Expression and Identification of TNF Vaccine and Study of TNF Vaccine on Down-Regulation of Intestinal Inflammatory Responses in Mice

by

Allan Guohao Ma

A Thesis submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfilment of the requirements of the degree of

Master of Science

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Of

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Abbreviations

ELISA  Enzyme-linked immunosorbent assay
HBcAg  Hepatitis B core antigen
IBD    Inflammatory bowel disease
IFN-γ  Interferon-γ
IL     Interleukin(s)
mAb    Monoclonal antibodies
KLH    Keyhole limpet hemocyanin
PBS    0.02 M phosphate buffered saline, pH 7.2
PBST   PBS supplemented with 0.05% Tween 20
SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TNBS   2,4,6-trinitrobenzene sulphonic acid
TNF    Tumour necrosis factor formally named tumour necrosis factor alpha
ABSTRACT

**Background & Aims:** Tumour necrosis factor alpha (TNF) plays an important role in mediating the inflammation of inflammatory bowel disease (IBD), which includes, in particular, Crohn's disease. Strategies aimed at reducing TNF in patients with IBD include administration of monoclonal antibodies (mAb) against TNF, e.g. infliximab. These reagents have been shown to be effective clinically in the treatment of IBD. However, a short half-life, extremely high cost, and side effects such as development of antibodies to the infused mAb and infusion reactions limit the use of the passive neutralizing approach. We have previously developed 4 recombinant mouse TNF peptide-based vaccines. Here, we sought to identify whether the 4 vaccines were able to induce long-lasting auto-antibodies to TNF and if administration of such a vaccine could down-regulate intestinal inflammation in a murine model of colitis, which was induced by intrarectal administrations of trinitrobenzene sulfonic acid (TNBS).

**Methods:** Four recombinant TNF peptide-based vaccines, named mT1, mT2, mT3 and mTx, were expressed by E. coli, purified by a combination of ammonium sulfate precipitation and chromatography, and identified by SDS-PAGE and immunoblotting. The immunogenicity of the vaccines were tested by immunization of mice with each of the vaccines and testing serum TNF-specific IgG antibodies. One vaccine (mT1) was selected for *in vivo* evaluation in a newly developed mouse model of TNBS-induced acute and chronic colitis. In the *in vivo* study, mice were immunized with the vaccine three times, and then intrarectally administered with 2 mg of TNBS once (in acute colitis) or eight times (in chronic colitis). Mice receiving a vaccine carrier protein or saline served as controls. Symptoms and body weight were monitored after TNBS
administration. Mice were sacrificed one week after the last TNBS administration. Sera and colons were then collected for various analyses.

**Results:** All 4 vaccines were presented as virus-like particles and elicited TNF-specific IgG responses. Among them, mT1 and mTx vaccines reacted with polyclonal anti-TNF antibodies in Westernblotting and induced high tittered TNF-specific IgG responses. As the mT1 vaccine induced the highest TNF-specific IgG responses, it was selected for *in vivo* study. In both acute and chronic colitis, mT1-vaccinated mice had less body weight loss and significantly increased TNF-specific IgG responses when compared with controls. In acute colitis, 4 out of 6 mice died in the carrier group, while only one died in the vaccine group. Also, less colon inflammation was found in vaccinated mice as shown by reduced myeloperoxide activity and by decreased infiltration of neutrophils and other features of inflammation in H&E staining. In chronic colitis, vaccinated mice exhibited decreased soluble collagen and expression of TNF and IL-12/23p40 in colon tissues when compared with controls.

**Conclusions:** TNF peptide-based vaccines induce auto-antibodies to TNF. Administration of the vaccine improves symptoms, inflammation, and colon fibrosis of colitis, suggesting that this may be a potential therapeutic approach in the treatment of Crohn’s disease.
I  INTRODUCTION

I.1  Inflammatory bowel disease (IBD)

(1)  Epidemiology

Inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis, is a lifelong disease occurring early in life in both males and females. The incidence and prevalence of IBD markedly increased over the second half of the 20th century and, at the beginning of the 21st century, is considered one of the most prevalent gastrointestinal diseases. Estimates indicate that as of 2005, about 1.4 million Americans have been diagnosed with IBD. Roughly 10% of them are children and adolescents under the age of 17. Recent estimates from a population-based, multiple province-wide study in Canada indicate that approximately 0.5% of Canadians have IBD (about 170,000 or approximately 1 in 180) and that incidence rates across the country are among the highest in the world.

(2)  Pathogenesis

Inflammatory bowel disease is thought to result from inappropriate and ongoing activation of the mucosal immune system driven by the presence of normal luminal flora. This aberrant response is most likely facilitated by defects in both the barrier function of the intestinal epithelium and the mucosal immune system. The pathogenesis of IBD is multifactorial. Affected individuals often have a genetic predisposition to develop Crohn’s disease or ulcerative colitis. Studies have shown evidence for a genetic predisposition to IBD. First-degree relatives of patients with IBD have a 4- to 20-fold increased risk and a 7% absolute risk. Among family
members with Crohn’s disease, there is strong concordance within disease category and disease location.\textsuperscript{6} Studies in several different animal models and humans have also demonstrated that luminal flora is required for IBD to develop in a susceptible host.\textsuperscript{6,7} In a genetically predisposed host, an environmental trigger appears to precipitate the development of IBD. The environmental triggers implicated in the pathogenesis of IBD act by altering the luminal flora or disrupting the mucosal barrier. These environmental factors include antibiotics, diet, nonsteroidal anti-inflammatory drugs, acute infections as well as stress and smoking which may allow luminal antigens to activate the mucosal immune system.

