Doctor's Thesis

Prevention of experimental autoimmune encephalomyelitis by transfer of genetically modified ES cell-derived dendritic cells

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博士（医学）論文

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（遺伝子改変 ES 細胞由来の樹状細胞による
実験的自己免疫性脳脊髄炎の抑制）

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2005 年 3 月

1. Contents

2. Summary

Experimental autoimmune encephalomyelitis (EAE) is caused by activation of myelin antigen-reactive $CD4^+$ T cells. In the current study, I tested a strategy to prevent EAE by pre-treatment 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 TNF-related apoptosis-inducing ligand (TRAIL) or Programmed Death-1 ligand (PD-L1). For genetic modification of DC, I used a recently established method to generate DC from mouse 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 antigen (OVA) plus TRAIL, or OVA plus PD-L1, or co-injection with ES-DC expressing MOG plus ES-DC-expressing TRAIL or PD-L1 had no effect to reduce the disease severity. On the other hand, immune response to irrelevant exogenous antigen (KLH) was not impaired by treatment with any of the genetically modified ES-DC. Furthermore, the severity of myelin basic protein-induced EAE was also reduced by ES-DC-TRAIL/MOG but not by PDL1/MOG. The adoptive transfer of $CD4^+$ T cells from ES-DC-TRAIL/MOG-treated mice protected the recipient naïve mice from subsequent MOG-induced EAE. These results demonstrate the prevention of an autoimmune disease by treatment with ES-DC expressing target autoantigen along with immune-suppressive molecules, and implicate future clinical application of this technology.

3. Publication list

1. Hirata, S., Senju, S., Matsuyoshi, H., Fukuma, D., Uemura, Y. and Nishimura, Y. Prevention of experimental autoimmune encephalomyelitis by transfer of ES cell-derived dendritic cells expressing MOG peptide along with TRAIL or PD-L1. ***J. Immunol.*** 174: 1888-1897, 2005

2. Matsuyoshi, H., Senju, S., Hirata, S., Yoshitake, Y., Uemura, U., and Nishimura, Y. Enhanced priming of antigen-specific CTL in vivo by transfer of ES cell-derived dendritic cells expressing chemokine along with antigenic protein; application to anti-tumor vaccination. ***J. Immunol.*** 172: 776-86, 2004

3. Uemura, Y., S. Senju, K. Maenaka, L. K. Iwai, S. Fujii, H. Tabata, H. Tsukamoto, S.Hirata, Y. Z. Chen, and Y. Nishimura. 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: 947-960, 2003

4. Senju, S., S. Hirata, H. Matsuyoshi, M. Masuda, Y. Uemura, K. Araki, K Yamamura, and Y. Nishimura. Generation and genetic modification of dendritic cells derived from mouse embryonic stem cells. ***Blood*** 101:3501-3508, 2004

5. 平田真哉，千住覚，西村泰治「抗原提示細胞をターゲットとした免疫抑制法」，***Mebio***，メジカルビュー社(東京), 21(10): 33-41, 2004.

6. 平田真哉，千住覚，西村泰治「自己免疫疾患の細胞移入治療：樹状細胞療法の可能性」，**先端医療シリーズ アレルギー・リウマチ・膠原病の最新医療**，先端医療技術研究所(東京), 19: 228-233, 2003.

7. 平田真哉，西村泰治「HLA-DOによるHLA- DMの制御と抗原提示」，**臨床免疫**，　科学評論社（東京）, 39(3): 365-370, 2003

8. 平田真哉，西村泰治「疾患感受性遺伝子：HLA-DRB1遺伝子」，**日本臨牀2005年増刊：関節リウマチ**，日本臨牀社（大阪），印刷中

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5. Abbreviations

CBF1, (CBA x C57BL/6) F1

cDNA; complementary DNA

CLIP, class II-associated invariant chain peptide

DC, dendritic cell

EAE, Experimental autoimmune encephalomyelitis

ELISA; enzyme-linked immunosorbent assay

ES cell, embryonic stem cell

ES-DC, ES cells-derived dendritic cell

GM-CSF; granulocyte-macrophage colony stimulating factor

IFN; interferon

Ii, invariant chain

IL; interleukin

i.p.; intraperitoneal injection

IRES, internal ribosomal entry site

KLH, keyhole limpet hemocyanin

mAb; monoclonal antibody

MBP, myelin basic protein

MHC; major histocompatibility complex

MOG, myelin oligodendrocyte glycoprotein

mRNA; messenger ribonucleic acid

neo-R; neomycin resistant

OVA; ovalbumin

PBS; phosphate-buffered saline

PCC, pigeon cytochrome c

PD-L1, Programmed Death-1 ligand

PEF; primary embryonic fibroblast

PLP, myelin proteolipid protein

RT-PCR; reverse transcription-polymerase chain reaction

TCR; T cell receptor

TNF; tumor necrosis factor

TRAIL; TNF-related apoptosis inducing ligand

6. Introduction

6-1) Experimental autoimmune encephalomyelitis (EAE), as a model of organ-specific autoimmune disease

Autoimmune disease occurs when a specific adaptive immune response is mounted against self antigens. The normal consequence of an adaptive immune response against a foreign antigen is the clearance of the antigen from the body. Virus-infected cells, for example, are destroyed by cytotoxic T cells, whereas soluble antigens are cleared by formation of immune complexes of antibody with antigen, which are taken up by cells of the mononuclear phagocytic system such as macrophages. When an adaptive immune response develops against self antigens, however, it is usually impossible for immune effector mechanisms to eliminate the antigen completely, and so a sustained response occurs. The consequence is that the effector pathways of immunity cause chronic inflammatory injury to tissues, which may prove lethal.

The mechanisms of tissue damage in autoimmune diseases are essentially the same as those that operate in protective immunity and in hypersensitivity disease. Adaptive immune responses are initiated by the activation of antigen-specific T cells, and it is believed that autoimmunity is initiated in the same way. T-cell responses to self antigen can induce tissue damage either directly or indirectly. Cytotoxic T-cell responses and inappropriate activation of macrophages by Th1 cells can cause extensive tissue damage, whereas inappropriate T-cell help to self-reactive B cells can initiate harmful autoantibody responses. Autoimmune responses are a natural consequence of the open repertories of both B-cell and T-cell response, which allow them to recognize any pathogen. Although these repertories are excluded of most receptors that bind with high affinity to self antigens encountered during development, they still include receptors of lower affinity reactive to some self antigens. It is not known what triggers autoimmunity, but both environmental and genetic factors, especially MHC genotype, are clearly important. Transient autoimmune responses are common, but it is only when they are sustained and cause lasting tissue damage that they attract medical attention.

EAE, an animal model of human multiple sclerosis, is characterized by neurological impairment resulting from de-myelination in the central nervous system caused by myelin antigen-reactive $CD4^+$ T cells (Figure 1). This disease model is induced by immunization of animals with myelin antigens such as MOG, or myelin

basic protein (MBP) or myelin proteolipid protein (PLP). In addition, it is known that appropriate antigen used for EAE induction is different between the species and strains of animals.

Figure 1

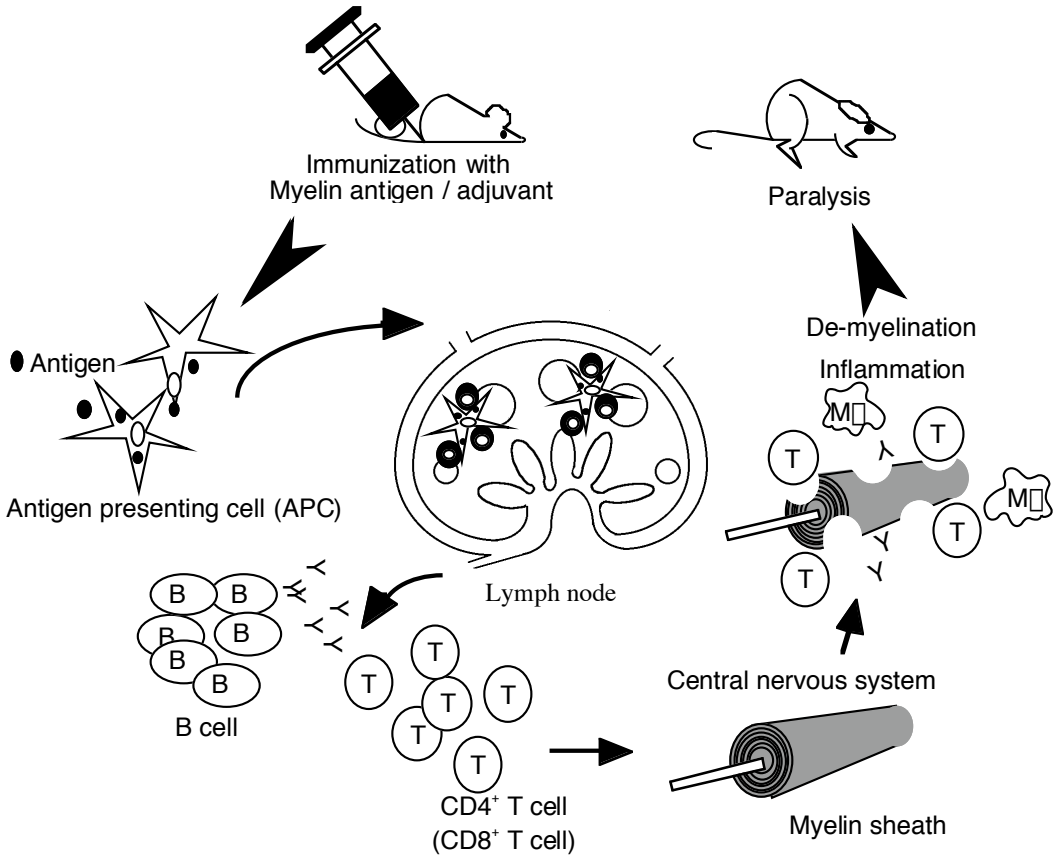

Figure 1. Experimental autoimmune encephalomyelitis (EAE)
This disease is produced in experimental animals by injecting them with isolated spinal cord (such as myelin antigen) homogenized in complete Freunds' adjuvant. EAE is due to an inflammatory reaction in the central nervous system that causes a progressive paralysis affecting first the tail and hind limbs before progressing to forelimb paralysis and eventual death. Plaques active disease show infiltration of nervous tissue by lymphocytes, plasma cells, and macrophages, which cause destruction of the myelin sheaths that surround nerve cell axons in the brain and spinal cord.

6-2) Dendritic cell

DCs are antigen-presenting cells with the unique capacity to take up and process antigens in the peripheral blood and tissues. They subsequently migrate to draining lymph nodes, where they present antigen to resting lymphocytes. Immature DCs are particularly good at antigen ingestion and processing, but for a productive T-cell response they must mature to fully activated DCs, which express high levels of cell-surface major histocompatibility complex (MHC) antigen complexes and costimulatory molecules. DCs of various phenotypes serve as sentinel cells in virtually all tissues—including the peripheral blood, where they are continuously exposed to antigens. Very small numbers of activated DCs are highly efficient at generating immune responses against viruses, other pathogens and endogenous tumors. In addition, it was reported that DC are also involved in the maintenance of immunological self-tolerance, promoting T cells with regulatory functions or inducing anergy of T cells.

DCs can be generated in vitro by culture of DC precursors obtained from bone marrow (rodents) or peripheral blood (humans and nonhuman primates) with different cytokine cocktails. In vivo transfer of antigen-loaded DC with a tolerogenic character is regarded as a promising therapeutic means to negatively manipulate immune response in an antigen-specific manner. Presently, two different approaches for the selective enhancement of the tolerogenic properties of DCs are under investigation: (1) the use of immature DCs or the pharmacological arrest of the maturation of DCs; and (2) the use of genetically engineered DCs expressing immunosuppressive molecules (Figure 2).

Various culture procedures used to generate DC with a tolerogenic character have been reported (1-6). Mouse bone marrow-derived DC generated in the presence of IL-10 and/or TGF-□ or in the low dose of GM-CSF showed immature phenotypes, a low level expression of cell surface MHC and co-stimulatory molecules, and induced T cell-anergy in vitro and tolerance to specific antigens or allogeneic transplanted organs in vivo. In human monocyte-derived immature DC loaded with antigenic peptides and transferred in vivo have been shown to cause the antigen-specific immune suppression (7).

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 (FasL), indoleamine 2, 3-dioxygenase (IDO), IL-10, or CTLA4Ig by gene-transfer has

been also reported (8-11). 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 (12).

