

Article

Protective Effects of Overexpression TCR V β 5.2-HSP70 and TCR V β 8.2-HSP70 against Collagen-Induced Arthritis in Rats

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Collagen-induced arthritis (CIA) is an animal model, which closely resembles human rheumatoid arthritis (RA) in pathogenesis and pathology. Evidence suggests that the inhibition of T lymphocytes or their functions can alleviate the progression of arthritis. So the administration of arthritogenic T cell receptor (TCR) variable region peptide or DNA vaccines encoding pathogenic TCR V β variable region may provide useful information for designing specific immunotherapies against autoimmune diseases. Heat shock proteins (HSPs) have the function of raising antigenic immunogenicity and HSP70 has a protective effect against arthritis. We previously demonstrated the presence of pathogenic predominant T cell receptor V β 5.2 and V β 8.2 clonotypes in the joints of CIA rats. In this study, we constructed the recombinant eukaryotic expression vectors pTARGET-TCR V β 5.2/8.2-HSP70, and evaluated their protective effects on CIA rats. Protective effects were observed in CIA rats by injecting these recombinant DNA vaccines, which could alleviate arthritis index, decrease the levels of IFN- γ and anti-CII antibody in serum, and increase the levels of IL-4. Pathological changes were not as serious as those observed in control CIA rats. The rat injected with two combined vaccines showed better protective effects than CIA rats administered with individual vaccine. These results showed that recombinant DNA vaccines pTARGET-TCR V β 5.2-HSP70 and pTARGET-TCR V β 8.2-HSP70 could significantly alleviate the arthritic symptoms of CIA rats, and better protective effects could be achieved if these two vaccines were used in combination. *Cellular & Molecular Immunology*. 2007;4(6):439-445.

Key Words: collagen-induced arthritis, HSP70, DNA vaccine

Introduction

Rheumatoid arthritis (RA) is a T cell-mediated autoimmune disease that is characterized by destructive polyarthritis. Although the etiological agent of RA remains unclear, there is some evidence that type II collagen might act as an autoantigen. It has been reported that some antigen-reactive T lymphocytes are specifically activated in the articular synovial membrane and fluid of RA patients, and some T lymphocytes have the same T cell receptor (TCR) V β clonotype as those in the polyarthritic joints (1-3). Therefore,

DNA vaccines encoding pathogenic TCR V β variable regions can induce specific immune response directly by the way of expressing protein antigen in RA patient's body, and play a therapeutic role by recognizing TCR of pathogenic T cells and kill them or make them lose activity. Collagen-induced arthritis (CIA) is an autoimmune model that shares genetical background, histological and immunological features with RA.

We have previously determined the presence of pathogenic predominant T cell receptor V β 5.2 and V β 8.2 clonotypes in the joints of CIA rats (4) and constructed recombinant plasmids of TCR V β 5.2 and TCR V β 8.2 with the pTARGETTM vector respectively (5, 6). Heat shock proteins (HSPs) are called stress proteins, so the expressions of self-HSPs are upregulated at sites of inflamed synovial tissue in rats with CIA or patients with RA (7). Thus the

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production of HSPs at a specific site is closely related to the amount of ongoing inflammation. Self-HSP reactive T cells are normally controlled by the immune system in healthy body, but these cells may effectively play a potentially protective role against arthritis in the situations of inflammation (8). In this study, we extended our strategy to construct DNA vaccines encoding TCR V β 5.2-HSP70 and TCR V β 8.2-HSP70, and investigated their immunotherapeutic effects on CIA rats.

Materials and Methods

Animals

Female BALB/c mice, 4 to 6 weeks of age, 18-22 g, were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences. Female inbred Lewis rats, 4 to 5 weeks of age, 50-70 g, were purchased from the Experimental Animal Company, Weitonglihua, Beijing. These animals were maintained at the animal facilities in the Department of Immunology, Capital Medical University, China.

