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Autoantibodies to T Cell Costimulatory Molecules in Systemic Autoimmune Diseases

Toshihiro Matsui,* ‡ Manae Kurokawa,† Tetsuji Kobata,* ‡ Shinji Oki,§ Miyuki Azuma,§ Shigeto Tohma,† Tetsufumi Inoue,† Kazuhiko Yamamoto,† Kusuki Nishioka,* and Tomohiro Kato‡*

To determine whether antilymphocyte Abs to T cell costimulatory molecules are generated in patients with autoimmune diseases and, if they exist, to clarify the mechanism of their production and pathological roles, we investigated the presence of autoantibodies to CTLA-4 (CD152), CD28, B7-1 (CD80), and B7-2 (CD86) in serum samples obtained from patients with various autoimmune diseases and from normal subjects using recombinant fusion proteins. In ELISAs, anti-CD28, anti-B7-1, and anti-B7-2 Abs were rarely seen, whereas anti-CTLA-4 Abs were detected in 8.2% of the patients with systemic lupus erythematosus, 18.8% of those with rheumatoid arthritis, 3.1% of those with systemic sclerosis, 31.8% of those with Behcet’s disease, 13.3% of those with Sjögren’s syndrome, and 0% of healthy donors. This reactivity was confirmed by immunoblotting. More importantly, the purified anti-CTLA-4 Abs reacted with CTLA-4 expressed on P815 cells by flow cytometry. In addition, we found at least three epitopes on the CTLA-4 molecule. Furthermore, among the patients with Behcet’s disease, uveitis was seen significantly less frequently in the anti-CTLA-4 Ab-positive patients. Taken collectively, these data indicate that anti-CTLA-4 autoantibodies are generated in systemic autoimmune diseases by an Ag-driven mechanism and may modulate the immune response in vivo by binding to CTLA-4 on T cells. The Journal of Immunology, 1999, 162: 4328–4335.

Autoantibodies to surface molecules on lymphocytes have been described in various immune conditions, such as autoimmune diseases, infections, blood transfusions, and multiple pregnancies (reviewed in Refs. 1 and 2). For instance, the presence of anti-lymphocyte Abs (ALAs) has been correlated with disease activity (3), lymphopenia (4), lymphocyte subset distortions (5, 6), and various functional abnormalities of T cells, B cells, and monocytes in systemic lupus erythematosus (SLE) (7–10). However, the pathogenic roles of ALAs remain unclear mainly because ALAs are usually detected by the microcytotoxicity test (1, 2), which cannot differentiate target molecules. To further clarify the roles of ALAs, it is essential to identify their individual determinants. Only a few targets of ALAs, including CD45 (11, 12), lymphocyte antigen 6 (13), and HLA class I molecules (14), have been described. Other surface molecules may also be targets. Thus, we tried to identify individual targets of ALAs. Specifically, we studied whether ALAs to T cell costimulatory molecules CTLA-4 (CD152), CD28, B7-1 (CD80), and B7-2 (CD86) are generated in autoimmune diseases, since T cells play central roles in the immune response.

T cells require at least two distinct signals for full activation: one is through TCR, and the other is through costimulatory molecules. TCR signal transduction without the costimulatory signals does not activate T cells but, rather, induces anergy (15). The best characterized costimulatory signaling systems are CD28 and CTLA-4 on T cells and their ligands, B7-1, and B7-2 on APCs (16, 17). CD28 gives a positive signal for T cell activation, but the signal through CTLA-4 remains controversial. Early studies suggested that CTLA-4 delivers a synergistic signal with CD28 (18). However, recent studies using anti-CTLA-4 Abs and CTLA-4-deficient mice have demonstrated that CTLA-4 is a negative regulator of T cell activation (19–22). Furthermore, CTLA-4 has been reported to be involved in immune disorders as follows. First, in vivo administration of anti-CTLA-4 Abs exacerbated the severity of experimental autoimmune encephalomyelitis in murine models (23–25). Second, lymphocyte infiltration into various organs of CTLA-4-deficient mice, is similar to that seen in autoimmune mice (21, 22). Third, a skewed CTLA-4 gene polymorphism has been reported in patients with Grave’s disease (26, 27) and autoimmune diabetes mellitus (27).

