Development of A Recombinant Xenogeneic TNF-α Protein Vaccine to Protect Mice From Experimental Colitis

Yang Wan a,b, Meng Li b, Hailong Zhang c,d, Xiuran Zheng b, Chaoheng Yu b, Gu He b, Yan Luo b, Li Yang b#, Yuquan Wei b

Department of Geriatric Medicine, No. 4 Teaching Hospital, Sichuan University, Chengdu, PR China a
State Key Laboratory of Biotherapy / Collaborative Innovation Center for Biotherapy, West China Hospital, West China Medical School, Sichuan University, Chengdu, PR China b
Department of Cell Biology, Institute of Immunology, Medical College of Henan University, Kaifeng, PR China c
Department of Immunology, Key Laboratory of Cellular and Molecular Immunology, Henan University, Kaifeng, PR China d

#Address correspondence to Li Yang, yl.tracy73@gmail.com.

Abstract

Previous studies have highlighted the efficacy of tumor necrosis factor alpha (TNF-α) inhibitors, including monoclonal antibodies and soluble receptors, in the treatment and management of the intestinal bowel disease (IBD). However, because of the immunogenicity of xenogenic TNF-α inhibitors, anti-drug antibodies (ADAs) can be triggered after repeated administration. An alternative way to target TNF-α is active
immunization, to elicit the production of high titers neutralizing antibodies. In this study, we prepared a xenogeneic TNF-α protein vaccine, and studied the protective effects in experimental colitis models. The xenogeneic TNF-α protein vaccine could overcome self-tolerance and induce TNF-α-specific neutralizing antibody. Moreover, the xenogeneic TNF-α protein vaccine could protect mice from acute and chronic colitis induced by dextran sodium sulfate (DSS). One possible explanation for this protective effect is the production of TNF-α specific neutralizing antibody, which absorbed the biological activity of mTNF-α, and failed to induce T lymphocytes apoptosis. In summary, the xenogeneic TNF-α protein vaccine may be a potent therapeutic strategy for IBD.
Introduction

The intestinal bowel disease (IBD), characterized as chronic relapsing inflammatory disorders of the gastrointestinal tract (1), is primarily a syndrome of the developed world (2). However, an increasing of prevalence rate has been observed in traditionally low-incident regions such as Asia, South America and southern and eastern Europe (3). The conventional treatments are limited to anti-inflammatory drugs and immune-suppressive medications. However, their application has been restricted by problems with long-term efficacy and safety issues (4).

Previous studies have highlighted the efficacy of tumor necrosis factor alpha (TNF-α) inhibitors, including monoclonal antibodies and soluble receptors, in the treatment and management of IBD, especially in patients who are refractory to or intolerant of the conventional treatment regimens (5). TNF-α, a pleiotropic proinflammatory cytokine, has an increased expression in the mucosa of inflamed intestine (6-8). However, because of the immunogenicity of the xenogenic TNF-α inhibitors, anti-drug antibodies (ADAs) can be triggered after repeated administration, leading to treatment resistance (9). The reported loss of response (LOR) rates ranged between 11% and 48% (10). Furthermore, these therapeutic approaches are expensive and cumbersome. These limitations prompted investigations of alternative strategies, including active anti-TNF-α immunization.

However, because of immune tolerance, the immunity to self-antigens is difficult to elicit. Our previous studies have explored the feasibility of immunotherapy of tumors with xenogeneic homologous molecules as vaccine against those on autologous cells in a cross-reaction between the xenogeneic homologous and self-molecules (11-14). However,
this xenogeneic vaccination strategy has not been tested in inflammatory diseases yet. In this study, we prepared a xenogeneic TNF-α protein vaccine, and studied the protective effects in mice IBD model.

Materials and methods

Experimental Mice. Male 6-8-week-old C57BL/6 mice were bred and kept under pathogen-free conditions. All animal protocols were approved by the Animal Care and Use Committee of State Key Laboratory of Biotherapy.