Construction of the recombinant plasmids

DNA fragments encoding TCR V β 5.2/8.2-HSP70 fused genes were amplified using Taq DNA polymerase (Promega, Madison, WI) with one of the primers specific for V β 5.2-HSP70 (5'-ATG ATT ACC GAC GCG GTT ATC ACG ACG CCC GCC TAC TTC AAT GAC GCC TCA AAC ACT GCC CTC TCT-3'), and another specific for V β 8.2-HSP70 (5'-ATG ATT ACC GAC GCG GTT ATC ACG ACG CCC GCC TAC TTC AAT GAC GCC GAA GCT GCA GTC AC-3'), and the C β primer (5'-TCA TGC TTC TGA TGG CTC AAA C-3'). All of the forward primers were designed to include an in-frame ATG codon. PCR products were cloned into the pTARGETTM vector (Promega) according to the manufacturer's instructions. Colonies were selected and recombinant plasmids were isolated using Plasmid Miniprep Kit (Promega). Colonies with an insert of the right length were screened by PCR. The nucleotide sequence of each clone was determined to confirm that insert had the right sequence with an in-frame ATG. PCR was performed with dNTP and Taq DNA polymerase for 30 cycles (94°C for 40 s, 55°C for 50 s, 72°C for 1 min) followed by a 7-min extension at 72°C. The PCR products were electrophoresed in 1.7% agarose gel and visualized by ultraviolet light.

DNA vaccination of the mice

Large-scale preparation of plasmids was performed using the Plasmid Maxiprep Kit (Vigorous, Beijing, China). The purified plasmids were resuspended in sterile PBS. Spectrophotometric analysis revealed the ratio of the absorbance at 260 and 280 nm was between 1.8 and 1.9. To facilitate the expressions of RNA and protein, BALB/c mice were pretreated with 0.75% bupivacaine (1 μ l/g body weight) by injection into tibialis anterior muscles 3 days before vaccination (9). Then, 100 μ g of DNA was injected into the same site according to the indicated protocol. A booster

vaccination was given with the same method once every 2 weeks, for a total of three times.

RT-PCR analysis

The muscular tissues were removed from the injected sites 3 days after the last vaccination and total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using oligo-dT primers and M-MuLV reverse transcriptase (Sangon, Shanghai, China) from 5 μ g of each RNA sample.

Immunohistochemical staining analysis of the TCR protein expressed in the muscle

Muscles from the injection points were fixed with 4.0 g/L formaldehyde and embedded in paraffin. Four micrometre sections were consecutively cut and subjected to deparaffinage. To inactivate the endogenous peroxidase before antibody application, the sections were incubated in 3% hydrogen peroxide for 10 min at room temperature and rinsed several times in distilled water. The antigens were prepared in 0.01 M citrate buffer, and nonspecific binding was blocked with 0.5% BSA for 20 min. The antigens were incubated with the mouse anti-rat TCR α/β antibody (Serotec, Oxford, UK) at a dilution of 1:50 for 1-2 h at 37°C. Biotinylated goat anti-mouse immunoglobulin (1:50) and streptavidin-horseradish peroxidase (HRP) complex (Serotec) were applied, followed by 3-3'-diaminobenzidine (DAB) until a brown reaction product was observed. To suppress any remaining peroxidase, the slides were incubated in 3% hydrogen peroxide for 3 min. After being washed three times, the sections were dehydrated gradually by alcohol and made transparent using dimethyl benzene. Finally, these sections were mounted using neutral resin and observed through a microscope.

DNA vaccination of the rats

The rats were pretreated with 0.75% bupivacaine (1 μ l/g body weight) by injection into tibialis anterior muscles 3 days before vaccination. Then, 150 μ g of DNA was injected into the same site to the vaccinated groups or the pTARGET group, and 300 μ g to the combined vaccine group. A booster vaccination was given once every 2 weeks for a total of three times (10). CIA was induced 12 days after the last injection of DNA.

CIA induction and assessment

Bovine Collage II (bCII) (Sigma-Aldrich, St. Louis, MO), prepared from calf cartilage, was dissolved in 0.1 M acetic acid at 2 mg/ml by stirring overnight at 4°C. An emulsion was made with an equal volume of incomplete Freund's adjuvant (IFA). Test rats were injected with 200 μ g of bCII in a volume of 200 μ l emulsion intradermally at the base of the tail. The rats were challenged with an identical protocol a week later. The day of CIA induction was designated as day 0. The clinical development of CIA was scored by daily observations where the inflammation of all four paws were graded from 0 to 4 as described below: grade 0, no arthritis; grade 1, erythema and/or mild swelling of the paw; grade 2,