In this study we prepared four recombinant proteins of CTLA-4, CD28, B7-1, and B7-2 and investigated the presence of autoantibodies to these molecules in the sera of patients with various autoimmune disorders. Anti-CTLA-4 Abs were detected frequently. In contrast, Abs to the remaining three molecules were rare. In addition, we investigated the epitope regions on the CTLA-4 molecule and the actual binding of purified anti-CTLA-4 Abs to native CTLA-4 expressed on mammalian cells.

Materials and Methods

Patients and healthy donors

Serum samples were obtained from a total of 162 patients with systemic autoimmune diseases (138 women and 24 men; mean age, 50.2 yr; age...
range, 20–79 yr). They included 49 patients with SLE (46 women and 3 men; mean age, 42.3 yr; age range, 20–72 yr), 48 patients with rheumatoid arthritis (RA; 39 women and 9 men; mean age, 56.1 yr; age range, 22–79 yr), 32 patients with systemic sclerosis (SSc; 28 women and 4 men; mean age, 52.2 yr; age range, 29–71 yr), 22 patients with Behçet’s disease (14 women and 8 men; mean age, 50.9 yr; age range 24–78 yr), and 15 patients with Sjogren’s syndrome (Sjs; 15 women; mean age, 49.4 yr; age range, 32–66 yr). Four patients with both RA and Sjs were included in the respective groups. The diseases were diagnosed according to the standard criteria of each disease (28–32). Control serum samples were obtained from 82 healthy donors (68 women and 14 men; mean age, 49.5 yr; age range, 22–82 yr). The patients were being treated at the hospital of the University of Tokyo or the hospital of St. Marianna University School of Medicine.

Preparation of CTLA-4, CD28, B7-1, and B7-2 cDNA

Based on the previously reported nucleotide sequences of human CTLA-4 (33), CD28 (34), B7-1 (35), and B7-2 (36, 37), four sets of oligo-DNA primers were synthesized (Table I), and PCR was performed. Each primer pair was used to amplify the DNA fragments that encode the full protein-coding region of CTLA-4, CD28, B7-1, and B7-2. All the PCR products were confirmed by sequencing.

Construction of the expression plasmids of CTLA-4, CD28, and B7-1

The cDNA fragments that encode CTLA-4 (672 bp) and B7-2 (972 bp) were each subcloned into pTEX7 (38), which is a derivative of the previously reported plasmid expression vector pEX2 (39). In this expression system, the proteins are expressed as β-galactosidase fusion proteins. The cDNA fragments that encode CD28 (867 bp) and B7-1 (921 bp) were each subcloned into pTEX7-β-gal, which is a derivative of the plasmid expression vector pTEX7. With this plasmid, a continuous stretch of six histidines (His)_6, which is a tag for affinity purification, was included in the coding region of CTLA-4, CD28, and B7-1.

Expression and purification of the recombinant fusion proteins

Expression of the recombinant fusion proteins in E. coli, confirmed by sequencing, was prepared with S. typhimurium (POP2136) transformed with each of these recombinant plasmids. The recombinant proteins were induced, were lysed in 6 M guanidine buffer and sonicated. After centrifugation, the lysate was applied to a column with ProBond resin (NiCl2; Invitrogen, San Diego, CA) in a binding buffer (8 M urea, 20 mM sodium phosphate, and 500 mM NaCl, pH 7.8). The column was then developed in buffer (8 M urea, 20 mM sodium phosphate, and 500 mM NaCl, pH 6.0 and pH 5.3). The His-tagged recombinant proteins were eluted with an elution buffer (8 M urea, 20 mM sodium phosphate, and 500 mM NaCl, pH 4.0).

ELISA

Each well of a multiliter plate (Cook, Dynatech, Alexandria, VA) was coated with 10 μg/ml of the individual purified fusion protein or β-galactosidase (as a background) in a carbonate buffer (50 mM sodium carbonate, pH 9.6), followed by blocking with PBS containing 3% BSA and 0.1% Tween 20. To adsorb the reactivity of the serum samples to bacterial proteins and β-galactosidase, the serum samples were diluted and incubated with 20 μg/ml of bacterial lystate containing nonrecombinant pTEX7 product in PBS containing 3% BSA and 0.1% Tween 20 for 2 h at room temperature before incubation with the coated recombinants. The serum dilution that exhibited the highest specific binding was determined to be 1/7000 for CTLA-4 and B7-2 and 1/2000 for CD28 and B7-1. After reacting with the coated recombinants for 2 h, the wells were washed 10 times in PBS with 0.1% Tween 20. Next, the bound Abs were incubated with horseradish peroxidase-conjugated goat anti-human IgG (specific for γ-chain; Zymed, San Francisco, CA) at a 1/4000 dilution for 8 h at 4°C, then reacted with o-phenylenediamine as a substrate, and quantitated using a microplate photometer at 492 nm.

