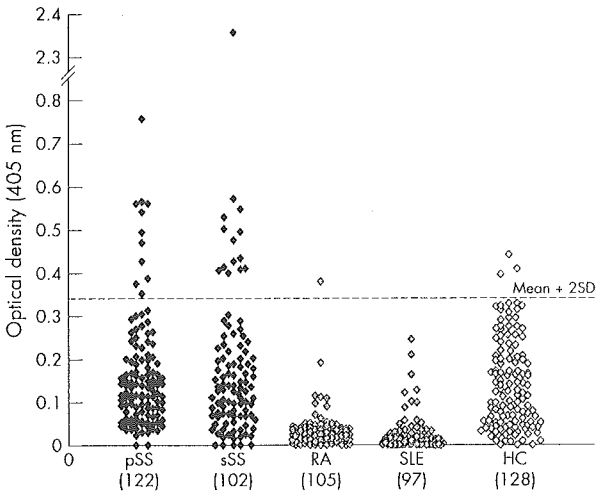

Figure 1 Optical density. pSS, primary Sjögren's syndrome; sSS, secondary Sjögren's syndrome, RA, rheumatoid arthritis, SLE, systemic lupus erythematosus, HC, healthy controls. Numbers in parentheses represent the number of patients in each group.

activate nitric oxide synthase coupled to the lachrymal gland M3R, suggesting that anti-M3R Abs are a new marker of dry eye SS.[4] The M3Rs are expressed on salivary and lachrymal glands, and thus they should be key receptors involved in the production of saliva and tears after stimulation of acetylcholine. Thence, autoantibodies against M3R could interfere with the production of saliva and tears. To test this hypothesis we analysed the prevalence of anti-M3R Abs in patients with SS.

Approval for this study was obtained from the local ethics committee and written informed consent was obtained from all patients and volunteers who participated in this study.

METHODS

Serum samples were collected from 122 Japanese patients with primary SS and 102 Japanese patients with secondary SS followed up at the Department of Internal Medicine, University of Tsukuba Hospital, Japanese Red Cross Mito Hospital, and Shimosizu National Hospital. All patients with SS satisfied the Japanese Ministry of Health criteria for the classification of SS. We also recruited 105 patients with rheumatoid arthritis, 97 with systemic lupus erythematosus, and 128 healthy subjects from our University.

A 25mer peptide (KRTVPPGECFIQFLSEPTITFGTAI) corresponding to the sequence of the second extracellular loop domain of the human M3R was synthesised (Kurabo Industries, Osaka, Japan). As a negative peptide, a 25mer peptide (SGSGSGSGSGSGSGSGSGSGSGS) was also synthesised (Kurabo Industries). Peptide solution (100 µl/well at 10 µg/ml) in 0.1 M Na_2CO_3 buffer, pH 9.6, was adsorbed to a Nunc-Immuno plate (Nalge Nunc International, Rochester, NY) at 4°C overnight, and blocked with 5% bovine serum albumin (Wako Pure Chemical Industries, Osaka) in phosphate buffered saline (PBS) for 1 hour at 37°C. Serum at 1:50 dilution in blocking buffer was incubated for 2 hours at 37°C. The plates were then washed three times with 0.05% Tween 20 in PBS, and 1 µl of alkaline phosphatase conjugated goat antihuman IgG (Fc; American Qualex, San Clemente, CA) diluted 1:1000 in PBS was added for 1 hour at room temperature. After extensive washing, 100 µl of *p*-nitrophenyl phosphate (Sigma, St Louis, MO) solution (final concentration 1 mg/ml) was added as alkaline phosphatase substrate. Plates were incubated for 1 hour at room temperature and the optical density at 405 nm was measured by plate spectrophotometry (Bio-Rad Laboratories, Hercules, CA; fig 1). Determinations were performed in triplicate and standardised between experiments.

RESULTS AND DISCUSSION

The 25mer synthetic amino acid encoding the second extracellular domain of M3R was used as the antigen, because this portion has an important role in intracellular signalling.[6] The binding activity of Abs to the second extracellular domain of M3R is dependent on the concentration of Abs using serial-diluted quantitative assay (data not shown). Figure 1 shows that Abs against M3R were more commonly detected in the serum of patients with primary (11/122 (9%), $p<0.05$) and secondary SS (14/102 (14%), $p<0.05$) than in those with other autoimmune diseases such as rheumatoid arthritis (1/105 (1%)) and systemic lupus erythematosus (0/97 (0%)), or healthy subjects (3/128 (2%)). These results clearly showed that autoantibodies against M3R are specifically present in SS, suggesting that anti-M3R Abs could be used as a diagnostic marker in a subgroup of patients with SS (9–14%). The proportions of patients positive for anti-M3R Ab and anti-SSA Ab, anti-SSB Ab, rheumatoid factor, and antinuclear factor were 68%, 29%, 57%, and 83%. In contrast, the proportions of patients negative for anti-M3R Ab with these autoantibodies were 65%, 6%, 59%, and 76%, respectively. Thus, anti-SSB Ab is strongly associated with anti-M3R Ab ($p<0.05$), although the homology between SSB and the M3R molecule is very low and the detailed mechanism remain unclear. The clinical feature is not significantly different between in patients with SS positive for anti-M3R Ab and negative patients.

In conclusion, we detected autoantibodies against M3R in a subgroup of patients with SS, suggesting that anti-M3R Ab could be used as a new diagnostic marker for SS. Further experiments on the functional analysis of anti-M3R Abs in SS using M3R transfectant cell lines should shed light on the relationship between the presence of anti-M3R autoantibodies and the pathogenesis of SS.

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CONCISE REPORT

Association of mannose binding lectin (MBL) gene polymorphism and serum MBL concentration with characteristics and progression of systemic lupus erythematosus

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Objective: To determine whether occurrence, characteristics, and progression of systemic lupus erythematosus (SLE) are associated with polymorphism of the mannose binding lectin (MBL) gene and with serum MBL concentration.
Methods: Codon 54 MBL gene polymorphism of 147 patients with SLE and 160 healthy controls was determined by polymerase chain reaction-restriction fragment length polymorphism. Serum concentration of MBL was measured by enzyme immunoassay. Fluctuations of serum MBL were analysed with respect to disease characteristics and activity.
Results: Frequency of homozygosity for codon 54 minority allele was 6% (9/147) in patients with SLE, and significantly higher than in controls (p=0.0294, Fisher's exact test). MBL polymorphism in patients with SLE was not significantly associated with disease characteristics or immunological phenotypes. Patients homozygous for the B allele tended to have a higher risk of infection during treatment. Levels of C3 and CH_{50} were slightly, but significantly, associated with serum MBL concentration in patients with SLE homozygous for the majority allele. During the course of SLE, serum MBL concentration increased in 6/14 patients, and decreased in 7 after initiation of immunosuppressive treatment.
Conclusions: MBL gene polymorphism influences susceptibility to SLE, but has no direct effect on disease characteristics. Serum MBL levels fluctuate during the course of SLE in individual patients. MBL genotyping may be useful in assessing the risk of infection during treatment of SLE.

Mannose binding lectin (MBL) is a molecule that shares many features with C1q. MBL comprises a trimer of three identical polypeptides, and several trimers further combine to form a bouquet-like structure.[1] MBL mediates lectin dependent activation of the complement pathway,[1] and has an important role in host defence against micro-organisms. People lacking this protein could develop severe episodes of bacterial infections from early life.[2] Several polymorphisms have been reported for the MBL gene, and a large interindividual difference in serum MBL concentration is caused by the possession of variant alleles. Codon 52, 54, and 57 polymorphisms are all on exon 1 of the MBL gene, and the presence of any of the minority alleles significantly reduces serum MBL concentration. Furthermore, homozygosity for minority alleles results in almost complete deficiency of serum MBL.[3] This has been attributed to increased degradation of the mutated protein.[4]

Recently, several studies have suggested that possession of MBL minority alleles may be associated with occurrence of systemic lupus erythematosus (SLE).[5 6] It is known that C1q deficiency is associated with severe symptoms of SLE.[7] Two possible explanations for associations between MBL or C1q deficiency and occurrence of SLE can be proposed: (*a*) MBL and C1q can bind to and initiate uptake of apoptotic cells into macrophages,[8 9] and abnormal clearance of apoptotic cells caused by MBL or C1q deficiency may result in overexpression of autoantigens; (*b*) viral infection is believed to be one of the causes of SLE,[10] and MBL or C1q deficiency may lead to more frequent infections.

