Using transforming growth factor-beta 1 (TGF-β1) vaccines to suppress TNBS-induced chronic bowel inflammatory responses in mice

BY

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ABSTRACT

Background: Epidemiologic data provides strong evidence of a steady rise of autoimmune diseases over the past 50 years. One such disease is Crohn's disease, which affects millions of people worldwide and requires long term treatment. Fibrosis is a major complication in Crohn's disease. Transforming growth factor-β (TGF-β), a multifunctional cytokine, plays a critical role in the fibrogenic process.

Hypothesis: Over-expressed TGF-β1 can be down regulated by active immunization with a TGF-β1 vaccine. This strategy can be used to ameliorate bowel inflammatory responses

Specific objectives of this experiment were to express and purify a TGF-β1 vaccine which induces high levels of auto-antibodies to TGF-β, and then evaluate the *in vivo* effects of the vaccine in the down-regulation of bowel inflammatory responses using a mouse model of chronic colitis.

Methods: A recombinant mouse TGF-β1 peptide-based vaccine was expressed by *E. coli* and purified using ammonium sulphate precipitation. Bowel inflammatory responses were induced with multiple administrations of a chemical (TNBS). Mice were immunized with the vaccine 3 times before (for preventive study) or after (treatment study) TNBS administration commenced. Mice receiving vaccine carrier protein and saline served as controls. Body weight, serum antibody levels, and inflammation and collagen deposition in the colon tissue were examined.

Results: A recombinant TGF-β1 vaccine was successfully expressed and purified. Mice immunized with TGF-β1 vaccine had high levels of antibodies to TGF-β1, less body weight loss, and significantly lower collagen deposition as compared to controls ($P<0.05$) in both preventive and treatment studies.

Conclusions: Administration of TGF-β1 vaccine may be a potential therapeutic approach in the treatment of inflammatory bowel disease.

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ABBREVIATIONS USED

Ab/Abs	Antibody/antibodies
ANOVA	Analysis of variance
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
HBcAg	Hepatitis B core antigen (carrier of peptide – functions as antigen
IBD	Inflammatory bowel disease
IgG	Immunoglobulin G
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
LB(+AMP)	Lysogeny broth with ampicillin
mAbs	Monoclonal antibodies
PBS	Phosphate buffered saline
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TGF-β	Transforming growth factor-β
Th1	Type 1 helper T cells
Th2	Type 2 helper T cells
TNBS	2,4,6,-trinitrobenzene sulphonic acid (induced colitis)

1. INTRODUCTION

1.1 Immune disorders

The term 'immunity' is derived from the Latin *immunitas* meaning exempt. Classical immunology studies the relationship between the body systems, pathogens, and immunity. The earliest written mention of immunity can be traced back to the plague of Athens in 430 BCE. The historian Thucydides noted that people who had recovered from a previous bout of the disease could nurse the sick without contracting the illness a second time. By the 19th and 20th centuries this theory had developed into scientific practice. In developed countries, new treatments and vaccines have allowed many infectious diseases to enter remission or become almost completely eradicated. In 2000, mumps, measles and tuberculosis have an almost zero percent incidence of infection compared to nearly 100 percent in 1950. Yet in that same time period, immune disorders have shown a complete opposite trend (Figure 1). Within the past 50 years, epidemiological data have provided strong evidence of a steady increase in allergic and autoimmune diseases in developed countries. Asthma, diabetes and inflammatory bowel disease have increased exponentially. Today, almost 20% of the world's population has such severe asthma that it has become resistant to steroid treatment, and Crohn's disease has more than tripled in Europe since the 1950s (Bach, 2002).

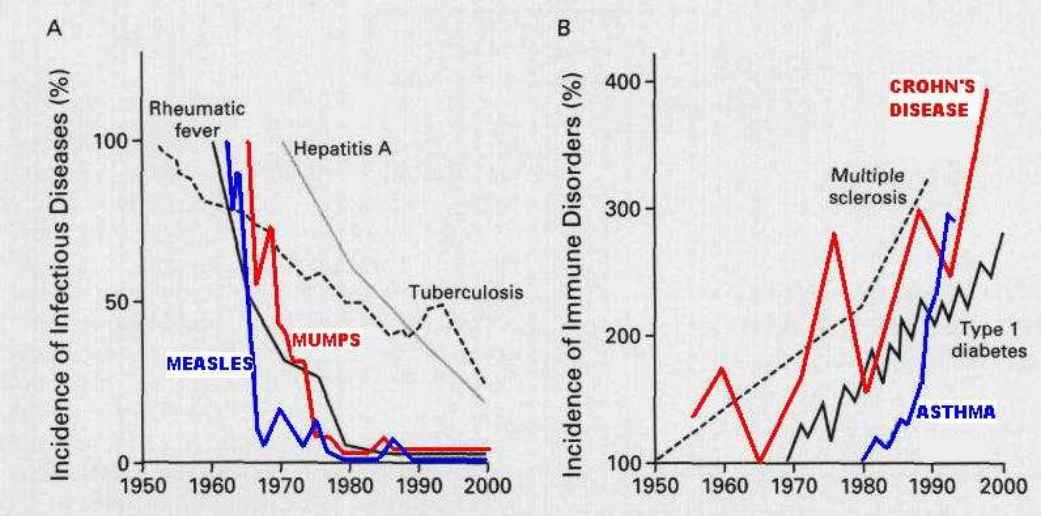


Figure 1: Inverse relation between the incidence of prototypical infectious diseases (A) and incidence of immune disorders (B) from 1950 to 2000 (Bach, 2002)

Protective immunity against microbes is mediated by the early reactions of innate immunity and the later responses of adaptive immunity. The immune system possesses several properties which are of fundamental importance for its normal functions: specificity for different antigens, a diverse repertoire capable of recognizing a wide variety of antigens, memory for antigen exposure, specialized responses to different microbes, self-limitation, and the ability to discriminate between foreign antigens and self antigens. Without all these qualities, the immune system would be unable to function effectively (Abbas and Lichtman, 2005).

Normally the immune system recognizes the foreign antigens and produces specific immune responses that protect the body from the harmful and foreign substances meanwhile keeps ignore to self antigens. An autoimmune disorder is a condition that occurs when the immune system mistakenly attacks and destroys healthy body tissue. Under certain conditions, the immune system cannot tell the difference between healthy body tissue and foreign antigens, and thus the body

mounts an abnormal immune attack with antibodies and/or T cells, against a person's own self-tissue antigens. The result is an immune response that destroys normal body tissues (Abbas and Lichtman, 2005; Prescott *et al.*, 2005). Because there is such a high morbidity rate in autoimmune disorders, it is apparent that a treatment or therapy that reduces or eliminate these problems must be found.

Cytokines are signalling proteins and glycoproteins that are used extensively in cellular communication. Researches have found that the development of autoimmune diseases is connected with over-expressed cytokines such as interleukin-12 (IL-12) or interferon-gamma, produced by type 1 helper T cells (Th1) and the development of allergic diseases is dominated by over-expressed cytokines such as IL-4, 5, 13 produced by type 2 helper T cells (Th2) (Prescott *et al.,* 2005). This provides promising therapy targets for intervening the development of severe autoimmune diseases.

1.2 Inflammatory bowel disease (IBD)

The digestive system is a set of organs that convert the foods we eat into nutrients and then absorbs these nutrients into the bloodstream to fuel our bodies. We seldom notice its function unless something goes wrong. It is estimated that several million people in the world have inflammatory bowel disease (IBD), and that those numbers are increasing every year (Table 1). Although it occurs most frequently in people ages 15 to 30, it is known to affect all age groups (Podolsky, 1991).

Table 1: The change in incident and prevalence rates of ulcerative colitis (UC) and Crohn's disease (CD) in Manitoba, Canada between 1989 and 2000 (Berstein *et al.*, 1999).

	UC Incidence Rate (per 100,000)	UC Prevalence (per 100,000)	CD Incidence Rate (per 100,000)	CD Prevalence (per 100,000)
1989-1994	14.5	167	14.6	197
1998-2000	15.4	248.6	15.4	271.4

Inflammatory bowel disease refers to two diseases that cause chronic inflammation of the intestines: ulcerative colitis and Crohn's disease. Although these diseases have common features, there are some important differences (Podolsky, 1991).