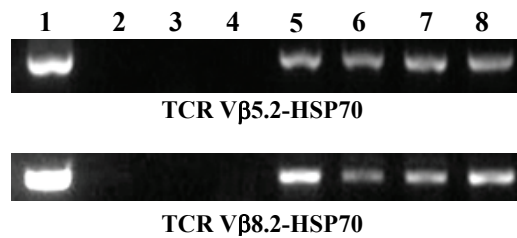


Figure 1. RT-PCR analysis of TCR V β 5.2-HSP70 and TCR V β 8.2-HSP70 mRNA expressions in mouse skeletal muscle. BALB/c mice were pretreated with 0.75% bupivacaine (1 μ l/g body weight) by injection into tibialis anterior muscles. Three days later, 100 μ g of DNA was injected into the same site, and a booster vaccination was given once every 2 weeks for a total of 3 times. The muscular tissues were removed from the injected sites 3 days after the last vaccination. TCR V β 5.2-HSP70 (upper) or TCR V β 8.2-HSP70 (lower) mRNA levels were determined by RT-PCR. Lane 1, positive control; Lane 2, blank control; Lane 3, PBS control; Lane 4, pTARGET control; Lanes 5-8, TCR V β 5.2-HSP70 or TCR V β 8.2-HSP70 mRNA expressions in different mice.

medium swelling of the paw and ankle; grade 3, severe swelling of the paw and ankle; grade 4, severe swelling and ankylosis (11). Each paw was graded, and the four scores were totaled so that the maximum possible score per rat was 16.

Cytokine assay

The spleens were removed aseptically from rats 24 days after CIA induction. Rat IFN- γ and IL-4 were quantified in the culture supernatants of spleen cells by enzyme-linked immunospot assay (ELISPOT) kit (U-CyTech, Utrecht, The Netherlands) according to the manufacturer's instruction. Briefly, ELISPOT plates were coated overnight with anti-rat cytokine monoclonal capture Abs. Spleen cells from rats were suspended to 1×10^6 cells/ml (1×10^5 cells/well). After 24 h of incubation at 37°C, cells were removed by washing the plates, and the site of cytokine secretion was detected using biotinylated anti-rat cytokine monoclonal detection Abs and streptavidin-alkaline phosphatase conjugate. The enzyme reaction was developed with 3-amino-9-ethyl-carbazole (AEC) substrate. Spot-forming cells (SFCs) were counted using KS ELISPOT system.

Immunoassay of antibodies to CII

All rat sera were obtained at the same time by cardiac bleeding. Anti-collagen antibodies were measured using the enzyme-linked immunosorbent assay (ELISA) method. Briefly, 96-well flat-bottom ELISA plates were coated overnight at 4°C with bCII (50 μ g/ml). After washing with PBS-Tween, the plates were blocked with PBS containing 1% bovine serum albumin (BSA) for 1 h. The plates were washed again, and serum samples (1:100 dilution) were added and incubated for 1 h at 37°C. After washing, peroxidase-labeled goat anti-rat IgG (Sigma) diluted 1:1,000 was added and reacted with the plates for 1 h at 37°C. Color

reaction was induced by 0.01% tetramethyl benzidine (TMB), and was stopped 15 min later by adding 50 μ l of 2 M H₂SO₄. The optical density was read at a wavelength of 450 nm by spectrophotometry.

Joint histology

The rats were killed at day 24, the peak of CIA, and their hind paws were removed and fixed in 10% buffered formalin, then paraffin embedded. Five-micrometre midline sagittal sections were stained with hematoxylin and eosin using a micrometer-attached microscope.

Statistical analysis

Data were expressed as mean \pm SEM, and analyzed with SPSS12.0 software. Comparisons of numerical data between groups were performed by the One-Way ANOVA test. *p* values less than 0.05 were considered statistically significant.

Results

Detection and expression of recombinant plasmids

To test the expression efficiency after the DNA vaccines were injected into tibialis anterior muscles of BALB/c mice, the transcripts corresponding to the plasmid sequence plus insert were detected by RT-PCR and the TCR protein expression by immunohistochemical staining. As shown in Figure 1, TCR V β 5.2/8.2-HSP70 mRNA was expressed in the recombinant plasmid injected tibialis anterior muscles (Figure 1). And the muscles of recombinant plasmid-vaccinated groups were brown-stained, but not the PBS and pTARGET control groups (Figure 2). These results indicated that TCR V β 5.2/8.2-HSP70 were successfully expressed in the injected muscles.

