Table 2
Comparison of deletion frequency between anti-GPI Abs (+) and anti-GPI Abs (−)

Anti-GPI Abs	39 deletions	Deletions/GPI groups		p values
HC(−)				
1	ds:2	2/13	5/78 (6.4%)	$p < 0.001$
2	d:1	1/19		
3	0	0/16		
4	d:1, ds:1	2/15		
5	0	0/15		
HC(+)				
1	d:4, ds:1	5/16	23/73 (31.5%)	
2	ds:1	1/12		
3	d:5, ds:1	6/12		
4	d:3, ds:3	6/20		
5	d:1, ds:4	5/13		
RA(−)				
1	0	0/18	1/63 (1.6%)	$p < 0.0001$
2	ds:1	1/5		
3	0	0/8		
4	0	0/12		
5	0	0/20		
RA(+)				
1	d:1, ds:2	3/14	22/77 (28.6%)	
2	d:2, ds:2	4/10		
3	ds:2, d:2	4/22		
4	d:6, ds:2	8/17		
5	d:1, ds:2	3/14		

p value by χ^2 test. Anti-GPI Ab Pos. vs. Neg. $p < 0.0001$.
d, deletion with no flame shift. ds, deletion with flame shift was found.

autoantibodies against intact GPI are produced through an epitope spreading mechanism, especially in the inflammatory arthritides [25].

Why does this modification occur? Matsumoto et al. [5] reported that soluble GPI protein level decreased with age in a K/BxN arthritis model, and that such decrease triggered T cell perturbation toward GPI [5]. These changes suggested that protein modification can activate autoreactive T cells. Several groups identified the presence of modified GPI proteins in patients with various conditions [17–22], although we did not identify such proteins in our subjects. Deleted protein of myelin proteolipid protein (PLP) in patients with multiple sclerosis is highly expressed in the thymus, and such expression is associated with induction of autoantibody production by autoreactive T cells [26,27]. It is possible that the spontaneous post-transcriptional modification of autoantigens alters the activation of autoreactive T cells, and hence autoantibody production. In fact, we have already identified GPI reactive T cells in the PBMC of patients with GPI variants (Kori et al., submitted).

In conclusion, our results provided evidence that the presence of GPI variants is associated with the production of autoantibodies against GPI. Although the mechanism of post-transcriptional alteration of GPI antigen remains unclear, the GPI variants seem to play a key role in the generation of anti-GPI Abs. Further research using recombinant GPI variant proteins should shed more light on the mechanisms underlying the production of pathogenic antibodies against autoantigens.

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Expression of Tristetraprolin (G0S24) mRNA, a Regulator of Tumor Necrosis Factor-α Production, in Synovial Tissues of Patients with Rheumatoid Arthritis

AKITO TSUTSUMI, EIJI SUZUKI, YOSHIHIRO ADACHI, HIDEYUKI MURATA, DAISUKE GOTO, SATOSHI KOJO, ISAO MATSUMOTO, LEI ZHONG, HIROSHI NAKAMURA, and TAKAYUKI SUMIDA

ABSTRACT. ***Objective.*** To determine the significance of tristetraprolin (TTP) gene expression in synovial tissues of patients with rheumatoid arthritis (RA).

Methods. Gene expression was examined in synovial tissue and peripheral blood lymphocytes of a patient with RA by differential display-polymerase chain reaction (PCR). One of the identified genes, TTP, was selected for further analysis. cDNA was prepared from synovial tissues of 22 patients with RA and 22 with osteoarthritis (OA). Expression of TTP and tumor necrosis factor-α (TNF-α) genes was measured by TaqMan real-time semiquantification PCR. In RA samples, expression of TTP mRNA was compared with TNF-α mRNA, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and steroid and/or disease modifying antirheumatic drug use.

Results. Expression of TTP gene was significantly higher in synovial tissues of RA patients than in OA. There was no apparent relationship between expression of TTP and TNF-α genes. TTP gene expression had a tendency to be inversely correlated with serum CRP, measured immediately before surgery. In addition, CRP was higher in patients with a low TTP/TNF-α gene expression ratio ($p = 0.0071$, Spearman rank correlation).

Conclusion. A low TTP/TNF-α gene expression ratio could indicate failure of RA patients to produce adequate amounts of TTP in response to increased TNF-α production. Inappropriate TTP production may be one factor that contributes to higher RA disease activity. (J Rheumatol 2004;31:1044–9)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
DIFFERENTIAL DISPLAY
TRISTETRAPROLIN
TUMOR NECROSIS FACTOR-α

Rheumatoid arthritis (RA) is a chronic disorder of unknown pathogenesis associated with polyarthropathy. Persisting inflammation in the joints may lead to total destruction of joints, causing a great reduction of quality of life of patients with RA. Among a number of cytokines involved in the pathogenesis of arthritis in RA, tumor necrosis factor-α (TNF-α) is one of the most important. TNF-α is produced by macrophages and synovial cells, and induces an array of inflammatory cytokines, chemokines, adhesive molecules, and proteinases. TNF-α is present in synovial fluids of patients with RA[1-3], but not in those of patients with osteoarthritis (OA)[3]. Interestingly, while transgenic mice carrying the complete human TNF-α gene develop normally, transgenic mice carrying a 3' modified human TNF-α transgene have dysregulated TNF-α expression and develop chronic inflammatory arthritis resembling human RA, which could be completely prevented by treatment with anti-TNF-α antibodies[4]. Thus, dysregulated TNF-α production is responsible for arthritis in this animal model, and the 3' region of the TNF-α gene is important for properly controlled production of this cytokine. Subsequent studies and the recent introduction of anti-TNF-α therapies have shown that this cytokine plays a key role in the pathogenesis of human RA, and is a potential target for therapy. However, a better understanding of the disease is needed to develop new methods of therapies, and to prevent total destruction of the joints.

To investigate the molecular mechanisms involved in the pathogenesis of RA, and to search for possible targets for RA-specific therapies, we focused on identifying genes that are specifically expressed in synovial tissues of patients with RA. We used the differential display-polymerase chain reaction (DD-PCR) method to identify known and unknown genes specifically expressed in a given tissue[5,6]. Among a

From the Division of Rheumatology, Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, Tsukuba; and Institute of Clinical Sciences, St. Marianna University, Kawasaki, Japan.

A. Tsutsumi, MD, Associate Professor; E. Suzuki, MD, PhD Student; Y. Adachi, MD, Lecturer; H. Murata, MD, Lecturer; S. Kojo, PhD, Laboratory Scientist; I. Matsumoto, MD, Lecturer; D. Goto, MD, Instructor; L. Zhong, MD, Laboratory Scientist; T. Sumida, MD, Professor and Head, Division of Rheumatology, Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba; H. Nakamura, MD, Lecturer, Institute of Clinical Sciences, St. Marianna University.

Address reprint requests to Dr. A. Tsutsumi, Division of Rheumatology, Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan.
E-mail: atsutsum@md.tsukuba.ac.jp

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number of candidate genes identified, we focused on tristetraprolin (TTP), since TTP is an intracellular protein involved in the degradation of TNF-α mRNA. TTP binds to the 3' untranslated region (3'-UTR) of the TNF-α mRNA, and induces its instability, as reviewed by Blackshear[7]. Thus, TTP is a natural regulator of TNF-α production[8], and theoretically may have a protective role in the inflammatory process of RA. We report that TTP mRNA is highly expressed in RA synovial tissues, compared to its expression in OA synovial tissues. We also examined the relationships between TTP gene expression and TNF-α gene expression, serum C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and rheumatoid factor (RF) in patients with RA before surgery.

MATERIALS AND METHODS

Differential display-PCR. Samples from a patient who fulfilled the American College of Rheumatology criteria for classification of RA[9] were used with written informed consent. Total RNA was extracted from synovial tissue from the operated knee joint and Ficoll (Amersham Bioscience Japan, Tokyo, Japan) separated peripheral blood mononuclear cells (PBMC), using Isogen (Nippon Gene, Tokyo, Japan), following the manufacturer's recommended protocol. RNA was reverse transcribed to cDNA using a RevertAid first-strand cDNA synthesis kit (Fermentas, Hanover, MD, USA) following the manufucturer's instructions. Gene expression in synovial and PBMC samples was compared by DD-PCR. DD-PCR was carried out using the delta-differential display kit (Clontech, Palo Alto, CA, USA) as described in the manual, with the exception that we visualized the bands on the polyacrylamide gel by silver stain. Bands visible only in amplified products from the synovial sample were excised from the gel, and were cloned into pCRII vectors (Invitrogen Japan, Tokyo, Japan) and sequenced using standard protocols by a sequencer (ABI Prism 310 gene analyzer; Perkin Elmer, Wellesley, MA, USA). A total of 105 genes considered to be specifically expressed by the synovial tissue sample were analyzed in this way. Sequences were searched for homologies by the NCBI BLAST system on the Internet [cited January 20, 2004; available from http://www.ncbi.nih.gov/blast/].

