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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

Shinya Hirata, Satoru Senju, Hidetake Matsuyoshi, Daiki Fukuma, Yasushi Uemura, and Yasuharu Nishimura

Experimental autoimmune encephalomyelitis (EAE) is caused by activation of myelin Ag-reactive CD4+ T cells. In the current study, we tested a strategy to prevent EAE by pretreatment of mice with genetically modified dendritic cells (DC) presenting myelin oligodendrocyte glycoprotein (MOG) peptide in the context of MHC class II molecules and simultaneously expressing TRAIL or Programmed Death-1 ligand (PD-L1). For genetic modification of DC, we used a recently established method to generate DC from mouse embryonic stem cells (ES cells) in vitro (ES-DC). ES cells were sequentially transfected with an expression vector for TRAIL or PD-L1 and an MHC class II-associated invariant chain-based MOG epitope-presenting vector. Subsequently, double-transfectant ES cell clones were induced to differentiate to ES-DC, which expressed the products of introduced genes. Treatment of mice with either of the double-transfectant ES-DC significantly reduced T cell response to MOG, cell infiltration into spinal cord, and the severity of MOG peptide-induced EAE. In contrast, treatment with ES-DC expressing MOG alone, irrelevant Ag (OVA) plus TRAIL, or OVA plus PD-L1, or coinjection with ES-DC expressing MOG plus ES-DC-expressing TRAIL or PD-L1 had no effect in reducing the disease severity. In contrast, immune response to irrelevant exogenous Ag (keyhole limpet hemocyanin) was not impaired by treatment with any of the genetically modified ES-DC. The double-transfectant ES-DC presenting Ag and simultaneously expressing immune-suppressive molecules may well prove to be an effective therapy for autoimmune diseases without inhibition of the immune response to irrelevant Ag. The Journal of Immunology, 2005, 174: 1888–1897.

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Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; DC, dendritic cell; Ii, invariant chain; CLIP, class II-associated Ii peptide; PD-L1, Programmed Death-1 ligand; ES cell, embryonic stem cell; ES-DC, ES cell-derived DC; PLP, myelin proteolipid protein; MBP, myelin basic protein; IRES, internal ribosomal entry site; PCC, pigeon cytochrome c; KLH, keyhole limpet hemocyanin.

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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 F1 (CBF1) mice, and all in vivo experiments were done using CBF1 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 (MEVGWYRSPFSRVVHLNYK), mouse myelin proteolipid protein (PLP) p109–209 (SKTASIGLCA/DARM YOVL), and mouse myelin basic protein (MBP) p35–47 (TGILDSI YGVL), and mouse myelin basic protein (MBP) p35–55 epitope, 5′-TTAGGGGATGGACCTTTGAC-3′ and 5′-AAATATGTTGCTAAAGGTT-3′, were synthesized using the F-nuc method on an automatic peptide synthesizer (PSSM8; Shimadzu) and purified using HPLC (23–25).

**Cytotoxicity assay and proliferation assay of T cells stimulated**

For detection of apoptosis of splenic CD4+ T cell, Annexin V/propidium iodide was used. For blocking experiments, anti-TRAIL (clone N2B2; rat IgG2a; BD Pharmingen), R-PE-conjugated hamster IgG control (clone G155-178; BD Pharrnigen), FITC-conjugated mouse IgG2a control (clone G155-178; BD Pharmingen), R-P-E-conjugated hamster IgG control (Immunotech), R-P-E-conjugated rat IgG2a control (clone LO-DNP-16; Caltag), biotinylated anti-mouse TRAIL (clone N2B2; rat IgG2a; eBioscience), anti-mouse anti-human CD74 (clone M-B741; mouse IgG2a; BD Pharmingen), and PE-conjugated streptavidin (Molecular Probes; Invitrogen Life Technologies). In some experiments, the DC fraction was gated by forward and side scatter. For detection of apoptosis of splenic CD4+ T cell, Annexin V/7-AAD apoptosis detection kits (BioVision) were used. In brief, splenic 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.

**RT-PCR to detect transgene products**

Total cellular RNA was extracted using a UV 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 calculated by primer set for GAPDH (forward primer 5′-CCGGTGATGGAAGTTGGTTGGTATCGTT-3′ and reverse primer 5′-GCTTGTAGGC3′) and 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′-ACCATGCCAACCTCTGTCTG-3′ and 5′-CGGGTTGTGTGGTGTGTGAGA-3′.

**Flow-cytometric analysis**

Staining of cells and analysis on a flow cytometer (FACScan; BD Biosciences) were done as described (2). Abs and reagent used for staining were as follows: anti-I-Ab (clone 3JP; mouse IgG2a), R-PE-conjugated anti-mouse CD11c (clone N148; hamster IgG; Chemicon), R-PE-conjugated anti-mouse CD68 (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-P-E-conjugated hamster IgG control (Immunotech), R-P-E-conjugated rat IgG2a control (clone LO-DNP-16; Caltag), biotinylated anti-mouse TRAIL (clone N2B2; rat IgG2a; eBioscience), anti-mouse anti-human CD74 (clone M-B741; mouse IgG2a; BD Pharmingen), and PE-conjugated streptavidin (Molecular Probes; Invitrogen Life Technologies). In some experiments, the DC fraction was gated by forward and side scatter. For detection of apoptosis of splenic CD4+ T cells, Annexin V/7-AAD apoptosis detection kits (BioVision) were used. In brief, splenic 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.

