

Prediction and identification of B-cell epitopes for tumor necrosis factor- α

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Abstract. The aim of the present study was to predict and identify B-cell epitopes for mouse tumor necrosis factor- α (mTNF- α). DNASTar and BcePred software were used to predict B-cell epitopes for mTNF- α . A predicted eight-branch multiple antigenic polypeptide (MAP) was synthesized and used to immunize BALB/c mice, combined with a promiscuous helper interleukin-1 β epitope (VQGEESNDK, amino acids 163-171). The serum titer was measured. The specificity and avidity were determined by western blotting and indirect enzyme-linked immunosorbent assay (ELISA). Amino acids 54-65 (MAP1) and 78-92 (MAP2) of mTNF- α were predicted as most likely to be B-cell epitopes. Dynamic monitoring of antibody concentration demonstrated that MAP1 and MAP2 may induce the production of specific antibodies with a higher antibody level for MAP2. Furthermore, MAP1 and MAP2 were confirmed to induce mTNF- α -specific antibodies by western blotting. Indirect ELISA was used to confirm that MAP2 had the highest affinity with commercial anti-mTNF- α antibody. Amino acids 54-65 and 78-94 of mTNF- α are B-cell epitopes, wherein amino acids 78-94 have the strongest immunogenicity. The present study provides a theoretical basis for further research into the mTNF- α polypeptide antibody and a B-cell MAP vaccine.

Introduction

Tumor necrosis factor-alpha (TNF- α) is a cytokine with complicated bioactivity. It is also a therapeutic target of auto-antigens (1,2). In addition to killing tumor cells *in vitro* and *in vivo*, TNF- α may also induce inflammation, defend viral infection, regulate immunity, and promote cell proliferation

and activation (3-5). When TNF- α is overexpressed, it leads to various diseases, such as inflammatory bowel disease (3-5). These damages may be mitigated after neutralizing excess TNF- α using exogenous antibodies or soluble receptors. A monoclonal antibody against TNF- α , infliximab, is the most commonly used exogenous antibody that has been approved for the treatment of Crohn's disease. Clinical trials demonstrated that infliximab also has a positive role in the treatment of ulcerative colitis (UC) (6,7). However, using synthetic infliximab is a passive immunotherapy associated with many inherent defects, such as short duration and easy-to-induce hypersensitivity. Therefore, it would be a major breakthrough for the treatment of UC if the vaccine induced natural anti-TNF- α antibodies *in vivo* via active immunization.

The biological characteristics of TNF- α have no significant species specificity. The amino acids of murine TNF- α (mTNF- α) are highly homologous to those of human TNF- α with a similar tertiary structure (8). Its precursor has 235 amino acid residues containing a signal peptide with 79 amino acid residues. Based on the primary structure of TNF- α , bioinformatics was used to predict mTNF- α B-cell epitopes. Multiple antigenic polypeptides (MAPs) were synthesized using an eight-branch design (9). Interleukin-1 β (IL-1 β) peptide 163-171, as a promiscuous helper epitope peptide (T-helper epitope), enhances immunity and humoral immune response of other antigen peptides (10). With combined immunization of animals, it induced the production of polyclonal antibodies (9,10). A potential B-cell epitope peptide was obtained by indirect enzyme-linked immunosorbent assay (ELISA), western blotting and immunohistochemistry identification of the polyclonal antibody titer. The aim of the present study was to provide an initial experimental basis for active immunotherapy research for mTNF- α targeting UC in mice.

Materials and methods

Materials. The mTNF- α sequence was obtained from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) as follows: *MSTESMIRDVELAEALPQKMGGFQNSRRCLCLSLFSFLLVAGATTLFCLLNFGVIGPQRDEKFPNGLPLISSMAQTLTLRSSQNSSDKPVAHVVANHQVEEQLEWLSQRANALLANGMDLKDNLVVPADGLYLVYSQVLFKGQ*

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GCPDYVLLTHTVSRFAISYQEKVNLSSAVKSPCKDTP
GAELKPWYEPIYLGGVFQLEKGDQLSAEVNLPKYLDFAE
SGQVYFGVIAL.