In the screening of autoantibodies to CTLA-4, CD28, B7-1, and B7-2, serum samples from 162 patients with systemic autoimmune disease and 82 sex- and age-matched healthy donors (68 women and 14 men; mean age, 49.5 yr; age range, 22–82 yr) were prepared. In the screening of autoantibodies to CTLA-4 in the respective diseases, serum samples from 40 of the healthy donors (38 women and 2 men; mean age, 43.0 yr; age range, 26–66 yr) for SLE, from 40 of the healthy donors (32 women and 8 men; mean age, 55.9 yr; range, 28–82 yr) for RA, from 40 of the healthy donors (35 women and 5 men; mean age, 52.2 yr; age range, 31–71 yr) for SSc, from 40 of the healthy donors (26 women and 14 men; mean age, 49.5 yr; age range, 22–73 yr) for Behçet’s disease, and from 40 of the healthy donors (40 women; mean age, 49.8 yr; age range, 30–73 yr) for Sjs were prepared.

The reactivity to the fusion protein in ELISA was expressed in units according to the formula: sample (binding unit) = [OD sample */(mean OD sample* + 3 SD of normal sera)] × 100. For each sample, the OD value of β-galactosidase was subtracted from the OD value of the fusion protein to obtain OD sample*. According to this formula, 100 binding units is the cutoff point.

Immunoblotting

Immunoblotting was performed as previously described (40). Briefly, 5 μg of each purified fusion protein or β-galactosidase (as a control), separated by 8% SDS-PAGE, was transferred onto nitrocellulose membranes. After blocking with PBS containing 5% BSA and 0.1% Tween 20 for 1 h and washing in PBS with 0.1% Tween-20 for 30 min, each membrane was then incubated with the respective protein-specific Ab (goat anti-human CTLA-4 Ab, goat anti-human CD28 Ab, goat anti-human B7-1 Ab, or goat anti-human B7-2 Ab; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit

Table I. PCR primers used to amplify the DNA fragments encoding T cell costimulatory molecules

<table>
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<tr>
<th>Molecule</th>
<th>Primers (5′→3′)</th>
<th>Reference</th>
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<td>29</td>
</tr>
<tr>
<td>B7-1</td>
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<td>30</td>
</tr>
<tr>
<td>B7-2</td>
<td>AAAGGATCCATGGCTTGCCTTTGGATTTCA</td>
<td>31, 32</td>
</tr>
</tbody>
</table>

Each clone was confirmed by DNA sequencing.
anti-β-galactosidase Ab (Chemicon, Temecula, CA), or serum samples diluted adequately in PBS containing 3% BSA and 0.1% Tween-20 for 1 h. In the case of the serum samples, they were diluted at 1/500 and preincubated with 20 μg/ml of the bacterial lysate containing nonrecombinant pTEX7 product for 2 h at room temperature before incubation with the membranes. After three washings in PBS with 0.1% Tween 20, the bound Abs were reacted with horseradish peroxidase-conjugated rabbit anti-goat IgG (American Qualex, San Clemente, CA), goat anti-rabbit IgG (Medical & Biological Laboratories, Nagoya, Japan), or goat anti-human IgG (Zymed) diluted 1/5000 for 30 min. Finally, the bound Abs were visualized with diaminobenzidine.

