

**Targeting IL-12 and/or IL-23 by employing peptide-based vaccines
in the amelioration of murine colitis**

By

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Abstract

Overexpression of IL-12 and IL-23 has been implicated in the pathogenesis of Crohn's disease. Targeting these cytokines with monoclonal antibodies has emerged as an effective therapy, but one with adverse reactions. In this study, we sought to develop peptide-based virus-like particle vaccines specific to p40 unit (shared by IL-12 and IL-23) or IL-12 (p35) or IL-23 (p19) and evaluate the effects of the vaccine in 2,4,6-trinitrobenzene sulphonic acid (TNBS)- and dextran sodium sulfate (DSS)-induced acute and chronic murine colitis.

Three vaccines against p40 induced high-titered and long-lasting antibodies to IL-12, IL-23 and p40 without the use of adjuvants. Vaccine-induced antibodies could block IL-12- and IL-23-induced biological functions *in vitro* dose-dependently. One of the three p40 vaccines was selected for further evaluation in acute and chronic colitis.

Administration of the vaccine before or after the commencement of TNBS or DSS delivery, significantly improved body weight loss and decreased inflammatory scores, collagen deposition, and the expression of p40, IL-12, IL-23, IL-17 and TNF in colon tissues, compared with mice receiving carrier protein (HBcAg) or saline.

Moreover, in mesenteric lymph nodes, vaccinated mice exhibited a trend to lower percentages of Th1 cells in acute colitis and of Th17 cells in chronic colitis compared to carrier and saline controls. Vaccinated mice also had higher ratios of Treg/Th1 and Treg/Th17 and higher percentages of apoptosis in Th1 and Th17 cells than controls.

Vaccine treatment decreased the infiltration of CD11c⁺ cells into the gut, but promoted the production of IL-10 from these cells. Safety evaluation indicated that vaccine immunization did not increase the susceptibility to the infection of *Chlamydia muridarum*.

Two vaccines specific to IL-12 (against p35) and one vaccine to IL-23 (against p19) were also developed. They induced specific antibodies against IL-12 and IL-23, respectively. IL-23p19 vaccine immunization, not IL-12p23 vaccine, ameliorated TNBS-induced chronic colitis.

In summary, IL-12/IL-23p40 vaccine treatment ameliorated murine colitis through rebalancing Th1/Th17/Treg responses, promoting Th1 and Th17 apoptosis, and promoting IL-10 production, and did not increase the severity of chlamydia muridarum infection. This vaccine strategy may provide a novel long-term treatment for Crohn's disease.

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Abbreviations

APCs	Antigen presenting cells
CD	Cluster of differentiation
CTL	Cytotoxic T cells
DAI	Disease activity index
DC-SIGN	DC-specific ICAM-3 grabbing non-integrin
DCs	Dendritic cells
DSS	Dextran sodium sulfate
ELISA	Enzyme linked immunesorbent assay
FACS	Fluorescent activated cell sorting
FcR	Immunoglobulin Fc receptors
Foxp3	Forkhead box P3
GATA-3	GATA-binding protein 3
GALT	Gut associated lymphoid tissue
GWAS	Genome-wide association studies
HBcAg	Hepatitis B core antigen
HBSS	Hank's balanced salt solution
H&E	Hematoxylin and eosin
HSCT	Haematopoietic stem-cell transplantation
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iTregs	Inducible regulatory T cells
Jak	Janus kinase
KLH	Keyhole limpet hemocyanin

LAK	large activated killer cells
LPMC	Lamina propria mononuclear cells
LPS	Lipopolysaccharide
mAb	Monoclonal antibodies
MHC	Major histocompatibility complex
MDP	Muramyl dipeptide
MLN	Mesenteric lymph nodes
MPO	Myeloperoxidase
MoPn	Chlamydia muridarum
mRNA	Messenger RNA
NF- κ B	Nuclear factor kappa B
NKT	Natural killer T cells
NLRs	Nod-like receptors
NOD	Nucleotide-binding oligomerization domain
nTreg	Naturally occurring regulatory T cells
OX40L	OX40 ligand
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern-recognition receptors
PBS	Phosphate buffered saline
PMA	Phorbol myristate acetate
R	Receptor
RT-PCR	Real-Time Reverse-Transcription Polymerase Chain Reaction
SNP	Single-nucleotide polymorphisms
Stat	Signal transducer and activator of transcription
T-bet	T-box transcription factor
TCR	T cell receptor
TGF	Transforming growth factor

Th	Helper T cells
TL1A	TNF-like ligand 1A
TLRs	Toll-like receptors
TNBS	2,4,6-trinitrobenzene sulphonic acid
TNF	Tumor necrosis factor
Treg	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
VLP	Virus-like particle

Part One. Introduction

I. Introduction of inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract, which clinically contains Crohn's disease, ulcerative colitis and other conditions.¹ The inflammation of the intestinal mucosa in IBD is characterized by episodes of abdominal pain, diarrhea, bloody stools, weight loss, and the influx of neutrophils and macrophages that produce cytokines, proteolytic enzymes and free radicals that result in inflammation and ulceration.^{1,2}

IBD 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 since the beginning of the 21st century IBD has been considered one of the most prevalent gastrointestinal diseases.³⁻⁶ Estimates indicate that as of 2005, about 1.4 million Americans and several millions persons in the worldwide have been diagnosed with IBD. Roughly 30% are children and adults between 10 to 30 years of age.⁷ The incidence of Crohn's disease in the North America has been estimated at between 3.1 and 14.6 per 100,000, with a prevalence of between 26.0 and 198.5 per 100,000.¹ For ulcerative colitis, both incidence and prevalence are estimated at between 2.2 and 14.3, and 37.5 and 229 per 100,000, respectively.¹ A population-based, multiple province-wide study in Canada by Bernstein C. *et al.* indicates that approximately 0.5% of Canadians have IBD (about 170,000 or approximately 1 in 180); and incidence rates across the country are among the highest in the world.⁸

Crohn's disease usually involves the terminal ileum, cecum, peri-anal area and colon, but it can affect any region of the intestine in a discontinuous pattern.⁹⁻¹¹ In

contrast, ulcerative colitis involves the rectum and can affect part of the colon or the entire colon in a continuous pattern.⁹⁻¹¹ Crohn's disease is exhibited histologically a thickened submucosa, transmural inflammation, fissuring ulceration and granulomas, whereas the inflammation in ulcerative colitis is limited to the mucosa and submucosa with cryptitis and crypt abscesses.^{10, 11}

II. Pathogenesis of IBD

Although the cause of IBD remains unknown, considerable progress has been made in recent years to unravel the pathogenesis of this disease. Studies have provided evidence that the pathogenesis of IBD is associated with genetic susceptibility of the host, intestinal microbiota, other environmental factors, and immunological abnormalities.^{12, 13}

1. Genetic factors

Genome-wide association studies (GWAS) have identified 99 non-overlapping genetic risk loci, including 71 identified in Crohn's disease and 47 in ulcerative colitis.^{11, 14, 15} Of these genetic risk loci, 28 are shared between Crohn's disease and ulcerative colitis. The analysis of the genes and genetic loci identified in IBD indicates that several pathways play important roles in maintaining intestinal homeostasis, such as epithelial barrier function, innate mucosal defence, immune regulation, cell migration, autophagy, adaptive immunity and metabolic pathways associated with cellular homeostasis.¹¹ The permeability of the epithelial barrier enables microbial incursion, which is recognized by the innate immune system, which then launches appropriate tolerogenic, inflammatory and restitutive responses partially by secreting extracellular mediators that recruit other cells, including adaptive immune cells.¹¹

Nucleotide-binding oligomerization domain 2 (NOD2) is the first gene found to be associated with Crohn's disease, which is frequently mutated in patients with Crohn's disease, occurring in around one third the patients.^{7, 16} For instance, Crohn's disease patients associated with 1007fs mutation in the NOD2 gene show a much more severe disease phenotype than other Crohn's disease patients; while R702W and G908R mutations lead to increase inflammatory cytokine responses.⁹ NOD2, a member of the cytosolic Nod-like receptor (NLR) family based on their triggers and the signaling pathways that they control, is one of the two important and distinct detection systems to sense microbial invaders.⁹ NLR proteins are found in the cytoplasmic compartment, and the other detection system is membrane-bound receptors, termed toll-like receptors (TLRs). NOD2 can recognize the minimal bioactive fragment of peptidoglycan found in the cell wall of both Gram-negative and Gram-positive bacteria, called muramyl dipeptide (MDP).^{9, 17, 18} Thus NOD2 is thought to be important as an intracellular sensor of bacterial components.^{9, 17, 18} Upon binding to its ligand-MDP, a conformational change of NOD2 occurs that allows it to bind the caspase recruitment domain of the adaptor protein RIP2.^{9, 17} RIP2 then induces the polyubiquitination of nuclear factor kappa B (NF- κ B) essential modulator-I κ B γ , which is the key scaffolding protein of NF- κ B.¹⁷ It then activates NF- κ B, leading to secretion of some proinflammatory cytokines, such as IL-12. It can also activate MAPK signaling pathway.^{9, 17}

NOD2 has also been implicated in the initiation of autophagy.^{11, 19} Autophagy is a highly conserved recycling process involving the degradation of cytosolic contents and organelles, as well as resistance against infection and the removal of intracellular microbes.^{11, 19} MDP stimulation can activate the autophagy process leading to

confinement of intracellular bacteria within autophagosomes and subsequent control of infection.²⁰ Following bacterial recognition, NOD2 serve as molecular scaffolds for the nucleation of the autophagy machinery by interacting with ATG16L1.²⁰ ATG16L1 is essential for all forms of autophagy. Interestingly, ATG16L1 polymorphisms are also linked to Crohn's disease like NOD2.²⁰

Recently, GWAS has identified numerous single-nucleotide polymorphisms (SNP) in *IL-23R*, with high association for Crohn's disease and ulcerative colitis.^{21, 22} Of interest, Arg381Gln, an uncommon allele at a highly conserved amino-acid polymorphism, confers a protective effect in patients with Crohn's disease or ulcerative colitis.²²

2. Microbial factors

IBD appears to result from abnormal host immune responses to the intestinal microbiota.²³ Intestinal microbiota is the major environmental driver of IBD. The gastrointestinal tract of the human body is colonized at birth by a vast range of microorganisms that numerically exceed host cells by around 10 times.^{23, 24} These microorganisms contain around 100 fold as many genes as are present in the human genome.^{23, 24} This intestinal microbiota is necessary for intestinal homeostasis and function, health and disease.^{23, 24} Tolerance to intestinal microbiota must be maintained to benefit from their coexistence; on the contrary, colonization with specific pathogenic microbes might be detrimental to the host, leading to disease.²⁴ The coexistence with the microbiota can be beneficial to host metabolism and gastrointestinal development.^{23, 24} In addition, the commensal microorganisms are required for the development and differentiation of the local and systemic immune system and nonimmune components.^{23,}
²⁴ And they can protect the host from enteric pathogenic infections via colonization

resistance and via synthesis of factors promoting mutualism.²³ For example, induction of a transforming growth factor (TGF)- β -rich environment by indigenous *Clostridium species*, enhances regulatory T cell (Treg) numbers and function in the colon and the resistance to DSS-induced murine colitis.^{23,25} Therefore, the host has evolved numerous mechanisms to maintain the homeostasis.

Both commensal and pathogenic microorganisms determine the consequence of an infection. Bacteria can be detected by the recognition by pattern-recognition receptors (PRRs, including TLRs, NLRs and RIG-like receptors) of pathogen-associated molecular patterns (PAMPs), which are found in many species of microorganisms.^{23,24} The recognition of PRRs activates the innate immune system, leading to the activation of NF- κ B, which stimulates the production of pro-inflammatory cytokines and chemokines and this can also enhance tissue homeostasis and mucosal tolerance in the absence of barrier broken.²⁴ The PAMPs are small molecular motifs conserved within many species of non-pathogenic and pathogenic microbes. Therefore, PRRs recognition is largely unable to distinguish between non-pathogenic and pathogenic microbes.^{24,26} It leads to recognition of innate immunity to commensal microorganisms, having a crucial role in the maintenance of intestinal homeostasis, and critical for the protection against gut injury and associated mortality.^{24,26} The imbalance of these interactions can contribute to the development of intestinal inflammation.

Due to the complexity and multiplicity of the intestinal microbiota, our understanding is developing slowly on the roles of commensal and pathogenic microorganisms in establishing a healthy intestinal epithelial barrier and in disrupting the intestinal homeostasis.^{23,24} And the specific roles of the intestinal flora in inflammatory

conditions and the effects of specific microorganisms, which can initiate IBD, have not been systemically encapsulated. Based on the studies in human and animal infection models, it is unlike that a single infection causes or triggers the IBD in humans. But the intestinal microbiota clearly promotes the development of IBD.^{23, 24} For instance, the presence of *Mycobacterium avium* subsp *paratuberculosis*, and adherent-invasive *Escherichia coli* is increased in Crohn's disease patients; the presence of *Clostridium difficile* is increased in both Crohn's disease and ulcerative colitis patients in relapse and remission states.²⁷ And the increased mucosal bacterial counts and decreased anti-inflammatory commensal *Faecalibacterium prausnitzii* are also found in Crohn's disease patients.²⁷

In summary, microbial factors play important roles in the pathophysiology of IBD through impacting the immune systems in major ways, and affecting host metabolism and gastrointestinal development.^{23, 24}

3. Other environmental factors

The important role of other environmental factors in the pathogenesis of IBD is supported by recent trends in IBD epidemiology. The frequency of Crohn's disease has significantly increased in the more developed countries over the past 50 years, and the recognition of the disease corresponding with progressive industrialization in the less developed countries has also increased.^{28, 29}

Food intake is another important factor that affects the development of IBD.³⁰ Studies have provided evidence that intake of fast foods containing many fat and sugar-rich foods may exacerbate the development of Crohn's disease.³⁰ One study also shows that medium-chain fatty acids are more effective in accelerating intestinal inflammation

than long-chain fatty acids.³¹ In most of western developed countries, sugar-rich foods have been recognized as one of the risk factors for Crohn's disease.³⁰

Smoking is another example of a disease-specific modifier that seems to worsen Crohn's disease while being protective against ulcerative colitis.^{28,29} Smoking has been shown to affect cellular and humoral immune responses and to promote colonic mucus production.^{28,30} Nicotine, an essential content of cigarettes, has an inhibitory effect on Th2 cell function, but has no effect on Th1 cells function.²⁸ Evidence also suggests that smoking impairs autophagy, a process thought to be involved especially in Crohn's disease.³²

There are other environmental factors that influence the development of IBD, including psychological stress, appendectomy, diet, antibiotics, etc. For example, appendectomy is an independent risk factor for developing Crohn's disease, while it is protective for ulcerative colitis.³³ Intestinal flora and mucosal defense systems also play major roles in the pathogenesis of IBD.

4. Immunological abnormalities

The immunological dysregulation in IBD is characterized by epithelial damage (abnormal mucus production, defective repair), expansion of inflammation driven by intestinal flora and a large number of cells infiltrating into the lamina propria including T cells, B cells, macrophages, DCs, neutrophils, and a failure of immune regulation to control the inflammatory response.^{3,34} The activated lamina propria cells produce high levels of proinflammatory cytokines in the local tissue, including TNF, IL-1 β , IFN- γ and cytokines of the IL-23/Th17 pathway.^{3,10}

Innate and adaptive immunity: The intestinal immune system is divided into innate immunity and adaptive immunity. Innate immunity includes the barrier function of the intestinal mucosa, antibacterial proteins (complement, defensins, etc.), the acid PH value of stomach to limit microbial growth, innate immune cells (neutrophils, macrophages, dendritic cells (DCs) and natural killer T cells, etc.), and innate cytokines and molecules (IL-1, TNF, defensins).³⁴ Adaptive immunity is pathogen-specific, and is usually initiated under the circumstances in which the innate immune responses cannot circumvent the stimulation of a pathogen.³⁴ After exposure to a pathogen, it usually takes several days to finally activate adaptive immune responses, including T and B cells.³⁴ The initiation of immune response to intestinal flora is tightly regulated, and this regulation determines the occurrence of immune tolerance or a defensive inflammatory response. Disturbance of the balance of these responses can cause IBD.¹⁰

4.1 Dysregulation of innate immune system

Dysregulation of Intestinal epithelial barrier

The 400-mm² single layer of intestinal epithelial cells (IEC) is the primary cellular barrier. It functions as a selective barrier to confine the entry of antigens to the mucosal immune system for the aim of inducing oral tolerance to commensal microorganisms or food antigens, and for the aim of host defense against pathogens.^{16, 35} Therefore, the IEC play important roles in the gut in an immunological context through providing the antigen-sampling machinery, expressing PPRs (eg, TLR, NLR), and involving in the establishment of the tolerogenic environment in the intestine and controlling of the immune system in the gut associated lymphoid tissue (GALT).³⁶ The

tight junctions between epithelial cells allow for the selective penetration of nutrients, fluids, and microorganisms. Normal gastrointestinal permeability relies on the intact epithelium, surface mucus, peristalsis and the production of host protective factors.³⁷

Epithelial integrity is disturbed in IBD patients, and mice that have deficient epithelial barrier functions develop colitis.¹⁶ Proinflammatory cytokines, secreted during intestinal inflammation such as TNF or IFN- γ , can increase the epithelial permeability by regulating tight junctions and promoting apoptosis.¹⁶ IFN- γ increases paracellular permeability and induces endocytosis of tight junction transmembrane proteins.³⁸ Increased permeability to macromolecules has been found in IBD patients.³⁹ The high apoptotic rate of epithelial cells also leads to diminished epithelial barrier function observed in IBD. Studies have shown that apoptotic rate is increased in mildly to moderately inflamed colon of Crohn's disease and ulcerative colitis.¹⁶ And apoptosis allows the loss of ions, water and the entry of small antigens.⁴⁰ IL-13, a key effector Th2 cytokine in ulcerative colitis, also shows the ability to impair epithelial barrier function by affecting epithelial apoptosis, tight junctions and reconstitution velocity.⁴¹ The reduced velocity of restitution can play a role in the response of an epithelial layer to naturally occurring or pathogen-induced small lesions.⁴¹

The intestinal epithelium is also responsible for electrolyte transport. Disrupted electrolyte transport may lead to diarrhea.¹⁶ Around 50% of Crohn's disease patients and almost 100% of ulcerative colitis patients have diarrhea as a symptom. The deficiencies of electrolyte transport in IBD contain hyporesponsiveness of electrogenic anion secretion, reduced synthesis of epithelial sodium channels, reduced NaCl absorption, and alteration of electrochemical gradient.¹⁶

The intestinal epithelium may also be improved, protected and repaired by growth factors and cytokines.⁴² These growth factors and cytokines play vital roles in the regulation of cell proliferation, differentiation, angiogenesis, inflammation, intestinal defense mechanisms, and intestinal wound repairs.¹⁶ Currently, at least 30 different peptide growth factors have been shown to be involved in the maintenance of intestinal mucosal integrity, including epidermal growth factor, the TGF- β family, the insulin-like growth factor family, the fibroblast growth factor family, the colony-stimulating factor family, etc.⁴² Of these factors, epidermal growth factor, insulin-like growth factor family, fibroblast growth factor family and colony-stimulating factor family appear promising in the treatment of IBD and are being evaluated in clinical trials.¹⁶

Dendritic cells

Dendritic cells (DCs) are hemopoietic bone marrow progenitor derived leukocytes, which are widely distributed throughout the body in small numbers.^{43,44} Although DCs were first described by *Paul Langerhans* in the late nineteenth century, their role as a central coordinator was not established until 1973 by *Ralph Steinman* et al.^{45,46} DCs are professional antigen presenting cells (APCs) specialized in antigen capture, process and presentation to T cells. DCs are considered to be the most potent APCs that orchestrate innate and adaptive immune responses.⁴⁷

DCs are found throughout the gut, including the lamina propria, isolated lymphoid follicles, Peyer's patches and mesenteric lymph nodes (MLNs).⁴⁴ DCs have been documented both in the maintenance of immune tolerance to the commensal microorganisms and food antigens and in the initiation of host defence against pathogens.⁴⁸ In the intestine, DCs subtypes have been characterized into conventional

DCs and plasmacytoid DCs, similar to those in other peripheral lymphoid organs.^{36, 43}

Conventional DCs are further divided into: CD11b⁺CD8 α ⁻ DCs in the subepithelial dome, preferentially secreting IL-10 and inducing Th2 cells; CD11b⁻CD8 α ⁺ in the interfollicular regions, and CD11b⁻CD8 α ⁻ subsets in both areas, preferentially secreting IL-12 and inducing Th1 cells.^{36, 43} In the steady-state lamina propria, two major DCs subsets have been characterized based on the reciprocal expression of CD103 and CX₃CR1.^{36, 43}

DCs are present in an immature state with high phagocytic ability localized in peripheral tissues and in discrete regions of organized secondary lymphoid organs.⁴³

Immature DCs constitutively acquire foreign and self-antigens from the intestinal lumen through: 1) microfold (M) cells which transcytose antigens from the lumen to the mucosa;⁴³ 2) CX₃CR1⁺ DCs extending dendrites between IEC and into the intestinal lumen to directly capture antigens and present them to CD4⁺ T cells, which differentiate into effector T cells and secrete proinflammatory cytokines;^{43, 44, 48} 3) direct sample antigens as a result of breaches in the epithelial integrity as seen in intestinal inflammation;^{43, 48} 4) mechanisms mediated by the fetal Fc receptor;^{43, 48} 5) lamina propria CD103⁺ CX₃CR⁻ DCs receiving conditioning from epithelial cells and serving as the inducer of Treg cells.^{43, 44, 48}

After capturing antigens, immature DCs migrate from the Peyer's patch and lamina propria to the draining MLN, where they present the antigens to naïve T cells.³⁶ During the migration, DCs gradually become mature with the expression of costimulatory molecules. In addition, the lamina propria DCs constitutively transport antigens from apoptotic IECs or commensal microorganisms to the draining MLN to interact with T and B cells to initiate tolerogenic responses.³⁶ In particular, CD103⁺ DCs isolated either from

the lamina propria or from the MLN promote the development of Foxp3⁺ Tregs, which rely on retinoic acid and TGF-β.⁴⁴ And DCs conditioned in the presence of IEC-secreted thymic stromal lymphopoietin (TSLP) are less capable of secreting IL-12, and promoting Th2 responses.³⁶

In the presence of pathogens, the migration of DCs to the MLN increases.

Activated DCs trigger a protective immune response including activating effector cells, and determining which CD4⁺ T helper cells (e.g., Th1, Th2, or Th17) will predominate.⁴⁸

In patients with IBD, DCs are attracted by the upregulated chemokines such as CCL20 or addressins such as mucosal vascular addressin cell adhesion molecule-1, and accumulate at inflammatory sites. Correlated with large amounts of DCs accumulation in the intestine, plasmacytoid DCs and myeloid DCs are down-regulated in the peripheral blood of patients with active IBD.^{44, 48} In the lesions of Crohn's disease, the numbers of CD83⁺ DC and DC-specific ICAM-3 grabbing non-integrin (DC-SIGN)⁺ populations are significantly increased, while IL-12 and IL-18 are only detected in DC-SIGN⁺ DC and not in CD83⁺ DC.⁴⁹ DCs from MLN of patients with Crohn's disease preferentially induce the Th1 response.⁵⁰ Three types of DC are identified in the MLN of Crohn's disease and ulcerative colitis patients, including mature DCs, myeloid DCs and plasmacytoid DCs.⁵⁰ Myeloid DCs from MLN of patients with Crohn's disease produce high levels of IL-23 and low levels of IL-10.⁵⁰

Besides reacting inappropriately to captured antigens, intestinal DCs might also receive inappropriate signals from IEC during intestinal inflammation.⁴⁴ IEC isolated from about 70% of patients with Crohn's disease do not express TSLP mRNA and cannot control the DC-mediated proinflammatory response, leading to upregulated production of

IL-12 by DCs, which then polarizes Th1 responses.⁵¹ NOD2 expression on DCs may also play a critical role in their responses to microbes, because DCs derived from NOD-2 deficient Crohn's disease patients have an impaired ability to induce IL-17 production upon MDP challenge.⁵²

Evidence from animal models also demonstrates the role of DCs in the chronic intestinal inflammation.^{44, 48} Large amounts of activated DCs accumulate in the lamina propria and MLN in murine models of colitis.^{44, 48} In the CD45RB^{hi} CD4⁺ T cells transfer model of colitis, large amounts of CD11c⁺ DCs expressing activation marker OX40 ligand (OX40L) are found in the MLN, and transferred T cells create aggregates with CD11c⁺ DCs in the lamina propria.⁴⁴ Blocking OX40-OX40L interaction ameliorates colitis.^{53, 54} Analysis of the DC phenotype in murine colitis has shown that colonic lamina propria mature DCs express higher levels of costimulatory molecules (CD40, CD80, and CD86) and increase productions of IL-12p40 and IL-23p19 upon CD40 ligation.⁵⁵ IL-12p40 and IL-23p19 form IL-23, which is important for the stabilization of Th17 cells activation. When DCs are selectively ablated in mice before developing dextran sodium sulfate(DSS)-induced colitis, colitis is exacerbated compared with that of untreated mice.⁴³

Taken together, these data indicate that DCs play an important role in the pathogenesis of IBD through influencing the tolerance to the commensal microflora and dietary antigens, and affecting immune responses.⁴⁸

Macrophages and NKT cells

Macrophages are white blood cells that reside in the tissues, which have critical roles in the host immune defenses.⁵⁶ Macrophages are differentiated from monocytes after emigrating from blood vessels in response to different stimuli.^{56, 57}

Intestinal macrophages are the most abundant mononuclear phagocytes in the intestine, especially in the large intestine, where they account for around one-fifth of all leucocytes.^{48, 58, 59} Most of the macrophages are found underneath the epithelium of lamina propria of the intestine, and some can also extend transepithelial dendrites into the intestinal lumen.⁵⁹ Intestinal macrophages play critical roles in maintaining intestinal homeostasis, and are also drivers of the pathology associated with IBD.⁵⁸ Resident macrophages in the lamina propria immediately capture and clear the bacteria that breach the epithelial layer without initiating an inflammatory response, and thus are vital for maintaining homeostasis.⁵⁸ For instance, they efficiently eradicate phagocytosed enteric bacteria such as *Salmonella typhimurium* and *Escherichia coli*. They might also eliminate apoptotic and senescent cells and other cellular debris.^{48, 58} Moreover, resident macrophages in the lamina propria have a unique surface markers' expression pattern- low expression of costimulatory molecules, Fc receptors for IgA and IgG, complement receptors and integrin $\alpha 2\beta 1$.⁴⁸ It suggests that these macrophages do not function as professional APC, unlike macrophages from other body compartments.^{48, 57, 60, 61} And these macrophages do not secrete proinflammatory cytokines in reaction to cytokines or PAMPs, or following phagocytosis of apoptotic cells.^{48, 57}

On the other hand, many inhibitory receptors are expressed on intestinal macrophages, including CD172a, CD200R1, IL-10R and TGF- β receptors.⁵⁸ So the function of intestinal macrophages is influenced by corresponding soluble factors, such as

IL-10 and TGF- β , secreted by a wide range of cell types, including epithelial cells, fibroblasts, subepithelial myofibroblasts and lymphocytes.⁴⁸ Macrophages also have roles in tolerance through inducing anergic T cells or Tregs, and can impact the differentiation of naïve T cells into Th1, Th2 or Th17 cell types.⁶²

Studies have indicated the role of macrophages in the pathogenesis of IBD. In IBD patients, the number of macrophages increases in the inflamed mucosa which can initiate a rapid response to luminal microbial antigens, unlike the resident macrophages.⁴⁸ And many of the phenotype and functions of the macrophages in the inflamed sites differ from those in physical conditions. For instance, they express high levels of costimulatory molecules, such as CD40, CD86, CD80 and CD40.⁶³ In addition, aberrant CD14-expressing macrophages isolated from the mucosa of IBD patients produce high levels of IL-12 and IL-23 in vitro under the microbial stimulation.⁶⁴

Animal models of IBD also support the role of dysregulated macrophages in the pathogenesis of IBD.⁴⁸ IL-10^{-/-} mice spontaneously develop colitis in which macrophages preferentially differentiated into proinflammatory subsets that produce high levels of IL-12 and IL-23. Deficiency of macrophages in IL-10^{-/-} mice prevents the ongoing of colitis.⁶⁵

Natural killer T (NKT) cells are another cell type involved in the pathogenesis of IBD.¹³ NKT cells are a subset of lymphocytes that co-express TCR along with typical surface receptors of natural killer cells and share the features of both innate and adaptive immune cells.^{66, 67} NKT cells recognize phospholipids or glycolipids that are presented by CD1d on the APC resulting in a rapid innate response through producing large amounts of Th1, Th2 and Th17 cytokines that then initiate most of branches of the innate and

adaptive immune systems.¹³ NKT cells can be activated through multiple mechanisms, including direct activation by the recognition of CD1d on self- or microbial-derived lipids, and indirect activation via cytokines, such as IL-12 and IL-18.⁶⁶ Increased numbers of T cells expressing the NK marker CD161 are found in the inflamed lamina propria of ulcerative colitis patients, not in Crohn's disease. These cells can respond to CD1d with increased production of IL-13.⁶⁸ In consistent with this, deficiency of CD1d and NKT cells prevents the development of oxazolone-induced murine colitis, resembling like ulcerative colitis.⁶⁹

Innate immune cytokine pathways

In IBD, there is a markedly increased local production of various non-specific inflammatory mediators, such as free radicals, leukotrienes, chemokines, and pro-inflammatory cytokines (eg, TNF and TNF related cytokines, IL-6 family of cytokines), which follow the influx of inflammatory cells into the intestinal tissue.¹²

TNF and TNF related cytokines (TL1A): TNF is a 17 kDa pro-inflammatory cytokine mainly secreted by monocytes, macrophages and T cells that can impact proliferation, differentiation and functions of multiple types of cells.⁷⁰ TNF has multiple biological functions, including stimulation of the acute phase response, cachexia, cytotoxicity, and potentially lethal shock.⁷⁰ TNF can also promote the production of IL-1 and IL-6, enhance the expression of adhesion molecules, and stimulate fibroblast proliferation.⁷⁰ TNF exists as a transmembrane protein, named membrane-bound TNF, where it's cleaved to a soluble form by TNF converting enzyme.⁷¹ Secreted TNF employs its biological functions via binding to two distinct cell surface receptors, the 55 kDa TNFR1(p55) and the 75 kDa TNFR2 (p75).⁷¹ The binding of TNF to its receptors lead to

activate one of three pathways: a death domain pathway results in apoptosis; another activates JNK, which is involved in cell differentiation and proliferation; and the third pathway activates NF- κ B.⁷²

TNF has been implicated as an inflammatory mediator in many autoimmune diseases, such as rheumatoid arthritis, IBD, and multiple sclerosis.⁷³ Evidence has shown that the levels of TNF are increased in the intestinal mucosa, stool, and blood samples of IBD patients.⁷² Moreover, the levels of TNF are correlated with clinical disease activity of Crohn's disease patients.⁷² Several animal colitis models also demonstrate the role of TNF in the pathogenesis of intestinal inflammation. And anti-TNF monoclonal antibodies induce beneficial responses in some patients with IBD.⁷² Anti-TNF blockade can not only promote the apoptosis of activated T cells, but can also protect epithelial cells from apoptosis and tight junction compromise in the gastrointestinal epithelium.⁷²

More recently, TNF-like ligand 1A (TL1A) has been shown to be an important mediator of intestinal inflammation.⁷⁴ TL1A secretion is induced in both APC by TLR ligands and FcR cross-linking, and in T cells by TCR stimulation. The signaling pathway of TL1A is mediated through DR3, a TNF-family receptor that is mainly expressed on T cells.⁷⁵ TL1A synergistically increases the capacities of IL-12, IL-4, or IL-23 in the differentiation of Th1, Th2 and Th17 cells.⁷⁴ For instance, DR3 is selectively increased on Th17 cells, and TL1A enhances the proliferation of Th17 effector cells. While, DCs derived from TL1A-deficient mice show a reduced capacity in promoting Th17 differentiation and proliferation.⁷⁶

The role of TL1A in the pathogenesis of IBD has been indicated.⁷⁵ The levels of TL1A are increased in IBD patients. Lamina propria CD14⁺ macrophages in Crohn's

disease patient produce higher level of TL1A and TL1A promotes alloantigen-induced IL-17 and IFN- γ production from T cells.⁷⁷ Furthermore, it has been demonstrated that polymorphisms in the TL1A gene (TNFSF15) are associated with increased risk for IBD.⁷⁸

Consistent with studies of TL1A in IBD patients, animal studies also demonstrate a role for TL1A.^{79, 80} Administration of exogenous TL1A to mice with DSS-induced colitis, up-regulate both Th1 and Th17 responses in inflamed colonic tissue.⁷⁷ And administration of anti-TL1A antibodies partially ameliorates DSS-induced murine colitis, and completely prevents the development of TNBS-induced murine colitis.^{79, 80}

IL-6: There is accumulating evidence that IL-6 plays a pivotal role in the pathogenesis of IBD.^{71, 81} Studies have shown that the levels of IL-6 are increased in the serum and the intestinal mucosa of patients with active Crohn's disease.⁸² Moreover, the level of IL-6 is positively correlated with the clinical disease activity, frequency of relapses, and the severity of endoscopic and histopathological signs of inflammation in Crohn's disease.^{71, 83, 84} Macrophages and T cells in lamina propria are likely to be the main producers of IL-6.^{71, 85}

In intestinal inflammation, IL-6 exerts its effect through binding to the soluble form of its corresponding receptor (sIL-6R), not through the membrane-bound receptor for IL-6 (IL-6R).^{71, 86} The levels of sIL-6R and IL-6/sIL-6R complex are increased in the serum of IBD patients. Then the IL-6/sIL-6R complex activates gp130-positive T cells lacking IL-6R, leading to the translocation of STAT-3 and subsequent activation of transcription of the anti-apoptotic genes Bcl-2 and Bcl-xl.⁸⁷ Therefore, this pathway confers resistance against apoptosis of intestinal T cells in IBD patients, and in animal

models of colitis as well.⁷¹ A clinical trial shows that Tocilizumab, a humanized anti-IL-6R monoclonal antibody, induce significantly higher clinical response rate in active Crohn's disease than that of the placebo group.⁸⁸ It indicates that anti-IL-6R antibody may represent another therapeutic strategy for the management of IBD.^{71, 81}

4.2 Adaptive immune system dysregulation

Dysregulation of the innate immune system causes functional abnormalities of the adaptive immune system, which reveals many characteristics of chronic inflammatory processes in IBD.⁴⁸

CD4⁺ Th cells play a critical role in orchestrating adaptive immune responses to various infections microbes.^{89, 90} They are also involved in the pathogenesis of autoimmune and allergic diseases. Upon activation by T-cell receptor complex, naïve CD4⁺ T cells may differentiate into four major types of Th cells in the presence of different cytokines, including Th1, Th2, Th17 and inducible T-regulatory (iTreg) cells. They can be characterized by their special cytokine production profiles, transcription factors and their functions.⁹¹ Under the stimulation of IL-12, naïve CD4⁺ T cells differentiate into Th1 cells, mainly producing IFN- γ and vital for protective immunity against intracellular viral and bacterial infections.⁹¹ Under the stimulation of IL-4, naïve CD4⁺ T cells differentiate into Th2 cells, producing IL-4, IL-5, IL-13, IL-9 and IL-25, and critical for eliminating extracellular parasites such as helminths.⁹¹ TGF- β and IL-6 induce naïve CD4⁺ T cells to differentiate into Th17 cells, producing IL-17, IL-17F, IL-21 and IL-22, and important for controlling extracellular bacterial and fungi infections.⁹¹ In the presence of TGF- β without IL-6, naïve CD4⁺ T cells differentiate into iTreg cells.

iTreg together with naturally occurring T-regulatory (nTreg) cells, are vital for maintenance of immune tolerance, and regulation of lymphocyte homeostasis, activation and function.⁹¹ Transcription factors also play important roles in the differentiation of Th cells and production of cytokines. The vital transcription factors of Th lineage are T-bet/Stat4 for Th1, GATA-3/Stat5 for Th2, ROR γ t/Stat3 for Th17, and Foxp3/Stat5 for iTreg.⁹¹

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 to bacterial antigens in genetically predisposed individuals.^{12, 37, 92-94} 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.^{95, 96} On the contrast, ulcerative colitis is usually considered as a “Th2-like” disease characterized by increased amounts of IL-5, IL-13.⁷⁵

Th1 cells: A number of observations indicate Th1 cells are involved in the pathogenesis of Crohn's disease.^{75, 97} T cells in the colonic lamina propria of Crohn's disease patients produce large amounts of IFN- γ , and increase the expression of IL-12R β 2, T-bet and STAT4.⁹⁷ And IFN- γ producing lamina propria lymphocytes are accumulated in the mucosa of patients. Macrophages in Crohn's disease patients produce high levels of IL-12.⁹⁷ At the initial phase of Crohn's disease, mucosal T cells mount a typical Th1 response that resembles an acute infectious process, and gradually disappear with progression to late Crohn's disease.⁹⁸ In addition, clinical responses are induced in a subcohort of patients with Crohn's disease treated with anti-IFN- γ antibody.^{75, 97} In

animal colitis model, abrogation of IFN- γ in the CD4⁺ CD45RB^{hi}/Rag^{-/-} transfer model potently prevents the development of colitis;⁷⁵ T-bet-deficient CD4⁺CD45RB^{hi} cell cannot induce the colitis in Rag^{-/-} recipients.⁹⁹ These results indicate that Th1 play a role in the pathogenesis of Crohn's disease.

Th17 cells: With the finding of the IL-23/Th17 pathway, more recently, studies highlight the role of this pathway in the pathogenesis of IBD.¹⁰⁰ Studies have shown that large amounts of IL-17-producing cells are mainly accumulated in the lamina propria of ulcerative colitis patients, and in the submucosa and muscularis propria of Crohn's disease patients.¹⁰¹ Flow cytometry analysis of mucosal cells also show that the number of IL-17 producing T cells is increased in Crohn's disease patient than that in normal controls, but some of these cells co-express IFN- γ .¹⁰² Gut biopsies grown ex vivo and LPMC cultured in vitro also produce high levels of IL-17 in IBD patients than in controls.¹⁰³ Other Th17 cytokines, such as IL-21, IL-22 and IL-23 are also increased in the inflamed tissue of IBD patients.¹⁰⁴

The role of Th17 cells in the pathogenesis of IBD has also been evaluated in animal models. IL-17 is shown to be elevated in the IL-10 knockout and RAG1 knockout mouse models of IBD, respectively.^{105, 106} Anti-IL-17 Ab ameliorates the severity of intestinal inflammation in RAG1 knockout mice reconstituted with IL-10 knockout CD4⁺ T cells.¹⁰⁶ And deficiency of IL-17R (receptor) prevents the development of TNBS-induced murine colitis, including improving body weight loss, decreasing productions of IL-6 and local macrophage inflammatory protein-2, ameliorating colonic inflammation, and reducing tissue myeloperoxidase activity.¹⁰⁷ IL-17F-deficiency improves the development of DSS-induced murine colitis, whereas IL-17-deficiency exaggerates the

development of DSS-induced murine colitis, indicating that IL-17F rather than IL-17A is important in sustaining DSS colitis.¹⁰⁸

Enhanced production of IL-17 in the gut is also found in the C3H/HeSnJ SCID transfer colitis model, and adoptive transfer of IL-17-producing T cells to SCID recipients leads to severe colitis.¹⁰⁹ In the model of CD8⁺ T cell-dependent colitis, it shows that a single adoptive transfer of naïve CD8⁺ T cells into syngeneic RAG-deficient mice results in severe colitis, with rapid spontaneous proliferation of these CD8⁺ T cells in MLN.¹¹⁰ These CD8⁺ T cells in the MLN co-express IL-17 and IFN- γ . And adoptive transfer of naïve CD8⁺ T cells isolated from either IL-17 or IFN- γ deficient mice induced a remarkably less severe colitis, suggesting IL-17 and IFN- γ can cooperate to cause colitis in this model.¹¹⁰

A role for IL-21 in the murine colitis is also indicated.¹¹¹ DSS colitis and TNBS-relapsing colitis are significantly decreased in IL-21-deficient mice, which is associated with reduced expression of Th17 cell-related genes (IL-17, IL-17F and ROR γ t) in the colon tissue.¹¹¹ Furthermore, blockade of IL-21 using a specific IL-21R-fusion protein improves intestinal inflammation and down-regulates Th17 responses during the course of DSS colitis.¹¹¹ Taken together, these data indicate that Th17 pathway plays an important role in the pathogenesis of IBD.