The following reagents and antibodies were purchased: Commercial recombinant mTNF- α protein (eBioscience; Thermo Fisher Scientific, Inc., Waltham, MA, USA), commercial polyclonal rabbit anti-mouse full-length TNF- α protein antibody (cat. no. 17590-1-AP). ProteinTech Group, Inc., Chicago, IL, USA), Freund's complete adjuvant (Sigma-Aldrich; Merck, KGaA, Darmstadt, Germany), incomplete adjuvant (Sigma-Aldrich; Merck KGaA), enzyme-labeled goat anti-mouse IgG-horseradish peroxidase II (cat. no. EK1003). Wuhan Boster Biological Technology, Ltd., Wuhan, China), western blotting chemiluminescence kit (Shanghai Kangcheng Sheng Biological Engineering Company, Shanghai, China) and protein marker (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Mouse H22 hepatoma cells were obtained from Huazhong University of Science and Technology (Wuhan, China). A total of 80 female BALB/c mice (age, 4-5 weeks; weight, 18-25 g) were purchased from Shanghai Laboratory Animal Center (Shanghai, China; certificate no. SCXK20080115). The mice were placed in a plexiglass feeding box and kept on the clean shelf laminar flow. Each feeder box contained 3-4 mice under the following conditions: Constant temperature, 25-27°C; humidity, 45-50%; fresh air; high-level dust sterilization; and specific pathogen-free environment under a 12-h light/dark cycle. The animals had free access to sterilized water and food. The animal studies were approved by the ethics committee of Zhejiang Medical University (approval no. X1002623; Hangzhou, China). Live animal surgery was performed using 0.3% sodium pentobarbital (30 mg/kg). Upon completion of the experiment, the experimental animals were sacrificed by cervical dislocation.

B-cell epitope prediction. DNASTAR software version 7.1 (DNASTAR, Inc., Madison, WI, USA) and BcePred software (<http://www.imtech.res.in/raghava/bcepred/>) were used to analyze 235 amino acid sequences of mTNF- α to evaluate the physical and chemical properties of the encoded protein, such as hydrophilicity, accessibility, and plasticity, which was used for assessing the mTNF- α molecule antigenicity in the highly hydrophilic region. The two analyses were combined, and the overlapping result area was selected as the candidate mTNF- α B-cell epitope.

MAP synthesis. The MAP of the B-cell epitope peptide was synthesized by the Chinese Peptide Company Ltd. (Hangzhou, China). Based on the selected amino acid peptide and lysine core matrix, an eight-branch polypeptide was designed. Synthesis was performed on a peptide synthesizer using the solid-phase method, where amino acid peptides were linked with the 8 amino terminals of the 4 lysine located at the end of the MAP core structure. According to the amino acid sequence of the peptide, they were sequentially connected one by one from the carboxyl terminal to the amino terminal. Synthesized polypeptides purified by high-pressure liquid chromatography had a purity of >97%.

Binding force between MAP peptide and whole-protein mTNF- α antibodies. A 96-well ELISA plate was coated with