This study was conducted on the premise that occurrence, characteristics, and progression of SLE are associated with polymorphism of the MBL gene and with serum MBL concentration. To our knowledge, this is the first study that has measured serum MBL concentration before and after immunosuppressive treatment in patients with newly diagnosed SLE.

PATIENTS AND METHODS

Samples from 147 Japanese patients with SLE followed up at our hospital, were used for the study. All patients fulfilled the 1997 American College of Rheumatology Classification Criteria for SLE. Samples from 160 Japanese healthy volunteers served as controls.

Genomic DNA was purified from peripheral blood leucocytes using the DNAQuick DNA purification kit (Dainippon Pharmaceuticals, Osaka, Japan), and stored at −30°C. Typing of the MBL gene allele was performed by polymerase chain reaction-restriction fragment length polymorphism, according to the method of Madsen *et al*.[3] The wild-type allele was designated allele A, and codon 54 substitution (glycine to aspartic acid) was designated allele B. Previous studies have shown that codon 52 and 57 polymorphisms are not present or extremely rare in the Japanese population.[11] Serum concentration of MBL was measured by a specific enzyme immunoassay using two rabbit polyclonal anti-MBL antibodies as described previously.[12]

Table 1 Codon 54 genotypes of the MBL gene in patients with SLE and healthy controls

	SLE	Healthy controls	p Value
AA + AB	138 (AA; 84, AB; 54)	158 (AA; 101, AB; 57)	0.0294
BB	9	2	
Total	147	160	

Allele A, codon 54 wild type majority allele; allele B, codon 54 variant minority allele.
p Value by Fisher's exact test.

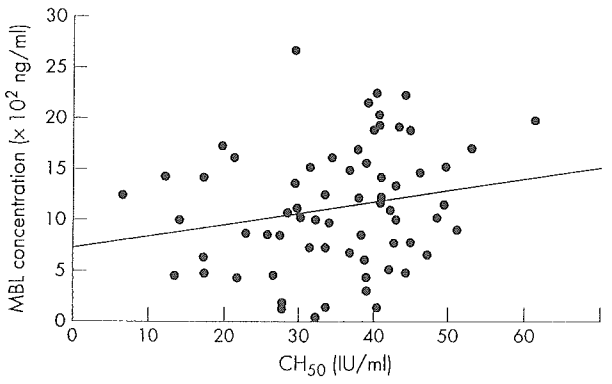

Figure 1 Relationship between serum MBL concentration and CH_{50} in genotype AA patients with SLE. $r_s = 0.253$, $p = 0.0412$ by Spearman's rank correlation test.

Fisher's exact test was used to compare the frequencies of genotypes AA/AB and BB, between disease and control groups, and to compare clinical characteristics between patients with genotypes AA/AB and those with BB. Mann-Whitney's U test was used to compare ages at diagnosis of SLE between patients with genotypes AA/AB and those with BB, and to compare serum MBL concentration between patients and controls of the same genotype. Spearman's rank correlation test was used to compare serum MBL concentration and the levels of anti-DNA antibody, C3, C4, and CH_{50}. Values of $p \leqslant 0.05$ were considered significant.

RESULTS

MBL gene genotypes were studied in patients with SLE and healthy controls (table 1). Among 147 patients with SLE, 9 were homozygous for allele B, which was significantly increased compared with controls ($p = 0.0294$).

We analysed the difference in disease characteristics among patients with SLE categorised by MBL genotypes. Ages (mean (SD)) at diagnosis of SLE tended to be younger in patients with allele B (AA: 32.5 (14.8); AB: 30.7 (15.2); BB: 23.4 (13.3)), but no significant differences were seen ($p = 0.0681$). Clinical characteristics, serological, and immunological measures did not significantly differ between genotype BB patients and other patients with SLE. This is

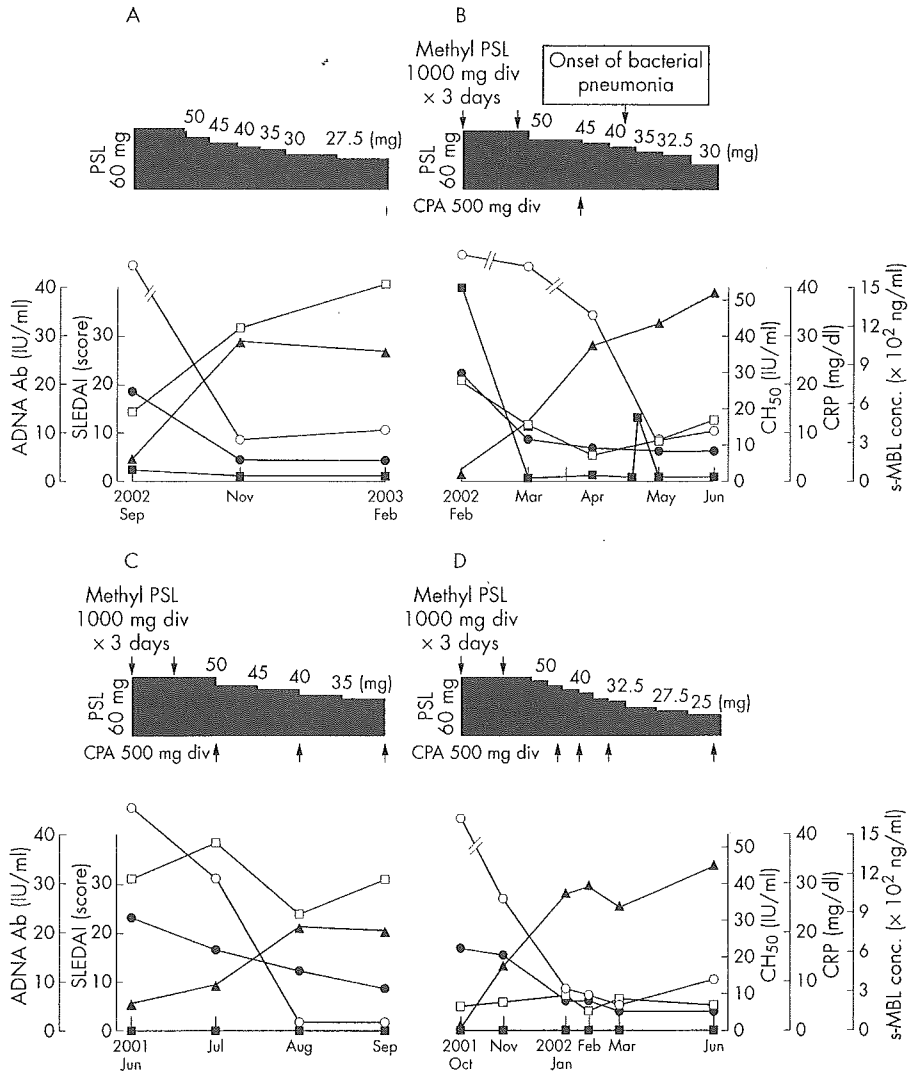

Figure 2 Fluctuation of serum MBL concentration and clinical variables during immunosuppressive treatment in patients with newly diagnosed SLE. Open squares, serum MBL concentrations (s-MBL); closed squares, C reactive protein (CRP); open circles, anti-DNA antibody (ADNA Ab); closed circles, SLE Disease Activity Index (SLEDAI); closed triangles: CH_{50}. PSL, prednisolone; CPA, cyclophosphamide.

most probably because of the small size of the BB cohort. However, incidence of infections requiring admission to hospital was significantly higher in patients with genotype BB than in other patients (genotype AA +AB; 35/132 patients, BB; 5/8 patients, $p = 0.0287$).

Serum MBL concentration reflected the MBL genotype of the individual subject, in accordance with previous reports[3] (data not shown). Among subjects with the same genotype, patients with SLE tended to have a higher MBL concentration than controls, but without statistical significance. The level of CH_{50} was weakly but significantly associated with serum MBL concentration in patients with SLE with genotype AA ($p = 0.0412$) (fig 1). In genotype AA patients, C3 was also associated with serum MBL concentration, although C4 was not (C3; $p = 0.0494$, C4; $p = 0.4265$). No significant relationship between anti-DNA antibody titre and serum MBL was found. In patients with other genotypes, no significant association was seen between serum MBL concentration and levels of anti-DNA antibody or complement components (data not shown).