Treg cells: The gut-associated lymphoid tissue (GALT) is believed to be the primary site where naïve conventional CD4⁺ T cells convert to iTregs after exposure to oral antigens and in a lymphopenic environment.^{112, 113} This conversion is dependent on TGF- β and retinoic acid producing CD103⁺ DCs in the GALT.^{111, 112} It has been

supposed that nTregs mainly protect against autoimmunity in situ, but iTregs primarily inhibit immune responses against environmental and food antigens in the gut.¹¹⁴

The dysfunction of Tregs in IBD is usually believed to be due to the defective numbers of Tregs or their suppressive function which cannot control the intestinal inflammation.¹¹³ For instance, patients with a *FOXP3* gene mutation have defective Tregs and always suffer from intestinal inflammation.¹¹⁵ When compared with healthy controls, the numbers of Treg are decreased in peripheral blood but increased in inflamed colons of patients with IBD.¹¹³ And the ratio of Tregs to Th17 in peripheral blood is reduced in IBD patients when compared with controls.¹¹⁶ However, the increased number of Tregs in the colon lamina propria of IBD patients is still lower than that of patients with infectious enteritis or diverticulitis.¹¹⁷ Tregs isolated from inflamed colon or peripheral blood maintained normal cell-contact-dependent, cytokine-independent suppressive capacity in vitro.¹¹⁷⁻¹¹⁹ But effector T cells from IBD patients display relative resistance to Treg-mediated suppression, because effector T cells express high levels of Smad7 which is an inhibitor of the TGF- β signalling pathway.¹²⁰ These data indicate that Treg dysfunction might be due to an extrinsic milieu of activated cells that are resistant to suppression.¹¹³

Animal models of colitis also demonstrate the role of Tregs in the control of intestinal inflammation.¹¹³ Adoptive transfer of naïve T effector cells in the absence of Tregs into SCID mice leads to colitis, whereas co-transfer of T effector cells and Tregs does not induce colitis.¹¹³ Furthermore, adoptive transfer of CD4⁺CD25⁺ Tregs cures established CD4⁺CD45RB^{hi} transfer colitis.¹²¹ In this model, Tregs are capable of

suppressing colonic inflammation by down-regulating Th1 and Th17 responses depending on the presence of IL-10 and TGF- β .^{122, 123}

More recently, a new type of iTregs, called iTR35, has been identified which mainly produce the suppressive cytokine IL-35, not IL-10 or TGF- β .^{124, 125} Adoptive transfer of IL-35-deficient Tregs cannot cure CD4⁺CD45RB^{hi}-induced murine colitis.¹²⁵ Whereas adoptive transfer of iTR35 generated in vitro can significantly improve the intestinal inflammation.¹²⁴ And IL-35 also shows strong function in controlling intestinal inflammation. Administration of recombinant IL-35 significantly reduces the development of several forms of experimental colitis and reduces levels of cytokines of Th1 and Th17 cells.¹²⁶

Both iTreg and Th17 differentiation require TGF- β which induces Foxp3 and ROR γ t, so there is a fine balance existed between these two types of cells under the control of many factors.¹³ For instance, low concentrations of TGF- β together with IL-6 and IL-21 induce the expression of IL-23R, and promote the differentiation of Th17 cells;¹³ On the contrast, high concentrations of TGF- β inhibit the expression of IL-23R and promote the development of iTregs.¹³ Foxp3 directly interacts with ROR γ t to suppress its function, but IL-6, IL-21 and IL-23 down-regulate the Foxp3-mediated suppression of ROR γ t.^{13, 127} On the other hand, there is a close relationship between these two types of cells. Recent data have documented that memory Tregs can convert into Th17 cells under inflammatory conditions, in which IL-1 is the key molecule in promoting conversion.^{128, 129} A hybrid subpopulation of memory Tregs co-expressing Foxp3 and ROR γ t has been found which exert suppressive functions but concomitantly secrete IL-17 ex vivo.¹³⁰⁻¹³² In the presence of IL-1, IL-2, IL-23 and TGF- β , human Th17

cells preferentially differentiate from natural naïve regulatory cells, rather than from conventional CD4⁺CD25⁻ naïve T cells.¹³³

Taken together, the pathogenesis of IBD is associated with genetic susceptibility of the host, intestinal microbiota, other environmental factors, and immunological abnormalities.

III. Cytokines IL-12 and IL-23

IL-12

IL-12 is a heterodimeric cytokine predominantly produced by DCs, monocytes, and macrophages following recognition of pathogenic structures by toll-like receptors and other receptors.^{134, 135} IL-12 induces the production of IFN- γ , favours the differentiation of Th1 and forms a link between innate and adaptive immunity.¹³⁶

IL-12 is a cytokine with a molecular weight of 70 Kda, composed of two disulphide-linked subunits designated p35 and p40, which are expressed on different chromosomes.¹³⁷ Expression of the p35 and p40 subunits of IL-12 is independently regulated. Although p35 transcripts are detected in many cell types, free p35 is not secreted without the p40 subunit. The p35 requires co-expression of p40 for secretion of the biologically active cytokine from the cell.¹³⁷

The biological functions of IL-12 are mediated through binding to its receptor complex which is composed of two chains-IL-12 receptor β 1 (IL-12R β 1) and IL-12R β 2.¹³⁷⁻¹³⁹ IL-12R is expressed predominantly by activated T cells and NK cells. IL-12R is also detected on other cells, such as DC and B-cell lines.^{136, 138} Activation of T cells through the binding of TCR with peptide-MHC complex promotes the expression of

both chains of IL-12R, and this promotion-especially for the $\beta 2$ chain expression-is enhanced by IL-12 itself, IFN- γ , TNF and the co-stimulation through CD28.^{136, 138} As to T cells, the expression of IL-12R $\beta 2$ is confined to Th1 cells, and its expression correlates with responsiveness to IL-12.^{134, 136} Both receptor chains are required to mediate maximal signalling, but these two chains have different roles. IL-12R $\beta 1$ is required for high-affinity binding to IL-12p40 subunit and activates the Janus kinase (Jak) family member Tyk-2, whereas IL-12R $\beta 2$ chain mediates the signalling pathway through acting as the docking site for STAT4 and activates Jak-2.¹³⁷ After STAT4 binds to the receptor chain, it will be phosphorylated, homodimerized and shuttled into the nucleus where they bind to STAT binding sites in the IFN- γ promoter, and induce the transcription of IFN- γ gene.¹³⁸

IL-12 is mainly secreted by activated DCs, monocytes, neutrophils, macrophages, microglia cells and, to a lesser extent, by B cells.^{136,137} PAMPs such as lipopolysaccharide (LPS), teichoic acid, peptidoglycan and CpG DNA, can induce the production of IL-12.¹³⁷ CD8 α^+ DCs, not macrophages, are the first cells to produce IL-12 in the spleen of mice in response to LPS or a soluble extract of *Toxoplasma gondii*.¹⁴⁰ The initiation of IL-12 production is independent of IFN- γ and signals from T cells,¹⁴¹ even though the production of IL-12 is facilitated by stimulation of CD40L on T cells.¹³⁶ Other pathogens, such as *Brucella abortus* and Bacteria CpG DNA, can induce both CD8 α^- and CD8 α^+ DCs to generate the early production of IL-12.¹³⁶ Plasmacytoid DCs or CD8 α^- CD11b $^+$ myeloid DCs can also produce IL-12 in response to viruses.¹³⁶ The production of IL-12 by DCs seems to be much less dependent on the presence of IFN- γ or other enhancing cytokines than is produced by phagocytes.¹³⁶

The production of IL-12 is strictly regulated by positive and negative regulatory mechanisms involving Th1 cytokines, Th2 cytokines and type I IFN.^{136, 142} IFN- γ promotes the production of IL-12 heterodimers, thus forming a positive-feedback mechanism during inflammatory and Th1 responses.¹³⁶ The two Th2 cytokines, IL-4 and IL-13, can also promote the synthesis of IL-12. But during the first 24 hours of treatments using these two cytokines, they inhibit p40 production, and then at later times, they strongly enhance the production of IL-12.¹⁴³ T cells enhance the production of IL-12 through both the secreted cytokines such as IFN- γ and IL-4, and direct cell-cell interactions, especially the interaction of CD40L on activated T cells with CD40 on DCs or macrophages.¹⁴⁴ Some other cytokines, including IL-10, TGF- β , IFN- α and IFN- β , can inhibit the production of IL-12.¹³⁶

IL-12 has multiple biological functions and bridges the innate and adaptive arms of immune responses.¹³⁶ IL-12 plays an important role in the differentiation of naïve CD4⁺ T cells into Th1 cells. IL-12 does not induce the proliferation of resting peripheral-blood T cells or NK cells, but it has direct effects on the proliferation of pre-activated T cells and NK cells.^{136, 145} IL-12 synergistically with CD28 facilitates T cell proliferation and IFN- γ production after antigen presentation to T cells by APC.¹⁴⁶ IL-12 also has synergistic effects with IL-18 in the differentiation of Th1 cells, and IL-12 and IL-18 reciprocally upregulate each other's receptors.¹⁴⁷ IL-12 promotes the generation of cytotoxic T cells (CTL) and large activated killer cells (LAK), and it enhances the cytotoxicity of CTL and NK cells through inducing the transcription of genes encoding perforin and granzymes, and through upregulating the expression of adhesion molecules.¹⁴⁸ In addition, IL-12 is also involved in promoting T cell trafficking and

migration by inducing expression of functional adhesion molecules such as E- and P-selectin ligand on Th1 cells, not Th2 cells,¹⁴⁹ and by inducing the expression of chemokine receptors such as CCR5 and CXCR3 on T cells.^{137, 150}

IL-12 is also essential for host immune defence against infections, especially for bacterial and parasitic infections, through the generation of Th1 responses and IFN- γ production, and through activating DCs and increasing macrophages' antimicrobial activity.¹³⁷ A number of studies have also demonstrated the anti-tumour roles of IL-12. Endogenous IL-12 is vital for suppressing transplantable tumors and carcinogenesis-induced fibrosarcomata.¹⁵¹ Administration of IL-12 has an obvious anti-tumor effect on mouse tumors through inducing regression of established tumors or through suppressing establishment of tumors.¹⁵² The mechanisms of anti-tumor function of IL-12 are complex and include innate and adaptive immunity.¹⁵³ For instance, IL-12 augments the cytotoxicity of CTL and NK cells; and it also induces the production of IFN- γ and other pro-inflammatory cytokines which have the direct toxic effects on tumors and might activate anti-angiogenic mechanisms.¹³⁶

The IL-12p40 subunit can also be secreted in a monomer p40 and in a homodimer p80.^{154, 155} They can antagonize IL-12 as they competitively bind to the IL-12 β 1.¹³⁹ p40 is secreted at a 50-fold higher level than IL-12p70 in a murine shock model and at a 10-20 fold excess by stimulated human peripheral blood cells.¹³⁹ Natural p80 accounts for 20-40% of the total p40 in the serum of a murine model of systemic infection.¹³⁹ Natural p80 is also detected in the bronchoalveolar lavage of asthmatic patients.¹⁵⁶ Studies have shown that p80 can act as a macrophage chemoattractant and an inducer of DC migration.^{157, 158} Recombinant murine p80 functions as the chemoattractant for both

mouse and rat macrophages over a dose range of 10-1000 ng/ml.¹⁵⁷ And Russell et al demonstrate that intratracheal delivery of recombinant murine p80, not recombinant murine IL-12 or p40, promoted the recruitment of macrophage into the airway.¹⁵⁹ Khader et al show that migration of DCs from the lung to the draining lymph nodes after exposure to *Mycobacterium tuberculosis* is defective in p40-deficient mice, not in IL-12p35-deficient mice lacking.¹⁵⁸ *Mycobacterium tuberculosis*-induced DC migration and the ability of p40-deficient DCs to activate naive T cells can be restored by treating p40-deficient DCs with p80.¹⁵⁸

IL-23

IL-23 is a heterodimeric cytokine composed of a 19-kDa fourfold helical core α chain (p19), disulfide linked to IL-12p40 subunits. It is also predominantly produced by activated dendritic and phagocytic cells.^{135, 160, 161} Similar to IL-12, generation of biologically active IL-23 requires synthesis of both p19 and p40 subunits within the same cell.¹⁶¹ The sequence of human p19 has around 70% homology with mouse p19, both proteins contain four α -helix and five cysteine residues.^{134, 159, 160} And p19 shows 40% homology with the IL-12p35 subunit.¹⁶²

IL-23 production is stimulated via the activation of TLRs by their corresponding ligands (LPS, bacterial CpG DNA, peptidoglycan et al), via endogenous signals like prostaglandin E2, and via stimulation via CD40L.¹⁶³ And the regulation of IL-12 and IL-23 production in response to TLRs signals is different: TLR2 stimulation alone or in combination with NOD2 stimulation induces production of IL-23;^{161, 163} in contrast, TLR4, TLR3 or TLR8 stimulations induce the production of IL-12.^{161, 163} Cytokines IL-

1 β , TNF and IL-17 have been shown to promote the gene expression of the IL-23p19 subunit.¹⁶²

IL-23 exerts its biological function through binding to its membrane receptor which is composed of a unique IL-23R subunit, and the IL-12R β 1 subunit shared with IL-12 receptor.¹⁶¹ IL-23R is mainly expressed on T cells, NK cells, DCs and macrophages.¹⁶³ IL-23R binds to IL-23p19 subunit, while IL-12R β 1 binds to the p40 subunit. IL-23 binding to its receptor complex induces the tyrosine phosphorylation of Jak 2 and Tyk 2, which in turn, activates STAT3 and STAT4.¹⁶³ The phosphorylated STATs are subsequently dimerized and translocated into the nucleus, and activate the transcription of target genes. When compared with IL-12, IL-23 can strongly induce the formation of STAT3 homodimer and STAT3-STAT4 heterodimers, and weakly induce STAT4 homodimer.¹⁶² STAT3 signaling is required for the development of Th17 cells.¹⁶³

IL-23 has multiple biological functions, including roles in Th differentiation, infection, tumor and autoimmunity, and it is a key cytokine that bridges innate and adaptive immune responses.^{90, 164, 165} IL-23 plays an important role in stabilizing/amplifying Th17 proliferation.^{166, 167} IL-23 cannot drive the differentiation of naïve T cells into Th17 cells because naïve T cells don't express IL-23R.¹⁶⁸ For mouse and human Th17 cells, TGF- β with IL-6 or IL-21 drive the differentiation of naïve T cells into Th17 cells, and upregulate the transcription factor ROR γ t.¹⁶⁷ The presence of IL-6 or IL-21 upregulates the expression of IL-23R, suppresses the generation of Foxp3.¹⁶⁷ High TGF- β concentrations inhibit the expression of IL-23R and promote the differentiation of Foxp3⁺ iTreg cells; while low concentrations of TGF- β with IL-6 or IL-

21 enhance the expression of IL-23R and promote Th17 differentiation.^{127, 162, 169, 170} IL-23 can also promote the differentiation of Th1 cells.¹⁶⁹

The IL-23/Th17 pathway has been shown to be involved in the microbial infections.^{161, 171} In the infection model of *Citrobacter rodentium*, its infection induces a temporary distal colitis. After 14-21 days, the bacteria are cleared and the lesions resolve with induction of potent Th1 and Th17 responses.^{172, 173} Mice deficient in IL-23p19 fail to clear this infection, similar to mice deficient in IL-12p40. It indicates that IL-23 provides the protection against *Citrobacter rodentium*.¹⁷⁴ Studies have shown Th1 and Th17 cell responses play important roles in the host defence against *Klebsiella pneumonia* infection.¹⁷⁵⁻¹⁷⁷ The production of IL-17 from *Klebsiella pneumonia* pulsed DCs depend on IL-23 *in vitro*. Mice deficient in IL-12/IL-23p40 are extremely sensitive to intrapulmonary *Klebsiella pneumonia* infection, and mice deficient in IL-23p19, IL-12p35 or IL-17R also increase the susceptibility to this infection.¹⁷⁵ However, the anti-pathogen functions of IL-23/Th17 pathway can be detrimental to the host in some situations.¹⁶¹ Both IL-23 and IL-17 impair the antifungal immune resistance to *Candida albicans* and *Aspergillus fumigatus*, promote neutrophil inflammation and regulate the killing activity of neutrophils.^{178, 179}

With the finding of the IL-23/Th17 pathway, the roles of this pathway in autoimmunity has been highlighted.¹⁸⁰ Some of the autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and IBD, is ever thought that IL-12/Th1 pathway play the more important role in the pathogenesis, but now it's usually thought IL-23/Th17 pathway is more important, not IL-12/Th1.^{161, 171, 181, 182} For instance, administration of recombinant murine IL-12 promotes the development of collagen-induced arthritis, while

treatment of mice with anti-IL-12 slightly ameliorates collagen-induced arthritis.¹⁸³ Treatment of mice with the combination of anti-IL-12 (IL-12p40) and anti-TNF significantly suppresses the progression of murine collagen-induced arthritis when compared with anti-IL-12 treatment alone, including decreasing the clinical score and inflammation etc.¹⁸⁴ And then through using IL-12p35-deficient mice or IL-23p19-deficient mice, it's found that deficiency of IL-23p19 protects against the onset of collagen-induced arthritis, while deficiency of IL-12p35 exacerbates arthritic inflammation with over-expression of IL-17.¹⁸⁵ It's also been found that IL-23 is strongly expressed in the inflamed joints; and IL-23 stimulates the production of IL-17 from Th17 cells, then IL-17 stimulates the production of IL-1, TNF and receptor activator of NF- κ B leading to the aggravation of the synovial inflammation and osteoclast differentiation, resulting in bone erosion and joint destruction.¹⁶² In rheumatoid arthritis patients, the expressions of IL-17 and IL-23p19 mRNA and protein are increased in the serum, synovial fluid and synovial tissues.¹⁶² And IL-1 β and TNF can increase the expression of IL-23 in fibroblast-like synoviocytes isolated from patients.¹⁸⁶⁻¹⁸⁸ The serum concentrations of IL-23 in rheumatoid arthritis patients are correlated with serum concentrations of IL-17, DAS28 score and number of tender joint.¹⁶² And results of GWAS also demonstrate that the polymorphism of IL-23 and IL-23R genes are associated with the pathogenesis of rheumatoid arthritis.¹⁶²

IV. The roles of IL-12 and IL-23 in the pathogenesis of Crohn's disease

Studies have demonstrated that IL-12 and IL-23 play important roles in the pathogenesis of Crohn's disease. In active Crohn's disease patients, expression of IL-12

receptor is increased on mucosal cells.^{189, 190} Multiple studies indicate that IL-12 is overproduced in the gastric mucosa, LPMC, and macrophages in Crohn's disease, and macrophages isolated from the inflammatory lesions of patients with Crohn's disease produce increased amounts of IL-12 *ex vivo*.¹⁹¹⁻¹⁹⁴ In TNBS-induced colitis mice, administration of an IL-12 antagonist, the IL-12p40-IgG2b fusion protein¹⁹⁵ or anti-mouse IL-12 antibodies,^{196, 197} abrogates mucosa inflammation, prevents body weight loss, and results in a nearly normal histological appearance of the colon, through a mechanism of induction of Fas-mediated apoptosis of Th1 cells.¹⁹⁶ This treatment also reduces TNF α and increases IL-10 secretion in mice.¹⁹⁵

More recently, studies have highlighted the roles of IL-23 in the pathogenesis of Crohn's disease.^{3, 106, 198, 199} The colonic level of IL-23 is increased in patients with Crohn's disease.²⁰⁰ The myeloid DCs from the MLN of Crohn's diseases patients secrete high levels of IL-23.⁵⁰ The expression of IL-23R is upregulated in lamina propria isolated from Crohn's disease.²⁰¹ And the upregulated expression of IL-23R is correlated with IFN- γ .²⁰¹ Recently, GWAS has identified numerous SNP in *IL-23R*, with high association for Crohn's disease and ulcerative colitis.^{21, 22} Of interest, Arg381Gln, an uncommon allele at a highly conserved amino-acid polymorphism, conferred a protective effect in patients with Crohn's disease or ulcerative colitis.²² Yen and his colleagues used IL-10 knockout mice, a spontaneous IBD model, and showed that the development of colitis was suppressed by IL-23p19 deficiency but not IL-12p35 deficiency in IL-10^{-/-} mice; administration of IL-23 accelerated the onset of colitis and promoted inflammation through IL-17- and IL-6-dependent mechanisms.¹⁰⁶ Anti-IL-23 monoclonal antibody can prevent and reverse active colitis in a T cell-mediated colitis mouse model, with down-

regulation of a broad array of inflammatory cytokines and chemokines in the colon.¹⁰⁹ Using *IL-23R*-deficient mice, the Fiona Powrie group demonstrates that through direct signalling, in the intestine IL-23 promotes the proliferation and accumulation of Th17 cells, enhances the generation of IL-17⁺IFN- γ ⁺ T cells, and suppresses the differentiation of iTreg and IL-10 production from T cells.²¹ These results demonstrate that IL-23 plays important roles in the pathogenesis of Crohn's disease.

However, these findings do not exclude the role of exaggerated Th1 responses in Crohn's disease since IL-12/IFN- γ , and IL-23/IL-17 may be parallel pathways involved in inflammatory response.³⁷ These Th1 cytokines are probably critical in generating and perpetuating the chronic intestinal inflammation of Crohn's disease.⁹³ 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.^{202, 203}

V. Current biological therapies of IBD

Currently, there are several treatment modalities for IBD, including medication and surgery. Drugs such as 5-ASA derivatives, corticosteroids and immunomodulators are currently the front line therapies for Crohn's disease.²⁰⁴ However, only around 50% of patients achieve sustained remission using these drugs. The biological therapies are a newer form of treatment that has shown promising results which have attracted huge attentions.²⁰⁵ Current biological therapies for IBD include monoclonal antibodies against cytokines, cytokines, blockade of leukocyte migration, anti-T cells activation, small molecules, hematopoietic stem-cell transplantation and growth factors.²⁰⁵

Monoclonal antibodies (mAb) against cytokines

Using human or humanized mAbs to block over-produced proinflammatory cytokines involved in the pathogenesis of IBD, has become an effective biological therapy for IBD.²⁰⁵ Several cytokines have been targeted using this strategy, including TNF, IL-12/IL-23p40, and IL-6.

mAb to TNF: Infliximab is the first biological agent to be approved for the therapy of IBD, and has shown the established efficacy and safety.²⁰⁶ Infliximab is a chimeric (75% murine, 25% human) IgG1 κ subclass antibody that binds to TNF and neutralizes its activity. The early studies show that a single infusion results in a great clinical response in moderate-to severe Crohn's disease patients (48% remission rate compared with 4% in the placebo group).²⁰⁷ Then two randomized, double-blind, multicenter trials-ACCENT I and ACCENT II test the effects of infliximab as maintenance therapy in patients with or without fistulizing Crohn's disease. It shows that infliximab induces the sustained remission, improvement in their colitis, or enhancement in the quality of life for Crohn's disease patients with/without fistulizing disease.⁷²

Other anti-TNF blocking agents, such as Adalimumab, certolizumab pegol (CDP870), CDP571 and soluble TNF receptors (etanercept and onercept), are also approved for the management of Crohn's disease or are in clinical trials.²⁰⁵

The mechanisms of anti-TNF blocking agents include their binding to soluble TNF and membrane-bound TNF to block its functions, restoring epithelial barrier integrity, inducing of apoptosis of activated T cells, and down-regulating VCAM-1 expression on intestinal vascular endothelium.²⁰⁶

mAb to IL-12/IL-23p40: ABT-874 is a fully humanized antibody against IL-12/IL-23p40 subunit which demonstrates the efficacy for Crohn's disease. A random, double-blind phase II clinical trial evaluates the safety and efficacy of ABT-874 in 79 patients with active moderate-to-severe Crohn's disease.²⁰⁸ Patients in cohort one (first and second injections with four-week delays) achieve a remission and response rate of 50% at the end of follow-up (week 18) at a dose of 3 mg/kg, compared with 13% and 25% of the placebo group. Patients in cohort two (weekly injection for 7 weeks) achieve a remission and response rate of 38% and 69%, compared with 0% and 25% in the placebo group.²⁰⁶ Down-regulation of production of IL-12, IL-23, IFN- γ , and TNF by mononuclear cells of the colonic LPMC is associated with clinical improvement in patients receiving ABT-874.^{208, 209}

Ustekinumab is another human monoclonal antibody that specifically binds to IL-12/23p40, which has demonstrated excellent control of psoriasis in the phase III clinical trial study.²⁰⁶ In a randomized, double-blinded cross-over Phase IIa clinical trial with moderate-to-severe active Crohn's disease patients, ustekinumab also induce a clinical response, especially in patients whom were previous given infliximab.²¹⁰ 25 subjects are weekly given subcutaneous ustekinumab at weeks 0-3, then placebo at weeks 8-11; 26 subjects are weekly given subcutaneous placebo at weeks 0-3, then ustekinumab at weeks 8-11; 26 subjects are given intravenous ustekinumab at week 0, then placebo at week 8; or 27 subjects are given intravenous placebo at week 0, then ustekinumab at week 8.²¹⁰ Clinical response rates for the combined groups given ustekinumab are 53% at weeks 4 and 6, compared with 30% of placebo group ($P = 0.02$). In a subgroup of 49

patients treated previously with infliximab, clinical response to ustekinumab is significantly greater than those for placebo ($P < 0.05$) through week 8.^{205, 210}

Blockade of leukocyte migration

Immune responses within gut involve the recruitment of activated T cells to sites of inflammation, and the selective migration of naïve T cells into draining lymph nodes, which are vital in maintaining the inflammatory responses.²⁸ The homing and trafficking of leukocytes is mediated by adhesion molecules expressed on the surface of circulating leukocytes and endothelial cells, and chemokines.²⁸ Selective T cells homing to gut Peyer's patches is mediated via the interaction of $\alpha 4\beta 7$ integrin and mucosal addressin cell adhesion molecule-1.²⁸ Therefore, blocking leukocyte migration has been as one strategy for management of IBD.

Natalizumab, a humanized monoclonal antibody targeting the $\alpha 4$ integrin, inhibits leukocyte adhesion and migration into inflamed tissue, and has been approved by FDA for use of Crohn's disease treatment in the US under a limited prescription program.^{206, 211} Randomized clinical trials have shown that Natalizumab induces a statistically increased rate of remission in patients with active Crohn's disease. And it's also been found that migration of lymphocytes and monocytes into the intestine is effectively inhibited with increased numbers in the peripheral blood.²¹¹ However, several severe side-effects are reported, including fatal progressive multifocal leucoencephalopathy.²⁰⁶

Vedolizumab is a monoclonal antibody specially targeting the $\alpha 4\beta 7$ integrin responsible for T cell trafficking to the gut tract, without cross-reaction with the

individual component monomers.²⁰⁶ A recent study evaluates the effect of infusion of vedolizumab on patients with moderate-to-severe active Crohn's disease. Vedolizumab induces the response rate of 53%, 49% at 2.0 mg/kg and 0.5 mg/kg respectively, compared with 41% in the placebo group.²¹² Vedolizumab is well tolerated in the patients. Currently, this antibody is under study in phase III clinical trial for both patients with moderate-to-severe active ulcerative colitis and Crohn's disease.²⁰⁶

Anti-T cell activation

The activation of naïve T cells requires at least two signals. The first signal is mediated by the recognition of TCR complex on naïve T cells to MHC-peptide complex presented by APC. The second signal is mediated by co-stimulatory molecules expressed on T cells and APC, such as CD28-B7, CD40-CD40L. Activation of T cells without co-stimulation will lead to T cell anergy, render T cells resistant to further stimulation.²¹³ Inhibiting T cell activation by targeting co-stimulatory molecules is an effective way to induce immune tolerance, and appears to be a new strategy for IBD treatment.²⁰⁵

Anti-CD40 mAb: Chimeric 5D12 (Ch5D12) is a molecularly engineered human IgG4 antibody containing the variable domains of the heavy and light chains of mAb 5D12 (anti-human CD40).²¹⁴ In an open-label dose-escalation phase I/IIa study, Ch5D12 is given to 18 patients with moderate-to-severe Crohn's disease.²¹⁴ Ch5D12 induce the overall response and remission rates with 72 and 22%, respectively. Furthermore, Ch5D12 treatment reduces microscopic disease activity and intensity of LPMC infiltration, but doesn't alter percentages of T and B cells in the peripheral blood.²¹⁴

Haematopoietic stem-cell transplantation (HSCT)

Autologous HSCT has been successfully used in the treatment of many autoimmune diseases since the late 1990s, including multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and Crohn's disease.²¹⁵ The rationale of autologous HSCT for autoimmune diseases is to reset the immune system, that is to generate a new and antigen-naïve immune systems after chemotherapy-induced elimination of self- or auto-reactive lymphocytes.²¹⁶

The first demonstration of the therapeutic efficacy of autologous HSCT for Crohn's disease was done in 2003. The first four patients all achieved clinical remission with no significant adverse event from the transplantation.^{216, 217} A phase I HSCT study in 12 patients with refractory Crohn's disease showed that 11 of 12 patients achieved an early and sustained clinical remission (CDAI < 150) after a median follow-up of 18.5 months.^{216, 218}

HSCT also shows a good alternative to conventional therapies. The second phase I-II study recruit four patients with active moderate-to-severe Crohn's disease refractory or intolerant to multiple drugs including infliximab.²¹⁹ Three months after HSCT transplant, clinical remission is achieved in all patients, and complete endoscopic remission is achieved in two of three patients.²¹⁹ Three of the four patients maintain both clinical and endoscopic remission after a median follow-up of 16.5 months without any drugs, and complete fistula closure is observed in all affected patients. No mortality is observed in these patients.^{205, 216, 219}

Other strategies for IBD treatment are under investigation, such as helminth ova (promoting Treg generation, and IL-10 and TGF- β production), small molecules (Janus kinase inhibitors, NF- κ B inhibitors).²⁰⁵

Part Two. Rationales and specific aims for the development of the vaccines

I. The designs of vaccines against cytokines

A vaccine is a biological preparation that improves immune responses to a particular disease through active immunization. There are several kinds of vaccines, including killed organism vaccines, live attenuated organism vaccines, subunit vaccines, toxoids and DNA vaccines. Vaccines can be used for prophylaxis, or therapeutics.

Since the use of vaccines against microorganisms, many infectious diseases have been well-controlled, such as measles, mumps and hepatitis A; even smallpox infection has been eradicated in the world.²²⁰ Concomitantly, epidemiologic data show that the incidence of allergic and autoimmune diseases, such as asthma, IBD, multiple sclerosis, insulin-dependent diabetes mellitus, has steadily increased in developed countries over the past three decades.²²⁰ The over production of cytokines play an important role in the pathogenesis of these chronic diseases.²²¹

The treatments with monoclonal antibodies (mAb) against human cytokines or with soluble cytokine receptors provide a novel strategy to neutralize over-expressed cytokine and have achieved great efficacy clinically.²²¹ However, these agents all function as passive blockers or better antagonists with short half-lives. And it usually requires repeated intravenous or subcutaneous injections with relative large doses to maintain the effects.²²¹ To overcome these disadvantages, a new approach that may provide relatively long-term efficacy is being investigated.^{222, 223} In this approach, by using vaccine strategy, auto-antibodies against endogenous cytokines will be induced. The rationale of this strategy is to induce high affinity auto-antibodies against cytokines via active

immunization with vaccines, which circulate in the stromal compartment of inflamed tissues, locally neutralize the interaction of the excess self-cytokine with its receptors, resulting in the improvement of cytokine-induced pathogenesis.^{221, 224} This vaccine strategy has several advantages, including simplicity and lower costs. And the induction of this autoimmune response looks like more physiologic.²²⁴

Owing to immune tolerance, immune responses against self-molecules are usually not generated. B cell antigen receptors to self-molecules are normally eliminated from the repertoire to induce tolerance and avoid autoimmune responses.^{221, 225, 226} But immature B cells undergoing tolerance induction are extremely sensitive to T cell help. With T cell help, these immature B cells will be rescued from the induction of tolerance and be promoted to mature.^{221, 227} Immune tolerance against self-molecules can be overcome by incorporating a strong Th cell epitope within the self-molecule.^{228, 229} Four broad experimental approaches have been employed to design such vaccines.

The first strategy is to modify the intact self-protein by inserting a foreign peptide containing immunodominant Th epitopes.^{229, 230} Dalum et al show that active immunization of modified mouse TNF molecules induces high levels of polyclonal auto-antibodies against mouse TNF. Mouse TNF is modified via inserting a Th epitope from ovalbumin which binds to the mouse MHC II molecule H-2A^d; or by inserting a Th epitope from hen egg-white lysozyme which binds to H-2E^k.²²⁹ Vaccination with these modified mTNF molecules reduces experimental cachexia in mice, ameliorates collagen-induced arthritis, and improves allergic airway inflammation through blocking the activity of TNF by induced auto-anti-TNF antibodies.^{229, 230}

The second strategy is to link the intact self-protein to a heterologous carrier

protein.²³¹ The commonly used carrier proteins are bacterial proteins (such as Tetanus toxoid or Diphtheria toxoid), Keyhole limpet hemocyanin (KLH), ovalbumin and virus-like particles (VLP).²²¹ This vaccine strategy will induce carrier-specific Th cell proliferation helping the self-antigen-specific B cells to produce antibodies.²²⁴

Vaccination of mice with human TNF coupled to KLH adjuvanted in incomplete Freund's adjuvant, elicit high titers of antibodies which block the biological functions of hTNF but don't cause a cellular response to hTNF.²³² And the mice immunized with this vaccine are resistant to TNF-driven shock and protective from spontaneous arthritis.²³²

Another cytokine-mouse IL-17, is coupled to VLP bacteriophage Q β to make conjugate vaccine.^{233, 234} Vaccination with this conjugate vaccine induces high levels of antibodies against mouse IL-17, which can reduce autoimmune myocarditis and heart autoantibody responses, and prevent collagen-induced arthritis and experimental autoimmune encephalomyelitis in mice.^{233, 234} IL-17 has also been coupled to ovalbumin. Mice immunized with IL-17-ovalbumin induce antibodies against IL-17, and are completely protected against experimental autoimmune encephalomyelitis.²³⁵

The third strategy is to use a peptide of the target cytokine coupled to KLH or VLP.^{224, 236} Compared with the second strategy, the antibodies induced by this vaccine strategy recognize the specific region of the target cytokine, and might decrease possible cross-reactivities and opportunistic infections.^{224, 236} Spohn G et al show that vaccination of mice with VLP of the Q β coupled with either the full soluble TNF protein or a 20-aa peptide derived from TNF, induce specific antibodies which protect from collagen-induced arthritis.²³⁶ However, mice immunized with full TNF based vaccine increase the susceptibility to *Listeria monocytogenes* infection, and promote reactivation of latent

Mycobacterium tuberculosis; whereas mice immunized with TNF peptide-based vaccine are not immunocompromised with respect to infection with these pathogens.²³⁶ They also find the difference caused by the recognition of both transmembrane and soluble TNF by antibodies induced by full TNF based vaccines and a selective recognition of only soluble TNF by antibodies induced by TNF peptide-based vaccine.²³⁶ Our group also show that one peptide derived from murine IL-13 is inserted into VLP hepatitis core antigen (HBcAg) to make IL-13 peptide-based vaccine. Immunization of this peptide-based vaccine induces relative long-lasting antibodies against IL-13, which ameliorate ovalbumin-induced murine allergic airway inflammation.²²³ A phase I/II study has shown the safety and tolerability of the vaccine composed of a TNF peptide coupled to VLP Q β for the treatment of psoriasis.²²⁴ All patients treated with this vaccine raise antibodies against TNF and show a temporary improvement of the disease.²²⁴

The fourth strategy is to use DNA vaccination.²²⁴ Gene immunization with plasmid DNA encoding TNF leads to the generation of immunological memory to its target gene product. This memory effectively suppresses the development of adjuvant-induced arthritis and collagen-induced arthritis.^{237, 238} Although the mechanisms are not clear, it is thought that the existence of CpG sequences may play a role in breaking the tolerance, serving as adjuvants.²²⁴

II. Rationales for development of IL-12/IL-23p40 peptide-based vaccines

As described above, studies have shown that the pathways IL-12/Th1 and IL-23/Th17 play important roles in the pathogenesis of Crohn's disease. And these two

pathways have been the therapeutic target for Crohn's disease, by blocking the overproduced cytokines and inhibiting T cell activation. IL-12 and IL-23 play important roles in the differentiation of Th1 and Th17 respectively, and IL-12 and IL-23 share the p40 subunit. Therefore, through blocking the IL-12/IL-23p40 subunit, it might be possible to block the differentiation of Th1 and Th17 cells.

Administration of an IL-12 antagonist, the IL-12p40-IgG2b fusion protein¹⁹⁵ or anti-mouse IL-12/IL-23p40 antibodies,^{196, 197} ameliorate TNBS-induced intestinal inflammation. And clinical trials have documented the efficacy of the blockage of IL-12/IL-23p40 for Crohn's disease patients.^{208, 210} Taken together, these data demonstrate the feasibility of targeting IL-12/IL-23p40 for treatment of Crohn's disease.

Since Crohn's disease is a chronic inflammatory disease, it usually needs long-term treatment; while monoclonal antibodies have short half-life. For instance, the half-life of infliximab is 9.5 days, that of adalimumab is 18 - 20 days and that of ustekinumab is estimated around 15-32 days.^{239, 240} Repeated injections are required to maintain their effects, since improvements seen are reversed upon the discontinuation of treatment. On the other hand, vaccine can induce relatively long-lasting antibodies, so this strategy might be used as a supplement for monoclonal antibody treatments, especially useful for the maintenance treatment of chronic inflammatory diseases. Natural autoantibodies against cytokines have been detected in healthy individuals at very low levels without notable effects.²⁴¹ So it might be feasible using vaccine to raise autoantibodies and to enhance their affinity and neutralizing activity.²⁴¹

To overcome B cell tolerance, vaccines against excessive endogenous cytokines have been developed mainly by either inserting a foreign peptide into the intact cytokine

molecule²³⁰ or linking the intact cytokine to a heterogeneous carrier such as ovalbumin²⁴². Studies also show that full entire TNF-based vaccine ameliorates collagen-induced arthritis, but increases susceptibility to *Listeria monocytogenes* infection, and promotes reactivation of latent *Mycobacterium tuberculosis*.²³⁶ And immunization of mice with mouse IL-12 coupled to PanDR epitope peptide or ovalbumin, prevents the development of experimental autoimmune encephalomyelitis,²⁴³ or attenuates atherosclerosis,²⁴⁴ but increases the susceptibility of *Leishmania major* infections.²⁴³

Antibodies raised by this entire cytokine vaccines act against multiple antigen determinants of the target cytokine. As such, their use may be hindered by undesirable cross-reactions with other self-proteins containing similar epitopes. This is of particular concern when this strategy is used in humans.^{221, 245} Furthermore, for induction of high titers of autoantibody with such vaccines, the use of adjuvant has always been required. These issues largely limit their application, efficacy and safety.^{221, 245} Compared with full entire TNF-based vaccine, TNF peptide-based vaccine also ameliorates collagen-induced arthritis without increasing susceptibility to *Listeria monocytogenes* infection.²³⁶ A clinical trial also demonstrates the safety and tolerability of the vaccine composed of a TNF peptide coupled to VLP Q β for the treatment of psoriasis.²²⁴ All patients treated with this vaccine raise antibodies against TNF and show a temporary improvement of the disease.²²⁴ Therefore, in this study, peptide-based VLP vaccine strategy will be used.

