Original article

Inhibition of collagen-induced arthritis by DNA vaccines encoding TCR Vβ5.2 and TCR Vβ8.2

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Keywords: arthritis, collagen-induced; T cell receptor; vaccines, DNA

Background  Arthritogenic T lymphocytes with common T cell receptor (TCR) Vβ clonotypes, infiltrating in the articulars of rheumatoid arthritis (RA) patients, play a central role in the pathogenesis of RA. TCR Vβ5.2 and TCR Vβ8.2 are the main pathogenic T cell clonotypes in the course of collagen-induced arthritis (CIA) progression in Lewis rats. To investigate a TCR-based immunotherapy for RA, we constructed recombinant DNA vaccines encoding TCR Vβ5.2 and TCR Vβ8.2, and evaluated the inhibitive effects of the two vaccines on CIA rats.

Methods  Genes encoding TCR Vβ5.2 and TCR Vβ8.2 were amplified by RT-PCR from spleen lymphocytes of Lewis rats and cloned into the eukaryotic expression vector pTargetT. The expression of vaccines was confirmed by RT-PCR and immunohistochemistry. The inhibitive effects of the vaccines on articulars of CIA rats were assessed with arthritis index evaluation and histology. Interferon γ (IFN-γ) and interleukin (IL)-4 production by spleen lymphocytes were tested with enzyme-linked immunospot assay (ELISPOT) technique, the changes in peripheral CD4+ and CD8+ lymphocyte populations were tested by flow cytometry, and the level of anti-CII antibody in serum was assayed by enzyme-linked immunosorbent assay (ELISA).

Results  Recombinant DNA vaccines pTargetT-TCR Vβ5.2 and pTargetT-pTCR Vβ8.2 were successfully constructed. Both vaccines inhibited CIA, which alleviated the arthritis index score (P < 0.05), decreased the level of IFN-γ (P < 0.05), and reduced the ratio of CD4+/CD8+ lymphocytes (P < 0.05) and the anti-CII antibody in serum (P < 0.05). In addition, the histological change in DNA-vaccinated rats was less serious than CIA rats. Compared to pTCR Vβ 8.2 and pTCR Vβ 5.2 groups, the group that was injected with a combination of the two vaccines showed stronger inhibitive effects on CIA than either individual vaccine.

Conclusion  The recombinant plasmids pTargetT-TCR Vβ5.2 and pTargetT-pTCR Vβ8.2 have obvious inhibitory effects on CIA rats and better effects could be achieved when the vaccines were used in combination.

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Rheumatoid arthritis (RA) is a chronic, inflammatory, systemic autoimmune disease of unknown etiology. It is characterized by symmetric inflammation of synovial joints leading to progressive erosion of cartilage and bone, joint pain, deformities and destruction of the joints. Patients with RA are at risk of decreased physical capacity and functional ability. Consequently, they have increased susceptibility for comorbidity conditions such as cardiovascular disease or osteoporosis.1-4

The factors triggering RA are thought to be a combination of genetic, infectious, environmental and hormonal factors which are all involved in complex, interrelated ways.5 The central role of antigen-specific T cells in the pathogenic immune response in RA has been described elsewhere.6 Antigen recognition by T cells activates a host of pathways, which lead to the production of cytokines, prostanolinds, leukotrienes and oxygen free radicals, as well as further recruitment of immune cells.7 These factors act in concert to exacerbate the response. The result of this process is full-blown disease and tissue destruction leading to the clinical manifestations of RA.8

Understanding of the mechanism of RA has already generated several effective therapies, including some targeted therapy approaches such as neutralization of proinflammatory cytokines by monoclonal antibody (mAb) or soluble receptors. These targeted therapies are based on inhibition of the proinflammatory cytokines and can effectively control RA, revealing the potential of modulating the cytokine balance as a therapeutic strategy for controlling RA.9-12 However, these therapies often result in systematic suppression of the immune system.10,12 Another promising immunotherapeutic approach is to target the inflammatory Th1 cells that initiate and propagate damage in the synovium directly.13,14 Experiments have revealed that there are arthritogenic antigen-reactive T lymphocytes, which are specifically activated, infiltrating in the articular synovium and fluids, and there are common T cell

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receptor (TCR) Vβ clonotypes in the articulars of RA patients. Thus, examination of the TCR repertoire of arthritic Th1 cells, identification of the expansion of T cell clonotypes, and administration of arthritogenic TCR variable region peptides or DNA vaccines encoding the pathogenic TCR Vβ region may provide useful information for the design of specific immunotherapy against RA. Compared with TCR peptide vaccines, TCR DNA vaccines have higher specificity, better safety and are easier to produce. Although there are reports about the therapeutic effect of TCR Vβ peptide vaccines on some RA patients, there have been few reports about the effects of TCR DNA vaccines on RA.