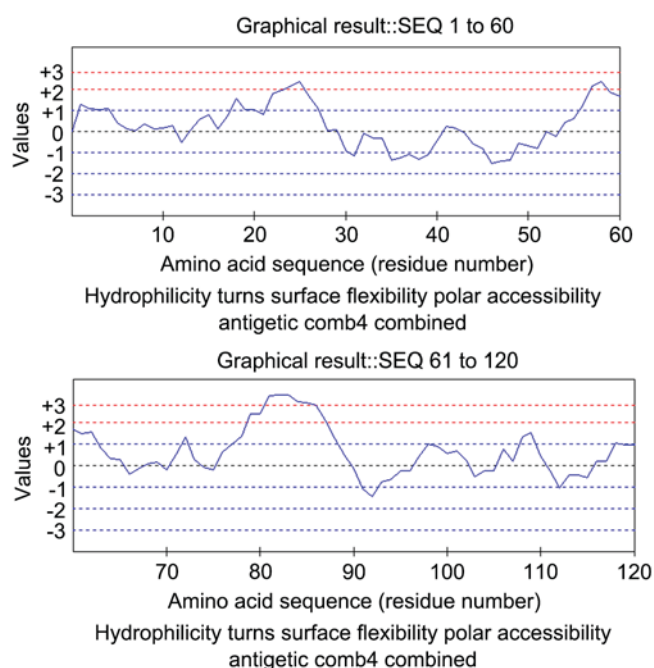


Figure 1. B cell epitopes of mouse tumor necrosis factor- α were predicted using Bcepred software. Amino acids 54-65 (GVIGPQRDEKFP) and 78-92 (LTLRSSQNSSDKPV) exhibited good hydrophilicity, accessibility and plasticity.

100 μ l of different MAPs or recombinant TNF- α protein at a concentration of 10 mg/l and maintained overnight at 4°C. After blocking in 1% bovine serum albumin (BSA), a rabbit anti-mouse full-length TNF- α antibody (1:4,000; 100 μ l/well; cat. no. 17590-1-AP; ProteinTech Group, Inc.) was added, with normal BALB/c mouse serum (1:4,000) serving as a control. The results were expressed as the mean of three experiments. The absorbance of the plates were measured at a wavelength of 490 nm using an Imark Micoplate Absorbance Reader 13550 (Bio-RAD, Hercules, California, USA).

Material grouping. Eighty male BALB/c mice (age, 3-4 weeks; weight, 18-25 g), were randomly divided into four groups as follows: MAP1 group (amino acids 54-65), MAP2 group (amino acids 78-92), mTNF- α group, and phosphate-buffered saline (PBS) control group, with 20 mice in each group.

Immunization. The MAP1 and MAP2 groups were treated with eight-branch MAP immunogen. The mice were injected four times at 2-week intervals. The mice were first immunized with Freund's complete adjuvant (Sigma-Aldrich; Merck KGaA) and boosted with incomplete Freund's adjuvant. The quantity of MAP used for the first immunization and boosting was 0.2 mg per mouse. The mTNF- α control group was immunized with commercial recombinant mTNF- α protein at a dose of 20 μ g per mice. An equal quantity of T helper (TH) linear peptide was mixed, dissolved in 0.5 ml PBS, and emulsified with 0.5 ml Freund's adjuvant. Multi-point injections were performed intradermally on the backs of the mice. The PBS control group was also injected in the same way with 0.5 ml PBS emulsified with an equal volume of Freund's complete adjuvant.

