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Development of a Recombinant Xenogeneic Tumor Necrosis Factor Alpha 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 West China Teaching Hospital, Sichuan University, Chengdu, People's Republic of China[a]; State Key Laboratory of Biotherapy/Collaborative Innovation Center for Biotherapy, West China Hospital, West China Medical School, Sichuan University, Chengdu, People's Republic of China[b]; Department of Cell Biology, Institute of Immunology, Medical College of Henan University, Kaifeng, People's Republic of China[c]; Department of Immunology, Key Laboratory of Cellular and Molecular Immunology, Henan University, Kaifeng, People's Republic of China[d]

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 intestinal bowel disease (IBD). However, because of the immunogenicity of xenogeneic TNF-α inhibitors, antidrug 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 of 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 mouse TNF-α (mTNF-α) and failed to induce T lymphocyte apoptosis. In summary, use of the xenogeneic TNF-α protein vaccine may be a potent therapeutic strategy for IBD.

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 rate of prevalence has been observed in traditionally low-incidence 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 (MAbs) 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, shows increased expression in the mucosa of inflamed intestine (6–8). However, because of the immunogenicity of the xenogeneic TNF-α inhibitors, antidrug antibodies (ADAs) can be triggered after repeated administration, leading to treatment resistance (9). The reported rates of loss of response (LOR) 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, immunity to self-antigens is difficult to elicit. Our previous studies have explored the feasibility of immunotherapy of tumors by treatment with xenogeneic homologous molecules as vaccines 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 yet been tested in inflammatory diseases. In this study, we prepared a xenogeneic TNF-α protein vaccine and studied the protective effects in a mouse IBD model.

MATERIALS AND METHODS

Experimental mice. Male 6-to-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 TNF-α (hTNF-α) and mouse TNF-α (mTNF-α) open reading frames (ORF) were purchased from InvivoGen (San Diego, CA, USA). cDNA fragments coding soluble mTNF-α (residues 80 to 235) and hTNF-α (residues 77 to 233) were amplified by *Ex Taq* DNA polymerase (TaKaRa Biotechnology, Dalian, China). Primers used in this study were as follows: for human TNF-α, 5′-GGGGTACC*GACGACGACGACAAG*GTCAGATCATCTTCTCGAAC-3′ (sense primer) and 5′-CCG**CTCGAG**TCACAGGGCAATGATCCCA-3′ (antisense primer); and for mouse TNF-α, 5′-GGGGTACC*GACGACGACGACAAG*CTCAGATCATCTTCTCAAAATTC-3′ (sense primer) and 5′-CCG**CTCGAG**TCACAGAGCAATGACTCCAAAG-3′ (antisense primer). The KpnI site is underlined, the XhoI site is shown in bold, and the enterokinase site coding sequence is shown in italics. The PCR product was digested with KpnI and XhoI (NEB, Ipswich, MA, USA) and was subcloned into a pET32a(+) vector (Novagen, Madison, WI, USA). The resultant recombinant protein

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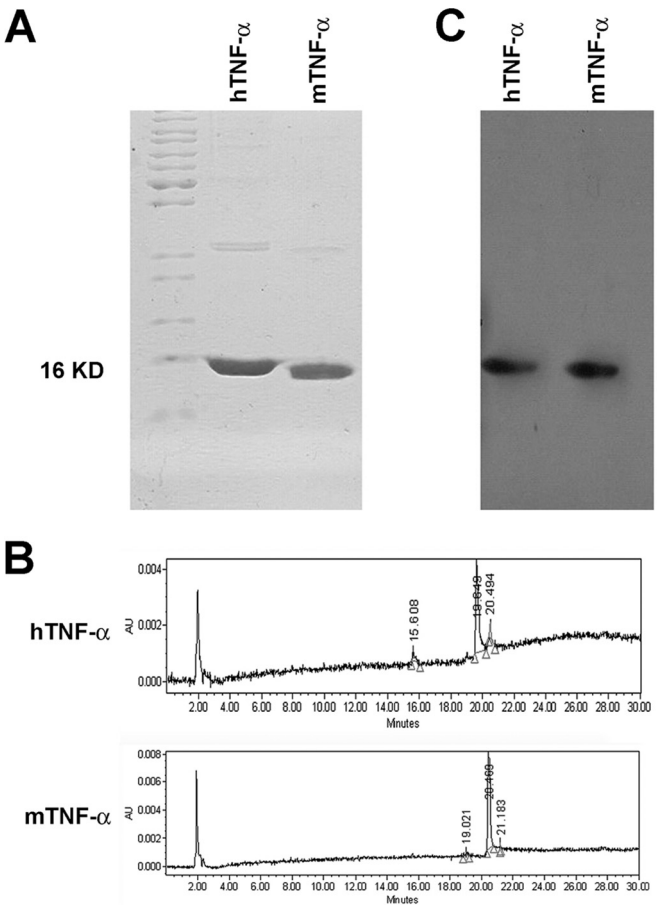