Ulcerative colitis is an inflammatory disease of the large intestine, also called the colon. In ulcerative colitis, the mucosa of the intestine becomes inflamed and develops ulcers. Ulcerative colitis is often the most severe in the rectal area and can cause frequent diarrhea. Mucus and blood often appear in the stool if the lining of the colon is damaged (Monteleone *et al.*, 2006).

Crohn's disease differs from ulcerative colitis in the areas of the bowel it affects. It generally tends to involve the entire bowel wall, whereas ulcerative colitis affects only the lining of the bowel. Crohn's disease most commonly affects the last part of the small intestine - the terminal ileum - and parts of the large intestine. It causes inflammation that extends much deeper into the layers of the intestinal wall than ulcerative colitis does (Monteleone *et al.*, 2006).

Both diseases have tissue damage as a result of exaggerated immune response to antigens. Inflammation signals are amplified and maintained as a result of active cross-talk between immune and non-immune cells (Gordon *et al.*, 2005).

However, Crohn's disease is characterized by chronic, relapsing and remitting intestinal inflammation and an increase in fibrosis and collagen deposition. Patients suffer from abdominal pain, bloody diarrhea, weight loss and recurrent fever. This makes it a very difficult disease to live with. There is still no therapy apart from counteracting the symptoms using anti-inflammatory drugs or surgical resection of the affected intestine. Thus, the importance of determining disease pathogenesis and a more effective way of treating it becomes apparent (Hume *et al*., 2006).

1.3 Fibrosis and transforming growth factor – β (TGF- β)

Developments have shown that Crohn's disease is a Th1 cell mediated response. Th1 cell derived cytokines like tumor necrosis factor-α (TNF-α) are produced in excess by macrophages in fibrotic tissue (Monteleone, 2006). Fibrosis is a complex tissue disease that leaves a somewhat irreversible state of scar tissue. Long-term activation of fibroblasts results in massive extracellular matrix (ECM) deposition affecting normal wound healing. In tissue fibrosis, the net accumulation of collagen is a result of an imbalance between enhanced production and deposition and impaired degradation of ECM components. Collagen is the most abundant protein in mammals, making up about 25% of the whole-body protein content. There are 28 types of collagen described in literature. Type I collagen is the most abundant collagen of the human body. It is present in scar tissue, the end product when tissue heals by repair. Abnormalities in any step of type I collagen production may result in abnormally elevated synthesis of type I collagen which, in turn, causes tissue fibrosis (Torrego, *et al.* 2007).

Due to the increase in fibrosis in IBD, attention is now focusing on the role of growth factors, a group of diverse molecules derived from blood cells, which contribute to the fibrogenic process. TGF-β and connective tissue growth factor (CTGF) are considered master switches for the induction of the fibrotic program, by being key regulators of ECM assembly and remodeling. Specifically, TGF-β isoforms have the ability to induce the expression of ECM proteins in mesenchymal cells, and to stimulate the production of protease inhibitors that prevent enzymatic breakdown of the ECM. Excessive and abnormal deposition of ECM is the usual characteristic of fibrosis (Verrechia and Mauviel, 2007).

TGF-β belongs to a family of multi-functional polypeptides produced by a wide variety of lymphoid and non-lymphoid cells. More than 60 TGF-β family members have been identified in multicellular organisms. They exist in five different isoforms, three of which are expressed in mammals and designated as TGF-β1, TGF-β2 and TGF-β3 (Campell and Reece, 2002; Verrecchia and Mauviel, 2007). All have an important role in the regulation of immune cells. Specifically, elevated TGF-β1 expression in affected organs, and subsequent deregulation of TGF-β1 functions, correlates with the abnormal collagen tissue deposition observed during the onset of fibrotic diseases (Verrecchia and Mauviel, 2007; Makinde *et al.*, 2007). Down-regulating TGF-β1 could create a possible treatment to Crohn's disease.

1.4 Current available monoclonal antibodies in treatment of IBD

Blocking cytokines and inhibiting non-specific inflammation has had therapeutic value in autoimmune disorders. Through studies and trials, some treatments have been found. New biological therapies for Crohn's disease include anti-TNF-α monoclonal antibodies (mAbs) such as Infliximab and Adalimum, as well as an antibody neutralizing the sub-unit p40 of IL-12. These mAbs have been highly effective in treating active Crohn's disease, maintaining remission, closing fistulas, and maintaining fistula closure. Unfortunately, there are disadvantages to these treatments. Not only are they expensive ($40,000 per year), a great deal needs to be injected (750mg) fairly frequently (2-4 weeks). Furthermore, the clinical response decreases after about 10 weeks due to the development of antibodies against the infused monoclonal antibodies; and infusion reactions are also found in one third of patients receiving Infliximab treatment (Sands *et al.,* 2004). For these reasons, new therapies need to be found.

1.5 Active immunization against self-proteins

Two broad experimental strategies have been used to design vaccines against self-proteins: 1) to modify the intact self-protein by inserting a foreign peptide containing helper T cell epitopes (antigenic determinant recognized by the immune system); and 2) to chemically couple the intact self-protein, or a considerable part thereof, to an immunogenic carrier protein (Zuany-Amorim *et al.*, 2004). By these approaches, the self-component is recognized as foreign by the

immune system of the host, and autoantibodies against self-epitopes are generated. However, polyclonal antibodies induced by such vaccines, which are against multiple self-antigen determinants, may cross-react with other self-proteins that contain similar epitopes. This is of particular concern when this strategy is used in humans as it may result in the wrong self-protein being attacked (Vernersson *et al.*, 2002).

In an effort to overcome the inherent disadvantages of the therapeutic approaches outlined previously, a cytokine peptide-based vaccine has been designed, which limits the potential for possible cross-reactivity. The vaccine was constructed by inserting a small peptide derived from the receptor binding site of the target cytokine into a carrier, hepatitis B core antigen (HBcAg), via gene recombination methods. Figure 2 compares the differences between the peptide-based vaccine and the regular cytokine auto-vaccine. The peptide-based vaccine presents as virus-like particles and induces auto-antibodies to the target cytokine (Peng *et al.*, 2007; Ma Y. *et al.*, 2007). Administration of an interleukin-13 peptide-based vaccine has down-regulated airway inflammatory responses in mice (Ma A.G. *et al.*, 2007). Moreover, in a pilot study, administration of a tumour necrosis factor-α peptide vaccine reduced symptoms in mice with TNBS-induced colitis, suggesting that administration of relevant cytokine vaccines may be an alternative therapy in IBD (Zhou *et al.,* 2007).

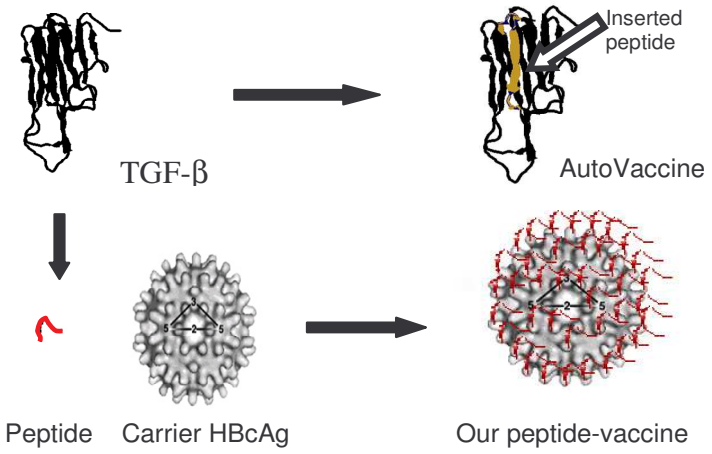


Figure 2: A comparison of a usual AutoVaccine and the proposed new peptide-based vaccine

Compared with currently used mAbs to cytokines, this active immunization strategy using vaccines has the following advantages: 1) It provides *long-term protection* with a few injections, while mAbs such as Infliximab must be administered every 8 weeks; 2) The induction of autoantibodies can be focused on *targeting to the highly active* and antigenic epitopes; 3) *Costs are low***.** The vaccine itself is less costly in comparison with Infliximab, which is estimated at over $40,000 annually per patient at a low dose of 5 mg/kg; 4) *Less side effects***,** such as avoidance of acute infusion reactions and the induction of anti-chimeric antibodies, occur. Table 2 summarizes the differences between active versus passive anti-cytokine antibody therapies and the differences between anti-cytokine and anti-microbe vaccines. Figure 3 shows the two different approaches of immunization (Ma Y. *et al.*, 2007).

