along with OVA induced the most potent antitumor immunity. Thus, α-GalCer-loaded ES-DC expressing tumor-associated antigen along with SLC can stimulate multiple subsets of effector cells to induce a potent therapeutic effect against peritoneally disseminated tumor cells. The present study suggests a novel way to use α-GalCer in immunotherapy for peritoneally disseminated cancer. (*Cancer Sci* 2005; 96: 889–896)

A means to induce the close cooperation of both innate and acquired immunity would be necessary for the induction of efficient antitumor therapy. Recent studies have shown DC to be potent stimulators of both innate and acquired immunity. The *in vivo* transfer of DC presenting tumor-associated antigens has proven to be efficient in the priming of CTL specific to the antigens. α-GalCer presented by DC efficiently stimulates NKT cells,[1–4] a subset of T cells implicated in the innate immunity against infection and cancer.[5–7] In addition, NKT cells stimulated by the *in vivo* administration of α-GalCer secondarily stimulate conventional T cells.[8,9] It is thus presumed that the *in vivo* transfer of DC simultaneously loaded with tumor-associated antigens and α-GalCer may stimulate both tumor-reactive T cells and NKT cells, thus resulting in a potent antitumor immunity.

Chemokines mediate leukocyte adhesion and homing, and the concordant migration of specific leukocyte subsets induced by chemokines is pivotal for the development of proper immune responses. SLC/CCL21 attracts both T cells and DC to lymphoid tissues through its receptor CCR7, and the effect of SLC is essential for the priming of naive T cells in the initiation phase of the immune response. CXCR3 and its ligands, Mig/CXCL9 and IP-10/CXCL10, mediate the migration of effector/memory T cells and NK cells to the site of inflammation. In addition, a recent study revealed that these chemokines and their receptors also mediate the migration of some subpopulations of NKT cells.[10–12]

As a means for loading the tumor-associated antigens to DC, genetic modification to express antigenic proteins has several advantages. The expression of tumor antigens by DC circumvents the need for identifying specific CTL epitopes within the protein, and by that the antigens are continuously supplied for presentation as opposed to a single pulse of peptides or tumor cell lysates.[13] For the efficient gene transfer to DC, the use of virus-based vectors is required because DC are not easy to genetically modify. Considering the clinical application, however, there are several problems related to the use of virus vectors. These include the inefficiency of gene transfer, the instability of gene expression, and the potential risk accompanying the use of virus vectors. In addition, in many countries, legal restrictions have been placed on the use of virus vectors outside of carefully isolated laboratories.

We and others have established methods to generate dendritic cells *in vitro* from mouse ES cells.[14,15] ES-DC have the

---

[3]To whom correspondence should be addressed.
E-mail: senjusat@gpo.kumamoto-u.ac.jp

Abbreviations: Ab, antibody; BM-DC, bone marrow cell-derived dendritic cell; CBF1, (CBA × C57BL/6) F1; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; ES cell, embryonic stem cell; ES-DC, ES cell-derived dendritic cell; α-GalCer, α-galactosylceramide; GM-CSF, granulocyte macrophage colony-stimulating factor; HLA, human histocompatibility leukocyte antigen; IFN-γ, interferon-gamma; IL, interleukin; i.p., intraperitoneally; IP-10, IFN-γ-inducible 10 kDa protein; mAb, monoclonal antibody; Mig, monokine induced by IFN-γ; NK, natural killer; OVA, ovalbumin; s.c., subcutaneously; SLC, secondary lymphoid tissue chemokine.

doi: 10.1111/j.1349-7006.2005.00123.x
© 2005 Japanese Cancer Association

capacity to stimulate T cells, present antigen and migrate to lymphoid tissues upon *in vivo* administration, and these capacities of ES-DC are comparable to those of BM-DC. The genetic modification of ES-DC can be carried out without the use of virus vectors by introducing exogenous genes by electroporation into undifferentiated ES cells and the subsequent induction of their differentiation into ES-DC. We can generate multiple gene-transfectant ES-DC by the sequential transfection of ES cells with vectors bearing different selection markers.[16,17] In a previous study, we generated double-transfectant ES-DC expressing SLC or Mig along with a model tumor antigen.[16] Using these double-transfectant ES-DC, we demonstrated that the coexpression of SLC or Mig enhanced the capacity of *in vivo*-transferred ES-DC to activate antigen-specific CTL and to protect the recipient mice from a tumor cell challenge.

In the present study, we evaluated the capacity of α-GalCer-loaded ES-DC to stimulate NKT cells both *in vitro* and *in vivo*, in comparison to that of BM-DC. We next evaluated the antitumor effect of simultaneous stimulation of NKT cells and antigen-specific conventional T cells by the *in vivo* administration of α-GalCer-loaded ES-DC expressing a model tumor antigen, namely OVA. Furthermore, we addressed whether coexpression of SLC or Mig with the antigen by ES-DC could enhance the synergic antitumor effect of NKT cells and conventional T cells.