FIG 1 Characterization of recombinant hTNF-α and mTNF-α proteins. (A and B) The purified proteins were analyzed by SDS-PAGE (A) and HPLC (B). (C) Western blot showing that the recombinant proteins were recognized specifically by the anti-TNF-α antibodies. AU, arbitrary units.

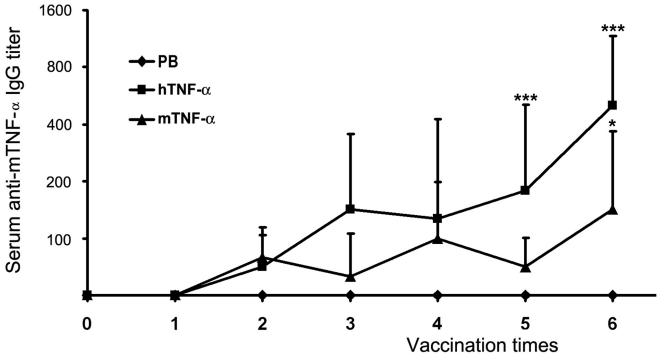

FIG 2 mTNF-α-specific IgG responses after TNF-α protein vaccination. C57BL/6 mice were immunized subcutaneously with mTNF-α or hTNF-α proteins in combination with 2% Alhydrogel as the adjuvant 6 times at 1-week intervals. Sera were collected 5 days after immunization and at death. Data represent geometric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

consisted of thioredoxin (Trx), His tag, the cleavage sequence of thrombin, S tag, and soluble TNF-α.

Protein expression and purification. The *Escherichia coli* BL21(DE3) strain bearing the expression plasmids was induced with IPTG (isopropyl-β-D-thiogalactopyranoside) 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 processing 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 secondary Ni-chelating Sepharose affinity chromatography, and the use of HiTrap Q HP ion exchange columns (GE Healthcare). The protein concentration was estimated by the use of 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. Both proteins were negative for endotoxin contamination in the limulus amebocyte lysate (LAL) test. Moreover, the recombinant proteins were characterized by Western blotting assay with rabbit monoclonal anti-TNF-α antibodies (CST, Danvers, MA, USA), and peptide mass fingerprinting were determined by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis 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 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 identified by crystal violet staining.

The neutralizing activity of TNF-α-specific Abs was similarly determined after incubation 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 combination with Alhydrogel (Brenntag Biosector, Frederikssund, Denmark) as the adjuvant six times at 1-week intervals. As a negative control, mice in the third group (phosphate buffer [PB] group) were immunized with 100 μl of PB in a combination with Alhydrogel. Sera were collected 5 days after immunization and at death. Moreover, weight loss, ruffling of fur, behavior, and feeding data were recorded twice a week during TNF-α vaccination.

Anti-hTNF-α Ab titer assay. Specific anti-mTNF-α antibody titers in sera of immunized and control mice were determined by enzyme-linked immunosorbent assay (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 being washed and blocked, the plates were incubated with serial dilutions of sera from immunized and control mice for 2 h at 37°C. The plates were washed and incubated with 100 μl horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:10,000 dilution) for 1 h at 37°C followed by 100 μl/well Sureblue TMB (3,3′,5,5′-tetramethylbenzidine) substrate (KPL, Inc., Gaithersburg, MD, USA) for 15 min. The color development was stopped by adding 1 M H_2SO_4 (50 μl/well), and the optical density (OD) value was measured at 450 nm using a Multiskan MK3 microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The results were considered positive when the ratio of absorbency in the antiserum group versus the control serum group was greater than 2.1.