Collagen-induced arthritis (CIA), inducible in susceptible strains of mice and rats by immunization with type II collagen (CII), is characterized by peripheral joint lesions and shares many features with human RA. It has been widely used to investigate the pathogenic mechanisms of RA and to evaluate potentially new therapeutic agents. Many experiments have demonstrated that both the cellular and humoral immune response influence the development of CIA. However, the activation of self-reactive T lymphocytes is the main stimulator that causes the damage of tissues in CIA. Therefore, inhibition of self-reactive T lymphocytes can inhibit not only the cellular response mediated by T cells but also the humoral immune response mediated by B cells.

We recently demonstrated by RT-PCR/SSCP that TCR Vβ5.2 and TCR Vβ8.2 predominate in the synovium in the course of CIA progression in Lewis rats, whereas irrelevant TCRs are diverse. We have also shown that TCR Vβ5.2 and TCR Vβ8.2 are pathogenic by adoptive transfer of lymphocytes and reported the effect of TCR Vβ5.2/8.2-HSP DNA vaccines on CIA. To investigate the inhibitory effect of TCR DNA vaccines on CIA rats, we extend our strategy to construct DNA vaccines encoding TCR Vβ5.2/8.2-HSP and TCR Vβ5.2/8.2 DNA vaccines. In this study we investigate the effect of TCR Vβ5.2/8.2 DNA vaccines on CIA rats.

METHODS

Animals
Inbred female Lewis rats, aging 4–5 weeks and weighing (80±10) g, were purchased from the Experimental Animal Company, Weitonglihua, Beijing. Animals were raised and maintained under pathogen-free conditions in the Animal Breeding Centre of this institute. Experiments were conducted under the supervision and guidelines of the Animal Welfare Committee.

Construction of recombinant DNA plasmids
Total RNA was extracted from spleen cells of normal Lewis rats and reverse transcribed into cDNA. The CDNA was then amplified by PCR using Taq polymerase with primers specific for TCR Vβ5.2 (5'TCT GTTCTGTAGGCTCA-3'), TCR Vβ8.2 (5'CAAAC-ACATGGAAGCTGCA-3') and Cβ primer (5'TCT GCTTCTGACTGCG-3'). All of the forward primers were designed to include an in-frame ATG codon. The PCR products were cloned into pTargetT according to the manufacturer's instructions. After transformation into E. coli JM109 (kept in our laboratory), colonies grown in competent cells were picked and recombinant DNA plasmids were isolated using the Plasmid Miniprep procedure (Sigma, USA). The colonies were screened for correct insert direction and length with bacteria colony PCR and digestion with Pst I. Selected clones were sequenced by Shanghai Sangon Company (China) to confirm sequence authenticity and presence of the in-frame ATG codon.

Expression of TCR Vβ in injected muscles of Lewis rats
RT-PCR analysis
To verify the expression of TCR Vβ5.2 and TCR Vβ8.2, inbred female Lewis rats were pretreated with 0.75% bupivacaine (1 µg/g body weight) by injecting into quadriceps using a sterile syringe. Five days later 150 µg recombinant DNA plasmid was injected into the same site according to the protocol indicated above. The control rats were injected with PBS or empty plasmid pTargetT (Promega, USA). Booster vaccination was given twice with the same method, once every 2 weeks. The quadriceps were removed from the injected site 12 days after the last vaccination, and RNA was extracted using Trizol, following the manufacturer's protocol. First strand cDNA synthesis was achieved with an oligo(dT)6 primer using Moloney murine leukemia virus (M-MuLV) reverse transcriptase. Using a forward primer specific for the plasmid sequence upstream to the insert and Cβ primer, the PCR program consisted of 4 minutes at 93°C, followed by 30 cycles of 1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C. The final extension step consisted of 7 minutes at 72°C.