Table 2: A comparison of antibodies (Ab) in passive and active immunization (Ma Y. *et al.*, 2007).

Passive Immunization	**Active Immunization**	
Ab induced by chimeric humanized mAb	By self-vaccines	By conventional microbe vaccines
Antigen not required	Self-protein derivatives	Foreign microbes
Contains 5% mouse Ab	100% of human Abs	100% human Abs
Recombinant or chimeric	Nature human molecules	Human molecules
Monoclonal Ab	Polyclonal Ab	Polyclonal Ab
Injected passively	Induced by the host	Induced by the host
Short duration (2 - 3 wk)	Long term (> 4 months)	Long (> 1 yr)
Large dose (5mg/kg)	Low	Low
Therapeutic	Therapeutic	Preventive
May have anti-infused mAb response	No	No
Possible infusion reactions	No	No
Extremely high cost	Low cost	Low cost

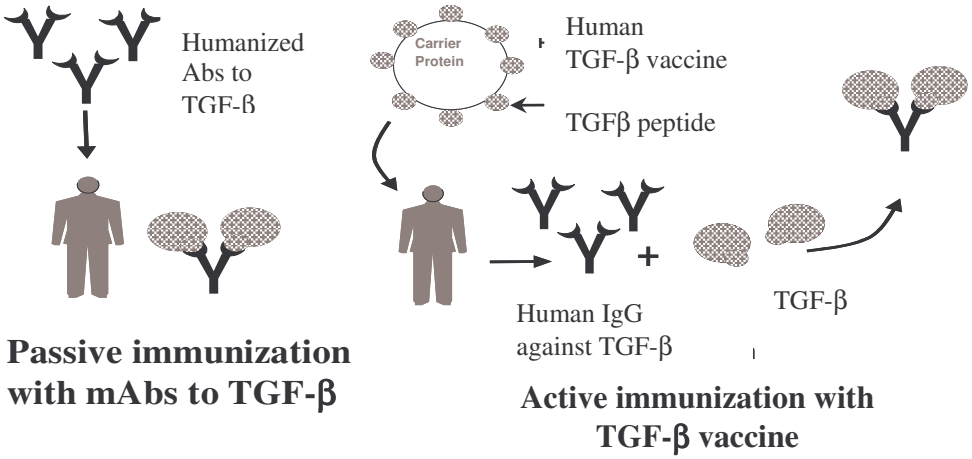


Figure 3: A comparison of passive immunization with monoclonal Ab and the proposed active immunization with TGF-β vaccine

1.6 Preparation of an effective peptide-based vaccine

To design an effective vaccine, the following factors must be taken into consideration.

(1) The size of the peptide. Small antigenic peptides should be selected from TGF-β1 because they have several advantages for limiting any possible cross-reactivities (Jegerlehner *et al.*, 2002).

(2)Location and cross-reaction of the peptide. It is safer to choose peptides derived from receptor binding sites to avoid possible activation of cells when antibodies react with the cell-bound TGF-β1. The possibility of no cross-reactivity of the chosen peptides to self-proteins will be ensured (Schirmbeck *et al.*, 2001).

(3) The carrier protein. The most commonly used carrier proteins are bacterial proteins that humans encounter normally, such as Tetanus toxoid. Virus-like particles can induce potent B cell responses because they improve the presentation of the epitopes to cells of the immune system. Therefore, the inserted foreign peptide is natively arrayed in a highly repetitive and ordered fashion on the surface of the virus-like particles (Netter *et al.*, 2001). Dr. Peng's lab has successfully extended the application of hepatitis B core antigen (HBcAg) as a vaccine carrier into the area of breaking self-immune tolerance and inducing high titered auto-antibodies (Peng *et al.,* 2007). Therefore, HBcAg was chosen as the carrier protein.

2. HYPOTHESIS

It was hypothesised that over-expressed TGF-β1 can be down regulated by neutralising auto-antibodies induced by active immunization using anti-TGF-β1 vaccines. This strategy can be used to ameliorate chronic bowel inflammatory responses in mice.

3. SPECIFIC OBJECTIVES

1. To express, purify and identify recombinant mouse TGF-β1 peptide-based virus-like particle vaccine.

2. To *in vivo* test the effects of TGF-β1 vaccine in the down-regulation of TNBS-induced chronic colonic inflammatory responses in mice.

4. EXPERIMENTAL DESIGN, MATERIALS, AND METHODS

All procedures were completed by a research team in Dr. Peng's laboratory.

4.1 Selection of antigenic peptides and construction of recombinant DNA plasmids

An antigenic peptide containing 15 amino acids and corresponding to the receptor binding site of mouse TGF-β1 was selected according to antigenicity prediction and structure analysis of TGF-β1 receptor complex. Antigenic peptides were successfully selected and identified in Dr. Peng's lab. Two plasmids were also prepared: 1) plasmid HBcAg, which expressed a carrier composed of truncated HBcAg; and 2) plasmid HBcAg-TGFβ-1, which expressed a carrier composed of HBcAg and TGFβ-1 peptide.

4.2 Vaccine preparation and identification

4.2.1 Transformation

Bacteria transformation refers to a stable genetic change brought about by taking up naked DNA (DNA without associated cells or proteins), and competence refers to the state of being able to take up exogenous DNA from the environment. In this experiment, artificial competence was used to ensure DNA entrance into the an *E. coli* cell. Artificial competence is not encoded in the cell's genes. Instead it is induced by laboratory procedures in which cells are passively made permeable to DNA, using conditions that do not normally occur in nature. Chilling cells in the

presence of divalent cations prepares the cell walls to become permeable to plasmid DNA. *E. coli* cells are incubated on ice with the DNA and then briefly heat shocked which causes the DNA to enter the cell.

A pipet was used to transfer 10µl of plasmid TGFβ-1 peptide vaccine into 200 µl of competent *E. coli* cells. The tubes were then placed in an ice-bath for 30 minutes, transferred into 42ºC water-bath for 90 seconds and then placed back into an ice-bath for one minute. The competent cells were then added to a 13ml tube containing 1ml Lysogeny Broth (no ampicillin). This was placed in a shaker at 150rpm at 37ºC. After 35 minutes in the shaker, 200 µl of the culture was spread onto an ampicillin agar plate and incubated overnight at 37ºC.

4.2.2 Expression of recombinant TGF-β1 proteins

A colony was taken from the ampicillin agar plate and added to a beaker of Lysogeny Broth with ampicillin (LB+AMP). Following incubation at 37ºC overnight, other LB+AMP beakers (5% by volume) were inoculated with the new cell culture. The beakers were shaken for approximately three hours. To induce gene expression, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added, 0.2µg/1ml, to each beaker and shaken again for three and a half hours. Material was transferred to large jars and centrifuged for 20 minutes at 2500rpm and 4ºC. The supernatant was discarded and pellet stored at 4ºC.

4.2.3 Purification and identification

To purify the induced protein, the recovered cells were re-suspended with 1-2ml of phosphate-buffered saline (PBS) and lysed by ultra-sonication at amplitude 40 for two minutes. After centrifugation at 14000rpm at 4ºC for 20 minutes, the supernatant was collected in a 13ml tube. Suspension, sonication and centrifugation were repeated. The collected supernatants were precipitated by adding ammonium sulphate to a concentration of 40% saturation (0.242g/ml of supernatant). The suspension sat 30 minutes and then was centrifuged at 14000RPM at 20ºC for 10 minutes. The remaining pellet was completely suspended with 20% of saturation ammonium sulphate and centrifuged again at 14000RPM at 20ºC for 15-20minutes. The supernatant was discarded and the last step repeated. The residual pellet was re-suspended with 1ml PBS and centrifuged at 14000RPM at 20ºC for 15 minutes. The procedure was repeated three times, using less PBS each time. The recovered protein (supernatant) was purified further by combined chromatography procedures which consisted of Macro-Prep Ceramic Hydroxyapatite, Polymysin, and Sepadex G25, and then placed in the freezer to preserve.