**Affinity purification of autoantibodies to fusion proteins**

Adsorption and affinity purification were performed essentially according to the method of Olinstedt (41). The partially purified recombinant proteins were separated by SDS-PAGE, then transferred onto nitrocellulose membranes. Next, the recombinant protein bands, visualized with a Ponceau S solution (Sigma, St. Louis, MO), were cut out and used for affinity purification. Each serum sample that had been preincubated with bacterial lysate containing nonrecombinant pTEX7 product for 2 h was diluted in PBS containing 3% BSA and 0.1% Tween 20 (1/500 dilution) and incubated for 2 h with the proteins fixed on the nitrocellulose membrane, after which the membrane was recovered. The bound Abs were eluted with 4 ml of 0.2 M glycine HCl buffer, pH 2.5, containing 0.15 M NaCl and 3% BSA, followed by neutralization with 600 μl of 2 M Tris base.

**Establishment of stable transfectant**

Human CTLA-4 cDNA was obtained from anti-CD3-activated PBMC by PCR using the sense primer (GATCCCGAGATGCTTGCGCTTG GATTTC) and the antisense primer (GATCCCGAGATGCTTGCGCTTG GATGGAATAAA), where the recognition sites for the restriction enzymes used are underlined. The cDNA was cloned into the XhoI- and i-digested BMCGSneo expression vector containing a neomycinycin gene (42). Transfection of murine mastocytoma P815 cells was achieved by electroporation as described by Azuma et al. (43). The drug-resistant cells were harvested and cloned, and cells with the highest cell surface expression of CTLA-4 were selected by flow cytometry.

**Indirect immunofluorescence and flow cytometry**

Binding of the purified anti-CTLA-4 Abs to native CTLA-4 was detected by indirect immunofluorescence. To block the binding of IgG to mouse Fc receptors, the P815 cells and the CTLA-4-transfected P815 cells were preincubated with mouse serum diluted 1/10 for 1 h at 4°C. After washing the cells in a staining buffer (PBS containing 2% BSA), the cells were suspended in the staining buffer and reacted with Abs purified from the patients’ sera or anti-CTLA-4 mAb (Biostride, Palo Alto, CA) for 30 min on ice. After washing in the staining buffer, the cells were incubated with phycoerythrin-conjugated goat anti-human IgG (Southern Biotechnology Associates, Birmingham, AL) or phycoerythrin-conjugated rabbit anti-mouse Ig (Southern Biotechnology Associates), respectively, for 30 min on ice. The fluorescence intensity was measured by FACSCalibur (Becton Dickinson, Mountain View, CA).

**Statistical analysis**

Laboratory data are expressed as the mean ± SEM. The Mann-Whitney U test was used to examine differences in the clinical parameters of patients with or without anti-CTLA-4 Ab. Fisher’s exact test was used to examine the correlation between clinical features of the patients and the presence of anti-CTLA-4 Ab.

**Results**

**Expression of four recombinant costimulatory molecules and their antigenicities**

DNA fragments encoding each of the human CTLA-4, CD28, B7-1, and B7-2 molecules were obtained by PCR from cDNA of a healthy donor PBL and then expressed as a β-galactosidase fusion protein. Thus, we obtained sufficient amounts of each fusion protein that had the expected m.w. (Fig. 1). We first confirmed the antigenicity of each of these recombinant proteins by immunoblotting using the respective protein-specific Abs. As shown in Fig. 1, the recombinant CTLA-4, B7-1, and B7-2 proteins were each stained as a clear band. In the case of CD28, staining with its specific Ab produced two major bands. Since the upper band had the expected m.w., the lower band was thought to be a degraded product of the full-length CD28 fusion protein. In addition, since approximately half the purified recombinant CD28 was the full-length CD28 fusion protein, the protein was thought to be of sufficient quality for detecting autoantibodies in ELISA.

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Reactivity of the serum of patients with various systemic autoimmune diseases with the recombinant CTLA-4, CD28, B7-1, and B7-2 proteins

The recombinant CTLA-4, CD28, B7-1, and B7-2 proteins were used to detect the presence of the respective autoantibodies in the sera of patients with various systemic autoimmune diseases by ELISA. As shown in Fig. 2, IgG-type anti-CTLA-4 Ab was detected in the sera of 21 of the 162 patients (13.0%); however, the sera of only three patients were positive for anti-B7-2 Ab, and no patient was positive for anti-CD28 or for anti-B7-1 Ab. Autoantibodies to each of the four molecules were not detected in the sera of any of the healthy donors. In addition, the presence of IgM-type anti-CTLA-4 Ab was determined in a similar way and was detected in the sera of only three patients (data not shown).