(3) Immune mechanisms

Although the cause of IBD remains unknown, considerable progress has been made in recent years to unravel the pathogenesis of this disease. Evidence indicates that a dysregulation of mucosal immunity in the gut causes an overproduction of pro-inflammatory cytokines and aggregation of immune cells, especially T cells, in intestinal mucosa, which leads to uncontrolled mucosal inflammation.\textsuperscript{8,9}

Three CD4+ T helper subsets, Th1, Th2, and Th17, have been distinguished on the basis of their cytokine production patterns.\textsuperscript{10-12} Th1 responses are characterized by IL-12, IFN-γ, and TNF; Th2 dominated responses are biased towards production of IL-4, IL-5, IL-9, and IL-13 cytokines;\textsuperscript{12} whereas newly identified helper T cells - Th17 cells - are characterized by production of IL-17, IL-21, and IL-22 cytokines.\textsuperscript{13} Th1 cytokines mediate delayed-type hypersensitivity responses against intracellular pathogens and inhibit Th2 responses. Th2 cytokines are relevant in humoral immune responses and are associated with atopy and asthma.\textsuperscript{14} Th17 cells are highly pro-inflammatory and induce severe autoimmunity.\textsuperscript{13}
It has been widely accepted that Crohn's disease is caused by an overly aggressive Th1 immune response and, recently found, excessive IL-23/Th17 pathway activation in response to bacterial antigens in genetically predisposed individuals.\textsuperscript{8, 9, 15-17} The resulting infiltration of the bowel by granulocytes and macrophages leads to a release of enzymes, reactive oxygen intermediates, and pro-inflammatory cytokines, all of which cause discontinuous ulceration and full thickness bowel wall inflammation often including granulomas.\textsuperscript{18, 19} These Th1 cytokines are likely critical in generating and perpetuating the chronic intestinal inflammation of Crohn's disease.\textsuperscript{17} The IL-23/Th17 pathway (IL-23 promoting the differentiation of Th17 cells to produce IL-17) is critical for the development of chronic intestinal inflammation.\textsuperscript{20, 21} For these reasons, targeting these cytokines has emerged as a new biological therapy in IBD.\textsuperscript{1, 22}

I.2 TNF in IBD

(1) TNF

As a Th1 cytokine, TNF is considered to play a pivotal role in Crohn's disease.\textsuperscript{23, 24} TNF is a 17 kDa pro-inflammatory cytokine produced by monocytes, macrophages and T cells that can affect proliferation, differentiation and function of virtually every cell type. The biological effects of TNF include stimulation of the acute phase response, cytotoxicity, cachexia, and potentially lethal shock.\textsuperscript{25} Furthermore, TNF stimulates IL-1 and IL-6 production, expression of adhesion molecules, procoagulant activity, and fibroblast proliferation.\textsuperscript{26} Secreted TNF forms a bioactive 51 kDa trimer and binds as a soluble ligand to two cell-bound transmembrane TNF receptors, the 55 kDa TNF-α-R1(p55) and the 75 kDa TNF-R2 (p75).
(2) **TNF and Crohn's disease**

TNF is considered the prototypical proinflammatory cytokine secreted by activated macrophages and monocytes and chronically activated T lymphocytes. Several studies have detected increased TNF levels in mucosal biopsies from patients with CD.\(^{27}\) The mechanisms of TNF regulating inflammation in the gut of Crohn’s disease are complex and multifactorial: 1) TNF-\(\alpha\) functions early in the inflammatory cascade; 2) TNF-\(\alpha\) augmentation of Th1 function in the mucosa is critical for disease pathogenesis.\(^{28}\)

In conclusion, there are some evidences supporting the idea that TNF plays a central role in the pathogenesis of mucosal inflammation in CD.\(^{28,29}\) (1) TNF is elevated in the blood, stool, and intestinal tissue, and its concentrations correlate with clinical disease activity in patients with Crohn's disease;\(^{30,31}\) (2) several animal models of IBD show significant amelioration of mucosal inflammation when treated with anti-TNF antibodies;\(^{32}\) (3) deletion of elements from the 3' untranslated region of the mouse TNF gene leads to transmural inflammation highly reminiscent of human Crohn's disease;\(^{33}\) and (4) anti-TNF monoclonal antibodies have produced beneficial responses among some patients with Crohn's disease.\(^{34,35}\)

(3) **TNF antagonists**

Therapeutic strategies towards reducing TNF include the administration of monoclonal antibodies (mAb) to TNF (infliximab, adalimumab, certolizumab) and TNF soluble receptors (etanercept and onercept). In the past decade, anti-TNF therapy has emerged as an alternative to corticosteroids, azathioprine, and other traditional treatment. These mAbs have been highly effective in treating active Crohn's disease, maintaining remission, closing fistulas, maintaining fistula closure, and treating ankylosing spondylitis.\(^{34-41}\) Administration of infliximab induced
significant improvement of symptoms and quality of life in 65% of patients refractory to 5-ASA drugs, corticosteroid and/or immunomodulators. This therapy also resulted in an approximate 60% reduction from baseline in endoscopic lesions, accompanied by a marked reduction of inflammatory infiltrate in mucosal biopsies. It also resulted in corticosteroid withdrawal in 73% of patients. In addition to Crohn’s disease, anti-TNF therapy with infliximab is effective in treating ulcerative colitis, suggesting that a Th1 response may also be involved in the pathogenesis of ulcerative colitis. These TNF antagonists represent a novel approach in the management of Crohn’s disease and, perhaps, ulcerative colitis.