We studied fluctuation of serum MBL concentration during immunosuppressive treatment in patients with newly diagnosed SLE (fig 2). In patient 1 with genotype AA (fig 2A), serum MBL increased in parallel with CH_{50} after initiation of methylprednisolone treatment, while the SLE Disease Activity Index (SLEDAI) and anti-DNA antibody decreased. In patient 2 with genotype AA (fig 2B), serum MBL concentration decreased after initiation of methylprednisolone pulse therapy, while CH_{50} increased. After CRP decreased to normal levels, MBL gradually increased in parallel with CH_{50}. In patient 3 with genotype AA (fig 2C), serum MBL did not show a clear trend, although disease activity steadily decreased. In patient 4 with genotype AB (fig 2D), serum MBL was low throughout, reflecting the MBL genotype. When the serum MBL concentration before and after immunosuppressive treatment was compared in 14 newly diagnosed patients, it increased in 6/14 patients (genotype AA: 2, AB: 4), and decreased in 7 patients (genotype AA: 5, AB: 2). There was no significant association between increase or decrease of serum MBL concentration and genotypes and clinical phenotypes in patients with SLE (data not shown).

DISCUSSION

Several studies have indicated that MBL gene polymorphism influences susceptibility to SLE.[5 6] When the components of the classical pathway of complement (C1q, C1r, C1s, C4, or C2) are deficient, it has been suggested that abnormal clearance of not only immune complexes[13] but also apoptotic cells[8] contributes to the occurrence of SLE. It has been indicated that inappropriate levels of apoptotic nuclei may be a major source of autoantigens in SLE.[14] Recently, it was reported that MBL can bind to apoptotic cells and initiate their uptake by macrophages,[9] and thus, abnormal clearance of apoptotic cells due to MBL deficiency may provide a source of autoantigens in SLE. However, deficiency of MBL is not an extremely high risk factor, in contrast with deficiencies of other complement molecules such as C1q.[7] The precise consequences of MBL deficiency for the onset and progression of SLE remain unclear. The lag time between occurrence of the first symptom attributable to SLE and diagnosis of definite SLE was reported to be significantly shorter for variant allele carriers than in those with genotype AA.[6] Therefore, the MBL gene may be a disease modifier locus rather than a true SLE susceptibility locus. Although no significant correlation between disease characteristics and MBL genotypes was seen, genotype BB was significantly associated with occurrence of infection in our patients, in accord with a previous report.[6] MBL genotyping may help in assessment of the risk of opportunistic infections in patients with SLE.

The balance of MBL production and consumption determines serum MBL levels. As the presence of MBL deposits in tissues of autoimmune patients has been demonstrated,[15 16] we expected that MBL would be consumed during active disease, and that serum MBL concentration might reflect disease activity and pathological features of SLE in individual patients. To test this hypothesis, we measured serum MBL concentration during immunosuppressive treatment in patients with newly diagnosed disease. As shown in fig 2, serum MBL concentration did fluctuate during the course of immunosuppressive treatment in patients with SLE, especially in genotype AA patients. In patients 1 and 2, the increasing phase of serum MBL concentration may reflect the decreased consumption of MBL while SLE activity gradually decreased, and the decreasing phase may reflect reduced production of MBL because MBL is an acute phase inflammatory protein.[17] Thus, MBL levels appear to reflect disease activity in some patients. The weak but significant association between serum MBL concentration and serum C3 or CH_{50} levels supports this view.

In conclusion, frequency of homozygosity for a minority allele of the MBL gene was increased in patients with SLE compared with controls, confirming previous studies. MBL gene polymorphism may have no direct effect on disease characteristics, but patients homozygous for the minority allele had significantly more frequent episodes of infections. Serum MBL levels did fluctuate during the course of SLE in individual patients, although the mechanism of their fluctuation and their consequences in SLE are unclear. The value of serum MBL monitoring in clinical practice should be determined in future studies.

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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[1]

Shinya Hirata, Satoru Senju, Hidetake Matsuyoshi, Daiki Fukuma, Yasushi Uemura, and Yasuharu Nishimura[2]

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.**

Currently, corticosteroids and other immune suppressants are commonly used for treatment of subjects with autoimmune diseases. The medication with these drugs often leads to systemic immune suppression and consequent opportunistic infections. Thus, it is desirable to develop a therapeutic means to down-modulate immune responses in an Ag-specific manner without causing systemic immune suppression.

Experimental autoimmune encephalomyelitis (EAE),[3] an animal model for human multiple sclerosis, is characterized by neurological impairment resulting from demyelination in the CNS caused by myelin Ag-reactive CD4$^+$ T cells. This disease model is induced by immunization with myelin Ags such as myelin oligodendrocyte glycoprotein (MOG). In the current study, we wanted to try to prevent MOG-induced EAE by treatment of mice with genetically modified dendritic cells (DC). We generated double-transfectant DC presenting MOG peptide in the context of MHC class II molecules and simultaneously expressing molecules with T cell-suppressive property. We tested a strategy to down-modulate the immune response in an Ag-specific manner by in vivo transfer of such genetically modified DC to prevent development of the disease.

For efficient presentation of MOG peptide in the context of MHC class II molecules, we used a previously devised expression vector in which cDNA for human MHC class II-associated invariant chain (Ii) was mutated to contain antigenic peptide in the class II-associated Ii peptide (CLIP) region (1). An epitope inserted in this vector is efficiently presented in the context of coexpressed MHC class II molecules (2). Because they are molecules with a T cell-suppressive property, we tested TRAIL and Programmed Death-1 ligand (PD-L1). TRAIL, a member of the TNF superfamily, is constitutively expressed in a variety of cell types, including lymphocytes, NK cells, and neural cells (3, 4). TRAIL$^{-/-}$ mice are hypersensitive to collagen-induced arthritis and streptozotocin-induced diabetes (5). PD-L1, a ligand for PD-1 and member of the CD28/CTLA-4 family, is expressed on DC, IFN-γ-treated monocytes, activated T cells, placental trophoblasts, myocardial endothelium, and cortical thymic epithelial cells (6, 7). PD-1$^{-/-}$ mice spontaneously develop a lymphoproliferative/autoimmune disease, a lupus-like disease, arthritis, and cardiomyopathy (8, 9). Thus, abrogation of either of these two molecules make mice autoimmune prone, suggesting that these molecules play significant roles

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[3] 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.

0022-1767/05/$02.00

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

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

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

Materials and Methods

Mice

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

Peptides, protein, cell lines, and cytokines

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

Plasmid construction

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

Transfection of ES cells and differentiation of DC from ES cells

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

RT-PCR to detect transgene products

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

Flow-cytometric analysis

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

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

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

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

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

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

Induction of EAE and treatment with ES-DC

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

Immunohistochemical analysis

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

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

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

Statistical analysis

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

Results

Induction of EAE in CBF_1 mice

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

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

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

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

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

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

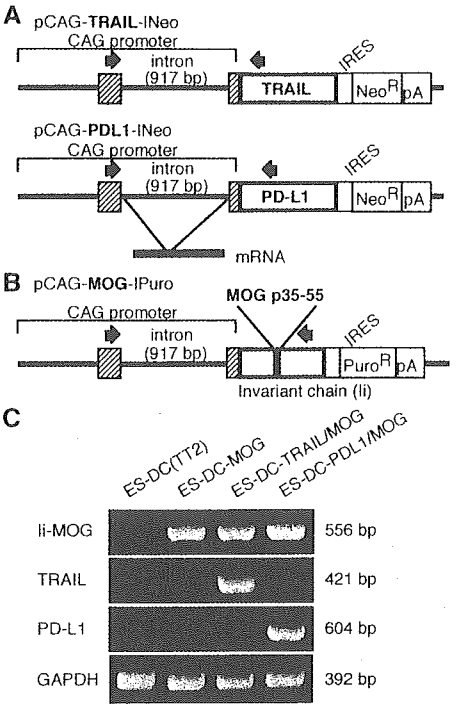

FIGURE 1. Genetic modification of ES-DC to express TRAIL, PD-L1, and Ii-MOG. *A*, The structures of pCAG-TRAIL-INeo and pCAG-PDL1-INeo, the expression vectors for TRAIL and PD-L1, and PCR primers for RT-PCR to detect transgene products are shown. Primer pairs (arrows) were designed to span the intron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