Plasmid construction. Human and mice TNF-α ORF were purchased from InvivoGen (San Diego, CA, USA). cDNA fragments coding soluble mice TNF-α (80–235 residues) and human TNF-α (77–233 residues) were amplified by Ex Taq DNA polymerase (Takara Biotechnology, Dalian, China). Primers used in this study were shown as follow:

for human TNF-α, sense primer, 5'-GGGGTACCGACGACGACGACAGGTTCAGATCATCTTTCTGAAC-3', and anti-sense primer, 5'-CCGCTCGAGTCACAGGGCAATGATCCCA-3'; for mice TNF-α, sense primer, 5'-GGGGTACCGACGACGACGACAGCTCAGATCATCTTTCTCAAAATTC-3', and anti-sense primer, 5'-CCGCTCGAGTCACAGAGCAATGACTCCAAAG-3'. Kpn I site is underlined, Xho I site is shown in bold, and Enterokinase site coding sequence is shown in Italic. The PCR product was digested with Kpn I and Xho I (NEB, Ipswich, MA, USA), and was subcloned into a pET32a(+) vector (Novagen, Madison, WI, USA). The resultant recombinant protein consisted of thioredoxin (Trx), Histag, the cleavage sequence of thrombin, S-tag and soluble TNF-α.
Protein expression and purification. The E.coli strain BL21 (DE3)-bearing the expression plasmids was induced with IPTG for protein production. The bacteria were lysed using a high-pressure homogenizer (GEA Niro Soavi, Parma, Italy). mTNF-α or hTNF-α protein was purified via four process steps, which included Ni-chelating Sepharose affinity chromatography (GE Healthcare, Piscataway, NJ, USA), excision of the Trx-His6-tag by Enterokinase, removal of the Trx-His6-tag with second Ni-chelating Sepharose affinity chromatography, and HiTrap Q HP ion exchange columns (GE Healthcare). The protein concentration was estimated by a protein assay reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard, and the purity was estimated by SDS–PAGE and High Performance Liquid Chromatography (HPLC) analysis. The both proteins were negative for endotoxin contamination in the limulus amebocyte lysate (LAL) test. Moreover, the recombinant proteins were characterized by Western blot assay with a rabbit monoclonal anti-TNF-α antibodies (CST, Danvers, MA, USA), and peptide mass fingerprinting were determined by MALDI-TOF as described before (15, 16).

TNF-α bioassay and the neutralizing activity of TNF-α-specific Abs. The activity of the recombinant proteins was determined using TNF-α sensitive L929 fibroblasts as described before (17). L929 cells were treated with serial dilutions of the recombinant TNF-α for 24 h in the presence of actinomycin D (Sigma-aldrich, St. Louis, MO, USA, 1 μg/ml). The viable cells were determined by the crystal violet staining.

The neutralizing activity of TNF-α-specific Abs was similarly determined after incubating with serially diluted sera.

Immunizations. Male C57BL/6 mice were randomized into 3 groups, and immunized
subcutaneously with 10 μg mTNF-α or hTNF-α in a total volume of 100 μL in (BRENNTAG Biosector, Frederikssund, Denmark) as adjuvant for 6 times at 1-week intervals. As negative control, mice in the third group (PB group) were immunized 100 μL of PB combination with Alhydrogel. Sera were collected 5 days after immunization and at death. Moreover, weight loss, ruffling of fur, behavior, or feeding were recorded twice a week during TNF-α vaccination.

**Anti-hTNF-α Ab titer assay.** Specific anti-mTNF-α antibody titer in sera of immunized and control mice was determined by ELISA. Briefly, 96-well Polystyrene plates were precoated with 1 μg/well mTNF-α in coating buffer (carbonate-bicarbonate, pH 9.6) overnight at 4°C. After washed and blocked, the plates were incubated with serial dilutions of sera from immunized and control mice for 2 hours at 37°C. The plates were washed, and incubated with 100 μl HRP-conjugated goat anti-mouse IgG (1:10,000 dilution) for 1 hour at 37°C, followed with 100 μl/well Sureblue TMB substrate (KPL, Inc., Gaithersburg, MD, USA) for 15 minutes. The color development was stopped by adding 1 M H₂SO₄ (50 μl/well) and the OD value was measured at 450 nm using Multiskan MK3 microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The results were considered positive when the ratio of absorbency in antiserum group versus control serum group is greater than 2.1.

**Induction of Colitis.** Male C57BL/6 mice were allowed free access to 2.5% DSS (dissolved in sterile, distilled water, weight/volume, mol. wt. 36,000–50,000; MP Biomedicals, LLC, Eschwege, Germany) solution as drinking water for 7 days. Chronic colitis was induced by three cycles of administration of 2% DSS for 5 days, alternating with DSS-free drinking water for 5 days.
Determination of Disease Activity Index (DAI). Animal body weight, stool consistency, and the presence of occult or gross blood were recorded into a scale to evaluate clinical disease severity, as described previously (1, 18). The scores were defined as follows: change in body weight (0: none, 1: 1-5%, 2: 5-10%, 3: 10-15%, 4: >15%), stool consistency (0: normal, 2: loose stool, 4: diarrhea) and stool blood (0: negative, 2: positive, 4: gross bleeding). The clinical DAI, ranging from 0 to 4, was the sum of scores for these parameters divided by three.