(4) Disadvantages of currently used TNF antagonists

As the over-expressed cytokines play important roles in the development of IBD, one intriguing strategy aims at limiting production of these cytokines and their accessibility to their respective receptors, so as to control the pathogenic process. To date, many new biological agents as therapeutic modifiers have emerged as important new treatments. Among current anti-cytokine antagonists, anti-TNF biologic agents are the most successful in the treatment of IBD patients. A chimeric anti-TNF monoclonal antibody, infliximab, has become the standard therapy for Crohn’s diseases has also been a beneficial therapy for ulcerative colitis. Although the mAbs to TNF provide a novel treatment for Crohn’s disease, both mAbs and soluble receptors act passively with short half lives (9.5 days for infliximab, 18-20 days for adalimumab). As such, repeated injections are necessary and any improvements seen are reversed upon discontinuation of treatment. Moreover, one study found that 61% of patients receiving infliximab formed antibodies to the infused mAb. The infliximab-anti-infliximab complexes lead to a failure to respond to further infliximab therapy. One third of patients receiving the
therapy has developed acute infusion reactions. Another highly relevant concern is the extremely high cost associated with such passively administered therapeutics. These disadvantages limit the usefulness of this approach, as IBD is chronic and requires long-term treatment.

I.3 Active immunization against self-proteins

(1) Research done in this field

To overcome the disadvantages, active immunization with vaccines against self-molecules is currently being investigated. Due to immunological tolerance, immune responses against self-proteins are usually not generated. Studies have demonstrated that if a self-protein is coupled to a foreign protein or modified by inserting a foreign peptide containing T cell epitopes, the self-component in the conjugate or the modified self-protein is recognized as foreign by the immune system of the host, and auto-antibodies against self-epitopes are generated. Based on providing effective T cell help, two broad experimental strategies have been used to design such vaccines: 1) to modify the intact self-protein by inserting a foreign peptide containing Th epitopes; \(^{47}\) 2) to chemically couple the intact self-protein,\(^{48, 49}\) or a considerable part thereof,\(^{50}\) to an immunogenic carrier protein. Administration of these vaccines induce high titers of auto-antibodies to the target self molecule, leading to the improvement of the disease.\(^{51, 52}\) Compared with passive immunization with monoclonal antibodies, active immunization using vaccines has the following advantages: 1) It provides long-term treatment with few injections; 2) The antibodies induced by the vaccine are natural human antibodies which are more effective and long lasting than the artificial chimeric antibodies or receptors; 3) Side effects such as the
development of antibodies against the infused mAb and infusion reactions can be avoided; 4) The approach is inexpensive.

(2) Relevant research done in our group

Dr. Peng's lab has designed self-molecule peptide-based and virus-like particle vaccines which limit the potential for possible cross-reactivity and induce sufficient auto-antibodies to the target without use of adjuvants. The vaccine is constructed by inserting a small peptide derived from the target cytokine into a carrier protein, hepatitis B core antigen (HBcAg). The peptide-based vaccine presents as virus-like particles and elicits sufficient auto-antibodies to the target cytokine without the need of an adjuvant.53-56 Using this approach, we have shown that administration of a human IgE vaccine, which cross-reacts to the IgE of rodents, prevents the increase of serum IgE and reverses elevated IgE in sensitized rodents,56 while administration of an IL-13 or IL-4 vaccine effectively suppresses all features of acute airway allergic inflammation in asthmatic mice,53-55 confirming the effectiveness of this strategy. In addition, 4 TNF peptide-based vaccines have been constructed in the laboratory, but not yet identified.
II. HYPOTHESIS

It is critical to investigate whether or not the application of a TNF peptide-based vaccine is able to suppress pathogenic cytokines in IBD as an alternative therapy. We hypothesize that active immunization strategy using vaccines against over-expressed endogenous TNF can be applied to ameliorate intestinal inflammation in mice.
III. SPECIFIC AIMS

1. To express, purify, and identify (in vitro and in vivo) 4 mouse TNF vaccines.

2. To examine whether administration of a TNF vaccine will down-regulate intestinal inflammatory responses in mice
IV. MATERIALS AND METHODS

IV.1 Expression, purification, and identification of 4 recombinant TNF peptide-based vaccines

(1) Expression and purification of recombinant TNF vaccines

Four antigenic peptides (named mT1, mT2, mT3 and mTx) ranging between 8 and 23 amino acids were selected from mouse TNF (Fig. 2A) based on the use of antigen predictive software. Four vaccine plasmids, each containing a carrier protein [hepatitis B Core antigen (HBcAg)], a mouse TNF peptide, and one carrier plasmid containing truncated HBcAg (amino acids 1-149), were constructed using the methods as previously described.\textsuperscript{53,54} These vaccines presented as virus-like particles under an electron microscope (Fig. 2B)
Briefly, using the vector pThio-His, these plasmids were transformed into *Escherichia coli* DH5α cells, respectively. 0.5 µl of each plasmid was added to 200 µl competent cells in a 1.5 ml microfuge tube. After heat shock at 42°C for 1.5 minute, all tubes were returned to ice for 1 minute. With the addition of 1 ml LB to each tube, all tubes went through a 37°C water bath for 45 minutes. 200 µl DH5α cells were plated from each tube onto separate agar plates containing ampicillin and incubated at 37°C overnight. Single colonies were picked into LB media and incubated overnight at 37°C. The cultures were then transferred to fresh LB at a rate of 5 % total volume and incubated at 37°C. Expression was induced in all cases by adding 1 mmol/L IPTG when OD₆₀₀ arrived at 0.6-0.8, and then incubating at 37°C for another 4 hours. Induced cells were harvested by centrifugation at 3000 rpm at 4°C for 20 minutes. Pellets were resuspended in PBS and further lysed by ultra-sonication. Supernatants of the lysates were collected after centrifugation. The expression of HBcAg or recombinant HBcAg-TNF peptide in the supernatants was analyzed by SDS-PAGE and immunoblotting using a polyclonal biotinylated goat anti-mouse TNF antibody.

To purify the recombinant proteins, ammonium sulfate was added to the supernatants to 40% saturation and kept in room temperature for one hour. The precipitate was washed with 20% saturation ammonium sulfate in PBS twice and then suspended in PBS, which contained recovered target proteins. Target proteins were further purified by loading them onto a Sepharose CL-4B gel filtration chromatography column, while the main protein peak was collected and used for immunization.