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

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

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

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

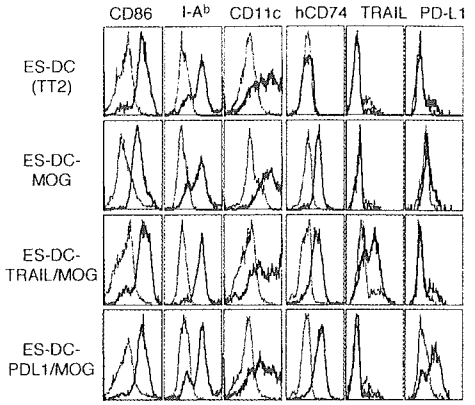

FIGURE 2. Surface phenotype of genetically modified ES-DC. Expression of cell surface CD86, $I\text{-}A^b$, CD11c, TRAIL, and PD-L1 on transfectant ES-DCs was analyzed by flow-cytometric analysis. Expression of mutant human Ii (hCD74) bearing MOG peptide was examined using intracellular staining. Staining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

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

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

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

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

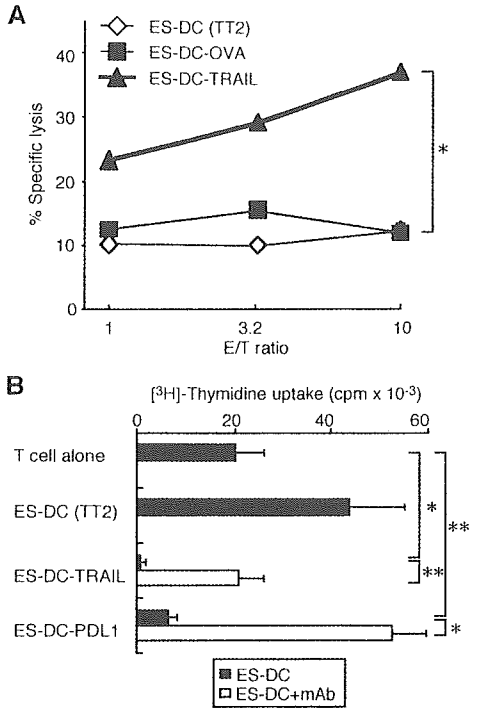

FIGURE 3. Expression of functional TRAIL or PD-L1 in ES-DC transfectants. *A*, The activity of TRAIL expressed in ES-DC was analyzed based on cytotoxicity against L929 cell. ^{51}Cr-labeled target cells (5×10^3 L929 cells) were incubated with ES-DC (TT2), ES-DC-OVA, or ES-DC-TRAIL as effector cells at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

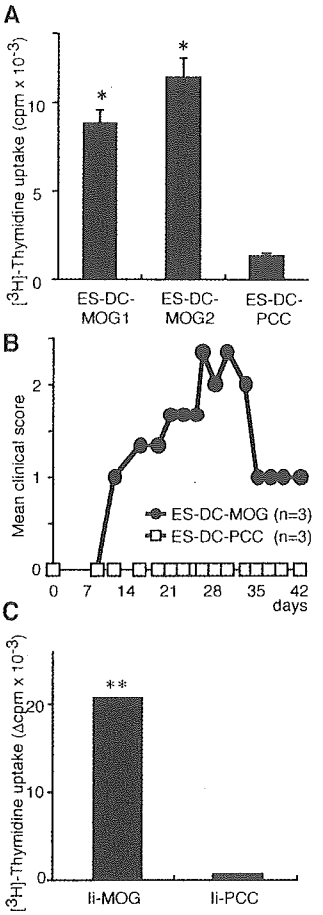

FIGURE 4. Presentation of MOG epitope by ES-DC introduced with Ii-based MOG epitope-presenting vector. *A*, T cells (1.5×10^5) isolated from inguinal lymph nodes of CBF_1 mice immunized according to the protocol for EAE induction were cocultured with one of two independent clones (2×10^4) of ES-DC-MOG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

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

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

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

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

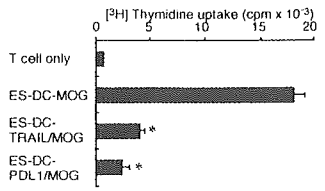

FIGURE 5. Decreased proliferative response to MOG peptide of MOG-reactive T cells cocultured with ES-DC expressing MOG plus TRAIL or MOG plus PD-L1. T cells (2×10^5) isolated from inguinal lymph nodes of CBF_1 mice immunized according to the protocol for EAE induction were cocultured with irradiated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

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

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

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

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

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

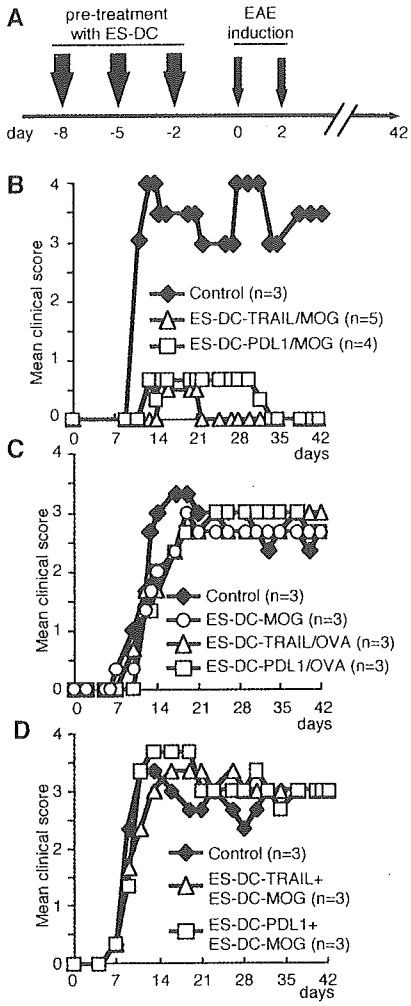

FIGURE 6. Prevention of MOG-induced EAE by pretreatment of mice with ES-DC expressing MOG plus TRAIL or MOG plus PD-L1. *A*, The schedule for pretreatment and induction of EAE is shown. CBF_1 mice (three to five mice per group) were i.p. injected with ES-DC (1×10^6 cells/injection/mouse) on days −8, −5, and −2. EAE was induced by s.c. injection of MOG peptide plus *M. tuberculosis* H37Ra emulsified in IFA on day 0, and i.p. injection of *B. pertussis* toxin on days 0 and 2. *B–D*, Disease severity of mice treated with ES-DC-TRAIL/MOG, ES-DC-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.

absence of Ag) for that of untreated, ES-DC-MOG, -TRAIL/MOG, and -PDL1/MOG-treated mice were 2.8, 2.4, 1.3, and 1.0, respectively. These results suggest that treatment with ES-DC-TRAIL/MOG or -PDL1/MOG inhibited the activation of MOG-specific T cells or reduced their number in mice immunized with MOG peptide and adjuvants.

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

Table II. *Suppression of EAE induction in CBF_1 mice treated with ES-DC*[a]

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

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

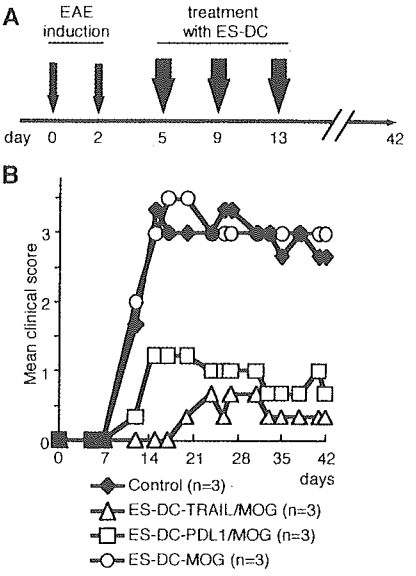

FIGURE 7. Inhibition of MOG-induced EAE by treatment with ES-DC expressing MOG plus TRAIL or MOG plus PD-L1 after immunization with MOG. *A*, The schedule for induction of EAE and treatment is shown. CBF_1 mice (three mice per group) were immunized on days 0 and 2 according to the EAE induction schedule described above, and subsequently i.p. injected with ES-DC (1×10^6 cells/injection/mouse) on days 5, 9, and 13. *B*, Disease severity of mice treated with ES-DC-TRAIL/MOG, ES-DC-PD-L1/MOG, ES-DC-MOG, or RPMI 1640 medium (control) is shown. The data are each representative of two independent and reproducible experiments, and data of all experiments are summarized in Table II.