Effects of recombinant DNA vaccine on articular of CIA rats

After the induction of CIA, Lewis rats of the CIA positive control group and pTARGET group showed symptoms of arthritis from day 14.9 ± 0.7 , and the swelling and edema reached their peak levels after 7 to 9 days. By day 23, most

Table 1. Anti-CII antibody assay following DNA vaccination

Group	anti-CII antibody (OD ₄₅₀)
Normal	0.099 \pm 0.009 [#]
CIA	1.571 \pm 0.023 ^{Δ#}
pTARGET	1.590 \pm 0.016 ^{Δ#}
pTARGET-TCR V β 5.2-HSP70	1.493 \pm 0.019 ^{*Δ}
pTARGET-TCR V β 8.2-HSP70	1.515 \pm 0.018 ^{*Δ#}
Combined-vaccined	1.489 \pm 0.034 ^{*Δ}

*Compared with CIA group or pTARGET group, *p* < 0.01; #compared with combined-vaccined group, normal, CIA, and pTARGET groups, *p* < 0.01, pTARGET-TCR V β 8.2-HSP70, *p* < 0.05; ^Δcompared with normal group, *p* < 0.01.

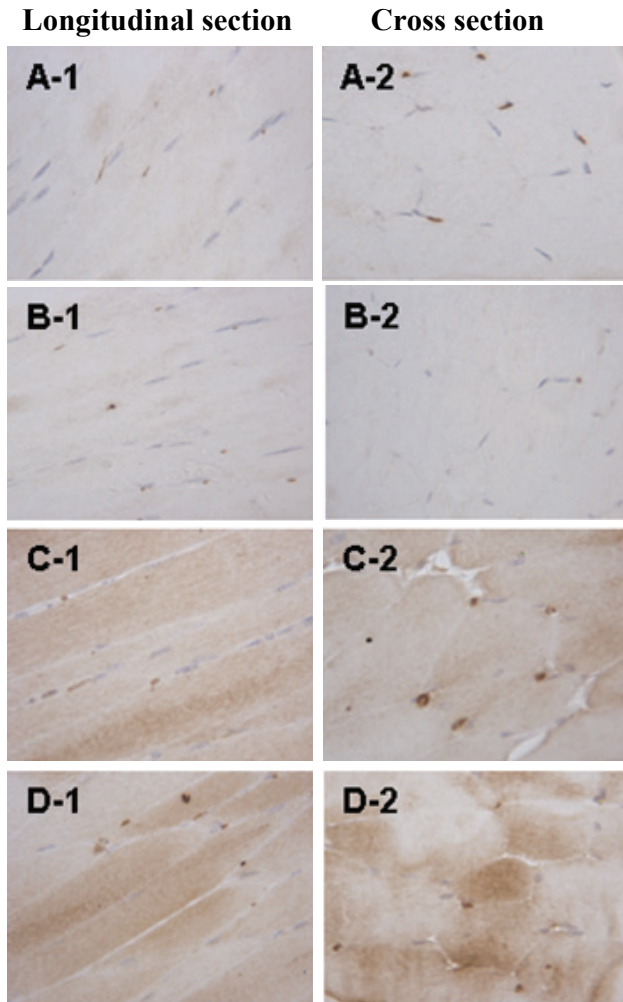


Figure 2. The expressions of TCR V β 5.2-HSP70 and TCR V β 8.2-HSP70 in mouse skeletal muscle by immunohistochemical staining ($\times 400$). Mouse skeletal muscles from the injection points were fixed, paraffin embedded, 4 μ m serially sectioned, and then deparaffinaged. After blocking, the antigens were incubated with the mouse anti-rat TCR α/β antibody at a dilution of 1:50 for 1-2 h at 37°C. Biotinylated goat anti-mouse immunoglobulin (1:50) and streptavidin-HRP complex were applied, followed by DAB until a brown reaction product was observed. (A) PBS control group; (B) pTARGET control group; (C) pTARGET-TCR V β 5.2-HSP70-vaccinated group; (D) pTARGET-TCR V β 8.2-HSP70-vaccinated group.