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

Standard 3Cr 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 CBF1 mice, and T cells were purified using nylon wool columns. X-ray-irradiated (35 Gy) ES-DC (2 × 10^7) and the T cells (1 × 10^7) were seeded into wells of 96-well flat-bottom culture plates precoated with anti-CD3 mAb (145-2C11; eBioscience) and cultured for 4 days. [3H]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 [3H]thymidine was measured using scintillation counting. For blocking experiments, anti-TRAIL (clone N2B2) or anti-PD-L1 (clone MH5) 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 normal immunized according to protocol for EAE induction described below using nylon wool columns. X-ray-irradiated (35 Gy) ES-DC as stimulator cells (2 × 10^6) were cocultured with the MOG-reactive T cells (1.5–2 × 10^6) in wells of 96-well culture plates for 3 days. Proliferation of T cells in

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 (50 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.
the last 12 h of the culture was quantified based on [\textsuperscript{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\textsubscript{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 \textmu g of MOG p35–55 peptide and 400 \textmu g 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 \times 10\textsuperscript{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 \times 10\textsuperscript{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-\mu m 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 diaminobenzidine.

**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 \textmu g of KLH protein (Sigma-Aldrich) emulsified in CFA. After indicated days, inguinal lymph node cells and spleen cells were isolated and cultured (5 \times 10\textsuperscript{5} cells/well) in the presence of MOG peptide (0, 8, 2.5, or 80 \textmu g/ml) or KLH (16, 50, or 160 \textmu g/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 \textsuperscript{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 \mu M 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-\gamma.

**Statistical analysis**

Two-tailed Student’s \textit{t} test was used to determine the statistical significance of differences. A value of \textit{p} < 0.05 was considered significant.

**Results**

**Induction of EAE in CBF\textsubscript{1} mice**

To date, we found no study in which EAE had been induced in CBF\textsubscript{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\textsubscript{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 \mu g of MOG p35–55 and 400 \mu g of *M. tuberculosis* toxin on days 0 and 2, EAE is reproducibly induced in CBF\textsubscript{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\textsubscript{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-Neo) or PD-L1 (pCAG-PDL1-Neo), as shown in Fig. 1A. 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-Ipuo (Fig. 1B). In this vector, a cDNA for human \textit{li} 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 \textit{li}
MOG
TRAIL
of transgene-derived mutant human mRNA for CAG-MOG. Designed to generate PCR product of 556 bp originating from transgene-tide at the CLIP region, are shown as in MOG-IPuro, the expression vector for mutant human Ii bearing MOG peptide (NeoR)-polyadenylation signal sequence (pA). TRAIL or PD-L1 are followed by the IRES-neomycin-resistance gene (Neo5)-polyadenylation signal sequence (pA). The structure of pCAG-MOG-IIPuro, the expression vector for mutant human Ii containing the MOG peptide, TRAIL, and PD-L1 in ES-DC was confirmed by RT-PCR (Fig. 1C) 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 and flow-cytometric analysis confirmed by RT-PCR (Fig. 1C).

**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 region. B. The structure of pCAG-MOG-IIPuro, 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 (li-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 transfected 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.

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. 3A, 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) nor ES-DC-TRAIL showed manifest killing activity against L929. In contrast, neither ES-DC (TT2) nor ES-DC-TRAIL showed manifest killing activity against L929.

**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. 4A, 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. 4B).

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 containing the MOG peptide, TRAIL, and PD-L1 in ES-DC was confirmed by RT-PCR (Fig. 1C) 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 transfected 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.
from the mice and cultured in the presence of MOG peptide. As shown in Fig. 4C, 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. 6A. As shown in Fig. 6B and Table II, EAE was almost
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. 6D and Table II, coinjection of ES-DC-TRAIL/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. 7A, mice were immunized according to the protocol for EAE induction and, after that, injected with ES-DC on days 5, 9, and 13 (1 × 10⁶ 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. 7B and Table II).

Decreased T cell response to MOG in mice treated with ES-DC-TRAIL/MOG or -PDL1/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. 6A), we isolated inguinal lymph node cells and analyzed their proliferative response upon restimulation in vitro with MOG peptide. As shown in Fig. 8A, 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

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 × 10⁶) isolated from inguinal lymph nodes of CBF1 mice immunized according to the protocol for EAE induction were cocultured with irradiated ES-DC-MOG, TRAIL/MOG, or PDL1/MOG (2 × 10⁶) for 3 days, as in Fig. 4A. 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.

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. CBF1 mice (three to five mice per group) were i.p. injected with ES-DC (1 × 10⁶ 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-PDL1/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.
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. 8B), 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 transfected 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-dioxygenase, 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.
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. 8A). 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, PD-L1/MOG, or untreated and subsequently immunized according to the protocol for EAE induction as shown in Fig. 6A. 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-PD-L1/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+, or Mac-1+ cells per 1 mm2 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.
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 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.

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References