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

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

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

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

Discussion

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

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

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

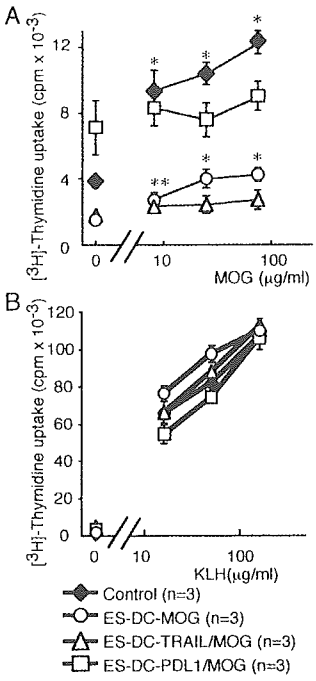

FIGURE 8. Inhibition of activation of MOG-reactive T cells and no effect of activation of KLH-specific T cell by treatment of mice with ES-DC expressing MOG plus TRAIL or PD-L1. *A*, Inguinal lymph node cells (3×10^5) were isolated from CBF_1 mice (three mice per group) of various treatment groups at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

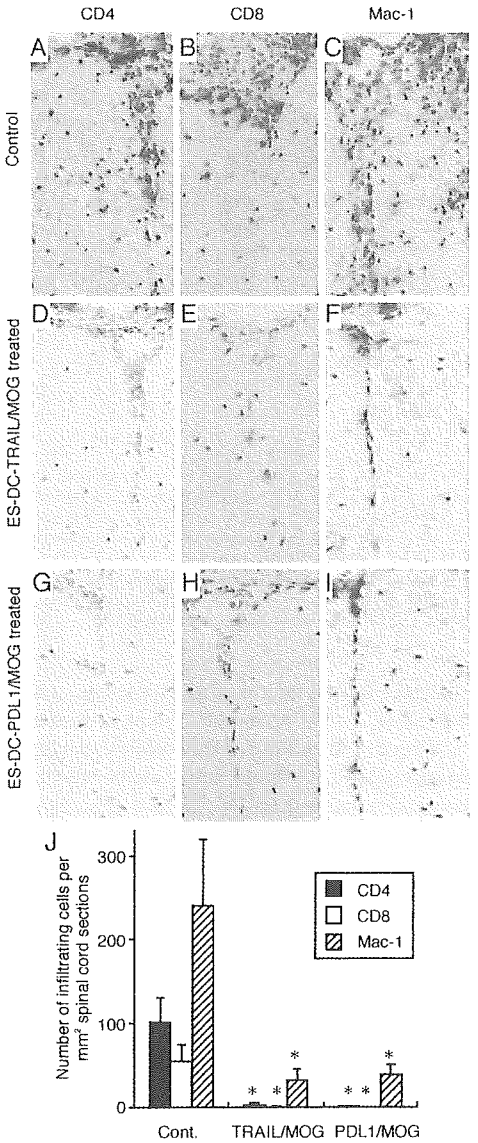

FIGURE 9. Inhibition of infiltration of $CD4^+$ T cells, $CD8^+$ T cells, and Mac-1^+ macrophages into spinal cord by treatment of mice with ES-DC expressing MOG plus TRAIL or PD-L1. Mice were pretreated with ES-DC-TRAIL/MOG, PDL1/MOG, or untreated and subsequently immunized according to the protocol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

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

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

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

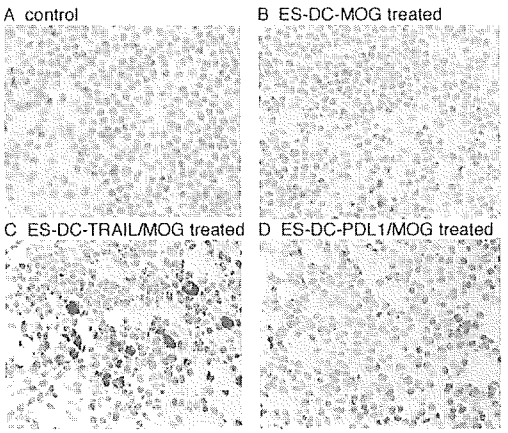

FIGURE 10. Induction of apoptosis of spleen cells by treatment of mice with ES-DC expressing TRAIL along with MOG peptide. Mice were treated with the indicated ES-DC and immunized with MOG peptide, following the schedule described in Fig. 6*A*. On day 11, spleens were isolated from the mice, and apoptotic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

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

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

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

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

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

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A role of kinase inactive ZAP-70 in altered peptide ligand stimulated T cell activation

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Abstract

T cell activation signals induced by altered peptide ligands (APLs) are different from those induced by the original agonistic peptide. The characteristics of the former are partial phosphorylation of TCR-ζ and no tyrosine-phosphorylation of ζ-associated protein-70 (ZAP-70). To analyze further those signaling pathways, we introduced a dominant negative (DN) form of ZAP-70 into a human $CD4^+$ T cell clone in which fully and partially agonistic peptide ligands have been well characterized. We found that some over-expressed partially agonistic ligands (OPALs) induced T cell responses without tyrosine-phosphorylation and kinase activation of ZAP-70. However, those responses were inhibited in T cells expressing DN ZAP-70, which could associate with partially phosphorylated TCR-ζ. In OPAL-stimulated T cells, PLC-γ1 was phosphorylated and it was suppressed by DN ZAP-70 expression, suggesting that the ZAP-70-TCR-ζ association mediates the activation of PLC-γ1 leading to T cell responses even in the absence of kinase activation of ZAP-70.

Keywords: Altered peptide ligand; TCR-ζ; ZAP-70; Phospholipase Cγ1; T cell activation

The engagement of the TCR with an antigenic peptide bound to class II MHC molecules activates the intracellular signaling cascade of biochemical events that trigger cytokine production, changes in the expression levels of cell surface molecules, and cell proliferation. Early signal transduction through the TCR is initiated by the phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCR-ζ chains by Src kinases [1–3]. Subsequently the doubly phosphorylated ITAMs of TCR-ζ chains provide binding sites for ZAP-70 through the interaction of its tandem Src homology 2 (SH2) domains to recruit ZAP-70 to the TCR-ζ [4–7]. The recruitment and activation of ZAP-70 molecules contribute to the activation of a cascade of downstream signals that are crucial for the initiation of cellular responses.

Altered peptide ligands (APLs), which have modifications in the original antigenic peptide, can be divided into different classes based on the potency for the induction of T cell responses; full agonist, partial agonist, and antagonist [8–11]. Cytokine production [12–15], the up-regulation of some cell surface molecules [16–18], and down-modulation of TCR [19–22] correlated with the capacity of each APL to induce TCR signaling. The difference in the TCR signal transduction between partial agonists and full agonists is characterized by the phosphorylation status of TCR-ζ and ZAP-70. While fully agonistic stimulation induces two forms (p21 and p23) of phosphorylated TCR-ζ and ZAP-70 phosphorylation, the partially agonistic stimulation induces the incomplete phosphorylation (only p21 form) of TCR-ζ and no tyrosine-phosphorylation of ZAP-70 [23–25]. Subsequently, the partially agonistic ligands thereby induce only a partial activation of T cells.

We previously found that stimulation with L cell clones over-expressing partially agonistic ligand (OPAL) covalently linked with HLA-DR4 induced proliferation and cytokine production of the cognate T cell clone without tyrosine-phosphorylation and activation of ZAP-70 [26].

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The question arose as to whether or not T cell activation stimulated with OPAL was independent of ZAP-70. To answer this question, we utilized the human $CD4^+$ T cell clone expressing dominant negative (DN) ZAP-70. Notably, the expression of DN ZAP-70 markedly inhibited T cell activation induced with OPAL. We therefore presume that the incompletely phosphorylated TCR-ζ chain associated with ZAP-70 plays an important role in the TCR signaling cascade leading to the observed T cell activation stimulated with OPAL.