Histological Scoring. After mice were sacrificed, the entire colon was removed and fixed in 10% buffered formalin for histological analysis. Sections (4-μm thick) were prepared and subjected to staining with hematoxylin and eosin (H&E). Histological scoring was performed using a previously described scoring system by two experienced pathologists in a blinded fashion (1, 19). Three independent parameters were measured (severity of inflammation, depth of injury, and crypt damage) and scored from 0 (normal) to 3 (scored as: 1, severe; 2, transmural injury; 3, inflammation with entire crypt and epithelium loss). The score of each parameter was multiplied by a factor reflecting the percentage of tissue involvement (1, 0 - 25%; 2, 26 - 50%; 3, 51 - 75%; and 4, 76 - 100%) and summed to obtain a histological injury score.

Furthermore, liver, kidneys, lungs, spleen, or heart were removed, and pathological studies were tested to investigate potential adverse effects.

Adoptive transfer of immune sera. Serum from mice vaccinated with hTNF-α or mTNF-α protein respectively was obtained one week after the last immunization, and stored at 4°C until use. Mice were induced acute colitis as described above, and were injected i.p. with 0.2 mL of the serum from vaccinated mice on day 0, 2,
4, 6 after DSS treatment. Animals treated with serum from control mice served as controls.

Cytokine quantification. Serum mTNF-α was measured with a commercially available ELISA kit (Neobioscience, Shenzhen, China) according to the manufacturer’s instructions.

Detection of Apoptosis in vitro. To measure the effects of mTNF-α specific anti-serum on apoptosis of T lymphocytes, splenocytes were dissociated by crushing the spleen with a 2-ml syringe piston on a 70-μm filter (cell strainer; BD Biosciences, San Jose, CA, USA) and were then were separated by Ficoll solution according to the manufacturer’s protocol. Purified lymphocytes were incubated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum in the presence of 2 μg/mL of anti-CD3 antibody (BD Biosciences) for 48 hours. Activated T lymphocytes were washed and resuspended in culture medium in the presence of sera at dilution of 1:100. After 24 hours, cells were double stained with annexin V staining and propidium iodide (PI) to detect apoptosis. Data was acquired using the Novocyte flow cytometer (ACEA Biosciences, San Diego, CA, USA).

Statistical Analysis. Parametric data were analyzed statistically with one-way analysis of variance (ANOVA) followed by tukey’s multicomparison test when appropriate. In all cases, a $P$ value of 0.05 was determined to be significant.

Results

Expression, Purification and Characteration of recombinant proteins. hTNF-α and mTNF-α recombinant proteins were expressed in the E. coli expression system, and
purified as described above. The purified proteins were analyzed by SDS–PAGE and HPLC, and the results indicated that the purity of both recombinant proteins was above 90% of total protein amount (FIG 1A&B). The recombinant proteins were recognized specifically by the anti-TNF-α antibodies as shown in Western blot assay (FIG 1C). Moreover, both recombinant proteins were characterized by MALDI-TOF (data not shown).

**hTNF-α vaccine Induces of Anti-TNF-α Abs.** C57BL/6 mice were immunized subcutaneously with 10 μg mTNF-α or hTNF-α in a total volume of 100 μL in combination with Alhydrogel as adjuvant. As shown in Fig. 2, after the last vaccination, hTNF-α vaccine could overcome self-tolerance and induce mTNF-α-specific Abs in all mice, whereas immunization with mTNF-α elicited only a much lower anti-mTNF-α Abs in 4 out of 6 mice (P = 0.023).

**hTNF-α Vaccination Protects Mice from Development of colitis.** We firstly tested the protective effect of TNF-α vaccine in acute colitis model. One week after the last immunization, mice were fed 2.5% DSS for 7 days to induce acute colitis. mTNF-α vaccinated mice or control mice showed body weight loss, diarrhea, and bleeding in stool, resulting in a sharp increase of DAI. In contrast, hTNF-α vaccinated mice exhibited a significantly reduced DAI from days 4 to 7 (P < 0.05, FIG 3 A).

A well-established histopathological event, including infiltration of inflammatory cells, ulceration, and crypt damage was detected on H&E staining of colonic tissue sections from mTNF-α vaccinated mice or control mice. In contrast, the colons from hTNF-α vaccinated mice were relatively normal, exhibiting only mild evidence of inflammatory cells infiltration and mucosal injury (FIG 3B). Furthermore, colonic tissue sections from
hTNF-α vaccinated mice also showed significantly lower histological score than that from mTNF-α vaccinated or control mice ($P = 0.006$, FIG 3C).