Materials and Methods

Mice

CBA and C57BL/6 mice were obtained from Clea Animal Co. (Tokyo, Japan) or Charles River (Hamamatsu, Japan) and kept under specific pathogen-free conditions. Male CBA and female C57BL/6 mice were mated to produce CBF1 mice and all *in vivo* experiments were carried out using the CBF1 mice. The animal experiments in this study were approved by the animal experiment committee of Kumamoto University (permission number A16-074).

Reagents

Recombinant mouse GM-CSF was purchased from PeproTech EC (London, UK) and α-GalCer was kindly provided by the Kirin Brewery Co. (Tokyo, Japan). Mouse IL-4 and IFN-γ ELISA kits were purchased from eBioscience (San Diego, CA, USA). Polyclonal rabbit anti-asialo GM1 Ab was purchased from Wako Chemicals (Tokyo, Japan).

Cell lines and preparation of DC

The ES cell line TT2, derived from CBF1 blastocysts,[18] was maintained as described previously.[19] MO4[20] was generated by the transfection of C57BL/6-derived melanoma B16 with the pAc-neo-OVA plasmid. The procedure for inducing the differentiation of ES cells into DC has been reproted previously.[15] ES-DC expressing OVA (ES-DC-OVA) and ES-DC expressing chemokine, SLC or Mig, along with OVA (ES-DC-OVA/SLC or ES-DC-OVA/Mig) were generated as reported previously.[16] ES-DC recovered after a 14-day culture in bacteriological Petri dishes were used for both *in vivo* and *in vitro* assays. To generate BM-DC, bone marrow cells were isolated from CBF1 mice and cultured in RPMI + 10% fetal calf serum + GM-CSF (5 ng/mL) for 7 days, according to the method reported by Lutz *et al.*[21]

Analysis of the activation of NKT cells by DC loaded with α-GalCer

Embryonic stem cell-derived dendritic cells or BM-DC were cultured in the presence of α-GalCer (100 ng/mL) or vehicle (0.00025% Polysorbate-20) alone for 22 h, washed twice, and cocultured with splenic T cells of syngeneic CBF1 mice (5×10^4 DC + 2.5×10^6 T cells/well in 24-well culture plates). Splenic T cells were isolated using nylon-wool columns, as described previously.[16] After 24 h or 5 days, the cells were recovered and analyzed on their cytotoxic activity by a 4-h ^{51}Cr-release assay using YAC-1 cells (1×10^4 cells/well) as targets in 96-well round-bottomed culture plates at the effector : target ratio indicated. The amount of IL-4 and IFN-γ in the culture supernatant was measured by ELISA. In the analysis of the stimulation of NKT cells *in vivo*, ES-DC or BM-DC loaded with either α-GalCer (100 ng/mL) or vehicle alone, as described above, were injected i.p. into syngeneic CBF1 mice (1×10^6 cells/mouse). After 24 h, the mice were killed and the cytotoxic activity of whole spleen cells against YAC-1 cells was analyzed, as described above.

Tumor challenge experiments

Indicated numbers of MO4 cells were injected s.c. into the shaved left flank region, or i.p. on day 0. On day 3, 1×10^5 genetically modified ES-DC preloaded with either α-GalCer or vehicle alone were transferred i.p. into the mice. The survival rate of the mice was monitored and, in s.c. challenge experiments, the tumor sizes were also determined biweekly in a blinded fashion. The tumor index was calculated as:

Tumor index (mm) = square root of (length × width).

In vivo depletion experiments

The mice were challenged i.p. with 1×10^5 MO4 cells on day 0 and they were injected i.p. with 1×10^5 ES-DC-OVA preloaded with α-GalCer on day 3. To deplete the specific types of cells, the mice were given a total of 14 i.p. injections (days −4, −1, 2, 5, 10, 13, 15, 19, 26, 33, 40, 47, 54 and 61) of mAb, ascites (0.1 mL/mouse/injection) from hybridoma-bearing nude mice, or polyclonal rabbit anti-asialo GM1 Ab (50 μg/mouse/injection). The mAbs used were rat antimouse CD4 (clone GK1.5), rat antimouse CD8 (clone 2.43) and mouse anti-NK1.1 (clone PK-136). Normal rat IgG (Sigma, St Louis, MO, USA) was used as a control (200 μg/mouse/injection).

Statistical analysis

Two-tailed Student's *t*-test was used to determine the statistical significance of differences in cytolytic activity and the tumor growth between the treatment groups. A value of $P < 0.05$ was considered to be significant. The Kaplan–Meier plot for survival was used to determine any significant differences in tumor challenge experiments, using the Breslow–Gehan–Wilcoxon test. In some experiments, the difference in the survival rate between treatment groups was assessed for significance using the χ^2-test. Statistical analyses were made using the StatView 5.0 software (Abacus Concepts, Calabasas, CA, USA).

doi: 10.1111/j.1349-7006.2005.00123.x
© 2005 Japanese Cancer Association