Active immunisation targeting soluble murine tumour necrosis factor alpha is safe and effective in collagen-induced arthritis model treatment

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Abstract

Objective

TNF-α has been proved to be an effective target in rheumatoid arthritis treatment. So far, all the commercialised TNF-α antagonists function as passive immunotherapy. The aim of this study was to design a complex which can trigger active immunisation and overcome self-tolerance to elicit antibodies against murine TNF-α.

Methods

The complex (KLH-TNF) was chemically synthesised by linking a selected peptide $TNF\alpha_{4-23}$ from murine soluble TNF-α to a carrier protein, keyhole limpet haemocyanin (KLH). We evaluated its safety and antibody eliciting performance. We also evaluated its disease-regulating ability on collagen-induced arthritis models. Furthermore, the immune cells responses were analysed by T cell proliferation assay and B cell memory experiments.

Results

The complex was safe without cytotoxity. The anti-mTNF-α antibody titers of the KLH-TNF group were 400 times greater than the control groups ($p<0.0001$). The elicited antibodies could combine with soluble TNF-α. The antibody response was independent of autologous TNF-α and could be reinforced by booster immunisation. Moreover, the complex did not trigger T cell activation and B cell memory response against native TNF-α. In animal experiments, KLH-TNF immunised mice showed a lower arthritis score ($p<0.001$) and better weight gain ($p<0.01$). Histological evaluations showed milder inflammation and cartilage depletion.

Conclusion

Active immunotherapy against cytokine TNF-α is feasible by conjugating cytokine peptide with carrier protein. The elicited antibodies could combine with the native TNF-α and inhibit its activity. Importantly, the antibody response is reversible and independent of autologous TNF-α.

Key words

rheumatoid arthritis, TNF-α therapeutic immunisation, cytokine peptide, soluble TNF-α

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Introduction

Rheumatoid arthritis (RA) is an immune disorder disease, the primary pathogenesis of which is the overproduction of cytokine TNF-α (1). It has been proved that TNF-α antagonists are effective in RA treatment (2). However, the long-term use of TNF-α inhibitors has potential risks. This has prompted us to explore alternative strategies against TNF-α (3). Of all the new strategies, active immunisation is the most intriguing (4, 5).

Active immunisation utilises antigenic and immunogenic protein to produce target antibodies, the protein called immunogen. The immunogen in our study was designed by conjugating a selected peptide from soluble murine TNF-α (mTNF-α) to carrier protein, keyhole limpet haemocyanin (KLH) (6). The derived complex (KLH-TNF) belonged to a biologically inactive but immunogenic cytokine derivatives family (7). The coupled peptide belonged to B cell epitope, currently, the analysis of which was carried out by computational software specifically designed for protein crystal structure (8).

In this study, we evaluated the antibody eliciting performance of KLH-TNF. We also evaluated its disease-regulating ability on mouse collagen-induced arthritis (CIA) model. Furthermore, the immune cell responses were analysed by T cell proliferation assay and B cell memory experiments.

Materials and methods

Animals and reagents

DBA/1 mice (Shanghai SLAC Animal Laboratory); Recombinant mTNF-α (PeproTech); Bovine type II collagen, actinomycin D, KLH, and complete/incomplete Freund's adjuvant (CFA/IFA) were from Sigma Aldrich. Cell counting kit-8 (CCK-8) was from DOJINDO, Japan. The candidate antigen peptide, SSQNSSDKPVAHVVANHQVE, was the 4th to 23rd sequence of soluble mTNF-α from C-terminal (TNF-α_{4-23}). Chemical synthesis was carried out by GenScript Inc.

Immunogen safety test

The biological activity of KLH-TNF was assessed by L929 cytotoxicity assay. Serial dilutions of KLH-TNF and mTNF-α (containing 1ug/ml actinomycin D) were separately added to 96-well plates which had incubated 5×10^4/ml L929 cells for 24 hours. The number of survival cells was determined by CCK-8 after incubating for 18 hours.