Induction of colitis. Male C57BL/6 mice were allowed free access to a solution of 2.5% DSS (MP Biomedicals, LLC, Eschwege, Germany) (dissolved in sterile, distilled water; molecular weight, 36,000 to 50,000 [wt/vol]) 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 DAI score. Animal body weight, stool consistency, and the presence of occult or gross blood were recorded using 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% to 5%; 2, 5% to 10%; 3, 10% to 15%; 4, >15%), change in stool consistency (0, normal; 2, loose stool; 4, diarrhea), and change in stool blood status (0, negative; 2, positive; 4, gross bleeding). The clinical disease activity index (DAI) score, ranging from 0 to 4, represented the sum of scores for these parameters divided by 3.

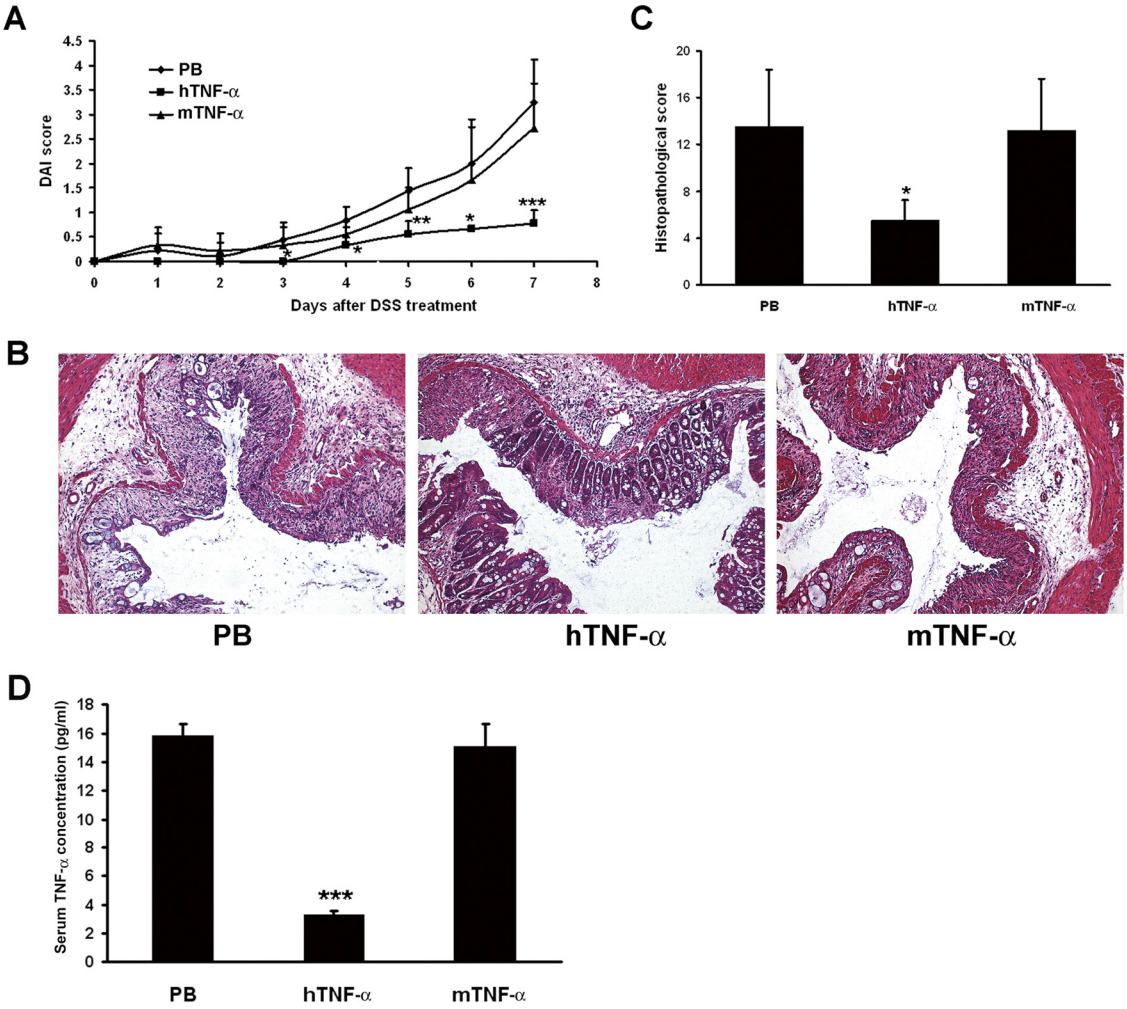

FIG 3 Protective effect of TNF-α vaccine in acute colitis model. One week after the last immunization, mice were fed 2.5% DSS for 7 days to induce acute colitis. (A) The DAI score was recorded every 2 days. Data represent mean DAI scores ± SD for $n = 6$ mice/group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with the results from the 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 blind fashion. Data represent mean histological scores ± SD for $n = 6$ mice/group. *, $P < 0.05$ (compared with the results from the 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 the PB control group).