Figure 2

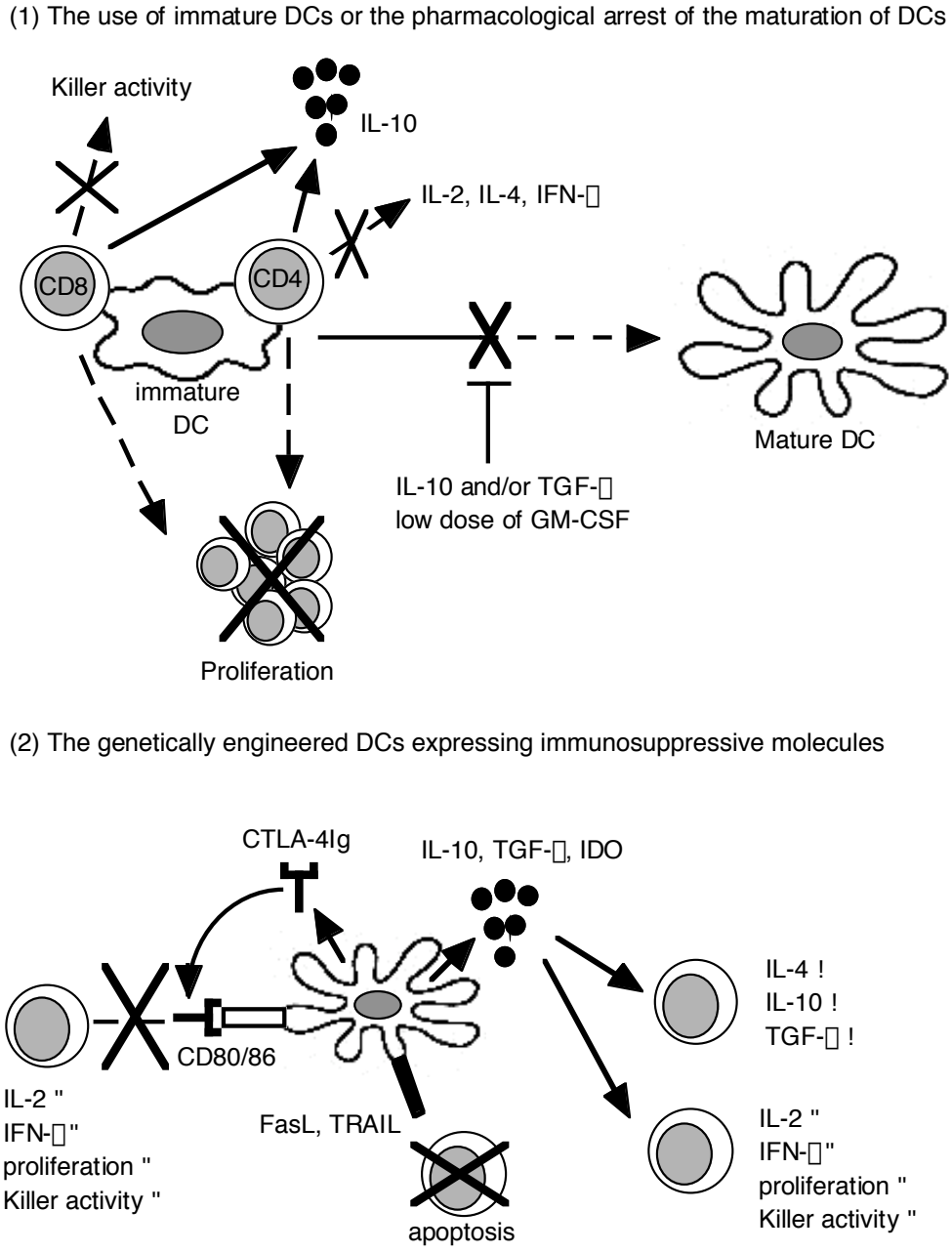

Figure 2. Immuno suppression by modified DC

6-3) Antigen-processing pathways for the MHC class II-restricted antigen presentation

The class II major histocompatibility complex (MHC) molecules, expressed in antigen presenting cells (APCs), present peptides originating from exogenous proteins, membrane proteins or intravesicular pathogens that enter the cell through the endocytic route to $CD4^+$ T cells (13). APCs include the dendritic cells initiating primary response of naive T cells, macrophages specialized to engulf particulate materials, and B cells that efficiently internalize specific antigen by receptor-mediated endocytosis of the antigen bound to their surface immunoglobulin (14, 15).

As shown in Figure 3, proteins incorporated into cells through endocytosis become enclosed in endosome, which become increasingly acidic as they progress into the interior of the cell, eventually fusing with lysosomes. The endosomes and lysosomes contain proteases, known as acid proteases, which are activated at low pH and eventually degrade the protein antigens contained in the vesicles. These acid proteases include the cysteine proteases cathepsins; B, D, and E. The biosynthetic pathway for MHC class II molecules starts with their translocation into the endoplasmic reticulum, and they must therefore be prevented from binding prematurely to peptides transported into the endoplasmic reticulum lumen or to the cell's own newly synthesized polypeptides. The binding is prevented by the assembly of newly synthesized MHC class II molecules with a protein known as the MHC class II-associated invariant chain (Ii) (16-19). The Ii have another function, a delivery of the MHC class II molecules to a low-pH endosomal compartment where peptide loading onto MHC class II molecules can occur. In such vesicles, proteases including cathepsins S and L cleave the invariant chain, leaving the class II-associated invariant chain peptide bound to the MHC class II molecules.

Pathogens and their proteins are cleaved down into peptides within acidified endocytic vesicles, but these peptides cannot bind to MHC class II molecules that are occupied by class II-associated invariant chain peptide (CLIP). The class II like molecule, human histocompatibility leukocyte antigen (HLA)-DM (H-2M in mice), binds to MHC class II/CLIP complexes, catalyzing the release of CLIP and the binding of antigenic peptides. HLA-DO (H-2O in mice) is produced in thymic epithelial cells and B cells and acts as a negative regulator of HLA-DM. HLA-DO interacts with HLA-

DM to inhibit both the HLA-DM-catalyzed release of CLIP from, and the binding of other peptide to, MHC class II molecules.

Figure 3

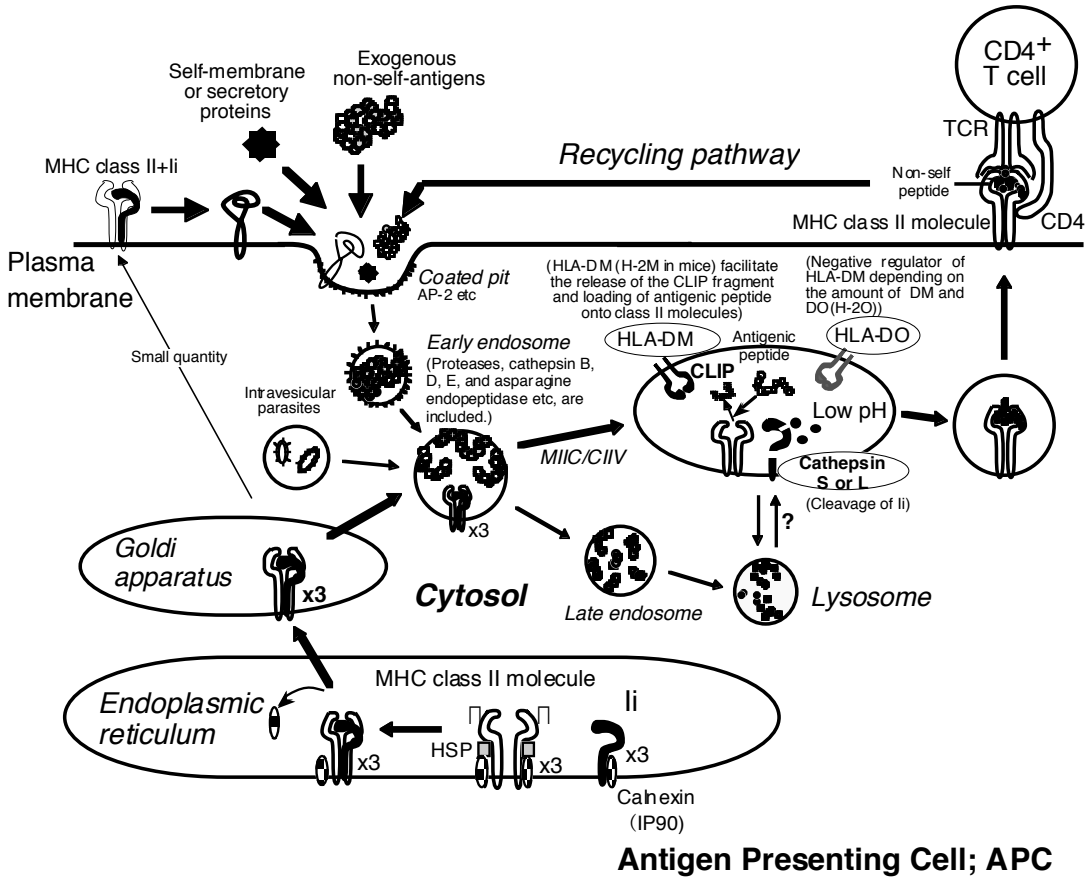

Figure 3. Antigen-processing pathways for the MHC class II-restricted antigen presentation.

6-4) Structure and function of invariant chain

The invariant chain (Ii), a type II integral membrane glycoprotein, play a critical role in MHC class II-restricted antigen presentation pathway by stabilizing peptide-free class II □□ heterodimers in a nonameric (□□ Ii) complex (Figure 4) (17) and has the following two important functions with regard to antigen presentation by MHC class II molecules: 1) Ii associates with newly synthesized MHC class II □ and □ dimers in the endoplasmic reticulum to form a complex and directly prevents peptides in the endoplasmic reticulum from binding to MHC class II molecules, using a domain

termed CLIP that occupies the peptide-binding groove of class II □□ dimers (Figure 4), 2) This □□-Ii complex is composed of one Ii trimer and three □□ dimers and transported via the Golgi apparatus to the endosomal pathway where peptide loading of class II takes place, by the targeting signal; di-leucine motif [(D/E)XXX(L/M)(L/I/V)] (20, 21), in the cytoplasmic domain of Ii. In this pathway, Ii is proteolytically degraded from the C-terminus and the class II □□-CLIP complex is generated. In specialized endocytic organelles enriched in MHC class II molecules and designated MHC class II compartments (MIICs) or class II vesicles (CIIVs) (Figure 3) (22-24), HLA-DM catalyzes the dissociation of CLIP from class II □□ dimer and binding of other peptides (25, 26). The CLIP region of Ii, roughly residues 81-104, is one of two segments shown to interact with HLA-DR molecules. The other segment, Ii 118-216 is C-terminal to CLIP, mediates trimerization of the ectodomain of Ii and interferes with DM/class II binding.

Figure 4

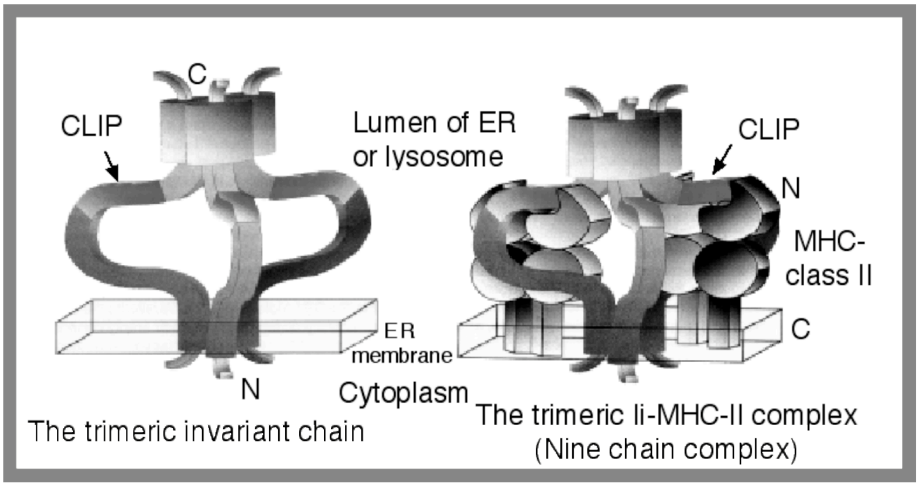

Figure 4. Model of the trimeric invariant chain bound to MHC class II □:□ heterodimers.

In the ER, the MHC class II □ and □ heterodimers associate with an invariant chain (Ii), to assemble into nonameric complexes comprising three □-, three □-, and three Ii-chain subunits. A lumenal domain of Ii, known as CLIP interacts with peptide-binding groove of MHC class II, rending the groove inaccessible to peptides present in the ER.