Immunisation and booster

Six-week-old DBA/1 mice were grouped randomly to KLH-TNF, KLH, and TNF-α_{4-23} group. Each mouse received 10 ug respective immunogen emulsified in IFA on day 1, 14, and 28 subcutaneously. On day 64, KLH-TNF group was randomly subdivided into two subgroups, one subgroup received a booster and the other received PBS.

Antibody detection and binding ability

Sera were serially diluted and applied to ELISA plates which had been coated with 1 ug/ml mTNF-α. Before this, the coated plates were sealed with blocking buffer (PBS, 2% BSA, 0.05% Tween 20) for 2 hours to inhibit the non-specific adsorption. Reactivity of serum antibody with mTNF-α was determined by peroxidase rabbit anti-mouse secondary Ab. The antibody titer was expressed as the reciprocal of the highest dilution ratio that displayed an OD of 0.5. KLH-TNF immune sera were diluted to 1/1000 and then incubated with different concentrations of soluble mTNF-α for 2 hours before added to plates which had been coated with 0.1ug/ml mTNF-α. The rest of serum Abs that bound to plate mTNF-α were detected with rabbit anti-mouse secondary Ab.

T cell proliferation assay

Purified spleen lymphocytes from KLH-TNF, KLH, and TNF-α_{4-23} group mice were added to 24-well plates 1×10^5/ml per well, supernatants were collected after incubating with 20ug/ml KLH, 2ug/ml TNF-α_{4-23} and 1ug/ml mTNFα for 72 hours to determine the IL-2.

Animal experiments

Six-week-old male DBA/1 mice were injected intradermally with 200 ug collagen mixed with CFA. 21 days later, collagen was given by emulsifying with IFA. 16 CIA models were grouped randomly to KLH-TNF and KLH group.