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 blind fashion (1, 19). Three independent parameters were measured (severity of inflammation, depth of injury, and crypt damage) and scored from 0 (normal) to 3 (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% to 25%; 2, 26% to 50%; 3, 51% to 75%; 4, 76% to 100%), and the scores were summed to obtain a histological injury score.

Furthermore, liver, kidney, lung, spleen, or heart tissues 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 was obtained 1 week after the last immunization and stored at 4°C until use. Acute colitis was induced in mice as described above, and the mice were injected intraperitoneally (i.p.) with 0.2 ml of the serum from vaccinated mice on days 0, 2, 4, and 6 after DSS treatment. Animals treated with serum from control mice served as controls.

Cytokine quantification. Serum mTNF-α levels were 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 antiserum on apoptosis of T lymphocytes, splenocytes were dissociated by crushing the mouse spleen with a 2-ml syringe piston on a 70-μm-pore-size filter (cell strainer; BD Biosciences, San Jose, CA, USA) and were then were separated by the use of 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 h. Activated T lymphocytes were washed and resuspended in culture medium in the presence of sera at a dilution of 1:100. After 24 h, cells were doubly stained with annexin V staining and propidium iodide (PI) to detect apoptosis. Data were acquired using a Novocyte flow cytometer (ACEA Biosciences, San Diego, CA, USA).

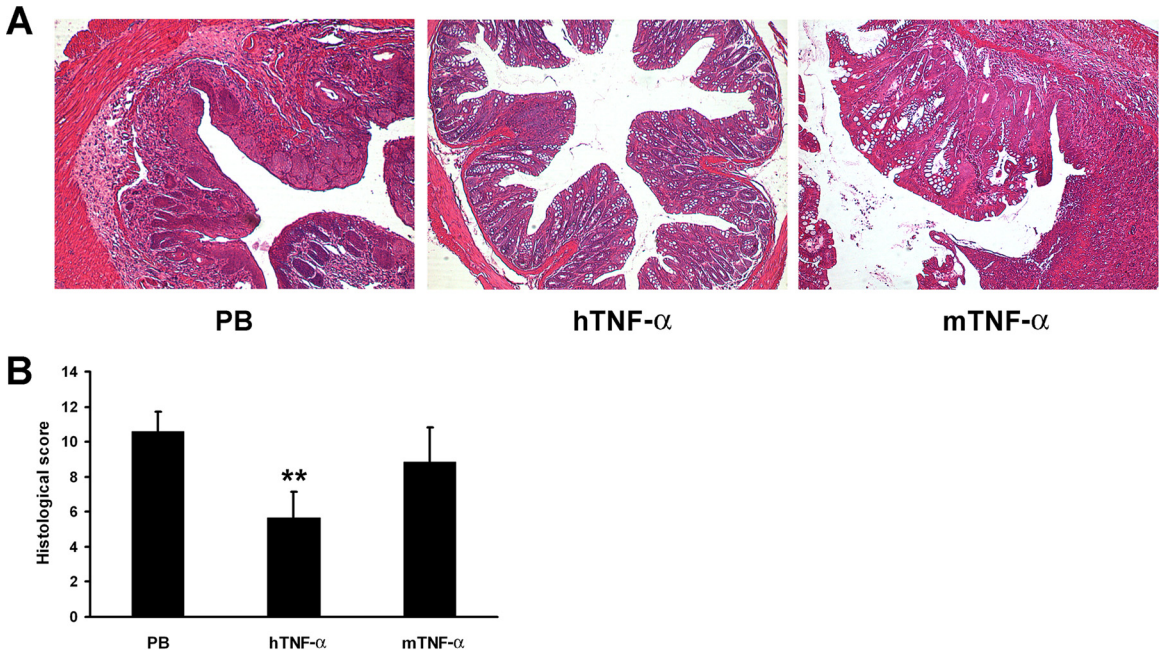

FIG 4 Protective effect of TNF-α vaccine in chronic colitis model. One week after the last immunization, mice were fed by three cycles of administration of 2% DSS in drinking water for 5 days, alternating with 5-day periods of recovery. (A) Photomicrographs of representative H&E-stained colon sections. 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 blind fashion. Data represent mean histological scores ± SD for $n = 6$ mice/group. **, $P < 0.01$ (compared with the results from the PB control group).