All samples were checked with SDS-PAGE to assess the purity of the recovered protein. Samples were loaded into individual wells (100µl/well) and run at 200V for 30 minutes.

4.3 Animals

Female BALB/c mice, aged 6-8 weeks were purchased from Charles River Laboratories and housed in the Central Animal Care at the University of Manitoba. All animals were used in accordance with the guidelines issued by the Canadian Council on animal care, and all animal care utilization protocols were approved by the Animal Care Committee at the University of Manitoba. All mice were anesthetized with isoflourane gas prior to injections and exposures.

4.4 Immunization and TNBS administration

Four groups of mice were injected subcutaneously to test the degree and duration of TGF-β1 specific responses induced by the vaccine: (1) normal control group (n=6): injected subcutaneously with (PBS); (2) saline group (n=12): injected with PBS and then subjected to intrarectal sensitization with TNBS; (3) carrier group (n=12): immunized subcutaneously with 100µg carrier (HBcAg) and then TNBS; and (4) the vaccine group (n=12): immunized with 100µg vaccine (HBcAg - TGF-β1) and then TNBS.

Experimental mice were subcutaneously immunized three times at a two-week interval with vaccines, while the control mice received saline or the vaccine carrier (native HBcAg). Intrarectal sensitization with TNBS was achieved through administration via a 3.5 F catheter into the rectum until the tip was 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 one minute after the intrarectal injection.

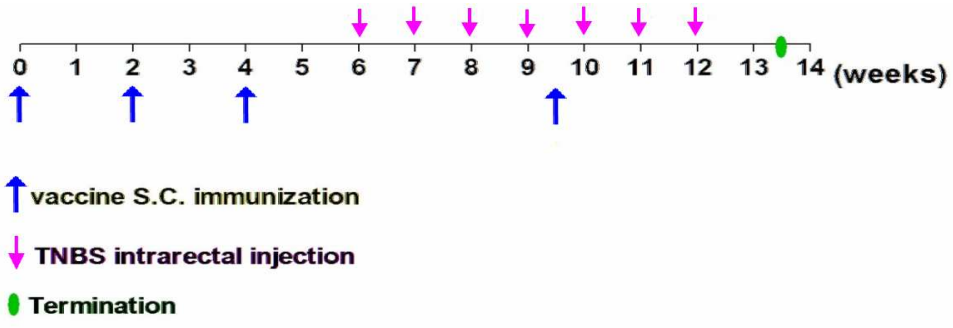
4.4.1 Murine colitis induction protocol

To study the effect of the vaccine, mouse models of chronic colitis were used, as chronic models are more relevant to human IBD and to the study of the effect of cytokine vaccines. Hapten-induced colonic inflammation is a widely used animal model of human Crohn's disease (Jurjus *et al.*, 2004; Ebach *et al.*, 2005; Te Velde *et al.*, 2006). This model is believed to be mediated by an excessive Th1 response, constituting a model for Crohn's disease. Intrarectal delivery of a 2,4,6,-trinitrobenzene sulphonic acid (TNBS) induced colitis by haptenation of colonic proteins, leading to a delayed-type hypersensitivity reaction caused by $CD4^+$ Th1 cell responses to self-antigens. TNBS (0.5 mg) was administered seven to eight times at a one-week interval (Elson *et al.*, 1996; Fichtner-Feigl *et al.,* 2006; Lawrance *et al.,* 2003).

4.4.2 Immunization protocols

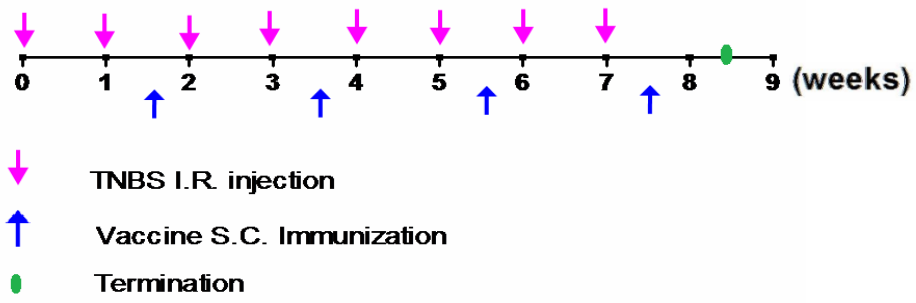
Preventative study

Vaccination was completed by subcutaneous immunization of mice with the TNF-β1 vaccine, carrier or saline (50μg/mouse) for three times at a two-week interval. Two weeks after the last immunization, mice were administered with TNBS for seven times at a one week interval. Mice were immunized at week nine again.



Treatment study

In the treatment study, the TNF-β1 vaccine began to be administered three days after the second administration of TNBS (eight times at a seven week interval). They were subcutaneously immunized with 50 μg/mouse of vaccine, carrier, or saline four times at a two-week interval.



4.5 Measurements

4.5.1 Body weight

After administration of TNBS, mice were assessed by measurement of body weight every day and then averaged per week. The per-week weight was then calculated as a body weight percentage, with weight on day 0 being 100%.

4.5.2 Histological analysis of colon tissue

After the allotted time period, the animals were sacrificed under anesthetic to obtain colon tissue. To prepare the tissue, colons were harvested from BALB/c mice, their stool removed, and colons weighed. Colons were fixed overnight in

formalin (Fisher Scientific, catalogue #305-510). Tissues were processed using the protocol described in Table 3 with a Citadel® Tissue Processor from Shandon Inc. Each colon was embedded separately in paraffin wax. The tissues were then sectioned using a Microtome sectioner (Shandon Inc.). Sections were 6µm thick and placed on slides with two sections per slide. The slides were incubated overnight at room temperature to fix the sections to the slide.

Table 3: Procedure for processing colon tissue

Step	**Solution**	**Time**
1	70% ethanol	4 hours
2	70% ethanol	4 hours
3	80% ethanol	4 hours
4	95% ethanol	2 hours
5	95% ethanol	2 hours
6	100% ethanol	2 hours
7	100% ethanol	2 hours
8	100% ethanol/xylene	20 minutes
9	Xylene	20 minutes
10	Xylene	20 minutes
11	Paraffin wax	2 hours
12	Paraffin wax	2 hours

Deparaffinization and rehydration

Prior to any staining, colon sections underwent a process to remove the paraffin wax and rehydrate the tissue. The protocols for deparaffinization and rehydration are described in Table 4.

Table 4: Procedure for deparaffinization and rehydration

Step	**Solution**	**Time**
Deparaffinization		
1	Xylene	3 minutes
2	Xylene	3 minutes
3	Xylene	3 minutes
Rehydration		
4	100% ethanol	3 minutes
5	100% ethanol	3 minutes
6	100% ethanol	3 minutes
7	95% ethanol	3 minutes
8	95% ethanol	3 minutes
9	95% ethanol	3 minutes
10	80% ethanol	3 minutes
11	80% ethanol	3 minutes
12	80% ethanol	3 minutes
13	Deionized water	At least 5 minutes

Masson's Trichrome stain

Masson's Trichrome staining was used to visualize fibrotic tissue in the colon. Prior to staining, slides were deparaffinized and rehydrated. Slides were allowed to mordant in preheated Bouin's solution (Sigma Aldrich, lot# 095K4341) at 56ºC for 30 minutes. During this time, Wiegert's Iron Hematoxylin (Sigma Aldrich, lot# 045K4342) and Phosphotungstic/Phosphomolybdic acid solutions (Sigma Aldrich, lot# HT709-1SET) were prepared according to manufacturer's instructions (Appendix 9.1.1 and 9.1.2). After incubation in Bouin's solution, the slides were cooled in tap water for five minutes and then flushed under running tap water to remove the yellow colour from the tissue sections. Slides were placed in Wiegert's Iron Hematoxylin for ten minutes, flushed under running tap water for ten minutes, and then rinsed in distilled water. Slides were stained Biebrich Scarlet-Acid Fuschin (Sigma Aldrich, lot# 065K4340) for 15 minutes and then rinsed in distilled water. The slides were then placed in working

Phosphotungstic/Phosphomolybdic acid solution for five minutes, followed by Aniline Blue (Sigma Aldrich, lot# 065K4340) for seven minutes. Slides were placed in 1% acetic acid for five minutes, rinsed in distilled water, dehydrated and cleared.