Semiquantitative PCR. Genes identified by DD-PCR were studied in the literature for possible relationships with cell proliferation or survival, inflammation, and immunological functions such as antibody or cytokine production. TTP and several other genes that were considered of possible interest were selected and their expression in synovial tissues from 22 patients with RA and 22 with OA were measured using the TaqMan PCR real-time semiquantification method. All samples were taken with donors' written informed consent, and the study was approved by the local ethical committee. Sera from these patients were obtained 0–2 days before surgery. At the time of serum sampling, patients were taking 0–10 mg/day prednisolone and 0–3 disease modifying antirheumatic drugs (DMARD) including methotrexate (8 mg/week maximum), salazosulfapyridine (1000 mg/day maximum), and 100–200 mg/day bucillamine, a DMARD commonly used in Japan. Total RNA was extracted from synovial tissues from operated knee joints, and cDNA was synthesized using the RevertAid first-strand cDNA synthesis kit. Synthesized cDNA samples were amplified with specific primers and fluorescence-labeled specific probes for the gene of interest, and accumulation of amplified products was monitored with an ABI 7700 sequence detector (Applied Biosystems Japan, Tokyo, Japan). PCR mixture (qPCR Mastermix) was purchased from Eurogentec (Seraing, Belgium); magnesium concentration was 5 mM final, primer concentrations 200 nM final, and the probe concentration was 100 nM final. Thermal cycler conditions were 50°C for 2 min, 95°C for 10 min, then 55 cycles of 95°C for 15 s, and 60°C for 1 min. Standard samples were included and standard curves for the gene of interest and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were generated in every assay. All measurements were done in triplicate. The level of gene expression was calculated from the standard curve, compensated with that of GAPDH gene, and was expressed as a ratio. The sequences of specific primers and probes are as follows: TTP forward: 5'GGCGACTCCCCATCTTCAAT3', TTP probe: 5'TCTGAGTGACAAAGTGACTGCCCGGTC3', TTP reverse: 5'CAGTGCAAGAGACGTGGCTC3'; mortality factor 4 forward: 5'TGCCGAAATTCTTG CAGATT3', mortality factor 4 probe: 5'TCCCGATGCACCCATGTCCC3', mortality factor 4 reverse: 5'AGATGTGGCACTCCATACACC3'; CD63 forward: 5'TTCTTGCTCTACGTCCTCCTG3', CD63 probe: TGGCCTTTTGCGCCTGTGC, CD63 reverse: 5'CACGGCAATCAGTCCCAC3'. Ki autoantigen forward: 5'AAAGCCGCAGACCCTGG3', Ki autoantigen probe: 5'CTCTGGTGGCTAGGGATGTACTCATGCTCA3', Ki autoantigen reverse: 5'TGTCCAAGCGTGCACACAT3'; TNF-α forward: 5'TGGAGAAGGGTGACCGACTC3', TNF-α probe: 5'CGCTGAGATCAATCGGCCCGACTAT3', and TNF-α reverse: 5'TCCTCACAGGGCAATGATCC3'. Primers and the probe for GAPDH were purchased from Applied Biosystems.

The Mann-Whitney U test was used to compare gene expressions in RA and OA samples. Spearman's rank coefficient was used to examine the relationship between expressions of 2 different genes, and the relationship between gene expressions and CRP, ESR, or RF. $P < 0.05$ was considered significant.

RESULTS

High expression of TTP gene in RA synovial tissue. Using DD-PCR, we selected and sequenced 105 genes from samples of one patient with RA. Identified genes were: complement C1r, ferritin L chain, collagen type 1, chitinase, TTP (G0S24), epididymal secretory protein, cytosolic selenium-dependent glutathione peroxidase, ubiquinol-cytochrome c reductase binding protein, NADH dehydrogenase subunit 2, 17-beta-hydroxysteroid dehydrogenase, IgG1 heavy chain, Ki autoantigen, CD63, sphingolipid activator, mortality factor 4, p47, cytochrome P450 IIIA4, and immunoglobulin-binding protein 1, others being either genes of unknown function or not in the databases. Among these genes, we focused on TTP, originally reported as a member of a set of genes (putative G0/G1 switch regulatory genes) that are expressed transiently on human PBMC, after addition of lectin or cycloheximide[10]. CD63, immunoglobulin binding protein 1, mortality factor 4, and Ki nuclear autoantigen genes were arbitrarily chosen for real-time PCR analyses, since we considered that the functions of these genes may also be related to the pathogenesis of RA. To determine whether TTP gene expression is indeed enhanced in RA synovial tissues, expression of TTP gene in synovial tissues of 22 RA and 22 OA patients was measured by TaqMan real-time semiquantification PCR. Expression of TTP gene was significantly higher in RA synovial tissues compared to OA synovial tissues ($p = 0.0128$, Mann-Whitney U test; Figure 1). Expression of CD63, immunoglobulin binding protein 1, mortality factor 4, and Ki nuclear autoantigen genes showed no significant differences between RA and OA synovium samples (data not shown).

TTP/TNF-α gene expression ratio correlates inversely with CRP. To determine whether the expression level of TTP

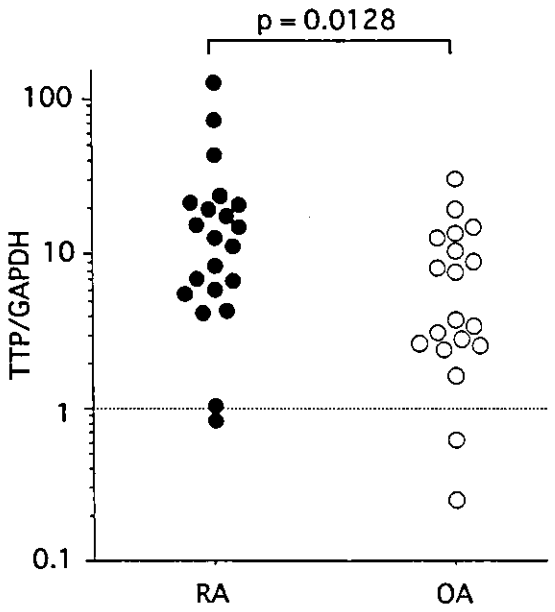

Figure 1. Expression of TTP mRNA in synovial tissues of patients with RA and OA. P value calculated by Mann-Whitney U test.

gene directly correlated with TNF-α gene, we analyzed expression of TNF-α gene using real-time semiquantification PCR, and the results were compared to the expression of TTP gene. Standard curves were generated that indicated the validity of the real-time semiquantification PCR used in this study (Figure 2). There was no significant correlation between the expression of TNF-α and TTP genes (Figure 3). To further examine the relevance of TTP gene expression in RA, we determined the relationships between TTP gene expression and CRP, ESR, or RF. CRP tended to be higher in patients with higher TNF-α gene expression, but without statistical significance (r = 0.306, p = 0.2071, Spearman rank correlation; Figure 4A). CRP also tended to be higher in patients with lower TTP gene expression, although this was statistically insignificant (r = –0.429, p = 0.0771, Spearman rank correlation; Figure 4B). However, when the ratio of TNF-α and TTP gene expression was calculated, and compared with CRP, a significant inverse relationship was observed (r = –0.653, p = 0.0071, Spearman rank correlation; Figure 5A). ESR showed a similar trend, although statistically insignificant (r = –0.441, p = 0.0692, Spearman