Materials and methods

Cell lines and cell culture. A human $CD4^+$ T cell clone, T5-32 derived from *Herpesvirus saimiri* transformed T cell clone (YN5-32), was maintained in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 20% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 U/ml of recombinant human IL-2 kindly provided by Dr. Tomoko Ejima of Ajinomoto Co., Inc. YN5-32 cells recognize and respond to streptococcal peptide M12p54-68 (NRDLEQAYNELSGEA) in the context of HLA-DR4 (DRA/DRB1*0406) and were established, as previously described [16]. Mouse L cells expressing HLA-DR4 alone (L-DR4), HLA-DR4 covalently linked with either peptide M12p54-68 (M12DR4) or with its analogues Q59GDR4 or Y61VDR4 were established, as previously described [26]. The linker for activation of T cells (LAT) and the SH2 domain containing leukocyte-specific phosphoprotein of 76 kDa (SLP-76) deficient Jurkat cell lines, JCaM2.5 and J14, were donated by Dr. Arthur Weiss.

Antibodies. The following antibodies were used in this study: anti-PLC-γ1 mAb, and anti-phosphotyrosine mAb, 4G10 (Upstate Biotechnology, Lake Placid, NY), anti-FLAG mAb (Sigma, St. Louis, MO), anti-human ZAP-70 mAb (Transduction Laboratories, San Diego, CA), anti-ZAP-70 Ab (Santa Cruz, CA), anti-phospho-PLC-γ1 (Tyr783) Ab (Cell Signaling Technology, Beverly, MA), goat anti-mouse IgG Ab (PIERCE, Rockford, IL), PE-conjugated anti-IFN-γ mAb (Immunotech, Marseille, France), PE-conjugated mouse IgG, anti-TCR-αβ mAb, and anti-CD3ε mAb (PharMingen, San Diego, CA).

Plasmids, generation of pseudovirus, and infection. The retroviral vector (pMX-IRES-GFP) and MLV-gagpol-IRES-bsr [27] were a kind gift from Dr. Toshio Kitamura of The University of Tokyo. The amphotropic envelope glycoprotein expression vector, SV-A-MLV-Env [28], was kindly provided by Dr. Nathaniel R. Landau of Salk Institute. The dominant negative (DN) ZAP-70 has amino acid residues 1–276 of ZAP-70 consisting of two tandem SH2 domains without the kinase domain followed by FLAG tag and the cDNA was subcloned into pMX-IRES-GFP. The R190K (a non-functional control of DN ZAP-70) has the same construct as that of DN ZAP-70 except for containing a single residue substitution of Arg^{190} to Lys [29,30]. Fifteen micrograms of retroviral vector (pMX-IRES-GFP) was co-transfected with 10 μg MLV-gag-pol expression vector (pGag-pol-IRES-bsr) and 10 μg SV-A-MLV-Env into 293 T cells using Lipofectamine 2000 reagent (Invitrogen). The supernatants were collected at 72 h after transfection. After cell debris had been removed by low-speed centrifugation (2000*g*, 10 min), the supernatants were then further centrifuged at 12,000*g* for 12 h at 4 °C. The pellets were suspended and were added to 1×10^6 of T5-32 in the presence of 6 μg/ml polybrene (Sigma). The expression of the recombinant proteins was monitored by GFP expression and a Western blot analysis using an anti-FLAG mAb.

Flow cytometry. T cells were stimulated by co-culturing with L cells expressing each peptide/HLA-DR4 complex in FCS free medium as previously described [26]. The surface markers were analyzed using FACScan (Becton–Dickinson, Mountain View, CA) and PE-conjugated anti-TCR-αβ mAb. To monitor IFN-γ production, intracellular staining using IntraPrep (Immunotech) was done according to the manufacturer's recommendations. T5-32 cells were stimulated with the L cells as described above in the presence of 20 μg/ml Brefeldin A (Sigma) for 5 h. The cells stained with PE-conjugated anti-IFN-γ were analyzed using FACScan and CELLQuest software (Becton–Dickinson).

Immunoprecipitation and Western blotting. For immunoprecipitation and Western blotting, T5-32 cells (1×10^7) stimulated with each L cell transfectant confluently grown in 15 cm dishes or Jurkat T cells (1×10^7) stimulated with anti-CD3ε mAb were recovered and lysed on ice for 30 min in lysis buffer (20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% NP40, 1 mM Na_3VO_4, 10% glycerol, and a protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany)). The lysates were immunoprecipitated for 3 h at 4 °C with the indicated antibody followed by collection with protein-A beads (Pierce). The immunoprecipitates or whole cell lysates were separated by SDS–PAGE and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h in TBS (150 mM NaCl, 20 mM Tris, pH7.6) containing 5% skim milk, 0.5% bovine serum albumin, and 0.1% Tween 20, and incubated with the indicated primary antibody for 2 h at room temperature. The blots were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig or anti-rabbit Ig antibodies (Amersham Biosciences, Piscataway, NJ). In some experiments, the membranes were stripped and reprobed with respective antibodies followed by incubation with the HRP-conjugated second Ab. Blots were visualized using enhanced chemiluminescence (ECL, Amersham Biosciences).

Results

Differences in the tyrosine-phosphorylation of ZAP-70 and its association with TCR-ζ in T5-32 stimulated with L cell transfectant expressing each HLA-DR4/peptide complex

To investigate the tyrosine-phosphorylation status of ZAP-70 and its association with TCR-ζ in T cells stimulated with the OPALs (Y61VDR4 and Q59GDR4), the T cell clone T5-32 was co-cultured with mouse L cells expressing each HLA-DR4/peptide complex. The L cell transfectant over-expressing fully agonistic ligand (M12DR4) stimulated tyrosine-phosphorylation of ZAP-70 coupled with two forms (p21 and p23) of phosphorylated TCR-ζ (Fig. 1). In contrast, stimulation with L cell

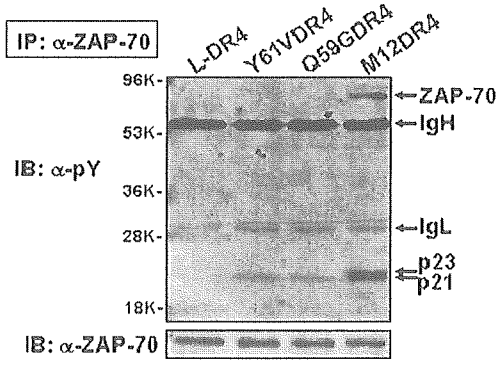

Fig. 1. The induction of tyrosine-phosphorylation of ZAP-70 after the stimulation of T cell clone (T5-32) with each L cell transfectant clone. T5-32 cells (1×10^7) were incubated with the L cell transfectant expressing each HLA-DR4/peptide complex for 5 min at 37 °C. Tyrosine-phosphorylation of TCR-ζ and ZAP-70 in the stimulated T5-32 cells was visualized after immunoprecipitation (IP) with anti-ZAP-70 mAb (α-ZAP-70) and immunoblotting (IB) with anti-phosphotyrosine mAb, 4G10 (α-pY). The positions of ZAP-70 protein, mouse immunoglobulin heavy (IgH) and light (IgL) chains of immunoprecipitating Ab, and phosphorylated TCR-ζ chains (p21 and p23) are indicated on the right. Labels on the left side of the panel indicate the approximate molecular sizes of the marker proteins.

transfectant over-expressing Y61VDR4 or Q59GDR4 did not induce ZAP-70 phosphorylation. Interestingly, the p21 form of phosphorylated TCR-ζ was observed in the ZAP-70 immunocomplexes, thus suggesting that unphosphorylated ZAP-70 could be recruited to the TCR complex by associating with the p21 form of phosphorylated TCR-ζ (Fig. 1). L-DR4 expressing only HLA-DR4 without a covalently linked cognate peptide did not induce the phosphorylation of ZAP-70 nor its association with TCR-ζ. Depending on the phosphorylation status of ZAP-70 and the accompanying TCR-ζ, we distinguished M12DR4-stimulated activation exhibiting fully agonistic properties from either Y61VDR4- or Q59GDR4-stimulated activations exhibiting partially agonistic properties (Table 1). The partially agonistic activation pattern showed incomplete phosphorylation (p21 form) of TCR-ζ and no tyrosine-phosphorylation of ZAP-70, which is in accordance with the findings of previous reports [23–25].