6-5) Targeting antigens to MHC class II presentation pathway using invariant chain

The presentation of antigenic peptides by MHC class II to $CD4^+$ T cells is crucial to initiate immune responses. Several groups including our group, developed a new system for delivery of an antigenic peptide to the MHC class II pathway, using the Ii (27-29). They designed a mutated human p33-form Ii, CLIP-substituted Ii, in which streptococcal M12p55-68 (RDLEQAYNELSGEA) was substituted for CLIP (27). They examined the peptide presenting function of this construct, in comparison with the previously reported C-terminal fused Ii, in which a cathepsin cleavage site and M12p54-68 was ligated to the C-terminus of Ii. Mouse L cell transfectants expressing either of these two mutated Ii along with HLA-DR4 could process and present M12p55-68 to the peptide specific and DR4-restricted $CD4^+$ T cell clone. CLIP-substituted Ii was much more efficient in antigen presentation than was the C-terminal fused Ii. Similar to the wild-type Ii, the CLIP-substituted Ii was associated intracellularly with DR4 molecules. These results indicate that the peptide substituted for CLIP of Ii p33 bound to the groove of DR molecules in the same manner as CLIP and it was preferentially presented to the $CD4^+$ T cell clone in the absence of HLA-DM molecules. This system may prove useful for immunotherapy with DNA vaccines or for construction of an antigen presenting cell library with diverse peptides.

For efficient presentation of autoantigenic peptide in the context of MHC class II molecules, we used this expression vector in which cDNA for human MHC class II-associated invariant chain (Ii) was mutated to contain antigenic peptide, myelin oligodendrocyte glycoprotein (MOG) p35-55, in the CLIP (class II-associated invariant chain peptide) region, as shown in Figure 5. An epitope inserted in this vector is efficiently presented in the context of co-expressed MHC class II molecules (30).

Figure 5

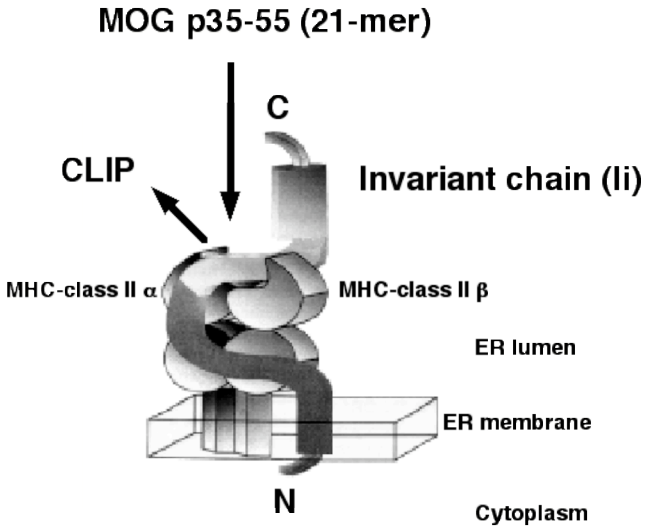

Figure 5. Strategy for targeting antigenic peptide to MHC class II pathway.
The use of an invariant chain with targeting signals to endosome is a pertinent strategy for antigen delivery to MHC class II restricted antigen presentation pathway. The Ii chain with CLIP replacement exploit the natural assembly pathway of class II-Ii complex to obtain loading of MOG p35-55 into peptide-binding groove of MHC class II molecules.

6-6) TRAIL and PD-L1 as the immuno-suppressive molecules

As molecules with a T cell-suppressive property, I utilized TNF-related apoptosis-inducing ligand (TRAIL) and Programmed Death-1 ligand (PD-L1) in this study. TRAIL, a member of the TNF superfamily, is constitutively expressed in a variety of cell types, including lymphocytes, NK cells, and neural cells (31, 32). TRAIL$^{-/-}$ mice are hypersensitive to collagen-induced arthritis and streptozotocin-induced diabetes (33). PD-L1, a ligand for PD-1 and member of the B7 family, is expressed on DC, IFN-γ-treated monocytes, activated T cells, placental trophoblasts, myocardial endothelium, and cortical thymic epithelial cells (34, 35). PD-1$^{-/-}$ mice spontaneously develop a lymphoproliferative / autoimmune disease, a lupus-like glomerulonephritis disease and arthritis on C57BL/6 mice, and cardiomyopathy in BALB/c mice (36, 37). Thus, abrogation of either of these two molecules make mice autoimmune-prone, suggesting that these molecules play significant roles in maintaining immunological self-tolerance in physiological situations (38-46). (Table I)

Table I. TRAIL and PD-L1

TRAIL (TNF- Related Apoptosis-Inducing Ligand)	PD-L1/B7-H1 (Program Death 1 Ligand)
30kDa, TNF family members, Type II membrane protein	62kDa, B7 family members, Type I membrane protein
Expressed in many tissues, especially, in spleen, lung, and prostate. Immature DCs ±, Mature DCs ++ DCs infected with CMV++	Expressed in Heart, Lung, Thymus, Kidney etc. Dendritic cell, Monocytes, T cells and B cells, also and Keratinocytes after IFN-γ stimulation Immature DCs ±, Mature DCs ++
・Suppression of cell cycle progression in T cells ・Suppression of antibody production in B cells ・Induction of apoptosis in tumor cells	・Inhibition of T cell responses including proliferation and cytokine production of IFN- γ, IL-4 and IL-10
TRAIL$^{-/-}$ mice are prone to autoimmunity ・Hypersensitive to collagen-induced arthritis ・Hypersensitive to streptozotocin-induced diabetes, ・Development of heightened autoimmune responses	PD-1 $^{-/-}$ mice are prone to autoimmunity ・Glomerulonephritis and arthritis in C57BL/6-PD-1$^{-/-}$ ・Accelerated autoimmunity in terms of severity and time onset in C57BL/6$^{lpr/lpr}$-PD-1$^{-/-}$ ・Dilated cardiomyopathy in BALB/c-PD-1 $^{-/-}$

6-7) ES cell-derived dendritic cell

For introduction of multiple expression vectors into DC, I utilized a method for ES cell-mediated genetic modification of DC. Recently, we and another group established culture procedures to generate DC from mouse ES cells (30, 47). ES cell-derived DC (esDC or ES-DC) have the capacity comparable to bone marrow-derived DC to process and present protein antigens to T cells, stimulate naïve T cells, and migrate to lymphoid organs in vivo (48, 49). A recent study using the method revealed the role of Notch signaling in differentiation of DC (50). 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 (48).

7. Objectives of this study

In the current study, I tried to prevent MOG-induced EAE by treatment of mice with genetically modified DC. I generated double-transfectant DC presenting MOG peptide in the context of MHC class II molecules and simultaneously expressing molecules with T cell-suppressive property. I tested a strategy to down-modulate the immune response in an antigen-specific manner by in vivo transfer of such genetically modified DC to prevent development of the disease. (Figure 6).

Figure 6

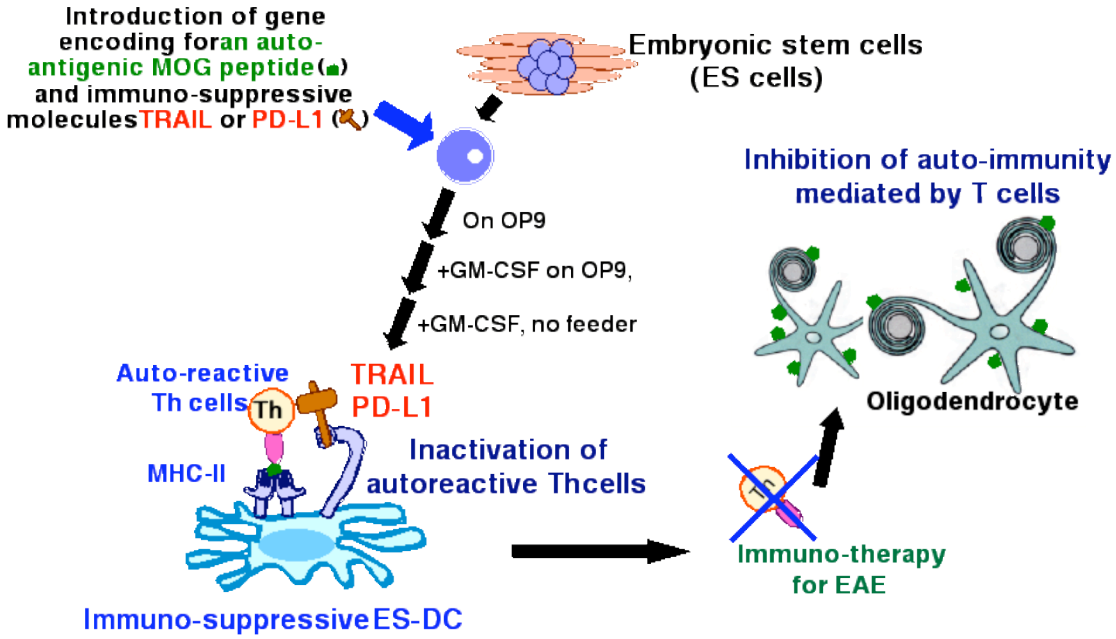

Figure 6. Prevention of EAE by genetically modified ES-DC.
The genetically modified ES-DCs expressing TRAIL or PD-L1 along with MOG peptide inactivate MOG-reactive T cells and prevent from EAE.

8. Materials and Methods

8-1) Mice

CBA, and C57BL/6 mice obtained from Clea Animal Co. (Tokyo, Japan) or Charles River (Hamamatsu, Japan) 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.

8-2) Peptides, protein, cell lines, and cytokines

The mouse MOG p35-55 (MEVGWYRSPFSRVVHLYRNGK), mouse myelin proteolipid protein (PLP) p190-209 (SKTSASIGSLCADARMYGVL), and mouse myelin basic protein (MBP) p35-47 (TGILDSIGRFFSG), were synthesized using the F-MOC method on an automatic peptide synthesizer (PSSM8, Shimadzu, Japan) and purified using HPLC (51-53). Bovine MBP was purchased from Sigma-Aldrich Co. (St. Louis, MO). The ES cell line, TT2, derived from CBF1 blastocysts, and the M-CSF-defective bone marrow-derived stromal cell line, OP9, were maintained, as described (30). 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 Co. (Tokyo, Japan) and was purchased from PeproTech EC Ltd. (London, UK).

8-3) Plasmid construction

Mouse TRAIL cDNA was prepared by RT-PCR amplification from total RNA of mouse spleen with PCR primers 5'-AACCCTCTAGACCGCCGCCACCATGCCTT CCTCAGGGGCCCTGAA -3' and 5'-AAAGGGATATCTTTACTGGTCATTTAGTT-3'. The design of these primers results in cloning of TRAIL cDNA downstream of the Kozak sequence.(48) The PCR products were subcloned into a pGEM-T-Easy vector (Promega, Madison, WI), 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, Japan) (35). 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'-CCGGTGATGGAAGTTGGT TGGTATCGTTCTCCATTCTCTCGTGTTGTTCATCTTTATCGTAACGGTAAGCTG CCCATGGGAGCT-3', was inserted into the previously reported human Ii-based epitope presenting vector, pCI30 (30). 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 (30).

8-4) Transfection of ES cells

Transfection of TT2 ES cells were done as previously described (30, 48). In brief, ES cells suspended in DMEM at a concentration of $2.5x10^7$/ml, and 1 x 10^7 cells were mixed with 40 □g of linearlized plasmid DNA, pCAG-TRAIL-INeo or pCAG-PDL1-INeo, and electroporated in a 4 mm-gap cuvette under the condition of 200 V and 950 mF. Transfected ES cells were cultured on feeder cell layers of primary mouse embryonic fibroblasts (PEF) in 90-mm culture dishes and selected with G418 (250 □g/ml, GIBCO), and drug-resistant colonies were picked up on day 9. Subsequently, TRAIL or PD-L1 transfected-ES cell clones or parental TT2 ES cells were introduced with pCAG-MOG-IPuro or pCAG-OVA-IPuro, and selected with puromycin (2 □g/ml). ES cell colonies resistant to both neomycin and puromycin were picked up and expanded (Figure 7).