Immunohistochemistry analysis
Expression of TCR protein was detected by immunohistochemical staining. Twelve days after the last vaccination of pTarget TCR Vβ5.2 and pTarget TCR Vβ8.2, the injected muscles of rats were dissected out, fixed in 40 g/L formaldehyde, embedded in paraffin, consecutive cuts of 4 µm were taken and subjected to deparaffinization. To neutralize endogenous peroxidase activity before antibody application, we incubated the sections for 10 minutes in 30% hydrogen peroxide at room temperature and then rinsed the sections three times in distilled water. Citrate buffer (0.01 mol/L) was used to repair antigens, and nonspecific binding was blocked with 0.5% bovine serum albumin for 20 minutes. Primary mouse polyclonal antibody of mouse anti-rat TCRα/β (Serotec, UK) at a dilution of 1:80 was incubated for 1–2 hours at 37°C with the sections. After washing three times with PBS, the coverslips were incubated in horseradish peroxidase-conjugated goat anti-mouse (Serotec, UK, 1:300) for 2 hours, followed by 3,3-diaminobenzidine (DAB) until a
brown reaction product was observed. After being washed three times, the sections were gradually dehydrated with alcohol, and made transparent using dimethyl benzene. The sections were mounted by neutral resin and the staining was observed through a microscope (Olympus, Japan).

DNA immunization
Large-scale preparations of recombinant DNA plasmids pTCR Vβ5.2, pTCR Vβ8.2 and empty plasmid pTargeT were made using the plasmid Maxiprep kit (Vigorous Biotechnology Corporation, China). Plasmid DNA was resuspended in sterile PBS. Spectrophotometric analysis showed that the 260/280 nm ratios of all three preparations were between 1.8 and 2.0, indicating no contamination of RNA or proteins. The quality of the DNA preparation was confirmed on a 0.7% agarose gel. The concentrations of plasmids DNA were adjusted to 1 g/L using sterile PBS.

Thirty-six healthy inbred female Lewis rats were randomly assigned to six groups: pTCR Vβ5.2 group, pTCR Vβ8.2 group, pTCR Vβ5.2 + pTCR Vβ8.2 group, pTargeT group, normal group and CIA group. For DNA vaccination, rats were pretreated with 0.75% bupivacaine (1 µl/g body weight) by injection into the bilateral quadriceps, with a plastic collar to limit needle penetration to 2 mm. The tested DNA vaccines were administered at the same site 5 days after the bupivacaine pretreatment according to the indicated protocol. Each immunization of unilateral quadricepses was carried out as follows: for pTCR Vβ5.2 group, 150 µg pTCR Vβ5.2 was injected; for pTCR Vβ8.2 group, 150 µg pTCR Vβ8.2 was injected; for pTCR Vβ5.2 + pTCR Vβ8.2 group, 150 µg pTCR Vβ5.2 and 150 µg pTCR Vβ8.2 were injected; for pTargeT group, 150 µg pTargeT was injected. Booster vaccination was given once every 2 weeks using the same procedure. The normal control rats were injected with PBS.

CIA induction and assessment
CIA was induced 12 days after the last DNA vaccination. For induction of CIA, bCII was solubilized in 0.1 mol/L acetic acid to a concentration of 2 mg/ml with constant mixing overnight at 4°C. Then bCII was emulsified with an equal volume (1:1) of Freund’s incomplete adjuvant (IFA), and 300 µl of the emulsion was injected intradermally at the back and the base of the tail of each rat under light ether anesthesia. The day of CIA induction was designated as day 0. The induction of CIA was boosted with the same volume 7 days later. Disease severity was assessed by direct observation of all four limbs in each rat. A relative score between 0 and 4 was assigned to each limb based on the degree of joint inflammation, redness, and deformity; thus, the maximum possible score for an individual rat was 16.

Joint histology
The rats were killed at day 24, the peak of CIA, and their legs and hind paws were removed and fixed with 10% neutral formalin for 48 hours, decalcified in 5% formic acid, embedded in paraffin, and 5 µm slices were prepared for analysis. Sections were stained with hematoxylin and eosin (HE).

Flow cytometric analysis
Peripheral blood of CIA rats was aspirated via cardiac puncture at day 24 and mixed with PE-conjugated mouse-anti-rat CD8 antibody (BD, USA) and FITC-conjugated mouse-anti-rat-CD4 antibody (BD, USA) for 20 minutes in the dark. The reaction was preceded by adding red blood cell lysis buffer for 10 minutes at 37°C. The samples were then centrifuged at 1000 r/min for 8 minutes. The precipitated cells were washed twice with PBS, and resuspended in 0.5 ml PBS. The stained cells were analyzed by FACScan flow cytometry.