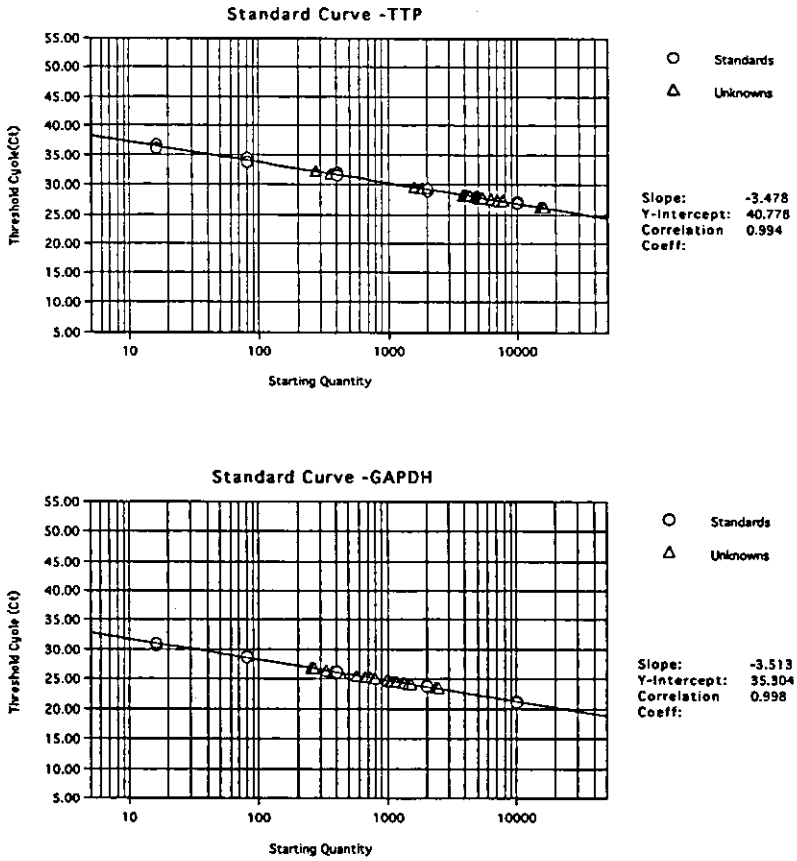

Figure 2. Standard curves generated for real-time semiquantification PCR. Upper panel: TTP; lower panel: GAPDH. All measurements were in triplicate, and sample values that were not within the standard curve were diluted and remeasured. Similar standard curves were obtained for all PCR analyses (data not shown).

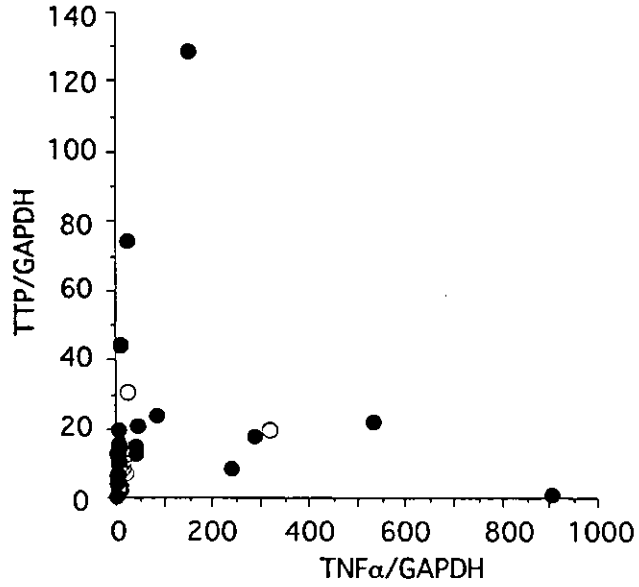

Figure 3. Relationship between expression of TTP mRNA and TNF-α mRNA. ●: RA, ○: OA.

rank correlation; Figure 5B). Steroid and/or DMARD usage and RF did not significantly correlate with TTP gene expression (data not shown).

DISCUSSION

Our results suggest that TTP gene expression may play an important role in RA disease activity. Elucidation of the role of TTP in the pathogenesis of RA may be helpful in the search for new therapies for RA.

DD-PCR is a powerful tool for identifying genes that are highly expressed in one of the 2 samples compared. Using this technique, we identified TTP as a possible candidate gene whose expression may have a role in the pathogenesis of RA. Our strategy was to compare samples from the same patient, to avoid detecting differences between individuals, then use real-time PCR to determine whether the identified genes are highly expressed in synovial tissues from RA patients compared to those from OA patients. Applying DD-PCR to synovial tissues from RA and OA samples is an another possible approach, which may give us a completely different result.

We found that the expression of TTP gene was significantly higher in synovial tissues from RA patients than those from OA patients. In addition, in RA patients the magnitude of TTP gene expression was lower in patients with higher serum CRP, an inflammation marker commonly used to monitor RA activity. Although this relationship was not quite statistically significant ($p = 0.0771$), a significant relationship was observed when TNF-α gene expression was taken into account ($p = 0.0071$). A similar relationship for ESR was also observed ($p = 0.0692$), although it was not

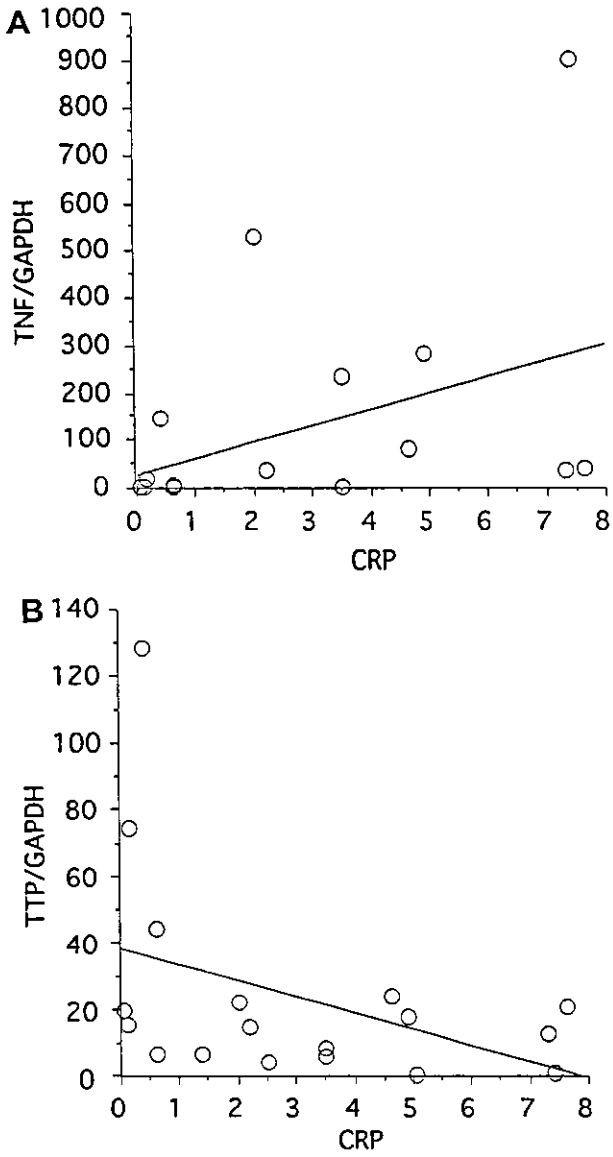

Figure 4. Relationship between serum CRP and expressions of TNF-α mRNA and TTP mRNA. A. CRP and TNF-α mRNA ($p = 0.2071$, Spearman rank correlation). B. CRP and TTP mRNA ($p = 0.0771$, Spearman rank correlation).

statistically significant. The finding that TTP gene expression is higher in RA samples is not merely due to more inflammatory cells in RA samples than OA samples, although this may partly account for the finding. If enhanced TTP gene expression reflects only the increment of inflammatory cells in the synovium, one would expect that, in RA synovium, TTP gene expression would correlate with TNF-α gene expression and inflammatory markers. Such a relationship was not observed. Instead, TTP gene expression seemed to be lower in synovium from patients with more active inflammation.