(2) *in vitro* identification of the vaccines using SDS-PAGE and immunoblotting
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were used to examine whether the expressed recombinant vaccines could specifically bind to polyclonal anti-TNF antibodies. SDS-PAGE was performed in a discontinuous system according to Laemmli using a Bio-Rad mini slab gel apparatus.

Samples were prepared in SDS-PAGE loading buffer (0.0625 M Tris, pH 6.8, 2.5% SDS, 6% glycerol, 5% β - mercaptoethanol, 0.5% bromophenol blue) by first boiling for 10 minutes, then loading onto 12% polyacrylamise gel (10 µl/well). Proteins in the samples were separated by electrophoresis at 150 V for 45 minutes. The gel was then stained with 0.25% Coomassie brilliant blue in 10% acetic acid and 45% methanol for 1 hour. The gels were de-stained in 30% methanol and 10% acetic acid solution until proteins bands were clearly visualized. Molecular weights of the components were estimated with reference to the mobilities of prestained protein standards (Bio-Rad Laboratories, Mississauga, ON, Canada).

Induced bacterial cells were centrifuged in sample amounts of 3 ml at 12,000 rpm for 30 seconds. The pellets were re-suspended in 200 µl PBS (20 mM PB, 0.13M NaCl, pH7.4), and then equal volume of electrophoresis sample buffer (2X = 250 mM Tris-HCl pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, and 2% β-mercaptoethanol) was added to each sample. After heating at 95 °C for 5-10 minutes, 15 µl of each sample (5-100 µg total protein) were loaded into each well of 15% polyacrylamisde gel and electrophoresed for 1.5 hr at voltage 150. One gel was stained with 0.25% Coomassie brilliant blue in 10% acetic acid and 45% methanol for 1 hour. The gel was de-stained in 30% methanol and 10% acetic acid solution until proteins bands were clearly visualized. Molecular weights of the components were estimated with reference to the mobilities of prestained protein standards (Bio-Rad Laboratories, Mississauga, ON, Canada).
Proteins on another gel were electronically transferred to a nitrocellulose membrane using a Trans-Blot cell (BioRad) at 10 V at 4°C overnight. The membrane was removed and rinsed in PBST (20 mM PBS, supplemented with 0.05% Tween 20, pH 7.2) and then incubated with blocking solution (3% BSA in PBST) for 3 hours at 4°C. This was followed by incubation of the membrane with biotinylated goat anti-mouse TNF polyclonal antibodies (PeproTech, Rocky Hill, NJ, USA) diluted (0.2 µg/ml) in PBST containing 1% BSA for 1 hour with agitation at room temperature. After washing for 30 minutes with agitation in PBST (changing the buffer every 5 minutes), the membrane was incubated with alkaline phosphatase-conjugated avidin (1:5,000) (Sigma, Saint Louis, Missouri, USA) for 30 minutes at room temperature. After washing, color was visualized by incubation of the membrane with the substrate solution (one tablet Sigma Fast BCIP/NBT dissolved in 10 ml water). The tray containing the membrane was agitated for 1-5 minutes until the bands displayed clearly. The development was terminated by rinsing with water.

(3) *in vivo* examination of the antigenicity of the vaccines

i. Mice

Female BALB/c mice (7-8 weeks old) purchased from Charles River Laboratories (Saint-Constant) were maintained at the Central Animal Care Services, University of Manitoba. The experiments were approved by the Animal Care and Use Committee at the University of Manitoba and the investigators adhered to Canadian Council on Animal Care (CCAC) guidelines for humane treatment of animals.
ii. Protocol of immunization of mice with the vaccines

To determine whether the recombinant vaccines were able to induce high titers of neutralising antibodies, eight groups of mice (4 mice each group, aged 7-8 weeks old) were immunized subcutaneously with 4 vaccines at 2 doses (mT1 was 45 µg and 90 µg/mouse, the others were 30 µg/mouse and 60 µg/mouse) 3 times at two-week intervals. Mice receiving the carrier (native HBcAg) served as controls. Sera were obtained from the saphenous vein every 2-3 weeks until week 22. At week 17, as the titres of TNF-specific IgG were reduced to near baseline, a booster injection of the vaccine was administered. All serum samples were assayed for TNF-specific IgG levels using an enzyme linked immunosorbent assay (ELISA).

iii. Measurement of TNF-α-specific IgG by ELISA

96-well flat bottom plates (Corning Costar Corp. MA, USA) were used in the ELISA. TNF (PeproTech) was diluted in 0.05 M, pH 9.6, carbonate buffer (1µg/ml) and coated (50 µl/well) on the plates overnight at 4°C. After rinsing with washing buffer (0.02 M, pH 7.4 PBS containing 0.05% Tween 20 and 0.02% NaN₃) three times, plates were blocked with blocking buffer (washing buffer containing 2% bovine serum albumin) for 1 – 2 hours. Test serum samples were diluted with dilution buffer (washing buffer containing 0.2 % bovine serum albumin) and added to each well. When the results were expressed using optical density at 405 nm (OD₄₀₅), the samples were diluted at 1:4,000. When the results were expressed using titers, samples were 2-fold series diluted with a starting dilution of 1:4,000. The plates were incubated at 37°C for 1.5 hours. After rinsing 3 times, goat anti-mouse IgG conjugated with alkaline phosphatase (dilution 1:3,000, Zymed Lab. Inc. San Francisco, CA) was added. After incubation at 37°C for 1.5 hours and washing, colour was developed by addition of the enzyme substrate
solution, p-nitrophenyl-phosphate. Optical density at 405 nm (OD$_{405}$) was read on a micro-plate reader. The results were expressed using either the value of OD$_{405}$ or the titer that was determined as the reciprocal of the highest dilution in which the OD$_{405}$ was twice that of the corresponding control serum when its OD$_{410}$ was 0.10.