We further studied the effect of hTNF-α vaccination on serum TNF-α concentrations at the end of acute colitis experiment. As shown in FIG 3D, TNF-α concentrations of serum from hTNF-α vaccinated mice were significantly reduced ($P < 0.001$).

The IBD are characterized by chronic relapsing inflammatory disorders of the gastrointestinal tract, so we further tested protective effect of TNF-α vaccine in chronic colitis model. Similar to the results in acute colitis model, hTNF-α vaccination protected mice from crypt loss, erosions, inflammatory cell infiltrations, as well as the DSS-induced increase in histological score (FIG 4).

Moreover, no change of weight loss, ruffling of fur, behavior, or feeding was recorded during TNF-α vaccination. And no pathological changes in the liver, kidneys, lungs, spleen, or heart were observed (suppl FIG 1).

**Self-specific Abs developed in hTNF-α vaccinated mice are neutralizing in vitro.** To evaluate the Abs’ ability to neutralize TNF-α *in vitro*, L929 cells were treated with mTNF-α and serially diluted sera. As shown in FIG 5A, serum at high dilution from hTNF-α vaccinated mice abolished the cytotoxic activity of mTNF-α on L929 cells, whereas there were no protective effects of sera from mTNF-α vaccinated or control mice.

Furthermore, a sera adoptive transfer experimental was performed in this study. Mice were treated with DSS to induce acute colitis and administrated with sera from hTNF-α or mTNF-α vaccinated mice. Mice were sacrificed on day 7 after DSS treatment, and...
colons were collected for histological examination. Serum from mTNF-α vaccinated mice showed no protective effects on colitis, whereas serum from hTNF-α vaccinated mice significantly reduced DAI and histological damage score (FIG 5 B&C).

**TNF-α specific anti-serum fails to induce T lymphocytes apoptosis.** It was reported that besides neutralizing effect, Infliximab, a chimeric anti-TNF-α monoclonal antibody could induce apoptosis in activated T lymphocytes (20, 21). We further investigated the effects of anti-serum on T lymphocytes apoptosis. As shown in FIG 6, no obvious increase of T lymphocytes apoptosis was detected after treated with serum from hTNF-α vaccinated mice.

**Discussion**

Recently, the introduction of TNF-α inhibitors highlighted the pivotal role in the pathogenesis of IBD (22). TNF-α, which is mainly produced by activated macrophages, monocytes, eosinophils and T cells, is increased in mucosa and serum of IBD patients (23). TNF-α is a pleiotropic proinflammatory cytokine that binds to its receptors TNFR1 and TNFR2 followed by the intracellular activation of nuclear factor-κB (NF-κB)(7), resulting the activation of immune cells, increased angiogenesis, the induction of Paneth cell death, the production of matrix metalloproteinases (MMPs) by myofibroblasts, and the direct damage of intestinal epithelial cells (24-27). These studies indicated that inhibition of TNF-α by monoclonal antibodies or soluble receptors is one of the effective approaches for IBD treatment (28). However, because of the immunogenicity of the xenogenic TNF-α inhibitors, ADAs can be triggered after repeated administration. Previous studies have investigated ADAs formation and its influence on infliximab
concentrations and clinical outcome (29, 30). ADAs might reduce the drug efficiency by competing with the endogenous ligands and/or by forming immune complexes, which accelerates the clearance of the drug from the circulation (31).

In the study, we investigated the efficiency of protein vaccine against TNF-α in IBD treatment, by eliciting the production of neutralizing antibodies. This strategy allows the production of polyclonal autologous anti-TNF-α antibodies potentially bypassing the risk of ADAs formation. However, it is difficult to elicit the immune responses to self-antigens because of immune tolerance (32). Recently, a heterocomplex vaccine, called human TNF-α kinoid (TNF-K) was developed by Neovacs (Paris, France), consisting of biologically inactive but immunogenic hTNF-α protein conjugated to a carrier protein, keyhole limpet hemocyanin (KLH). This compound is capable of inducing the production of neutralizing anti-hTNF-α antibodies and avoiding the risk of ADAs induction. The results of Phase I/II clinical trial of TNF-K showed good efficiency and safety (33, 34). However, the carrier protein KLH can not be reproduced synthetically, and can only be purified from the keyhole limpet Megathura crenulata, which may limit the future clinical usage.