Cytokine assays
CD4+ T cell responses in vivo were usually evaluated by both IFN-γ (Th1) and IL-4 (Th2) assay. Therefore, rat IFN-γ and IL-4 were quantified in the culture supernatants of spleen cells with ELISpot according to the manufacturer's instructions (U-CyTech Company, NL). Briefly, the spleens were removed aseptically from rats at day 24 after CIA induction. ELISpot plates were coated overnight with anti-rat cytokine monoclonal capture antibodies. Nonspecific binding was blocked by incubation with 1% bovine serum albumin for 1 hour at room temperature. Then spleen lymphocytes from rats were added at a concentration of 1×10⁷ cells/ml (1×10⁶ cells/well). After incubation at 37°C for 24 hours, cells were removed by washing the plates, and the site of cytokine secretion was detected using biotinylated anti-rat cytokine monoclonal detective antibodies and streptavidin-alkaline phosphatase conjugate. Spots were developed by the addition of AEC substrate. Spot-forming cells (SFCs) representing the level of secreted cytokines by lymphocytes were enumerated using KS ELISpot system.

Indirect ELISA
Blood were obtained from the rats at day 24 by cardiac puncture under ether anesthesia, and the level of anti-CII antibody in serum was determined by indirect ELISA. Briefly, 96-well flat-bottom ELISA plates were coated with bCII at 50 µg/ml in carbonate buffer by incubation overnight at 4°C. After washing with PBST (PBS+0.05% Tween-20) three times, the plates were blocked with 1% bovine strum albumin-PBS for 1 hour. The plates were then washed three times and serum samples (1:100 dilution in PBS) were added for 1 hour at 37°C. After washing with PBST the plates were incubated for 1 hour at 37°C with peroxidase-conjugated goat anti-rat IgG (Sigma, USA) diluted 1:1000 in PBS. The reaction was preceded by 3,3,5,5-tetramethylbenzidine (TMB) for 15 minutes and stopped by adding 2 mol/L H₂SO₄. The optical density was measured at 450 nm with an ELISA-reader.
Figure 1. DNA vaccine construction and verification. A: Acquisition of TCR Vβ8.2 and TCR Vβ5.2 gene fragments. Lane 1: DNA marker (GeneRuler™ 100 bp DNA ladder); Lane 2: TCR Vβ5.2, approximately 500 bp; Lane 3: TCR Vβ8.2, approximately 400 bp. B: Identification of positive clones by PCR. Lane 1: DNA marker; Lanes 2 and 3: positive band of TCR Vβ8.2; Lanes 4 and 5: positive band of TCR Vβ5.2; Lane 6: negative amplification. C: RT-PCR assay of TCR Vβ5.2 and TCR Vβ8.2 mRNA expression after injection into rat muscles. Lane 1: DNA marker; Lane 2: positive control of TCR Vβ5.2; Lane 3: positive control of TCR Vβ8.2; Lanes 4 and 6: positive band of TCR Vβ5.2; Lanes 5 and 7: positive band of TCR Vβ8.2; Lane 8: empty plasmid pTargT control; Lane 9: blank control.

Statistical analysis
Statistical analysis was performed using SPSS12.0 software. Values were expressed as the mean ± standard deviation of the mean (SEM) for each group if not otherwise indicated. The analysis of variance (ANOVA) was conducted to determine significant differences between different experimental groups. Differences with a P value less than 0.05 were considered statistically significant.

RESULTS
Construction of recombinant plasmids pTargeT-TCR Vβ5.2 and pTargeT-TCR Vβ8.2
cDNA fragments of TCR Vβ5.2 and TCR Vβ8.2 (about 400 bp and 500 bp, respectively) were obtained from spleen lymphocytes (Figure 1A), amplified by PCR and cloned into plasmid pTargeT. After transformation of E. coli, 15 randomly selected clones were screened for the expected length and orientation of the insert, and sequenced. Clones without sequence deviations were selected for further use (Figure 1B).

Expression of TCR Vβ in injected muscles of rats
To test expression efficiency, we injected pTCR Vβ5.2 and pTCR Vβ8.2 vaccines into the quadriceps of Lewis rats. The injected muscles were removed 12 days after the last vaccination. RT-PCR and immunohistochemistry analysis showed that both TCR Vβ5.2 and TCR Vβ8.2 gene fragments were successfully transcribed and expressed in the injected muscles of rats (Figures 1C and 2).

Inhibition of CIA with DNA vaccines
We evaluated the inhibitory effects of pTCR Vβ5.2 and pTCR Vβ8.2 DNA vaccines on the development of CIA, including their effects on the arthritis index and articular histological changes, IFN-γ and IL-4 production by spleen cells and the change of peripheral CD4⁺ and CD8⁺ lymphocytes. The level of anti-CII antibody in serum was assayed by indirect ELISA.