Dehydration and clearing

Table 5: Procedure for dehydration and clearing

Step	Solution	Time
1, 2, 3	95% ethanol	3 minutes each step
4, 5, 6	100% ethanol	3 minutes each
7, 8, 9	Xylene	5 minutes each

Slides may remain in xylene overnight to get a good clearing.

4.5.3 Quantitative assay of colonic collagen

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

4.5.4 Detection of antibodies to TGF-β1

Serum was collected from mice two weeks after last vaccination. This was then diluted 1:5000 and sent through enzyme-linked immunosorbent assay (ELISA) to determine antibodies. The plate was first coated with 100µl per well of 0.5µg/ml of the capture antibody (TGF-β1 protein), followed by a coat with 100µl

sera and then the 100µl goat anti-mouse IgG-alkaline phosphatase. After each coating, the plate was covered and incubated for one hour at room temperature. Between each coating, wells were washed three to five times with washing buffer (PBS/Tween), standing three minutes prior to dumping. Finally 100µl of substrate was added and wells were allowed to develop. Plate was then read at 405nm.

4.6 Statistical analysis

Statistical analysis was performed to assess differences of results between experimental groups using one-way analysis of variance (ANOVA), followed by the Newman-Keuls multiple comparison test (GraphPad Prism). Values were reported as X ± SE. P values <0.05 were considered statistically significant.

5. RESULTS

5.1 Vaccine construction and identification

This was completed by Dr. Peng's lab.

The vaccine, HBcAg-TGF-β1, was expressed efficiently in *E. coli* DH5α cells. This recombinant protein specifically reacted with polyclonal goat anti-mouse TGF-β1 antibody observed through western blotting. Analyzed by SDS-PAGE, the HBcAg-TGF-β1 and wild type HBcAg exhibited similar fractionation patterns after sucrose gradient centrifugation. This indicates that the chimeric protein HBcAg-TGF-β1 assembles into particles that are similar to native HBcAg. The presence of particles was further assessed by scanning electron microscopy.

5.2 TGF-β1 protein preparation

After inducement of gene expression through the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) and purification by washing with ammonium sulfate and then flowing through the chromatography columns, the recovered proteins were assessed for purity through SDS-PAGE. As seen in figure 4, expression of HBcAg and HBcAg-TGFβ1 are only visible after inducement by IPTG. They are also expressed at the appropriate size between 20 and 28 kD.

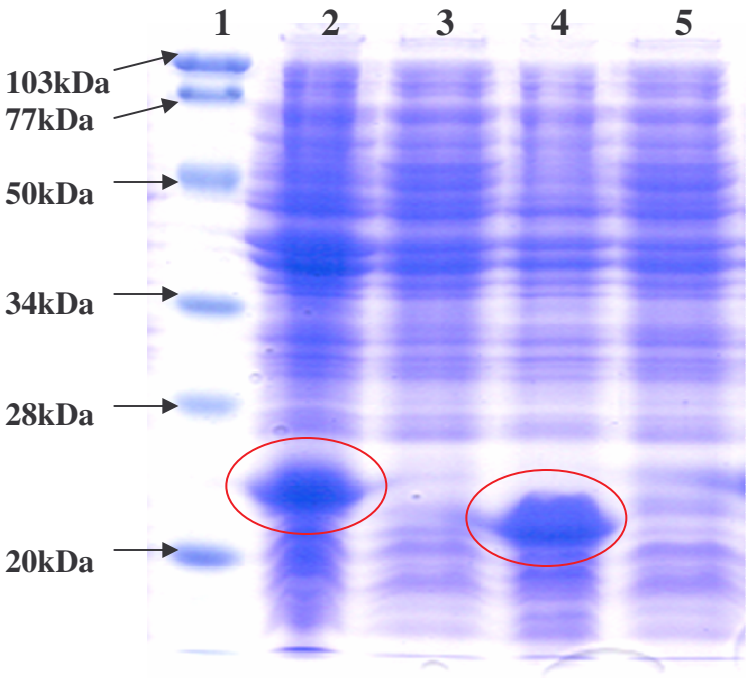


Figure 4: SDS-PAGE analysis of expressed recombinant protein in bacterial lyses

1. Pre-stained protein standards arranged by size (20 kiloDaltins (kDa), 28kDa, 34kDa, 50kDa, 77kDa, 103kDa)
2. **HBcAg-TGFbeta1, induction with IPTG**
3. HBcAg-TGFbeta1, non-induction
4. **HBcAg, induction with IPTG**
5. HBcAg, non-induction

5.3 TGF-β1 antibody response by vaccination

Mice immunized with vaccine produced high TGF-β1 specific antibody responses. This was determined by ELISA at a wavelength of 405nm (Figure 5). As observed, the density was high for the TGF-β1 vaccine (0.36 ±0.06) and low for the carrier HBcAg (0.05±0.003).

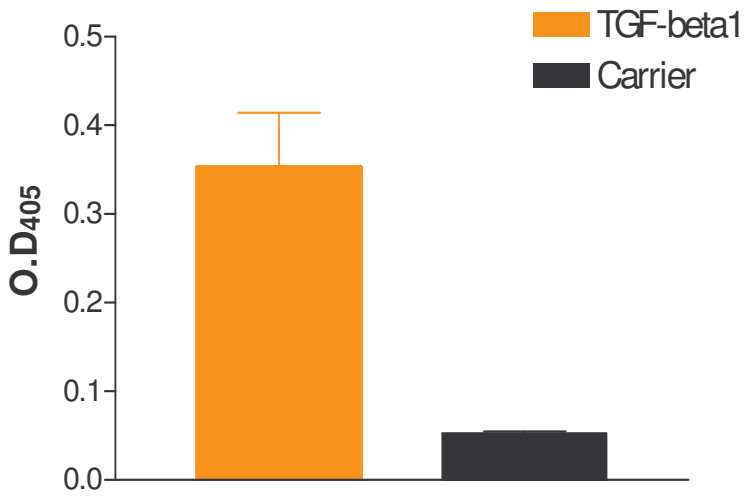


Figure 5: Serum TGF-β1 specific antibody was measured by ELISA. The results were expressed by optical density at a wavelength of 405nm (±SE).

Sera were obtained two weeks after the last immunization and diluted 1:5000. Antibodies were assayed through sandwich ELISA with TGF protein (0.5µg/ml), then sera, then goat anti-mouse IgG-alkaline phosphatase, then substrate.

5.4 TGF-β1 vaccine improved body weight of murine colitis

5.4.1 Preventative study

TGF-β1 vaccinated mice showed a similar pattern of weight gain compared to saline and control groups (Figure 6). After the first injection of TNBS, the saline, carrier and TGF-β1 groups decreased in body weight 2 – 3%±1. However, as the control and carrier groups continued to decrease at day 7, the TGF-β1 group returned to its original body weight.

At day 21, with an injection of the TGF-β1 vaccine, an increase in body weight of almost three percent in the TGF-β1 group is observed. While this increase was also seen in the saline and carrier groups, it was not as large. This suggested possible influence by the TGF-β1 vaccine in increasing recovery time from TNBS intrarectal injection.

After 49 days, the carrier and saline groups only increased an average of 4%±1 in body weight. According to previous research, the TNBS induced chronic colitis models should have less average weight gain as compared to a normal control group. Unfortunately, this was not the case in this study as the normal control mice had an unfortunate bout of illness and results were altered quite drastically.

Over all, the TGF-β1 vaccine group increased approximately 6%±2, 1% more than the saline group.