of rats manifested symptoms of arthritis and the incidence was 83.3% of total 6 rats. There were only one to two rats suffering from arthritis in recombinant plasmid groups. Besides swelling in hind limbs, some rats showed slight denude, diarrhea, falling off of nails, local ulcer in limbs and sluggish growth, but no incidence of death was observed in these rats. Compared to the CIA positive group, recombinant plasmid-vaccinated rats lagged behind about a week in exhibiting arthritic symptoms and manifested significantly milder symptoms. Inhibition of CIA was reflected as a diminished arthritis score, as shown in Figure 3. By day 15,

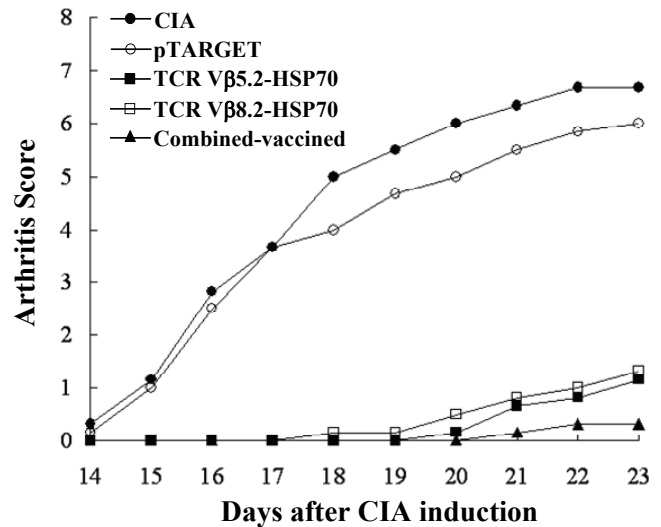


Figure 3. Reduction of arthritis scores of CIA by DNA vaccination with pTARGET-TCR V β 5.2-HSP70 and pTARGET-TCR V β 8.2-HSP70. The rats were injected with an emulsion of bCII-IFA intradermally at the base of the tail, containing 200 μ g of bCII. The rats were challenged with an identical protocol a week later. The day of CIA induction was designated as day 0. The clinical development of CIA was scored by daily observations where the inflammation of all four paws was graded from 0 to 4.

the arthritis score in the vaccinated groups was significantly lower than that of the CIA positive control group and pTARGET group.

Effects of recombinant DNA vaccine on cytokine secretion of CIA rats

The rats of the CIA positive control group showed a high level of IFN- γ secretion by spleen cells compared with the negative control rats ($p < 0.05$). However, no differences were observed between the CIA positive control group and the pTARGET group. The levels of IFN- γ secretion by recombinant plasmid groups were between the CIA positive control group ($p < 0.05$) and the negative control group (Figure 4). The results indicated that IFN- γ was associated with the pathogenesis of CIA and that the use of recombinant pTARGET-TCR V β 5.2-HSP70 and pTARGET-TCR V β 8.2-HSP70 vaccines could decrease IFN- γ levels and alleviate the symptoms of arthritis.

Spleen cells from the combined-vaccinated group secreted increased amounts of IL-4 compared with those of the CIA positive control and pTARGET groups ($p < 0.05$). Furthermore, the level of IL-4 in the combined-vaccinated group was significantly higher than that in the pTARGET-TCR V β 5.2-HSP70- or pTARGET-TCR V β 8.2-HSP70-vaccinated group ($p < 0.05$) (Figure 4). The results implied that the inhibition of CIA was associated with increased secretion of IL-4. A better protective effect could be achieved if the pTARGET-TCR V β 5.2-HSP70 and pTARGET-TCR V β 8.2-HSP70 DNA vaccines were used cooperatively.