Effects on articulars
After the induction of CIA, Lewis rats showed less activity and gradually lost weight. From day 13, edema, swelling and stiffness appeared in the hind paws of rats, with severe cases even accompanied by hemorrhage. The rats in the CIA control group and the pTargeT group showed evidence of arthritis from day 12 to day 14, and the swelling and edema reached a peak after 7–9 days. By day 22, all rats in the two groups had manifested arthritis symptoms. The incidence was 100% and arthritis scores showed no difference between the two groups. In contrast, there were only one or two rats suffering from CIA in the DNA-vaccinated groups. Besides swelling in the hind limbs, some rats showed slight denudation, diarrhea, loosening of nails, local ulcers in limbs and sluggish growth.

Compared with the CIA or pTargeT groups, progression of arthritis in rats immunized with pTCR Vβ5.2 or pTCR Vβ8.2 lagged behind about a week and manifested significantly milder symptoms, indicating the inhibitory effect of pTCR Vβ5.2 and pTCR Vβ8.2 on CIA development. Inhibition of CIA was reflected as a diminished arthritis score, as shown in Figure 3. By day 21 the mean arthritis score of the rats in pTCR Vβ5.2+pTCR Vβ8.2 group was also significantly lower than that of pTCR Vβ5.2 or pTCR Vβ8.2-vaccinated...
Effects on histologic change
Examination of the joints revealed that the histology of the CIA rats was characterized by subsynovial inflammation, synovial hyperplasia, pannus formation, cartilage erosion and destruction of cartilage. In contrast, pTCR Vβ5.2 and pTCR Vβ8.2-vaccinated rats manifested reduced bone and cartilage erosion and synovial inflammation. In addition, pTCR Vβ5.2+pTCR Vβ8.2 vaccinated rats showed far fewer infiltrating lymphocytes in the articular cavity and less severe proliferative synovitis (Figure 4). The results show that pTCR Vβ5.2 and pTCR Vβ8.2 DNA vaccines markedly reduce the joint inflammation of CIA rats and an even better arthritic-protecting effect can be achieved if pTCR Vβ5.2 and pTCR Vβ8.2 DNA vaccines are administered simultaneously. The result corresponds to the arthritic change of rats described above.

Effects on cytokine secretion
Lymphocytes isolated from the rats were tested for the secretion of IFN-γ and IL-4. The secretion of IFN-γ and IL-4 by rat lymphocytes was measured by the number of SFCs. Figure 5A shows the effect of DNA vaccination on IFN-γ secretion in CIA rats. The rats in CIA control group showed a higher level of IFN-γ secretion compared with the normal control (P<0.05), but there was no difference between CIA control group and pTargeT group. The level of IFN-γ secretion in the recombinant plasmid groups was lower than in the CIA and pTargeT control groups (P<0.05). The results indicate that IFN-γ secretion is closely associated with the pathogenesis of CIA, and the use of recombinant TCR Vβ5.2 and TCR Vβ8.2 vaccines can decrease its level.

Figure 5B shows that IL-4 secretion from spleen lymphocytes from rats in the pTCR Vβ5.2+pTCR Vβ8.2 group was increased compared with the CIA control and pTargeT groups (P<0.05). Also the level of IL-4 secretion in the pTCR Vβ5.2+pTCR Vβ8.2 group is significantly higher than the pTCR Vβ8.2 group (P<0.05), implying that inhibition of CIA is associated with increased secretion of IL-4.

Effects on T lymphocyte subtypes
Analysis of T lymphocytes by flow cytometry indicated

**Figure 3.** Reduction of CIA arthritis score after vaccination with pTCR Vβ5.2 and pTCR Vβ8.2.

**Figure 4.** Histological sections of hind limbs of rats (HE staining, original magnification ×100). Scale bar: 50 µm. A: normal control; B: CIA control; C: pTargeT control; D: pTCR Vβ5.2 group; E: pTCR Vβ8.2 group; F: pTCR Vβ5.2 + pTCR Vβ8.2 group.
that the number of CD8+ T lymphocytes in CIA control rats was significantly lower than normal rats (P < 0.05), and the CD4+/CD8+ ratio was higher than normal rats (P < 0.05). Flow cytometry analysis showed that the CIA control rats had significantly (P < 0.05) reduced numbers of CD8+ T lymphocytes and a significantly (P < 0.05) higher CD4+/CD8+ ratio compared to normal rats. This suggests that a disorder of T lymphocyte subtypes existed in the CIA rats. As shown in Table 1, in comparison with CIA or pTargetT control rats, the increase of CD8+ T lymphocytes and the decrease of the CD4+/CD8+ ratio in recombinant plasmid-vaccinated groups were both statistically significant (P < 0.05). Taken together, the data suggest that pTCR Vβ5.2 and pTCR Vβ8.2 DNA vaccines can to some extent correct the disorder of the T lymphocytes in CIA rats.