Our previous studies have explored the feasibility of immunotherapy of tumors with xenogeneic homologous molecules as vaccine against those on autologous cells in a cross-reaction between the xenogeneic homologous and self-molecules (11-14). In this study, we tested this strategy in mice IBD models. Our results showed that xenogeneic TNF-α protein vaccination, rather than homologous vaccine could elicit a neutralizing antibody response. Although the homology of the soluble form of TNF-α protein in human and mice was 79%, the differences between the two amino acid sequences were
sufficient to overcome immune tolerance in mice after xenogeneic vaccination.

Moreover, the xenogeneic TNF-α protein vaccine could protect mice from acute and chronic colitis induced by DSS. One possible explanation for this protective effect is the production of TNF-α specific neutralizing antibody which absorbs the biological activity of mTNF-α, as shown by neutralization assay and serum transfer experiment. However, it was reported that besides neutralizing ability, Infliximab could induce apoptosis in activated T lymphocytes (20, 21). We also tested the effect of anti-serum on lymphocytes apoptosis. As shown in FIG 6, TNF-α specific anti-serum failed to induce T lymphocytes apoptosis, suggesting neutralizing ability of TNF-α specific antibody may be possible mechanisms of the xenogeneic TNF-α protein vaccine.

Furthermore, potential adverse effects were tested in this study. Weight loss, ruffling of fur, behavior, or feeding were not changed in xenogeneic TNF-α protein vaccinated mice, and no obvious pathological changes were observed. In summary, the xenogeneic TNF-α protein vaccine may be a potent therapeutic strategy for IBD.

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Legends

FIG 1 Characterization of recombinant hTNF-α and mTNF-α proteins. The purified proteins were analyzed by SDS–PAGE (A) and HPLC (B). (C) Western blot showing the recombinant proteins were recognized specifically by the anti-TNF-α antibodies.

FIG 2 mTNF-α-specific IgG responses after TNF-α protein vaccination. C57BL/6 mice were immunized subcutaneously with mTNF-α or hTNF-α proteins in combination with Alhydrogel 2% as adjuvant 6 times at 1-week intervals. Sera were collected 5 days after immunization and at death. Data represent geometric mean antigen-specific IgG titres ± SD for n = 6 mice/group. *P < 0.05, ***P < 0.001, compared with the results from PB control group.

FIG 3 Protective effect of TNF-α vaccine in acute colitis model. One week after the last immunization, mice were fed 2.5% DSS for 7 days to induce acute colitis. (A) DAI score was recorded every two days. Data represent mean DAI score ± SD for n = 6 mice/group. *P < 0.05, ***P < 0.001, compared with the results from PB control group. (B) Photomicrographs of representative H&E stained colon sections. The colon section photographs are representative of 6 mice for each group (magnification 100×). (C) Histological scoring was performed by two experienced pathologists in a blinded fashion. Data represent mean histological score ± SD for n = 6 mice/group. *P < 0.05, compared with the results from PB control group. (D) The effect of hTNF-α vaccination on serum TNF-α concentrations. Data represent mean serum TNF-α concentrations ±
SD for n = 3 mice/group. ***P < 0.001, compared with the results from PB control group.

FIG 4 Protective effect of TNF-α vaccine in chronic colitis model. One week after the last immunization, mice were fed by three cycles of administration of 2% DSS in drinking water for 5 days, alternating with 5-day periods of recovery. (A) Photomicrographs of representative H&E stained colon sections. The colon section photographs are representative of 6 mice for each group. (magnification 100×). (B) Histological scoring was performed by two experienced pathologists in a blinded fashion. Data represent mean histological score ± SD for n = 6 mice/group. **P < 0.01, compared with the results from PB control group.

FIG 5 Neutralizing effects of TNF-α specific antibodies. (A) L929 cells were treated with mTNF-α and serially diluted sera and protective rate was calculated. Data represent mean protective rate ± SD for n = 3 mice/group. ***P < 0.001, compared with the results from PB control group. (B and C) Mice were treated with DSS to induce acute colitis and administrated with sera from hTNF-α or mTNF-α vaccinated mice. Mice were sacrificed on day 7 after DSS treatment, and colons were collected for histological examination. (B) Data represent mean DAI score ± SD for n = 6 mice/group. *P < 0.05, **P < 0.01, compared with the results from PB control group. (C) Data represent mean histological score ± SD for n = 6 mice/group. **P < 0.01, compared with the results from PB control group.

FIG 6 The effect of TNF-α specific anti-serum on activated T lymphocytes apoptosis. Activated T lymphocytes were treated with anti-serum at dilution of 1:100 for 24 hours, then cells were double stained with annexin V staining and propidium iodide (PI) to
detect apoptosis. Data shown are representative of 3 independent experiments.