IV.2. Evaluation of the effect of a TNF vaccine in TNBS-induced murine colitis

(1) Establishment of a TNBS-induced colitis mouse model

Hapten-induced colonic inflammation is a widely used animal model of human Crohn’s disease.$^{57,58}$ Intrarectal delivery of 2,4,6-trinitrobenzene sulphonic acid (TNBS) induces colitis by haptenation of colonic proteins, leading to a delayed-type hypersensitivity reaction by causing a Th1 reaction. This reaction leads to colitis similar to Crohn’s disease, with transmural mononuclear cell infiltrate, abnormal crypt architecture, ulcerations, and occasional granulomas.

Female BALB/c mice (7-8 weeks old) were obtained as mentioned in section IV.1 (3)iii. Mice were administered with 0.5 mg or 2.0 mg of TNBS. Briefly, mice were anesthetized by inhalation of 1-2% isoflurane and then intrarectally administered with 100 µl of 50% saline/ethanol (v/v) containing 0.5 or 2.0 mg of TNBS (Sigma-Aldrich) via a 3.5 F catheter purchased from Instech Solomon (Plymouth Meeting, PA. USA) affixed to a 1-mL syringe. The catheter was advanced into the rectum to a point 4 cm proximal to the anal verge. To ensure distribution of TNBS within the entire colon and cecum, mice were held in a vertical position for 50 seconds after TNBS administration (the figure in the right). Control mice were given 100µl saline/ethanol. After TNBS administration mice were monitored every day for signs of colitis.
including weight loss, diarrhea, rectal bleeding as well as systemic inflammation such as piloerection, lethargy and periorbital exudates. At day seven, mice were sacrificed. Colons were removed, fixed in 10 % formalin and embedded in paraffin. Paraffin-embedded colon sections were prepared and stained with H&E.

(2) Protocols of vaccination and TNBS administration

Among the 4 vaccines, the mT1 vaccine reacted with polyclonal anti-TNF antibodies (Fig. 1) and elicited high levels of TNF-specific IgG responses (Fig. 2C). As a result, mT1 was selected for in vivo evaluation in a murine model of TNBS-induced colitis.

Acute colitis. The protocol for the evaluation of acute colitis is shown in Fig. 4A. Three groups of mice (6-8/group) were subcutaneously immunized 3 times with the vaccine (50 µg/mouse), carrier (50 µg/mouse) or PBS, respectively. One week after the final immunization, mice were intra-rectally administered with 2 mg of TNBS in 50% ethanol (total volume of 100 µl), as described in section IV.2(1). Four mice that did not receive TNBS administration served as normal controls. Clinical symptoms and body weight were monitored daily. Mice were sacrificed 3 days after administration of TNBS. Serum samples were used for measurement of TNF-specific IgG levels by ELISA as described in section IV.1(3)iii. Each colon was dissected and divided into 2 parts. One part was frozen for an assay of myeloperoxides (MPO) activity and the other part was fixed with 10% buffered formalin for histological analyses.

Chronic colitis. The protocol for the evaluation of the vaccine in chronic colitis is shown in Fig. 5A. Three groups of mice (12/group) were subcutaneously immunized 4 times with the vaccine or carrier or saline. To induce chronic colitis, mice were weekly administered with
increasing doses of TNBS (1.0 – 2.5 mg in 45% ethanol) according to previous description. Serum samples were collected at weeks 5, 9 and 13 to measure TNF-specific IgG levels. At the end of the study, mice were sacrificed. Serum samples were used for measurement of TNF-specific IgG levels by ELISA as described in section IV.1(3)iii. Each colon was removed, divided into 2 parts, and frozen. One part was used for an assay of cytokines (TNF and IL-12p40) and the other part was used for a soluble collagen assay.

(3) Myeloperoxides (MPO) activity assay

MPO is an enzyme, found in neutrophils, which can be used as an index for tissue inflammation. Its activity was assayed using a modification of the method previously described. The colon tissue (100 mg tissue/ml) was homogenized in potassium phosphate buffer (50 mM, pH 6.0,) containing 5% hexadecyltrimethylammonium bromide, and then subjected to three cycles of freezing and thawing. After centrifuging, the supernatant was transferred to a 96-well plate (7μl per well, triplicate each samples). The enzyme reaction was carried out by adding 200 μl of phosphate buffer (50 mM, pH 6.0) containing 0.167 mg/ml o-dianisodine hydrochloride (Sigma) and 0.0005% H₂O₂. The kinetics of absorbance changes at 460 nm was measured at 0, 30, and 60 minutes in a microtiter reader. The MPO activity is presented as MPO units per gram of tissue.

(4) Soluble collagen assay

Colons were homogenized in 0.5 M acetic acid containing 1 mg of pepsin (at a concentration of 10 mg of tissue/5 ml of acetic acid solution). The resulting mixture was then incubated and
stirred for 24 h at 4°C. Total soluble collagen content of the mixture was then determined with a Sircol Collagen Assay Kit (Biocolor). Acid soluble type I collagen supplied with the kit was used to generate a standard curve.

(5) Measurement of the levels of colon TNF and IL-12p40 by ELISA

In addition to TNF, IL-12 also plays an important role in IBD. Levels of colon TNF and IL-12p40 were measured by ELISA. Frozen colonic samples were mechanically homogenized in a buffer containing 1M Tris-HCl, 3M NaCl, and 10% Triton supplemented with a protease cocktail (Sigma-Aldrich). Samples were then frozen (-70°C) and thawed (37°C) three times, followed by centrifugation at 14,000 rpm for 30 min at 4°C. Supernatants were frozen at -70°C until the assay was performed. Levels of TNF and IL-12p40 in colon tissue extracts were measured by ELISA according to the manufacturer’s instructions (BD Bioscience). Briefly, microplates were coated with mAb anti-mouse TNF or mAb against mouse IL-12p40, followed by sequential incubations with diluted test samples (1: 2), biotinylated mAb against TNF or biotinylated mAb against IL-12p40, and finally the streptavidin-alkaline phosphatase conjugate.