DN ZAP-70 inhibited tyrosine-phosphorylation of ZAP-70 and TCR down-modulation in T cells stimulated with M12DR4

As OPAL stimulation induced neither tyrosine-phosphorylation of ZAP-70 (Fig. 1) nor kinase activation of ZAP-70, as previously reported by us [26], the T cell responses were thus suggested to be independent of ZAP-70. To examine this possibility, we expressed DN ZAP-70 and its non-functional control R190K in T5-32 cells using the retroviral system (Fig. 2A). DN ZAP-70 is a kinase domain truncated mutant, consisting of only two tandem SH2 domains, and inhibits TCR signaling stimulated with anti-TCR mAb in Jurkat cells, while R190K (DN ZAP-70 carrying Arg^{190} to Lys substitution) has no capacity to inhibit TCR signaling [29]. It was previously reported that the $FLVR^{190}E$ sequence is involved in the phosphotyrosyl binding pocket in the SH2 domain of ZAP-70 and that the Arg^{190} to Lys mutation abolished its binding to the ITAM of CD8-ζ chimeric molecule [30]. The stable expression of DN ZAP-70 or R190K did not affect the expression level of endogenous ZAP-70 (Fig. 2B).

We first examined the effects of DN ZAP-70 and R190K on the tyrosine-phosphorylation of endogenous ZAP-70 when stimulated with each TCR ligand. After 72 h of infection, T cells, in which GFP positive cells were approximately 60–65%, were stimulated and ZAP-70 was immunoprecipitated from the lysate. When T cells expressing DN ZAP-70 were stimulated with M12DR4, the phosphorylation of endogenous ZAP-70 was suppressed in comparison to that of the T cells expressing R190K or the mock vector (Fig. 3A).

T cells stimulated with peptide–MHC complexes undergo TCR down-modulation [19–22] and recently the kinase activity of ZAP-70 has shown to be involved in this phenomenon [31]. As expected, the M12DR4 stimulation induced TCR down-modulation and this was markedly suppressed by the expression of DN ZAP-70 (Fig. 3B). On the other

Table 1
Characteristics of the HLA-DR4/peptide complexes used in this study and a summary of T cell responses to L cell clones expressing each HLA-DR4/peptide complex

L cell clones	Peptide sequence covalently linked with HLA-DR4[a]	Antigenicity of peptides pulsed on PBMCs[b]	T cell responses observed in recognition of L cell clones		
			ZAP-70 Phosphorylation	Phospho-TCR-ζ form associated with ZAP 70	T cell proliferation[c]
M12DR4	NRDLEQAYNELSGEA	Full agonist	Detected	p21, p23	+++
Q59GDR4	NRDLEGAYNELSGEA	Partial agonist	Not detected	p21	++
Y61VDR4	NRDLEQAVNELSGEA	Partial agonist	Not detected	p21	+/–
L-DR4	None	Null	Not detected	Not detected	–

[a] Each L cell clone was transfected with genes encoding for HLA-DR4 α-chain and HLA-DR4 β-chain covalently linked with a linker peptide and 15-mer peptide as previously described [26]. The underlined amino acid residues are substituted amino acid residues unique to APLs derived from M12p54-63 peptide. These cells express a large amount of each single species of the HLA-DR4/peptide complexes on their cell surface.
[b] Irradiated HLA-DR4$^+$ peripheral blood mononuclear cells (PBMCs) pulsed with each peptide acted on the YN5-32 T cell clone, from which the T5-32 T cell clone had originated, as indicated in the table as we previously reported [16].
[c] According to Irie et al. [26].

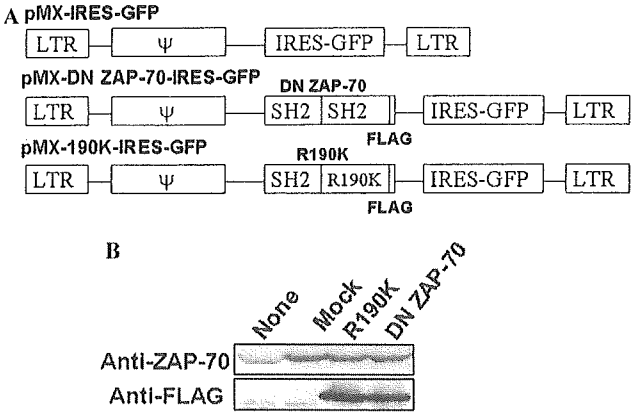

Fig. 2. The retroviral constructs used in this study and quantification of FLAG-tagged ZAP-70 mutants (lacking kinase domain) stably expressed in T cell transfectants in comparison with endogenous ZAP-70. (A) FLAG-tagged DN ZAP-70 and FLAG-tagged R190K (a non-functional control for DN ZAP-70) were subcloned into a retroviral vector containing the internal ribosome entry site (IRES) coupled to green fluorescent protein (GFP). ψ represents the psi sequence necessary for packaging full-length RNA and it also contains splice donor and acceptor sequences. (B) T cells were infected with retroviral vector alone, DN ZAP-70-IRES-GFP, or R190K-IRES-GFP. Two weeks later, 1×10^6 cells from the indicated transfectants were lysed, and the whole cell lysates were then analyzed by immunoblotting with anti-FLAG or anti-ZAP70 mAb. Because the anti-ZAP-70 mAb used recognizes kinase domain of ZAP-70, DN ZAP-70, and R190K could not be detected with the anti-ZAP70 mAb. T cells stably expressing the ZAP-70 mutants did not affect the expression level of endogenous ZAP-70.

hand, expression of R190K had no inhibitory effect on the TCR down-modulation, as observed with the expression of the mock vector. These results clearly showed that the expression of DN ZAP-70 in the T cells efficiently suppressed TCR induced activation of endogenous ZAP-70. The data support the idea that DN ZAP-70 competes with the endogenous ZAP-70 in terms of the binding to tyrosine-phosphorylated TCR-ζ, thus preventing endogenous ZAP-70 from both undergoing tyrosine-phosphorylation and the induction of its kinase activity. The binding of DN ZAP-70 but not R190K to tyrosine-phosphorylated TCR-ζ was confirmed as shown in Fig. 6.

Inhibition by DN ZAP-70 of TCR mediated IFN-γ production and TCR down-modulation in T cells stimulated with OPALs

In our previous study, the stimulation with some OPALs induced IFN-γ production in the cognate T cells without kinase activation of ZAP-70. Therefore, we investigated whether ZAP-70 was involved in the IFN-γ production in OPAL-stimulated T cells using T5-32 cells expressing DN ZAP-70 or R190K. Two weeks after retroviral infection, GFP-positive T cells were >50% (mock), >30% (R190K), and >30% (DN ZAP-70), as determined by flow cytometry. The percentage of intracellular IFN-γ-positive T cells stimulated with the M12DR4 was higher than that of T cells stimulated with Q59GDR4 (Fig. 4A). As shown in Fig. 4A, the IFN-γ production significantly decreased in the T cells expressing DN ZAP-70 in response to stimulation with the OPALs (Q59GDR4 and Y61VDR4) compared with the findings in T cells expressing either the mock control vector or the vector containing the R190K gene. The percentages of IFN-γ-producing cells in GFP-positive T cells stimulated with Y61VDR4 were 2% in DN ZAP-70 expressing T cells, 13% in R190K expressing T cells, and 12% in the mock infected T cells. While the percentages of IFN-γ-producing cells in GFP-positive T cells stimulated with