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 characterization 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 the total protein amount (Fig. 1A and B). The recombinant proteins were recognized specifically by the anti-TNF-α antibodies, as shown by the results of a Western blot assay (Fig. 1C). Moreover, both recombinant proteins were characterized by MALDI-TOF analysis (data not shown).

hTNF-α vaccine induces 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 the 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 much lower levels of anti-mTNF-α Abs in only 4 of 6 mice ($P = 0.023$).

hTNF-α vaccination protects mice from development of colitis. We first tested the protective effect of TNF-α vaccine in an 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 in the DAI score. In contrast, hTNF-α-vaccinated mice exhibited a significantly reduced DAI score from day 4 to day 7 ($P < 0.05$) (Fig. 3A).

A well-established histopathological event, including infiltration of inflammatory cells, ulceration, and crypt damage, was detected by 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 infiltration of inflammatory cells and mucosal injury (Fig. 3B). Furthermore, colonic tissue sections from hTNF-α-vaccinated mice also showed a significantly lower histological score than colonic tissue sections 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 the acute-colitis experiment. As shown in Fig. 3D, TNF-α concentrations of serum from hTNF-α-vaccinated mice were significantly reduced ($P < 0.001$).

IBD is characterized by chronic relapsing inflammatory disorders of the gastrointestinal tract, so we further tested the protective effect of TNF-α vaccine in a chronic colitis model. Similarly to the results seen in the acute colitis model, hTNF-α vaccination protected mice from crypt loss, erosions, and inflammatory cell infiltrations as well from as the DSS-induced increase in the 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 (see Fig. S1 in the supplemental material).

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

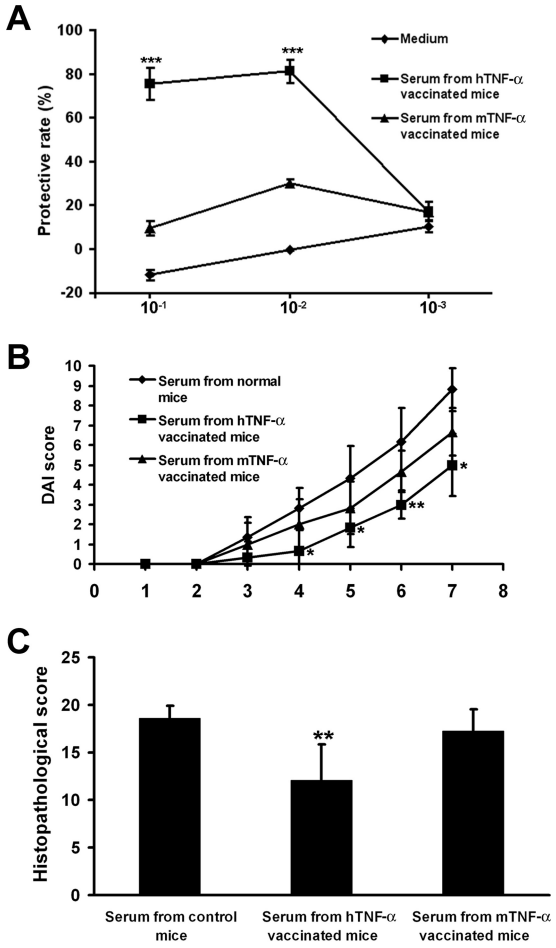

FIG 5 Neutralizing effects of TNF-α-specific antibodies. (A) L929 cells were treated with mTNF-α and serially diluted sera, and protective rates were calculated. Data represent mean protective rates ± SD for $n = 3$ mice/group. ***, $P < 0.001$ (compared with the results from the PB control group). (B and C) Mice were treated with DSS to induce acute colitis and administered 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 scores ± SD for $n = 6$ mice/group. *, $P < 0.05$; **, $P < 0.01$ (compared with the results from the PB control group). (C) Data represent mean histological scores ± SD for $n = 6$ mice/group. **, $P < 0.01$ (compared with the results from the PB control group).