TTP protein binds directly to the AU-rich element in the

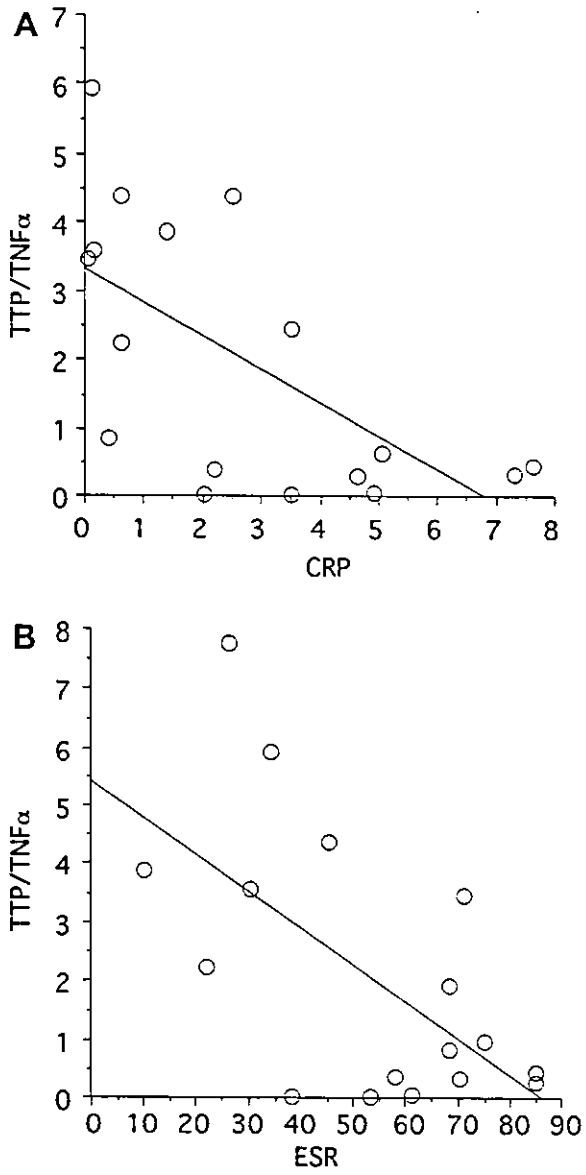

Figure 5. Relationship between expression of TTP/TNF-α gene expression ratio and CRP or ESR. A. TTP/TNF-α gene expression ratio and CRP (r = –0.653, p = 0.0071, Spearman rank correlation). B. TTP/TNF-α gene expression ratio and ESR (r = –0.441, p = 0.0692, Spearman rank correlation).

3'-UTR of TNF-α mRNA. Since binding of TTP leads to instability of TNF-α mRNA[8,11], it is conceivable that TTP is a physiological regulator of TNF-α production. It has been reported that TTP knockout mice develop erosive arthritis, dermatitis, conjunctivitis, glomerular mesangial thickening, and high titers of anti-DNA and antinuclear antibodies[12]. The pathological findings of erosive arthritis were similar to those observed in human RA. The phenotype seen in TTP knockout mice was reversed by administration of anti-TNF-α antibody[12], suggesting that TNF-α overproduction plays a major role in the pathogenesis of arthritis and other symptoms in these mice. In addition, TTP binds to AU-rich elements on mRNA of other genes such as interleukin 2 (IL-2), IL-3, granulocyte macrophage-colony stimulating factor (GM-CSF), and c-fos genes[13]. In T cells, TTP protein is increased after cell stimulation, and replaces HuA, an AU-rich element-binding protein, which stabilizes mRNA[13]. Thus, TTP is a natural suppressor of excessive cytokine production. We speculate that in patients with RA, the presence of TNF-α in synovial tissues could lead to enhancement of TTP production, and that patients who lack the potential to produce adequate amounts of TTP may develop more severe disease, leading to more intense inflammation and joint destruction. The level of TTP gene expression in the synovial tissue may be important in determining the disease activity of RA, making it a possible candidate for future therapeutic targets.

The lack of correlation between TTP mRNA and the amount of TNF-α mRNA does not necessarily indicate that the expression of TTP mRNA is not an important factor in TNF-α production in the synovial tissue of patients with RA. Physiologically, overproduction of TNF-α would lead to enhanced production of TTP, which in turn would suppress TNF-α production. On the other hand, inadequate production of TTP in RA patients would lead to overproduction of TNF-α, affecting the course of RA. Thus, the relationship between TTP mRNA production and TNF-α production may differ among individual patients. Indeed, the significant inverse relationship between TTP/TNF-α gene expression ratio and CRP suggests that an adequate TTP response could help in the control of inflammation that occurs in RA synovial tissues. Brooks, *et al*[14] reported the presence of TTP protein in RA synovial tissue. They also reported that human TTP binds to the 3'-UTR of TNF-α mRNA and reduces reporter gene expression. Their study emphasized the potential importance of posttranscriptional regulation of TNF-α production in the pathogenesis of RA. Our findings add support to their conclusions. Our study was done using synovial samples; it may be of interest to investigate whether a similar relationship can be observed in peripheral blood samples from RA patients. Studies at the protein level also should be done.

Our study does not indicate that elevated TTP gene expression is an RA-specific phenomenon. TNF-α is known to play important roles in various inflammatory diseases such as psoriatic arthritis, ankylosing spondylitis, Behçet's disease, and Crohn's disease. It is possible that interindividual differences in TTP production affect the arthritic condition in these diseases. TNF-α expression in cartilages from patients with OA has also been reported[15]. Thus, TNF-α and hence TTP may have a role in the progression of OA as well as RA. Whether TTP gene expression is elevated in OA synovium compared to healthy synovium, and whether this has implications for the severity of OA, are challenging

questions because of the difficulties of obtaining healthy synovial samples and assessing the severity of OA.

We speculate that TTP production is induced in patients with RA as a negative feedback of TNF-α overproduction, and that TTP may affect the course of RA by reducing the production of TNF-α in the synovium. Our current hypothesis is that compounds that mimic the properties of TTP or that enhance TTP gene expression, or even TTP gene therapies, may serve as a tool for controlling joint inflammation and destruction in severe RA.

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Polymorphisms of IL-1ß Gene in Japanese Patients with Sjögren's Syndrome and Systemic Lupus Erythematosus

YOSHIFUMI MURAKI, AKITO TSUTSUMI, REIKO TAKAHASHI, EIJI SUZUKI, TAICHI HAYASHI, YUSUKE CHINO, DAISUKE GOTO, ISAO MATSUMOTO, HIDEYUKI MURATA, EMIKO NOGUCHI, and TAKAYUKI SUMIDA

ABSTRACT. ***Objective.*** Interleukin (IL)-1ß is a proinflammatory cytokine involved in various immune responses. Five polymorphisms in the IL-1ß gene have been described, and relationships between these polymorphisms and some autoimmune diseases have been reported. Evidence suggests that IL-1ß may be involved in the destruction of salivary and lacrimal glands in Sjögren's syndrome (SS). We evaluated the significance of IL-1ß gene polymorphisms in SS.

Methods. Blood samples were taken from 101 patients with SS, 103 patients with systemic lupus erythematosus (SLE, excluding those with secondary SS), and 106 healthy volunteers. Each polymorphism of the IL-1ß gene was analyzed by polymerase chain reaction (PCR) amplification of the polymorphic site, followed by site-specific restriction digestion. Genotype frequencies of each polymorphism in SS patients were compared with those of the controls and SLE patients, and differences between primary and secondary SS patients were also compared.

Results. Genotypes CC, TT, and AA in positions –511, –31, and 3877, respectively, were significantly less frequent in SS patients than controls or patients with SLE. No significant differences were found in genotype frequencies of any of the polymorphisms between patients with primary SS and secondary SS.

Conclusion. IL-1ß gene polymorphisms may affect susceptibility to SS, but not SLE. (J Rheumatol 2004;31:720–5)

Key Indexing Terms:
INTERLEUKIN 1ß
SJÖGREN'S SYNDROME
GENE POLYMORPHISM
SYSTEMIC LUPUS ERYTHEMATOSUS

Interleukin (IL)-1ß is a proinflammatory cytokine involved in various immune responses, including autoimmune diseases[1]. The gene is located on chromosome 2q 13[2], and 5 polymorphisms have been described in the promoter regions (at –511: C/T)[3,4], TATA Box (at –31: T/C)[3,5], exon 4 (at +3263: C/T)[3,6], intron 4 (at +3877: A/G)[3,5], and exon 5 (at +3953: C/T)[3,7]. At least 3 of these, –511[8], –31[9], and +3953[7], are possibly related to IL-1ß production. It has been shown that the presence of the T allele at +3953 has a stimulatory effect on lipopolysaccharide (LPS)-induced IL-1ß protein production *in vitro*[7]. Santtila, *et al*[8] found that mononuclear cells derived from individuals with the T allele in the polymorphism at –511 and those without T allele in the polymorphism at 3953 showed slightly higher production of IL-1ß than other carriers, when stimulated by phorbol dibutyrate. The T allele at –31 of the IL-1ß gene comprises a TATA box-related gene structure, suggesting a role in gene expression[9].