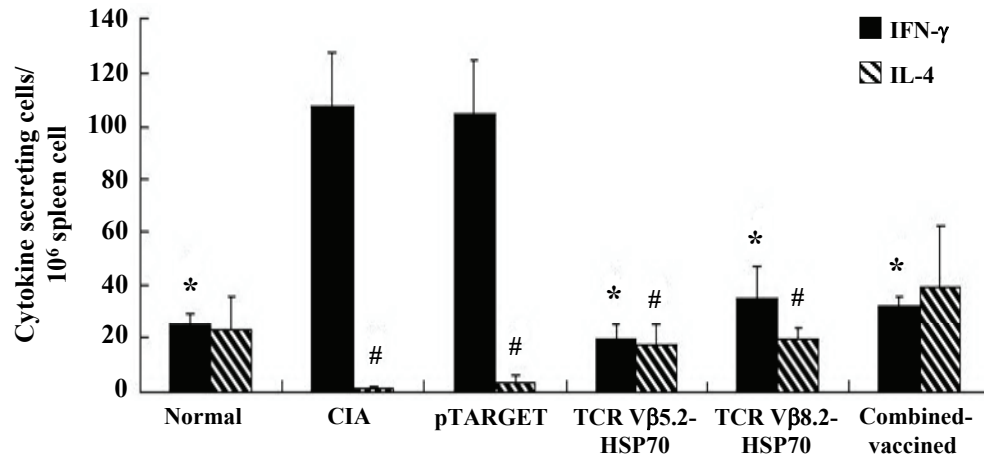


Figure 4. Effects of DNA vaccination on cytokine secretion of CIA rats. ELISPOT plates were coated overnight with anti-rat cytokine monoclonal capture Abs. Spleens were removed aseptically from rats 24 days after CIA induction. Spleen cells from rats were suspended at 1×10^6 cells/ml (1×10^5 cells/well). After 24 h of incubation at 37°C , cells were removed by washing the plates, and the site of cytokine secretion was detected using biotinylated anti-rat cytokine monoclonal detection Abs and streptavidin-alkaline phosphatase conjugate. The enzyme reaction was developed with AEC substrate. *, $p < 0.05$ compared with CIA control and pTARGET groups; #, $p < 0.05$ compared with combined-vaccinated group.

Effects of recombinant DNA vaccine on anti-CII antibody production of CIA rats

The level of anti-CII antibody in serum was assayed by ELISA. As indicated in Table 1, there was a significant difference in the antibody level ($p < 0.01$) between the CIA group or pTARGET group and recombinant plasmid group. The combined-vaccinated rats exhibited a lower level of anti-CII antibody than the pTARGET-TCR Vβ8.2-HSP70-vaccinated group ($p < 0.05$). Furthermore, the level of antibody in the normal group was much lower than those in the other five groups ($p < 0.01$). All of these results implied that the anti-CII antibody was arthritogenic. The symptoms of CIA could be alleviated by decreasing the level of anti-CII antibody. Statistical analysis indicated that the pTARGET-TCR Vβ5.2-HSP70 and pTARGET-TCR Vβ8.2-HSP70 DNA vaccines had the effect of decreasing anti-CII antibody levels and a better effect could be reached if the two vaccines were used in combination.

Effects of recombinant DNA vaccine on histological changes of CIA rats

Histological examination of the joints of rats revealed that the histology of CIA rats was characterized by proliferative synovitis and villiform pannus formation that eroded the adjacent cartilage and ultimately produced severe articular injury and ankylosis. The pTARGET-TCR Vβ5.2-HSP70-vaccinated and pTARGET-TCR Vβ8.2-HSP70-vaccinated rats manifested reduced bone and cartilage erosion and synovial inflammation (Figure 5). Moreover, there were much fewer infiltrating lymphocytes in the articular cavity and less proliferative synovitis in the combined-vaccinated rats. These results revealed that the pTARGET-TCR Vβ5.2-HSP70 and pTARGET-TCR Vβ8.2-HSP70 DNA vaccines could reduce the inflammation of CIA joints. A better protective