III. Hypothesis and specific aims of the present study

We hypothesize that active immunization strategy using vaccines against IL-12 and/or IL-23 can be applied to ameliorate intestinal inflammation in murine colitis. We

will develop IL-12/IL-23p40 specific peptide-based vaccines which induce neutralizing antibodies to the target cytokines. We will explore whether immunization with these vaccines can ameliorate acute and chronic murine intestinal inflammation and explore the underlying immune mechanisms involved. Then we will evaluate whether the IL-12/IL-23p40 peptide-based vaccine immunization can increase the susceptibility to lung chlamydia muridarum infection. And we will also develop IL-12/IL-23 specific peptide-based vaccines and explore their effects in murine intestinal inflammation.

Part Three. Materials & Methods

Selection of antigenic peptide

Antigenic peptide prediction was performed based on the following parameters: 1) possible receptor binding sites; 2) occurrence of amino acid residues in experimentally known segmental epitopes (<http://bio.dfci.harvard.edu/Tools/antigenic.html>); 3) surface probability; 4) flexibility; 5) hydrophobic property. Utilizing the information from the high-resolution solution structure of IL-12 and IL-23,²⁴⁶⁻²⁴⁸ priority was given to the peptide sequences located in the possible receptor binding sites and/or at the terminal regions, with the characteristics of high surface probability, high flexibility and high hydrophobic properties.

Construction and identification of peptide-based vaccines

The vector plasmid pThio-His-HBcAg containing an HBcAg encoding sequence was digested using KpnI restriction endonuclease sites at 37°C for 90 mins, and then treated with Alkaline Phosphatase at 50°C for 45 mins. After running a DNA gel, the corresponding DNA band was extracted and purified using the QIAGEN kit according to the protocol provided. The primers encoding the peptides' sequences were firstly treated with T4 Polynucleotide Kinase at 37°C for 60 mins and inactivated at 65°C for 15 mins. Then the primers encoding the peptides' sequences were subcloned into the digested vector plasmid pThio-His-HBcAg. After transformation, the possible colonies were taken from the ampicillin agar plate and added to a beaker with ampicillin. After incubating at 37°C overnight, 10 ml were transferred into the new 500 ml LB. The beakers were shaken for approximately three hours. To induce target protein expression, isopropyl β -D-1-

thiogalactopyranoside was added at the concentration of 0.2µg/ml and shaken again for three hours. After collecting the DH5α, the pellets were suspended with PBS to run SDS-PAGE Gel. By comparing with the size of marker and carrier protein, the possible positive clones were identified.

Preparation of the vaccines and the carrier HBcAg

The vector plasmid pThio-His-HBcAg containing truncated HBcAg (amino acids 1-149), and vaccine plasmid containing the truncated HBcAg and a selected peptide were transformed into *Escherichia coli* DH_{5α} cells.²⁴⁵ Expression of vaccine or carrier was induced, and the presence of virus like particles was confirmed by sucrose gradient centrifugation and SDS-PAGE. Recombinant proteins were purified by a combination procedure consisting of ultra-sonication lysis, ammonium sulfate precipitation, and size exclusion chromatography with Sepharose CL-4B (Sigma-Aldrich). Endotoxin in the recombinant proteins was removed with Affi-prep polymyxin Matrix (Bio-Rad).

Animals

Female BALB/c mice (7-8 weeks old) purchased from Charles River Laboratories (Saint-Constant) were maintained at Central Animal Care Services, University of Manitoba. All protocols used were approved by the University Animal Ethics Committee.

Animal models

Hapten-induced colonic inflammation is a widely used animal model of human Crohn's disease.²⁴⁹⁻²⁵¹ Intrarectal delivery of 2,4,6-trinitrobenzene sulphonic acid (TNBS)

induces colitis by haptination 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. Mice were lightly anesthetized with isoflurane, and then intrarectally administered TNBS in ethanol via a 3.5 F catheter affixed to a 1-mL syringe. The catheter was inserted into the rectum to a point 4 cm proximal to the anal verge, and TNBS was injected in a total volume of 100 μ l. To ensure distribution of TNBS within the entire colon and cecum, mice were held in a vertical position for 50 seconds after the injection.

DSS-induced murine colitis is another widely used colitis model. In this model, it is believed that DSS is directly toxic to epithelial cells of the basal crypts and affects the integrity of the mucosal barrier.²⁵² DSS-induced chronic colitis was induced by four-cycle administration of DSS drinking water. Briefly, female Balb/c mice received 4% (wt/vol) DSS drinking water for 7 days, followed by 7 days of regular drinking water. These mice continued to receive 4% DSS drinking water for another three cycles.

To evaluate whether immunization of IL-12/IL-23p40 peptide-based vaccine could increase the *Chlamydia trochomatis* infection, Chlamydia infection model was used. Studies have shown that Th1 and Th17 responses play important roles in host immune defense against chlamydia infection.²⁵³ Briefly, female Balb/c mice were anesthetized and inoculated intranasally with 1×10^3 inclusion-forming units (IFU) of *Chlamydia muridarum* in 40 μ l final volume of PBS. 8 days later, the mice were sacrificed to analyze the results.

***in vitro* inhibition tests**

Specific inhibition of IL-12 or IL-23 activity by the mouse antisera was performed *in vitro* by IL-12-induced IFN- γ secretion or IL-23-induced IL-17 secretion by activated splenocytes.²⁴⁵ BALB/c spleen cells were cultured for 72 h with 2 μ g/ml Con A and 2ng/ml IL-2. Blasts were restimulated with IL-12 (2 ng/ml) or IL-23 (2ng/ml), and IL-2 (2 ng/ml) for 48 hours in the presence of serum dilutions (1:100) obtained from vaccine-immunized mice or a pooled serum from carrier-immunized mice. Or BALB/c spleen cells were stimulated with anti-CD3 ϵ /CD28 for 4 days in the presence of IL-12, IL-23 or IL-35 with different serum dilutions or controls. Supernatants were collected to detect the expression levels of IFN- γ or IL-17 by ELISA. The inhibition percentage of each mouse anti-serum was calculated as follows:

$$\text{Inhibition (\%)} = \frac{\text{Cytokine of carrier serum} - \text{cytokine of test serum}}{\text{Cytokine of carrier serum}} \times 100\%$$

Protocols of vaccine immunization, induction of colitis and Chlamydial infection

Acute colitis: The vaccine was evaluated *in vivo* in a TNBS-induced acute colitis model in which mice were subcutaneously injected 3 times at a two-week interval with vaccine, vaccine carrier HBcAg or saline (100 μ g, 25 μ g and 25 μ g in 200 μ l, respectively) (n = 20). Two weeks later, mice were intra-rectally challenged with TNBS (Sigma-Aldrich) twice (1.5mg and 2.0mg, respectively) at a one-week interval to induce acute colitis according to the previous description.^{254, 255} Two days later, the mice were sacrificed to analyze.

Preventive chronic colitis: The vaccine was evaluated in TNBS-induced chronic colitis, in which mice were immunized 4 times with vaccine or carrier or saline (n = 12). Two weeks after the third vaccination, chronic colitis was induced by seven weekly administrations of increasing doses of TNBS (1.0 – 2.5 mg).²⁵⁵

Treatment chronic colitis: Mice were administrated weekly with TNBS eight times to induce chronic murine colitis. Four days later after the second TNBS administration, mice were immunized with vaccine, carrier or saline three times at two-week interval (n=16).

Two days (for acute colitis) or 10 days (for chronic colitis) after the last TNBS delivery, mice were sacrificed. Colons, mesenteric lymph nodes, and blood samples were collected and processed according to different assays. The evaluations were repeated once, and similar results were found.

DSS-induced chronic colitis: Mice were given 4% DSS drinking water for 7 days, followed by 7 days of regular water for 4 cycles to develop chronic murine colitis. 3 days after the first DSS drinking water, mice were immunized with vaccine, carrier or saline three times at two-week interval (n=16). 10 days later after the final DSS drinking water, mice were sacrificed to analyze.

Chlamydial infection: To evaluate whether vaccine immunization increases the susceptibility to lung chlamydial infection, mice were firstly immunized with vaccine, carrier or saline three times at two-week interval. Two weeks later, the mice were anesthetized and inoculated intranasally with 1×10^3 inclusion-forming units (IFU) of *Chlamydia muridarum* in 40 μ l final volume of PBS. 8 days later, the mice were sacrificed to analyze.

Body weight

Mouse body weight was monitored daily in acute colitis (before the delivery of TNBS on the delivery days and in the morning of remaining days) and infection model, and weekly in chronic colitis before each delivery of TNBS.

Clinical score evaluation

The clinical scoring of a disease activity index (DAI) for DSS-induced chronic colitis was based on stool consistency and bleeding.²⁵⁶ Stool consistency: normal, 0; pasty, semiformed, 1; sticky, 2; sticky with some blood, 3; completely liquid, bloody, or unable to defecate after ten minutes, 4. Rectal bleeding: no blood, 0; visible blood in rectum, 1; visible blood on fur, 2.

Histological examination

Colon tissue was fixed in 10% buffered formalin, processed using the protocol described in Table 1 with a Citadel® Tissue Processor from Shandon Inc., and embedded in paraffin. Then the embedded colon sections were cut in 6- μ m thick and stained with H&E or Masson's trichrome according to the protocol provided by Sigma-Aldrich. Histological scoring was evaluated by a pathologist blinded to the source of treatment based on the method previously described.²⁵⁷ During each histological examination, three different parameters were estimated: severity of inflammation (based on polymorphonuclear neutrophil infiltration; 0-3: none, slight, moderate, severe), depth of injury (0-3: none, mucosal, mucosal and submucosal, transmural), and crypt damage (0-4:

none, basal one-thirds damage, basal two-thirds damage, only surface epithelial intact, entire crypt and epithelium lost). All values were added to a sum, in which the maximum possible score was 10.

Soluble collagen assay

For quantitative measurement of collagen, a marker of chronic inflammation, 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 hours at 4°C. Total soluble collagen content of the mixture was determined with a Sircol Collagen Assay Kit (Biocolor).²⁵⁴ Acid soluble type I collagen supplied with the kit was used to generate a standard curve.

Myeloperoxidase (MPO) activity assay

MPO activity, an index of neutrophilic infiltration, was assayed using the method previously described²⁵⁸. 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 (7 µl 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-dianisidine 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.

Measurements of antibodies and cytokines by ELISA

Frozen colonic samples were mechanically homogenized in buffer containing 1M Tris-HCl, 3M NaCl, and 10% Triton supplemented with 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 minutes at 4°C. Supernatants were frozen at -70°C until assay.²⁵⁹

Serum p40-, IL-12-, and IL-23-specific IgG levels were assayed by using ELISA techniques established in our laboratory.^{223, 260} The results obtained from each sample were expressed using optical density at 405 nm (OD₄₀₅). The results obtained from sera pooled from each group were expressed using “titer”, the reciprocal of the highest dilution in which the OD₄₀₅ was twice that of the corresponding control serum when its OD₄₀₅ was 0.10. Cytokine concentrations in colon tissues were measured by ELISA according to the manufacturer’s instructions. IL-12p40, IL-17, IFN- γ , IL-13 and TNF- α assay were purchased from BD Bioscience; IL-23 and IL-10 assay were from eBioscience; TGF β 1 was from R&D.

Real-Time Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Briefly, total RNA was extracted from colons by Trizol (Invitrogen) according to the manufacturer’s instruction.²⁵⁵ Complementary DNA was synthesized using iScriptTM cDNA synthesis kit (Bio-Rad). Primer sequence was as follows: 5’-ggcaaattcaacggcacagt-3’ and 5’-agatggtgatgggcttccc-3’ for GAPDH; 5’-acttcgcagagatgtccagtca-3’ and 5’-tgccaagcgtcccctc-3’ for Bcl-2; 5’-

aacatctggaaccacgggcaactat-3' and 5'-gcattgcttgaggctgcgtatgat-3' for Foxp3; 5'-gtgacggcaaacatgacttcag-3' and 5'-gccatcgggcatctggta-3' for iNOS; 5'-ttccgaattcactggagcctcgaa-3' and 5'-tgcacctcagggagaagaatctggaa-3' for TNF; 5'-ctcacctgtgacacgcctga-3' and 5'-caggacactgaataacttctc-3' for IL-12/IL-23p40; 5'-tgaattccctgggtgagaagctga-3' and 5'-tggccttgtagacaccttggtctt-3' for IL-10. Real-time quantitative RT-PCR was assayed in special optical tubes of 96-well microtiter plates with ABI PRISM 7700 Sequence Detector Systems (Applied Biosystems, Foster City, CA). The reaction mixture consisted of SYBR Green PCR master mix (Applied Biosystems). PCR reaction conditions were 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles at 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 35 seconds, followed by a dissociation stage. Gene expression was calculated relative to the housekeeping gene GAPDH.

Isolation of lamina propria mononuclear cells (LPMC)

LPMC were isolated from freshly obtained colonic specimens using a modification of the method described.²⁶¹ In brief, colon specimens were washed in HBSS-calcium magnesium free (Invitrogen), cut into 0.5-cm pieces, and incubated in HBSS containing EDTA (0.37 mg/ml) and DTT (0.145 mg/ml) at 37°C for 30 min with shaking to release intraepithelial lymphocytes and epithelial cells. The tissue was then digested with 1 mg/ml collagenase D (Sigma, Canada) and 0.01 mg/mL deoxyribonuclease (Sigma, Canada) in a shaking incubator at 37°C for 40 min. Finally, the cells released from the tissue were layered on a 40–100% Percoll gradient (Pharmacia)

and spun at 2400 rpm for 20 mins to obtain the lymphocyte and monocyte-enriched populations at the 40–100% interface.

Intracellular staining for identification of Th1, Th17, Treg, and apoptosis of Th1 and Th17

To examine the percentages of Th1 and Th17 cells, single-cell suspensions containing 1×10^6 mesenteric lymph nodes cells (MLNs), or LPMC were cultured and stimulated for 6-7 hours with 50 ng/mL phorbol myristate acetate and 1 μ g/mL ionomycin (Sigma-Aldrich), with brefeldin A added for the last 4 hours of culture.²⁵⁵ Cells were harvested, washed, and stained with anti-CD4 (eBioscience). Surface-stained cells were fixed (2% paraformaldehyde) and resuspended in permeabilization buffer (0.5% saponin). This was followed by staining for intracellular IL-17 or IFN- γ (eBioscience). To evaluate the apoptosis of Th1 or Th17 cells, cells were stained with an antibody against activated caspase-3 (APO LOGIX, FAM-FMK kit; Cell Technology, USA) plus anti-IFN- γ or anti-IL-17A.¹⁰⁹ For Foxp3 staining, cells were fixed in eBioscience Fix/perm buffer after staining of surface molecules. Then the cells were permeabilized in eBioscience buffer and stained for Foxp3 according to the manufacturer's instruction. Cells were analyzed using a FACSCalibur (BD Biosciences).

Quantification of chlamydial in vivo growth

Mice were sacrificed at 8 days after infection, and the lungs were aseptically isolated and homogenized in SPG buffer.²⁶² Tissue suspensions were centrifuged at 1900 xg for 30 min at 4°C to remove debris and coarse tissues and frozen at -80°C until being

used.²⁶² Quantitation of *Chlamydia muridarum* (MoPn) was performed as described previously.²⁶² Briefly, HeLa 229 cells were cultured in 96-well plates in triplicate with 100 μ l of serially diluted lung tissue supernatants from mice infected with MoPn for 2 hours.²⁶² Then the plates were washed with PBS, and then each well was added 200 μ l of MEM containing cycloheximide, gentamicin, and vancomycin. After 48 hours incubation, the cell monolayers were fixed with absolute methanol.²⁶² To evaluate chlamydial inclusions, plates were incubated with a *Chlamydia* genus-specific murine mAb and then added HRP labeled goat anti-mouse IgG and developed with substrate (4-chloro-1-naphthol; Sigma-Aldrich).²⁶² The number of inclusions was calculated. The chlamydial levels in lung tissue were counted according to dilution titers of the original inoculum.²⁶²

Statistical Analysis

Values were expressed as mean \pm SD. Differences between experimental groups were assessed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test, or student t test (GraphPad Software, San Diego, California, USA). *P* values < 0.05 were considered statistically significant. In all figures, *represents *P*<0.05; ***P*<0.01; ****P*<0.001.

Part Four. Results

Result I Development and identification of IL-12/IL-23p40 peptide-based vaccines

Specific introduction

IL-12 is predominantly produced by dendritic cells, monocytes, and macrophages following recognition of pathogenic structures through toll-like and other receptors.^{134, 135} IL-12 is required for an effective polarization of naïve T helper cells to the Th1 phenotype characterized by the expression of IFN- γ . Expression of the p35 and p40 subunits of IL-12 is independently regulated. The p35 subunit requires the co-expression of p40 for secretion of the biologically active cytokine from the cell. Of interest, the p40 unit of IL-12 is also shared with IL-23, a new heterodimeric cytokine that consists of p40 and p19 units. IL-23 is also predominantly produced by activated dendritic and phagocytic cells.^{135, 160} Similar to IL-12, formation of biologically active IL-23 requires synthesis of both p19 and p40 subunits in the same cell. Moreover, IL-12 and IL-23 share the receptor subunit IL-12R β 1. IL-23 has been recently demonstrated to stabilize the proliferation of a pro-inflammatory subset called Th17 effector cells, which produce the pro-inflammatory mediators IL-17 and IL-6.^{90, 263}

It has become increasingly obvious that IL-12 and IL-23 play important roles in the regulation of inflammatory processes and exhibit strong autoimmune potentials. Over-expressions of IL-12 and IL-23 have been found in several autoimmune diseases, for example, Crohn's disease and multiple sclerosis.^{93, 264}

Crohn's disease is a chronic condition whose incidence and prevalence have markedly increased over the second half of the 20th century.³ It has been widely accepted that Crohn's disease is caused by an overly aggressive Th1 immune response, and a recently found excessive IL-23/Th17 pathway activation to bacterial antigens in genetically predisposed individuals.^{12, 37, 92-94} Both Th1 cytokines and the IL-23/Th17 pathway are critical for the development of chronic intestinal inflammation of Crohn's disease.^{93, 202, 203} Therefore, targeting these cytokines with humanized monoclonal antibodies has emerged as a new biological therapy in Crohn's disease.^{3, 265} In a multicenter, randomized, placebo-controlled, double-blind, phase 2 clinical trial, administration of a humanized monoclonal antibody against IL-12/IL-23 p40 induced clinical responses ($p=0.03$) and remissions ($p=0.07$) in patients with active Crohn's disease. This treatment is associated with decreases in Th1-mediated inflammatory cytokines at the site of disease.²⁰⁸

Like many autoimmune diseases, Crohn's disease is chronic, requiring long-term treatments. Recently, vaccines against endogenous cytokines have been investigated to ameliorate asthma and autoimmune diseases in animal models, since cytokine vaccines induce relatively long-lasting autoantibodies to the target cytokine, which may be used as a maintenance treatment for the diseases.^{223, 233-235} However, to induce antibodies against over-expressed endogenous cytokines by active immunization, immune tolerance to self-proteins must be overcome. Our laboratory has successfully designed cytokine vaccines by inserting a small peptide derived from the target cytokine into a carrier protein, 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.^{223, 260, 266-268}

Administration of the vaccines significantly reduces the levels of target cytokines, consequently resulting in the amelioration of the disease.

Given the previous success of this strategy and the prominent role of IL-12 and IL-23 in the immunopathogenesis of Crohn's diseases, we have, for the first time, developed mouse IL-12/IL-23 p40 peptide-based vaccines and explored the *in vivo* effects of these vaccines in the suppression of chronic colitis in mice.

Results

1. Selection of peptides from IL-12/IL-23p40

Based on the occurrence of amino acid residues in experimentally known segmental epitopes and by using DNASTAR software, seven peptides with high antigenic index, high flexibility, high surface probability and high hydrophilicity were selected from mouse IL-12/IL-23 p40 subunits (Table 1). And the corresponding human peptides of these seven peptides were tested in the crystal structures of human IL-12 and IL-23 to check their locations (Figure 1). The p40 subunit is composed of three domains (D1, D2, and D3). Of which D1 domain contains one N-terminal immunoglobulin like domain, and the D2 and D3 domains represent the canonical cytokine-binding region. In these 7 peptides selected, peptide C and F are located in the D1 domain, other peptides are located in the D2 or D3 domain.

Peptide	Sequence	Number of amino acid
A	²³⁶ PKNLQMKPLKNSQVEVSW ²⁵³	18
B	³¹⁵ QDRYYNSSCSKWACVP ³³⁰	16
C	⁵³ PEEDDITWTSDQRHGVIGS ⁷¹	18
D	¹⁶⁰ PDSRAVTCGMASLSAEKV ¹⁷⁷	18
E	¹¹⁹ NFKNKTFLKCEA ¹³⁰	12
F	³⁸ TPDAPGETV ⁴⁶	9
G	²⁷⁴ IQRKKEKMKETE ²⁸⁵	12

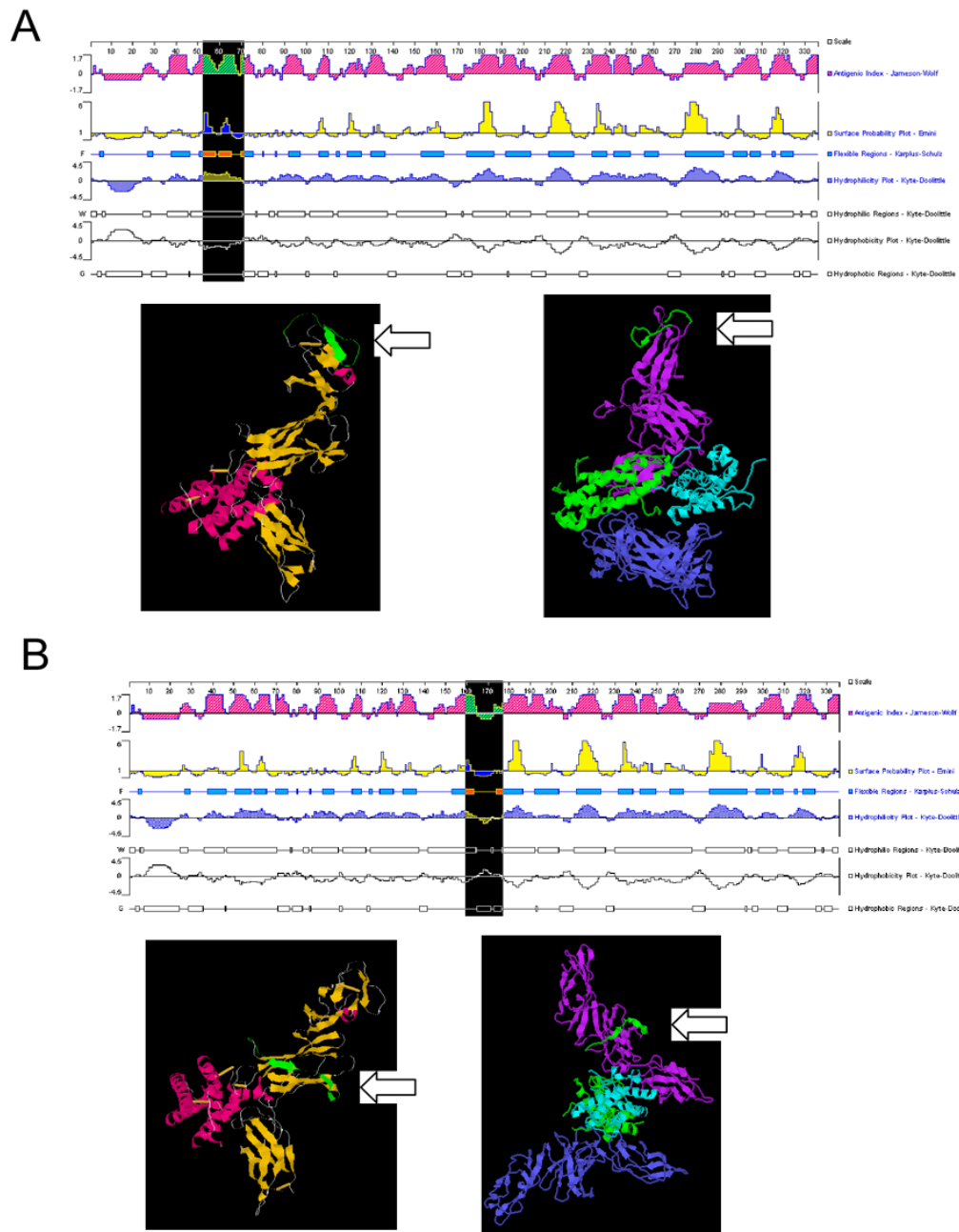


Figure 1. Selection of IL-12/IL-23p40 peptides. (A) top: peptide C predicted in the DNASTar software; bottom left: peptide C's corresponding peptide of human IL-12p40 in the crystal structure of IL-12; bottom right: peptide C's corresponding peptide of human IL-12p40 in the crystal structure of IL-23. (B) top: peptide D predicted in the DNASTar software; bottom left: peptide D's corresponding peptide of human IL-12p40 in the crystal structure of human IL-12; bottom right: peptide D's corresponding peptide of human IL-12p40 in the crystal structure of human IL-23. (Arrow indicated the positions of peptides selected)

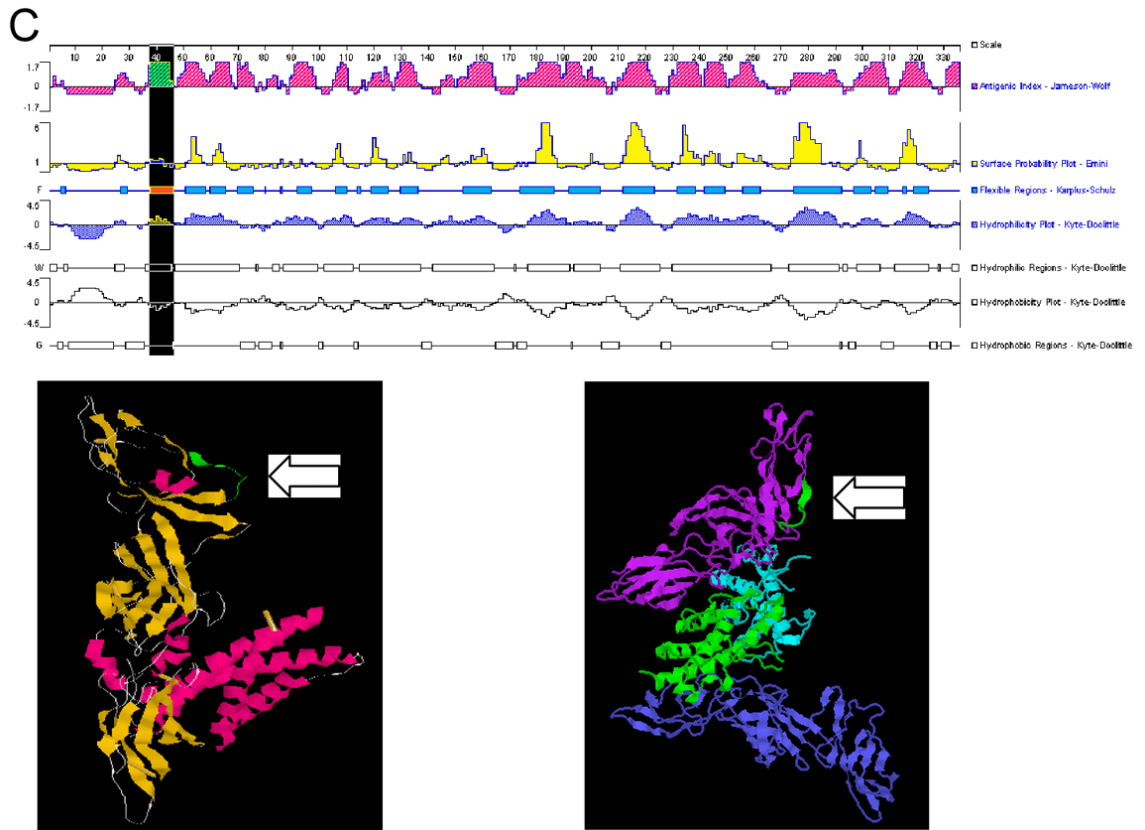


Figure 1. Selection of IL-12/IL-23p40 peptides. (C) top: peptide F predicted in the DNASTar software; bottom left: peptide F's corresponding peptide of human IL-12p40 in the crystal structure of human IL-12; bottom right: peptide F's corresponding peptide of human IL-12p40 in the crystal structure of human IL-23. (Arrow indicated the positions of peptides selected)

2. IL-12/IL-23 p40 peptide-based vaccines induce high titers of IgG to IL-12, IL-23 and p40

To construct peptide-based vaccines, the peptides selected were inserted into the vector plasmid pThio-HBcAg by gene recombination methods. The recombinant plasmids were then identified by restriction endonucleases digestion, and SDS-PAGE (Figure 2). Finally, based on the formation of virus-like particles, three separate p40 peptide vaccines, named vaccine C, D and F, were selected for further analysis.

To determine the ability of the vaccines to induce IL-12-specific IgG responses, mice were immunized with different doses of one of the three vaccines or the carrier protein. Sera were collected at indicated times to detect the antibody levels by ELISA. As shown in Figure 3, all vaccines induce high levels of IL-12 specific IgG antibodies after three immunizations, while mice received the carrier or saline injections have no detectable specific antibodies. The antibody levels remained high and lasted for at least over 6 weeks without a booster injection, which is similar to our previous reports on an IL-13 vaccine.²²³ At week 3, the levels of IL-12-specific IgG induced by 100 µg dose were higher than those induced by other doses. But, at week 7, the levels of IL-12-specific IgG induced by the three doses (100 µg, 50 µg, and 25 µg) were similar in the three vaccines. As a low dose of antigen induces high affinity antibodies, a 100 µg dose was used as the first immunization and a 25 µg dose was used as the next 2 immunizations in the following experiments.

Mice were then immunized with each of the vaccines, carrier or saline using this immunization dose protocol. The results showed that vaccine C, D, and F induced significantly high levels of specific IgG antibodies to IL-12, IL-23 and the p40 subunit,

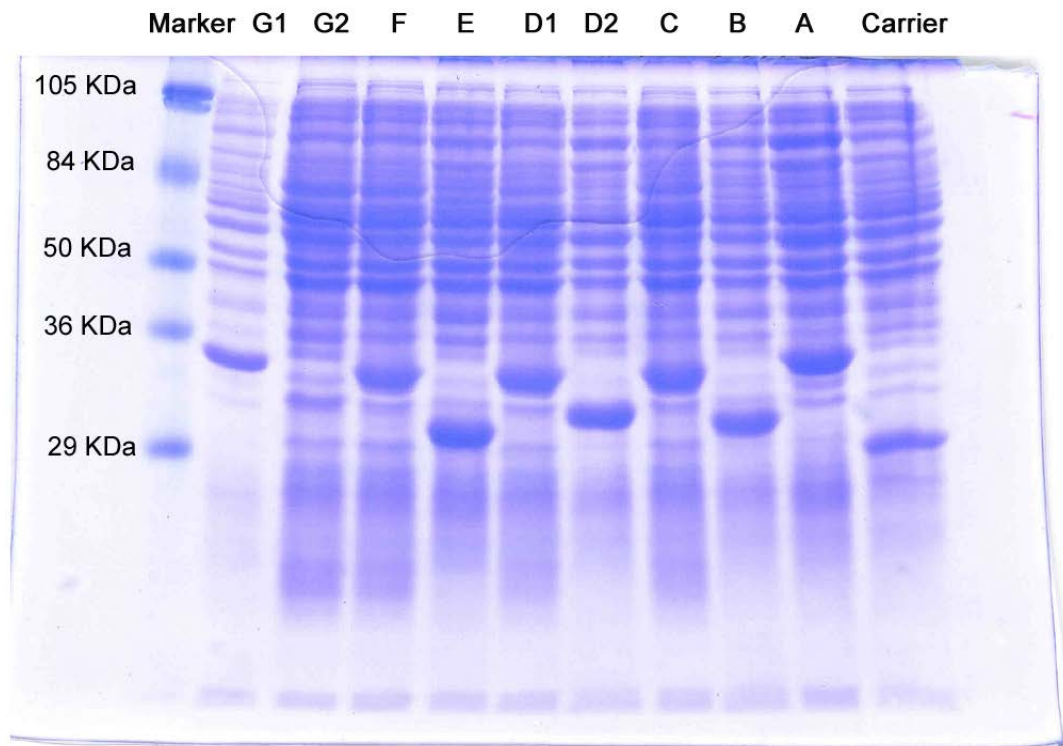


Figure 2. Identification of IL-12/IL-23p40 peptide-based vaccines constructed. The target peptides coupled into carrier were collected after induction with IPTG, and run SDS-PAGE gel to identify.

- Lane A: Recombinant vaccine containing peptide A
- Lane B: Recombinant vaccine containing peptide B
- Lane C: Recombinant vaccine containing peptide C
- Lane D1: Recombinant vaccine 1 containing peptide D
- Lane D2: Recombinant vaccine 2 containing peptide D
- Lane E: Recombinant vaccine containing peptide E
- Lane F: Recombinant vaccine containing peptide F
- Lane G1: Recombinant vaccine 1 containing peptide G
- Lane G2: Recombinant vaccine 2 containing peptide G

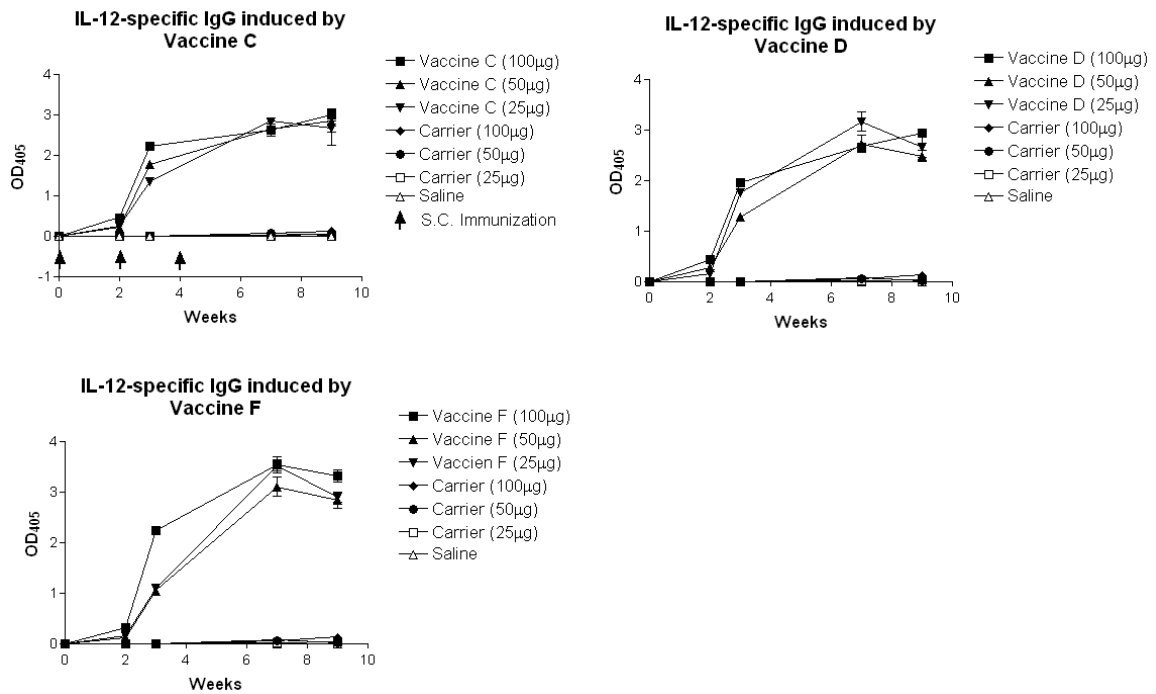


Figure 3. The levels of IL-12-specific IgG antibodies induced by different doses of vaccines. Female BALB/c mice (n=4) were subcutaneously immunized three times with 100 µg, 50 µg or 25 µg/200 µl of each vaccine, carrier or saline at a two-week interval. Sera were obtained from the mice at the indicated weeks and diluted 1/200 for determination of specific IgG levels by ELISA in which IL-12 (0.25 µg/ml) was coated on the plates.

compared with carrier and saline groups (Figure 4A). Among the three vaccine groups, the levels of the three specific IgG antibodies, induced by vaccine F, tended to be higher than the levels induced by vaccines C and D. As shown in Figure 4B, among the three specific IgG antibodies, p40-specific IgG has the highest titer, while IL-23-specific IgG has the lowest titer. The specific IgG antibodies induced by vaccine F consistently showed the higher titers than those induced by vaccines C and D; the titer was up to 496,000 for p40-specific IgG, 85,000 for IL-12-specific IgG, and 24,000 for IL-23-specific IgG.

These results indicated that the three IL-12/IL-23 p40 peptide vaccines were successfully constructed, and capable of inducing high levels of specific IgG to IL-12, IL-23 and the p40 subunit. Among them, vaccine F had the greatest immunogenicity.