Figure 7

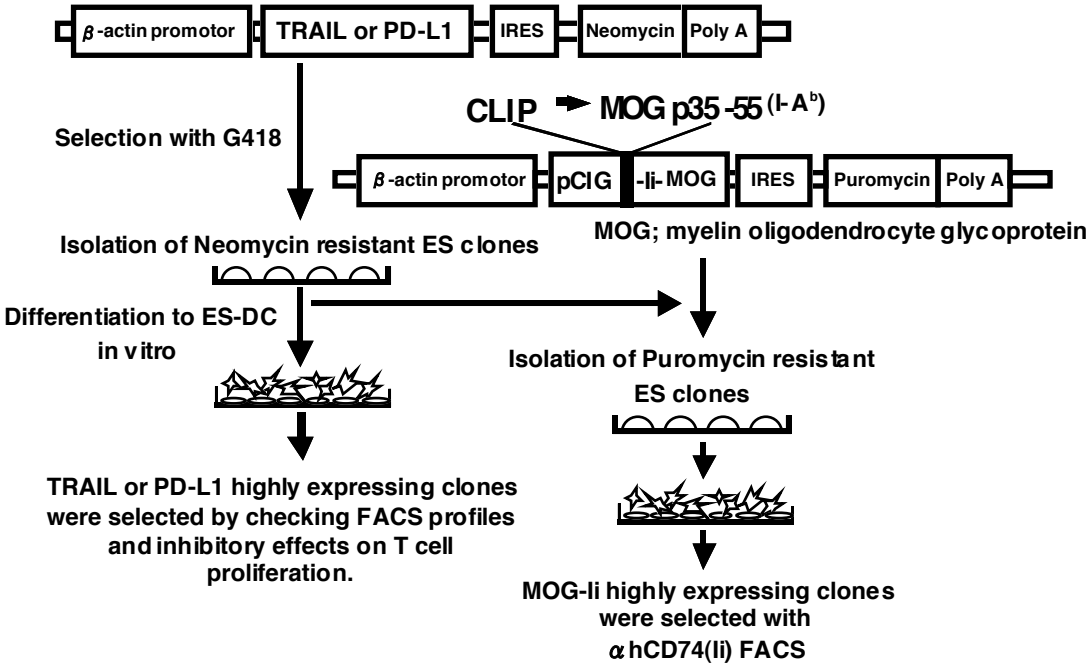

Figure 7. Transfection of ES cells

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 and selected with neomycin (G418) and puromycin. ES cell colonies resistant to both neomycin and puromycin were picked up and expanded. High expressing clones were selected by checking FACS profiles and biological function as described above.

8-5) Differentiation of DC from ES cells

The procedure for induction of differentiation of ES cells into DCs is shown in Figure 8. ES cells were suspended in □-MEM supplemented with 20% FCS and seeded (1.5 x10^4/2 ml medium/well) onto OP9 cell layers in 6-well plates. On day 3, half of the medium was removed and 2 ml of fresh medium was added to each well. On day 5, cells were harvested using PBS/0.25% trypsin/1mM EDTA, re-seeded onto fresh OP9 cell layers, and cultured in □-MEM supplemented with 20% FCS and GM-CSF (1,000 U/ml). At this step, cells recovered from 3 wells of 6-well culture plates were suspended in 20 ml of the medium and seeded into one 150-mm dish. On day 10 (5 days after the transfer), floating cells were recovered by pipetting. On average, 4-8 x 10^6 cells were recovered from one 150-mm dish, thus indicating 100-200 times increase in cell number from undifferentiated ES cells. The recovered cells were transferred to bacteriological Petri dishes (2.5 x 10^5 cells/90-mm dish) without feeder cells, and

Figure 8

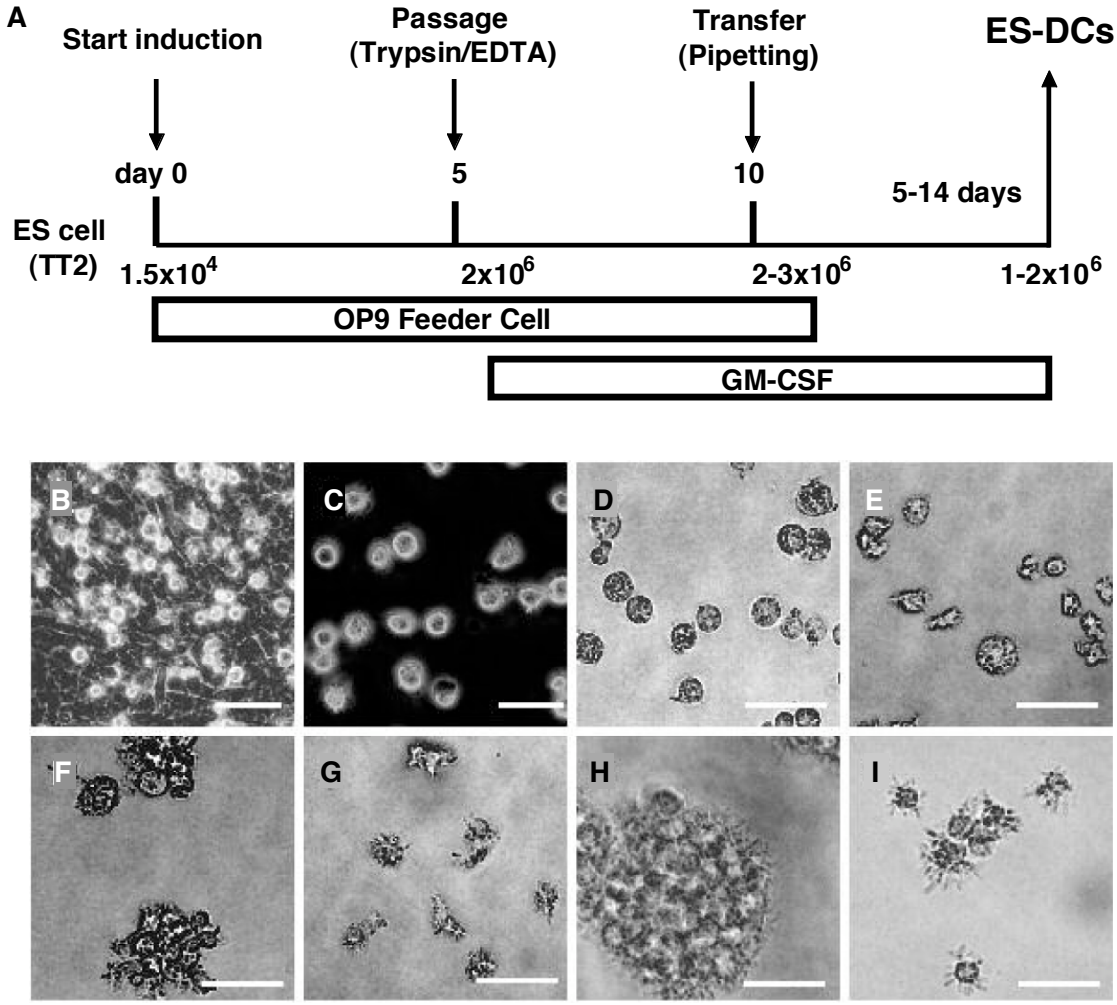

Figure 8. Differentiation and morphology of DC from ES cell.
Overview of the culture protocol for generation of DCs from ES cells (A). ES cell-derived cells on day 8 (B), day 12 (C, D), day 17(E, F), and day 27 (G) of differentiation culture are shown. Cells on day 24 were recovered and stimulated for 2 days with IL-4, TNF-□, plus agonistic anti-CD40 mAb (H) or with IL-4, TNF-□, plus LPS (I), as described in the materials and methods. Panel (B) and (C) are phase-contrast micrographs. Scale bars represent 20 □m.

cultured in RPMI-1640 medium supplemented with 12% FCS, GM-CSF (500 U/ml), and 2-ME. After days 17-19, 1.5-2 x 10^6 floating or loosely adherent cells were recovered per dish (ES-DCs) and used for experiments. In addition, to induce a complete maturation of ES-DCs, cells cultured for more than 10 days in Petri dishes were transferred to fresh Petri dishes and cultured in RPMI/10% FCS without GM-CSF. The next day, IL-4 (10 ng/ml), TNF-□ (5 ng/ml), plus anti-CD40 mAb (10 □g/ml, clone 3/23) or IL-4, TNF-□, plus LPS (1 □g/ml) were added. After 2 or 3 days, cells were

harvested by pipetting. Usually, some cells harvested on days 5 and 10 were freeze-stocked for future use.

8-6) 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. RP-PCR was done as described (48). 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'-CTGACTGACCGCGTTACTCCCACA-3' and 5'-GAAATGGTGTCCTGAAAGGTTC-3'; PD-L1, 5'-CTGACTGACCGCGTTACTCCCACA-3' and 5'-GCTTGTAGTCCGCACCACCGTAG-3'; and GAPDH, 5'-GGAAAGCTGTGGCGTGATG-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'-CGGGTTGTGTTGGTTGTAGA-3'.

8-7) Flowcytometric analysis

Staining of cells and analysis on a flowcytometer (FACScan, Becton Dickinson, CA) was done as described (30). Antibodies 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, Temecula, CA), R-PE-conjugated anti-mouse CD86 (clone RMMP-2, rat IgG2a, CALTAG, Burlingame, CA), FITC-conjugated anti-human CD74 (clone M-B741, mouse IgG2a, Pharmingen, San Diego, CA), FITC-conjugated goat anti-mouse Ig (Pharmingen), mouse IgG2a control (clone G155-178, Pharmingen), FITC-conjugated mouse IgG2a control (clone G155-178, Pharmingen), R-PE-conjugated hamster IgG control (IMMUNOTECH, Westbrook), R-PE-conjugated rat IgG2a control (clone LO-DNP-16, Caltag), biotinylated anti-mouse TRAIL (clone N2B2, rat IgG2a, eBioscience, San Diego, CA), anti-mouse PD-L1

(clone MIH5, rat IgG2a, eBioscience), rat IgG2a (CALTAG), biotinylated rat IgG2a (eBioscience), FITC-conjugated anti-rat Ig (Pharmingen) and PE conjugated-streptavidin (Molecular Probes, Invitrogen, Carlsbad, CA). In some experiments, the DC fraction was gated by forward and side scatters.

8-8) Cytotoxicity assay

Standard [^{51}Cr]-release assay was performed as described previously. Briefly, [^{51}Cr]-labeled L929 cells (5x10^3), as target cells, and effecter cells were mixed in 96-well flat-bottomed culture plates at indicated effecter : target (E:T) ratios. After 12 h of incubation, radioactivity in the cell-free supernatants was measured on a gamma counter. The percentage of specific lysis was calculated as described previously (32).

8-9) Proliferation assay of T cells stimulated with anti-CD3 mAb

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 x 10^4) and the T cells (1 x 10^5) were seeded into wells of 96-well flat-bottomed culture plates pre-coated 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 □Ci/well) in the last 16 h. At the end of culture, cells were harvested onto glass fiber filters (Wallac Gaithersburg, MD) 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 monoclonal antibody (5 □g/ml) was added to the culture.

8-10) 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 x 10^4) were co-cultured with the MOG-reactive T cells (1.5-2 x 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.

8-11) Induction of EAE

For EAE induction by synthetic peptides or purified protein, 6 to 8-week-old female CBF1 mice were immunized by giving a subcutaneous injection at the base of the tail with a 0.2 ml IFA/PBS solution containing 600 μg of MOG p35-55 peptide, MBP p35-47 peptide, or whole bovine MBP, and 400 μg *Mycobacterium tuberculosis* H37Ra (Difco Laboratories) on day 0. In addition, 500 ng of purified *Bordetella pertussis* toxin (CALBIOCHEM, Germany) were injected intraperitoneally (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 *Bordetella pertussis* toxin in 0.2 ml PBS on days 0 and 2. 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.

8-12) Treatment of EAE with ES-DC

For prevention of EAE, mice were injected i.p. with ES-DC (1×10^6 cells/mouse/injection) on days -8, -5 and -2 (pre-immunization treatment), or on days 5, 9 and 13 (post-immunization treatment). Mice were immunized according to protocol for EAE induction on day 0 and 2 as described above.

8-13) Immunohistochemical analysis

Freshly excised spinal cords were immediately frozen and embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co., Tokyo, Japan). Immunohistochemical staining of CD4, CD8 and Mac-1 was done, as described (48), 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, Tokyo, Japan).

8-14) Analysis of T cell response to MOG or keyhole limpet hemocyanin (KLH)

Immunization of mice and re-stimulation of draining lymph node cells in vitro was done as described (54), 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 Co.) emulsified in CFA. After indicated days, inguinal lymph node cells and spleen cells were isolated and cultured (5 x 10^5 cells/well) in the presence of MOG peptide (0, 8, 25 or 80 μg/ml) or KLH (16, 50 or 160 μg/ml) in 10% horse serum/RPMI 1640/2ME or 2% mouse serum/DMEM/2ME/insulin-transferrin-selenium-X (GIBCO, Invitrogen Co.), 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 (5x10^5 cells/well) in the presence of MOG peptide in 10% horse serum/RPMI 1640/2ME, and the proliferative response was quantified based on [^{3}H]-thymidine-uptake, as described above. In order to analyze production of cytokines of spleen cells isolated from mice treated with ES-DC, isolated spleen cells were stimulated with 10μM of 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.