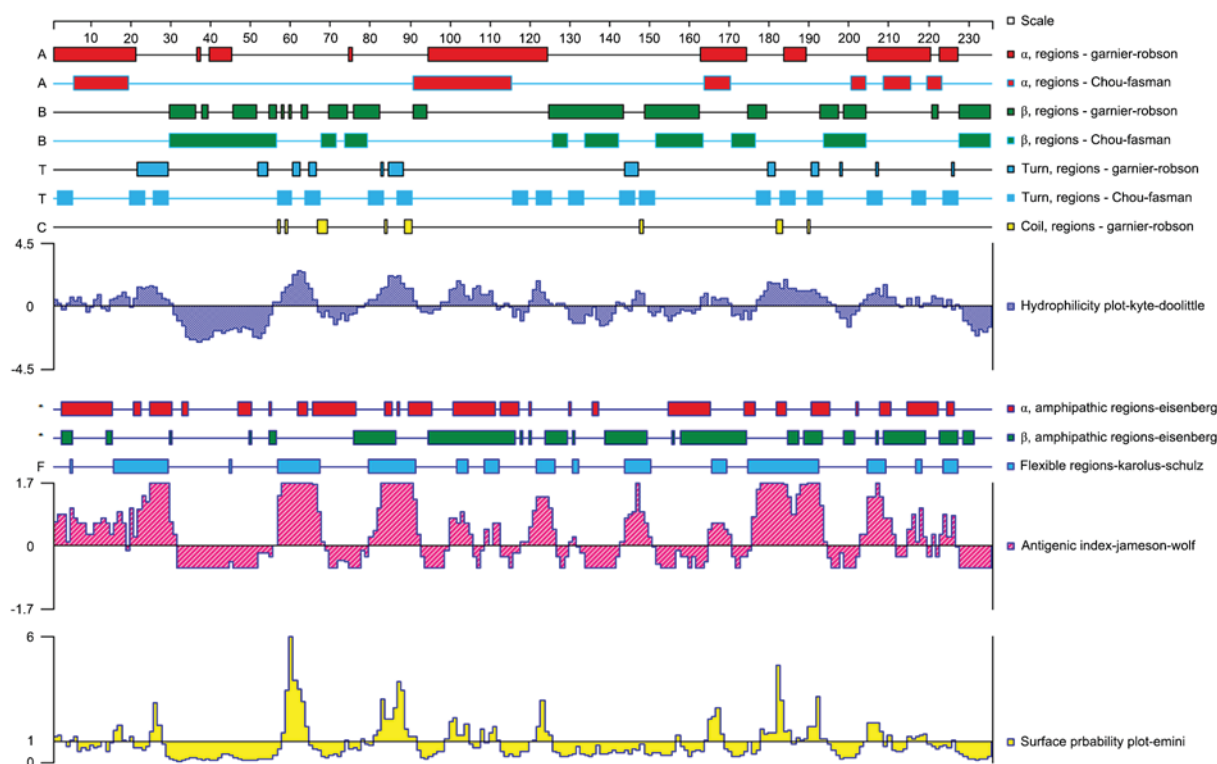


Figure 2. B cell epitopes of mouse tumor necrosis factor- α were predicted using DNASTar software. Amino acids 54-65 (GVIGPQRDEKFP) and 78-92 (LTLRSSQNSSDKPV) exhibited good hydrophilicity and accessibility, and were located in protein extended structures or with no coil in the secondary structure.

Specimen collection and antibody detection. Retro-orbital blood (0.5 ml per mouse) was collected prior to the first immunization and 2 weeks after each immunization. Blood was collected a total of five times, and the mice were sacrificed by cervical dislocation. Serum was centrifuged for 4 min at 1,000 \times g at 4°C and serum antibody titers were measured by standard indirect ELISA. The 96-well ELISA plate was coated with 10 mg/l MAP1, MAP2, mTNF- α protein, or TH full peptide, 100 μ l/well, and maintained overnight at 4°C. After blocking with 1% BSA, diluted test animal antiserum (dilutions ranging from 1:10,000 to 1:150,000) was used as the primary antibody for measurement, with pre-immune serum serving as a negative control. The results were presented as an A value (the mean of duplicated wells) and the experiments were repeated three times. The positive standard was >2.1, which was determined by the A value of the measured sample/negative control.

Protein electrophoresis and western blotting. H22 cells (1×10^9), which express mTNF- α stably and efficiently, were centrifugally washed. Pro-cooling suspended buffer solution (1 ml) was added to the cell pellet for dissociation. The cells were vortexed for 5 min and centrifuged for 5 min at 10,000 \times g at 4°C. The supernatant was discarded, and total protein was extracted with an equal volume of 2X sodium dodecyl sulfate. A total of 20 mg protein was analyzed by SDS polyacrylamide gel electrophoresis, then it was transferred to a polyvinylidene difluoride membrane at 400 mA for 90 min, and a commercial mTNF- α protein served as a control and a mouse pre-immune serum served as a negative control.

Glyceraldehyde-3-phosphate dehydrogenase was selected as an internal reference. Chemiluminescent western blot detection was performed according to the manufacturer's instructions. The antibodies used for western blotting were either commercially available antibodies against full-length mTNF- α (cat. no. 17590-1-AP; ProteinTech Group, Inc.) (1:2,000) or mouse antiserum against MAP or mTNF- α (1:3,000 dilution). The specificity of the protein detected by the antiserum was determined by the molecular weight of the bands. BandScan version 5.0 software (Glyko Inc., Novato, CA, USA) was used to compare the relative gray scale of the bands.