3. The avidity of the specific IgG antibodies to IL-12, IL-23 and p40 induced by vaccines

To evaluate the specific antibody binding affinity, the antibody avidity was measured by sodium thiocyanate (NaSCN)-displacement ELISA. NaSCN is a chaotropic agent which disrupts the antigen–antibody interaction. The binding of antibodies with greater avidity to the antigen is disrupted at higher concentrations of NaSCN than that of antibodies with lower avidity to the antigen. Figure 5 demonstrated that the effective concentration of NaSCN required to release 50% of the antibody (ED_{50}) was higher in vaccine C-immunized mice than those in vaccine F and D groups, while the ED_{50} from mice immunized with vaccine D was the lowest among these three vaccine groups. These results indicated that specific antibodies to IL-12, IL-23 and p40 subunit induced by

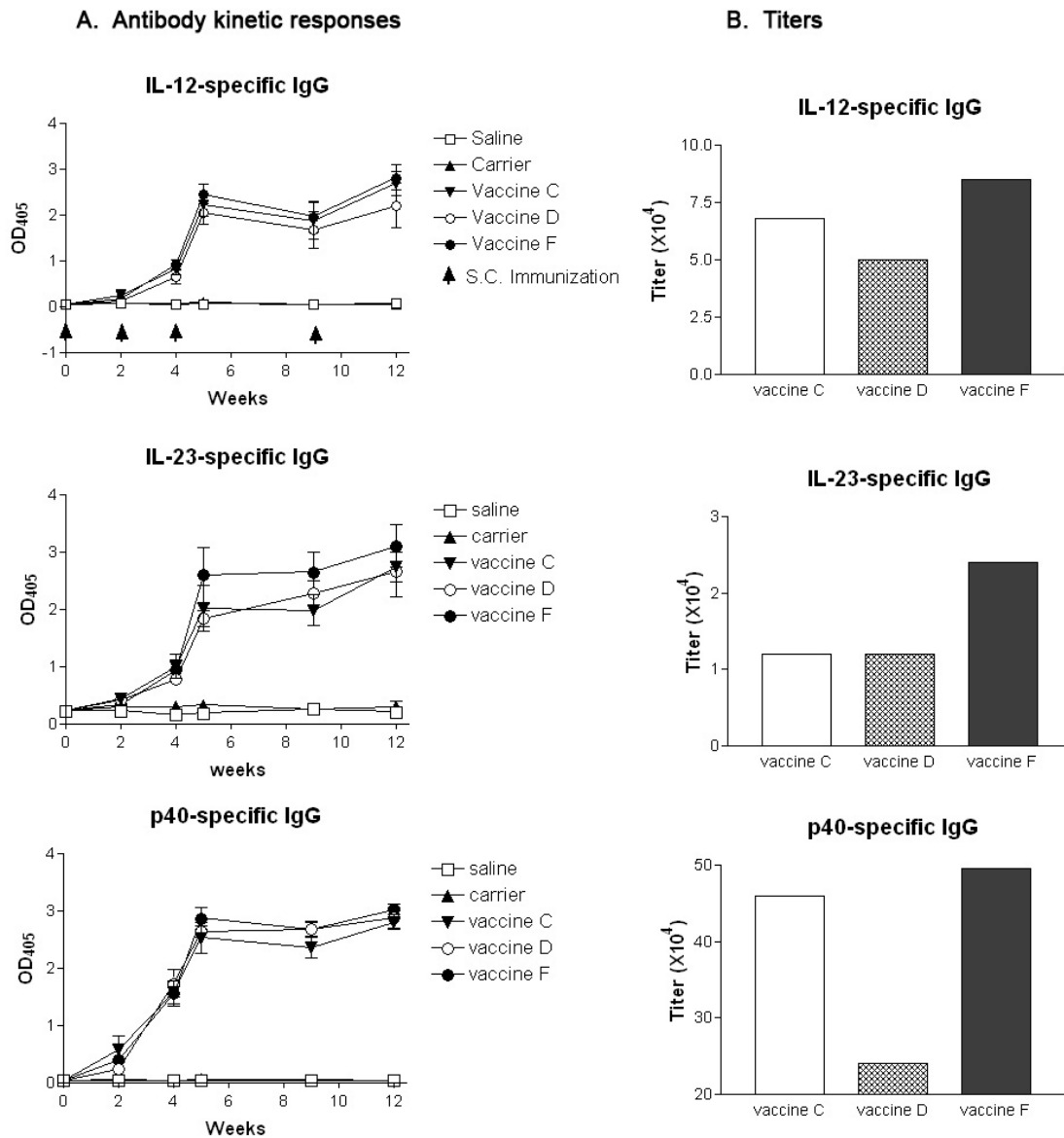


Figure 4. The levels and titers of specific IgG antibodies to IL-12, IL-23 and IL-12/IL-23p40 induced by vaccines. Female BALB/c mice ($n=8$) were subcutaneously primed with $100 \mu\text{g}/200 \mu\text{l}$ of each vaccine and carrier or $200 \mu\text{l}$ of saline, and boosted at weeks 2, 4 and 9 with $25 \mu\text{g}$ of the vaccine or the carrier. Sera were obtained from the mice at the indicated weeks and diluted $1/200$ for determination of specific IgG levels by ELISA in which IL-12, IL-23 or p40 ($0.25 \mu\text{g}/\text{ml}$) were coated on the plates, separately. The titer of the test sample was expressed as the reciprocal of the highest dilution in which the OD405 was twice that of the corresponding control serum (the carrier group) when its OD405 was 0.10. Data represent experiments repeated at least three times.

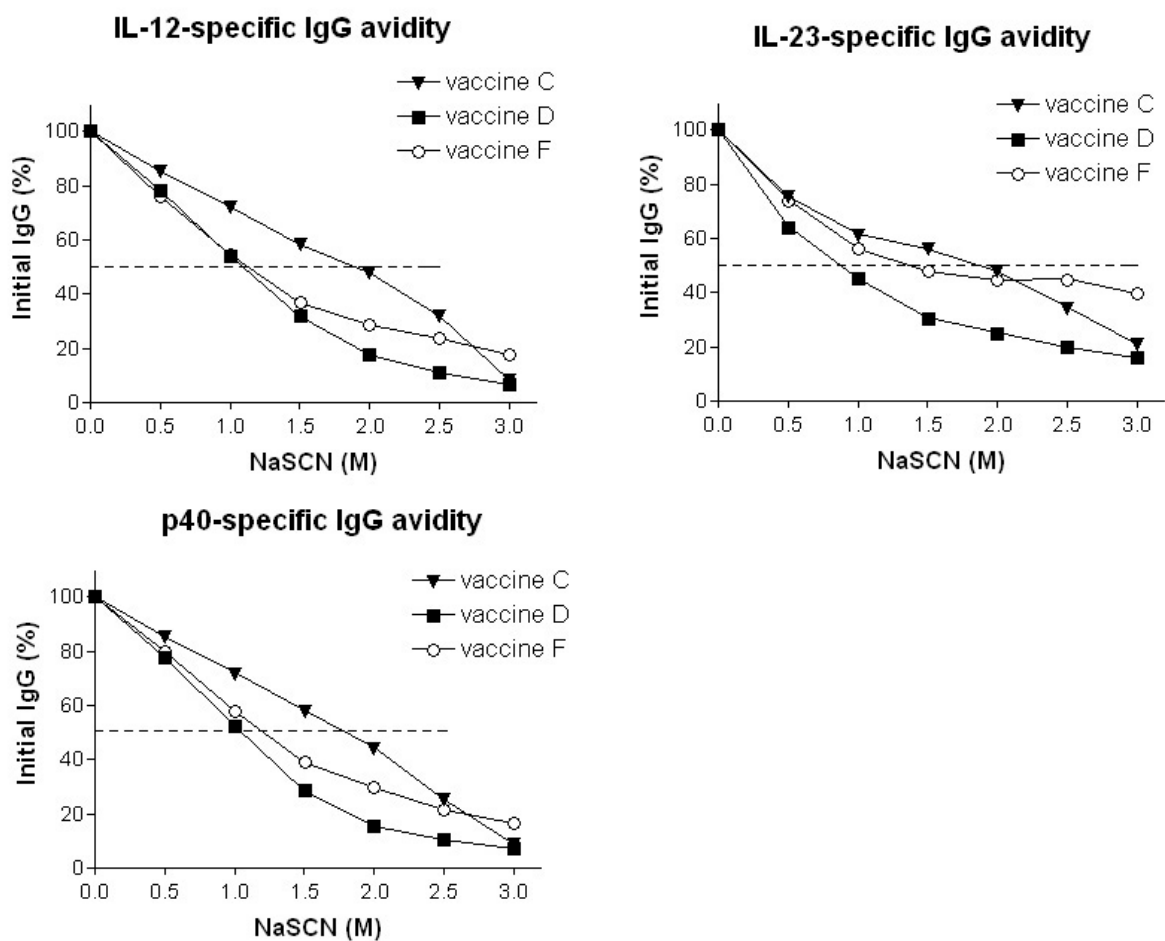


Figure 5. Avidity of specific antibodies induced by the three vaccines. Sera were obtained from the mice immunized with vaccine C or vaccine D or vaccine F as described in the legend of Fig. 4, and analyzed using a NaSCN displacement ELISA.

vaccine C had the highest avidity, Vaccine F had the second highest avidity, and vaccine D had the lowest avidity of the three groups.

4. Sera from vaccine-immunized mice inhibit IL-12-induced IFN- γ and IL-23-induced IL-17 secretion of splenocytes

Our results have shown that the p40 peptide-based vaccines can induce high levels of specific IgG antibodies to IL-12, IL-23 and p40. To evaluate whether the antisera could inhibit the biological functions of IL-12 and IL-23, IL-12-induced IFN- γ secretion and IL-23-induced IL-17 secretion of ConA blasts prepared from BALB/c spleen cells were tested. As shown in Figure 6A, antisera from mice immunized with vaccine C and F significantly inhibited IL-12-induced IFN- γ secretion of splenocytes, whereas sera from vaccine D-immunized mice showed no obvious inhibition. With regards to IL-23-induced IL-17 secretion, antisera from all vaccine groups had inhibitory effect. Sera from vaccine D-immunized mice showed a relative higher inhibitory effect than those from mice immunized with vaccines C or F (Figure 6B). These results indicated that the specific antibodies induced by the p40 peptide-based vaccines were able to inhibit the biological functions of IL-12 and IL-23.

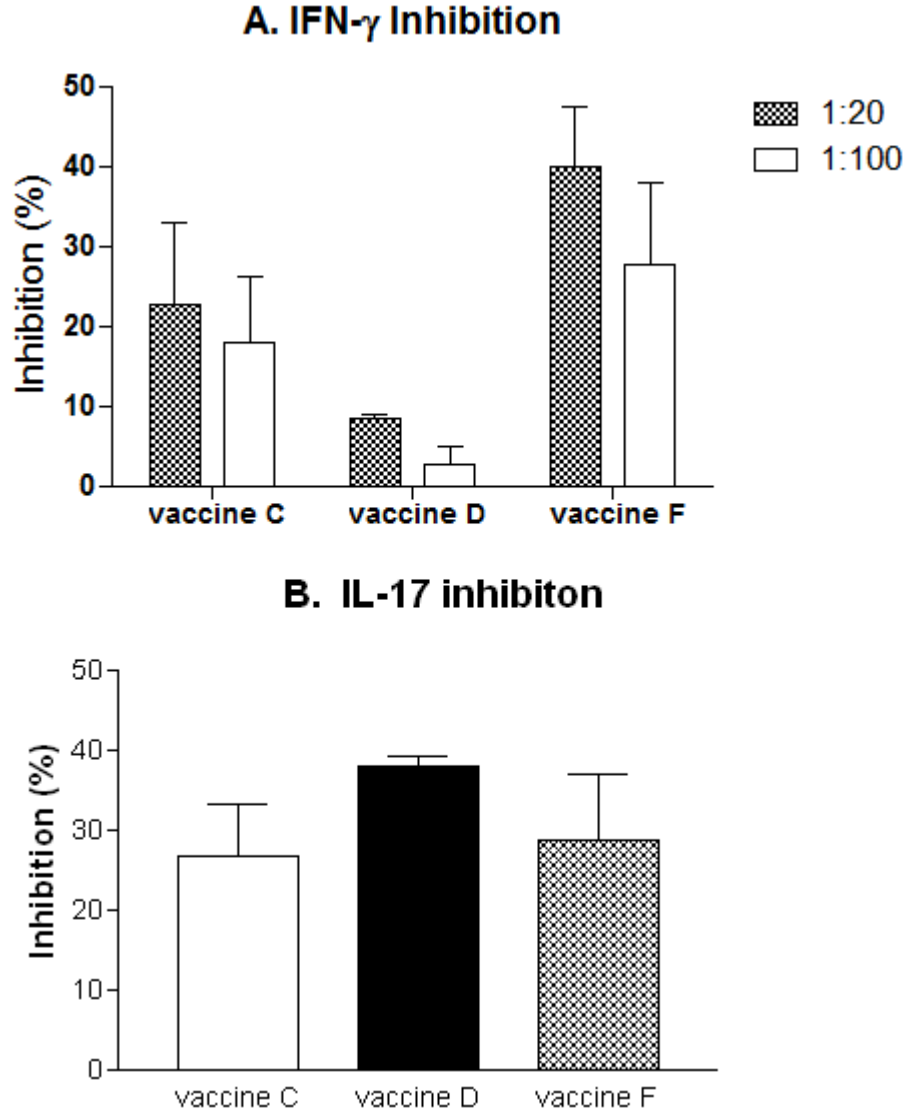


Figure 6. *In vitro* inhibition of vaccine-immunized antisera on IL-12-induced IFN- γ secretion and IL-23-induced IL-17 production from splenocytes. Splenocytes isolated from normal BALB/c mice were stimulated with Con A. After 72 h, the Con A-activated blasts were restimulated with IL-12 or IL-23 and IL-2 in the presence of various serum dilutions of mice receiving vaccine C, D, F or carrier. Forty-eight hours later, supernatants were collected for measurement of expression levels of IFN- γ or IL-17 by ELISA.

5. Vaccines C and F, but not vaccine D, suppress intestinal inflammation in chronic murine colitis

It has been shown that IL-12 promotes intestinal system autoimmunity through the activation/expansion of Th1 cells that produce IFN- γ , while IL-23 enhances intestinal system autoimmunity through expansion of Th17 cells that produce IL-17, IL-6 and TNF- α . We targeted the p40 subunit that is shared by IL-12 and IL-23. After identification of the three p40 vaccines, all of which were able to induce high titers of specific antibodies and inhibit the biological functions of IL-12 and IL-23, the three vaccines were further evaluated *in vivo* in a murine chronic colitis model.

Hapten-induced colonic inflammation is a widely used animal model of human Crohn's disease.²⁴⁹⁻²⁵¹ Intrarectal delivery of 2,4,6-trinitrobenzene sulphonic acid (TNBS) induces colitis by haptening 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. To evaluate the effects of IL-12/IL-23p40 peptide-based vaccines in the prevention of TNBS-induced chronic colitis, 5 groups of mice (8 mice per group) were immunized three times with each of the vaccines or the carrier or saline. After ensuring that high titers of specific IgG antibodies to IL-12, IL-23, and p40 were present, mice were then weekly administered with TNBS six times (1.0 - 2.3 mg per mouse, starting at 1.0 mg and gradually increasing the dose to 2.3 mg) (Figure 7A).

After repeated TNBS injections, colons from saline and carrier groups showed signs of severe inflammation, including inflammatory cell infiltration, goblet cell

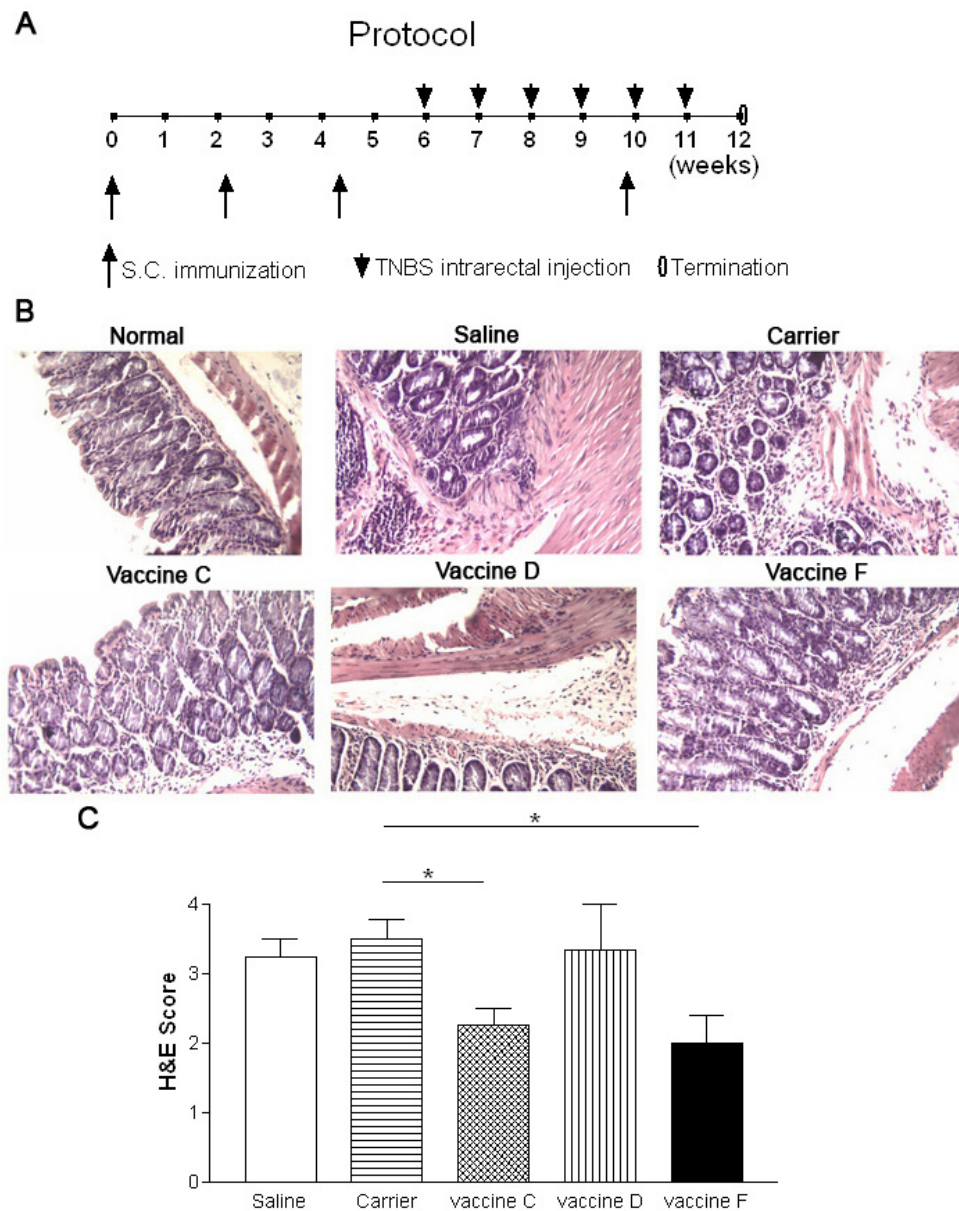


Figure 7. Effects of IL-12/IL-23p40 peptide based vaccines on histological inflammation in mice with TNBS-induced chronic colitis. (A) The protocol of vaccine immunization and TNBS intra-rectal injection. (B) Colonic specimens (n=6) were formalin-fixed and embedded in paraffin blocks, then 6- μ m sections were stained with hematoxylin and eosin. Representative histological images of samples from normal control and TNBS-treated mice following treatment with vaccines, carrier or saline are shown (original magnification x 100). (C) Semi-quantitative analysis was used to assess histological changes. H&E scores of 0-4 were given for each mouse. (*, $P < 0.05$)

reduction, and distorted tissue architecture (Figure 7B). Colon tissues from vaccine D group presented similar inflammatory findings as saline and carrier groups. However, colon tissues from mice immunized with either vaccine C or vaccine F showed reduced inflammation when compared to carrier and saline controls. Semi-quantitative analysis revealed that inflammatory scores in vaccine C and F groups were significantly lower than carrier controls ($P<0.05$) (Figure 6C), confirming that vaccines C and F were able to suppress TNBS-induced chronic intestinal inflammation.

6. Vaccines C and F inhibit fibrosis of chronic intestinal inflammation

As fibrosis is a characteristic of chronic inflammation, the collagen deposition in colons was evaluated using Masson's trichrome staining. In addition, soluble colon collagen was quantitatively measured using a Sircol Collagen Assay kit. As shown in Figure 8A, when compared to normal controls, the saline, carrier, and vaccine D groups show substantial collagen deposition in the intestinal subepithelial layer, deep in the lamina propria, and in the muscular layers. In contrast, vaccine C and F groups showed no significant increase in collagen deposition as compared to normal controls. Moreover, the soluble collagen assay revealed that the amounts of collagen were significantly reduced in vaccines C and F groups (Figure 8B). These results indicate that vaccines C and F are able to inhibit fibrosis formation during chronic intestinal inflammation.

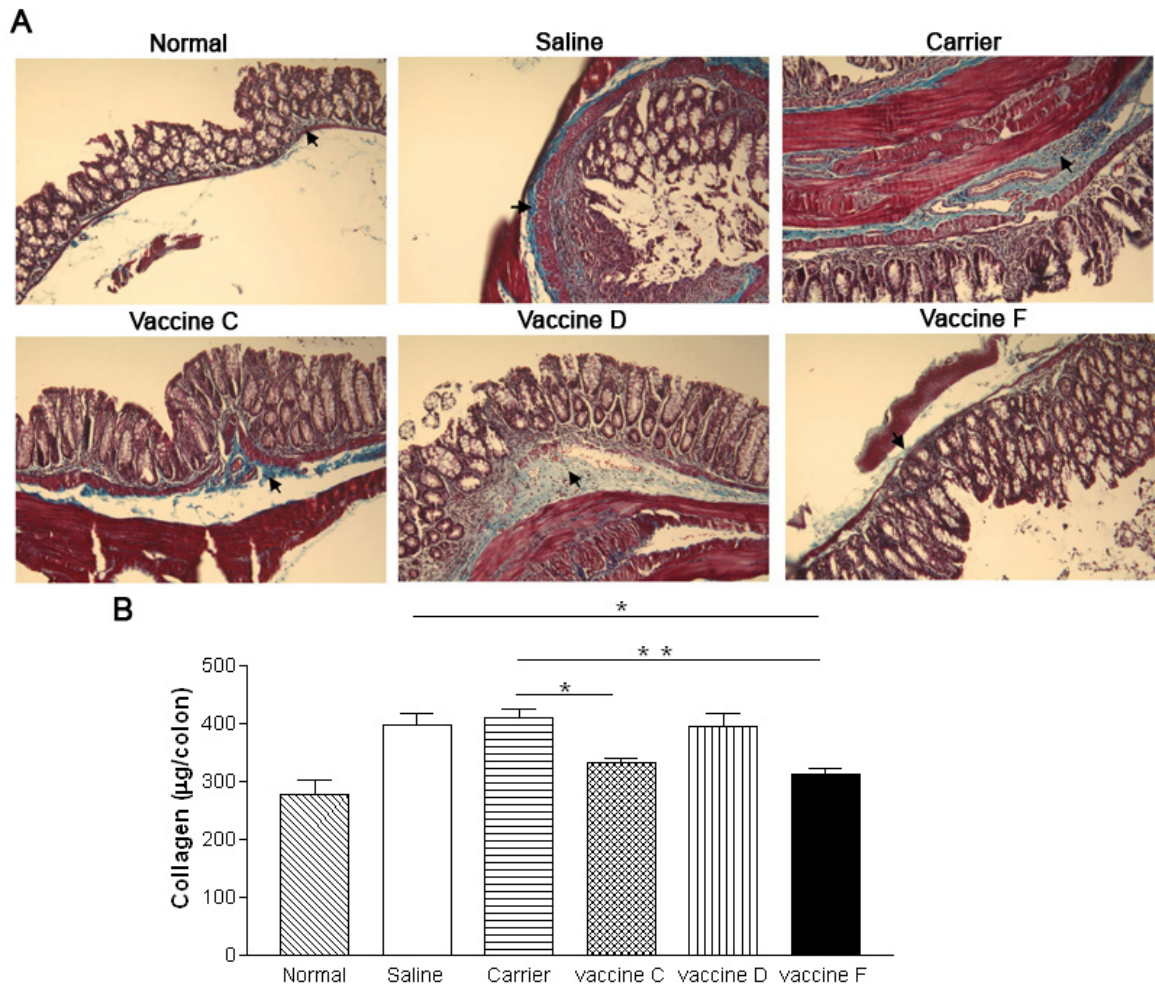


Figure 8. Effects of IL-12/IL-23p40 peptide based vaccines on collagen deposition in colon tissue of mice with TNBS-induced chronic colitis. (A) Masson's trichrome staining of representative colons from a normal control and TNBS-treated mice following treatment with vaccines, carrier or saline (original magnification x 100). (B) Quantitation of soluble collagens in colon tissue by a Sircol assay (n=6). (*, $P < 0.05$; **, $P < 0.01$)

7. Vaccines downregulate the levels of p40 subunit

Our vaccines were made to act directly against the p40 subunit that is shared by IL-12 and IL-23, both important inflammatory cytokines in Crohn's disease. To test the effectiveness of our vaccines, we analyzed samples from all groups for levels of the IL-12/IL-23 p40 subunit using ELISA. As shown in Figure 9, both carrier and saline groups had an increased expression of p40 when compared with normal mice. However, all vaccine groups showed decreased levels of p40, suggesting that IL-12/IL-23p40 vaccines are able to downregulate the production of the colonic proinflammatory cytokine IL-12 and IL-23 in TNBS-induced chronic colitis mice by reducing the levels of p40 in colon tissues.

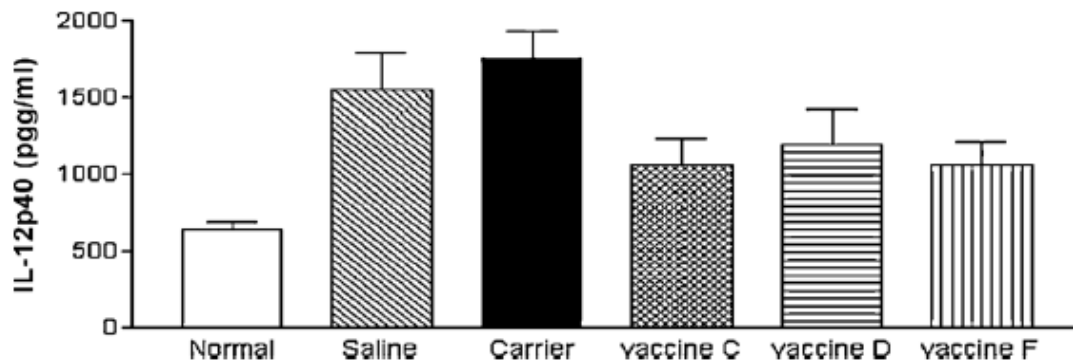


Figure 9. Effects of IL-12/IL-23p40 peptide based vaccines on p40 expression in colon of mice with TNBS-induced chronic colitis. Frozen colonic samples were mechanically homogenized in buffer supplemented with protease cocktail. Samples were centrifuged to get the supernatants. The concentration of IL-12/IL-23p40 in the supernatants was determined by ELISA. The results presented were a representative of two independent experiments. Values were expressed as mean \pm SD.

Discussion

The differentiation of Th1, Th2 and Th17 cells is a central paradigm in T cell-mediated immunity. Th2 cells have been shown to mediate allergic responses, whilst Th1 cells are generally associated with autoimmune diseases, including Crohn's disease, encephalitis and myocarditis. Recent studies have demonstrated that Th17 cells are also responsible for evoking many autoimmune processes formerly ascribed to Th1 cells, such as arthritis.²⁶⁹

The proinflammatory cytokines IL-12 and IL-23 have been found to play a prominent role in the pathogenesis of Crohn's disease. As such, targeting p40 subunit that is shared by IL-12 and IL-23 with humanized monoclonal antibodies has shown promise in the treatment of inflammatory bowel disease.²⁰⁸

The therapies with humanized monoclonal antibodies (mAb) against cytokines provide a novel technique to neutralize over-expressed cytokine levels, and these have achieved great success clinically.^{93, 270} But, these agents all act as passively administered antagonists with short half-lives. Repeated intravenous or subcutaneous injections with large doses of monoclonal antibodies are required to maintain the effects. Adverse reactions induced by administration of these monoclonal antibodies include development of antibodies against the infused mAb, infusion reactions, and anaphylaxis.²⁷¹⁻²⁷³ Another highly relevant concern is the extremely high cost associated with such passively administered therapeutics, which is estimated at \$35,000 - \$40,000 annually per patient. In the long term, the cost of down-regulating cytokine production using these passive blockages will be huge in the management of chronic diseases. To overcome these

disadvantages, a new strategy involving vaccines targeting cytokines is being investigated, which may offer long-term efficacy with fewer adverse effects.^{222, 223, 260, 274}

Due to immunological tolerance, immune responses against self-proteins are usually not generated. To overcome B cell tolerance, one strategy is to modify the intact self-protein by inserting a foreign peptide containing Th epitopes.²³⁰ The second strategy is to link the intact self-protein, or a considerable part thereof, to a heterologous carrier protein.²³¹ Polyclonal antibodies induced by such vaccines can have excellent neutralizing capacity, but because they are raised against multiple self-antigen determinants, 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. The present reported p40 peptide-based vaccines that contain 9 - 18 amino acids derived from the p40 have the unique advantage to avoid such possible cross-reactivity, as PubMed BLAST searching has shown that there is no similar sequences to the peptides existed in other molecules.

Selection of the appropriate carrier protein is another important consideration in the development of vaccines against self-proteins. The most commonly used carrier proteins are bacterial proteins, such as Tetanus toxoid or Diphtheria toxoid, commonly encountered by humans. Keyhole limpet hemocyanin and ovalbumin are also often used as vaccine carriers.²³⁵ Vaccines using these carriers have weak immunogenicity and strong adjuvants such as CFA/IFA are required to elicit sufficient antibody responses. As virus-like particles induce potent B cell responses even in the absence of adjuvants,^{223, 260, 266, 268, 275} as a carrier protein, they are more preferable to other proteins.²⁷⁶

The present reported peptide-based vaccines used HBcAg as a carrier and, therefore, presented as virus-like particles. The vaccine was prepared by inserting a peptide derived from the p40 subunit into the immunodominant epitope region of the carrier HBcAg using gene recombination methods. As the immunodominant epitope region of the carrier HBcAg has been broken down, antibodies induced by the carrier HBcAg itself were very low. The HBcAg virus-like particle 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 DCs and macrophage interactions.²⁷⁷ A single HBcAg particle consists of 180 or 240 HBcAg molecules, each of which is inserted with one p40 peptide. Therefore, a total of 180 or 240 p40 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,²⁷⁸ thus providing a unique advantage that leads to breaking of B cell tolerance. Another benefit to using HBcAg as a carrier is that the safety of using HBcAg as a carrier has been confirmed in a phase I clinical trial for a malaria vaccine,²⁷⁹ making this vaccine even more attractive for human use.

Through this active immunization, we aimed to induce high titered and long-lasting autoantibodies to the target cytokine, thus leading to the improvement of the disease. Our study demonstrated that active vaccination with IL-12/IL-23p40 peptide-based vaccines induced high titered and relative long-lasting neutralizing antibodies to IL-12, IL-23 and IL-12/IL-23p40. Using this strategy, we were able to decrease colonic inflammation and lessen the degree of collagen deposition in colon tissues. As to the

overall differing efficacies of the 3 vaccines, it may be due to their different locations and antigenicities of the chosen peptides within the p40 subunit.

These vaccines, by downregulating IL-12, are able to halt the differentiation of naïve T cells into mature Th1 cells, leading to a reduction in IFN- γ . In addition, the reduced presence of IL-23 may hinder the differentiation of naïve T cells into Th17 cells. As Th17/Treg cell imbalance has recently been demonstrated to be essential for the development and manifestations of Crohn's disease.²⁸⁰ These vaccines may act by partially correcting this balance. Further quantification of the Th1/Th17/Treg cell balance may be of value in helping to elucidate the action of these vaccines.

In summary, we have demonstrated that IL-12/IL-23p40 peptide-based vaccines, especially vaccines C and F, are capable of downregulating the inflammatory responses seen in chronic TNBS-induced murine colitis. By using an active immunization strategy, we offer an innovative, relatively long-term treatment approach capable of ameliorating the destructive effects of Crohn's disease.

Result II IL-12/IL-23p40 vaccine ameliorates acute and chronic murine colitis

Specific Introductions

It has been widely accepted that Crohn's disease is caused by an overly aggressive Th1 immune response and, recently discovered, excessive IL-23/Th17 pathway activation to bacterial antigens in genetically predisposed individuals.^{12, 37, 93} These Th1 cytokines, such as IL-12, and the IL-23/Th17 pathway are probably critical in generating and perpetuating the chronic intestinal inflammation of Crohn's disease.^{93, 202}

In the TNBS-induced chronic colitis model, Th1 responses are initiated after the first time TNBS challenge, and reach maximus after the second TNBS challenge with production of high levels of IL-12 and IFN- γ , which then gradually decline. After three weekly-TNBS challenges, Th1 responses have returned close to normal level, but then are supplanted by a Th17 response after 4-5 weeks with overproduced IL-23 and IL-17 in the colon tissue.²⁵⁴ Administration of an IL-12 antagonist (the IL-12p40-IgG2b fusion protein)¹⁹⁵ or anti-mouse IL-12p40 antibodies¹⁹⁶ abrogates TNBS-induced acute colitis, including lessening mucosa inflammation, improving body weight loss, and restoring a nearly normal histological appearance of the colon, through a mechanism of induction of Fas-mediated apoptosis of Th1 cells.¹⁹⁶ This treatment also reduces TNF levels and increases IL-10 secretion in mice.¹⁹⁵

Recently, with the finding of IL-23/Th17 pathway, many studies highlighted the roles of IL-23/Th17 pathway in the pathogenesis of Crohn's disease.^{3, 103, 106, 201, 259} For instance, the development of colitis is suppressed by IL-23p19 deficiency but not IL-

IL-12p35 deficiency in IL-10^{-/-} mice, and that administration of IL-23 accelerates the onset of colitis and promotes inflammation through an IL-17- and IL-6-dependent mechanism.¹⁰⁶ Anti-IL-23 monoclonal antibody can prevent and reverse active colitis in a T cell-mediated colitis mouse model, with the down-regulation of a broad array of inflammatory cytokines and chemokines in the colon, and promoting the apoptosis of Th17 cells.¹⁰⁹ Even so, these findings do not exclude the role of an exaggerated Th1 response in Crohn's disease, since IL-12/IFN- γ and IL-23/IL-17 may be parallel pathways involved in inflammatory responses.³⁷

These observations in mice are supported by successful clinical trials. Monoclonal antibodies (mAb) against IL-12/IL-23p40 have demonstrated efficacy for management of active moderate-to-severe Crohn's disease patients.²⁰⁸ Although the therapy with mAb may be useful clinically,^{93, 281} these agents all act as passively administered antagonists with short half-lives. Repeated intravenous or subcutaneous injections with large doses of mAb are required to maintain the effects.^{282, 283} Vaccines can induce relative long-lasting antibodies, and natural auto-antibodies against cytokines have been detected in healthy individuals, indicating that the vaccine strategy might be useful for the treatment of Crohn's disease.

In the previous chapter, we have developed and identified three recombinant mouse IL-12/IL-23 p40 peptide-based virus-like particle vaccines (C, D and F), which induce high levels of specific antibodies against p40, IL-12, and IL-23.²⁴⁵ The vaccine is constructed by inserting a peptide derived from mouse p40 subunit into a carrier protein, hepatitis B core antigen (HBcAg), using gene engineering methods. In this study, we systemically evaluated the effectiveness of the best vaccine (F) in the attenuation of

TNBS-induced both acute and chronic murine colitis. We further explored the possible immune mechanisms of vaccine-mediated improvement, especially Th1 and IL-23/Th17 responses.

Results

1. Vaccine induces high levels of specific IgG antibodies against p40, IL-12, and IL-23, and improves intestinal inflammation in acute colitis

After three immunizations, the p40 peptide-based vaccine induced significantly high levels of specific antibodies against p40, IL-12, and IL-23. The titers of the serum pooled from vaccinated mice were 150,000 for p40-specific IgG, 80,000 for IL-12-specific IgG, and 10,000 for IL-23-specific IgG. This was similar to our previous results.²⁴⁵

After mice developed high levels of antibodies against p40, IL-12, and IL-23, they were intrarectally challenged with TNBS twice to induce acute colitis (Figure 10A). As shown in Figure 10B, after each TNBS administration, mice of saline and carrier groups show obvious body weight loss, but vaccine-treated mice have significantly improved body weight loss ($P<0.05$) and their body weight quickly recovered to the level of normal mice on days 3 – 7.

Histological analysis revealed that untreated mice (saline and carrier groups) exhibited severe thickening of the muscularis, a marked mucosal inflammatory cell infiltrate, large numbers of focal lesions characterized by epithelial cell sloughing, increased neutrophilic infiltrate, and complete loss of crypt architecture, resulting in high inflammatory scores (Figure 10C,D). In contrast, colons from vaccine-treated mice showed much less inflammation than controls ($P<0.05$), indicating that vaccine treatment could ameliorate intestinal inflammation significantly.

The effectiveness of vaccination, seen in histological analysis, was further quantitatively confirmed by the results obtained from the measurement of MPO activity, an enzyme specific to granulocyte lysosomes, and, therefore, directly correlated with the

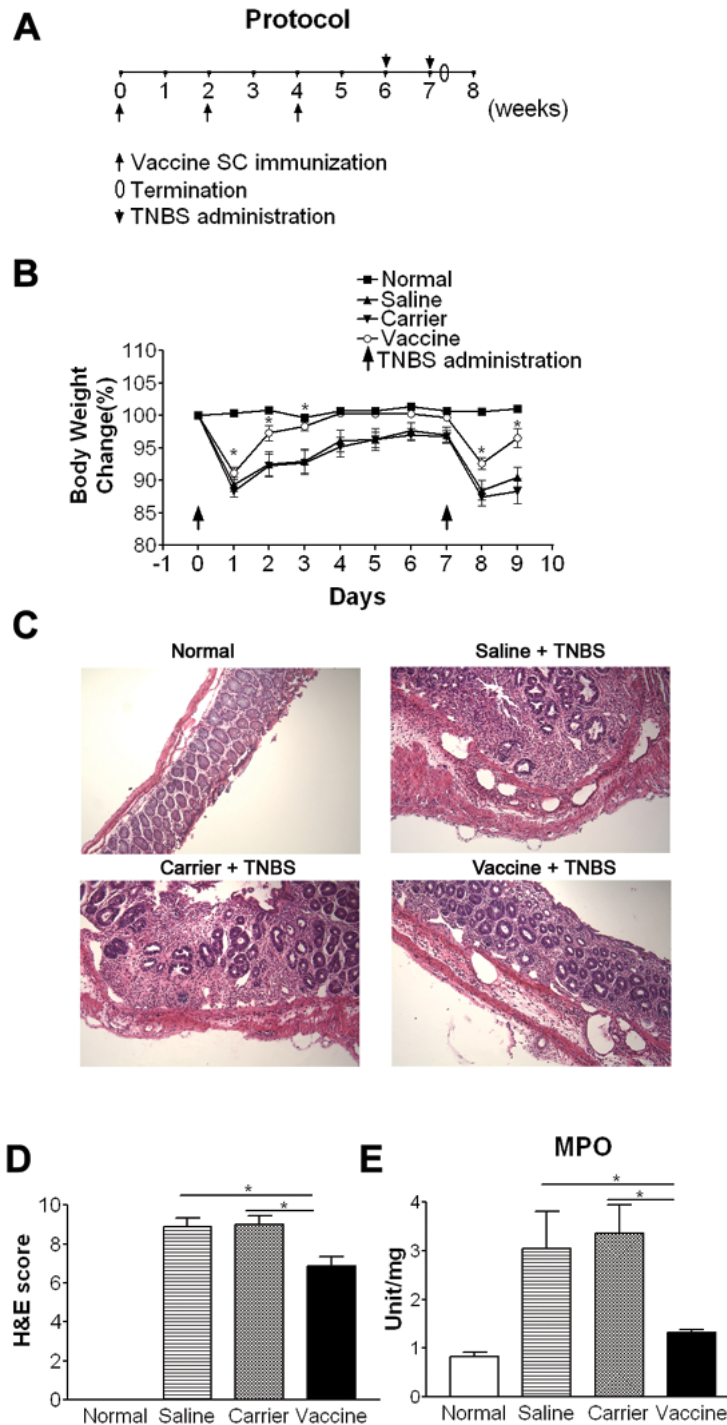


Figure 10. Vaccine ameliorates TNBS-induced acute colitis. BALB/c mice (n=16-20) were subcutaneously primed with vaccine, carrier or saline three times at a two-week interval. Two weeks later, the mice were challenged with TNBS to induce acute colitis. Mice were monitored daily. (A) Protocol. (B) Body weight. (C) Colon tissue sections (H&E staining). (D) Semi-quantitative analysis of histological inflammation. (E) MPO activity. (*, $P < 0.05$)

number of granulocytes. MPO activity was increased about 4-fold in carrier and saline groups compared with normal mice. In contrast, there was a significant decrease in the MPO activity in vaccine-treated mice ($P<0.05$) that was close to normal mice (Figure 10E).