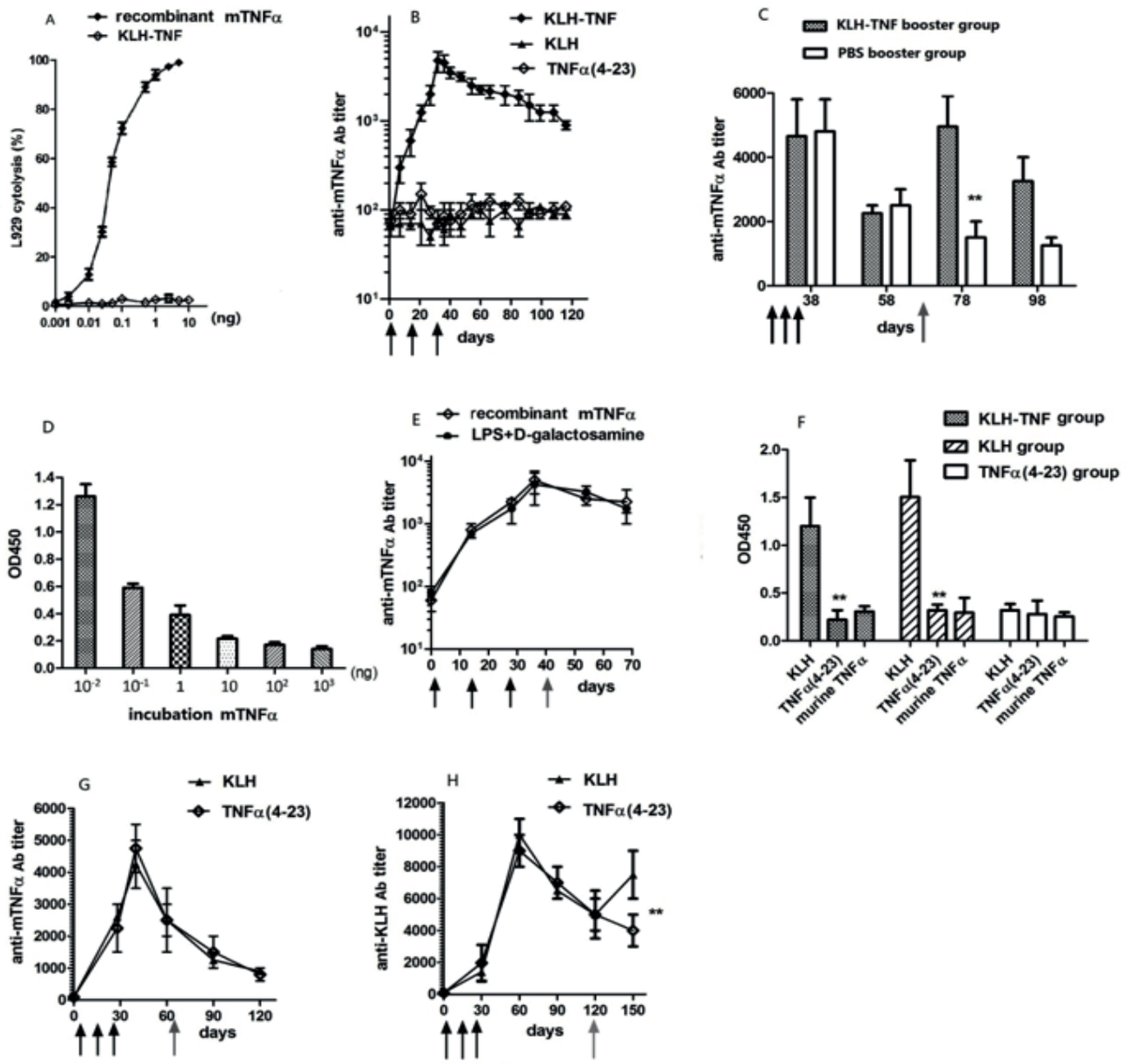

Fig. 1. **A**. The cytotoxity test of recombinant mTNF-α (◆) and KLH-TNF (◇). X-axis represents the dose of mTNF-α or KLH-TNF incubated with L929 cells, Y axis is the percentage of cytolysis. Values were shown as mean ± SEM (standard error of measurement), $p<0.001$ at the dose of 0.01 ng. **B**. Serum anti-mTNF-α antibody titer elicited by KLH-TNF (◆), KLH (▲), and TNF-$\alpha_{4\text{-}23}$ (◇). Black arrows represent immunisations on day 1, 14 and 28. Values were expressed as mean ± SEM. $p<0.0001$ for KLH-TNF *vs*. TNF-$\alpha_{4\text{-}23}$; $p<0.0001$ for KLH-TNF *vs*. KLH; $p>0.05$ for KLH *vs*. TNF-$\alpha_{4\text{-}23}$ (Newman-Keuls Multiple Comparison test). Results shown were representative of several independent experiments. **C**. Booster immunisation. Black arrows represent immunisations on day 1, 14 and 28. Grey arrow indicates the booster immunisation on day 64. Results were expressed as mean ± SEM. $^{**}p<0.001$. **D**. The combination with soluble mTNF-α. X-axis represents the dose of soluble mTNF-α incubating with 1/1000 diluted sera ($p<0.0001$ for 10^{-2}, 10^{-1}, and 1 ng groups). Similar results were obtained by repeating the test several times. **E**. The independence of antibody response. Ten male DBA/1 mice were immunised with KLH-TNF on day 1, 14, 28 (black arrows). Grouped the mice randomly on day 42 (grey arrow). No statistical significance was found between recombinant mTNF-α injection (◇) and LPS injection (•). **F**. T cell proliferation assay. X-axis shows specific stimuli that incubated with splenocytes; Y axis represents the OD value of ELISA for detecting IL-2. $^{**}p<0.001$ for KLH stimuli *vs*. TNF-$\alpha_{4\text{-}23}$ stimuli. **G**. Anti-mTNF-α antibody titers for B-cell memory experiments. On day 62, KLH-TNF immunised mice received KLH solution (▲) or TNF-$\alpha_{4\text{-}23}$ solution (◇) respectively. No statistical difference was found in anti-mTNF-α Ab titers. **H**. Anti-KLH antibody titers. On day 119 (grey arrow), KLH-TNF immunised mice received KLH solution (▲) or TNF-$\alpha_{4\text{-}23}$ solution (◇) respectively, $^{**}p<0.001$ on day 150.

Each mouse received respective immunogen emulsified in IFA three times. Record the arthritis severity and weight every 3–5 days. For prevention study, control group was pretreated with PBS. The collagen was given 21 days after first immunisation for both groups.