The prevalence of the anti-CTLA-4 autoantibody in each disease is shown in Fig. 3. The anti-CTLA-4 Ab was detected in the sera of 4 of 49 patients with SLE (8.2%), 9 of 48 patients with RA (18.8%), 1 of 32 patients with SSc (3.1%), 7 of 22 patients with Behçet’s disease (31.8%), and 2 of 15 patients with Sjs (13.3%). The positive serum samples were further tested by immunoblotting. Of the 21 samples that were positive by ELISA, 19 reacted to CTLA-4. The results of 5 representative autoimmune disease patients are shown in Fig. 4.

Recognition of native CTLA-4 by the autoantibodies

Our findings showed that a considerable percentage of the tested autoimmune disease sera contained anti-CTLA-4 Abs. However, the recombinant CTLA-4 produced in E. coli and solubilized in 8 M urea is denatured, and thus would not be expected to have the native conformation.

Therefore, we further investigated whether the anti-CTLA-4 autoantibodies in the patients could bind to the native form of CTLA-4 molecules. Specifically, we purified the Abs bound to the recombinant CTLA-4 and then examined whether these Abs bind to CTLA-4 molecules expressed on mammalian cells (P815) by
immunofluorescent staining followed by FACS analysis. The purified anti-CTLA-4 fusion protein Ab was able to bind to the TLA-4-transfected P815 cells, but not to P815 cells. A representative case is shown in Fig. 5. These data provide direct evidence that the anti-CTLA-4 autoantibodies of patients react with the native form of the CTLA-4 molecules expressed on the mammalian cell surface.

Epitope mapping of CTLA-4

To map the autoepitopes on the CTLA-4 protein, three pTEX7 plasmids with cDNA fragments F1, F2, and F3 (pTEX7-cDNA F1, pTEX7-cDNA F2, and pTEX7-cDNA F3) were constructed (Fig. 6A). We obtained the respective proteins shown in Fig. 6B. To confirm these reactivities, serially diluted serum samples were similarly tested by ELISA. Representative results (RA-5, ns-65, and ns-31) are shown in Fig. 7. Thus, most of the serum samples recognized multiple epitopes on CTLA-4.

Laboratory data and clinical features of Behçet’s disease patients whose sera do and do not contain anti-CTLA-4 Abs

Since anti-CTLA-4 Abs were detected most frequently in the sera of patients with Behçet’s disease, we compared the laboratory data and the clinical features, including past history of the anti-CTLA-4 Ab-positive and -negative Behçet’s disease patients. There was no significant difference in the laboratory data (erythrocyte sedimentation rate, peripheral lymphocyte count, and serum levels of IgG, IgA, and IgM) of the two groups of Behçet’s disease patients (Table III). Similarly, most of the clinical features of the two groups did not differ significantly. However, uveitis was detected in 13 of the 15 (86.7%) Ab-negative patients, but in only two of the seven (28.6%) Ab-positive patients, and this difference is statistically significant (Table IV).

Discussion

We have demonstrated for the first time that CTLA-4, a costimulatory molecule of T cell activation, is a target of ALAs. Furthermore, we showed that the autoantibody to CTLA-4 reacts with the native form of human CTLA-4 molecule expressed on the mammalian cell surface.

In our study, anti-CTLA-4 Abs were detected more frequently among patients with Behçet’s disease and RA than among those with SLE. This was unexpected, since ALAs are detected in a higher percentage of patients with SLE than in patients with Behçet’s disease and RA (2). This may be because CTLA-4 is a minor target of ALA (discussed below), and thus the prevalence of anti-CTLA-4 Ab would be different from the prevalence of total ALA previously reported (2). Alternatively, the discrepancy may be related to the detection method. The standard method for detection of ALAs is the microcytotoxicity test, in which the presence of ALA-mediated complement cytolysis is tested using PBL of healthy donors. This method may miss the ALAs to Ags that are

<table>
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a NT, not tested; ±, > mean OD + 2 SD of normal sera; +, > mean OD + 3 SD of normal sera.
b ns-2 and ns-48, the patients with RA and Sjs.
temporarily expressed or induced by activation, such as CTLA-4. Thus, ALA detection using recombinant proteins may produce different results from those obtained by the conventional method. Screening of a larger number of serum samples than that reported here would be needed to resolve this issue.