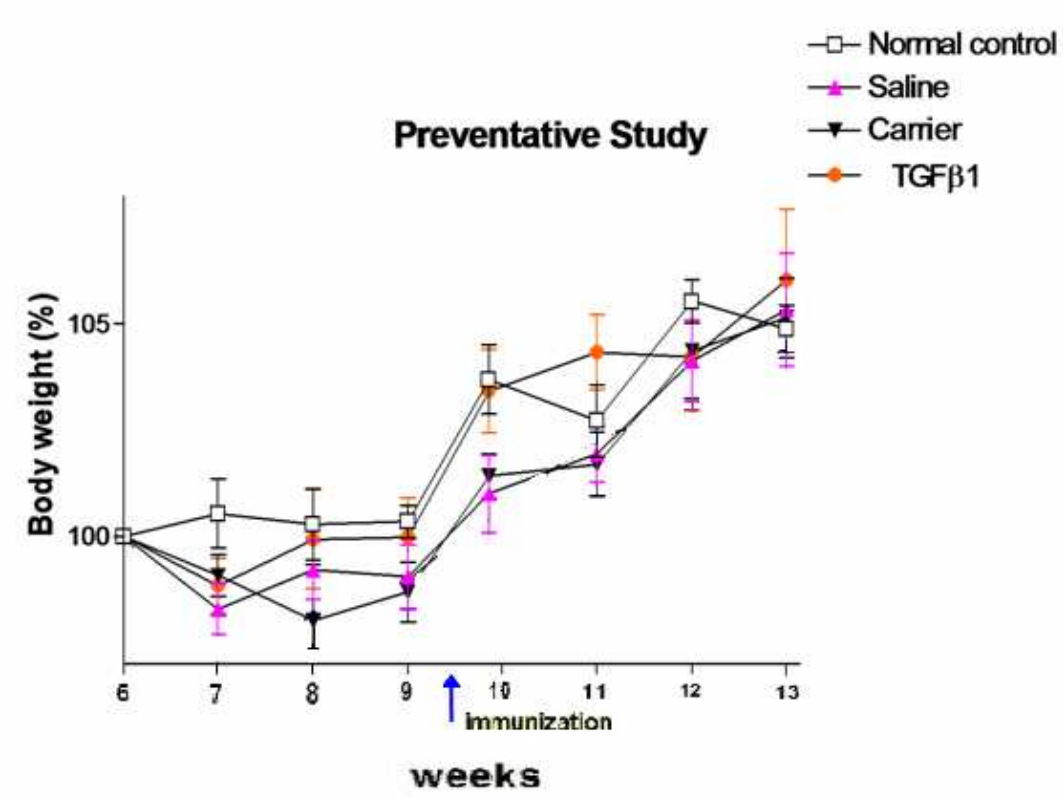


Figure 6: The mean change in percent body weight of mice over a seven week period in the preventative study (±SE).

Vaccination was completed by subcutaneous immunization of mice with the TNF-β1 vaccine, carrier or saline (50 μg/mouse) for three times at a four-week interval. Two weeks after the last immunization mice were administered with TNBS for seven times at a one week interval.

5.4.2 Treatment study

At day 0, all mice were injected with TNBS. Within three weeks, all groups displayed an increase in body weight of 10%±1 (Figure 7). However, after the second treatment of immunization on day 21, the TGF-β1 vaccine group showed a greater weight gain than the saline or carrier groups. The TGF-β1 group continued to rise until day 42, where it leveled. Then on day 49, the weight increased significantly again. Overall, the vaccine group recovered the weight lost faster than the saline or carrier groups.

After eight weeks, the TGF-β1 group had a body weight of 125%±2 while the saline group had a body weight of 115%±2. A difference of 10%±2 is observed between the treated and the non-treated.

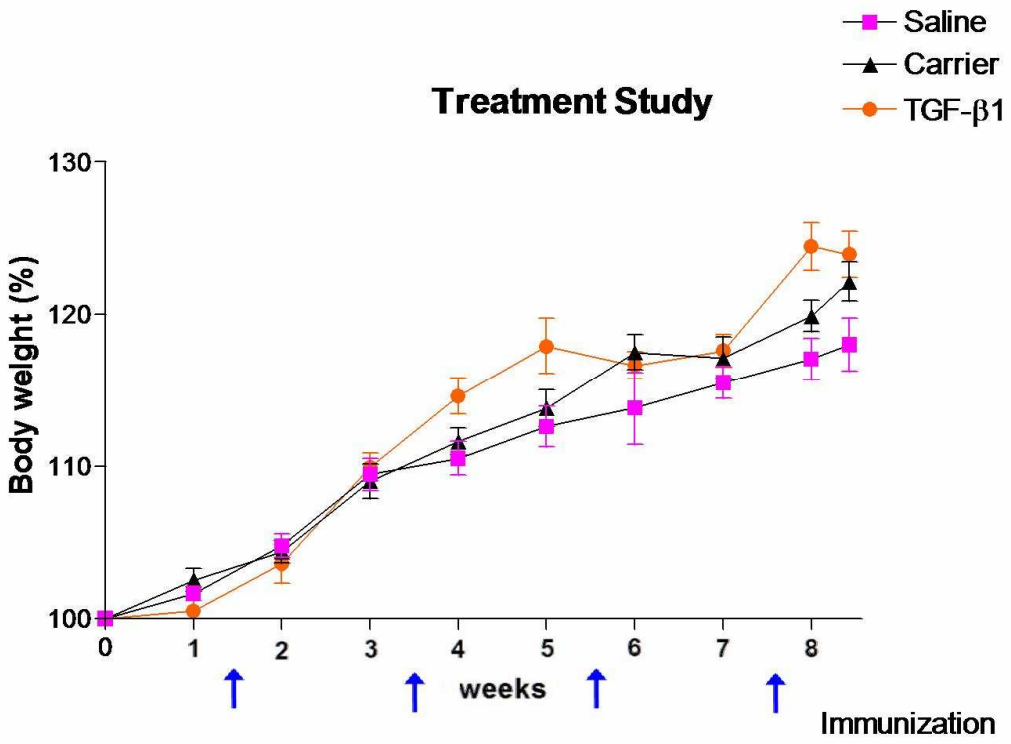


Figure 7: The mean change in percent body weight of mice over an eight week period in the treatment study (±SE)

Mice were induced with chronic colitis through TNBS eight times at a one week interval. The TNF-β vaccine began to be administered 3 days after the second administration of TNBS. They were subcutaneously immunized with 50 μg/mouse of vaccine, carrier, or saline 3 times at a two-week interval. Normal control can be seen in figure 6.

5.5 Colon appearance

Mice were sacrificed and colons harvested, weighed and measured. Colons induced with TNBS were noted to have a thicker appearance with increased inflammation (see Figure 8).

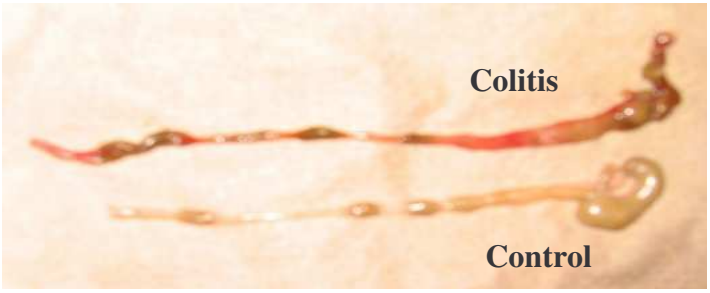


Figure 8: Comparison of a normal control mouse colon (bottom) with a TNBS induced chronic colitis mouse colon (top). The chronic colitis colon is more inflamed in appearance.

After comparing results of the two studies (preventative and treatment), the two had similar trends. The treatment study, however, had better results and for that reason shall be the main focus of the remaining results.

5.6 Histological analysis of colon tissue

Colon sections were stained with Masson's Trichrome for a more detailed look at colon structure in BALB/c mice. Masson's Trichrome stains nuclei black, collagen blue, and cytoplasm, erythrocytes and muscle strains various shades of red. Considerable differences in colon structure among the different groups were observed (see Figure 9).

The treatment study normal control group had little collagen present. Without chronic IBD, collagen production was normal and almost unnoticeable through slides. TNBS-induced chronic IBD however, resulted in excessive production of collagen as can be seen in the saline and carrier groups. This was expected as IBD produces excessive amounts of collagen due to fibrosis. The vaccine group had low observed collagen amounts similar in appearance ot the control group, possibly a result of down-regulation of TGF-β1.