Statistical analysis. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Quantitative data were presented as means \pm standard deviation. Pluralities of samples were compared using single factor analysis. The Student-Newman-Keuls post-test method was used for comparing variances between groups and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Prediction of B-cell epitope and peptide synthesis. The predictions of B-cell epitopes using BcePred and DNASTar are presented in Figs. 1 and 2, respectively. The results of the two methods were almost identical. Amino acids 54-65 (GVIGPQRDEKFP) and 78-92 (LTLRSSQNSSDKPV) exhibited good hydrophilicity, accessibility and plasticity, and were located in protein extended structures or with no coil in the secondary structure. They were presumed to be B-cell epitopes.

Table I. B cell epitopes of mTNF- α were predicted using BcePred (<http://www.imtech.res.in/raghava/bcepred/>) and DNASTar software (DNASTAR, Inc., Madison, WI, USA).

MAP	Amino acid	Peptide	Software
MAP1	54-65	GVIGPQ RDEKFP	BcePred, DNASTar
MAP2	78-92	LTLRSSSQ NSSDKPV	BcePred, DNASTar

mTNF- α , mouse tumor necrosis factor- α ; MAP, multiple antigenic polypeptide; MAP1, amino acids 54-65 of mTNF- α ; MAP2, amino acids 78-92 of mTNF- α .

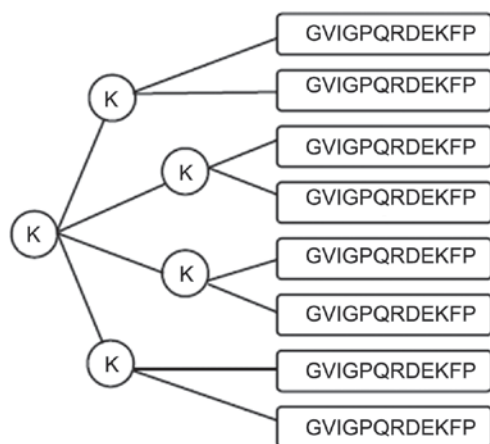


Figure 3. Schematic structure of eight-branched peptide multiple antigenic polypeptides. Amino acid peptides were linked with the 8 amino terminals of the 4 lysines located at the end of the MAP core structure. MAP, multiple antigenic polypeptide.

Therefore, two eight-branch MAPs (Fig. 3) were synthesized based on the predicted amino acid sequences (Table I).

Binding affinity of synthesized MAPs and mTNF- α full-length protein to anti-mTNF- α antibody. The binding affinity of synthesized MAP and mTNF- α full-length protein to anti-mTNF- α antibody was measured by indirect ELISA. On the basis of absorbance at a wavelength of 490 nm, MAP1 and MAP2 exhibited rather high affinity to the commercialized antibody. The binding of MAP2 and mTNF- α full-length protein to the antibody was stronger than that of MAP1 (Fig. 4).

Dynamic changes in mouse antibody titer. Specific antibodies were detected 2 weeks after the first immunization. The MAP1 antibody titer reached a plateau after 4 weeks with the mean highest measured antibody titer of 1:50,000. The MAP2 antibody titer reached the mean highest antibody titer of 1:130,000 6 weeks after immunization and peaked at 8 weeks. The mean highest antibody titer for MAP2 was 1:140,000. No antibodies were generated in the mTNF- α and PBS control groups (Fig. 5). Furthermore, no reactions between TH and the two MAP antisera, or cross-reactions between the two sera and two MAPs were observed.