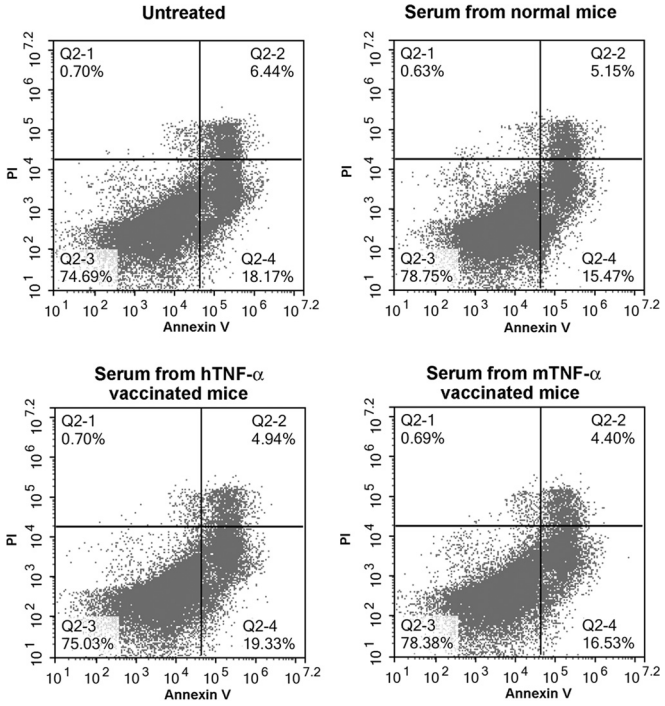

FIG 6 The effect of TNF-α-specific antiserum on activated T lymphocyte apoptosis. Activated T lymphocytes were treated with antiserum at a dilution of 1:100 for 24 h, and then cells were doubly stained with annexin V and propidium iodide (PI) to detect apoptosis. Data shown are representative of the results of 3 independent experiments.

protective effects of sera from mTNF-α-vaccinated or control mice.

Furthermore, a serum adoptive-transfer experiment was performed in this study. Mice were treated with DSS to induce acute colitis and administered 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 the DAI and histological damage scores (Fig. 5B and C).

TNF-α-specific antiserum fails to induce T lymphocyte apoptosis. It was reported that, in addition to exhibiting a 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 antiserum on T lymphocyte apoptosis. As shown in Fig. 6, no obvious increase of T lymphocyte apoptosis was detected after treatment with serum from hTNF-α-vaccinated mice.

DISCUSSION

Recently, the introduction of TNF-α inhibitors highlighted a pivotal role in the pathogenesis of IBD (22). Levels of TNF-α, which is mainly produced by activated macrophages, monocytes, eosinophils, and T cells, are increased in the 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 in 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 among the effective approaches for IBD treatment (28). However, because of the immunogenicity of the xenogeneic TNF-α inhibitors, ADAs can be triggered after repeated administration. Previous studies have investigated formation of ADAs and its influence on infliximab concentrations and clinical outcomes (29, 30). ADAs might reduce the efficiency of the drug by competing with the endogenous ligands and/or by forming immune complexes, which accelerates the clearance of the drug from the circulation (31).

In this study, we investigated the efficiency of a 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 bypass-

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

Our previous studies explored the feasibility of immunotherapy for treatment of tumors with xenogeneic homologous molecules as a vaccine against those on autologous cells in a cross-reaction between the xenogeneic homologous molecules 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 vaccination, could elicit a neutralizing antibody response. Although the homology level of the soluble forms 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 results. However, it was reported that, in addition to its neutralizing ability, infliximab could induce apoptosis in activated T lymphocytes (20, 21). We also tested the effect of antiserum on lymphocyte apoptosis. As shown in Fig. 6, TNF-α-specific antiserum failed to induce T lymphocyte apoptosis, suggesting that the neutralizing ability of TNF-α-specific antibody may derive from mechanisms of the xenogeneic TNF-α protein vaccine.

Furthermore, potential adverse effects were tested in this study. Characteristics of 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, use of the xenogeneic TNF-α protein vaccine may be a potent therapeutic strategy for treatment of IBD.

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