IV.3 Statistical analyses

Values were expressed as mean ± SD. Differences between experimental groups were assessed by one-way analysis of variance (ANOVA), followed by Newman-Keuls multiple comparison test (GraphPad Prism). P values of < 0.05 were considered statistically significant.
V. RESULTS

V.1. Expression, purification, and identification of 4 recombinant TNF peptide-based vaccines

(1) Expression, purification, and in vitro identification of recombinant TNF vaccines

All recombinant proteins were expressed efficiently by using *E. coli* DH5α cells as soluble proteins which had been previously identified as virus-like particles (Fig. 1 right). Among the 4 recombinant vaccines, mT1 and mTx specifically reacted with the polyclonal goat anti-mouse TNF antibody as shown in the immunoblotting (Fig. 1 left). After purification with ammonium sulfate precipitation and Sepharose CL-4B gel filtration chromatography, most unwanted proteins were removed. The resulting purity of the recombinant vaccines and the carrier was about 80%.
Figure 1. SDS-PAGE and Westernblot analysis of 4 recombinant mouse TNF vaccines and the carrier protein HBcAg. Arrows indicate recombinant proteins.

(2)  *in vivo* examination of the antigenicity of the vaccines

Immunization with each of the vaccines without the use of an adjuvant induced TNF-specific IgG responses in mice (Fig. 2C). mT1 and mTx vaccine induced high levels of TNF-specific IgG responses, while mT2 and mT3 vaccines induced moderate levels of antibody responses only after a boost injection at week 17. In mT1 and mTx vaccinated mice, the antibody levels could be maintained for a period of up to 13 – 17 weeks and were dramatically increased after the booster injection. Among the 4 vaccines, mT1 induced stronger TNF-specific antibody responses than others. The antibody titers induced by the mT1 vaccine were higher than 128,000.
Figure 2. Identification of 4 mouse TNF vaccines. Four antigenic peptides (mT1, mT2, mT3 and mTx) were selected from mouse TNF (A). The vaccine, which consists of a TNF peptide and a carrier HBcAg, presented as virus-like particles (B). To examine the immunogenicity of the vaccines, mice were immunized with each of the 4 vaccines 3 times and were boosted once with 2 different doses. Serum mouse TNF-specific IgG was measured using an ELISA (C).
V.2. Evaluation of the effect of a TNF vaccine in TNBS-induced murine colitis

(1) Establishment of a TNBS-induced colitis mouse model

As administration of 0.5 mg of TNBS did not change mouse body weight or produce any observable symptoms, a dose of 2.0 mg of TNBS was chosen to induce colitis. Mice received 2.0 mg of TNBS in 50% ethanol or 0.1 ml 50% ethanol as controls (day 1). One day after the TNBS administration (day 2), body weight was significantly reduced in TNBS administrated group, while control mice had only a mild loss in body weight (Fig. 3A), perhaps due to the stimulation of 50% saline/ethanol (v/v). TNBS administrated group had increased body weight loss compared to control group through all seven days. On the third day, body weight loss reached a maximum. During the period of Day 4 to Day 7, body weight loss was gradually recovered. Due to the limited number of experimental mice, no statistical difference was found between the 2 groups.

After sacrificing the mice, redness and swelling of the colon were found in the TNBS administrated group compared to the control group, as shown in Fig. 3B. H&E stained colon sections showed inflammatory signs such as neutrophils infiltration, reduction of goblet cells, and distorted architecture in TNBS-administered mice when compared with normal mice (Fig. 3C).
A. Body weight

B. Colons from normal and colitis mice

C. H&E staining of colon tissue from normal and colitis mice

**Figure 3.** Difference between normal and colitis mice. Mice were administered at Day 1 with 2.0 mg of TNBS in 50% ethanol and were sacrificed at day 7. Mice that received 50% ethanol served as controls. Daily body weight changes (A), colon appearance (B), and histological analysis of colon tissue (C) was examined in normal and TNBS-induced acute colitis mice.
(2) Evaluation of the effect of a TNF vaccine in TNBS-induced murine colitis

i. Acute colitis

ELISA was used to determine the levels of TNF-specific IgG in serum samples taken after sacrificing mice. As shown in Fig. 4B, vaccinated mice had significantly higher mean titer of TNF-specific IgG (30,000), while mice receiving carrier do not have any detectable TNF-specific IgG ($P < 0.001$). Although body weight was reduced in all three groups, mice receiving the vaccine had less body weight loss when compared with mice receiving carrier and mice receiving PBS (Fig. 4C). MPO measurement revealed that vaccinated mice had lower MPO levels than mice receiving carrier or PBS (Fig. 4D). As MPO is an enzyme released by neutrophils, reduced MPO levels indicate less neutrophil infiltration in the colon tissue of vaccinated mice. Due to a small number of mice in each group ($n = 6$), the difference between vaccine and either carrier or PBS groups did not reach statistical significance. The results obtained from MPO were supported by histological changes. As shown in Fig. 4F, the colon tissue of vaccinated mice indicates less infiltration of neutrophils and other features of inflammation such as mucosal erosion and loss of goblet cells than controls.
**Figure 4** Administration of a TNF vaccine in the down-regulation of intestinal inflammatory responses. Mice were immunized 3 times with a TNF vaccine (mT1) or the carrier or PBS, and then administered with TNBS intrarectally (A). Body weight was monitored for 3 days after TNF delivery and mice were then scarified. Serum TNF-specific IgG (B), daily body weight, and myeloperoxidase activity in colon tissue (D) were measured. Colon tissue histological inflammation (E) was also examined.
ii. Chronic colitis

i) TNF-specific IgG. Mice receiving vaccine mT1 had significantly high levels of TNF-specific IgG, which started at week 5 after 3 immunizations. When the levels of TNF-specific IgG declined at week 9, a booster injection of the vaccine induced significantly higher responses than those of week 5. The high levels of TNF-specific IgG remained until the end of the study (Figure 5B).