We examined cytokine expression levels in colon tissue by ELISA. The results showed that vaccine treatment suppressed the protein levels of p40, IL-12, IFN- γ , IL-17 and TNF, but increased IL-10 production, compared with saline and carrier groups ($P<0.05$) (Figure 11A). IL-23 levels were not significantly elevated in all colitis groups (data not shown). We further examined cytokine and proinflammatory molecules by using real time RT-PCR. Vaccine treatment also resulted in a decrease in the mRNA expression levels of p40, TNF, proinflammatory molecules inducible nitric oxide synthase (iNOS) and anti-apoptotic molecules Bcl-2 (Figure 11B). Consistent with the increased protein levels of IL-10 measured by ELISA, the mRNA levels of IL-10 and Foxp3 measured were increased in vaccine-treated group. Taken together, these data indicate that the p40 vaccine ameliorates intestinal inflammation in TNBS-induced acute colitis by inhibiting Th1 cytokines and inflammatory molecule expression and increasing IL-10 production.

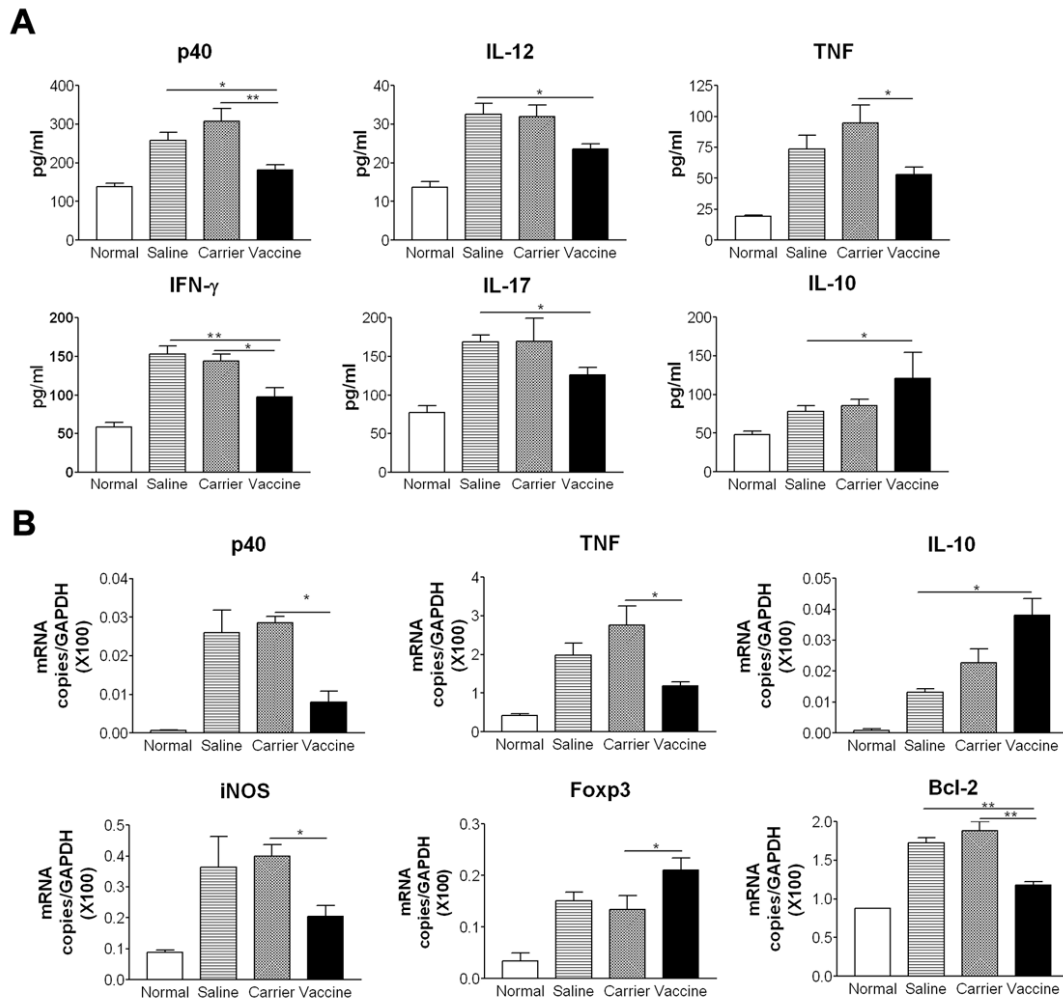


Figure 11. Vaccine down-regulates the levels of proinflammatory cytokines and other mediators involved in acute colitis, but up-regulates IL-10 production. (A) Frozen colonic samples were mechanically homogenized in buffer supplemented with protease cocktail. Samples were centrifuged to get the supernatants. The concentration of cytokines in the supernatants was determined by ELISA. (B) Total RNA was extracted from colons by Trizol according to the manufacturer's instruction. Complementary DNA was synthesized using iScript™ cDNA synthesis kit. Colon mRNA levels were evaluated by real-time PCR. (*, $P < 0.05$; **, $P < 0.01$)

2. Vaccine induces specific IgG antibodies and improves body weight loss in chronic colitis

Since IBD is a chronic disease, it would be more important and relevant to evaluate this vaccine strategy in a chronic colitis model, so we further explored the effects of the vaccine in TNBS-induced chronic model. As shown in Figure 12B, after two immunizations, the vaccine induces significantly high levels of specific antibodies against p40, IL-12, and IL-23, which reach a plateau after three immunizations and remain high levels in the entire experiment. These antibodies were undetectable in carrier and saline groups ($P<0.001$). The titers of the serum pooled from vaccinated mice were 200,000 for p40-specific IgG, 150,000 for IL-12-specific IgG, and 20,000 for IL-23-specific IgG, which were slightly lower than those in acute colitis. This may be due to the use of different batches of mice in the two experiments as immune responses are individually different. After mice developed high levels of antibodies, they were intrarectally challenged with TNBS seven times to induce a chronic colitis (Figure 12A). Consistent with the findings in acute colitis, in this chronic colitis model mice in saline and carrier groups showed obvious body weight loss after the first administration of TNBS that lasted from week 6 to week 9, and then gradually regained weight and recovered from other signs of chronic illness despite continued TNBS administrations after week 10 (Figure 13). In contrast, vaccine-immunized mice showed no obvious body weight loss compared with saline and carrier groups ($P<0.01$), and their body weight changes were similar to those of normal healthy mice and even higher than normal mice

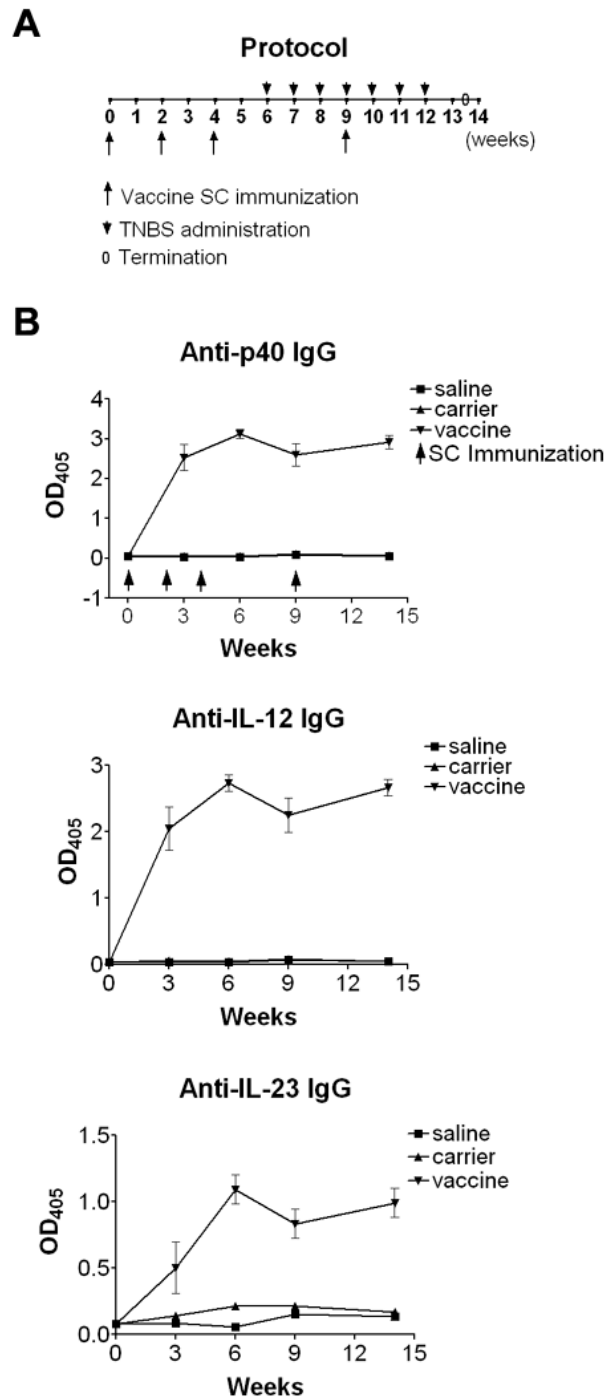


Figure 12. Immunization with the p40 vaccine induces specific IgG antibodies to p40, IL-12 and IL-23. (A) Protocol. Mice were subcutaneously primed with 100 $\mu\text{g}/200 \mu\text{l}$ of vaccine or carrier or 200 μl of saline, and boosted at weeks 2, 4 and 9 with 25 $\mu\text{g}/200 \mu\text{l}$ of vaccine or carrier or 200 μl saline. Chronic colitis was induced by seven weekly administrations of TNBS. (B) Serum specific IgG antibodies. Sera were obtained at the indicated weeks and diluted 1:200 for determination of specific IgG levels by ELISA.

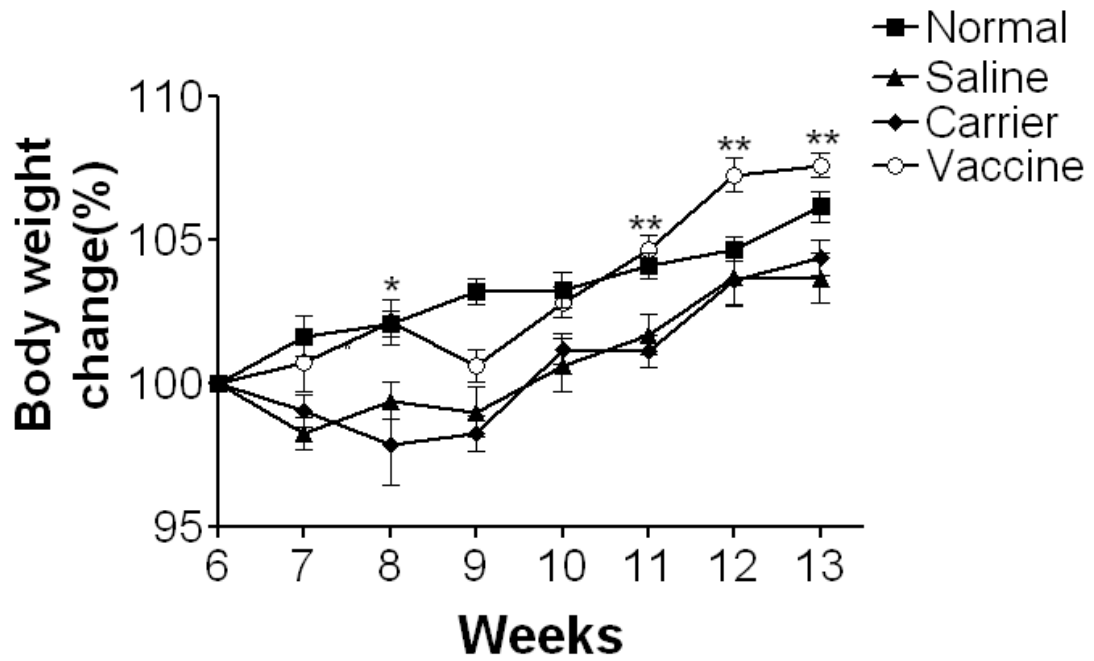


Figure 13. Vaccine improves body weight loss in chronic colitis. Body weight was monitored weekly on the day after TNBS administration. The results presented were a representative of two independent experiments. Values were expressed as mean \pm SD. (**, $P < 0.01$) (n=12)

between week 12 and week 13. These results indicated that the vaccine could lessen body weight loss in mice with TNBS-induced chronic colitis.

3. Vaccine suppresses intestinal inflammation in chronic colitis

In addition to the improvement of body weight loss, vaccine treatment also ameliorated other manifestations of chronic colitis. Histological analysis revealed that colons from saline and carrier groups exhibited obvious inflammation, including inflammatory cell infiltration, goblet cell reduction, and distorted architecture (Figure 14A). In contrast, colon tissues of vaccine-treated mice showed much less inflammation than controls. The vaccine-induced improvement of colon inflammation was further confirmed by semi-quantitative analysis in which vaccine-treated mice had significantly lower inflammatory scores than saline and carrier groups ($P < 0.001$) (Figure 14B).

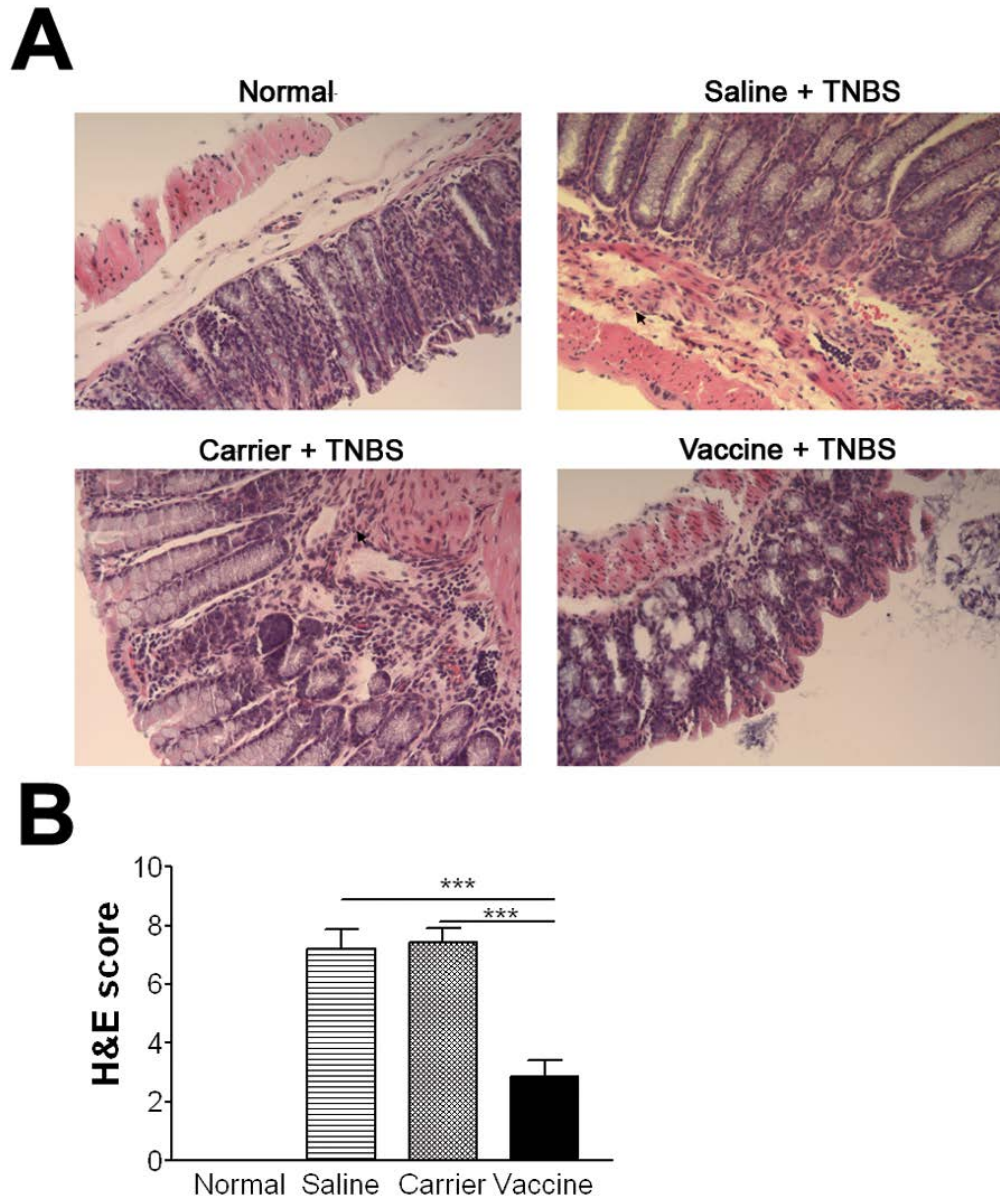


Figure 14. Vaccine ameliorates intestinal inflammation in chronic colitis. (A) Colonic specimens were formalin-fixed and embedded in paraffin blocks, and then 6- μ m sections were stained with hematoxylin and eosin. Representative histological images of samples from normal control and TNBS-treated mice following treatment with vaccines, carrier or saline are shown (original magnification x 100). (B) Semi-quantitative analysis was used to assess histological changes. H&E scores of 0-10 were given for each mouse. (***, $P < 0.001$)

4. Vaccine inhibits fibrosis in chronic colitis

As fibrosis is an important indicator for chronic inflammation, colon collagen deposition was evaluated. As shown in Figure 15A, compared with the normal group, the amount of collagen is increased in the subepithelium, in deeper layers of the colonic lamina propria, and in the muscular layer in saline and carrier groups. No significant increase in collagen deposition was observed in vaccine-immunized mice. The reduction in collagen deposition was further corroborated by the Sircol collagen assay that detects soluble collagen quantitatively. The amount of soluble collagen in vaccine-treated mice was significantly lower than that in saline and carrier groups, and was reduced almost to the normal level ($P<0.01$) (Figure 15B), confirming that vaccine immunization could decrease fibrosis in chronic colitis.

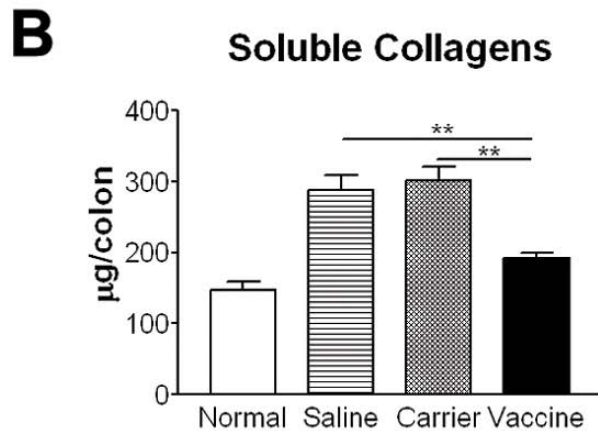
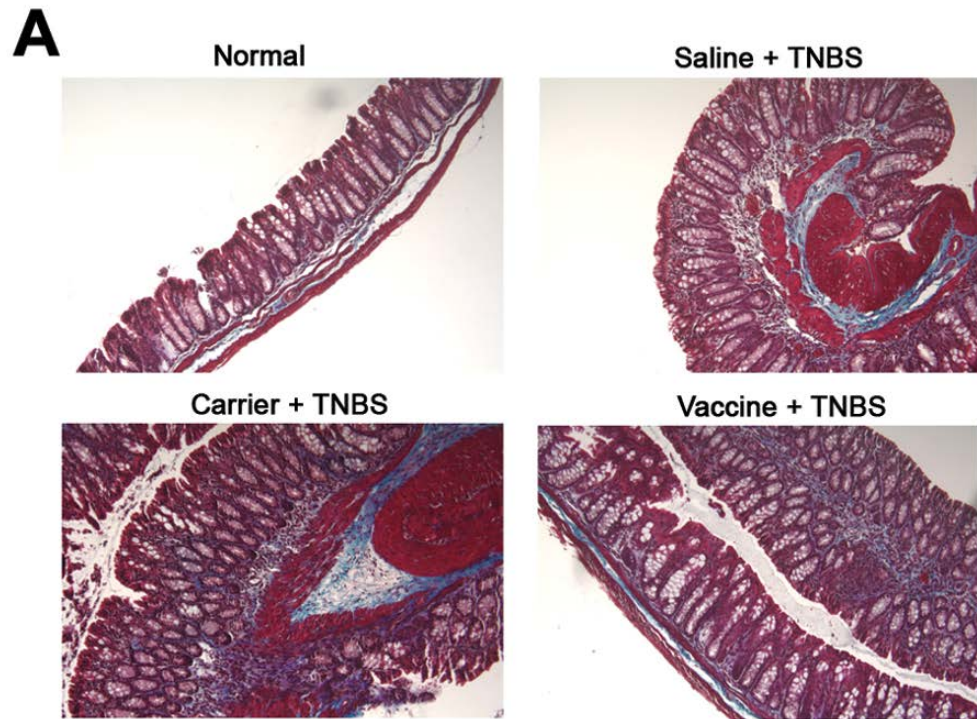


Figure 15. Vaccine reduces colon fibrosis in chronic colitis. (A) Representative colon sections stained with Masson's trichrome showing collagen deposition (the blue color) (original magnification x 100). (B) Quantitation of soluble collagens assayed by a Sircol assay. ($P < 0.01$)

5. Expression levels of cytokines in chronic colitis

Studies have provided evidence that the production of some proinflammatory cytokines (such as IL-23, IL-17 and TNF) and a profibrotic cytokine (TGF β 1), are increased in colon tissue of chronic colitis. As indicated in Figure 16, after administrations of TNBS, the protein levels of p40, IL-23, IL-17 and TGF β 1 in colon tissue of saline and carrier groups were significantly higher than those in normal control (Figure 16A). As expected, vaccine-treated mice had a decrease in p40, IL-23, IL-17 and TGF β 1 levels compared with carrier and saline groups ($P < 0.05$). The decreased p40 and TNF levels in the vaccine-treated group were further confirmed by the measurement of mRNA expression (Figure 16B). However, in contrast to the finding in acute colitis that IL-10 was increased in vaccinated mice, IL-10 levels were decreased in vaccinated mice in chronic colitis. The decreased IL-10 was supported by reduced IL-10 mRNA expression in colon tissue, compared with control groups. The mRNA expression levels of Bcl-2 and Foxp3 were also down-regulated in vaccine-treated group. The IFN- γ levels in normal control group were comparative with other groups (data not shown), which was similar to the results reported by Fichtner-Feigl S *et al.*²⁵⁴ We also detected the expression of IL-13, a typical Th2 cytokine, in the colon tissue. Our results showed that after TNBS challenge, IL-13 expression was significantly increased in saline, carrier and vaccine groups when compared with normal control; however, no significant differences were found between these three groups.

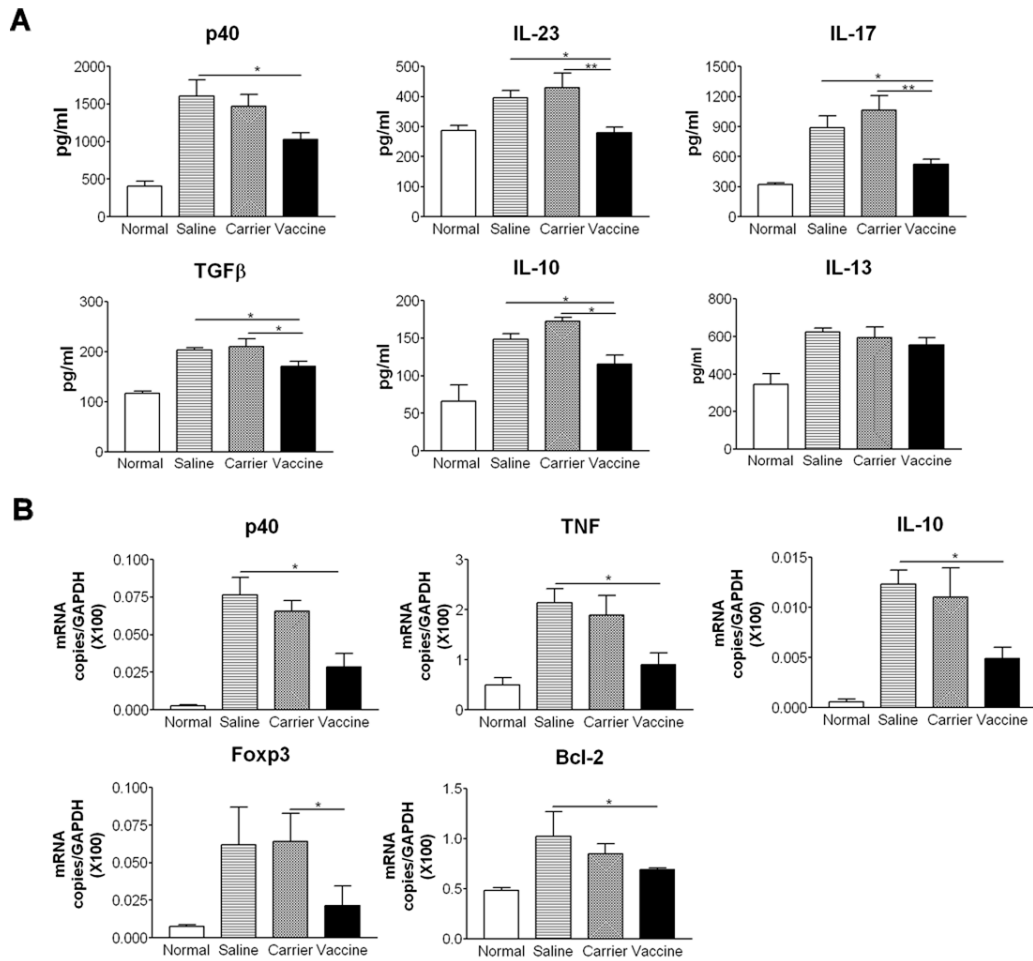


Figure 16. Vaccine inhibits colon proinflammatory cytokines production in chronic colitis. (A) Frozen colonic samples were mechanically homogenized in buffer supplemented with protease cocktail. Samples were centrifuged to get the supernatants. The concentration of p40, IL-23, IL-17, TGFβ1, IL-13 and IL-10 cytokines in the supernatants was determined by ELISA. (B) Total RNA was extracted from colons by Trizol according to the manufacturer's instruction. Complementary DNA was synthesized using iScript™ cDNA synthesis kit. Colon mRNA levels were evaluated by real-time PCR. (*, $P < 0.05$; **, $P < 0.01$)

6. Vaccine decreases the percentages of Th1 and Th17 in MLNs in chronic colitis

Studies have shown that IL-12 promotes the differentiation of Th1 cells and that IL-23 stabilizes the proliferation of Th17 cells. So, vaccination, by blocking IL-12 and IL-23, may inhibit the differentiation of Th1 and Th17 cells. As shown in Figure 17, in both acute (A) and chronic (B) colitis, the percentages of CD4⁺IFN- γ ⁺Th1 and CD4⁺IL-17⁺Th17 cells in MLNs, detected by flow cytometry analysis, tend to be higher in saline and carrier groups compared with normal mice. In contrast, the vaccine-treated group had lower percentages (close to that of normal mice) than saline and carrier groups. Although the differences didn't reach statistical significance, the trends were supported by the results obtained from ELISA and RT-PCR (Figures 11, 16). Furthermore, the mean percentage of Th1 cells was higher and that of Th17 cells was lower in acute colitis than those in chronic colitis. These data indicate that predominant Th1 cell responses may initiate the acute intestinal inflammation, while Th17 cell responses perpetuate the chronic intestinal inflammation, which are in agreement with previous findings.²⁵⁴ Taken together, vaccine treatment tends to decrease the percentage of Th1 and Th17 cells in MLN.

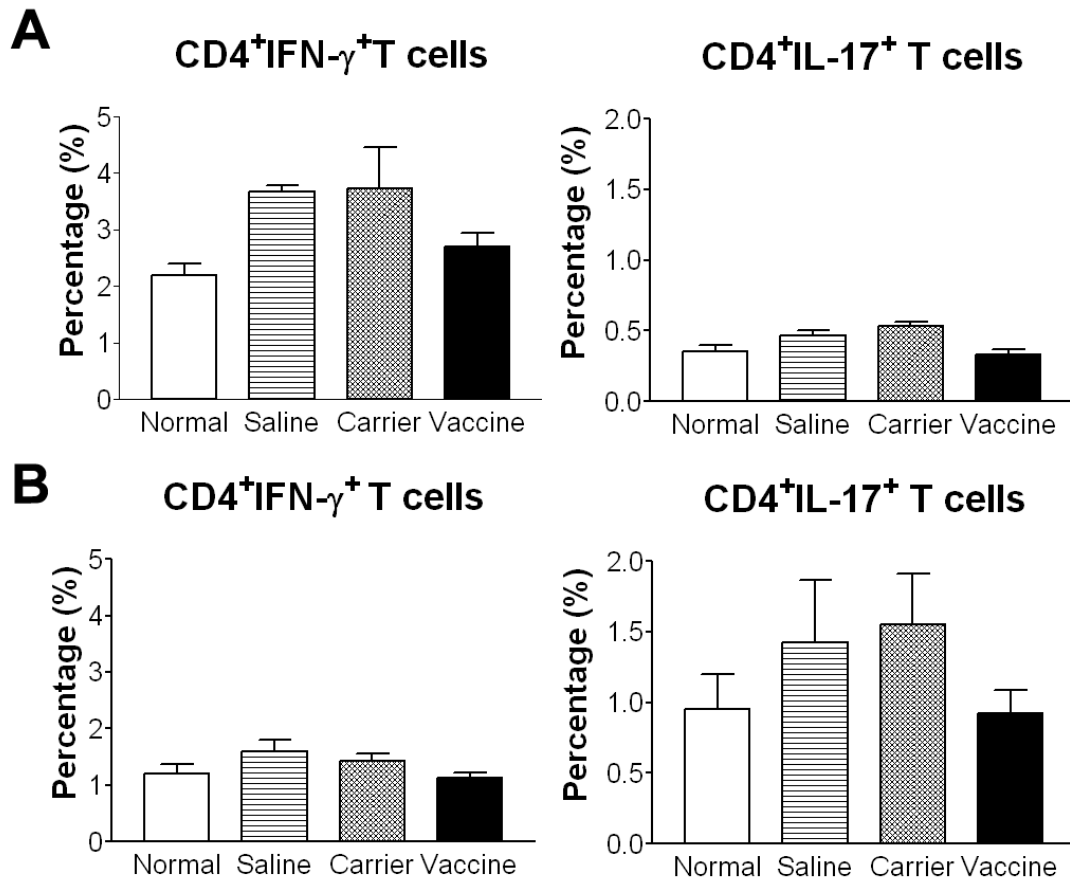


Figure 17. Vaccine treatment tends to reduce the percentages of Th1 and Th17 cells in MLNs. After stimulation with PMA and ionomycin, lymphocytes from MLNs were stained with anti-CD4, anti-IFN- γ and anti-IL-17 to detect the percentages of CD4⁺IFN- γ ⁺Th1 and CD4⁺IL-17⁺Th17 cells by flow cytometry. (A) Acute colitis. (B) Chronic colitis.

Discussion

We have previously developed a mouse IL-12/IL-23 p40 peptide-based virus-like particle vaccine that induced high titered and relative long-lasting antibodies to p40, IL-12 and IL-23, and the antisera could *in vitro* inhibit IL-12-induced interferon- γ ²⁴⁵ and IL-23-induced IL-17 production of splenocytes (data not shown). In this study, we systemically evaluated the *in vivo* effectiveness of the vaccine and the immune mechanisms involved in both TNBS-induced acute and chronic colitis models.

To date, to overcome B cell tolerance, vaccines against excessive endogenous cytokines are developed mainly by either inserting a foreign peptide into the intact cytokine molecule²³⁰ or linking the intact cytokine to a heterogeneous carrier such as ovalbumin.²⁴² Antibodies raised by these vaccines act against multiple antigen determinants of the target cytokine. As such, their use may be hindered by undesirable cross-reactions with other self-proteins containing similar epitopes. This is of particular concern when this strategy is used in humans. Cytokine peptide-based vaccines are currently under development; however, these vaccines use inappropriate carrier proteins such as keyhole limpet hemocyanin.²⁸⁴ As these vaccines are not virus-like particles, their antigenicity is low, requiring strong adjuvants, such as complete Freund's adjuvant, to elicit high titers of antibodies to the target cytokine. Virus-like particles induce potent B cell responses even in the absence of adjuvants,^{223, 260} and as carrier proteins, they are preferable to other proteins. More recently, cytokine peptide-based virus-like particle vaccines are being investigated by chemically conjugating synthesized cytokine peptides to bacteriophage Q β virus-like particles.²³⁶ Compared with the vaccines that are currently being investigated, the p40 vaccine used in the present study has unique advantages: (1) it

uses a small peptide avoiding possible cross-reaction with other self proteins; (2) it is highly antigenic eliciting long-lasting specific antibodies without the use of an adjuvant due to its virus-like particle feature; (3) it is a recombinant protein making it easier to prepare and to control the quality of the vaccine than those by chemical coupling;²³⁶ (4) the safety of the vaccine carrier HBcAg has been confirmed in a phase I clinical trial for a malaria vaccine used in humans,²⁷⁹ making this vaccine even more attractive for human use. For these reasons, the vaccine developed in the present study exhibits great advantages over other cytokine vaccine strategies.

In the TNBS-induced chronic colitis model, the initial Th1 response subsides after 3 weekly-TNBS challenges and, then, is supplanted by a Th17 response after 4-5 weeks.²⁵⁴ This pattern of immune response is confirmed in the present study in which CD4⁺IFN- γ ⁺ T cells, not CD4⁺IL-17⁺ T cells, tended to increase in the acute colitis, while CD4⁺IL-17⁺ T cells, not CD4⁺IFN- γ ⁺ T cells, were increased in chronic colitis (Figure 8). Although the differences didn't reach statistical significance, the trends were supported by the results obtained from ELISA and RT-PCR. In acute colitis, Th1 cytokines expression (IFN- γ , IL-12, TNF) were significantly increased after TNBS challenge (Figure 2); in chronic colitis, Th17 cytokines expression (IL-17, IL-23, TGF β 1) were significantly increased (Figure 7). As the p40 vaccine effectively targets both IL-12 and IL-23, it exerts its anti-inflammatory effects during the entire course of chronic colitis. We speculate that this vaccine, by downregulating IL-12, is able to halt the differentiation of naïve T cells into Th1 cells, leading to a reduction in IFN- γ and TNF. In addition, the vaccine, by blocking IL-23, may hinder the differentiation of naïve T cells into Th17 cells. Through these immune mechanisms, vaccine treatment indeed decreases the percentages

of Th1 and Th17 cells in the MLN after TNBS challenges, returning almost to levels in normal mice. Although the decrease is a trend, the significantly reduced Th1 and Th17 cytokine levels in colon tissue support the trend of the decreased percentages of Th1 and Th17 cells.

In the acute colitis model, the IL-17 level in colon tissue was elevated significantly (Figure 11) which is in agreement with previous reports.^{107, 285, 286} However, the percentage of Th17 in MLN cells was not significantly increased (Figure 17A right). Although IL-17 is described as a Th17 cell-secreted cytokine, much of the IL-17 released during an inflammatory response is produced by innate immune cells which include $\gamma\delta$ T cells, CD11b⁺ cells, macrophages and neutrophils, etc..²⁸⁷ Therefore, the significantly increased IL-17 levels in colon tissue might be produced by local innate immune cells, not the T cells in the mesenteric lymph nodes. Indeed, Fitzpatrick *et al.* has reported that TNBS administration resulted in increased staining cells for IL-17 and that the IL-17⁺ stained cells at the top of the muscularis mucosa appeared to be macrophages.²⁸⁶ In future, we will use immunohistological staining to identify IL-17 producing cells in colon tissue, which may be more relevant than the analysis of Th17 cells in MLN cells.

Studies have demonstrated that the main sources of IL-10 production are Treg and CD11b⁺ myeloid cells^{288, 289} and that IL-10 acts on Treg cells to maintain Foxp3 expression.²⁸⁹ In the present study, colon IL-10 levels were measured by both protein and mRNA expression. As regulatory T cells (Treg) are one of the important IL-10 producing cells, IL-10 levels may reflect the function of Treg. Our results showed that in acute colitis, IL-10 levels, measured two days after TNBS challenge, were higher in the vaccine-treated group than in the control groups (Figure 11), which coincides with results

reported by Stallmach A *et al.*¹⁹⁵ Recently, IL-23 has been found to restrain the activity of Treg to drive T cell-dependent colitis,²⁹⁰ and the homodimer of p40 is found to suppress the expression of CD25 and Foxp3.²⁹¹ We speculate that, by blocking p40 and IL-23, Treg might be activated or rapidly recruited into the local colon tissue to control inflammation in the acute phase of colitis. Therefore, p40 vaccine treatment might ameliorate colitis through improving the function and/or numbers of Treg in colon tissue in acute colitis. This is supported by the increased mRNA expression of Foxp3 and IL-10 in vaccinated mice. In chronic colitis, levels of IL-10 and Foxp3, measured 10 days after the last TNBS challenge, were significantly lower in vaccine-treated mice than those in saline and carrier controls (Figure 16). As at this time point vaccine-treated mice had significantly reduced colon inflammation, accompanied by significantly lower inflammatory cytokines such as p40, IL-23 and IL-17, Treg might not be required to exert their anti-inflammatory effects. This is supported by clinical observations that IL-10 expression in lamina propria mononuclear cells of patients with Crohn's disease had a tendency to decrease after treatment with monoclonal antibodies against IL-12/IL-23p40.²⁰⁸

Vaccine treatment reduced histological inflammation and MPO activity. This may be through the inhibition of IL-12. IL-12 has been demonstrated to be chemotactic for neutrophils and NK cells through induction of synthesis of platelet-activating factor.²⁹²²⁹³ It has also been reported that IL-12 is critical to neutrophil activation and resistance to polymicrobial sepsis induced by cecal ligation and puncture.²⁹⁴ Therefore, vaccine-induced specific antibodies blocked the bio-physical functions of IL-12, leading to less

migration and activation of neutrophils to the local colon tissue and, thus, ameliorating intestinal inflammation.

Our results showed that the colon inflammatory scores in the chronic colitis were slightly lower than those in the acute colitis. This is different from other reports where colon inflammatory scores are significantly higher in the chronic phase than those in the acute phase.^{285, 286} The reason may be due to the following factors: 1) Different detection times. In our acute colitis, mice were sacrificed two days after the second TNBS delivery; while in the chronic colitis, mice were sacrificed ten days after the last TNBS delivery. In our experience, after a TNBS challenge, the body weight shows an obvious loss in the first two days, and then gradually recovers during the next four days. At day seven, the body weight is close to that of baseline, accompanying with a decreased colon inflammation. 2) Different TNBS doses. In our acute colitis, mice were intra-rectally challenged with TNBS twice (1.5mg and 2.0mg, respectively), and in the chronic colitis, the mice were challenged with increasing doses of TNBS (1.0-2.5mg) over seven weeks. 3) Different experimental designs. In our study, the acute and chronic experiments were performed separately at different times with different mouse batches. This makes the data less compatible between acute and chronic colitis, when compared with other reports.^{285, 286} In future studies, we will combine the acute and chronic experiments as one experiment for kinetic observations.