8-15) Apoptosis detection

For detection of apoptosis of splenic $CD4^+$ T cell, Annexin V-FITC apoptosis detection kits (BioVision, Mountain View, CA) 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, Pharmingen), and subsequently analyzed by flowcytometry. Frozen sections of spleen were subjected to TUNEL staining by using ApopTag Fluorescein In Situ Apoptosis Detection Kits (Serologicals Corporation, Norcross, GA). In brief, sections were incubated with digoxigenin-conjugated nucleotides and terminal deoxynucleotidyl transferase, and subsequently with peroxidase-conjugated anti-digoxigenin antibody. The staining signals were developed using diaminobenzidene.

8-16) Adoptive transfer experiment

For the adoptive transfer experiments, donor CBF1 mice were i.p. injected with ES-DC ($1x10^6$ cells/injection/mouse) on days -10, -7, and -4. $CD4^+$ T cells were isolated from the spleen cells of donor mice using the MACS cell sorting system (Miltenyi Biotec) and i.v. injected into recipient mice (2.5×10^6 cells/ mouse) on day -2. The recipient mice were subjected to the procedure for EAE induction (on day 0 and 2) as described above.

8-17) 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.

9. Results

9-1) Induction of EAE in CBF1 mice.

To date I found no study that EAE had been induced in CBF1 mice. Therefore, prior to the study on therapeutic intervention, it was necessary to set up an experimental condition under which I could reproducibly induce EAE in CBF1 mice. I compared several induction protocols using protein or peptide antigen of MOG, MBP, and PLP. As a result, I found that, when mice were subcutaneously 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 *Mycobacterium tuberculosis* accompanying an i.p. injection of 500 ng of purified *Bordetella pertussis* toxin on days 0 and 2, EAE is reproducibly induced in CBF1 mice with an average peak clinical score of 3.3 (Table II). I 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 *Mycobacterium tuberculosis* and *Bordetella pertussis* toxin also induced EAE in CBF1 mice with a peak clinical score ranging between 2 and 3 (Table II).

Table II. EAE induction in CBF1 mice [1]

Exp.	Antigen	Antigen Dose	Disease incidence	Day of onset	Mean peak clinical score
1	MOG p35-55	200 μg x2[2]	1/2	9.0 ± 0	1.5 ± 0
2		400 μg	2/2	11.0 ± 0	4.0 ± 0
3		600 μg	44/44	10.2 ± 1.3	3.3 ± 0.5
4		800 μg	2/2	8.0 ± 0	3.0 ± 0
5	MBP p35-47	200 μg x2[2]	0/2	-	-
6		600 μg	8/8	5.5 ± 1.3	3.0 ± 0
7	MBP protein	200 μg x2[2]	0/2	-	-
8		600 μg	6/6	9.7 ± 1.8	3.0 ± 0
9	PLP p190-209	200 μg x2[2]	0/2	-	-
10		600 μg	2/2	5.0 ± 0	2.0 ± 0

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

9-2) 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 Figure 9A. 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 (Figure 9B). In this vector, a cDNA for human Ii was mutated to contain an oligo DNA encoding MOG p35-55 epitope in the CLIP region (27, 30, 55, 56). 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 containing the MOG peptide, TRAIL and PD-L1 in ES-DC was confirmed by RT-PCR (Figure 9C) and flowcytometric analysis (Figure 10). The mutant human Ii containing the MOG peptide was detected by intracellular staining with anti-human CD74 (Ii) mAb (Figure 9). ES-DC of similar morphology were generated from any of the transfectant ES cells. As shown in Figure 10, 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.

Figure 9

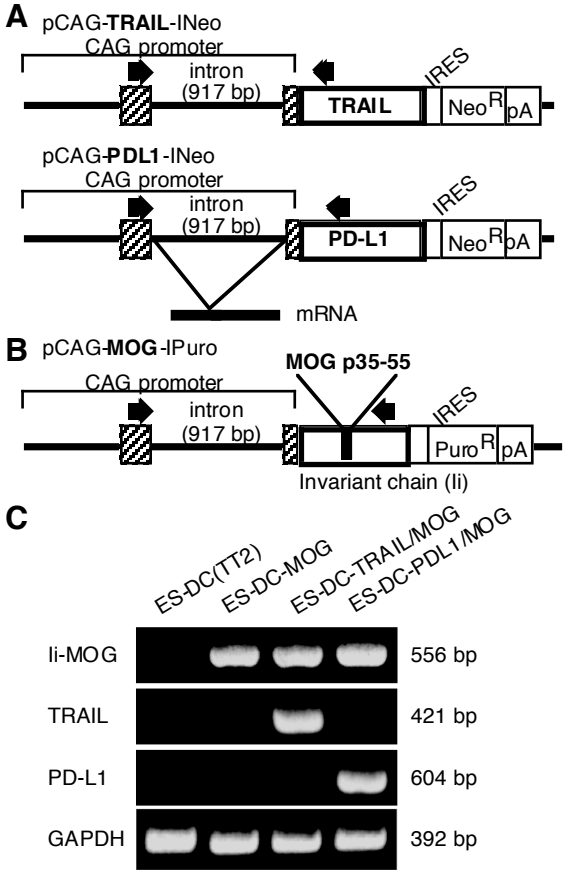

Figure 9. Genetic modification of ES-DC to express TRAIL, PD-L1 and Ii-MOG.

(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 bp and 604 bp, respectively) from genome-integrated vector DNA-origin. Hatched boxes indicate 5'-untranslated region of the rabbit b-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 (NeoR)-polyadenylation signal sequence (pA). (B) The structure of pCAG-MOG-IPuro, the expression vector for mutant human invariant chain 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 10

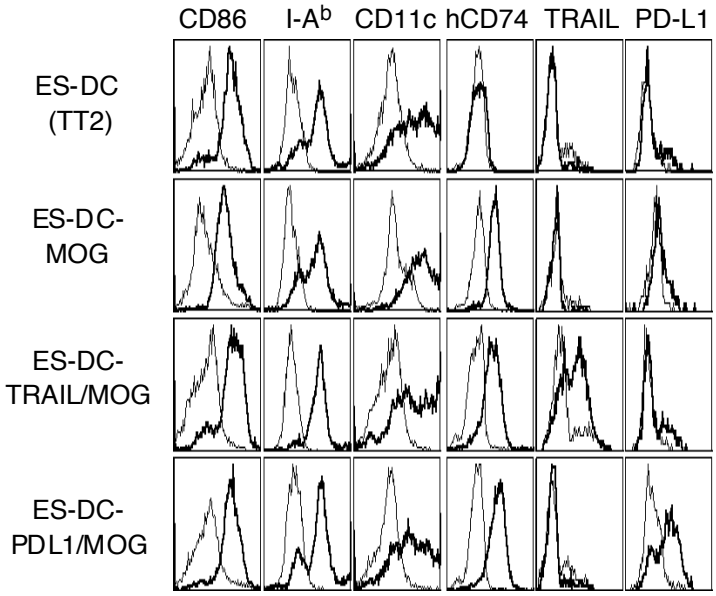

Figure 10. Surface phenotype of genetically modified ES-DC.
Expression of cell surface CD86, I-Ab, CD11c, TRAIL and PD-L1 on transfectant ES-DCs were analyzed by flowcytometric analysis. Expression of mutant human invariant chain (hCD74) bearing MOG peptide was examined using intracellular staining. Staining patterns with specific antibodies (thick line) and isotype-matched control (thin line) are shown.

9-3) 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 Figure 10A, ES-DC-TRAIL showed manifest killing activity against L929. On the other hand, 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 (Figure 10B). 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 (Figure 10B), 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.

Figure 11

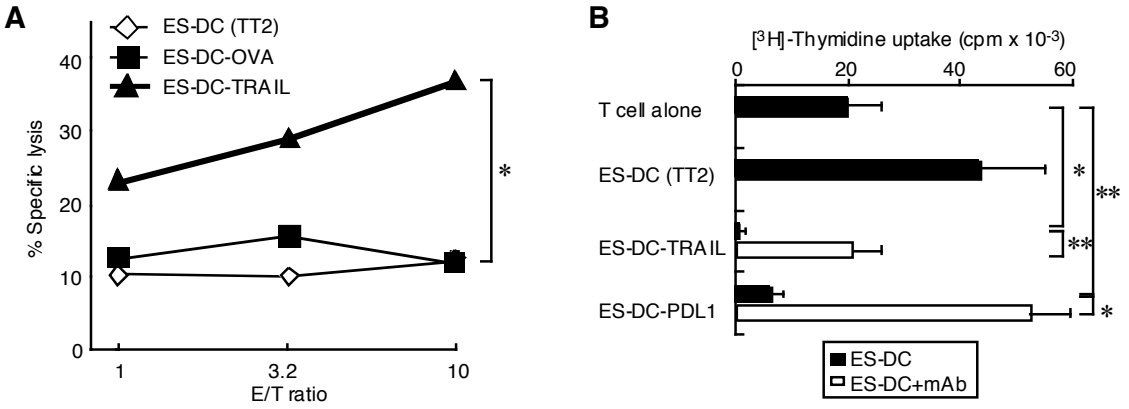

Figure 11. Expression of functional TRAIL or PD-L1 in ES-DC transfectants.
(A) The activity of TRAIL expressed in ES-DC was analyzed based on cytotoxicity against L929 cell. [^{51}Cr]-labeled target cells (5x10^3 L929 cells) were incubated with ES-DC (TT2), ES-DC-OVA, or ES-DC-TRAIL as effecter cells 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 (2x10^4 /well) were co-cultured with 1x10^5 syngeneic CBF1 splenic T cells in the presence (open bar) or absence (closed bar) of blocking antibody (anti-TRAIL mAb or anti-PD-L1 mAb, 5 □g/ml) for 4 days in 96-well flat-bottomed culture plates pre-coated 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.

9-4) 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 *Bordetella pertussis* toxin. Proliferative response of the MOG-reactive T cells upon co-culture with transfectant ES-DC was analyzed. As shown in Figure 12A, ES-DC-MOG stimulated the MOG-reactive T cells to induce proliferation. On the other hand, ES-DC carrying Ii-based PCC peptide expression vector (ES-DC-PCC) (30), as a control, did not do so. No proliferative response was observed when naïve splenic T cells isolated from syngeneic mice were co-cultured 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.

Figure 12

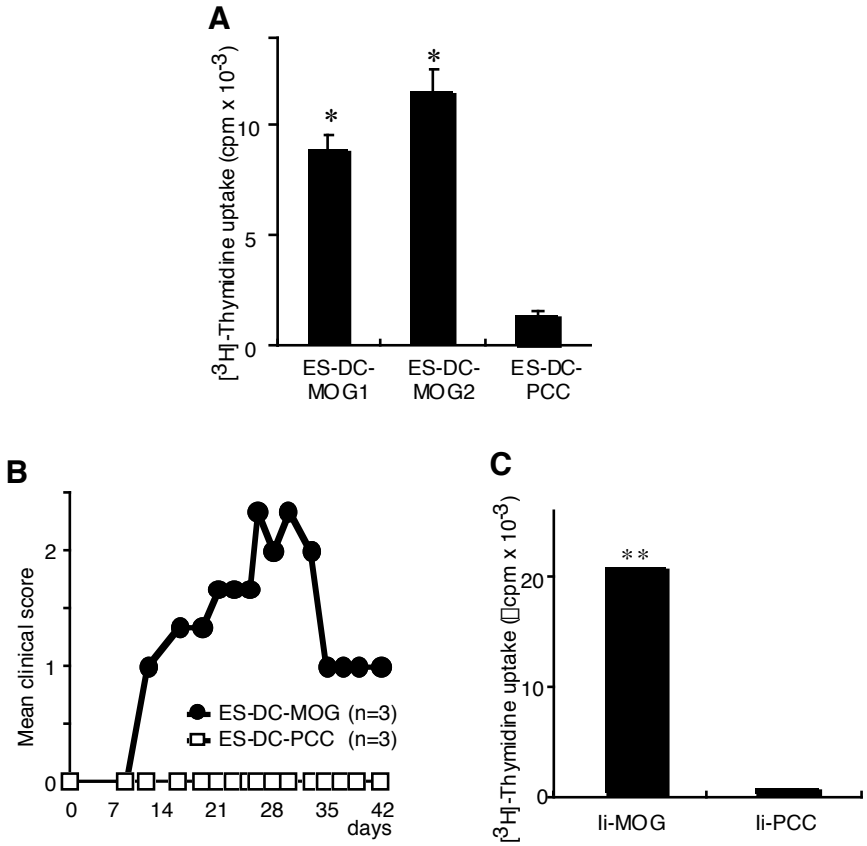

Figure 12. Presentation of MOG epitope by ES-DC introduced with Ii-based MOG epitope-presenting vector.