Arthritis follow-up

Arthritis severity of each paw was evaluated blindly according to the following criteria: 0 = normal; 1 = mild reddening or swelling of digits; 2 = pronounced reddening and swelling of digits and tarsal; 3 = strong swelling of entire paw. The scores of four limbs were added together to generate a total score ranging from 0 to 12 for each mouse.

Statistical analysis

GraphPad Prism was used to perform

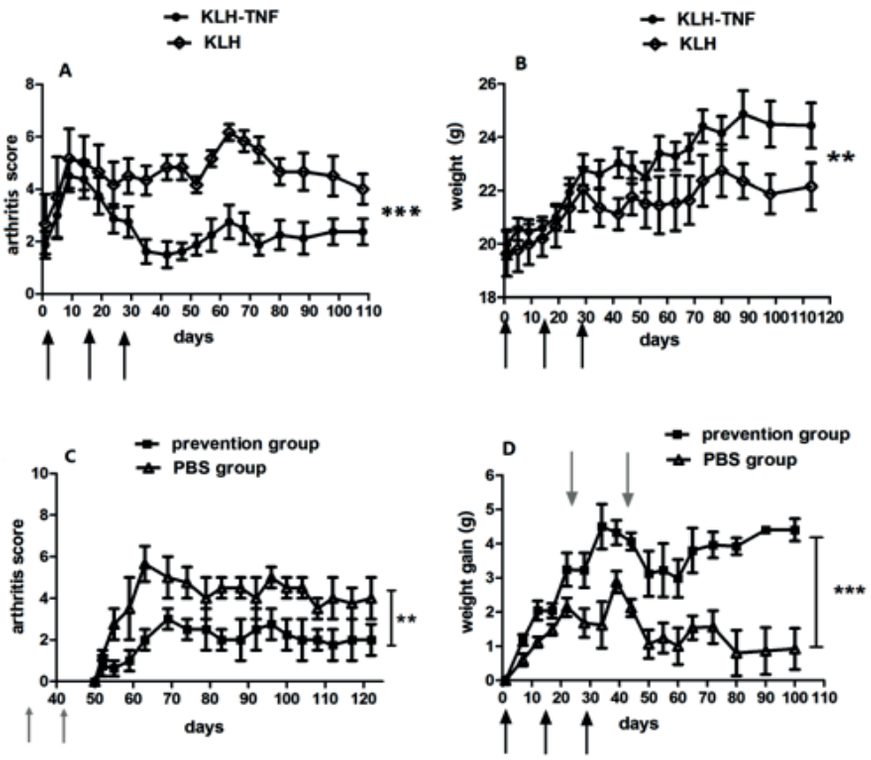

Fig. 2. Therapeutic and intervention experiments on CIA models.
A. Arthritis severity evaluation for therapeutic experiments. KLH-TNF (•), KLH (◇). Black arrows indicate three times immunisation on day 1, 14 and 28. ***p<0.0001 between day 24 and day 80. Results shown were representative of two independent experiments. **B**. Body weight in therapeutic experiments, p<0.001 between day 36 to 116. **C**. Arthritis severity evaluation for prevention study. Prevention group (■), PBS group (△). CIA models were made on day 21 and 42 (grey arrows). **p<0.001 for general arthritis severity comparison **D**. Weight gain ***p<0.0001. Results were representative of two independent experiments.

statistical analysis. Repeated measurements were analysed by one-way ANOVA. Group difference significance was analysed by Mann-Whitney test.

Results

The biological activity of KLH-TNF

KLH-TNF was biologically inactive in comparison with recombinant mTNFα in L929 cytolysis test. The percentage of cytolysis was as low as 1.28% even at the highest concentration (Fig. 1A).

KLH-TNF induced anti-mTNF-α antibody

The antibody titer of KLH-TNF group was almost 400 times greater than the other two groups, which peaked at 4500 from day 38. Anti-mTNF-α Ab titer gradually decreased and reduced by half on day 64 (Fig. 1B). KLH-TNF booster immunisation could increase the titer to 4800 14 days after booster. By contrast, the anti-mTNF-α titer of PBS group still dropped (Fig. 1C).