In summary, we have demonstrated that the IL-12/IL-23p40 peptide-based vaccine that induces specific antibodies to IL-12 and IL-23 is capable of significantly ameliorating experimental colitis, as seen by improved body weight loss and a decrease

of colon inflammation and collagen deposition, accompanied with reduced expressions of Th1 and Th17 cytokines and other inflammatory mediators in colon tissues.

Result III IL-12/IL-23p40 vaccine reverses ongoing chronic colitis

Specific Introduction

Studies have demonstrated that Th1 and Th17 cells play important roles in the pathogenesis of Crohn's disease.^{75, 104} IL-12 is required for an effective polarization of naïve T helper cells to the Th1 phenotype characterized by the expression of IFN- γ while IL-23 stabilizes the proliferation of Th17 cells. Overproduced IL-12 and IL-23 have been found in the Crohn's disease patients.⁷⁵ As IL-12 and IL-23 share p40 subunit, IL-12/IL-23p40 has been considered as the target for the treatment of Crohn's disease. Monoclonal antibodies against IL-12/IL-23p40 (ABT-874) have been developed and successfully used in clinic.²⁰⁸ Down-regulation of the production of IL-12, IFN- γ , and TNF by mononuclear cells of the colonic LPMC is associated with clinical improvement in patients receiving ABT-874.²⁰⁸

As Crohn's disease is a chronic inflammatory disease, long-term treatments are required. Monoclonal antibodies have a short half-life, and repeated injections are required to maintain their effects. Adverse reaction of the development of antibodies against the infused mAb occurs in up to 61 % of the patients receiving infliximab (mAb against TNF) therapy.²⁷¹ Vaccines against cytokines are currently being developed. The vaccine can induce relatively long-lasting antibody responses to the target cytokine. The vaccine strategy may provide an add-on supplement to the monoclonal antibody treatment, especially for the maintenance treatment of chronic inflammatory diseases.

In the previous two parts, we successfully developed IL-12/IL-p40 peptide-based vaccines which could induce relatively long-lasting antibodies against IL-12, IL-23 and p40. And the antibodies induced by these vaccines could partially block the biological

functions of IL-12 and IL-23 *in vitro*. Our results also demonstrated that immunization of mice with IL-12/IL-23p40 vaccine could ameliorate TNBS-induced acute and chronic colitis in the preventive studies. Crohn's disease is a chronic disease, requiring a long term treatment. To test whether the vaccine strategy could be used to reverse ongoing inflammation, which is more relevant to the clinic, in this chapter, we will evaluate the effects of IL-12/IL-23p40 peptide-based vaccine in mice with established TNBS-induced chronic colitis and DSS-induced chronic colitis.

Studies have demonstrated that IL-12 could inhibit Fas-mediated cell death of CD4⁺ T cells through the inhibition of caspase-3 activity and the upregulation of the anti-apoptotic molecule Bcl-2.²⁹⁵ Treatment of mice with monoclonal antibodies against IL-12/IL-23p40 promotes the apoptosis of Th1 cells in TNBS-induced colitis model, while treatment of mice with monoclonal antibodies against IL-23p19 promotes the apoptosis of Th17 cells in T cell-transfer colitis model.^{109, 196} In this section, we will evaluate whether IL-12/IL-23p40 peptide-based vaccine could influence the apoptosis of Th1 and Th17 cells in murine colitis.

IL-12 and IL-23 are important cytokines in host defense against microbial infections, such as chlamydia infection.²⁹⁶⁻²⁹⁸ The levels of IL-12 and IL-23 are increased in chlamydia infection.²⁹⁸ Neutralization of IL-12 inhibits chlamydia-specific delayed type hypersensitivity, which is required for chlamydia clearance, thus delays chlamydia clearance.²⁹⁹ In IL-12-deficient mice, the clearance of MoPn from the lung, and the antigen-specific systemic and lung IFN- γ production, are substantially reduced.³⁰⁰ Recently, evidence shows that Th17/IL-17 also contributes to protection against chlamydial lung infection.^{253, 301} Some studies have shown that cytokine-based vaccines

increase the susceptibility to some infections.²³⁶ Therefore, in this section, we will also evaluate whether IL-12/IL-23p40 peptide-based vaccine could increase the susceptibility to chlamydial lung infection.

Results

1. Vaccine induces specific IgG antibodies and improves body weight loss in TNBS-induced chronic colitis

The protocol was shown in Figure 18A. To induce chronic colitis, mice were intrarectally challenged with TNBS eight times at a one-week interval. Four days after the second injection of TNBS when intestinal inflammation was established, the mice were subcutaneously immunized with IL-12/IL-23p40 vaccine, carrier or saline three times at a two-week interval. After the first TNBS challenge, all mice showed obvious body weight loss at the first two days, and then gradually recovered (Figure 18B). Because the starting dose was only 0.5 mg per mouse, most of the mice recovered and even increased body weight at day 7. This differs from the prevention study (Figure 13), in which most of the mice didn't recover the body weight at day 7 after the first TNBS challenge, and the starting TNBS dose was relative high (1.5 mg per mouse).

After week 4, mice immunized with the vaccine showed significantly increased body weight when compared with mice immunized with saline or carrier, as after two immunizations, the vaccine had induced relatively higher levels of antibodies against IL-12, IL-23 and p40 (Figure 18C). This may explain why the body weight in the vaccine group was increased after week4 when compared with carrier and saline groups, indicating that vaccine treatment could improve body weight loss in ongoing chronic colitis.

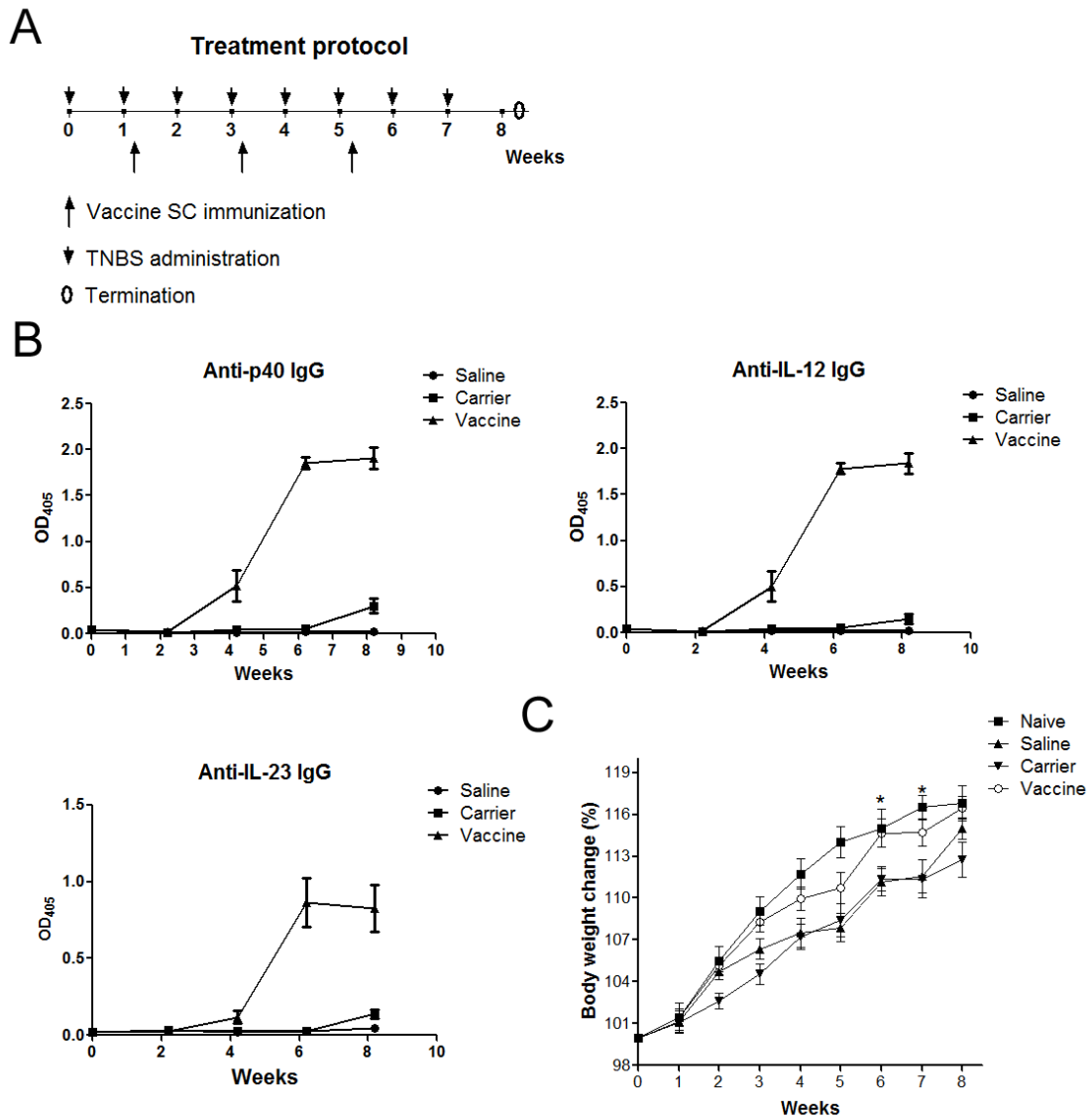


Figure 18. Vaccine treatment ameliorates body weight loss after TNBS challenges. The mice were weekly administrated with TNBS eight times to induce chronic colitis. Four days later after the second TNBS administration, mice were immunized with vaccine, carrier or saline three times at a two-week interval (n=16). Ten days later after the last TNBs delivery, mice were sacrificed to analyze. (A). Protocol of TNBS-induced chronic colitis model and vaccine immunization; (B) Antibody responses induced by IL-12/IL-23p40 peptide-based vaccine; (C) Body weight changes.

2. Vaccine ameliorates intestinal inflammation in TNBS-induced chronic colitis

In addition to the improvement of body weight loss, vaccine treatment also ameliorated TNBS-induced chronic intestinal inflammation. Histological analysis revealed that colons from saline and carrier groups exhibited obvious inflammation, including inflammatory cell infiltration, goblet cell reduction, and distorted architecture (Figure 19A) which is similar to that found in the chronic prevention study. In contrast, colon tissues of vaccine-treated mice showed much less inflammation than controls. The vaccine-induced improvement of colon inflammation was further confirmed by semi-quantitative analysis in which treated mice had significantly lower inflammatory scores than saline and carrier groups ($P < 0.05$) (Figure 19B).

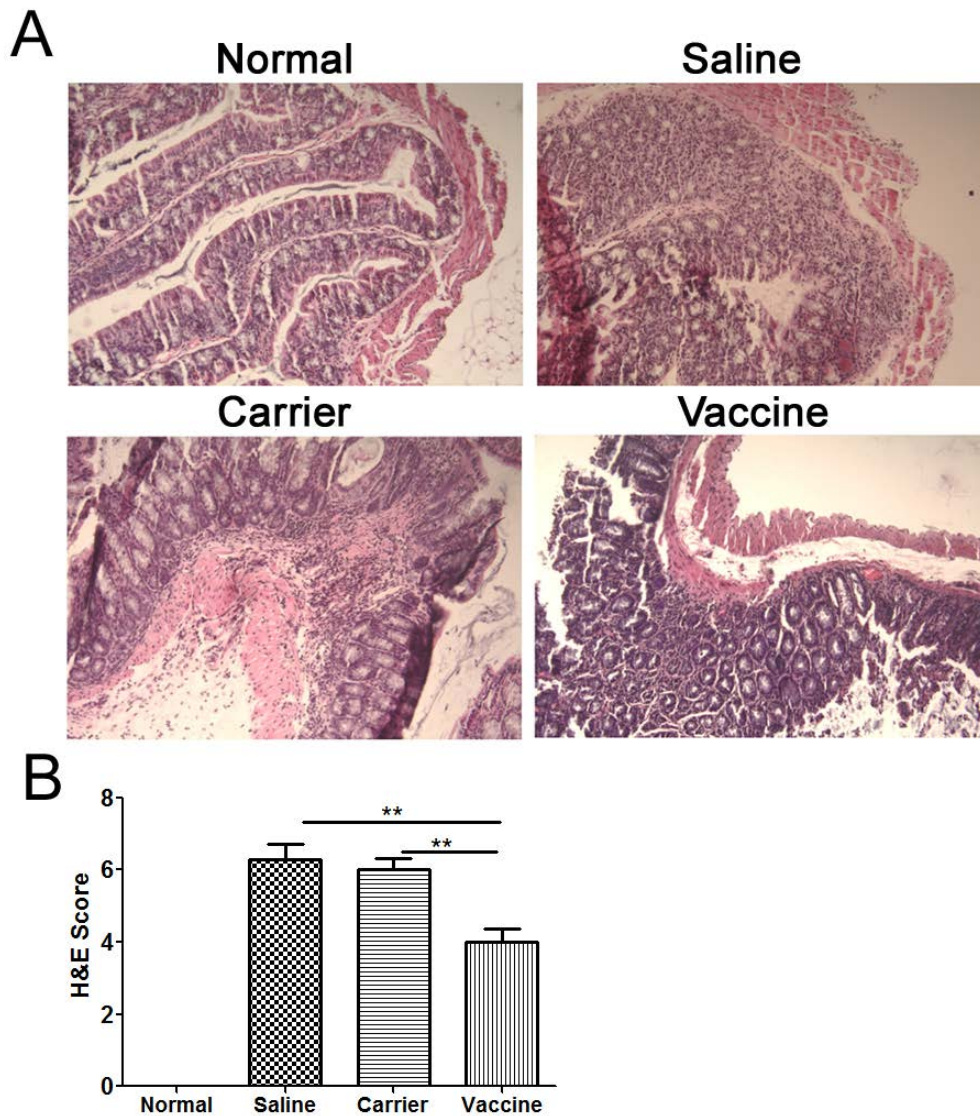


Figure 19. Vaccine treatment ameliorates intestinal inflammation in established TNBS-induced chronic colitis. (A) Colonic specimens were formalin-fixed and embedded in paraffin blocks, and then 6- μ m sections were stained with hematoxylin and eosin. Representative histological images of samples from normal control and TNBS-treated mice following treatment with vaccines, carrier or saline are shown (original magnification x 100). (B) Semi-quantitative analysis was used to assess histological changes. H&E scores of 0-10 were given for each mouse. (**, $P < 0.01$)

3. Vaccine treatment inhibits fibrosis in TNBS-induced chronic colitis

As shown in Figure 20A, compared with the normal group, the amount of collagen is increased in the subepithelium, deeper layers of the colonic lamina propria, and the muscular layer in saline and carrier groups. But, no significant increase in collagen deposition was observed in vaccine-immunized mice. The reduction in collagen deposition was further confirmed by quantitative measurement of soluble collagen. The amount of soluble collagen in vaccine-treated mice was significantly lower than those in the saline and carrier groups, and was reduced almost to the normal level ($P < 0.01$) (Figure 20B), confirming that vaccine immunization could decrease fibrosis in chronic colitis.

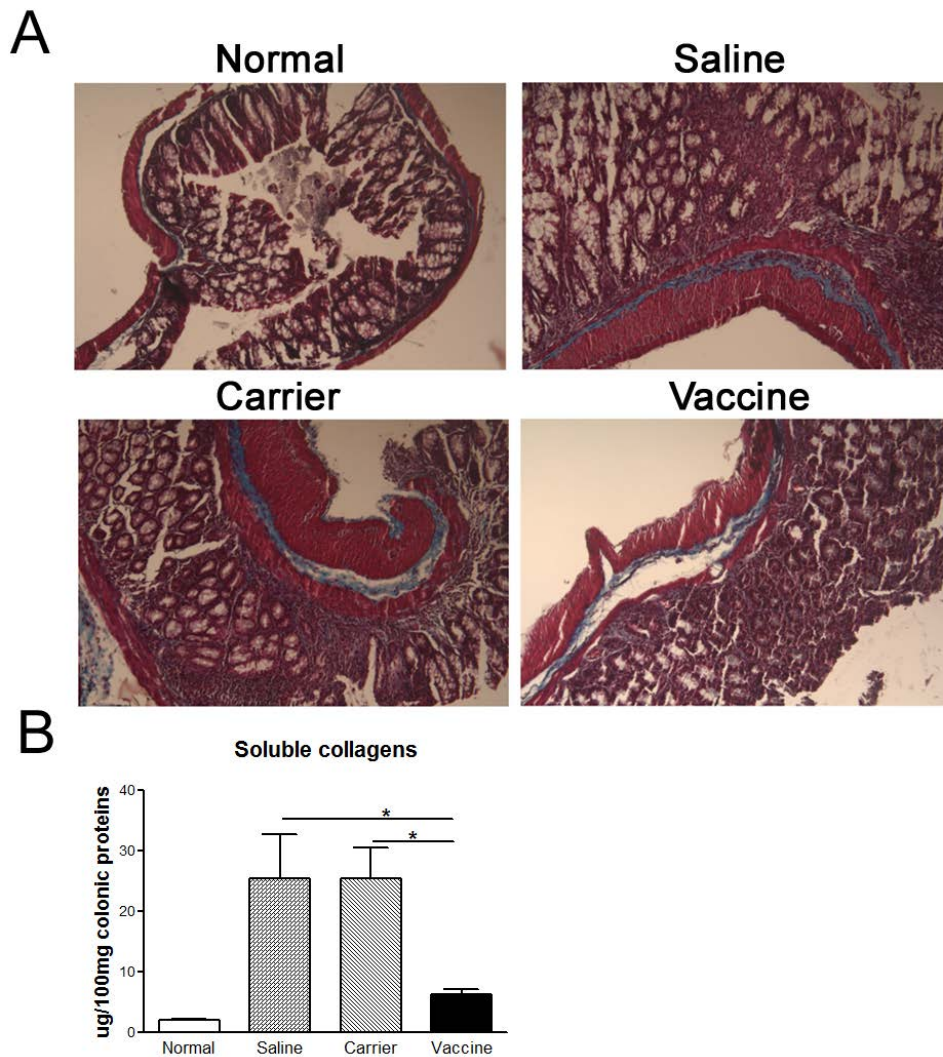


Figure 20. Vaccine treatment reduces colon fibrosis in established TNBS-induced chronic colitis. (A) Representative colon sections stained with Masson's trichrome showing collagen deposition (the blue color) (original magnification x 100). (B) Quantitation of soluble collagens in the colon tissue was measured by a Sircol assay. (*, $P < 0.05$)

4. Vaccine down-regulates colon cytokine expression in TNBS-induced chronic colitis

In the preventive study of chronic colitis, the levels of cytokines IL-23, IL-17, TNF, and IL-10 were increased in the colon tissue after repeatedly challenge with TNBS (Figure 16). In this treatment study, after administrations of TNBS, the protein levels of IL-23, IL-17, TNF and IL-10 in colon tissue of saline and carrier groups were also significantly higher than those in normal controls (Figure 21). As expected, vaccine-treated mice had a decrease level of IL-23, IL-17, TNF and IL-10, compared with carrier and saline groups ($P < 0.05$). It indicated that vaccine treatment down-regulated the cytokines production in the colon tissue of chronic colitis.

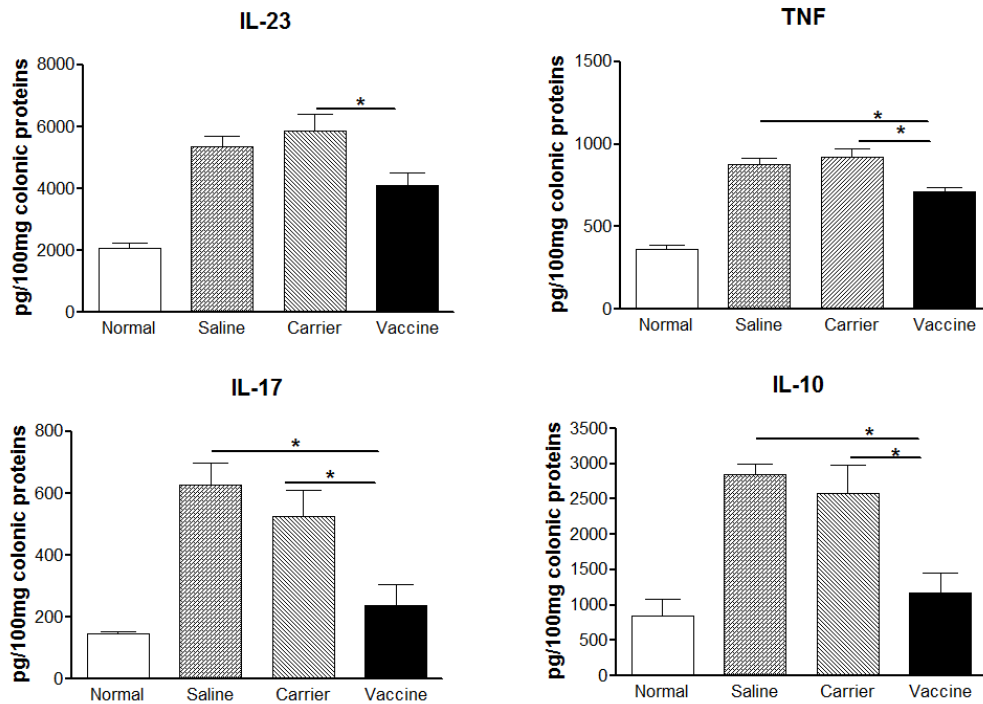


Figure 21. Vaccine treatment down-regulates colon proinflammatory cytokine production in established TNBS-induced chronic colitis. Frozen colonic samples were mechanically homogenized in buffer supplemented with protease cocktail. Samples were centrifuged to get the supernatants. The concentration of IL-23, IL-17, TNF and IL-10 in the supernatants was determined by ELISA. (*, $P < 0.05$)

5. Vaccine decreases clinical scores and colon inflammation in DSS-induced chronic colitis

We have demonstrated that immunization of IL-12/IL-23p40 vaccine could ameliorate intestinal inflammation in the prevention and treatment of TNBS-induced colitis model. DSS-induced murine colitis is another widely used colitis model. In this model, it is believed that DSS is directly toxic to epithelial cells of the basal crypts and affects the integrity of the mucosal barrier and the cytokine profiles are different in the two models.^{252, 285} And studies have shown that IFN- γ and IL-17 pathways are involved in DSS-induced chronic colitis.²⁸⁵ So, we evaluated the effects of IL-12/IL-23p40 vaccine in the treatment of DSS-induced chronic colitis.

As shown in Figure 22A, mice firstly received 4% (wt/vol) DSS drinking water for 7 days to induce intestinal inflammation. After the intestinal inflammation developed, mice were immunized with vaccine, carrier or saline three times at a two-week interval. Our results showed that after giving 4% (wt/vol) DSS drinking water, mice had slight diarrhea and bloody stool. With the continuation of drinking 4% DSS water, the symptoms became more severe. The clinical scores were significantly increased in saline and carrier groups. But, in vaccine treated mice, the clinical score was significantly decreased when compared with control groups ($P < 0.01$).

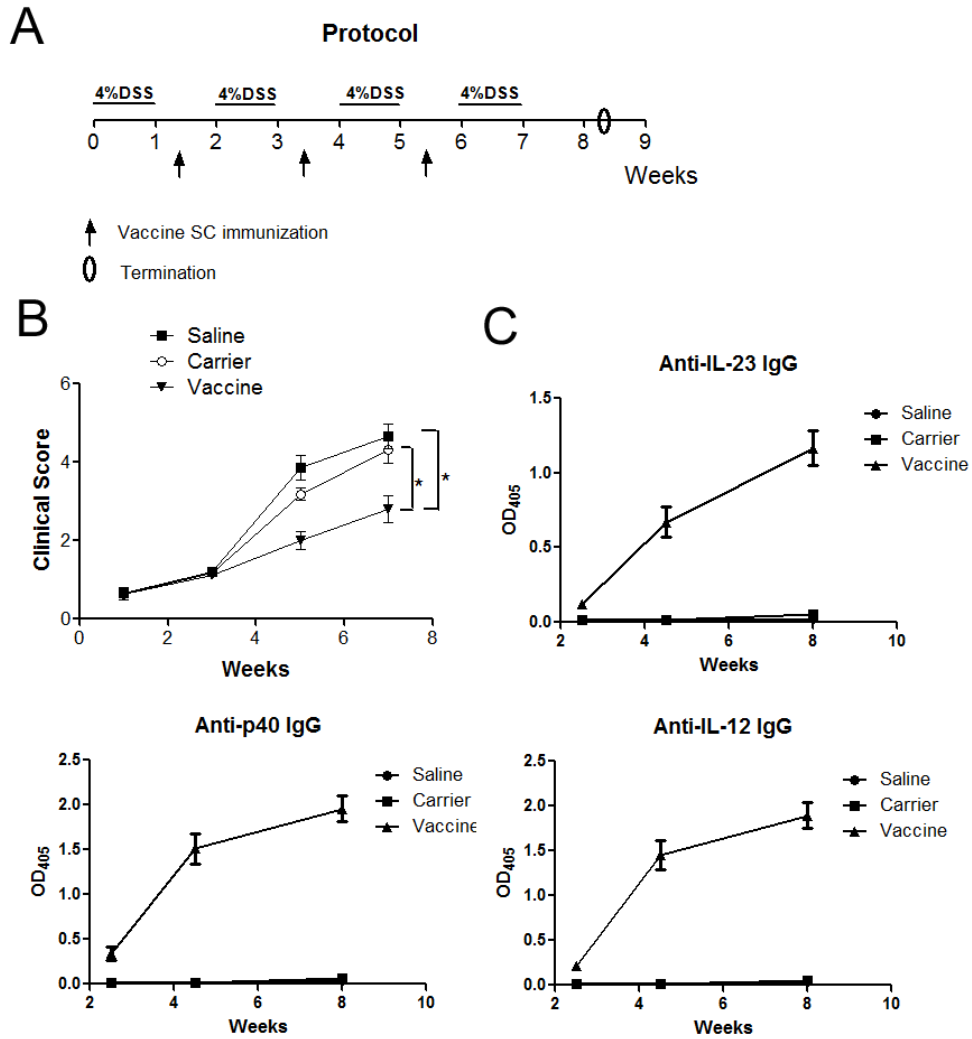


Figure 22. Vaccine treatment induces high levels of specific antibodies and down-regulates clinical scores of mice with established DSS-induced chronic colitis. Mice were given 4% DSS drinking water for 7 days followed by 7 days of regular water for 4 cycles to induce chronic colitis. Three days after the first DSS drinking water, mice were immunized with vaccine, carrier or saline three times at a two-week interval (n=16). (A) Protocol. (B) Clinical score. (C) The levels of specific antibodies against IL-12, IL-23 and p40 induced by vaccine.

As shown in Figure 23, when compared with naïve controls, mice with DSS-induced colitis in saline and carrier groups exhibited disruption of the epithelial barrier, a significant reduce of the number of crypts, and large amounts of inflammatory cells infiltration, mainly neutrophils, into the mucosa and sub mucosa of the colon tissue. However, mice immunized with IL-12/IL-23p40 vaccine had significantly reduced colon inflammation. This was further confirmed by semi-quantitative analysis in which vaccine-treated mice showed significantly lower inflammatory scores than saline and carrier groups ($P < 0.05$) (Figure 23B). Our results also showed that after repeated challenges with DSS, mice developed obvious collagen deposition in colon tissue (Figure 24). But, vaccine treatment decreased the degree of colon fibrosis when compared with saline and carrier groups. Quantitative measurement of soluble collagens further confirmed that vaccine treatment significantly reduced the production of soluble collagens in the colon tissue when compared with control groups.

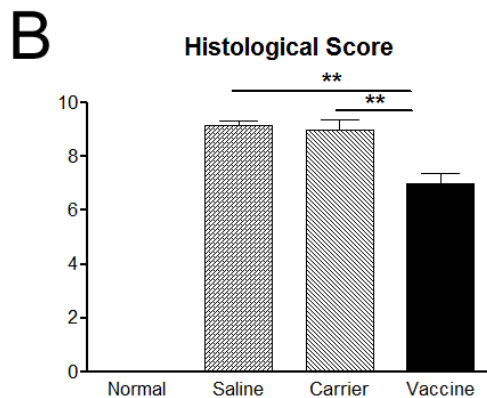
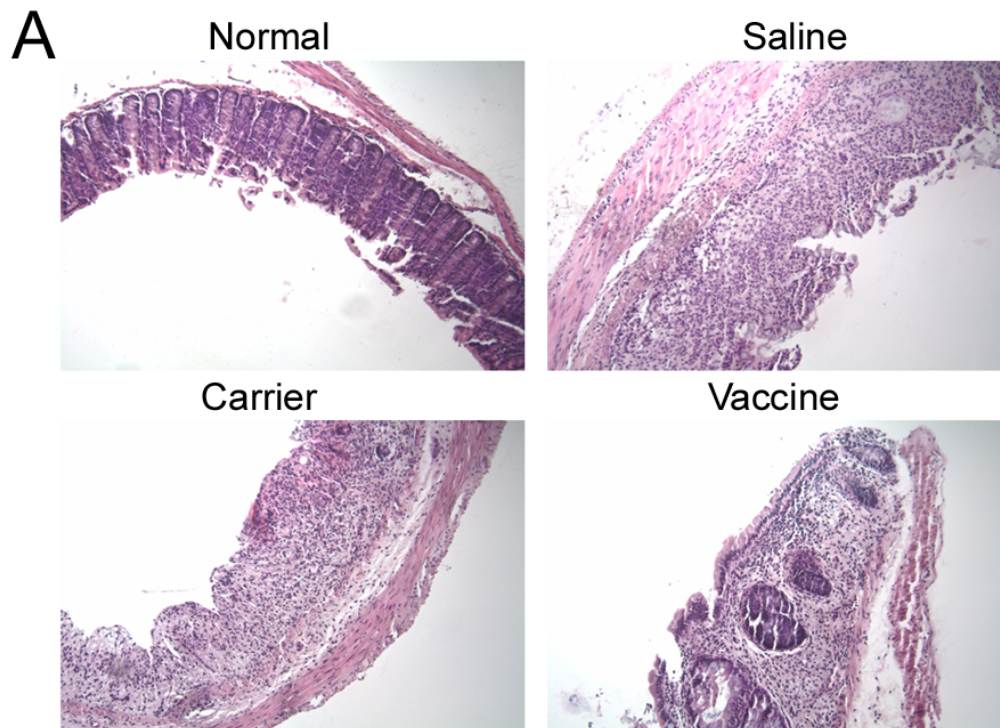


Figure 23. Vaccine treatment ameliorates intestinal inflammation in established DSS-induced chronic colitis. (A) Colonic specimens were formalin-fixed and embedded in paraffin blocks, then 6- μ m sections were stained with hematoxylin and eosin. Representative histological images of samples from normal control and TNBS-treated mice following treatment with vaccines, carrier or saline are shown (original magnification x 100). (B) Semi-quantitative analysis was used to assess histological changes. H&E scores of 0-4 were given for each mouse. (**, $P < 0.01$)

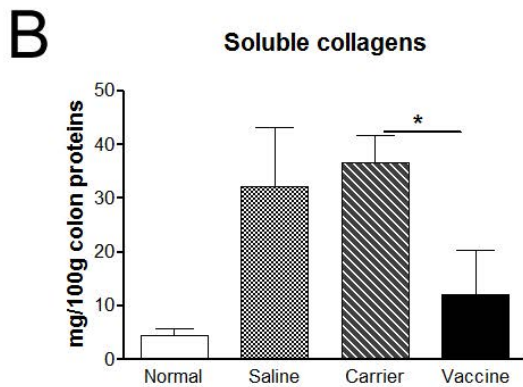
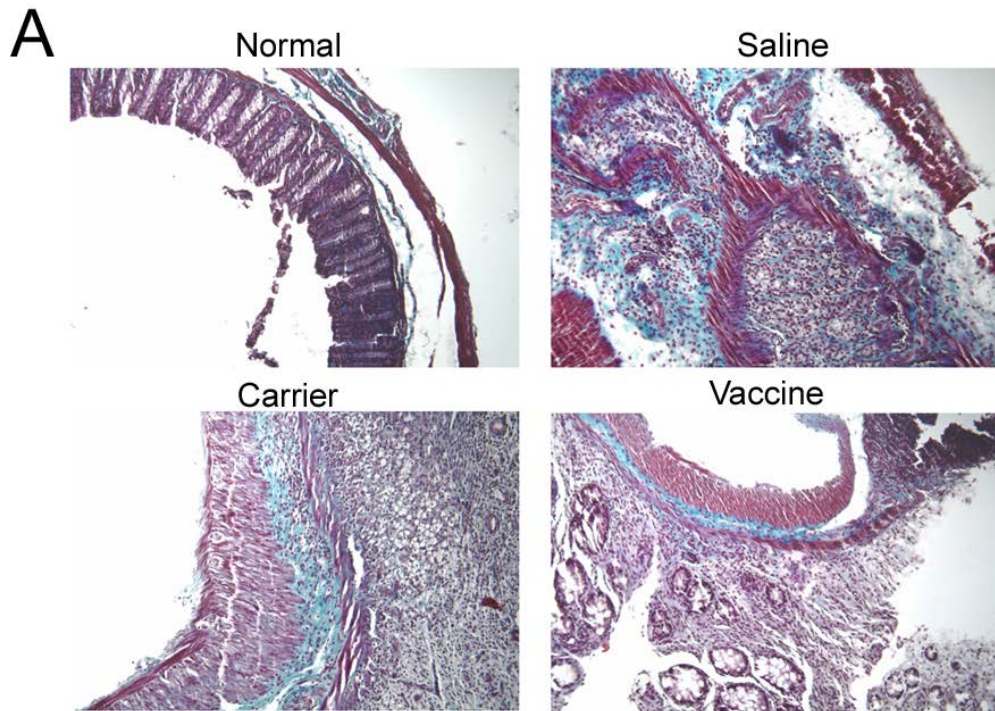


Figure 24. Vaccine treatment reduces colon fibrosis in established DSS-induced chronic colitis. (A) Representative colon sections stained with Masson's trichrome showing collagen deposition (the blue color) (original magnification x 100). (B) Quantitation of soluble collagens in the colon tissue was measured by a Sircol assay. (*, $P < 0.05$)

6. Vaccine down-regulates colon cytokine production in DSS-induced chronic colitis

Studies have shown that some proinflammatory cytokines, such as TNF and IL-17, are increased in the inflamed colon tissue in DSS-induced chronic colitis.²⁸⁵ As indicated in Figure 25, the levels of TNF, IL-17 and IL-12/IL-23p40 are significantly increased in the colon tissue after repeated DSS challenges. But, vaccine treatment down-regulated the expression levels of these cytokines in the colon tissue. Similar to the results of TNBS-induced colitis, vaccine treatment also significantly reduced IL-10 production in colon tissue after the last DSS challenge cycle.

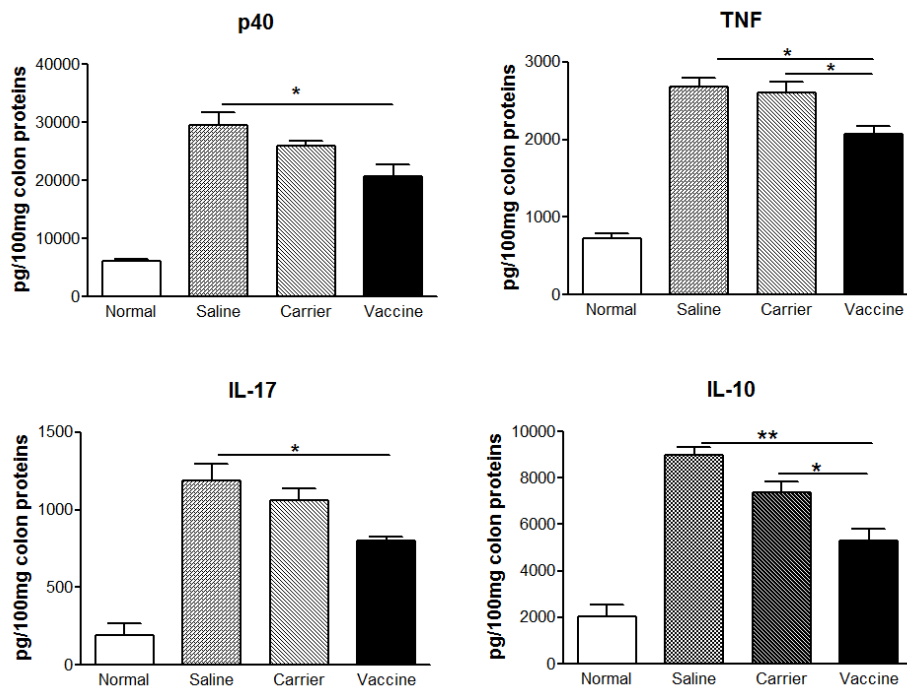


Figure 25. Vaccine treatment down-regulates colon proinflammatory cytokine production in established DSS-induced chronic colitis. Frozen colonic samples were mechanically homogenized in buffer supplemented with protease cocktail. Samples were centrifuged to get the supernatants. The protein levels of IL-23, IL-17, TNF and IL-10 were measured by ELISA. (*, $P < 0.05$; **, $P < 0.01$)

7. The effects of p40 vaccine on the percentages of Th1, Th17 and Treg cells in TNBS-induced colitis

Crohn's disease is usually described as a Th1 and Th17 mediated disease, in which Th1 and Th17 cells are increased.⁷⁵ As IL-12 and IL-23 play important roles in the differentiation of Th1 and Th17 cells, respectively, IL-12/IL-23p40 vaccine might employ its effects through inhibiting these two pathways. Our previous results showed that vaccine treatment down-regulated the expression of Th1 and Th17 cytokines in the colon tissue, suggesting that the vaccine might influence Th1 and Th17 cells. As indicated in Figure 26, the percentages of CD4⁺IFN- γ ⁺ Th1 and CD4⁺IL-17⁺ Th17 cells are increased in MLN after TNBS challenges when compared with naïve controls. However, vaccine treatment could down-regulate the percentages of Th1 and Th17. The percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in MLN was also analyzed. Our results showed that vaccine treatment could increase the percentage of Tregs compared with those in saline and carrier groups. And the ratios of Treg to Th17 and Treg to Th1 were significantly increased in vaccine treatment group when compared with saline and carrier groups. The ratios of Treg to Th17 and Treg to Th1 in LPMC also showed the same trend. These ratios were increased in the vaccine treatment group in contrast to saline and carrier groups (Figure 26). Taken together, these results indicated that vaccine treatment could re-balance the Th1, Th17 and Treg responses in murine colitis.