![Diagram of protocol and TNF-specific IgG responses in TNBS-induced chronic colitis.](image)

**Figure 5.** Protocol and TNF-specific IgG responses in TNBS-induced chronic colitis. A. The protocol of immunization and TNBS administration. B. Kinetic TNF-specific IgG responses during the study (serum dilution 1:1,000).
ii) **Body weight.** After the administration of TNBS commenced at week 6, all 3 groups of mice had an initial decrease of body weight between week 6 and week 8. Vaccinated mice started to gain weight at week 9, while carrier and saline controls started to gain weight at week 10 (Figure 6). It was apparent that vaccinated mice had consistently higher body weight than both carrier and saline control groups.

![Body weight graph](image)

**Figure 6.** Body weight in mice with TNBS-induced chronic colitis treated with TNF vaccine. TNF vaccinated mice gained more body weight than mice receiving the carrier or saline.

iii) **Colon collagen.** Collagen deposition in the colon is a feature of chronic inflammation. Soluble collagen in the colon tissue of mice with TNBS-induced chronic colitis which were only treated with saline or carrier was significantly increased when compared with normal mice (Figure 7A). In mice receiving the vaccine treatment, soluble collagen was found to be statistically reduced when compared with the saline and carrier groups ($P < 0.05$). The mean
level of collagen in vaccine group was close to that of normal mice. The lack of a significant difference between vaccinated and normal mice \((P > 0.05)\), suggesting that the TNF vaccine treatment is effective in reducing colon fibrosis in chronic colitis.

**iv) Cytokine levels in colon tissues.** TNF is a Th1 cytokine. Blocking TNF may influence the levels of other cytokines. IL-12 is required for an effective polarization of naïve T helper cells to the Th1 phenotype characterized by the expression of IFN-γ.\(^6\) Therefore, in addition to TNF, the levels of IL-12 p40 were also measured by ELISA. The results are shown in Figure 7. In vaccinated mice, the levels of TNF and IL-12 p40 were reduced. IL-12 is a heterodimeric protein comprised of covalently linked p35 and p40 subunits.\(^6\) The p40 unit of IL-12 is shared with IL-23, a new heterodimeric cytokine that consists of p40 and p19 units. A reduction in the p40 unit may also imply a reduction in IL-23, a cytokine that plays a critical role in the pathology of IBD.\(^6\)

**Figure 7.** Levels of soluble collagen, TNF, and IL-12p40 in colon tissue of vaccinated and control mice.
VI. DISCUSSION

We have successfully developed TNF peptide-based vaccines that induce high titers of autoantibodies against TNF. Administration of a TNF vaccine improved symptoms, colon inflammation, and soluble collagen in TNBS-induced murine colitis, suggesting that this strategy may provide a novel therapeutic approach for the long-term treatment of IBD.

Currently, different types of vaccines against over-produced endogenous cytokines are being developed. Due to immunological tolerance, immune responses against self-proteins are usually not generated. B cell antigen receptors to self-proteins are normally removed from the repertoire to induce tolerance and avoid autoimmunity. However, immature B cells undergoing the induction of tolerance are exquisitely sensitive to T cell help, which, if provided, can rescue B cells from the induction of tolerance and promote B cell development. Immunological tolerance against self-proteins can be bypassed via incorporating a strong Th cell epitope within a self-protein. To do so, one of the approaches is to modify the intact self-protein by inserting a foreign peptide containing Th epitopes. The other commonly used approach is to link the intact self-protein or a considerable part thereof to a heterologous carrier protein. In these vaccines, the self-component in the conjugate or the modified self-protein is recognized as foreign by the immune system of the host. Polyclonal antibodies induced by such vaccines can have excellent neutralizing capacity, but because they are raised against all self-epitopes of the target, their use may be hindered by undesirable cross-reactions with other self-proteins that contain similar epitopes. This is of particular concern when this strategy is used in humans. Furthermore, for induction of high titers of autoantibodies with such vaccines, the use of an adjuvant has always been required. These issues largely limit their application, efficacy, and
safety. To avoid the potential cross-reactivity, cytokine peptide-based vaccines have been investigated. Up to date, these vaccines are made by chemical coupling of synthesis peptides to a protein carrier keyhole limpet hemocyanin (KLH) \(^7\) or virus-like particles.\(^72,73\)

Selection of the appropriate carrier protein is important for increasing vaccine antigenicity. The most commonly used carrier proteins are bacterial proteins, such as Tetanus toxoid or Diphtheria toxoid, which are commonly encountered by humans. KLH and ovalbumin are also used as vaccine carriers. As such vaccines do not present as virus-like particles and therefore have low immunogenicity, requiring strong adjuvant (complete Freud’s adjuvant) to elicit sufficient immune responses.\(^74,75\) Virus-like particles can induce potent B cell responses even in the absence of adjuvants;\(^76\) they are more preferable as a carrier protein than other proteins.