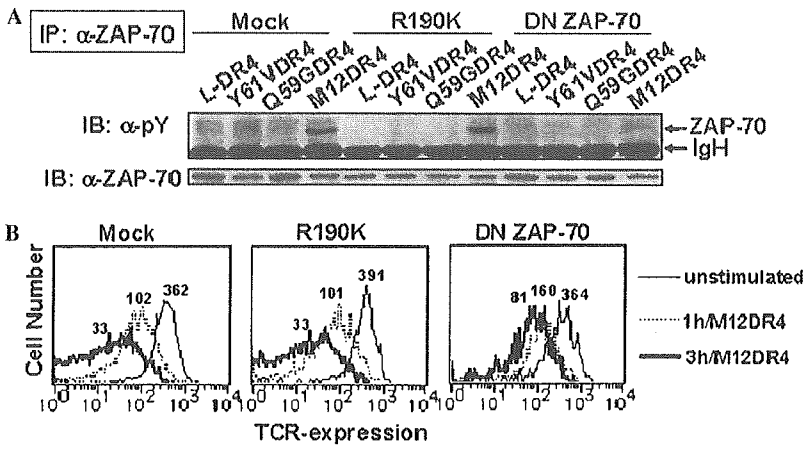

Fig. 3. The inhibitory effects of DN ZAP-70 on T cell activation. (A) The inhibition of endogenous ZAP-70 phosphorylation in T cells expressing DN ZAP-70. After 72 h of infection with retroviral vectors, 3×10^6 T cells (60% of cells were positive for GFP) were stimulated with each L cell transfectant for 10 min at 37 °C. To detect the phosphorylation of ZAP-70, ZAP-70 immunoprecipitates (IP) were analyzed by immunoblotting (IB) with anti-phosphotyrosine mAb. To confirm equal protein loading, the membranes were stripped and reprobed with anti-ZAP-70 mAb. (B) Inhibition of TCR down-modulation by DN ZAP-70. After the infection of the retroviral vectors, T cells were cultured for 2 weeks and then stimulated with L cells expressing M12p54-68/HLA-DR4 (M12DR4) for the indicated times. TCR down-modulation was analyzed using GFP positive cells. The numbers in each flow-cytometric profile indicate the mean fluorescence intensity (MFI) levels.

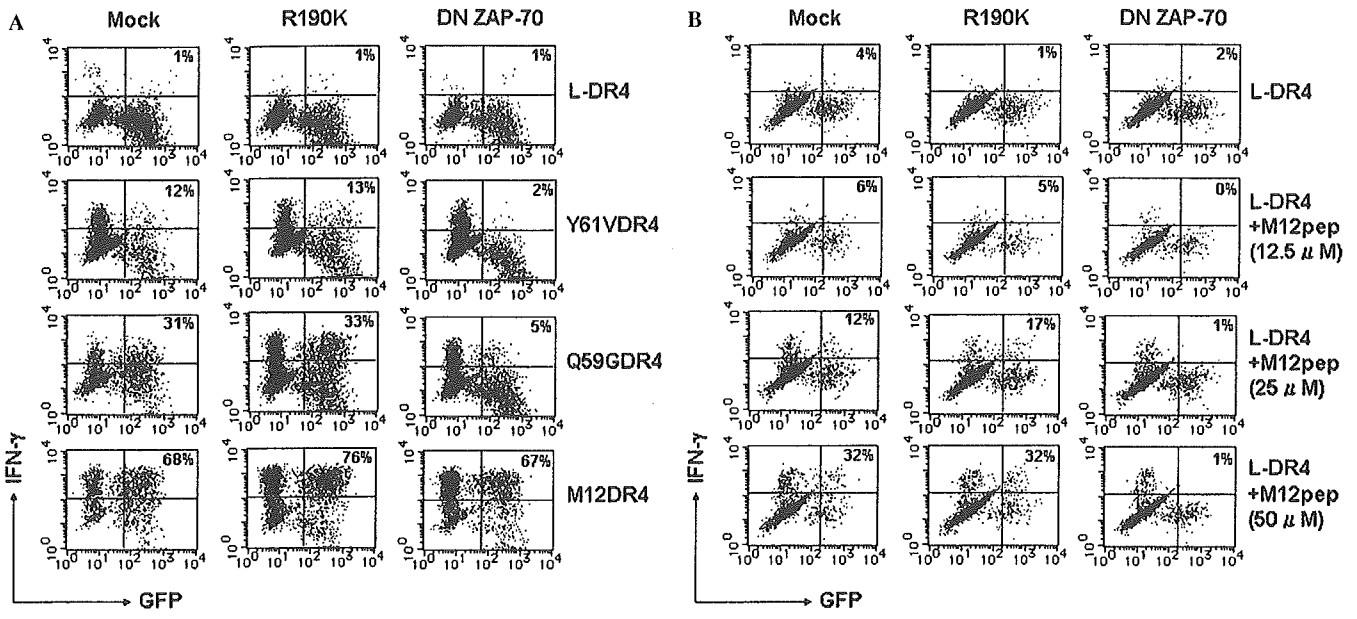

Fig. 4. Inhibition by DN-ZAP-70 of IFN-γ production in T cells stimulated with each L cell transfectant. After the infection of the retroviral vectors, T cells were cultured for 2 weeks and then were stimulated with each L cell transfectant. Intracellular IFN-γ was detected using flow cytometry after a 5 h co-culture of the T cells with each L cell transfectant in the presence of brefeldin A. (A) The effect on IFN-γ production in T cells stimulated with M12DR4 or each OPAL. (B) Effect on IFN-γ production in T cells stimulated with HLA-DR4-expressing L cells (L-DR4) prepulsed with M12p54-68 peptide. L-DR4 cells were prepulsed with the indicated dose of M12 peptide for 16 h. The percentages indicated in the given quadrants represent percentages of IFN-γ-producing cells in GFP positive T cells. The dot blots are representative of three independent and reproducible experiments.

Q59GDR4 were 33% in R190K expressing T cells, and 31% in mock infected T cells, it was markedly suppressed to 5% in the T cells expressing DN ZAP-70. The data indicate that the recruitment of kinase-inactive ZAP-70 to TCR complexes is involved in the IFN-γ production stimulated with the OPALs.

Compared with OPAL-stimulated T cells, no significant difference in the IFN-γ production was observed between DN ZAP-70 (67%) and R190K (76%) expressing T cells stimulated with M12DR4 (Fig. 4A). One reason for the absence of inhibitory effect of DN ZAP-70 on the IFN-γ production in M12DR4 stimulated T cells might be that the T cell activation stimulated with M12DR4 highly over-expressing HLA-DR4/M12p54-68 complexes is too strong to be inhibited by DN ZAP-70. To investigate the effect of DN ZAP-70 on IFN-γ production stimulated with the relatively small numbers of HLA-DR4/M12p54-68 complexes, we checked the effect of DN ZAP-70 on the T cells stimulated with L cells expressing HLA-DR4 (L-DR4) prepulsed with 12.5, 25, or 50 μM of fully agonistic peptide, M12p54-68, for 16 h at 37 °C. As shown in Fig. 4B, the IFN-γ production was abrogated in the T cells expressing DN ZAP-70 and stimulated with L-DR4 prepulsed with M12p54-68 peptide. The percentage of IFN-γ producing T cells expressing DN ZAP-70 and stimulated with the peptide-pulsed L-DR4 was similar to the background level as observed in T cells stimulated with L-DR4, whereas the IFN-γ production increased in a peptide dose-dependent manner in T cells expressing R190K or mock vector in recognition of the same TCR ligand. In our previous observation [26], L cells expressing HLA-DR4 prepulsed with 50 μM M12p54-68 induced proliferation and ZAP-70 phosphorylation of T5-32. These data support the idea that the stimulation with M12DR4 is too strong to be inhibited by the expression of DN ZAP-70.

To further investigate the effect of DN ZAP-70 on OPAL stimulation, we studied the TCR down-modulation in T5-32 cells expressing either DN ZAP-70 or R190K after TCR stimulation. The M12DR4 stimulation induced a strong TCR down-modulation as shown in Fig. 5. In contrast, the stimulation of OPALs (Q59GDR4 and Y61VDR4) induced a weak but definite TCR down-modulation. The expression of DN ZAP-70 inhibited the TCR down-modulation induced with both M12DR4 and OPAL stimulation while the expression of R190K was ineffective. These data indicate that ZAP-70 is thus involved in IFN-γ production and TCR down-modulation in T cells stimulated with OPALs.

Association of the DN ZAP-70 with tyrosine-phosphorylated TCR-ζ chain

The association of the tandem two SH2 domains of ZAP-70 with two phosphorylated tyrosines in the ITAMs of TCR-ζ chain is important in early TCR signal transduction. A previous report showed that DN ZAP-70 was bound to the hyperphosphorylated CD8-ζ chimeric molecule, suggesting that this mutant prevents