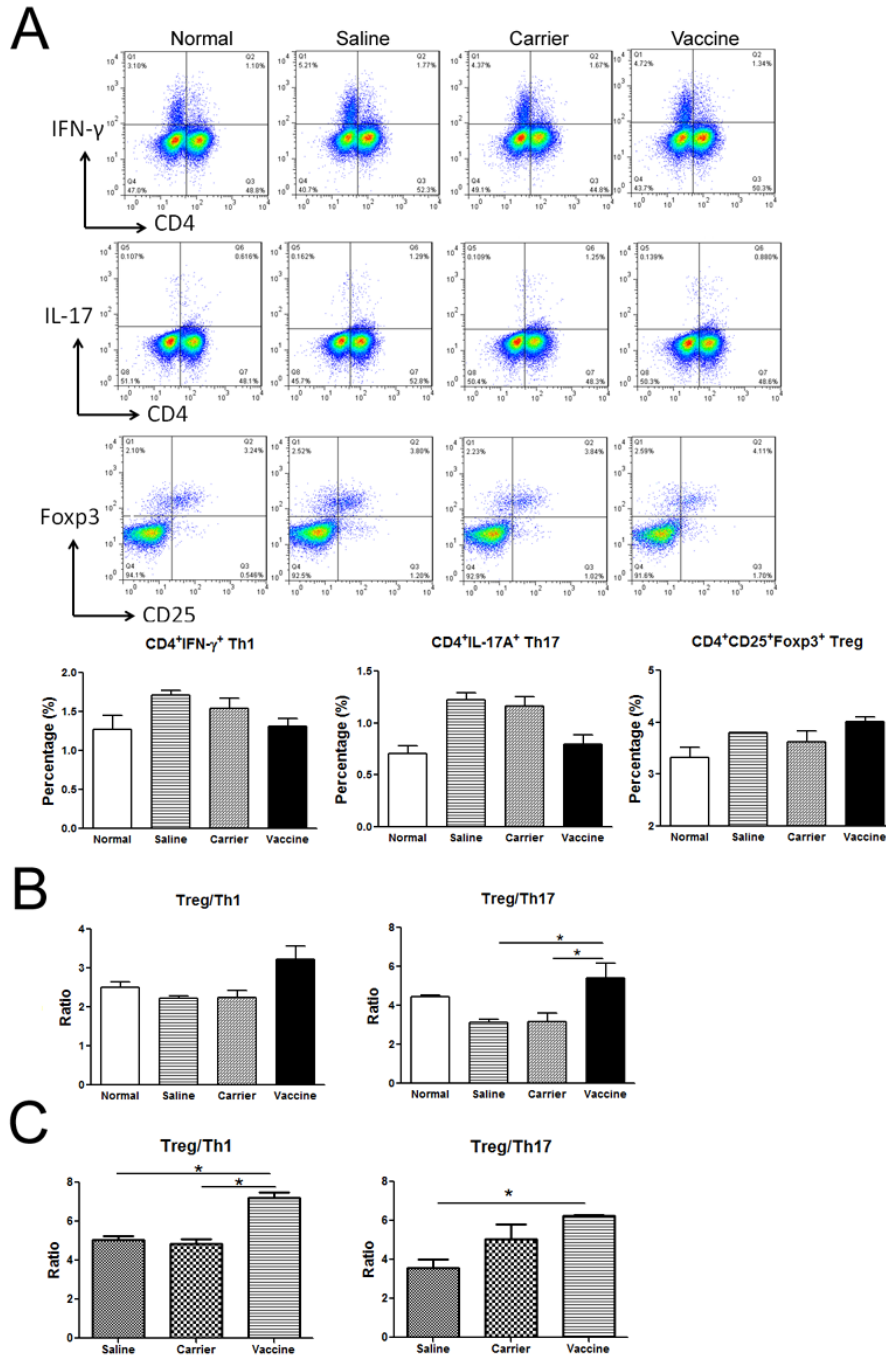


Figure 26. Vaccine treatment re-balances Th1/Th17/Treg responses in TNBS-induced chronic colitis. After stimulation with PMA and ionomycin, lymphocytes from MLNs or LPMC were stained with anti-CD4, anti-IFN- γ and anti-IL-17 to detect the percentages of CD4⁺IFN- γ ⁺Th1 and CD4⁺IL-17⁺Th17 cells by flow cytometry. CD4⁺CD25⁺Foxp3⁺ Treg cells were stained according to the manufacturer's manual. (A) The percentages of Th1, Th17 and Tregs in MLN. (B) The ratios of Tregs to Th1 and Tregs to Th17 in MLN. (C) The ratios of Tregs to Th1 and Tregs to Th17 in LPMC. (*, $P < 0.05$)

8. p40 vaccine promotes apoptosis of Th1 and Th17 cells in TNBS-induced colitis

IL-12 has been shown to prevent Fas-mediated apoptosis of lymphocytes by suppressing the activation of Caspase-3 and upregulating anti-apoptotic molecule Bcl-2. Vaccine treatment might also employ its effects through promoting Fas-mediated apoptosis of lymphocytes in the colitis. First, we used flow cytometry analysis to detect the percentage of active-caspase-3⁺ in Th1 and Th17 cells. As shown in Figure 27, the percentages of active caspase3⁺CD4⁺IFN- γ ⁺ cells are increased in saline and carrier groups, and the percentages of active caspase3⁺CD4⁺IL-17⁺ cells are also increased in the two groups. Interestingly, vaccine treatment significantly increased the percentages of active caspase 3⁺CD4⁺IFN- γ ⁺ and caspase3⁺CD4⁺IL-17⁺ cells when compared with saline and carrier controls. In Figures 11 and 16, our results also showed that vaccine treatment down-regulated the mRNA expression of anti-apoptotic molecule Bcl-2 in the colon tissue in contrast to saline and carrier groups. Taken together, these data indicated that mice immunized with IL-12/IL-23p40 promoted the apoptosis of lymphocytes.

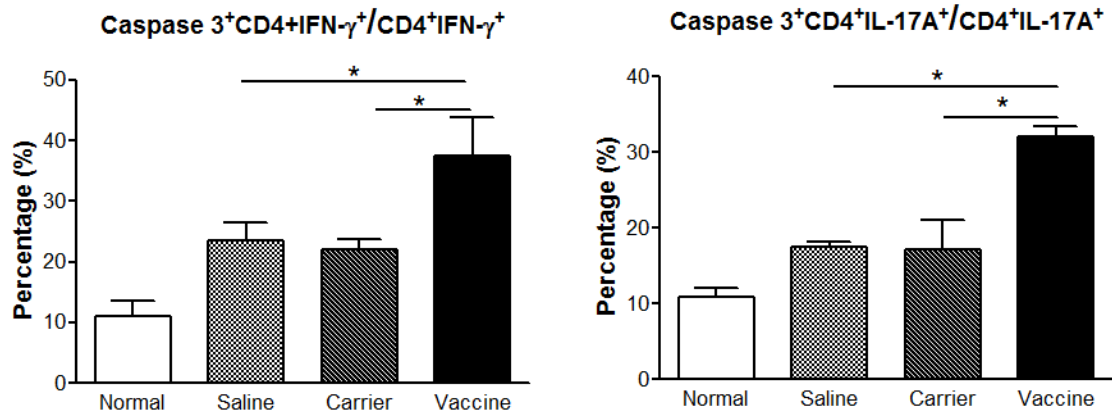


Figure 27. Vaccine treatment promotes the apoptosis of Th1 and Th17 cells in MLNs. After stimulation with PMA and inomycin for 6-7 hours, lymphocytes from MLNs were then stained with anti-CD4, anti-IFN- γ , anti-IL-17 and anti-activated caspase-3 and analyzed by flow cytometry gating on CD4⁺ T cells.

9. p40 vaccine promotes the production of IL-10 by increasing CD11c⁺ cells in LPMC of TNBS-induced colitis

IL-12 and IL-23 are mainly produced by activated professional APCs, such as DCs and macrophages. IL-12, IL-23 and p40 can also influence the function of these professional APCs, such as their migration and LPS-induced DC maturation.^{139, 302, 303} Here we evaluated the influences of vaccine treatment on macrophages and DC in LPMC. Compared with saline and control groups, the total number of LPMC was significantly decreased in vaccine treatment group. And the numbers of CD11b⁺ macrophages and CD11c⁺ DC in LPMC were also significantly decreased after vaccine treatment. To our surprise, the percentage of CD11c⁺IL-10⁺ cells in LPMC was increased after vaccine treatment. These results indicated that vaccine treatment decreased the infiltration of CD11b⁺ macrophages and CD11c⁺ DC cells into local inflamed tissue, but increased the production of IL-10 by CD11c⁺ DC cells.

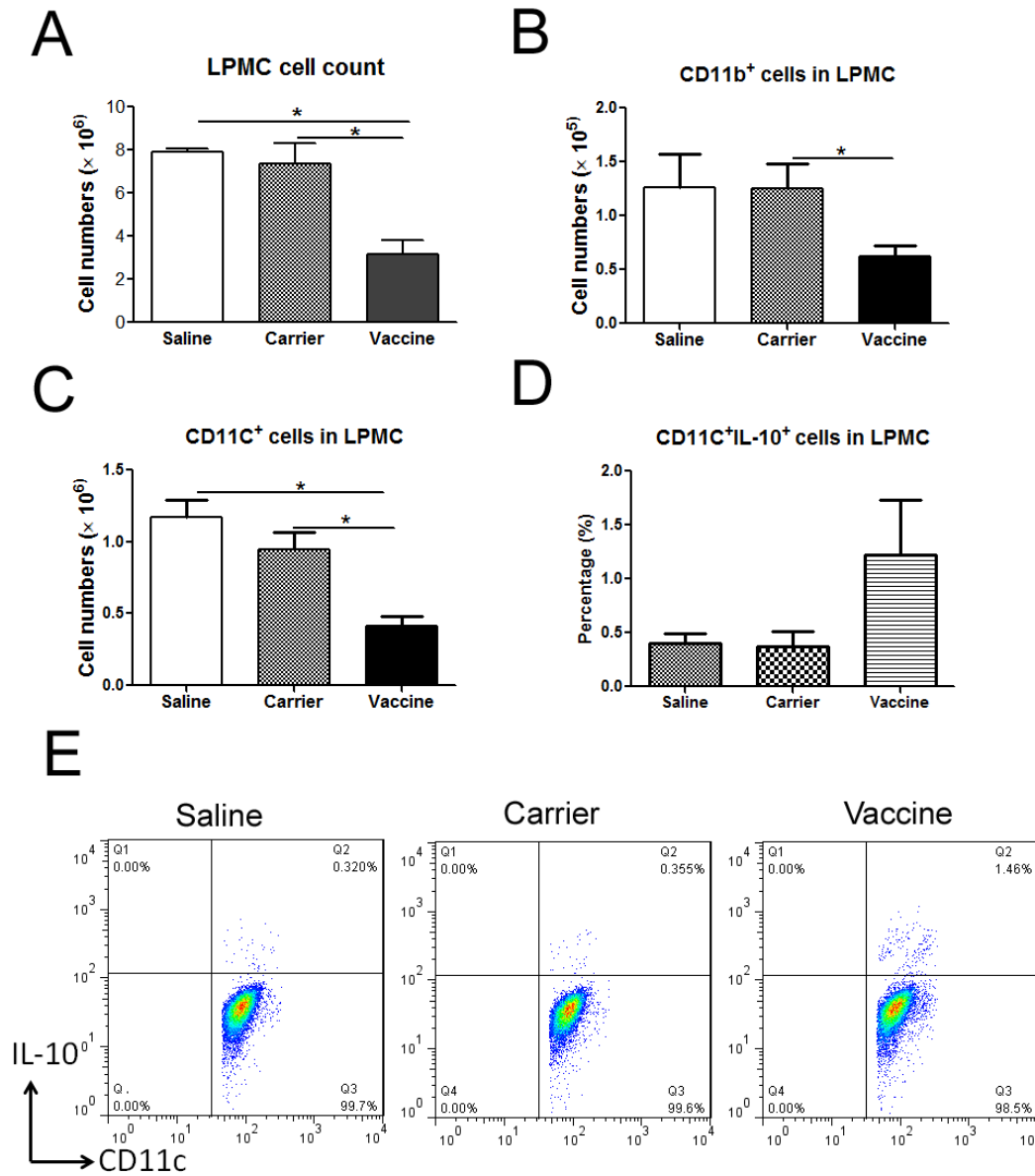


Figure 28. The influence of vaccine treatment on DCs and macrophages in LPMC. Colonic LPMC were isolated using the method described in the “Methods”. The total cell numbers of LPMC were counted. LPMC were then stimulated with PMA and ionomycin for 6 hours, and then stained with anti-CD11b, anti-CD11c and anti-IL-10 antibodies and analyzed by flow cytometry. (A) The total cell numbers of LPMC. (B) The cell numbers of CD11b⁺ macrophages in LPMC. (C) The cell numbers of CD11c⁺ DCs in LPMC. (D & E) The percentages of CD11c⁺IL-10⁺ cells in LPMC. (*, $P < 0.05$)

10. p40 vaccine does not increase the susceptibility to chlamydia infection

As IL-12 and IL-23 play important roles in the host defense against infections, the employment of IL-12/IL-23p40 vaccine might increase the susceptibility to infections, because of the relatively long-lasting antibodies induced. Studies have demonstrated that Th1 and Th17 cells are vital for the defense against chlamydial lung infection.^{253, 297, 299} Using this mouse model, we evaluated the effects of vaccine treatment on chlamydial lung infection. Mice were firstly immunized with vaccine three times to induce antibody responses. After establishing high levels of antibodies against IL-12, IL-23 and p40 (Figure 29A), mice were intranasally challenged with *Chlamydia muridarum* to induce lung infection. After *Chlamydia muridarum* infection, all mice showed obvious body weight loss. There was no significant difference among saline, carrier and vaccine groups (Figure 29B). The bacterial burden in the lung was examined. As indicated in figure 29, all mice infected had high levels of bacterial burden compared to those of naïve mice. To our surprise, vaccine treatment caused a slight decrease in bacterial burden, not an increase bacterial burden, when compared with saline and carrier groups (Figure 29C) ($P > 0.05$). Lung histological examination also confirmed our finding that there was no significant difference among saline, carrier and vaccine groups (Figure 29D), even though the lung inflammation was severe in these groups. Taken together, our results suggested that mice immunized with IL-12/IL-23p40 vaccine didn't have an increased susceptibility to chlamydia muridarum infection.

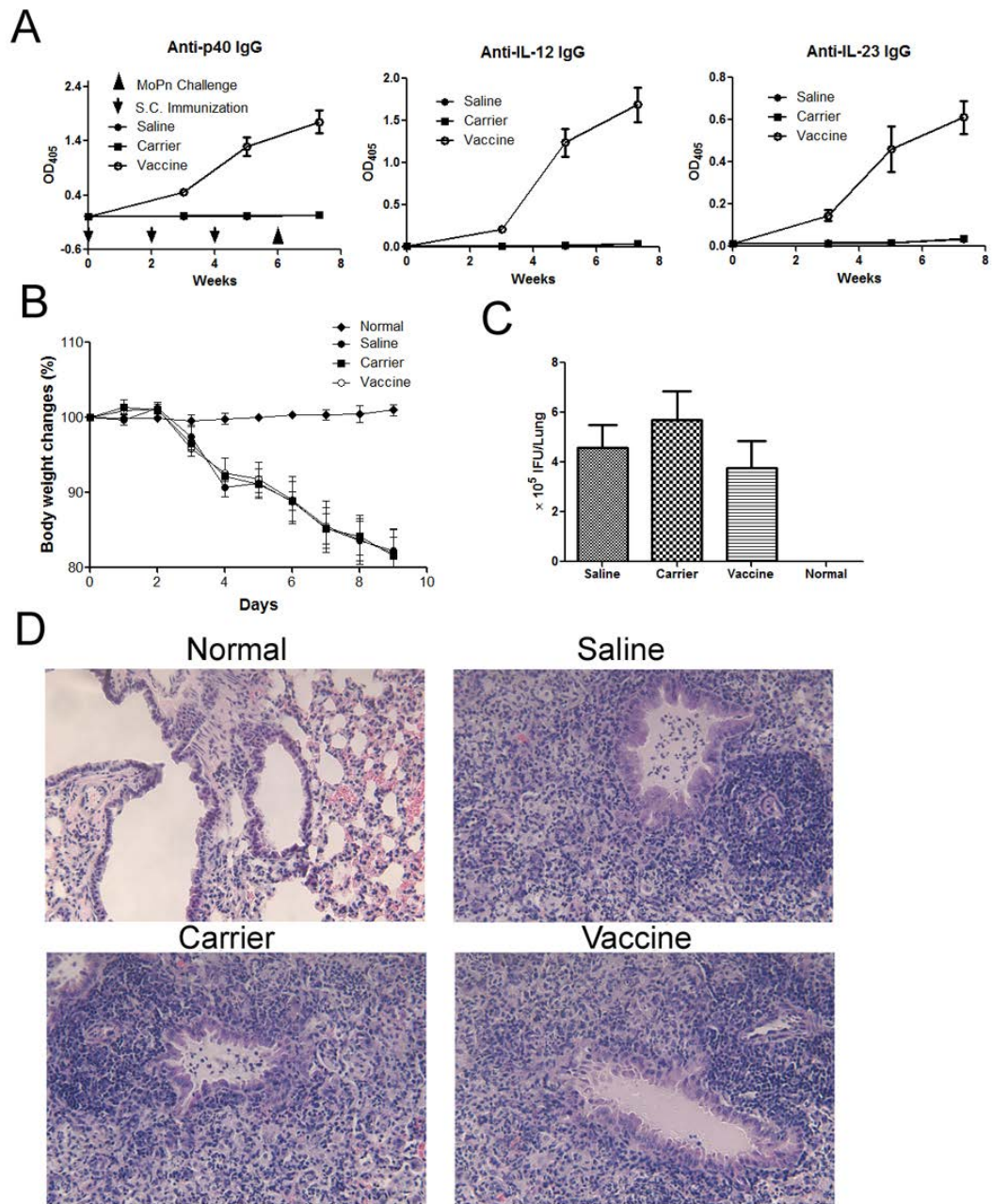


Figure 29. Vaccine treatment doesn't increase the susceptibility to chlamydia muridarum infection. Mice were immunized with vaccine, carrier or saline three times at a two-week interval (n=6). Two weeks later, the mice were challenged with MoPn to induce lung infection. Nine days later, mice were sacrificed to analyze. (A) The levels of specific antibodies against IL-12, IL-23 and p40 induced by the vaccine. (B) Body weight changes. (C) The burden of chlamydial growth *in vivo* in the lung tissue. (D) Lung sections, stained by H & E, original magnification x 100 under light microscopy.

Discussion

Our previous studies have confirmed that vaccination of mice with IL-12/IL-23p40 vaccine could prevent TNBS-induced acute and chronic colitis.²⁵⁵ In this section, we evaluated whether administration of this vaccine after the development of colitis would still be effective in both TNBS- and DSS-induced chronic murine colitis. Our results revealed that after receiving TNBS, vaccinated mice had significant decreases in body weight loss, intestinal inflammation and fibrosis, and levels of p40, IL-23, IL-17 and TNF in colon tissue, compared with saline and carrier groups. Similar results were obtained in DSS-induced colitis model. After establishing DSS-induced colitis, vaccinated mice developed high levels of antibody responses against IL-12, IL-23 and p40, along with significantly decreased clinical scores, intestinal inflammation and collagen deposition, and levels of proinflammatory cytokines in colon tissues, in contrast to saline and carrier groups.

Crohn's disease is usually believed to be caused by an overly aggressive Th1 immune response, and an excessive IL-23/Th17 pathway activation to bacterial antigens in genetically predisposed individuals.^{12, 37, 92-94} IL-12, vital for Th1 differentiation to produce IFN- γ , shares the p40 subunit with another cytokine-IL-23. IL-23 stabilizes the proliferation of Th17 cells and promotes the production of IL-17.⁹⁴ Therefore, blocking IL-12/IL-23p40 might suppress the differentiation of both Th1 and Th17 cells. Our results show that Th1 and Th17 cells are increased in murine colitis, confirming previous findings that Th1 and Th17 cells are involved in the pathogenesis of Crohn's disease. Similar to our previous results, mice immunized with IL-12/IL-23p40 vaccine also decrease the percentages of Th1 and Th17 cells in MLN in this treatment study.

Impaired balances of Th1/Treg and Th17/Treg responses are also reported in IBD.¹¹⁶ The dysfunction of Tregs in IBD is usually believed to be defective diminished number of Tregs or defective suppressive functions which can't control the intestinal inflammation. Recently, IL-23 has been found to restrain the activity of Treg to drive T cell-dependent colitis,²⁹⁰ and the homodimer of p40 is found to suppress the expression of CD25 and Foxp3.²⁹¹ Therefore, targeting IL-12/IL-23p40 using vaccine strategy may promote Tregs expansion. Our results indicated that the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs was slightly increased in MLN in saline and carrier groups after TNBS challenges. And vaccine treatment significantly increases the percentage of Tregs in MLN in contrast to that in naïve group. When compared with naïve mice the ratios of Treg to Th1 and Treg to Th17 decreased in saline and carrier group, especially for the ratio of Treg to Th17, which is coincident with the findings reported by Eastaff-Leung et al, in which a significant decrease of Treg/Th17 ratio is observed in the PBMC of IBD patients.¹¹⁶ However, vaccine treatment increases the ratios of Treg to Th1 and Treg to Th17 in contrast to those in naïve, saline and carrier groups (Figure 26). The above data indicate that IL-12/IL-23p40 vaccine-mediated improvement may be via re-balancing of Th1/Th17/Treg responses.

Our results also indicate that vaccine-mediated amelioration of murine colitis may be through the promotion of the apoptosis of activated Th1 and Th17 cells. IL-12 has been shown to inhibit Fas-mediated cell death of CD4⁺ T cells through the inhibition of caspase-3 activity and the upregulation of the anti-apoptotic molecule Bcl-2.²⁹⁵ Previous studies have reported that treating mice with IL-12/IL-23p40 mAb promotes the apoptosis of Th1 cells in TNBS-induced colitis model, and treatment of mice with IL-

23p19 mAb also promotes the apoptosis of Th17 cells in T cell-transfer colitis model.^{109,}
¹⁹⁶ Our previous results have shown that IL-12/IL-23p40 vaccine treatment down-regulate mRNA expression of anti-apoptotic molecule Bcl-2 in colon tissue, indicating that vaccine treatment may promote the apoptosis of T cells.²⁵⁵ In the present study, our results have demonstrated that the percentages of active caspase 3⁺CD4⁺IFN- γ ⁺ and active caspase3⁺CD4⁺IL-17⁺ cells are significantly increased in vaccine group, confirming that the increased apoptosis of T cells is involved in the vaccine-mediated improvement.

The effects of IL-12/IL-23p40 vaccine on macrophages and DC were also explored. p40 subunit contributes to both the IL-12 and IL-23 heterodimers. However, p40 can also be secreted as a monomer p40 and as a homodimer p80.^{154, 155} p40 is secreted at a 50-fold higher than IL-12 in a murine shock model and at a 10-20 fold excess by stimulated human peripheral blood cells.¹³⁹ Natural p80 exists at 20-40% of the total p40 in the serum in murine model of systemic infection.¹³⁹ Studies have shown that p80 can act as a macrophage chemoattractant and an inducer of DC migration.^{157, 158} Recombinant murine p80 can act as the chemoattractant for both rat and mouse macrophages over a dose range of 10-1000 ng/ml.¹⁵⁷ And Walter *et al.* have demonstrated that intratracheal delivery of recombinant murine p80, not recombinant murine IL-12 or p40, promotes the recruitment of macrophages into the airway.¹⁵⁹ It has also been reported that migration of DCs from the lung to the draining lymph nodes after exposure to *Mycobacterium tuberculosis* (Mtb) is defective in mice lacking p40, not in mice lacking IL-12p35.¹⁵⁸ The delivery of p80 to p40-deficient DCs restores Mtb-induced DC migration and the ability of p40-deficient DCs to activate naive T cells.¹⁵⁸ Our results

showed that IL-12/IL-23p40 vaccine treatment significantly decreased the total cell numbers and the numbers of macrophages and DCs in LPMC, suggesting that the infiltration of macrophages and DCs into the inflamed colon tissue was inhibited possibly via blocking the functions of IL-12/IL-23p40 or p80. Our results also showed that IL-10 production was increased in CD11c⁺ DCs in vaccine treated mice, suggesting that promoting IL-10 production from CD11c⁺ DCs may be another mechanism involved in vaccine-mediated improvement.

A potential concern with cytokine vaccines is that the long-lasting auto-antibodies against cytokines might increase the susceptibility to certain infections, as IL-12/IL-23 also plays an important role in the host defense against infections. Since Th1 and Th17 cells play important roles in the host defense against *Chlamydia muridarum* infection,^{253, 297, 299} we explored the effects of IL-12/IL-23p40 vaccine on the *Chlamydia muridarum* infection in the present study. Our results showed that immunization of IL-12/IL-23p40 vaccine did not exaggerate body weight loss, increase *Chlamydia muridarum* burden in the lung tissue, worsen lung inflammation or influence cytokine productions, in contrast to saline and carrier groups. They indicated that immunization with IL-12/IL-23p40 vaccine didn't increase the susceptibility to *Chlamydia muridarum* infections. In the future, we will explore whether IL-12/IL-23p40 vaccine increases other infections, such as *Leishmania major* infection.

In summary, our results indicated that administration of the IL-12/IL-23p40 vaccine could ameliorate on going chronic intestinal inflammation through rebalancing Th1/Th17/Treg responses, promoting apoptosis of Th1 and Th17 cells, and increasing IL-10 production from CD11c⁺ DC cells in LPMC.

Result IV Development of vaccines against IL-12p35 or IL-23p19 and evaluation of their effects in the amelioration of murine colitis

Specific introduction

In the 1990s, studies firstly demonstrated that IL-12 was increased in patients with active Crohn's disease and monoclonal antibodies against IL-12 (IL-12p40) could ameliorate established TNBS-induced murine colitis.^{193, 197} Since the finding of another cytokine, IL-23, many studies have highlighted the role of IL-23 in the pathogenesis of Crohn's disease as IL-12 and IL-23 share p40 subunit.^{3, 106, 198, 199, 304} In IL-10 knockout mice, a spontaneous IBD model, the development of colitis was suppressed by IL-23p19 deficiency but not IL-12p35 deficiency; administration of IL-23 accelerated the onset of colitis and promoted colon inflammation through an IL-17- and IL-6-dependent mechanism.¹⁰⁶ In a *Helicobacter hepaticus*-induced T cell-dependent colitis model, Kullberg M. *et al.* showed that adoptive transfer of wild type CD4⁺CD45RB^{hi} cells into p40 and Rag-double deficient mice failed to develop typhocolitis; in contrast, adoptive transfer of the same T cell populations into IL-12p35 and Rag-double deficient mice developed severe typhocolitis; and adoptive transfer of the same T cell populations into IL-23p19 and Rag-double deficient mice displayed attenuated typhocolitis.¹⁹⁹ Anti-IL-23 monoclonal antibodies could prevent and reverse active colitis in a T cell-mediated colitis mouse model, with the down-regulation of a broad array of inflammatory cytokines and chemokines in the colon.¹⁰⁹ Using IL-23R-deficient mice, Powrie's group demonstrated that through direct signaling, in the intestine, IL-23 promoted the proliferation and accumulation of Th17 cells, enhanced the generation of IL-17⁺IFN- γ ⁺ T

cells, and suppressed the differentiation of iTreg and IL-10 production from T cells.²¹ Recently, GWAS has identified numerous single-nucleotide polymorphisms (SNP) in IL-23R, with high association for Crohn's disease and ulcerative colitis.^{21, 22} These results demonstrate that IL-23 plays important roles in the pathogenesis of Crohn's disease.

In 2007, the biological functions of the new cytokine, IL-35, were discovered by two groups at the same time.^{125, 305} IL-35 is a heterodimeric cytokine which is composed of IL-12p35 subunit and IL-27Ebi3 subunit. The receptor of IL-35 and its signaling pathway have not been characterized yet.¹³⁷ IL-35 is mainly expressed by regulatory T cells, not by resting and activated CD4⁺ T cells. Loss of IL-35 leads to a significant reduction of the regulatory activity of Tregs *in vitro* and fails to cure murine colitis *in vivo*.¹²⁵ Recombinant IL-35 can suppress T cell proliferation.¹²⁵ Current data demonstrate that IL-35 is an important inhibitory cytokine.^{124, 306} As IL-12 and IL-35 share the p35 unit, the effects of IL-12 in the pathogenesis of IBD need to be re-considered. Since severe colitis is developed in IL-12p35-deficient mice, not in IL-23p19-deficient mice,^{106, 199} it is possible that IL-12p35-deficient mice cannot produce the inhibitory cytokine IL-35 which has a strong ability to improve colitis.

In the previous sections, we successfully developed an IL-12/IL-23p40 peptide-based vaccine which could induce relatively long-lasting antibodies to IL-12, IL-23 and p40. Administration of the vaccine significantly improved TNBS-induced murine colitis. In this section, we developed peptide-based vaccines specific to IL-12p35 (shared by IL-12 and IL-35) or IL-23p19 and explore the *in vivo* effects of the vaccines TNBS-induced chronic colitis.

Results

1. Selection of peptides from IL-12p35 and IL-23p19 subunits

Based on the occurrence of amino acid residues in experimentally known segmental epitopes and by using the DNASTar software, six and five peptides with high antigenic index, high flexibility, high surface probability and high hydrophilicity were selected from mouse IL-12p35 (table 2) and IL-23p19 subunits (table 3), respectively. The corresponding human peptides of these selected peptides were examined in the crystal structures of human IL-12 and IL-23 to check for their locations (Figure 30).

Table 2 Peptides selected from mouse IL-12p35 subunit

Peptide	Sequence	Number of amino acid
A	³⁵ SQSRNLLKTTDD ⁴⁶	12
B	⁴⁹ KTAREK ⁵⁶ KLK	8
C	⁶⁵ DHEDITRDQT ⁷⁵	11
D	⁹⁶ ETSS ¹¹³ TTRGSLPPQKTS	18
E	¹⁶⁹ NGETLRQKPP ¹⁷⁸	10
F	¹⁸⁰ EADPYRVK ¹⁸⁸	9

Table 3 Peptides selected from IL-23p19 subunit

Peptide	Sequence	Number of amino acid
A	²³ PRSSSPD ²⁹	7
B	⁵⁸ EEEDEETKNNVP ⁷¹	13
C	⁷⁶ GCDPQGLKDNSQ ⁸⁸	13
D	¹³⁹ EDHPRETQQ ¹⁴⁷	9
E	¹⁵³ SQQWQRPLL ¹⁶²	10

A

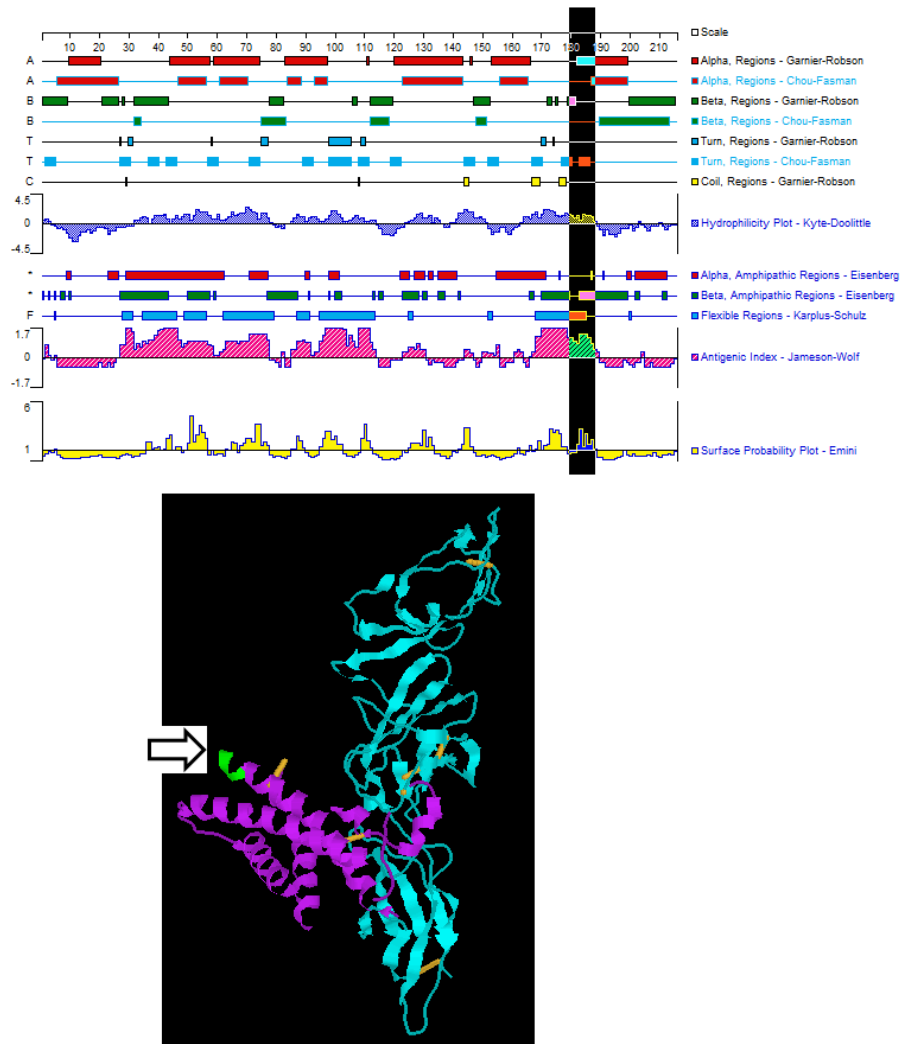


Figure 30. Selection of peptides from IL-12p35 or IL-23p19 subunit. (A) Peptide F from IL-12p35 subunit. Top: peptide F predicted in the DNASTar software; bottom: the corresponding peptide of human IL-12 (peptide F) in the crystal structure of human IL-12. (Arrow indicated the position of the peptide selected)

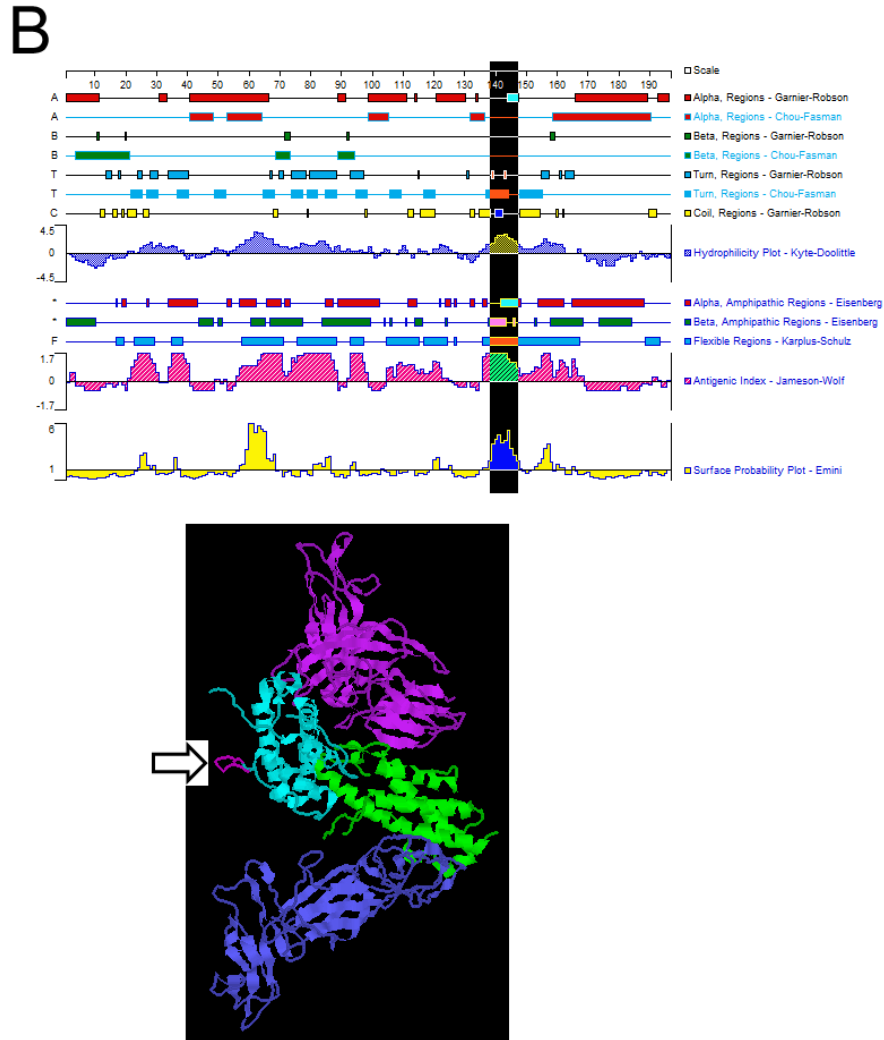


Figure 30. Selection of peptides from IL-12p35 or IL-23p19 subunit. (B) Peptide D from IL-23p19 subunit. Top: peptide D predicted in the DNASTar software; bottom: the corresponding peptide of human IL-23 (peptide D) in the crystal structure of human IL-23. (Arrow indicated the position of the peptide selected)

2. IL-12p35 and IL-23p19 peptide-based vaccines induce high titers of IgG to IL-12 and IL-23, respectively

To construct peptide-based vaccines, each peptide selected was inserted into the vector plasmid pThio-HBcAg by gene recombination methods. The recombinant plasmids were then identified by restriction endonucleases digestion and SDS-PAGE. Finally, based on the formation of virus-like particles, two separate IL-12p35 peptide vaccines (named vaccine C and F), and one IL-23p19 peptide vaccine (named vaccine D) were selected for further analysis.

To determine the ability of the vaccines to induce specific IgG response, mice were immunized with each vaccine or the carrier protein HBcAg for 4 times (Figure 31). Sera were collected at indicated times to detect the antibody levels by ELISA. As shown in Figure 31A, IL-12p35 vaccines C and F induced significantly high levels of specific IgG antibodies to IL-12 after three times of immunization. Antibodies to IL-12/IL-23p40 and IL-23 were undetectable (data not shown). IL-23p19 vaccine induced high levels of specific antibodies against IL-23 (Figure 31B) and no antibodies against IL-12 and IL-12/IL-23p40 (data not shown). Mice received carrier or saline injections had no detectable specific antibodies.

These results indicated that the two IL-12p35 peptide vaccines and one IL-23p19 peptide vaccine were successfully constructed, and capable of inducing high levels of specific IgG to IL-12 or IL-23, respectively.