Recent studies have described the relationships between these polymorphisms and various diseases such as rheumatoid arthritis (RA)[10-13], osteoporosis[6], osteoarthritis[14], inflammatory bowel diseases[15], gastric cancer[9], gastritis caused by *Helicobacter pylori*[16], multiple sclerosis (MS)[17], and alcoholic liver disease[18]. In autoimmune diseases, Buchs, *et al*[11] showed that patients with the T allele at +3953 show aggressive destructive arthritis compared to those without the T allele. On the other hand, Niino, *et al*[17] reported the lack of significant differences in the distribution of polymorphisms between Japanese patients with MS

From the Division of Rheumatology, Department of Internal Medicine, Institute of Clinical Medicine and the Department of Medical Genetics, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Japan.

Y. Muraki, MS; A. Tsutsumi, MD; R. Takahashi, MD; E. Suzuki, MD; T. Hayashi, MD; Y. Chino, MD; D. Goto, MD; I. Matsumoto, MD; H. Murata, MD; T. Sumida, MD, Professor, Division of Rheumatology, Department of Internal Medicine, Institute of Clinical Medicine; E. Noguchi, MD, Department of Medical Genetics, Institute of Basic Medical Sciences.

Address reprint requests to Professor T. Sumida, Division of Rheumatology, Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan.

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and controls. In addition, in their study, no association was observed between IL-1ß or IL-1ra gene polymorphisms and clinical characteristics. Huang, *et al*[19] also reported that the polymorphisms on promoter and exon 5 regions of the IL-1ß gene are not related to SLE in Taiwanese patients.

Sjögren's syndrome (SS) is an autoimmune disease characterized by multisystem inflammation mainly affecting the exocrine glands. There are common features between SS and systemic lupus erythematosus (SLE), but compared to SLE, primary SS (pSS) is a more organ-specific disease. Infiltrating mononuclear inflammatory cells inhibit the function of glandular cells in the salivary and/or lacrimal gland tissues. The typical symptoms of SS, dry eye (keratoconjunctivitis sicca) and dry mouth occur following destruction of these glands[20]. IL-1ß and tumor necrosis factor (TNF)-α are proinflammatory cytokines and seem to be involved in the destruction of acinar structure in human salivary glands[21,22]. In addition, IL-1ß may play a key role in the pathogenesis of keratoconjunctivitis sicca[23]. Although several genetic studies of autoimmune diseases including studies on cytokine polymorphisms, have already been reported[24], the relationships between IL-1ß gene polymorphisms and occurrence of SS have not been examined, and only one study is reported for SLE[19]. To investigate the effect of IL-1 gene polymorphisms on the susceptibility to SS, we analyzed the gene polymorphisms of IL-1ß by the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method, and compared the genotype frequencies of each polymorphism between SS and controls, SLE and controls, or SS and SLE. In addition, detailed analysis evaluated whether these polymorphisms affect the phenotypes of SS. Finally, we studied the relationships between gene polymorphisms and characteristics of these diseases.

MATERIALS AND METHODS

SS patients, SLE patients and healthy controls. 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. Blood samples were collected from 101 Japanese patients with SS and 103 Japanese patients with SLE followed at the Department of Internal Medicine, University of Tsukuba Hospital. All SS patients [43 with primary SS, 58 with secondary SS including 21 with rheumatoid arthritis, 20 with SLE, 11 with SSC, 4 with mixed connective tissue disease (MCTD), and 2 with Hashimoto's disease] satisfied the Japanese Ministry of Health criteria for the classification of SS[25], and all SLE patients (no patients with secondary SS, 10 men and 93 women) satisfied the 1997 revised American College of Rheumatology criteria for SLE[26]. As healthy controls, 106 Japanese volunteers (41 men, 65 women) were recruited from our institute.

IL-1ß polymorphism typing. Genomic DNA was isolated from 0.5 ml of anti-coagulated peripheral blood by using DNA QuickII DNA purification kit (Dainippon Pharmaceuticals, Osaka, Japan) and the instructions supplied with the kit. Five polymorphisms of the IL-1ß gene were analyzed by PCR-RFLP[4-6,13]. Briefly, 4 sets of PCR reactions were performed, and the amplified products were digested with appropriate restriction enzymes that cut one of the alleles in each polymorphism. The primers and restriction enzymes for each polymorphism were as follows. For -511: 5'-TGGCATTGATCTGGTTCATC-3', 5'-GTTTAGGAATCTTCCCACTT-3', and *Ava* I. For -31: 5'-TCATAGTTTGCTACTCCTTGC-3', 5'-CAAAAAGCTGAGAGAGGAGG-3', and *Alu* I. For +3263: 5'-TTTGAGAGGCAGGCTGTTTG -3', 5'-CTTGGTTGTCTTCCTACTATG-3' and *Mbo* II. For +3877 and +3953: 5'-GTTGTCATCAGACTTTGACC-3', 5'-TTCAGTTCATATGGACCAG-3', *BsoF* I for +3877 and *Taq* I for +3953. Digested fragments were run on 2.5% agarose gels and visualized with ethidium bromide (Figure 1).

Serum levels of IL-1ß. IL-1ß levels were measured with human IL-1ß US ELISA kit (BioSource International, Inc. Camarillo, CA, USA), using the instructions provided by the manufacturer.

Statistical analyses. Linkage disequilibrium analysis of these polymorphic sites in the IL-1ß gene was performed using Graphical Overview of Linkage Disequilibrium (GOLD) software package[27]. Chi-square analysis was used to compare genotypes or frequencies of alleles and phenotypes between patients and control groups. Fisher's exact test was used where appropriate. A p value of 0.05 was considered significant. For multiple comparisons (SS or pSS, healthy controls, SLE), a p value of 0.016 was considered as significant. Odds ratios were also calculated for disease susceptibility in carriers of a specific genotype. Correlations between each genotype in each polymorphism and clinical and biological variables were evaluated by analysis of variance or chi-square analysis.

RESULTS

Validation of the RFLP analyses. Fragments of predicted sizes were obtained for all polymorphisms (Figure 1), except for position +3263, where all samples were digested by *Mbo* II, indicating the absence or extreme rarity of the T allele in our Japanese population. Several randomly selected samples were sequenced to further confirm the accuracy of the RFLP analysis (data not shown).

Relationship between IL-1ß genotypes and SS and SLE. Comparison of SS group with other groups showed no evident differences in allele frequencies at any of polymorphisms (data not shown; can be calculated from Table 1A). In contrast, comparison of SS patients with other groups showed significant differences in genotype distribution at positions -511, -31, and 3877 (-511CC/CT/TT: SS vs controls and SLE: p = 0.053 and p = 0.028; -31 TT/TC/CC: SS vs SLE: p = 0.027; 3877 AA/AG/GG: SS vs controls and SLE: p = 0.039 and p = 0.054) (Table 1A). Specifically, the frequencies of genotypes CC, TT, and AA at positions -511, -31, and 3877, respectively, were lower in SS patients compared with the controls and the SLE group (-511, -31, 3877: SS/HC/SLE:18.8, 19.6, 22.8%; 32.1, 29.2, 36.8%; 34.0, 32.4, 35.9%). These differences were also evident when patients were categorized according to the presence or absence of these genotypes (-511CC/CT+TT: SS vs control and SLE: p = 0.029 and p = 0.014; -31 TT/TC+CC: SS vs control and SLE: p = 0.111 and p = 0.041: 3877 AA/AG+GG: SS vs control and SLE: p = 0.028 and p = 0.039, respectively, Table 1B). After adjustment for multiple comparisons according to Bonferroni, only the difference at 511 between SS and SLE remained significant. No significant differences were present between the SLE patients and controls (Table 1A-B). No significant differences in genotype frequencies were observed between primary and secondary SS (Table 1A). In agreement with previous