(A) T cells ($1.5x10^5$) isolated from inguinal lymph nodes of CBF1 mice immunized according to the protocol for EAE induction were co-cultured with one of two independent clones ($2x10^4$) 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) CBF1 mice (3 mice per group) were injected subcutaneously with ES-DC-MOG or ES-DC-PCC ($5x10^5$) on day 0, together with intraperitoneal injection of 500 ng purified *Bordetella 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 ($5x10^5$/ well) were cultured in the presence of 1 □g/ml of MOG peptide for 3 days. Proliferative response was quantified as in (A). Data were indicated as delta cpm (value in the presence of peptide – value in the absence of peptide (<$46x10^3$ cpm)), and SDs of triplicates were <9 % of mean value. The asterisks indicate that the differences in responses are statistically significant as 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.

It has been reported that transfer of bone marrow-derived DC pre-loaded with MOG peptide caused development of EAE in naïve mice (57, 58). I 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. I injected ES-DC-MOG or ES-DC-PCC, as a control, at the base of the tail of naïve mice and also gave i.p. 500 ng of *Bordetella pertussis* toxin on the same day and 2 days later. In results, EAE was developed in the mice transferred with ES-DC-MOG but not those transferred with ES-DC-PCC (Figure 12B).

I 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 *Bordetella pertussis* toxin, spleen cells were isolated from the mice and cultured in the presence of MOG peptide. As shown in Figure 12C, the spleen cells isolated from mice injected with ES-DC-MOG showed proliferative response to MOG peptide. On the other hand, 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 *Bordetella pertussis* toxin stimulated MOG-specific T cells to develop EAE.

9-5) Protection from MOG-induced EAE by treatment with ES-DC expressing MOG peptide along with TRAIL or PD-L1.

I 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 co-cultured with ES-DC-MOG, ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG. As shown in Figure 13, proliferative response of the MOG-reactive T cells co-cultured with ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG was significantly lower than those co-cultured with ES-DC-MOG, even though the three types of ES-DC expressed an almost equal level of MOG-Ii. These results indicate down-modulation of the response of MOG-reactive T cells in vitro by TRAIL and PD-L1 co-expressed together with MOG peptide on ES-DC.

Figure 13

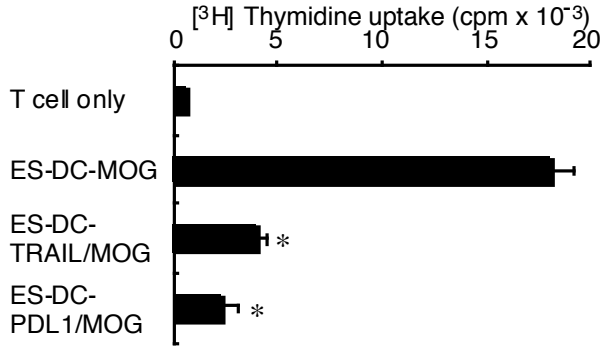

Figure 13. Decreased proliferative response to MOG peptide of MOG-reactive T cells co-cultured with ES-DC expressing MOG plus TRAIL or MOG plus PD-L1.
T cells ($2x10^5$) isolated from inguinal lymph nodes of CBF1 mice immunized according to the protocol for EAE induction were co-cultured with irradiated ES-DC-MOG, TRAIL/MOG or PDL1/MOG ($2x10^4$) for 3 days, as in Fig. 12A. The asterisks indicate that the differences in responses are statistically significant ($P<0.01$) as compared with ES-DC-MOG. The data are each representative of three independent and reproducible experiments with similar results.

I tested whether or not development of EAE would be prevented by pre-treatment 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 Figure 14A. As shown in Figure 14B and Table III, EAE was almost completely prevented by pre-treatment with either of these genetically modified ES-DC. On the other hand, pre-treatment with ES-DC-MOG, ES-DC-TRAIL/OVA (as irrelevant antigen) or ES-DC-PDL1/OVA had no effect (Figure 14C and Table III). 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, about 1×10^6 of genetically modified ES-DC as one injection dose is apparently necessary for the prevention of EAE under this experimental condition.

Figure 14

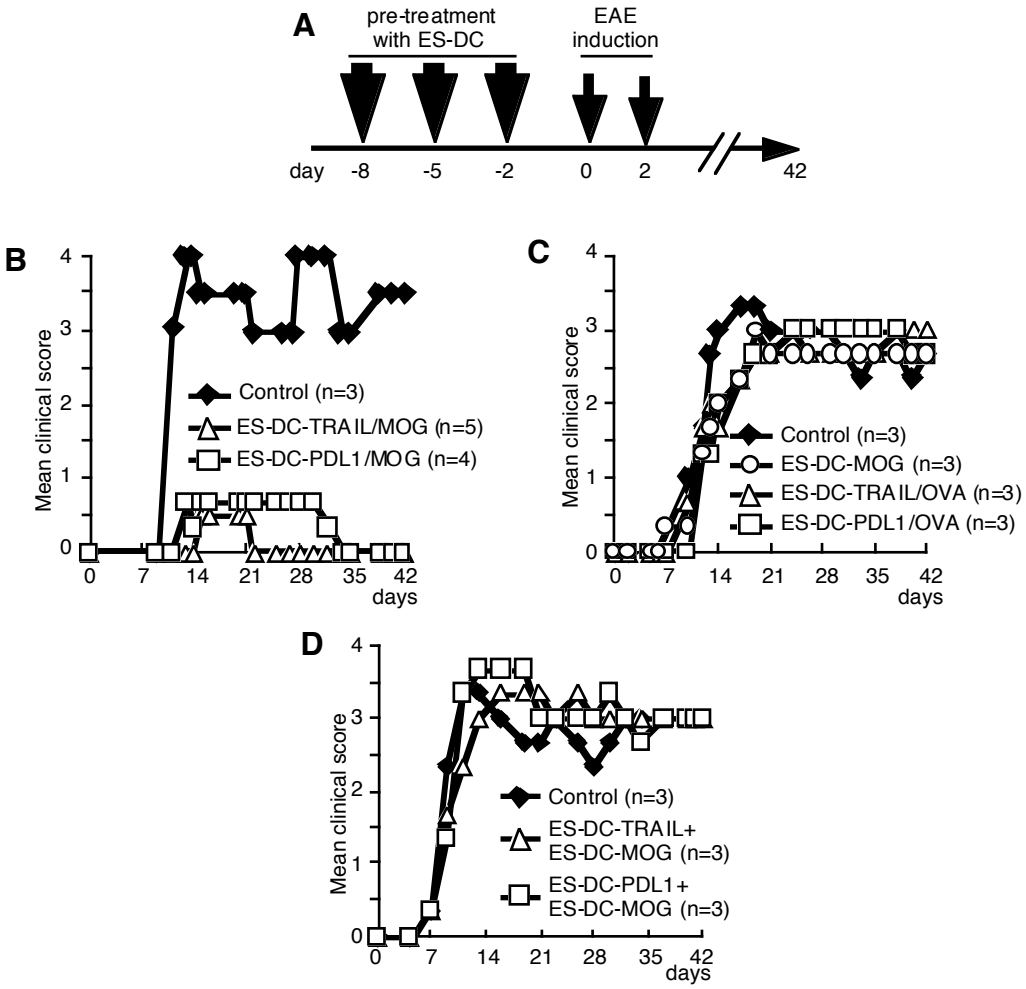

Figure 14. Prevention of MOG-induced EAE by pre-treatment of mice with ES-DC expressing MOG plus TRAIL or MOG plus PD-L1.

(A) The schedule for pre-treatment and induction of EAE is shown. CBF1 mice (3-5 mice per group) were i.p. injected with ES-DC ($1x10^6$ cells/injection/mouse) on days -8, -5, and -2. EAE was induced by s.c. injection of MOG peptide plus *Mycobacterium tuberculosis* H37Ra emulsified in IFA on day 0, and i.p. injection of *Bordetella pertussis* toxin on days 0 and 2. Disease severity of mice treated with ES-DC-TRAIL/MOG, ES-DC-PDL1/MOG, or RPMI medium (control) (B), ES-DC-MOG, ES-DC-TRAIL/OVA, ES-DC-PDL1/OVA, or RPMI medium (control) (C), co-injection with ES-DC-MOG plus ES-DC-TRAIL, ES-DC-MOG plus ES-DC-PDL1, or RPMI 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 III.

Figure 15

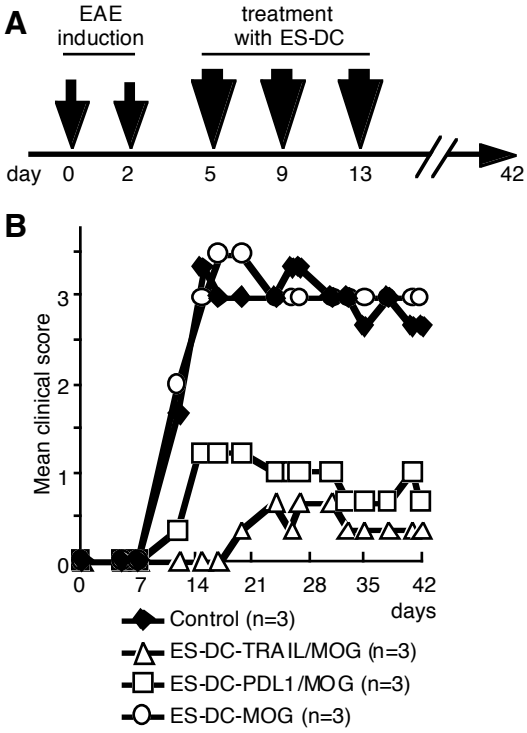

Figure 15. Inhibition of MOG-induced EAE by treatment with ES-DC expressing MOG plus TRAIL or MOG plus PD-L1 after immunization with MOG.

(A) The schedule for induction of EAE and treatment is shown. CBF1 mice (3 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 ($1x10^6$ cells/injection/mouse) on days 5, 9, and 13. (B) Disease severity of mice treated with ES-DC-TRAIL/MOG, ES-DC-PDL1/MOG, ES-DC-MOG, or RPMI medium (control) is shown. The data are each representative of two independent and reproducible experiments, and data of all experiments are summarized in Table III.

Table III. Suppression of EAE induction in CBF1 mice treated with ES-DC[1]

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[2]- 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[3]- 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

[1]Data are combined from a total of 10 separate experiments including those shown in Figs. 14 and 15. EAE was induced by subcutaneous injection at the tail base of a 0.2 ml IFA/PBS solution containing 400 μg *Mycobacterium tuberculosis* and 600 μg MOG peptide once (on day 0), together with intraperitoneal injections of 500 ng purified *Bordetella pertussis* toxin on days 0 and 2. For prevention of EAE, mice were injected i.p. with ES-DC (1 x 10^6 cells/mouse/injection) [2]on days -8, -5 and -2 (pre-immunization treatment), or [3]on days 5, 9 and 13 (post-immunization treatment).

I asked whether TRAIL or PD-L1 should be co-expressed by the same ES-DC as one presenting MOG peptide for their capacity to protect mice from EAE. As shown in Figure 14D and Table III, co-injection of ES-DC-MOG together with ES-DC-TRAIL or ES-DC-PDL1 did not reduce the severity of EAE. Thus, co-expression 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, I tested whether or not treatment with ES-DC after immunization with MOG would achieve some preventive effect on EAE. As shown in Figure 15A, mice were immunized according to the protocol for EAE induction and, after that, injected with ES-DC on days 5, 9 and 13 (1 x 10^6 cells/mouse/injection). Even in this post-immunization 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 (Figure 15B and Table III).

9-6) Decreased T cell response to MOG in mice treated with ES-DC expressing MOG plus TRAIL or MOG plus PD-L1.

I examined if 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 (Figure 14A), I isolated inguinal lymph node cells and analyzed their proliferative response upon re-stimulation in vitro with MOG peptide. As shown in Figure 16A, 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. On the other hand, that of lymph node cells from ES-DC-MOG-treated or untreated mice was increased with statistical significance. In the presence of 25 □g/ml of MOG peptide, stimulation index (count in the presence of MOG peptide / count in the absence of antigen) 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, I examined whether or not treatment with ES-DC would affect immune responses to an irrelevant exogenous antigen. I treated mice with ES-DC-MOG, -TRAIL/MOG, -PDL1/MOG, or RPMI medium (control) using the same schedule described above, and subsequently immunized the mice with KLH/CFA. Eleven days after the immunization, I isolated inguinal lymph node cells and analyzed their proliferative response upon re-stimulation with KLH in vitro. As a result, lymph node cells of ES-DC-treated and control mice showed the same magnitude of proliferative response (Figure 16B), thereby indicating that the treatment with such genetically modified ES-DC did not affect the immune response to irrelevant antigens.