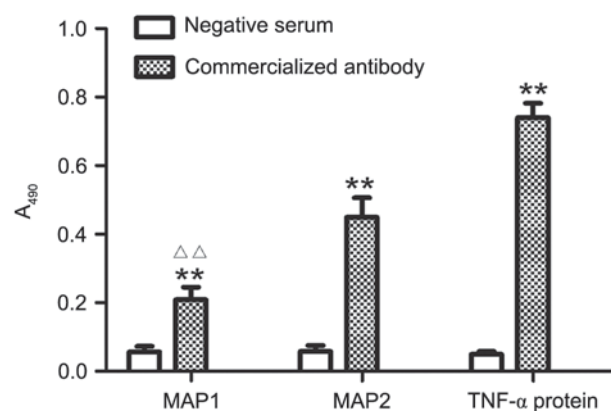


Figure 4. Binding affinity of MAP1 or MAP2 with the commercial anti-mTNF- α antibody (rabbit anti-mouse full-length TNF- α protein). MAP, multiple antigenic polypeptide; mTNF- α , mouse tumor necrosis factor- α ; MAP1, amino acids 54-65 of mTNF- α ; MAP2, amino acids 78-92 of mTNF- α . **P<0.01 vs. negative serum. ^ΔP<0.01 vs. MAP2 antiserum.

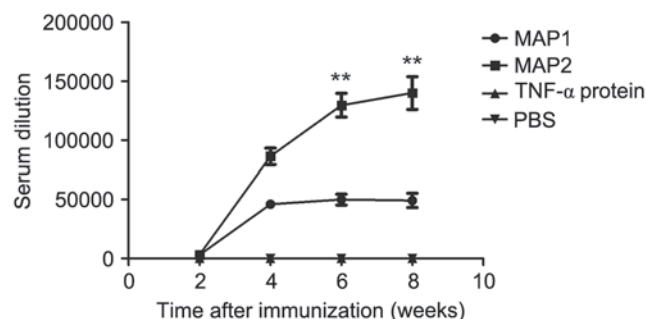


Figure 5. Dynamic changes of antibody titers in murine serum subsequent to immunization with different MAPs. MAP, multiple antigenic polypeptide; mTNF- α , mouse tumor necrosis factor- α ; MAP1, amino acids 54-65 of mTNF- α ; MAP2, amino acids 78-92 of mTNF- α ; PBS, phosphate-buffered saline. **P<0.01 vs. MAP1 antibody titer.

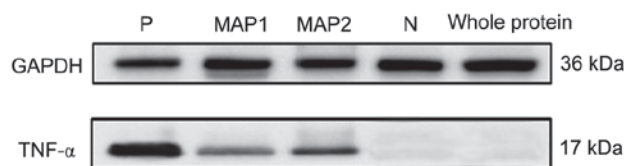


Figure 6. Specificity of the murine antisera against MAPs as detected by western blotting. P, positive control (commercialized TNF- α antibody); N, negative control (TH peptide antiserum); whole protein, recombinant TNF- α whole protein antiserum; MAP, multiple antigenic polypeptide; mTNF- α , mouse tumor necrosis factor- α ; MAP1, amino acids 54-65 of mTNF- α ; MAP2, amino acids 78-92 of mTNF- α .

Protein electrophoresis and western blotting. Total protein from H22 cells was used to evaluate the MAP1 antiserum, MAP2 antiserum and commercial mTNF- α antiserum. A commercial rabbit anti-mouse TNF- α antibody served as a positive control and a mouse pre-immune serum served as a negative control. Glyceraldehyde-3-phosphate dehydrogenase was selected as an internal reference. A very clear band was observed in a molecular weight of ~17 kDa from the commercial antibody. This was also observed for MAP1 and MAP2 antisera. Based upon a commercial antibody datasheet, 17 kDa indicated

the mTNF- α protein. The mTNF- α recombinant protein antiserum and negative controls did not exhibit a band (Fig. 6).

Discussion

The prevalence of UC has increased in China in recent years. Without effective treatment, its persistence and recurrent attacks severely affect the quality of life. TNF- α has emerged as a target for the treatment of UC (11,12).