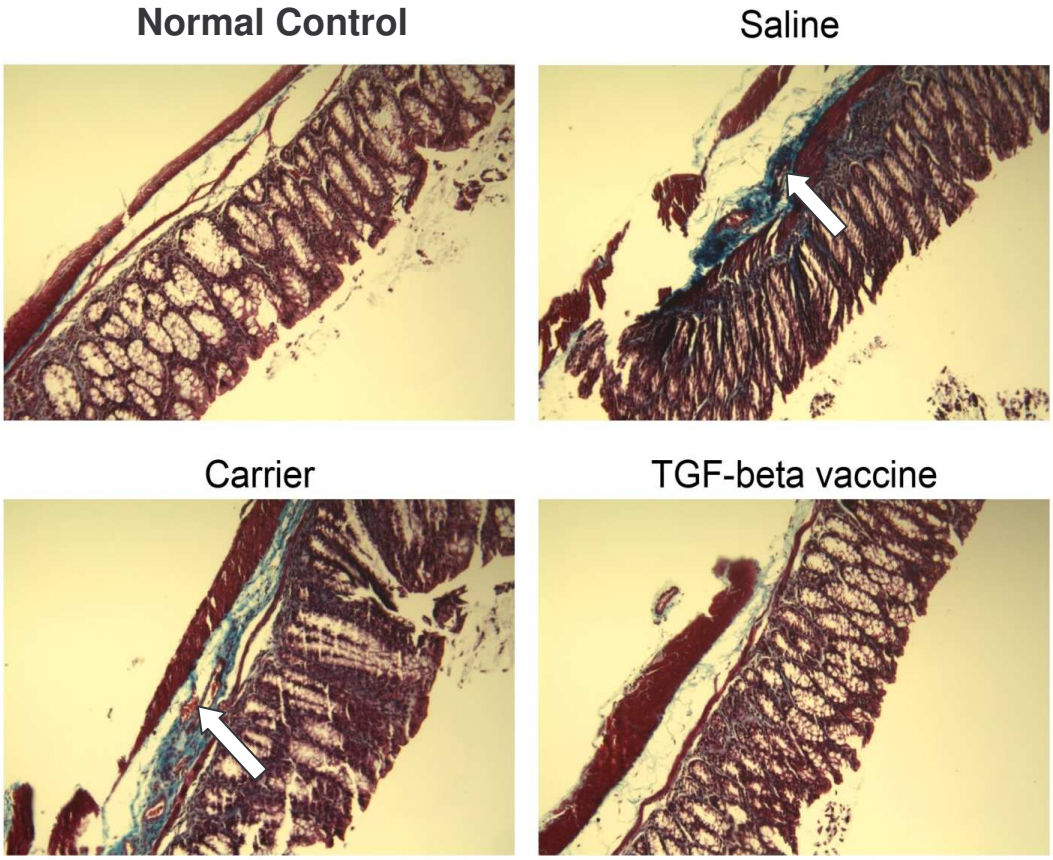


Figure 9: Masson's Trichrome staining of colon tissue sections from BALB/c mice. Photographs were originally taken at 40x magnification.

The saline and carrier groups showed an increase in collagen (blue) whereas the TGF-β1 vaccine had a decreased collagen level similar to the normal control. The normal control group consisted of no injections (no TNBS, no vaccine); the saline control group consisted of saline injections (in place of vaccine injections) and TNBS; the carrier control group consisted of injections of HBcAg and TNBS; and the vaccine group consisted of injections of TGF-β1 vaccine and TNBS.

5.7 Soluble collagen assay

The vaccine group had 844.4±93pg/colon weight and 683±124 pg/colon weight of less total collagen amount that the carrier and saline groups respectively (Figure 10). In fact, the TGF-β1 vaccine group displayed similar levels to the normal control group. This reduction in collagen amount, confirms the reduction in collagen seen in the Masson's Trichrome histological stain.

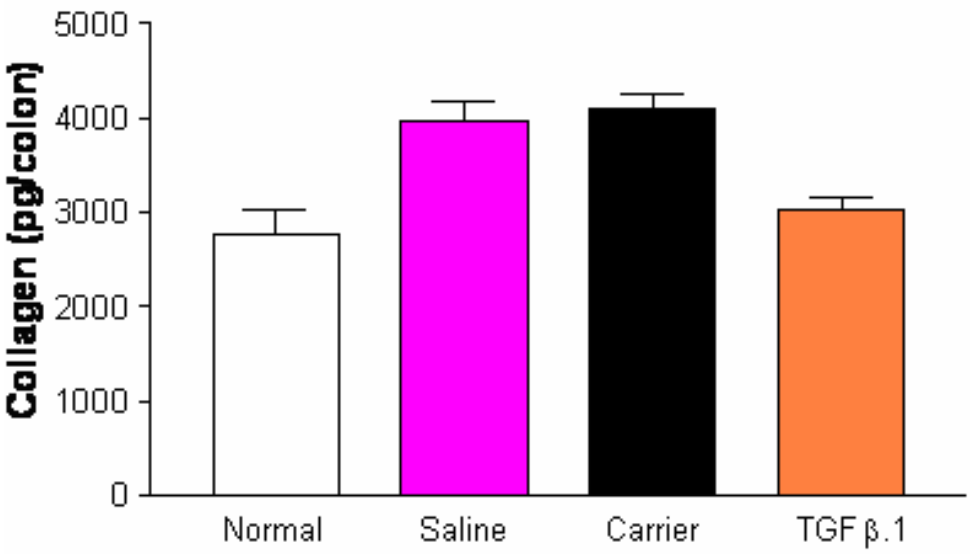


Figure 10: Mean total collagen amount (pg) in colon tissue for the normal, saline, carrier and TGF-β1 vaccine groups (±SE).
Total collagen amounts were 844.4±93pg/colon weight and 683±124 pg/colon weight less in TGF-β1 vaccine group than compared to the carrier and saline groups respectively ($P<0.05$).

One way analysis of variance (non-parametric) was performed, which resulted in statistically significant differences ($P<0.05$) in collagen results between normal and saline/carrier groups, and between saline/carrier and TGF-β1 vaccine groups (Table 6). Differences in results between the TGF-β1 vaccine group and the normal control group were not considered statistically significant with a P value greater than 0.05.

Table 6: One-way analysis of variance (non-parametric) for treatment study comparing collagen amounts between the various experimental groups. Results were considered statistically significant with a P value of less than 0.05.

	SS	Df	MS
Treatment (between columns)	4278000	3	1426000
Residual (within columns)	3112000	16	194500
Total	7390000	19	
Newman-Keuls Multiple Comparison Test	Mean Diff.	Q	**P value**
Normal vs Carrier	-1208	5.48	**P < 0.01**
Normal vs Saline	-1047	4.597	P < 0.05
Normal vs TGFβ-1	-363.9	1.65	**P > 0.05**
TGFβ-1 vs Carrier	-844.4	4.69	**P < 0.05**
TGFβ-1 vs Saline	-683	3.617	P < 0.05
Saline vs Carrier	-161.4	0.8547	P > 0.05

6. DISCUSSION

Since the introduction of vaccines against microbes, many infectious diseases have become well-controlled. However, self-molecule dysfunction associated diseases such as autoimmune diseases and some cancers have continued to increase significantly over the past several decades. The innovatory application of this vaccine may bring about a huge improvement in the treatment of these severe human immune diseases.

Although blocking only one molecule may not be enough to attain significant benefit on all clinical manifestations and in all patients, studies have shown that anti-key molecule therapy, if the molecule is deeply important in the pathogenesis of the disease, is effective in the treatment of the disease (Dalum *et al.*, 1999). Anti-TNFα therapy, with its monoclonal antibodies or soluble receptors, has been shown to be successful in the treatment of inflammatory bowel disease (Sandborn., 2005; Rutgeerts *et al.*, 2005) and severe asthma (Howarth *et al.*, 2005).