I 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 $Mac\text{-}1^+$ macrophages was observed in spinal cords of untreated control mice (Figure 16). 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.

Figure 16

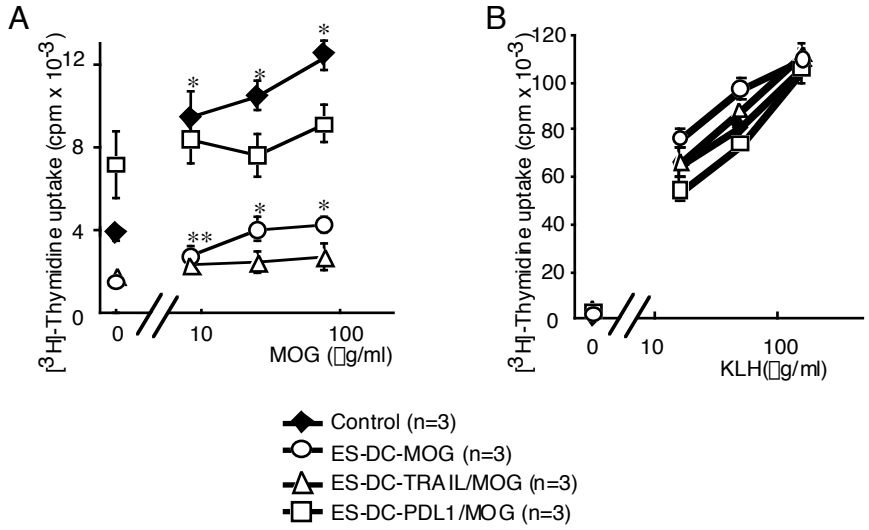

Figure 16. Inhibition of activation of MOG-reactive T cells and no effect of activation of KLH-specific T cell by treatment of mice with ES-DC expressing MOG plus TRAIL or MOG plus PD-L1.

(A) Inguinal lymph node cells ($3x10^5$) were isolated from CBF1 mice (3 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 as compared with count in the absence of antigen (*P<0.01, **P<0.05). The data are each representative of two independent and reproducible experiments with similar results. (B) CBF1 mice (3 mice per group) were i.p. injected with ES-DC ($1x10^6$ 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 re-stimulated with the indicated concentration of KLH in vitro. Proliferation of T cells was quantified as described above.

Figure 17

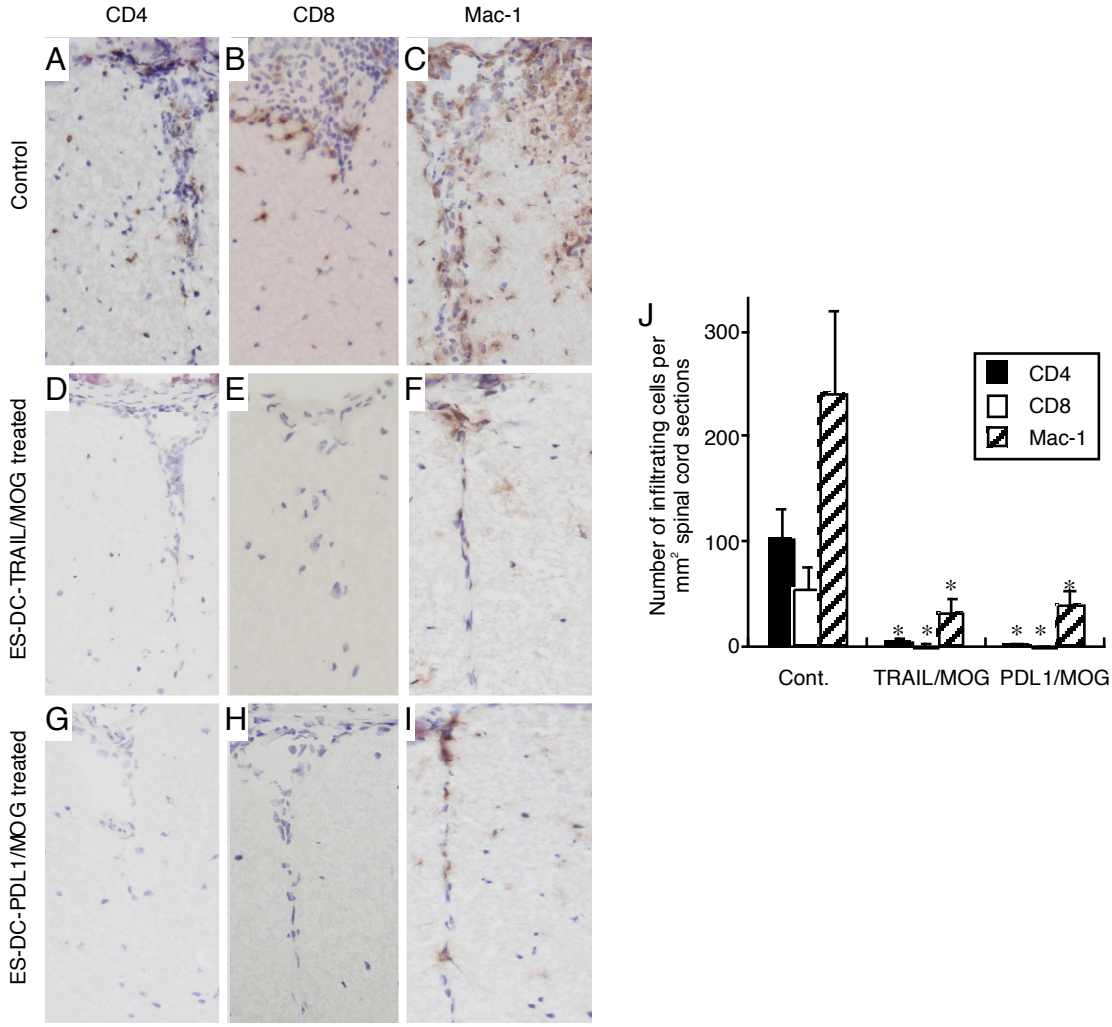

Figure 17. Inhibition of infiltration of CD4⁺ T cells, CD8⁺ T cells and Mac-1⁺ macrophages into spinal cord by treatment of mice with ES-DC expressing MOG plus TRAIL or MOG plus PD-L1.

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. 14A. 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, B and C), ES-DC-TRAIL/MOG treated- (D, E and F) and ES-DC-PDL1/MOG treated- (G, H and 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 $1mm^2$ tissue area of samples obtained from 5 mice. The asterisks indicate that the decreases in number of infiltrated cells are statistically significant ($P<0.01$) as compared with control.

9-7) Increased number of apoptotic cells in splenic $CD4^+$ T cells by treatment with ES-DC expressing MOG peptide along with TRAIL

With regard to the mechanism of prevention of EAE by transfectant ES-DC, I analyzed the apoptosis of $CD4^+$ T cell in spleens of mice treated with ES-DC by staining with Annexin V and subsequent flowcytometric analysis. In result, I observed that transfer of ES-DC-TRAIL/MOG caused an increase of apoptosis of $CD4^+$ T cells in recipient mice, compared with transfer of ES-DC-MOG, ES-DC-PDL1/MOG, or RPMI medium control (Table IV). In the experiments, 3 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 (Figure 18). The capacity of ES-DC-TRAIL/MOG to cause apoptosis of T cells may play some role in the protection from EAE.

Table IV. Apoptosis cell of $CD4^+$ splenic T cells isolated from ES-DC-treated mice.[1]

Treatment	Annexin V^+ cell (%)
No-treatment	10.2 ± 0.8
ES-DC-MOG	12.0 ± 0.4
ES-DC-TRAIL/MOG	**17.3 ± 2.5**
ES-DC-PDL1/MOG	12.2 ± 0.5

[1] Spleen cells were isolated from mice treated with each ES-DC or untreated (control) and subsequently immunized with MOG peptide for EAE induction. The apoptosis of $CD4^+$ T cell in the spleen cell were detected by staining with PE conjugated-anti CD4 antibody and FITC conjugated-Annexin V, and subsequent flowcytometric analysis. Frequency of Annexin V^+ cells in $CD4^+$ cells was calculated and mean percentage ± SD of 3 mice for each experimental group is indicated.

Figure 18

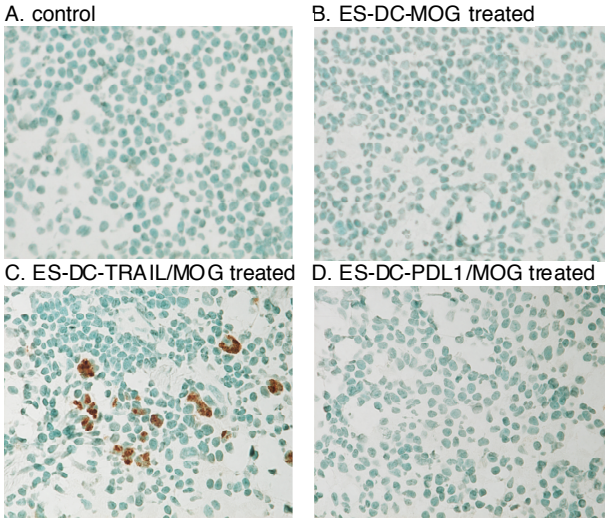

Figure 18. Induction of apoptosis of spleen cells by treatment of mice with ES-DC expressing MOG peptide along with TRAIL.
Mice were treated with the indicated ES-DC and immunized with MOG peptide, following the schedule described in the legend for Fig. 13A. On day 11, spleens were isolated from the mice and apoptotic cells were detected by in situ TUNEL staining. Original magnification, x200. 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 3 mice used in each experimental group and representative results are shown.

9-8) Prevention of MBP-induced EAE by transfer of ES-DC genetically engineered to express MOG peptide along with TRAIL.

Recently, it was reported that immature DC induce T regulatory type 1 (Tr-1) cells producing large amounts of IL-10 (59), and that proliferation of $CD25^+$ $CD4^+$ Treg is promoted by DC (60). As for the mechanism involved in the prevention of EAE by genetically modified ES-DC, we considered that not only inhibition of MOG-reactive effector T cells but also induction of MOG-reactive T cells with regulatory functions and their contribution to inhibition of the disease might occur. We hypothesized that, if this is the case, pre-treatment with ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG shows some effect also on EAE induced with MBP, because it may be possible that the T cells with regulatory activity were triggered in the central nervous system in recognition of MOG to suppress response of T cells autoreactive to MBP. To test this possibility, mice were pre-treated with ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG and subjected to EAE-induction by immunization with MBP (whole protein) or MBP p35-47. As shown

in Figure 19, we found that severity of MBP protein and peptide-induced EAE was significantly reduced by pre-treatment with ES-DC-TRAIL/MOG. On the other hand, pre-treatment with ES-DC-PDL1/MOG showed only a marginal effect on MBP protein-induced EAE, and ES-DC-MOG showed no effect.

Figure 19

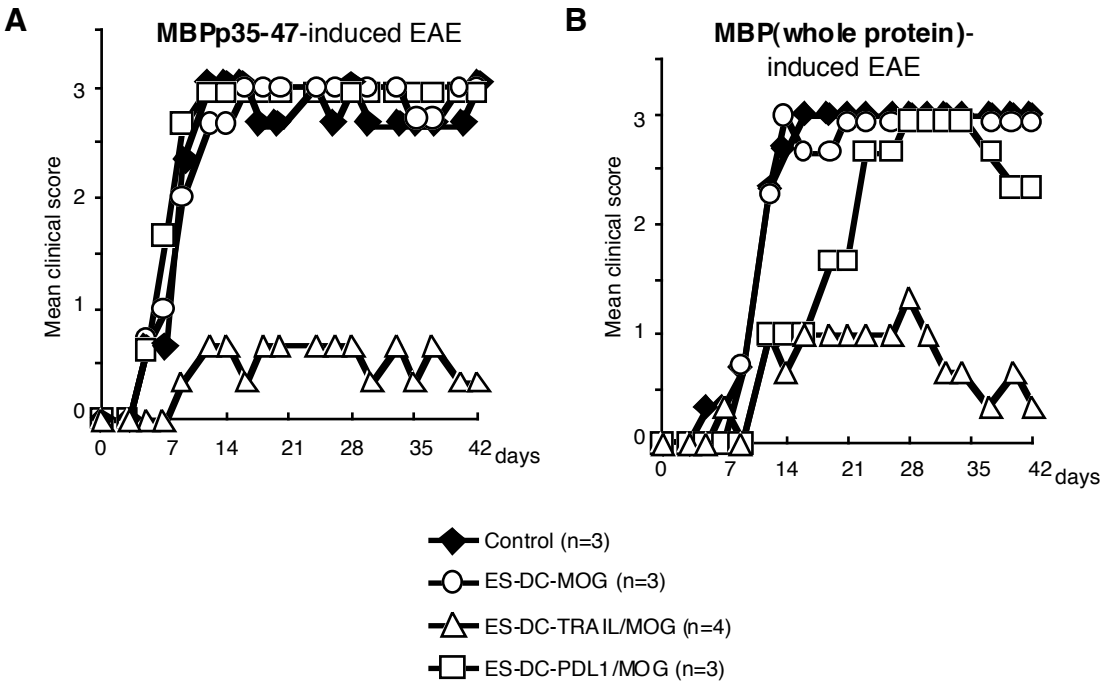

Figure 19. Prevention of MBP-induced EAE by pre-treatment of mice with ES-DC expressing MOG peptide along with TRAIL.