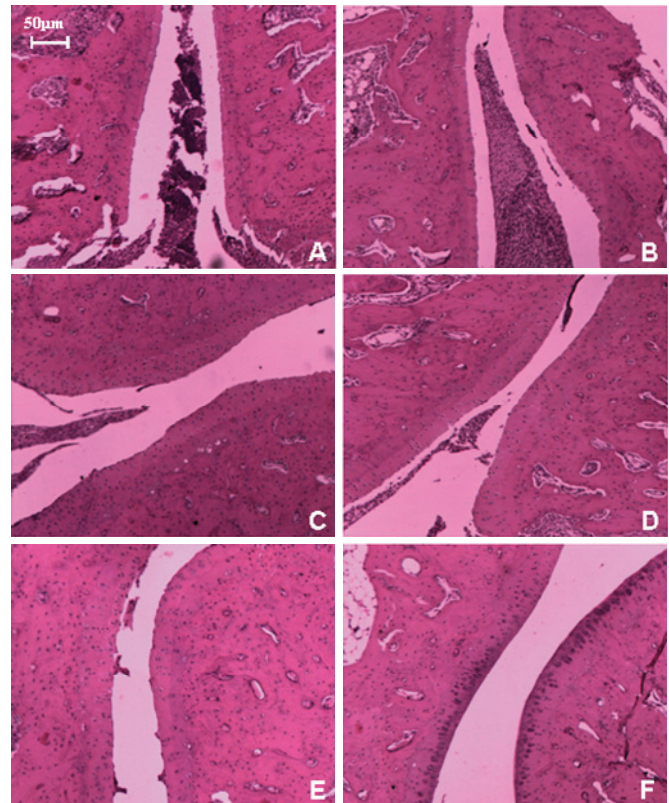


Figure 5. Histological changes of CIA rats ($\times 100$). The rats were killed at day 24, and their hind paws were removed and fixed in 10% buffered formalin, then paraffin embedded. Five-micrometre midline sagittal sections were stained with hematoxylin and eosin. (A) CIA group; (B) pTARGET control group; (C) pTARGET-TCR Vβ5.2-HSP70-vaccinated group; (D) pTARGET-TCR Vβ8.2-HSP70-vaccinated group; (E) combined-vaccinated group; (F) normal group.

effect could be achieved if the pTARGET-TCR V β 5.2-HSP70 and pTARGET-TCR V β 8.2-HSP70 DNA vaccines were administered simultaneously.

Discussion

RA is a chronic inflammatory disease characterized by persistent inflammation of the synovium, and the pathogenesis is inclined to be a self-antigen (such as CII) autoimmune disease, in which the self-antigen is not identified. CIA is an autoimmune model that shares a genetic background, histological and immunological features with RA (synovial hyperplasia, infiltration of lymphocytes, erosion of cartilage, bone reabsorption and reshaping) and therefore has been regarded as an important model for this human disease. VanderBorgh (12) reported that 30%-50% of inflammatory cells infiltrating the synovium are T lymphocytes, and most are reactive. Recent studies have found that the usage of some TCR V β clonotypes are preponderant in many infiltrating organs of human autoimmune diseases and their animal models, so the TCR V β clonotypes can often be used to analyze the relationship between T cells and disease (13-16). In earlier studies, we found that TCR V β 5.2 and TCR V β 8.2 are predominant in the synovium in the course of CIA induced in Lewis rats, and so we took them as candidate genes for our recombinant TCR DNA vaccines.

HSPs are highly immunogenic proteins with an exceptional degree of evolutionary conservation. They have a function in intracellular proteins undergoing cellular stress, such as during inflammation. They are grouped into four major families by their molecular weight: HSP90, HSP70, HSP60, and small HSPs. Members of the HSP70 family occur in all known organisms and display the highest level of interspecies conservation. Their conservation (and the consequent potential for the occurrence of molecular mimicry) makes self-HSPs obvious candidate targets for pathologic T cell responses (17, 18). Wendling (19) reported that exposing rats to this HSP70 conserved sequence (P111-125) *via* nasal peptide administration causes a significant reduction in arthritis. It is likely that the earlier-described arthritis protection by *Mycobacterium tuberculosis* (Mt) HSP70 was provoked by a conserved T cell epitope inducing T cells cross-reactive with self-HSP70. T cells raised against Mt HSP70 and this epitope induced strong expression of the anti-inflammatory cytokine IL-10, whereas the T cells specific for other conserved protein immunogens did not (20, 21).

In this study, the pTARGET-TCR V β 5.2/8.2-HSP70 recombinant plasmids were constructed and injected into the tibialis anterior muscles of BALB/c. Consequently, those transcripts corresponding to the plasmid sequence plus insert were successfully detected by RT-PCR and the TCR protein expression by immunohistochemical staining. Protective effects were observed in CIA rats by injecting pTARGET-TCR V β 5.2-HSP70 and pTARGET-TCR V β 8.2-HSP70 recombinant DNA vaccines, and better protective effects

could be achieved if these two vaccines were used in combination. This research will lay a foundation for further research into the protective effects of TCR DNA vaccines against CIA and other autoimmune diseases.

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- during disease progression. *Int Immunol.* 2004;16:131-138.
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