**Table 1.** Comparison of T lymphocyte subtypes following DNA vaccination

<table>
<thead>
<tr>
<th>Groups</th>
<th>Subjects (n)</th>
<th>CD4+ (%)</th>
<th>CD8+ (%)</th>
<th>CD4+/CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIA</td>
<td>6</td>
<td>60.30±6.31</td>
<td>22.55±4.76</td>
<td>2.79±0.74</td>
</tr>
<tr>
<td>Normal</td>
<td>6</td>
<td>54.90±7.60</td>
<td>27.27±2.71</td>
<td>2.01±0.17</td>
</tr>
<tr>
<td>pTargetT</td>
<td>6</td>
<td>61.67±4.56</td>
<td>24.80±3.41</td>
<td>2.51±0.22</td>
</tr>
<tr>
<td>pTCR Vβ5.2</td>
<td>6</td>
<td>62.07±2.96</td>
<td>28.23±2.07</td>
<td>2.21±0.15</td>
</tr>
<tr>
<td>pTCR Vβ8.2</td>
<td>6</td>
<td>46.71±14.81</td>
<td>29.73±2.14</td>
<td>1.56±0.43</td>
</tr>
<tr>
<td>pTCR Vβ5.2 + pTCR Vβ8.2</td>
<td>6</td>
<td>60.23±9.23</td>
<td>31.57±3.39</td>
<td>1.61±0.33</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with CIA control or pTargetT rats. **P < 0.05 compared with normal rats. ***P < 0.05 compared with pTCR Vβ5.2 + pTCR Vβ8.2 group.

**Effects on anti-CII antibody**

The level of anti-CII antibody in serum was assayed by indirect ELISA. As indicated in Table 2, the decrease in the level of antibody in recombinant plasmid-vaccinated groups was significant compared with the CIA and pTargetT control rats (P < 0.01). A lower level of anti-CII antibody was observed in pTCR Vβ5.2 + pTCR Vβ8.2 group compared with pTCR Vβ5.2-vaccinated rats (P < 0.01). Furthermore, the level of antibody in normal rats was far lower than the other five groups (P < 0.01). These results imply that anti-CII antibody is arthritogenic and we can alleviate the symptoms of CIA by decreasing its level. Statistical analysis indicates that pTCR Vβ5.2 and pTCR Vβ8.2 DNA vaccines can effectively inhibit the humoral immune response, thus decreasing the level of anti-CII antibody in CIA rats.

**Table 2.** Anti-CII antibody assay following DNA vaccination

<table>
<thead>
<tr>
<th>Groups</th>
<th>Subjects (n)</th>
<th>anti-CII antibody (AbsO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIA</td>
<td>6</td>
<td>0.417±0.007*</td>
</tr>
<tr>
<td>Normal</td>
<td>6</td>
<td>0.072±0.007**</td>
</tr>
<tr>
<td>pTargetT</td>
<td>6</td>
<td>0.450±0.013†</td>
</tr>
<tr>
<td>pTCR Vβ5.2</td>
<td>6</td>
<td>0.411±0.005‡</td>
</tr>
<tr>
<td>pTCR Vβ8.2</td>
<td>6</td>
<td>0.401±0.004*</td>
</tr>
<tr>
<td>pTCR Vβ5.2 + pTCR Vβ8.2</td>
<td>6</td>
<td>0.340±0.006*</td>
</tr>
</tbody>
</table>

*P < 0.01 compared with CIA control or pTargetT rats. **P < 0.01 compared with normal rats. ***P < 0.01 compared with rats in pTCR Vβ5.2 + pTCR Vβ8.2 group.

**DISCUSSION**

In this study we investigated the inhibitive effects of TCR DNA vaccines on the progression of CIA in Lewis rats on the basis of the finding that TCR Vβ5.2 and TCR Vβ8.2 predominate in the synovium in the course of CIA. Like other DNA vaccines, TCR DNA vaccines can induce immune responses directly by way of expressing protein antigens in the body. TCR DNA vaccines are characterized by high specificity and good safety and are easy to produce. They induce a specific immune response and may play a therapeutic role by recognizing and destroying or inactivating TCRs of pathogenic T lymphocytes.26,27 TCR DNA vaccine has been studied frequently for control of autoimmune diseases and has yielded promising results.28,30