In order to overcome the inherent disadvantages of the above vaccines, our group has designed recombinant cytokine peptide-based virus-like particle vaccines.\(^53-55\) Although cytokine peptide-based virus-like particle vaccines have been reported recently,\(^72,73\) they have been made by chemically coupling synthesis peptides to either papillomavirus virus-like particles or bacteriophage Q\(\beta\) virus-like particles. Although these vaccines present as virus-like particles, their antigenicity is limited because the peptides displayed on the surface of the particle are arranged in a non-uniform manner due to chemical coupling. Furthermore, it is not easy for quality control when chemically coupling method is used. By contrast, our TNF vaccines were made by a gene engineering method, where the peptide was inserted into the carrier truncated hepatitis B core antigen (HBcAg). Moreover, the safety of using HBcAg as a carrier has been confirmed in a phase I clinical trial for a malaria vaccine.\(^77\) HBcAg is effective in activating naive B cells as primary antigen-presenting cells, which are \(10^5\)-fold more efficient than what is
typically associated with dendritic cell and macrophage interactions. A single HBcAg virus-like particle consists of 180 or 240 HBcAg molecules, each of which is inserted with one cytokine peptide. Therefore, in our TNF vaccines, a total of 180 or 240 TNF peptides are displayed on the surface of a single virus-like vaccine particle in highly ordered and optimally spaced repeats. The highly repetitive ordered array of inserted self-polypeptides on the surface of virus-like particles may also abrogate the ability of the immune system to distinguish between foreign and self, thus providing a unique benefit that leads to the breaking of B cell tolerance. Our TNF peptide-based vaccines that presented as virus-like particles were successful in breaking self-immune tolerance and inducing high titers of auto-antibodies for TNF without the use of an adjuvant. In the 4 TNF vaccines, the vaccine made by peptide T1 is the most effective one. Perhaps this peptide is located at the receptor binding site of TNF and is more immunogenic. Below is an illustration that explains different types of current cytokine vaccines.

**Figure 8.** Types of current cytokine vaccines. KLH, keyhole limpet hemocyanin.
Through this active immunization strategy, we aimed to induce high titered and long-lasting autoantibodies to TNF which leading to the improvement of the disease. Our study demonstrated that active vaccination with the vaccine induced high titered and long-lasting autoantibodies to TNF. The mean titer of mT1 vaccine (Fig. 2C) reached a peak at week 5 and then gradually decreased to baseline at week 17. However, after a booster immunization, the titer was significantly increased (the mT1 vaccine). Using this strategy, we were able to decrease colonic inflammation in acute colitis as shown by reduced MPO that is released from neutrophils and by improved histological changes (Fig. 4). This strategy was also effective in ameliorating chronic colitis in which vaccinated mice had less body weight loss and significantly lower levels of soluble collagen in colon tissue. Due to chronic transmural inflammation and dysregulated wound repair leading to excessive deposition of extracellular matrix (ECM), patients with Crohn’s disease are prone to developing intestinal fibrosis and stricture formation. Despite the high incidence of bowel stenosis in Crohn’s disease, no effective measures are currently available to specifically treat or prevent intestinal fibrosis and obstructive stricture. In the present study, it shows that long-term down-regulation of TNF by using a vaccine may effectively improve colon fibrosis as seen by significantly reduced soluble collagen in colon tissue.

The TNF levels in colon tissue were reduced but the reduction did not reach a statistically significance. The levels of TNF were measured by an ELISA, in which the anti-TNF antibody was coated on the plates that bound both free TNF (the TNF that was not blocked by vaccine-induced antibodies) and the TNF that was bound with TNF-specific antibodies induced by the vaccine in the form of immune complex. This may occur because some of the immune complexes may not be cleared by the immune system and still exist in the serum. This phenomenon also occurs in other treatments with antibodies against self-molecules. For example,
the treatment with the monoclonal antibody against IgE results in significant improvements of IgE-mediated symptoms. This is accompanied with significantly reduced serum free IgE (measured using IgE receptor-capture ELISA), while total IgE (measure using anti-IgE-capture ELISA) remains unchanged. Because mouse TNF receptors are not commercially available, we could not measure free TNF in the study. Therefore, statistical difference could not be reached.

A potential concern with cytokine vaccines is that the injection of cytokine vaccines might induce a permanent immune condition, as some anti-bacterial and -virus vaccines do, eliminating all of the target cytokines that are required to maintain normal functions. In studies, cytokine vaccines down-regulate elevated cytokine levels to a level that is still higher than the normal level and the titers are reversible. In fact, the antibody titers induced by cytokine vaccines last about 4 months and are able to be adjusted by the frequency of immunization as the immunogenicity of cytokine vaccines is much less than that of microbe vaccines. Furthermore, anti-cytokine antibodies should only neutralize the pool of cytokines ectopically accumulated in the extracellular compartment, and should not interfere with cytokine processes occurring at the immunological synapse in normal tissues.

Anti-TNF therapy with mAb may increase the risk of severe infections, although the rate is low (the incidence of tuberculosis is up to 0.2% in treated patients) and has high rates of development of antibodies against the infused mAbs (61%) and of infusion reactions (1/3). Although the former side effect cannot be changed, the high rate of development of anti-infused mAbs and infusion reactions can certainly be avoided by using the vaccine strategy. The review of the benefit/risk profile has shown that TNF blockers currently used are effective and may be safely used for long-term management of rheumatoid arthritis and Crohn’s disease. The TNF vaccine should be safe when it is used with careful monitoring. In the remote event that the
above risk is real, it should be balanced against the benefits of treatment and the very real and often severe side effects of conventional drug and mAb therapies. It is only if the use of cytokine neutralizing antibodies was to provide significant benefit over the associated risk of infection that it would be safe to consider the use of cytokine vaccines.

In summary, we have demonstrated that the TNF peptide-based vaccine is capable of downregulating the inflammatory responses seen in the prevention of acute and chronic TNBS-induced murine colitis. The effects of this vaccine in the treatment of established experimental colitis are currently being investigated. By using an active immunization strategy, we offer an innovative and long-lasting therapeutic approach for the treatment of inflammatory bowel disease.

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