Pre-treatment and induction of EAE was done under the same condition as the experiment shown in Figure 14A, except that MBP was used instead of MOG for induction of EAE. CBF1 mice (3-4 mice per group) were injected with ES-DC-MOG, ES-DC-TRAIL/MOG, ES-DC-PDL1/MOG, or RPMI medium (control) before induction of EAE. (A) MBP p35-47 peptide (600 □g) was used for immunization. (B) Whole MBP protein (600 □g) was used for immunization. The data are each representative of at least two independent experiments with reproducible results.

9-9) Prevention of MOG-induced EAE by transfer of $CD4^+$ T cells from mice treated with ES-DC expressing MOG peptide along with TRAIL.

To test further the possibility that T cells with some regulatory activity was induced or expanded by the in vivo treatment with ES-DC-TRAIL/MOG, we did adoptive transfer experiments. We isolated $CD4^+$ T cells from spleens of mice treated with ES-DC and transferred them to naïve mice. Subsequently, the recipient mice were subjected to an immunization procedure for MOG-induced EAE (Figure 20A). Transfer of 2.5 x 10^6 $CD4^+$ T cells (Figure 20B) isolated from mice treated with ES-DC-TRAIL/MOG significantly reduced the severity of EAE of the recipient mice. On the other hand, $CD4^+$ T cells isolated from mice treated with ES-DC-PDL1/MOG or ES-DC-MOG, or those from untreated mice showed no effect. These results support the notion that $CD4^+$ T cells with some regulatory activity were induced or expanded by the treatment with ES-DC-TRAIL/MOG.

Figure 20

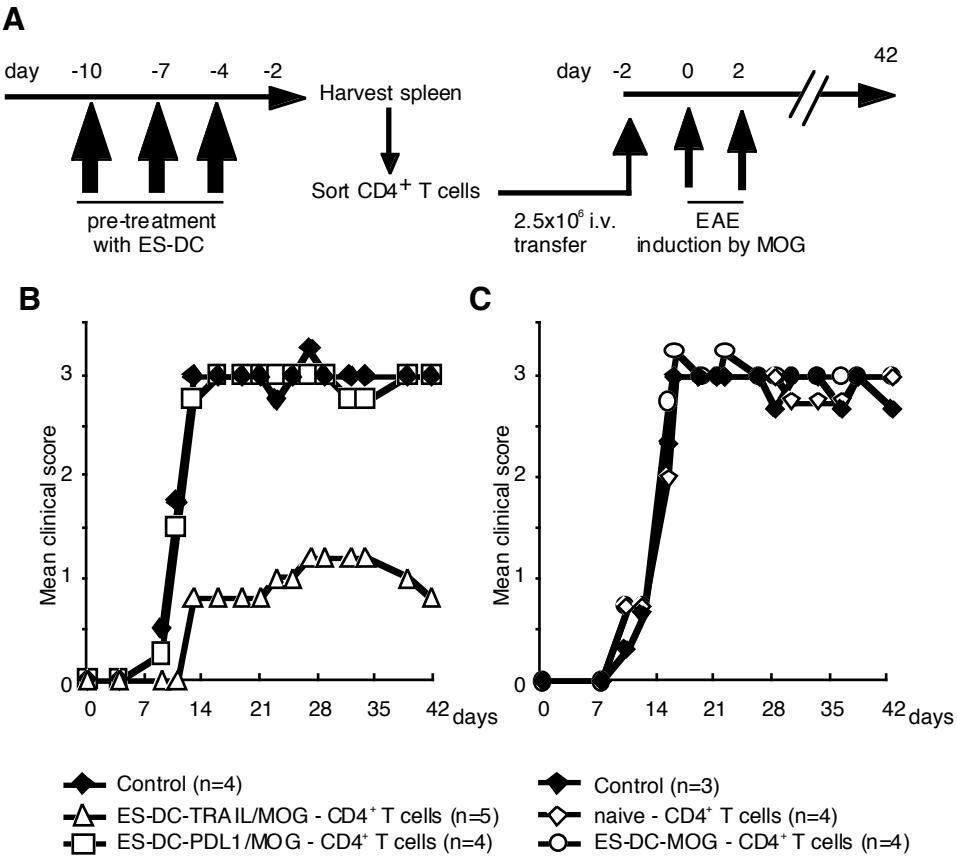

Figure 20. Protection from MOG-induced EAE by adoptive transfer of $CD4^+$ T cells from mice treated with ES-DC expressing MOG peptide along with TRAIL.

(A) The schedule for treatment of donor mice with ES-DC, adoptive transfer of $CD4^+$ T cells, and induction of EAE in recipient mice is shown. Donor CBF1 mice (3 mice per group) were i.p. injected with ES-DC ($1x10^6$ cells/injection/mouse) on days -10, -7, and -4. Splenic $CD4^+$ T cells were isolated from the mice and transferred to naïve mice ($2.5x10^6$ cells/mouse) on day -2. The recipient mice (5-3 mice per group) were immunized with MOG on days 0 and 2 according to the EAE-induction protocol described above. (B) Disease severity of mice transferred with $CD4^+$ T cells from mice treated with ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG, or mice injected with RPMI medium (control) is shown. (C) Disease severity of mice transferred with $CD4^+$ T cells from naïve mice or mice treated with ES-DC-MOG, or injected with RPMI medium (control) is shown. The data are each representative of two independent and reproducible experiments.

10. Discussion

Dendritic cells are the most potent antigen presenting cells 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 antigen-loaded DC with a tolerogenic character is regarded as a promising therapeutic means to negatively manipulate immune response in an antigen-specific manner. Various culture procedures used to generate DC with a tolerogenic character have been reported (1-6). 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 co-stimulatory molecules, and induced T cell-anergy in vitro and tolerance to specific antigens or allogeneic transplanted organs in vivo. In human monocyte-derived immature DC loaded with antigenic peptides and transferred in vivo have been shown to cause the antigen-specific immune suppression (7).

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 (FasL), indoleamine 2, 3-dioxygemase (IDO), IL-10, or CTLA4Ig by gene-transfer has been also reported (8-11). 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 (12).

Regarding methods for gene-transfer to DC, electroporation, lipofection, and virus vector-mediated transfection have been reported (8-12, 61). 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 antigen-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 antigen-specific manner, it is desirable to introduce multiple expression vectors to generate stable transfectant DC, which continuously present transgene-derived antigen and simultaneously express immuno-suppressive 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, I demonstrated that this methodology worked very effectively for induction of anti-tumor immunity, showing highly efficient stimulation of antigen-specific T cells by in vivo transfer of ES-DC expressing T cell-attracting chemokines along with antigen (48).

The present study demonstrates the usefulness of the genetically modified DC generated by this method for the treatment of subjects with autoimmune disease. I 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 post-treatment of mice with such ES-DC, I succeeded in preventing an autoimmune disease model, EAE induced by immunization with MOG peptide (Figure 14, 15 and Table III). Down-modulation of immune response by treatment with genetically modified ES-DC did not affect the immune response to irrelevant exogenous antigen, KLH (Figure 16B). Thus, I achieved the prevention of EAE without decrease in the immune response to an irrelevant antigen.

As for the function of TRAIL, induction of apoptosis has been reported by several groups (12, 31, 32, 62). I 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 medium (control), as shown in Figure 18. The result is consistent with a recent report by Liu, Z. et al.(12). 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 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 experiments suggest that ES-DC-TRAIL/MOG induced T cells with protective effects against EAE. I isolated splenic $CD4^+$ T cells from ES-DC-TRAIL/MOG-treated mice and adoptively transferred them to naïve mice. The severity of subsequently induced EAE in the recipient mice was significantly reduced by this treatment (Figure 20). 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 (Figures 6 and 21). However, to clarify the precise mechanism or character of the T cell with regulatory function, further investigations are necessary.

Figure 21

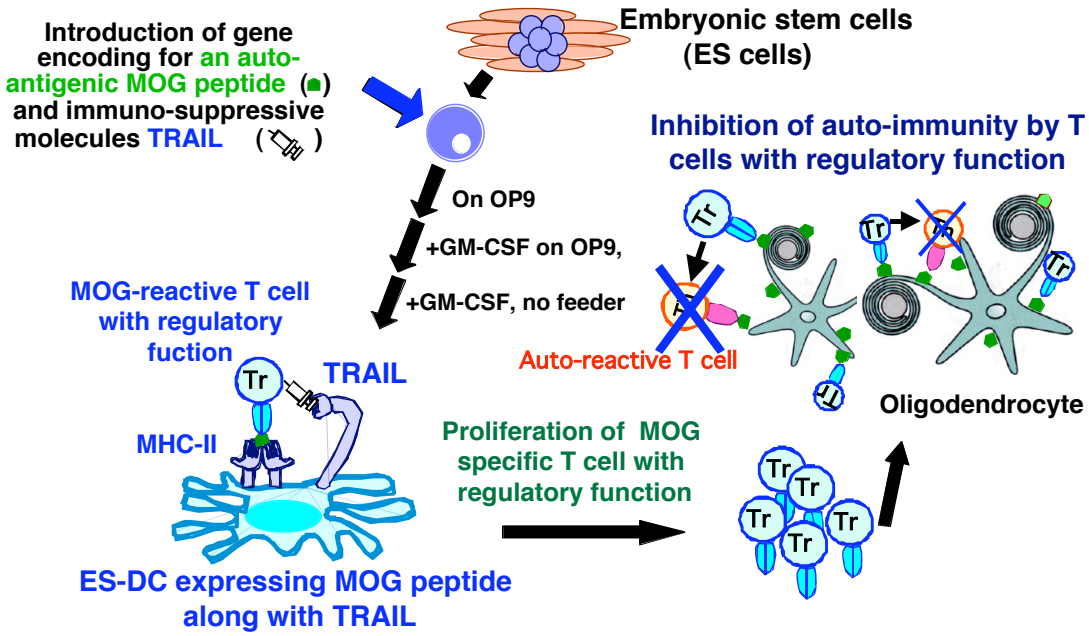

Figure 21. Prevention of EAE by T cells with regulatory function induced by ES-DC expressing MOG peptide along with TRAIL.

On the other hand, in case of treatment with ES-DC-PDL1/MOG, neither apoptosis of T cells nor induction of transferable disease-preventing T cells was observed (Figure 20B). I 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 (35, 42, 63-65).

To determine if the profile of cytokine production was altered by treatment with ES-DC, I did ELISA to quantify IL-10, IL-4 and IFN-□produced by spleen cells of ES-DC-treated mice upon stimulation with MOG peptide in vitro. I 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 months. 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(30). 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 non-human primate ES cells and also for genetic modification of them. I 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, antigen-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.

11. Conclusion

In this study, I established the genetically modified ES-DC presenting antigen peptide, MOG p35-55 peptide, in the context of MHC-II molecules and simultaneously expressing immune-suppressive molecules, TRAIL or PD-L1. I demonstrated a strategy to prevent EAE by pre-treatment of mice with genetically modified DC. In the result, 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 without any effects on response to irrelevant exogenous antigen (KLH). In addition, the severity of myelin basic protein-induced EAE was also reduced by pretreatment with ES-DC-TRAIL/MOG, and the adoptive transfer of $CD4^+$ T cells from ES-DC-TRAIL/MOG-treated mice protected the recipient naïve mice from subsequent MOG-induced EAE. These results demonstrate the prevention of an autoimmune disease by treatment with ES-DC expressing target autoantigen along with immune-suppressive molecules, and implicate future clinical application of this technology.

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