There are several ways of carrying out DNA vaccinations, one of which is by intramuscular injection. Intramuscularly injected plasmids are cytophagocytized by muscular cells and reach nuclei where they exist as non-replicating cyclical nuclear bodies, and their genes are transcribed in the presence of RNA polymerase II. mRNAs will cross the nuclear membrane and reach the cytoplasm where they are translated into proteins which will induce natural immune processes similar to those induced by cellular viral infections.26 The mechanism of antigen presentation falls into three different categories: (1) muscular cells that captured the DNA present the
antigen directly; (2) the antigen is emancipated by muscular cells, and captured and presented by APC (antigen presenting cells); (3) both muscular cells and APC can take up DNA and present the antigen. These cells activate Th and NK cells through MHC-I and II pathway, resulting in cytotoxic effects and formation of specific antibodies. In this study, we successfully detected expression of TCR Vβ5.2 and TCR Vβ8.2 genes which were induced by intramuscular injection of pTargeT-TCR Vβ5.2 and pTargeT-TCR Vβ8.2 with RT-PCR and immunohistochemistry analysis.

RA is a complex, debilitating, chronic, systemic autoimmune disease characterized by immunological, inflammatory and mesenchymal tissue reactions in the synovium, accompanied by polyarticular synovitis, and ultimately leading to the progressive destruction of articular and periarticular structures. Although the etiologic agent of RA remains unclear, there is convincing evidence that antigenic peptides presented by disease-associated HLA (predominately HLA-DRB1 *0101, *0401, *0404 and *0405) are involved in the pathogenesis of RA. These peptides might initially activate autoreactive T cells and mediate autoimmune responses of the disease. Since the main function of HLA class II molecules displayed on antigen-presenting cells is to present antigenic peptides to CD4+ T helper cells, this association suggests that RA is caused by arthritogenic antigens. In this respect, both endogenous and exogenous antigenic peptides have been implicated. There are a number of autoantigens in RA that have been identified, such as CII, HCgp-39 and BiP. Among these, CII has been extensively studied in recent years. It has been suggested that the CII263-272 peptide is one of the predominant antigenic peptides in RA. Experiments have identified specifically activated, arthritogenic antigen-reactive T lymphocytes that infiltrate in the articular synovium and fluids, and there are common predominant TCR Vβ clonotypes in the articulars of RA patients. Analysis of TCR has been thought by many investigators to be a promising approach to identify the pathogenic T cells on the basis of the antigen-driven nature of the response. In fact, experiments on RA with TCR Vβ peptide vaccination have produced promising results, suggesting a pathogenic role of these lymphocytes in disease progression. However, there have been a few reports on the treatment of RA or its animal models with only TCR DNA vaccines. To investigate the inhibitive effect of a TCR DNA vaccine on animal models of RA, we extend our strategy to observe the inhibitive effects of TCR-HSP DNA vaccines and TCR Vβ DNA vaccines on CIA rats, and we have reported the effect of TCR Vβ5.2/8.2-HSP DNA vaccines on CIA.

CIA is an autoimmune disease model that shares genetic background and histological and immunological features with RA (synovial hyperplasia, infiltration of lymphocytes, erosion of cartilage, bone reabsorption and reshaping, etc) and has therefore been widely used as an important model of this human disease. Both T and B cell activations are crucial in inducing CIA. Immune mechanisms that include both humoral and cellular immunity to CII have been implicated in the pathogenesis of the disease. At the onset of the disease, a Th1 cytokine profile has been reported to predominate, and the development of CIA is dependent on a T-cell-mediated activation of autoreactive B cells. The major role of B cells is production of arthritogenic anti-CII antibodies, which is clearly shown by the fact that antibodies reactive with CII can bind to cartilage and induce chronic arthritis. Besides, the transfer of T lymphocytes can induce synovitis. The inhibition of these T lymphocytes or their function can alleviate the progression of CIA. Experiments also showed that a mouse with its thymus removed and exposed to X ray irradiation could not rehabilitate its humoral immunity if only B cells were infused to its body. Only by infusing sufficient amounts of both T cells and B cells could the humoral responses to collagen be rehabilitated. Nor could a nude mouse secrete anti-CII antibody. These results suggest that both cellular and humoral immune response take part in the process and progression of CIA. Therefore, inhibition of self-reactive T lymphocytes not only inhibits cellular immunity but also helps to thwart humoral immune responses, as also shown in our study.