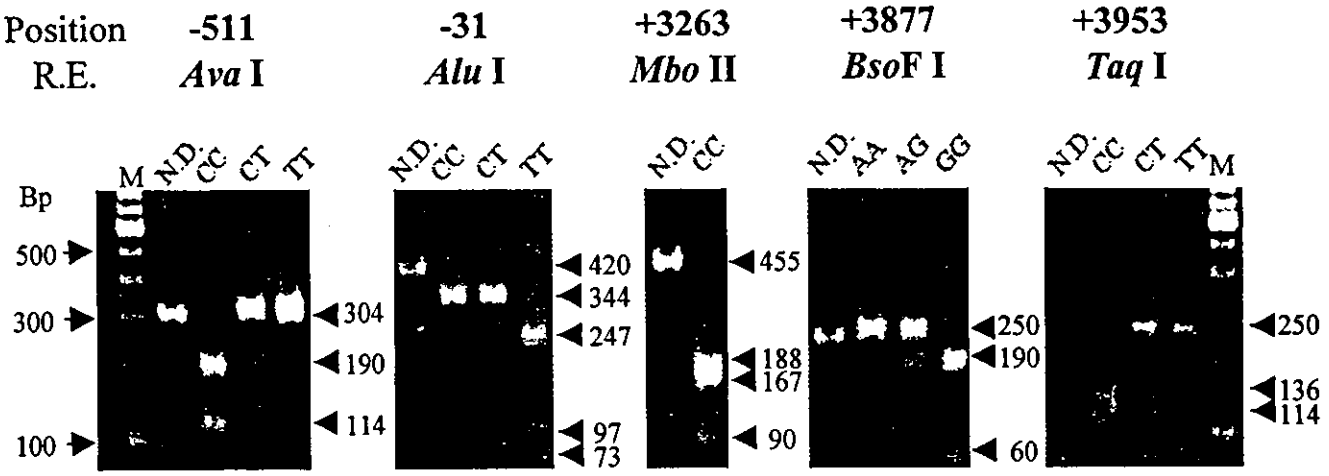

Figure 1. Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis of the 5 polymorphisms of the IL-1ß gene. cDNA was amplified with primers indicated in the text, and restriction digested by appropriate enzymes. Digested PCR products were run on agarose gels and visualized with ethidium bromide. RE: restriction enzyme. M: 100 bp marker. ND: non-digested PCR product.

Table 1A. Genotype distribution of IL-1ß gene polymorphisms in patients with SS and SLE and healthy controls.

Polymorphism	Genotype	HC n = 106 (%)	SS n = 101 (%)	pSS n = 43 (%)	sSS n = 58 (%)	SLE n = 103 (%)	p (chi-square)
	CC	34 (32.1)	19 (18.8)	7 (16.3)	12 (20.7)	35 (34.0)	
-511	CT	49 (46.2)	62 (61.4)	26 (60.5)	36 (62.1)	46 (44.7)	0.053
	TT	23 (21.7)	20 (19.8)	10 (23.3)	10 (17.2)	22 (21.4)	0.028*
	TT	31 (29.2)	19 (19.6)	7 (17.5)	12 (21.1)	33 (32.4)	
-31+	TC	52 (49.1)	57 (58.8)	23 (57.5)	34 (59.6)	41 (40.2)	0.246
	CC	23 (21.7)	21 (21.6)	10 (25.0)	11 (19.3)	28 (27.5)	0.027*
	AA	39 (36.8)	23 (22.8)	10 (23.3)	13 (22.4)	37 (35.9)	
3877	AG	48 (45.3)	63 (62.4)	24 (55.8)	39 (67.2)	50 (48.5)	0.039
	GG	19 (17.9)	15 (14.9)	9 (20.9)	6 (10.3)	16 (15.5)	0.054*
	CC	98 (92.5)	92 (91.1)	40 (93.0)	52 (89.7)	93 (90.3)	
3953	CT	8 (7.5)	9 (8.9)	3 (7.0)	6 (10.3)	9 (8.7)	
	TT	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.0)	

SS: Sjögren's syndrome; SLE: systemic lupus erythematosus; HC: healthy control; pSS: primary SS; sSS: secondary SS; +: 4 SS patients and 1 SLE patient were not genotyped. p: SS vs HC; *: SS vs SLE. p values of ≤ 0.016 were considered significant after Bonferroni corrections.

Table 1B. Genotype skewing of IL-1ß gene polymorphisms in patients with SS and SLE and healthy controls.

Polymorphism	Phenotype	HC n = 106 (%)	SS n = 101 (%)	pSS n = 43 (%)	SLE n = 103 (%)	p (chi-square)	p (Fisher's probability test)	OR (95% CI)
						0.029‡	0.038	0.49 (0.26–0.93)
-511	CC	34 (32.1)	19 (18.8)	7 (16.3)	35 (34.0)	0.053*	0.068*	0.41 (0.17–1.02)
	CT/TT	72 (67.9)	82 (81.2)	36 (83.7)	68 (66.0)	0.014**	0.017**	0.45 (0.24–0.86)
						0.031***	0.044***	0.38 (0.15–0.94)
	TT	31 (29.2)	19 (19.6)	7 (17.5)	33 (32.4)	0.111	0.142	0.59 (0.31–1.13)
-31*	TC/CC	75 (70.8)	78 (80.4)	33 (82.5)	69 (67.6)	0.041**	0.053**	0.51 (0.27–0.98)
	AA	39 (36.8)	23 (22.8)	10 (23.3)	37 (35.9)	0.028	0.034	0.51 (0.28–0.93)
3877	AG/GG	67 (63.2)	78 (77.2)	33 (76.7)	66 (64.1)	0.039**	0.046**	0.53 (0.28–0.97)
	CC	98 (92.5)	92 (91.1)	40 (93.0)	93 (90.3)			
3953	CT/TT	8 (7.5)	9 (8.9)	3 (7.0)	10 (9.7)			

SS: Sjögren's syndrome; SLE: systemic lupus erythematosus; HC: healthy control; pSS: primary SS; OR: odds ratio; 95% CI: 95% confidence interval; *: 4 SS patients and 1 SLE patient were not genotyped; ‡ SS vs HC; * pSS vs HC: ** SS vs SLE; *** pSS vs SLE. p values of ≤ 0.016 were considered significant after Bonferroni corrections.

reports[9,16], –511 and –31 polymorphisms were in almost complete linkage disequilibrium (Table 1A-B). Detailed analysis of linkage disequilibrium among the 4 polymorphisms examined in this study showed that these polymorphisms are indeed in linkage disequilibrium (D'= 0.622~1.000), and are not independent of each other.

Relationships between IL-1ß genotypes and serum IL-1ß. Serum IL-1ß was measured in 84 healthy controls, 43 patients with SS, and 101 patients with SLE. In these groups, measurable amounts of serum IL-1ß were detected in 11 controls, 4 patients with SS, and 5 patients with SLE. IL-1ß concentration in other samples did not exceed the detection limit (0.3 pg/ml). IL-1ß genotypes in individuals with measurable serum IL-1ß did not show any skewing toward a particular genotype (data not shown).

Relationships between IL-1ß genotypes and disease characteristics of SS and SLE. Disease characteristics and variables were examined in the context of IL-1ß genotypes in SS and SLE patients. Presence of anti-SSA antibody was slightly increased in patients with genotype CC and genotype TT at positions –511 and –31, respectively, (p = 0.040 and p = 0.025, Table 2). The presence of antinuclear antibody or anti-SSB antibody was not related to any of the genotypes examined (Table 2). Age at onset and presence of arthritis were also not associated with any of the genotypes examined (data not shown). In SS patients, serum IgG or presence of sicca syndrome was not associated with any of the IL-1ß genotypes studied (data not shown). In SLE patients, no significant relationship was observed between IL-1ß genotypes and disease variables or presence of symptoms including SLE Disease Activity Index[28], anti-DNA antibody, C_3, C_4, CH50, central nervous system lupus, serositis, and glomerulonephritis (data not shown).

DISCUSSION

Our major finding was that the genotype frequencies and/or the genotype skewings of 3 (–511, –31, +3877) of the 5 known IL-1ß gene polymorphisms were significantly different between SS patients and controls or SLE subjects. No such difference was observed between SLE patients and controls, which is in accord with a report on Taiwanese SLE patients [19]. In fact, in our study, significant differences were observed between patients with SS and SLE. Two (–511, –31) of these 3 polymorphisms were on the promoter region of the IL-1ß gene, and thus may affect IL-1ß gene expression and subsequent cytokine production. The third (+3877) was on intron 4, and we cannot speculate whether this polymorphism influences IL-1ß production or some other immunological or inflammatory functions, although this polymorphism is suggested to be an informative marker for disease association studies[5]. Although many of the significant differences were lost after adjustment of the p value for multiple comparisons, our results may provide a rationale for further studies on the role of the IL-1ß and the pathogenesis of SS. Analysis of linkage disequilibrium among the 4 polymorphisms observed in this study showed that these polymorphisms are in linkage disequilibrium and are not independent, and therefore, adjustment for multiple comparison was performed for comparisons between patients and healthy controls.