The bioactivity of TNF- α , a human self-antigen, is complicated. Studies have demonstrated that TNF- α overexpression co-produces various types of disease, such as inflammatory bowel disease (3,4,6,7). Anti-TNF- α monoclonal antibody (infliximab) is the most commonly used antibody for passive immunotherapy. It neutralizes TNF- α *in vivo* and alleviates pathological damage. Infliximab has been approved for the treatment of Crohn's disease, and its positive role in UC treatment has been confirmed by clinical studies (11,12). However, anti-TNF- α monoclonal antibody treatment has various drawbacks. It is associated with high medication volume use and high production cost. This type of treatment also requires long-term repeated use and is prone to causing hypersensitivity reactions (13,14). This has given a new direction for developing anti-self molecule immunotherapies, and active immunization vaccines against human self-proteins (autologous vaccines) rather than passive acceptance of monoclonal antibody drugs (15). Autoantigen epitope vaccines present as a better treatment modality for autoimmune disease. Due to their low molecular weight and unitary structure, self-antigens reduce immunogenicity and cannot induce the desired immune responses, particularly in B-cell epitope-mediated humoral immune responses (16). To solve this problem, short peptide vaccine epitope and carrier protein crosslinking methods were used to improve immunogenicity. However, carrier proteins are foreign antigen molecules and often induce antibodies against the carrier protein itself rather than the vaccine epitopes. In recent years, a design for MAPs has been proposed. Low-molecular weight and low-immunogenicity lysines are used as the core matrix coupling monomeric peptides (usually four or eight) to form a dendritic structure. Such a design mimics the natural conformational epitopes, but also activates humoral immunity and induces high-titer and high-affinity antibodies without coupling to a carrier protein (17). Therefore, the use of a branched MAP epitope complex designed using an MAP program may improve the quality of peptide vaccines with the removal of carrier protein defects.

The present study was based on previous investigations of a heparanase MAP vaccination strategy (18-22), with the establishment of a method to induce a high-titer humoral immune response (23). To achieve the inhibition of TNF- α , internet software was used to predict the TNF- α B-cell epitope and an optimized immunization strategy (MAP + T cell epitope peptide) was used to induce high titers of antibody *in vivo*. To validate the immunogenic specificity of the predicted B-cell epitopes, the corresponding MAP, together with T-helper epitope for polyclonal antisera, was synthesized and the BALB/c mice were immunized. ELISA results demonstrated that MAP1 and MAP2 induced TNF- α -specific antibodies *in vivo*, with a higher-titer antibody observed in MAP2, which

indicated a high affinity to the commercial full-length antibody. This suggested that MAP1 and MAP2 were possible epitope peptides, with MAP2 located in amino acids 78-92 and demonstrating a slightly higher immunogenicity. MAP2 is more likely an immunogen for inducing highly specific humoral immune response to TNF- α .

To further verify the specificity of the antibody produced by MAP immunization, total protein was extracted from mouse hepatoma H22 cells, that were stably expressing mTNF- α , and reacted with MAP1 antiserum, MAP2 antiserum, and commercial mTNF- α recombinant protein antiserum. A commercial rabbit anti-mouse mTNF- α antibody served as positive control, with the corresponding pre-immune mouse serum serving as a negative control. The results demonstrated a clear band at a molecular weight of ~17 kDa for MAP1 and MAP2, but no clear bands were observed for mTNF- α recombinant protein antiserum and the negative control. The western blotting results demonstrated that mTNF- α , as a self-antigen, could not induce a specific humoral immune response alone, whereas the MAP strategy could markedly enhance its B-cell epitope immunogenicity and induce polyclonal antibodies specifically for TNF- α .

In conclusion, the results of the present study indicated that amino acids 54-65 and 78-92 of mTNF- α were B-cell epitopes, with the strongest immunogenicity for amino acids 78-92. The study provided a theoretical basis for further investigations into a TNF- α polypeptide antibody and B-cell peptide vaccine. However, there were some limitations in this study, such as the optimal dose of MAP was not attentively selected, and the possibility of inducing autoimmune diseases or hypersensitivity reactions were not fully investigated. In addition, for TNF- α immunotherapy, further studies are required to clarify whether the above-mentioned B-cell epitopes are neutralizing epitopes of TNF- α , which inhibit TNF- α activity after binding and facilitate with the treatment of inflammatory bowel disease.

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