T-cell responses to CII immunization have been extensively studied in Lewis rats and mice having the I-Aq haplotype (e.g. the DBA/1 strain), which are highly susceptible to CIA. Intradermal injection of CII emulsified in Freund’s complete adjuvant (FCA) or FIA results in activation and expansion of antigen-specific CD4+ T cells having the Th1 phenotype, which initiate the harmful response. Preclinical phase of CIA mimics the Th1/Th2 imbalance that is abundantly documented in the literature on RA. That a Th1 response occurs is supported by the finding that IFN-γ and IL-12 worsen the disease when given early during CIA induction. Additional evidence that Th1 cells contribute to CIA comes from the consistent and long–lasting suppressive effect of exogenous Th2 lymphokines, such as IL-4, IL-10, and IL-13, which are well known to antagonize Th1 responses. Ample evidence indicates that Th2 cytokines inhibit macrophage secretion of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and IFN-γ. In this study we assayed the levels of TNF-α and IL-4 produced by the spleen lymphocytes of Lewis rats using the ELISPOT technique, and found that IFN-γ is highly associated with CIA progression. Compared with the CIA control group, the level of IFN-γ secretion by lymphocytes in pTCTR Vβ5.2 and pTCTR Vβ8.2 groups was reduced. On the other hand, the level of IL-4 secretion by the pTCTR Vβ5.2+pTCTR Vβ8.2 group was higher than in CIA control rats. The results indicate that the inhibitory effect of pTCTR Vβ5.2 and pTCTR Vβ8.2 on CIA is closely related to the down-regulation of IFN-γ and up-regulation of IL-4. It suggests that pTCTR Vβ5.2 and pTCTR Vβ8.2 inhibit clones of pathogenic T lymphocytes and promotes the deviation from Th1 to Th2...
cells. Besides, we analyzed the change in peripheral CD4+ and CD8+ lymphocytes by flow cytometry. Results showed that there was deviation in T lymphocyte subtypes distribution in CIA rats; the proportion of CD4+ cells and the ratio of CD4+/CD8+ were higher than in normal rats. In contrast, a lower proportion of CD4+ lymphocytes and a lower ratio of CD4+/CD8+ were observed in pTCR Vβ5.2 and pTCR Vβ8.2-vaccinated rats. This indicates that pTCR Vβ5.2 and pTCR Vβ8.2 can to some extent correct the disorder of T lymphocytes in CIA rats and thus exert arthritis-inhibiting effects.

In our work, we constructed recombinant plasmids pTCR Vβ5.2 and pTCR Vβ8.2 successfully. We then observed inhibitive effects of the two vaccines on the progression of CIA in Lewis rats. Rat quadriceps were injected with either pTCR Vβ5.2 or pTCR Vβ8.2, or both, before the challenge for CIA, resulting in alleviation of the arthritis symptoms, decrease of IFN-γ secretion, a reduced ratio of CD4+ to CD8+ lymphocytes and a low level of anti-CII antibody in serum. We found the main effect of pTCR Vβ5.2 was decreasing the level of IFN-γ while increasing the level of IL-4, but it seemed that pTCR Vβ8.2 had a better effect on correcting the disorder of T cells and reducing the level of anti-CII antibody in serum in CIA rats. In addition, the two vaccines seemed to have cooperative and complementary effects. In other words, compared with the pTCR Vβ5.2 and pTCR Vβ8.2 groups, the group of rats injected with a combination of the two vaccines showed better protective effects against CIA. Besides, compared with TCR-HSP DNA vaccines in our parallel research, we found that although HSP70 may inhibit inflammation, the TCR DNA vaccines can also have inhibitory effects on CIA rats. Therefore, we believe that TCR Vβ5.2 and TCR Vβ8.2 T lymphocytes are the main self-reactive T lymphocytes in CIA rats, and inhibition of them can alleviate the progression of CIA. This preliminary study lays a basis for research of new immune modulation and therapeutic methods and for further studies of TCR DNA vaccines for human RA.

In the present study, we have shown that rapid identification of pathogenic TCRs provides useful information for the design of TCR-based immunotherapy. Specific immunotherapeutic approaches to T cell-mediated autoimmune diseases are feasible through identification of pathogenic T cells and depletion or suppression of the T cell clonotypes with TCR DNA vaccines. The strategy employed in this study provides not only insights into a new specific prevention strategy for RA and other autoimmune diseases, but also supports the supposition that since depletion or suppression of one of two types of effector cells does not significantly improve the severity of the disease, a combined TCR-based DNA immunotherapy should be considered as primary for T cell-mediated autoimmune diseases. TCR-based immunotherapy may be applicable, not only to animal, but also to human autoimmune diseases whose pathological mechanism is still poorly understood.

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