The laboratory data and clinical features of patients did not correlate with the presence or the absence of anti-CTLA-4 Ab among patients with Behçet’s disease (Table II), those with RA, and those with SLE (data not shown). Previous reports indicated that the presence of ALAs correlates with lymphopenia (4); however, our data showed that the presence of anti-CTLA-4 Ab did not correlate with the lymphocyte count. As to clinical features, it is interesting that a negative correlation was found between uveitis and the presence of anti-CTLA-4 Ab in Behçet’s disease patients. Although the pathogenesis of Behçet’s disease remains unclear, immunological abnormalities, in particular infiltration of predominantly T lymphocytes in erythema nodosum-like lesions (44), oral ulcers (44), terminal ileum (45), and ocular lesions (46), have been reported. Thus, T cells play a role in the affected lesions. In this regard, the anti-CTLA-4 Ab detected in the Behçet’s disease patients may act to suppress the proliferation of uveitis-driving T cells. Decreased function of suppressor T cells and subsequent increased B cell function have been implicated in Behçet’s disease, and Sakane et al. suggested that ALAs might be responsible for the loss of suppressor T cell function (47). From this viewpoint, anti-CTLA-4 Abs may suppress the function of such suppressor T cells and dysregulate B cell function. In all patients with uveitis in our study, the uveitis was inactive at the time their serum samples were obtained. Therefore, further studies comparing disease activity with the titer of anti-CTLA-4 Ab may give us some clues to understanding the role of anti-CTLA-4 Ab in vivo. In RA, Verwilghen et al. reported that anti-CTLA-4 mAb had an inhibitory effect on the mixed lymphocyte cytotoxicity test when synovial fluid T cells from RA patients were used as stimulatory cells (48). These findings suggest that anti-CTLA-4 Ab in RA acts to suppress arthritis-driving T cell function, similar to its role in suppressing uveitis in Behçet’s disease.

Epitope mapping using three recombinant CTLA-4 fragments revealed that the CTLA-4 molecule contains at least three epitopes recognized by the IgG type of the anti-CTLA-4 Ab. Most of the anti-CTLA-4 Ab-positive sera reacted to more than one CTLA-4 fragment. This result indicates that the production of autoantibodies to CTLA-4 is induced by an Ag-driven mechanism, which is the main mode of production of many antinuclear Abs (49, 50). In addition, this diversity of anti-CTLA-4 Abs may indicate the existence of several kinds of anti-CTLA-4 Abs with different functions.

Among the four T cell costimulatory molecules tested, autoantibodies to CTLA-4 were frequently detected in patients with autoimmune disease, but autoantibodies to CD28, B7-1, and B7-2 were rare. In particular, CD28 shares 31% amino acid identity with CTLA-4 (51), but anti-CD28 Ab was not detected in any of the
patients. This fact suggests the existence of a CTLA-4-specific immune response among the four costimulatory molecules tested in this study. This may be related to the difference in the manner in which the molecules are expressed, i.e., activation-induced or constitutive expression. Further studies are needed to elucidate this mechanism.

To screen autoantibodies to the costimulatory molecules, we used proteins produced in bacteria. Therefore, autoantibodies to the natural form of the molecules could have been missed. To detect such kinds of Abs, transfecants can be useful. We tried to detect anti-CTLA-4 Abs with the CTLA-4-transfected P815 cells using whole sera instead of purified anti-CTLA-4 Abs from patients. However, this was not effective, since a considerable number of serum samples strongly reacted to the transfecants and also to the control P815 cells (data not shown). It is possible that other kinds of autoantibodies in the sera cross-reacted with the surface molecules of the P815 cells. Thus, the transfecants were useful in determining whether an Ab specifically purified from sera could react to the natural form of the molecule, but was incompletely screened for screening of ALA using whole sera.

Several studies in mice and humans have indicated that a lack or modification of negative signals through CTLA-4 is implicated in the autoimmune diseases (21–27). In addition, CTLA-4 was reported to modify the negative signals through CTLA-4 is implicated in the immune response among the four costimulatory molecules tested in the sera from patients. This fact suggests the existence of a CTLA-4-specific immune response in the sera from patients with autoimmune disease.

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References