Binding ability with soluble mTNFα

The KLH-TNF group sera were preliminarily incubated with different concentrations of soluble mTNF-α and then added to plates. When the amount of incubation mTNF-α ranged from 10^{-2} ng to 10 ng, the OD value dropped from 1.26 to 0.216, indicating that sera Abs had bound to soluble mTNF-α in the preliminary incubation process (Fig. 1D).

The independence of antibody production

To find out whether the antibody response induced by KLH-TNF could be affected by extraneous or endogenous mTNF-α, immunised mice were injected with 10 ng recombinant mTNF-α intravenously or 100 ng LPS mixed with 2 mg D-galactosamine intraperitoneally. The anti-mTNF-α Ab titers decreased as previously, indicating that neither extraneous mTNF-α nor endogenously produced TNF-α could affect the antibody response (Fig. 1E).

Immune cell response

10 ug KLH and TNF-$\alpha_{4\text{-}23}$ solution were separately injected without adjuvant when anti-KLH and anti-mTNF-α titers dropped. The anti-mTNF-α Ab titers still dropped as previously (Fig. 1G) but anti-KLH Ab titers increased (Fig. 1H). It proved that B-cell response to KLH was not accompanied by coupled peptide. T cell proliferation assay displayed that IL-2 OD value of KLH stimulated lymphocytes were significantly higher than that of TNF-$\alpha_{4\text{-}23}$ stimulated cells (1.38 *vs*. 0.269, p<0.01), indicating that KLH-TNF did not trigger obvious T cell response against peptide TNF-$\alpha_{4\text{-}23}$ but against KLH. The OD values of recombinant mTNF-α stimulated cells were comparable among three groups, which demonstrated that autologous TNF-α did not trigger T cell response either (Fig. 1F).

KLH-TNF ameliorated arthritis in CIA models

The average arthritis score of KLH-TNF group was not statistically different from that of KLH group on the first immunisation day (2.1±0.5 *vs*. 1.9±0.9, p>0.05), but the discrepancy began to increase after day 24 (Fig. 2A). The mean body weight of KLH-TNF treated group was comparable with that of KLH group during early 30 days but became significantly better in later stage (Fig. 2B). For prevention study, KLH-TNF pretreated group had lower arthritis score after disease onset (Fig. 2C) and better weight gain (Fig. 2D). Furthermore, KLH-TNF treated/pretreated mice showed lower degree of inflammation and cartilage depletion than KLH/PBS group (Fig. 3).

Discussion

In this study, we designed a chemically synthesised complex consisting of cytokine peptide TNF-$\alpha_{4\text{-}23}$ and carrier protein KLH. The soluble TNF-α, also called extracellular part, is a 17 kDa homotrimer which proved to be critical in TNF-α signalling pathway (9). The peptide TNF-$\alpha_{4\text{-}23}$ belongs to extracellular part, which is exactly the elicited antibody targeted. Our study proved that the produced antibodies could recognise the native soluble mTNF-α either. The