In SS, salivary and lacrimal glands are the main target organs for autoimmune tissue damage. IL-1ß and TNF-α are proinflammatory cytokines, and affect the destruction of acinar structure in salivary glands[21]. Messenger RNA of both Th1 and Th2 cytokines are detected in salivary glands of patients with SS, and may have important roles in the onset and progression of SS[22,29,30]. Interferon-γ, IL-2, IL-6, IL-10, and transforming growth factor-ß play essential roles in the induction of SS, while IL-4 and IL-5 may be involved in the progression of disease process[29]. IL-1ß has multiple biologic effects, which include induction of lymphokine synthesis (IL-2, -3, -4, -5, -6, -7, -10, and -12), and devel-

Table 2. Comparison of genotype distribution between patients positive for autoantibodies (ANA, SSA, SSB) and negative in SS and SLE.

Polymorphism	Genotype	ANA				Anti-SSA				Anti-SSB			
		SS (n = 97)		SLE (n = 89)		SS (n = 81)		SLE (n = 86)		SS (n = 65)		SLE (n = 85)	
		+	–	+	–	+	–	+	–	+	–	+	–
	CC	16	3	26	5	15	1	6	23	0	15	0	28
–511	CT	43	16	33	6	29	18*	10	31	7	29	0	41
	TT	15	4	16	3	14	4	6	10	2	12	1	15
	TT	16	3	26	4	15	1	6	22	0	15	0	27
–31	TC	41	14	27	6	26	18**	9	26	6	28	0	35
	CC	15	5	21	4	14	4	7	15	2	12	1	21
	AA	16	7	28	2	14	4	5	23	0	14	0	27
3877	AG	47	13	35	9	33	17	11	32	6	33	1	42
	GG	11	3	12	3	11	2	6	9	3	9	0	15
	CC	67	21	69	12	52	22	19	59	8	50	1	76
3953	CT	7	2	6	1	6	1	2	5	1	6	0	7
	TT	0	0	0	1	0	0	1	0	0	0	0	1

ANA: antinuclear antibody; SS: Sjögren's syndrome; SLE: systemic lupus erythematosus; * p = 0.040; ** p = 0.025 (n = 78), by chi-square test.

opment of Th2 type T cells[1]. IL-1 is known to initiate transcription and stabilize the mRNA of a variety of genes. Messenger RNA expression of inflammatory cytokines, such as IL-6 and 8, TNF-α, and granulocyte-macrophage colony-stimulating factor genes are upregulated by IL-1[1,31,32]. In this study, we found that the genotype skewings of 3 polymorphisms of the IL-1ß gene were significantly different in SS patients compared to those with SLE or controls. While adjustment for multiple comparison leads to the loss of statistical significance in many of the differences observed, we still believe that our data are of interest, since our primary focus was on SS, which has evidence suggesting the importance of IL-1ß in its pathogenesis, and not on SLE, which has no such evidence and which was included in this study rather as a comparator disease. We speculate that these polymorphisms may have direct or indirect impact on the onset or progression of SS.

In previous reports on IL-1ß polymorphisms and IL-1ß production, the relationship between the polymorphism and IL-1ß gene expression or IL-1ß production is not fully determined[8,33]. In our study, although we tried to evaluate the relationship between these polymorphisms and serum IL-1ß we could not find any relationship between the 4 polymorphisms of IL-1ß gene and concentration of serum IL-1ß, since only 11 individuals in the control group, 4 in the SS group, and 5 in the SLE group had measurable amounts of serum IL-1ß. These results were not unexpected, since it is difficult to detect IL-1ß in serum or plasma without *in vitro* stimulation by mitogens such as LPS because circulating levels of IL-1ß are very low and plasma normally contains IL-1sRII (IL-1 soluble receptor II), α-2-macroglobulin and complement, which bind to IL-1ß[1]. In addition, in most of our patients, disease activity was adequately controlled at the time of sampling. Therefore, to assess whether these polymorphisms are indeed associated with IL-1ß production, either a study with a large number of untreated patients or an *in vitro* study using a promoter assay would be necessary.

Genotypes at –511 and –31 of the IL-1ß gene were in almost complete linkage disequilibrium as reported by others[9,16]. Therefore, SS patients showed similar significant decreases of CC homozygous genotype at –511 and of TT homozygous genotype at –31. Presence of the T allele at –31 would make a TATA box-related sequence, which speculatively may lead to increased IL-1ß production. However, the number of individuals who are TT homozygous at –31 was significantly decreased in the SS patient group. Since the presence of this genotype was tightly linked with the probable high IL-1ß producing allele at –511[9,16], the significance of TATA box formation at –31 cannot be determined at this stage.

Polymorphism at +3877 in intron 4 of the IL-1ß gene, originally reported by Guasch, *et al*[5] was proposed as an adequate marker for genotype-disease association studies, because of its heterogeneity and low linkage to other polymorphisms. However, Langdahl, *et al*[6] reported the lack of any difference in genotype distributions at +3877 of the IL-1ß gene, or at +11100 of the IL-1ra gene between healthy controls and patients with osteoporosis. Furthermore, they found no relationships between these polymorphisms and disease variables of osteoporosis such as bone mass and bone turnover. In our study, we found that the G allele carriers at +3877 of the IL-1ß gene are significantly more frequent among SS patients compared to healthy controls or SLE patients. Since there is no information on the relationship between this polymorphism and gene expression, the significance of this finding remains unclear at present.

We found no significant skewing of genotype distribution in any of the polymorphisms studied with any of the disease characteristics including age at onset, severity of sicca syndrome, presence or absence of arthritis, anti-SSB antibody, and antinuclear antibody in both SS and SLE patients. The CC homozygous genotype at –511 and TT homozygous genotype at –31 were slightly increased in anti-SSA antibody-positive patients, compared to negative patients. Although IL-1ß and anti-SSA antibody production may be indirectly associated in some way in the complex steps of autoantibody production, considering the number of comparisons made in this study, this weak association should be retested in future studies.

Finally, the IL-1ß gene allele frequencies at positions –511 and +3953 observed in our study controls were similar to those obtained in healthy Japanese subjects studied by Takamatsu, *et al*[18] and in healthy Taiwanese subjects studied by Huang, *et al*[19], but were different from those obtained in healthy Caucasian subjects studied by Cantagrel, *et al*[13] because the allele T on +3953 is much more common in Caucasians than in the Japanese (frequency of TT homozygous healthy individuals was 0% in our study and in Takamatsu's[18], but was 4.7% in Cantagrel's[13]).

There are important steps, e.g., IL-1ß converting enzyme (ICE) digestion, competition with IL-1ra and IL-1a, and binding with the IL-1 receptor, before IL-1ß gene products exert their functions. Other cytokines and mediator molecules also affect this cascade. Therefore, the extent of IL-1ß function is dependent not only on IL-1ß gene expression. Nevertheless, our results showed significant differences in genotype distributions in 3 polymorphisms of the IL-1ß gene in SS patients compared with controls or patients with SLE. These findings imply that IL-1ß gene polymorphisms, and hence, IL-1ß function, may influence the onset and progression of SS. On the other hand, no such genotype skewing was observed in SLE patients, suggesting that this cytokine might not be an important factor in the onset of SLE.

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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: 0000–0000.

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

Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan

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[2] Address correspondence and reprint requests to Dr. Yasuharu Nishimura, Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Japan. E-mail address: mxnishim@gpo.kumamoto-u.ac.jp

[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.

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

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

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

Materials and Methods

Mice

CBA, and C57BL/6 mice obtained from CLEA Japan or Charles River were kept under specific pathogen-free conditions. Male CBA and female C57BL/6 mice were mated to generate 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′. AQ:

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). T1

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 F1

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.

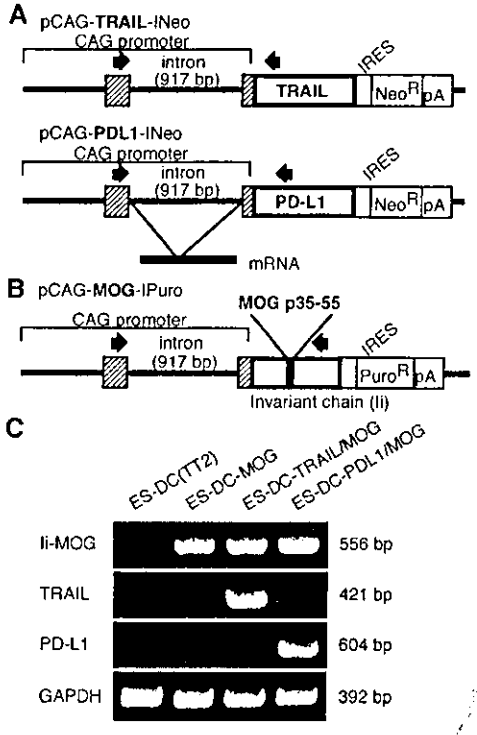

FIGURE 1. Genetic modification of ES-DC to express TRAIL, PD-L1, and Ii-MOG. *A*, The structures of pCAG-TRAIL-INeo and pCAG-PDL1-INeo, the expression vectors for TRAIL and PD-L1, and PCR primers for RT-PCR to detect transgene products are shown. Primer pairs (arrows) were designed to span the intron (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-A^b, or CD11c among ES-DC derived from parental TT2 ES cells, ES-DC-MOG, ES-DC-TRAIL/MOG, and ES-DC-PD-L1/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.