We sought to develop a TGF-β1 peptide-based vaccine, which has the unique advantage of inducing specific antibody responses dominantly to a key site thereby avoiding possible cross-reactions when compared with the vaccines based on the full length molecule. Herein, HBcAg particle was used as a carrier to enhance the immunogenicity of self-peptide. An HBcAg particle is a highly promising delivery system for foreign peptide-based vaccines (Pumpens and Grens, 2001). The highly

immunogenic features of HBcAg attribute to the fact that it can function as both a T cell dependent- and a T cell independent-antigen (Milich and McLachlan, 1986; Fehr *et al.*, 1998). This is associated with its capability of activating a high frequency of naïve B lymphocytes to function as primary antigen-presenting cells, presenting a 10^5 fold higher efficiency than typical dendritic cell and macrophage interactions (Milich and McLachlan, 1986). One virus-like HBcAg particle consists of 180 or 240 HBcAg molecules, each of which is inserted with one TGF-β1 peptide. Therefore, a total of 180 or 240 TGF-β1 peptides are displayed on the surface of a single vaccine particle in a highly ordered repeat of epitopes with optimal distances between each other. This unique structural feature, along with the well defined T cell epitopes in the primary sequence, also explains the high immunogenicity of the vaccine. Furthermore, the highly repetitive ordered array of inserted self-polypeptides on the surface, help eliminate the ability of the immune system to functionally distinguish between foreign and self, as well as provide extra benefits for breaking B cell tolerance (Chackerian *et al.,* 2002). Through extensive work, Dr. Peng's lab has successfully included in the application of HBcAg as a vaccine carrier, the ability to break self-immune tolerance and induce high titered auto-antibodies. The first objective was completed as the vaccine was purified and expressed.

The second objective of the project was aimed at testing *in vivo* the potential of TGF-β1 peptide based vaccines in inflammatory bowel disease treatment. For

this purpose, a well established chronic model of colitis (with multiple administrations of small amounts of TNBS) was used. This is because a chronic model is more relevant to human IBD (Elson *et al.*, 1996; Lawrance *et al.*, 2003; Fichnter-Feigl *et al.*, 2006). Experiments were performed using the BALB/c strain of mice. BALB/c mice are commonly used to model autoimmune responses as they show extreme responses following specific antigen or hapten sensitization (Chen *et al.,* 2005).

A number of evaluation methods – bodyweight, histological analysis of colon tissue, and total soluble collagen content – were used to evaluate the effects of the TGF-β1 vaccine. Research has shown that chronic mouse models of colitis gain less body weight over a period of eight weeks than normal, healthy mice and models in this study support those findings (Zhou *et al.,* 2007; Sandbornh 2005). Successful TGF-β1 vaccine would mean that this weight 'loss' would not be observed. This was the case, as the vaccine group gained 10% more body weight than the saline or carrier groups in the treatment study.

Collagen deposition is an important indicator for chronic IBD. Histological analysis of colon tissue revealed a marked decrease in collagen production in the colon in the vaccine group. With IBD, the amount of inflammation in the colon increases greatly, but down regulation of TGF-β1 with a TGF-β1 vaccine appears to have improved this. To confirm this, quantitative analysis through total soluble collagen assay revealed statistically significant differences ($P<0.05$).

As shown in figure 10, the total collagen per colon (pg/colon) in the carrier and saline groups increased significantly by 1208pg±93 and 1047±124, respectively, when compared to the normal control group ($p<0.01$). This was expected as the carrier group did indeed have chronic IBD and previous studies have proven an increase in collagen with TNBS-induced chronic colitis (Kanazawa *et al.*, 2001). Immunization with the TGF-β1 vaccine significantly inhibited collagen production by 844.4pg±52 and 683pg±52 when compared to immunization with carrier protein ($P<0.05$) or saline group ($P<0.05$) respectively. In fact, a comparison between the collagen amounts in the TGF-β1 vaccine group and the normal control group shows no statistical difference (which should be confirmed by a power analysis). Thus, TGF-β1 immunization has resulted in reduced collagen production, to the point that it is similar to disease free animals. A promising result, this creates not only a possible treatment for IBD, but also proves that down regulation of cytokines can have therapeutic potential.

Studies have voiced concerns with this vaccine as TGF-β1 is known to be a key suppressor of inflammation. Targeted disruption in the mouse TGF-β1 gene has resulted in a severe, multi-focal inflammatory response (Del Zotto *et al.*, 2003). Considering this, neutralizing TGF-β1 may exacerbate the inflammation responses. Interestingly, this was not the result in our studies using this peptide based vaccine. Hematoxylin and eosin (H&E) staining has shown that immunized animals have a suppressed amount of inflammation as compared to control groups after multiple

administrations of TNBS and a recovery period (data not shown here as it was not fully completed by time of printing). These results raise our interest to do further research to check the possible roles of TGF-β1 in the processes of acute inflammation and chronic inflammation responses. Future studies could clarify whether the vaccine acts by neutralizing TGF-β1 directly.

A potential concern with cytokine vaccines is that the injection of cytokine vaccines might induce a permanent autoimmune condition, as some anti-bacterial and -virus vaccines do, eliminating all of the cytokine that is required to maintain normal functions (Zagury and Gallo, 2004). Actually, cytokine vaccines are expected to down-regulate over-expressed cytokines, not those in normal tissues, because they target the cytokines ectopically accumulated in the extracellular compartment in inflammatory tissue where inflammation-induced angiogenesis benefit the accessibility of antibodies to cytokines. As well, some cytokines take effect by short-distance transfer between cells and since the antibodies cannot neutralize them in time they will not abolish normal physiological effects (Zuany-Amorim *et al.*, 2004; Tanaka *et al.,* 2001).

Additionally, as the immunogenicity of cytokine vaccines is less than that of microbe vaccines, the titers of cytokine vaccine-induced antibodies (which last about 4 months) are reversible and able to be adjusted by the frequency of immunization. In theory, cytokine vaccines should be safe and effective, and this has been documented by studies in animal and human trials. This encourages the

proposed approach of using cytokine peptide-based vaccines as a pioneering avenue for the treatment of IBD (Gringeri *et al.*, 1996; Zagury *et al.*, 2003).

Future work in this area of study is necessary. As a preliminary study, small sample sizes of the control group were used. Due to this small sample, an illness in the control group altered results, showing a smaller weight gain than previous studies. Further research would include a larger control sample so that more accurate comparisons could be made. As noted earlier, assessment of inflammation would be done throughout the study to check sufficiently the affects on the inflammation responses, produced by TGF-β1 vaccine. A more formal study would also include a dynamic display of the intensity and duration of the specific antibody throughout the study. Furthermore, an *in vitro* experiment, in which one uses sera from vaccinated mice to neutralize TGF-β1 activity on cultured sensitive cells, could be performed to identify whether the vaccine can take effect *in vivo* by directly blocking TGF-β1 signal transduction.

7. CONCLUSION

Preliminary studies show that in a murine model for chronic colitis, immunization with a TGF-β1 peptide-based vaccine decreases the amount of weight lost and reduces collagen deposition in the colon tissue. These observations support the hypothesis that TGF-β1 may play a role in the pathogenesis of inflammatory bowel disease and administration of a TGF-β1 vaccine could be a potential therapeutic approach in the treatment of chronic intestinal inflammation. This provides evidence that strongly suggests the administration of cytokine peptide-based vaccines hold excellent potential as an effective therapeutic approach in treating disorders where elevated cytokines are involved with the pathogenesis of the disease.

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9. APPENDIX

9.1 Recipes

9.1.1 Wiegert's Iron Hematoxylin

Wiegert's Iron Hematoxylin was prepared for Masson's Trichrome staining by mixing equal volumes of Part A and Part B from the Wiegert's Iron Hematoxylin Set (Sigma Aldrich lot# HT1079-1SET).

9.1.2 Phosphotungstic/Phosphomolybdic acid solution

Phosphotungstic/Phosphomolybdic acid solution was prepared for Masson's Trichrome staining by mixing one volume of phosphotungstic acid with 1 volume of phosphomolybdic acid and 2 volumes of distilled water. Solutions were provided with the Masson's Trichrome staining kit (Sigma Aldrich lot# HT15-1KT).