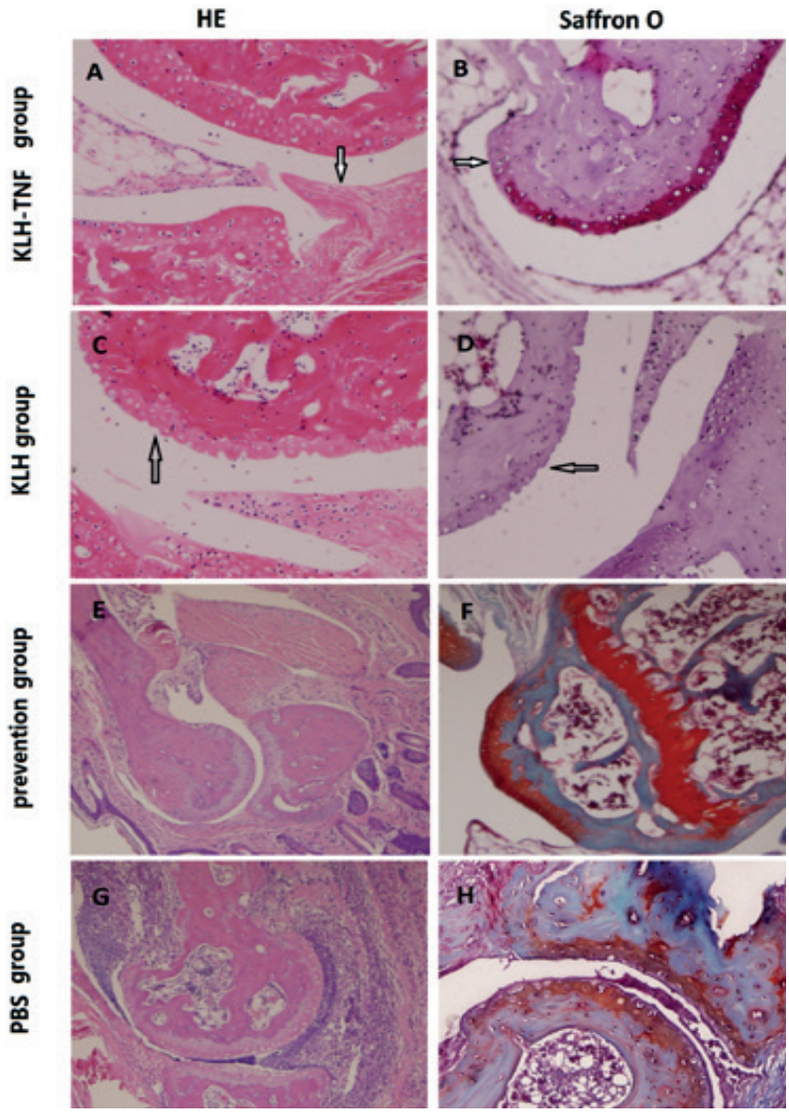

Fig. 3. Examples of histological sections for therapeutic (**A-D**) and prevention expeiments (**E-H**). **A**. KLH-TNF group shows small proliferation of synovium (white arrow) and mild infiltration of inflammatory cells around the ankle (HE). **B**. White arrow points to uncolored area on the lateral side of articular surface, indicating the depletion of cartilage (safranin O). **C**. KLH group exhibits complete bone erosion and entire cartilage loss (**D**). For intervention study, prevention group shows mild inflammation (**E**) and slight depletion of cartilage (**F**). PBS group shows severe inflammatory cells infiltration (**G**) and cartilage depletion from surface to central (**H**). Magnification: A-D and F, H x 200. E and G x 100.

soluble TNF-α was cytotoxic, however the cytokine derivative KLH-TNF was non-toxic proved by L929 cytolysis test. Besides, we did not find any adverse reactions in animal experiments.

KLH-TNF induced a high titer of anti-mTNF-α Ab but TNF-$\alpha_{4\text{-}23}$ failed. In T cell proliferation assay, TNF-$\alpha_{4\text{-}23}$ peptide failed to stimulate the IL-2 production but KLH did. These data demonstrated that KLH promoted carrier-specific T cell activation to help B cell producing antibodies against coupled peptide. Besides, KLH-TNF did not trigger T cell response against autologous TNF-α, indicating that the complex could not induce autoimmune response.

Our study proved that the antibody response was independent of either extraneous or endogenously produced TNF-α, the results of which also demonstrated that B cell did not evolve a memory response against autologous TNF-α. These data indicated that TNF-α produced in physiological or pathological situations would not affect the antibody response induced by preliminary given KLH-TNF. Although the immune system developed memory response against KLH, this memory did not affect the complex safety.

In vitro tests proved that elicited antibodies could combine with soluble TNF-α, we used CIA mice models to evaluate the disease regulating ability of KLH-TNF *in vivo*. The animal results showed that the complex was effective in restraining the arthritis development and improving the weights.

Conclusion

In this study, we demonstrated that cytokine derivative KLH-TNF could successfully overcome self-tolerance and induce B cell response against coupled peptide. The complex was nontoxic and antibody response could not be affected by autologous TNF-α. The elicited Abs could combine with soluble mTNF-α and inhibit its activity as tested by animal experiments.

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