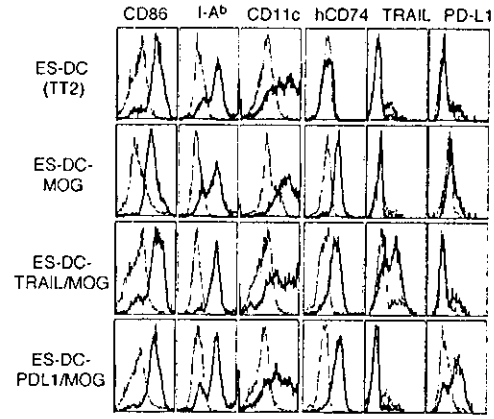

FIGURE 2. Surface phenotype of genetically modified ES-DC. Expression of cell surface CD86, I-A^b, CD11c, TRAIL, and PD-L1 on transfectant ES-DCs was analyzed by flow-cytometric analysis. Expression of mutant human Ii (hCD74) bearing MOG peptide was examined using intracellular staining. Staining patterns 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. F4

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

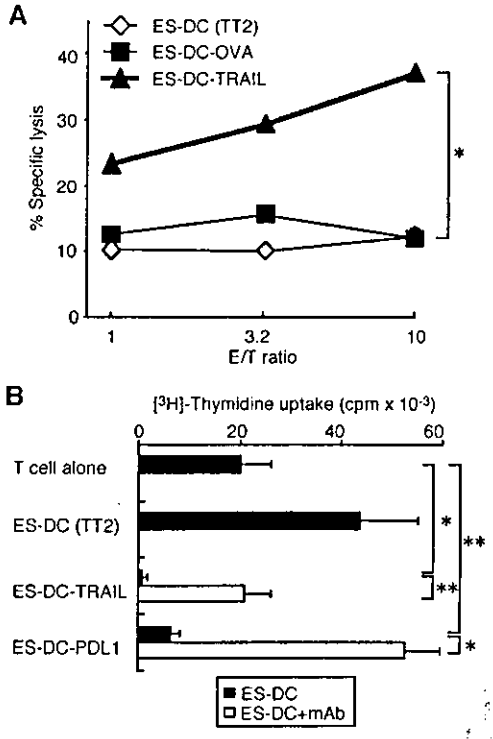

FIGURE 3. Expression of functional TRAIL or PD-L1 in ES-DC transfectants. *A*, The activity of TRAIL expressed in ES-DC was analyzed based on cytotoxicity against L929 cell. ^{51}Cr-labeled target cells (5×10^3 L929 cells) were incubated with ES-DC (TT2), ES-DC-OVA, or ES-DC-TRAIL as effector cells at 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.

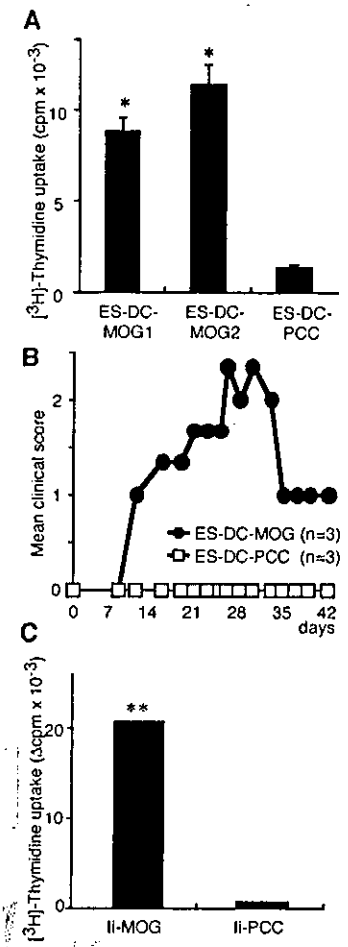

FIGURE 4. Presentation of MOG epitope by ES-DC introduced with Ii-based MOG epitope-presenting vector. *A*, T cells (1.5×10^5) isolated from inguinal lymph nodes of CBF_1 mice immunized according to the protocol for EAE induction were cocultured with one of two independent clones (2×10^4) of ES-DC-MOG 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 (<46×10^3 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-PD-L1/MOG. As shown in Fig. 5, proliferative response of the MOG-reactive T cells cocultured with ES-DC-TRAIL/MOG or ES-DC-PD-L1/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-PD-L1/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 completely prevented by pretreatment with either of these genetically modified

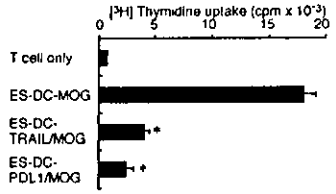

FIGURE 5. Decreased proliferative response to MOG peptide of MOG-reactive T cells cocultured with ES-DC expressing MOG plus TRAIL or MOG plus PD-L1. T cells (2×10^5) isolated from inguinal lymph nodes of CBF_1 mice immunized according to the protocol for EAE induction were cocultured with irradiated ES-DC-MOG, TRAIL/MOG, or PD-L1/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.

ES-DC. In contrast, pretreatment with ES-DC-MOG, ES-DC-TRAIL/OVA (as irrelevant Ag), or ES-DC-PD-L1/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-PD-L1/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-PD-L1/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-PD-L1 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-PD-L1/MOG reduced severity of the disease, but ES-DC-MOG did not do so (Fig. 7*B* and Table II).

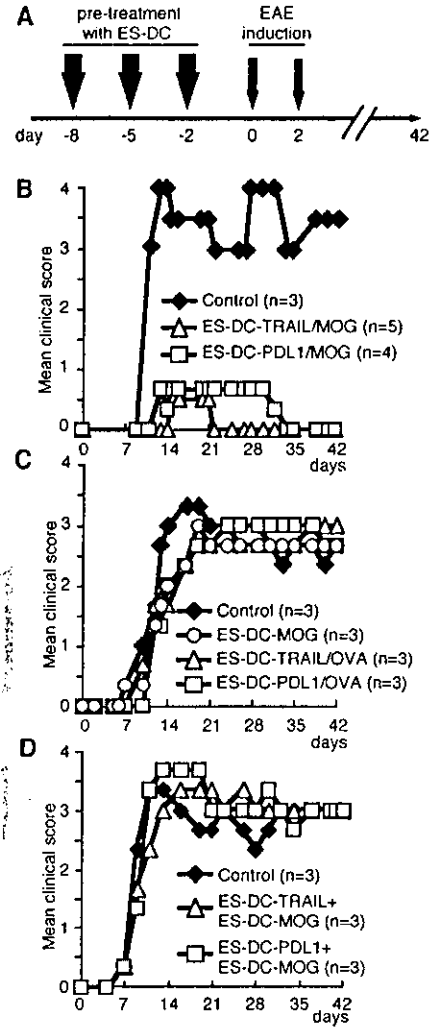

FIGURE 6. Prevention of MOG-induced EAE by pretreatment of mice with ES-DC expressing MOG plus TRAIL or MOG plus PD-L1. *A*, The schedule for pretreatment and induction of EAE is shown. CBF_1 mice (three to five mice per group) were i.p. injected with ES-DC (1×10^6 cells/injection/mouse) on days −8,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

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

We examined whether treatment with ES-DC-TRAIL/MOG or -PD-L1/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 -PD-L1/MOG was not increased in response to MOG peptide. In contrast, that of lymph node cells from ES-DC-MOG-treated or untreated mice was increased with statistical significance. In the presence of 25 μg/ml MOG peptide, stimulation index (count in the presence of MOG peptide/count in the absence of Ag) for that of untreated, ES-DC-MOG, -TRAIL/MOG, and -PD-L1/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 -PD-L1/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, -PD-L1/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 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

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