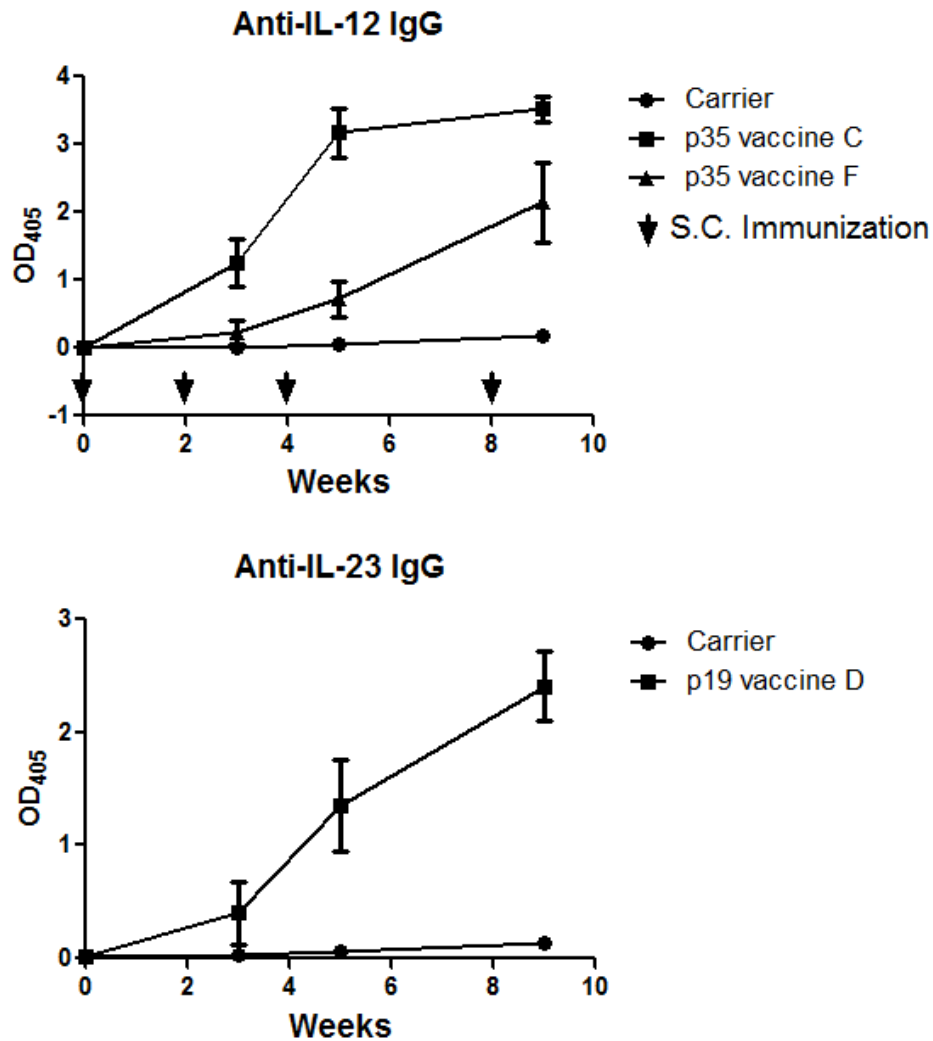


Figure 31. The levels of specific IgG antibodies to IL-12 or IL-23 induced by vaccines. Female BALB/c mice were subcutaneously primed with 100 μ g/200 μ l of each vaccine and carrier, and boosted at weeks 2, 4 and 8 with 25 μ g of the vaccine or the carrier. Sera were obtained from the mice at the indicated weeks and diluted 1/200 for determination of specific IgG levels by ELISA in which IL-12 or IL-23 (0.25 μ g/ml) were coated on the plates, separately.

3. Mice immunized with IL-23p19 peptide vaccine, not IL-12p35 peptide vaccines, ameliorates TNBS-induced chronic colitis

To evaluate the effects of these peptide-based vaccines in the prevention of TNBS-induced chronic colitis, 5 groups of mice were immunized three times with each of the vaccines or the carrier or saline. After ensuring that high titers of specific IgG antibodies to IL-12 or IL-23 were present, mice then received weekly treatments with TNBS six times (1.0 - 2.3 mg per mouse, starting at 1.0 mg and gradually increasing the dose to 2.3 mg) (Figure 32A).

Similar to our previous results, in this chronic colitis model, mice in saline and carrier groups showed obvious body weight loss after the administration of TNBS commenced (Figure 32B). In contrast, IL-23p19 vaccine-immunized mice exhibited improved body weight loss compared with saline and carrier groups ($P < 0.05$) during the last two weeks. However, IL-12p35 vaccine C- or vaccine F-immunized mice didn't show any improvement of body weight loss.

After repeated TNBS administrations, colons from saline and carrier groups showed signs of severe inflammation, including inflammatory cell infiltration, goblet cell reduction, and distorted tissue architecture (Figure 33A). Colon tissues from mice immunized with IL-12p35 vaccine C or F presented similar inflammatory findings as saline and carrier groups. But colon tissues from mice immunized with IL-23p19 vaccine showed significantly reduced inflammation when compared to carrier and saline controls. Semi-quantitative analysis revealed that inflammatory scores in the IL-23p19 vaccine group were significantly lower than controls ($P < 0.05$) (Figure 33B), confirming that IL-23p19 vaccine was able to suppress TNBS-induced chronic intestinal inflammation.

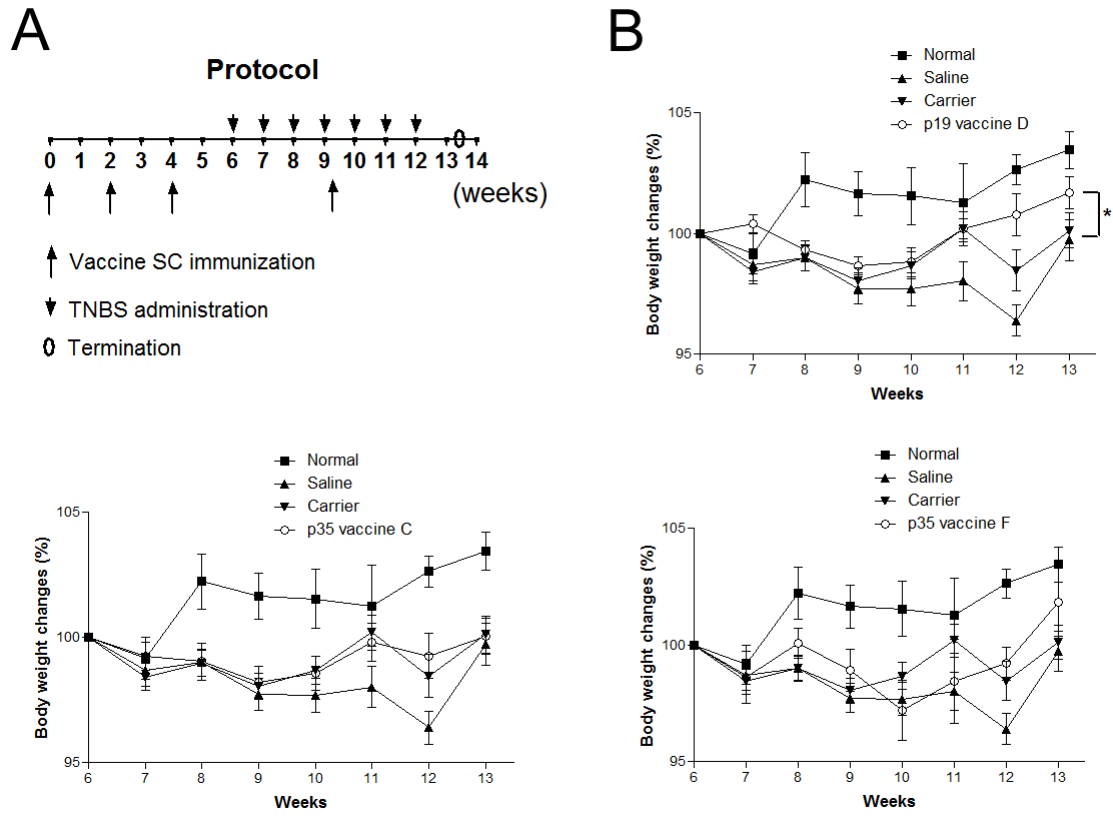


Figure 32. IL-23p19 vaccine, not IL-12p35 vaccines, improved body weight loss in TNBS-induced chronic colitis. Mice were first immunized with each vaccine, carrier or saline three times at two-week interval and booster immunized at week 9 (n=12). At week 6, mice were weekly challenged with TNBS 7 times to induce chronic colitis. Ten days after the last TNBS challenge, mice were sacrificed to analyze. Body weight of mice was monitored weekly. (A) Protocol. (B) Body weight changes. (*, $P < 0.05$)

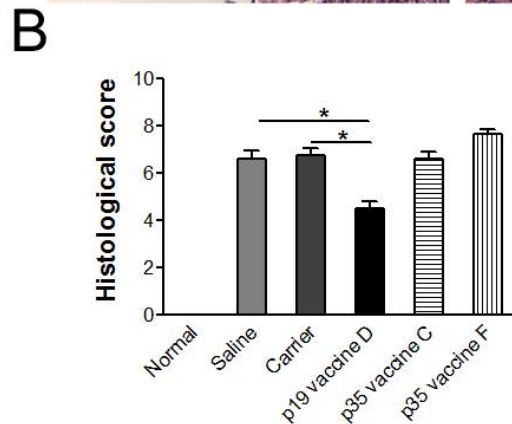
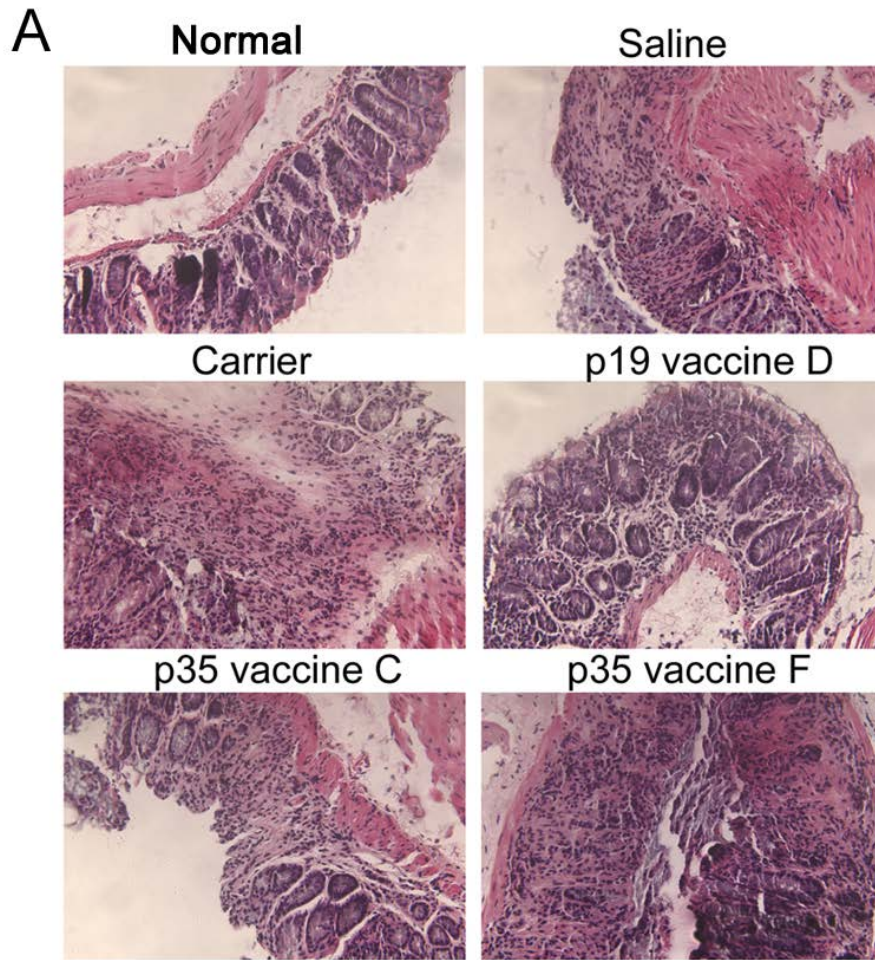


Figure 33. IL-23p19 vaccine, not IL-12p35 vaccines, reduces colon inflammation in TNBS-induced chronic colitis. (A) Colonic specimens were formalin-fixed and embedded in paraffin blocks, then 6- μ m sections were stained with hematoxylin and eosin. Representative histological images of samples from normal control and TNBS-treated mice following treatment with vaccines, carrier or saline are shown (original magnification $\times 100$). (B) Semi-quantitative analysis was used to assess histological changes. H&E scores of 0-10 were given for each mouse. (*, $P < 0.05$)

4. IL-23p19 vaccine, not IL-12p35 vaccines, inhibits fibrosis of chronic intestinal inflammation

As fibrosis is a characteristic of chronic inflammation, the collagen deposition in colons was evaluated using Masson's trichrome staining. In addition, soluble colon collagen was quantitatively measured using a Sircol Collagen Assay kit. As shown in Figure 34A, when compared to normal controls, the saline, carrier, and IL-12p35 vaccine groups show a substantial collagen deposition in the intestinal subepithelial layer, deep in the lamina propria, and in the muscular layers. In contrast, IL-23p19 vaccine group showed less collagen deposition as compared to saline and carrier controls. Moreover, the soluble collagen assay revealed that the amounts of collagen were significantly reduced in IL-23p19 vaccine group (Figure 34B). These results indicate that IL-23p19 vaccine is able to inhibit fibrosis formation during chronic intestinal inflammation.

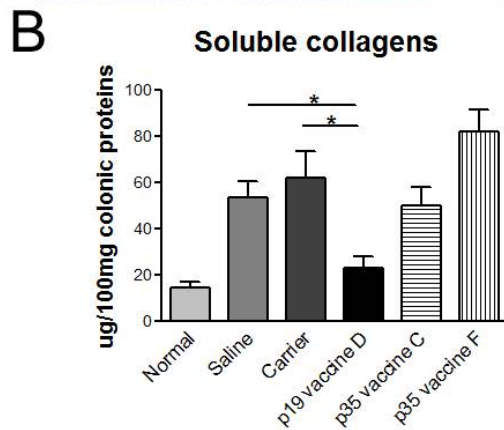
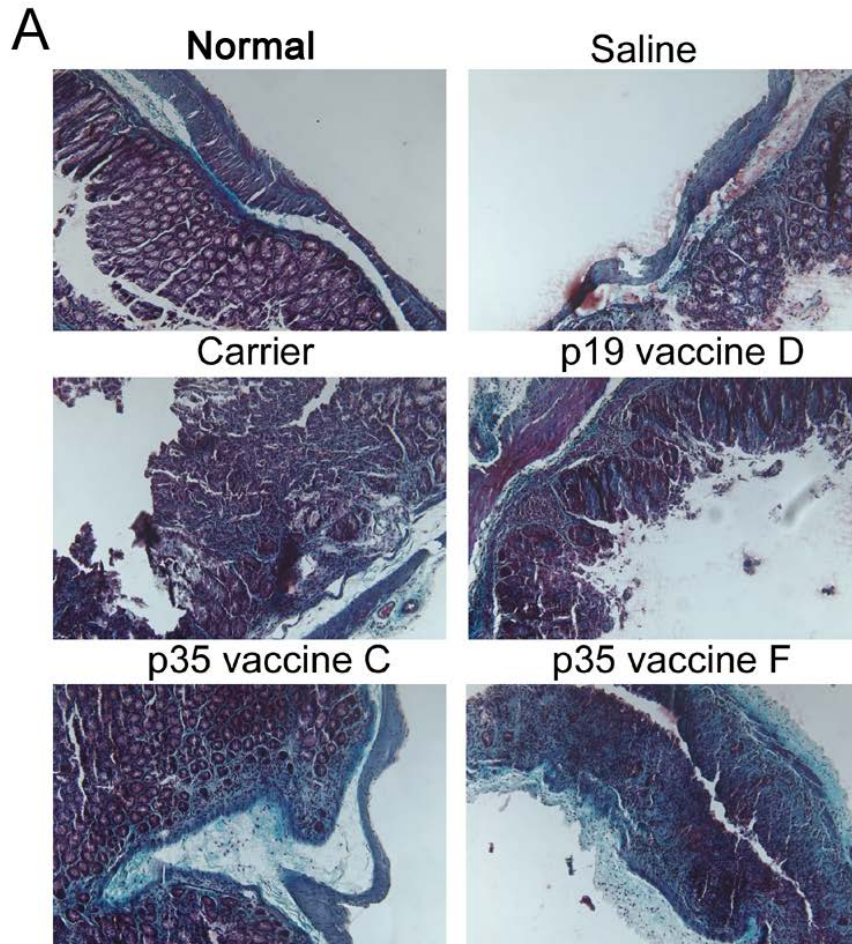


Figure 34. IL-23p19 vaccine, not IL-12p35 vaccines, reduces colon fibrosis in TNBS-induced chronic colitis. (A) Masson's trichrome staining of representative colon images (original magnification x 100). (B) Colon soluble collagens measured by a Sircol collagen assay (n=8-10). (*, $P < 0.05$)

5. IL-23p19 vaccine, not IL-12p35 vaccines, downregulates colon IL-12 and IL-23 levels

To test the effectiveness of vaccines, the levels of IL-12 and IL-23 in colon tissue were measured using ELISA. As shown in Figure 35, both carrier and saline groups have an increased expression of IL-12 when compared to normal mice. But, only mice immunized with IL-23p19 vaccine showed significantly decreased levels of IL-12 and IL-23, suggesting that this vaccine is able to downregulate not only IL-23 but also IL-12 levels in TNBS-induced chronic colitis mice.

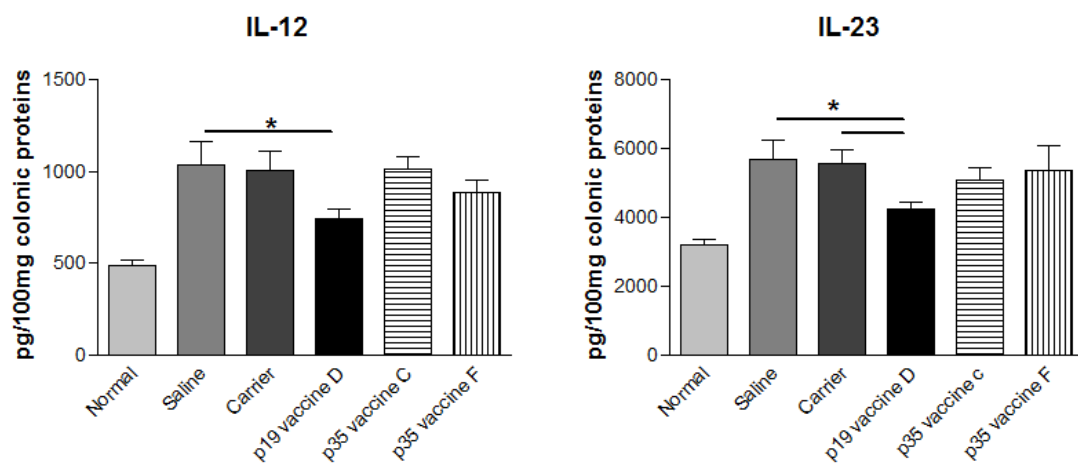


Figure 35. Expression of IL-12 and IL-23 in the colon tissue. After mice were sacrificed, the colon tissue was collected and homogenized. The supernatants of tissue homogenization were used to detect the expression of IL-12 and IL-23 by ELISA.

6. *in vitro* inhibition tests

Our results have clearly shown that IL-12p35 and IL-23p19 peptide-based vaccines induced specific antibodies responses. However, immunization with only IL-23p19 peptide-based vaccine, not IL-12p35 vaccines, ameliorated TNBS-induced chronic colitis, such as improving body weight loss and reducing intestinal inflammation. It might indicate antibodies induced by the IL-23p19 vaccine can efficiently block the biological functions of IL-23, and antibodies induced by IL-12p35 vaccines might block the biological functions of IL-35, not IL-12, or increase the biological functions of IL-12. To test this hypothesis, we performed *in vitro* inhibition tests. As we expected, the antibodies induced by IL-23p19 vaccine efficiently inhibited IL-23-induced production of IL-17 from activated lymphocytes in a dose-dependent manner (Figure 36). While, the antibodies induced by IL-12p35 vaccines C and F didn't obviously influence the IL-12-induced IFN- γ production from activated splenocytes, suggesting that the antibodies induced by IL-12p35 vaccine C or vaccine F couldn't block the biological functions of IL-12. Since IL-35 is not commercialized, at the moment we are unable to explore whether the antibodies induced by IL-12p35 vaccines could block the biological functions of IL-35. The above results indicated that the antibodies induced by IL-23p19 vaccine could *in vitro* block IL-23-induced biological functions; thus, this vaccine could efficiently ameliorate experimental colitis. In contrast, the antibodies induced by IL-12p35 vaccines failed to block IL-12-induced biological functions *in vitro*, therefore administration of the vaccines did not induce any improvement of intestinal inflammation.

IL-23p19 vaccine D

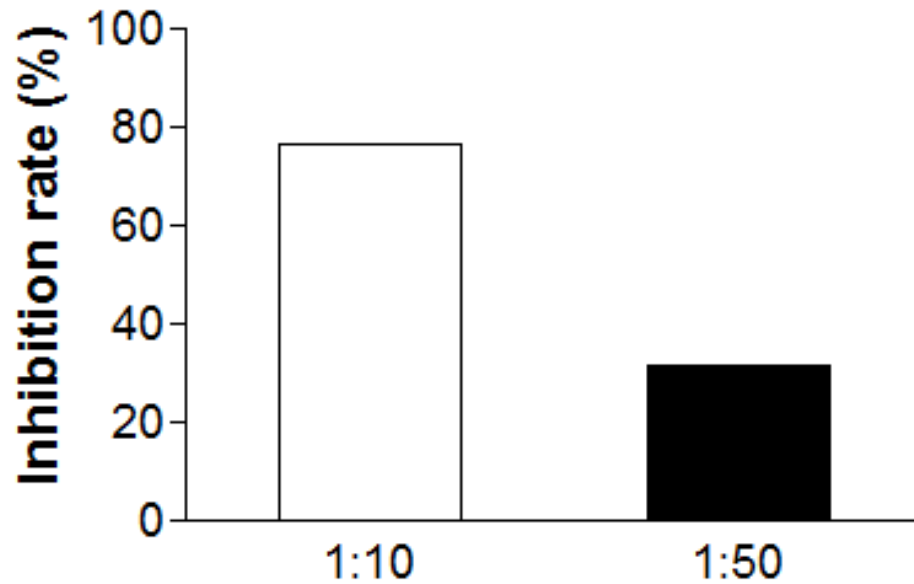


Figure 36. *In vitro* inhibition of vaccine-immunized antisera on IL-23-induced IL-17 production from splenocytes. Splenocytes isolated from normal BALB/c mice were stimulated with anti-CD3 ϵ /CD28 for 4 days in the presence of IL-6 (20 ng/ml), TGF- β (1 ng/ml) and IL-23 (10 ng/ml) with various dilutions of the sera from mice receiving IL-23p19 vaccine D or carrier. Supernatants were collected for measurement of expression levels of IL-17 by ELISA.

Discussion

In this section, IL-12p35 and IL-23p19 peptide-based vaccines were successfully developed which induced high levels of specific antibodies against IL-12 and IL-23, respectively. Mice immunized with IL-23p19 vaccine, not IL-12p35 vaccines, improved body weight loss, ameliorated TNBS-induced chronic intestinal inflammation, and down-regulated IL-12 and IL-23 expression in colon tissue when compared with saline and carrier groups.

The role of IL-23 in the pathogenesis of Crohn's disease has been demonstrated. For instance, IL-23p19 deficiency, not IL-12p35 deficiency, suppresses the development of colitis in IL-10^{-/-} mice;¹⁰⁶ anti-IL-23 monoclonal antibody prevents and reverses active colitis in a T cell-mediated colitis mouse model, with the down-regulation of a broad array of inflammatory cytokines and chemokines in the colon;¹⁰⁹ in the intestine, IL-23 promotes the proliferation and accumulation of Th17 cells, enhances the generation of IL-17⁺IFN- γ ⁺ T cells, and suppresses the differentiation of iTreg and IL-10 production from T cells.²¹ Therefore, IL-23 has been considered a target for the treatment of Crohn's disease. In the present study, using a vaccine strategy immunization with IL-23p19 peptide-based vaccine induced high levels of specific antibodies against IL-23, which could block the IL-23 induced biological function of IL-23 *in vitro*. And immunization with this vaccine improved body weight loss, and ameliorated TNBS-induced chronic intestinal inflammation and fibrosis. This indicates IL-23p19 peptide-based vaccine might be used to the treatment of Crohn's disease.

Just very recently, one group developed the first IL-23p19-based vaccines.³⁰⁷ Two peptides, selected from mouse IL-23p19 subunit, were chemically coupled to KLH to

obtain two peptide-based vaccines (IL-23k1 and IL-23k2).³⁰⁷ Immunization with these vaccines with incomplete Freund's adjuvant induced specific antibodies against IL-23, which could suppress IL-23-induced IL-17 production from splenocytes. Immunization with IL-23k1 vaccine strongly protected against collagen-induced joint destruction and inflammation.³⁰⁷ Comparing the peptides selected by this group with the ones used in our study, peptide E selected in our study (155-162aa) is close to IL-23k2 peptide (145-164aa). However, after the peptide E was inserted into the carrier protein HBcAg, the expressed recombinant protein failed to form virus-like particles. So, peptide E-based vaccine was not further explored. IL-23k1 peptide was not selected in our study. In the future, the IL-23k1 peptide will be examined to determine whether it forms virus-like particles after expression. Compared with the IL-23 peptide-KLH vaccine, our recombinant IL-23 peptide vaccine exhibits strong immunogenicity, as it induces high levels of antibody responses without the use of an adjuvant due to its virus-like particle form.

With the discovery of IL-35, a role for IL-12 in the pathogenesis of Crohn's disease can't be excluded. Studies indicate IL-23p19 deficiency, not IL-12p35 deficiency, suppresses the development of colitis in IL-10^{-/-} mice.¹⁰⁶ As IL-12 and IL-35 share p35 subunit, deficiency in IL-12p35 may lead to the lack of IL-35 that has been found to be an important regulatory cytokine. IL-35 is mainly expressed by regulatory T cells; the loss of IL-35 leads to significantly reduce regulatory activity of Tregs *in vitro* and fail to cure murine colitis *in vivo*.¹²⁵ Administration of IL-35 inhibits DSS/TNBS-induced murine colitis and collagen-induced arthritis through inhibiting Th1 and Th17 responses;^{126, 305} and improves allergic airway inflammation induced by dust mite through

inhibiting memory/effector Th2 cells.³⁰⁸ Treatment of naïve human or mouse CD4⁺ T cells with recombinant IL-35 induces the generation of a regulatory T cell population (iTR35) without expression of Foxp3, which mediates inhibitory functions via IL-35, not via IL-10 or TGF- β .¹²⁴ This type of regulatory T cells exhibits strong suppressive functions, is stable *in vivo*, and can be generated *in vivo* under inflammatory conditions such as *Trichuris muris* intestinal infection and within the tumor microenvironments.¹²⁴ Human Treg cells express IL-35 and require IL-35 for maximal suppressive capacity, and human Treg-mediated suppression results in the conversion of the suppressed conventional T cells into iTR35 which contribute to infectious tolerance.³⁰⁹ Therefore, deficiency in IL-12p35 induces colitis in IL-10^{-/-} mice, which might be due to deficiency of the suppressive function of IL-35, not IL-12.

As different peptides might have different effects in mediating biological functions of the host molecule, in the present study we used the peptide-based vaccine strategy to explore the role of IL-12/IL-35p35 in the TNBS-induced murine colitis. Our results showed that the two IL-12/IL-35p35 peptide-based vaccines induced specific antibody responses; however, they failed to inhibit IL-12-induced IFN- γ production. Administration of IL-12/IL-35p35 peptide-based vaccines did not ameliorate TNBS-induced chronic colitis. In the future, we will explore whether antibodies induced by IL-12p35 vaccines could block the biological functions of IL-35 *in vitro*.

In summary, we have demonstrated that IL-23p19 peptide-based vaccine is capable of downregulating inflammatory responses in TNBS-induced chronic murine colitis, suggesting that IL-23p19 vaccine strategy may be useful for the long-term treatment of Crohn's disease.

Part Five. Discussion, summary, significance, and future directions

For the past five years, my research has been primarily focused on the development and evaluation of cytokine peptide-based vaccines for downregulating intestinal autoimmune and allergic inflammatory responses, specifically on the vaccines against IL-12 and IL-23 in experimental murine colitis.

Crohn's disease is a chronic remitting and relapsing inflammatory bowel disease. Its incidence and prevalence have markedly increased over the second half of the 20th century.³ The pathogenesis of Crohn's disease is associated with genetic susceptibility of the host, intestinal microbiota, environmental factors, and immunological abnormalities.^{12, 13, 92} It has been widely accepted that Crohn's disease is caused by an overly aggressive Th1 immune response, and excessive IL-23/Th17 pathway activation to bacterial antigens in genetically predisposed individuals.^{12, 37, 92-94} Both Th1 cytokines and the IL-23/Th17 pathway are critical for the development of chronic intestinal inflammation of Crohn's disease.^{93, 202, 203} Because IL-12 and IL-23 play important roles in the differentiation of Th1 and Th17 cells, respectively, and share the p40 subunit, the p40 unit has been selected as a therapeutic target for Crohn's disease. Monoclonal antibodies against IL-12/IL-23p40 have been shown to be effective in murine colitis and in patients with active Crohn's disease.²⁰⁸ As Crohn's disease is chronic, requiring long-term treatment, while the monoclonal antibodies currently used have a short half-life, requiring continuous administrations to maintain their effects. In the present study, we developed vaccines against IL-12/IL-23p40, IL-23p19 and IL-12p35 which could induce relatively long-lasting antibodies and evaluated the effects of the vaccines in murine colitis.

Three IL-12/IL-23p40 peptide-based vaccines C, D and F were successfully developed, which could induce specific antibodies against IL-12, IL-23 and p40. The antibodies induced by these three vaccines could block the biological functions of IL-12 and IL-23 *in vitro* (Figure 6). Vaccine F showed the best effects in the induction of antibodies and in the amelioration of murine colitis. Our results showed that vaccine F immunization improved body weight loss, ameliorated intestinal inflammation and fibrosis, and down-regulated the expression levels of cytokines (IL-12, IL-23, p40, and IL-17, etc.) in the colon tissue in the preventive study of TNBS-induced acute and chronic colitis (Figures 10-16). More importantly, vaccine F immunization also significantly ameliorated ongoing chronic intestinal inflammation and fibrosis in contrast to saline and carrier groups, including improving body weight loss, lessening intestinal inflammation and collagen deposition, and decreasing cytokine productions in the colon tissue (Figures 18-25).

Our present study indicated that IL-12/IL-23p40 vaccine treatment could ameliorate fibrosis observed in the prevention and treatment of chronic colitis. This inhibition may be through the down-regulation of p40, TGF β 1 and IL-17 levels. Studies have shown that IL-12/IL-23p40 is overproduced during the establishment of an experimental fibrotic process.^{310, 311} And the profibrotic function of IL-12/IL-23p40 in experimental pulmonary fibrosis, may be possible through exacerbating macrophage recruitment.³¹² TGF β 1 involved in most of the processes of wound repair, is identified as a major profibrotic factor. IL-17, an important cytokine produced by Th17 cells, also shows the profibrotic function in the hepatitis B-related liver fibrosis, myocardial fibrosis and cystic fibrosis.³¹³⁻³¹⁵ In the present study, vaccine treatment decreased the expression

of IL-12/IL-23p40, IL-17 and TGF β 1 in colon tissue (Figure 16), and, therefore, ameliorated intestinal fibrosis.

Our results indicate that IL-12/IL-23p40 vaccine-mediated improvement may be obtained through re-balancing Th1/Th17/Treg responses. Studies have indicated that in Crohn's disease Th1 and Th17 responses are over-activated, and Treg responses are down-regulated, leading to an impaired Th1/Treg and Th17/Treg responses.⁹⁷ Studies have also indicated that IL-12 and IL-23 play important roles in the differentiation of Th1 and Th17 cells; IL-23 has been found to restrain the activity of Treg to drive T cell-dependent colitis,²⁹⁰ and the homodimer of p40 has been found to suppress the expression of CD25 and Foxp3.²⁹¹ Therefore, targeting IL-12/IL-23p40 may rebalance the impaired Th1/Th17/Treg cell responses. Our results showed that the ratios of Treg to Th1 and Treg to Th17 were decreased in saline and carrier groups when compared with naïve mice, indicating that the imbalance of Th1/Th17/Treg cell responses occurred in murine colitis. This is supported by the findings of Eastaff-Leung *et al*, in which a significant decrease of Treg/Th17 ratio is observed in the PBMC of IBD patients.¹¹⁶ However, vaccine treatment increased the ratios of Treg to Th1 and Treg to Th17 in contrast to those in saline and carrier groups (Figure 26), suggesting that IL-12/IL-23p40 vaccine-mediated improvement may be induced by the re-balance of Th1/Th17/Treg cell responses.

Vaccine treatment suppressed the infiltration of DCs and macrophages into the colon tissue, but increased the production of IL-10 from DCs. Studies have shown that p80 can act as a macrophage chemoattractant, and p40 and p80 can act as an inducer of DC migration.^{157, 158} Therefore, blocking p40 may negatively influence the functions of macrophages and DCs. This is further supported by a decrease in the total numbers of

LPMC and the numbers of macrophages and DCs in the LPMC in vaccine-treated mice (Figure 28). It suggested that vaccine treatment inhibited the infiltration of macrophages and DCs into the inflamed colon tissue partially via blocking the functions of IL-12/IL-23p40 or p80. In the present study, IL-10 production was increased in the CD11c⁺ cells by vaccine treatment. IL-10 has been shown to be beneficial on the incidence of colitis induced by CD45RB^{hi} T cells³¹⁶ and on Tregs to maintain Foxp3 expression.²⁸⁹ IL-10 also constrains Th17 cells development in the IBD patients through controlling IL-1 production from DC,³¹⁷ and dampening Th1 cell activity in TNBS-induced colitis model via indirectly enhancing the activity of TGFβ.³¹⁶ The main sources of IL-10 production are Treg, CD11b⁺ and CD11c⁺ myeloid cells in colitis.^{288, 289} Therefore, the promotion of IL-10 production by CD11c⁺ cells in vaccine-treated mice may be another mechanism involved in vaccine-mediated amelioration.

A potential concern with cytokine vaccines is that the level of the target cytokine may be reduced below normal baseline, thus impeding the normal functions of the cytokine, as some cytokines play important roles in immune defense against certain infections. First, as seen in our previous and present studies, the target cytokine levels are only downregulated to a level that is lower than that under the disease condition but still above the normal level. Second, vaccine-induced antibodies are capable of neutralizing the excessive target cytokine within the extracellular compartment in the pathologic tissues, thus inhibiting the cytokine reaction;^{241, 274} While in normal tissues, vaccine-induced antibodies may not be able to reach the homeostatically well-regulated cytokine process occurring within the immunological synapse.^{241, 274} Third, circulating natural anti-cytokine antibodies have been found in the sera of healthy individuals at low levels,

such as IL-6, IL-1, and IFN- γ .²⁴¹ And in patients treated with cytokines, the levels of specific antibodies against cytokine are increased slightly.²⁴¹ For instance, the levels of anti-IFN- α are increased in patients receiving IFN- α treatment. But the presence of autoantibodies is not associated with any notable effect.³¹⁸ Fourth, the antibody levels that persist for about 4 months are reversible and can be adjusted by the frequency of immunization as the immunogenicity of cytokine vaccines is much less than that of microbe vaccines.²⁷⁴ Therefore, the vaccine strategy may be safe when used with careful monitoring. Since Th1 and Th17 cells play important roles in the host defense against *Chlamydia muridarum* infection,^{253, 297, 299} we explored whether administration of IL-12/IL-23p40 vaccine would increase the severity of *Chlamydia muridarum* infection. Our results showed that immunization with IL-12/IL-23p40 vaccine did not exaggerate body weight loss, increase *Chlamydia muridarum* burden in the lung tissue, and worsen lung inflammation, in contrast to saline and carrier groups (Figure 29). It indicated that immunization with IL-12/IL-23p40 vaccine didn't increase the susceptibility to *Chlamydia muridarum* infections.

In our experience, the cytokine peptide-based vaccine has some unique characters. The antibodies induced by this kind of peptide-based vaccines don't always block the biological functions of the target cytokine; sometimes they enhance the biological functions of target cytokines. We previously developed an IL-10 peptide-based vaccine which induced high levels of antibodies against IL-10.³¹⁹ The antibodies induced by this vaccine enhanced, not blocked, the biological functions of IL-10 both *in vivo* and *in vitro*.³¹⁹ I also developed an IL-17 peptide-based vaccine which could induce specific anti-IL-17 antibodies. But the anti-IL-17 antibodies induced by this vaccine could

significantly promote IL-17 mediated TNF production from macrophages and IL-17 mediated IL-6 production from NIH 3T3 cell lines *in vitro*. Mice immunized with this IL-17 peptide-based vaccine exaggerated TNBS-induced chronic colitis (manuscript in preparation).

Second, the vaccines developed from different peptides of the same cytokine may have different effects. Our present results have shown that IL-12/IL-23p40 peptide-based vaccine F induced higher levels of specific antibodies against IL-12, IL-23 and p40, and immunization with vaccine F resulted in more improvement in ameliorating TNBS-induced intestinal inflammation than vaccines C and D. I also developed two IL-18 peptide-based vaccines A and D (manuscript in preparation). Vaccine A induced high titers of specific anti-IL-18 antibodies which had a strong suppressive function in IL-18-induced IFN- γ production from spleen cells, when compared with vaccine D. However, in the *in vivo* evaluation, vaccine D, not vaccine A, ameliorated TNBS-induced acute and chronic intestinal inflammation significantly; while vaccine A-immunized mice showed more severe intestinal inflammation when compared with saline, carrier and vaccine D groups. Five out of six mice in vaccine A group died during the first two TNBS injections. IL-18 has been found to play an important role in the maintenance of the colonic epithelial barrier and also in the pathogenesis of Crohn's disease, so our results might indicate that vaccine A immunization worsened TNBS-induced acute and chronic colitis through impairing the function of IL-18 in the maintenance of epithelial barrier; vaccine D ameliorated TNBS-induced acute and chronic colitis via neutralizing IL-18 function in promoting IFN- γ production (manuscript in preparation).

In summary, the significance of the present study is as follows: 1) For the first time, three IL-12/IL-23p40 peptide-based vaccines were successfully developed, which induce specific antibodies against IL-12, IL-23 and p40. The antibodies induced by these vaccines can partially block the biological functions of IL-12 and IL-23 *in vitro*. 2) *in vivo* evaluation of the vaccine in both acute and chronic TNBS-induced colitis demonstrates that immunization of IL-12/IL-23p40 peptide-based vaccine (F) significantly ameliorates murine colitis when the vaccine is given before the commencement of the colitis. 3) More importantly, in TNBS- and DSS-induced chronic colitis, immunization of the p40 vaccine also significantly ameliorates colitis when the vaccine is given after the colitis has been established. 4) IL-12/IL-23p40 vaccine-mediated amelioration of murine colitis is through re-balancing Th1/Th17/Treg responses, promoting the apoptosis of activated Th1/Th17 cells and enhancing the production of IL-10 from DC cells. 5) Immunization of IL-12/IL-23p40 vaccine does not increase Chlamydia infection. 6) IL-23p19 peptide-based vaccine induces IL-23-specific antibodies which inhibit IL-23-induced bioactivity *in vitro*. Immunization of this vaccine ameliorates TNBS-induced murine chronic colitis. 7) IL-12p35 vaccines elicit specific antibodies to IL-12 which failed to inhibit IL-12-induced bioactivity *in vitro*.

Immunization of these vaccines can't improve the symptoms of colitis.

In the future, we will modify the IL-12/IL-23p40 peptide-based vaccines and IL-23p19 peptide-based vaccines to enhance their functions, via inserting two or more peptides into 78-79 amino acid of carrier HBcAg or to the N-terminal/C-terminal of HBcAg. We will also continue to develop effective IL-12p35 peptide-based vaccines which can strongly suppress the biological function of IL-12.

More recently, one study demonstrated the imbalance of NKp44(+)/NKp46(+) NK cells in the intestinal mucosa of patients of Crohn's disease, of which NKp46(+) NK cells might exaggerate the intestinal inflammation of Crohn's disease through secreting IFN- γ .³²⁰ As IL-12 can activate NK cells to produce IFN- γ , we will evaluate the effects of vaccine immunization on the NK cells in murine colitis.

We will also evaluate whether a raise in the levels of IL-12/IL-23p40 by other stimuli (such as infections, tumor) will elicit the production of anti-IL-12/IL-23p40 antibodies after IL-12/IL-23p40 vaccine immunization. It's important for the safety of the vaccines. Therefore, we will use other mouse models of infections or tumors to evaluate the safety of the vaccines.

Overall, the present study offers an innovative inhibitory approach for the long-term treatment of IBD. This vaccine strategy may also be used in the treatment of other autoimmune diseases where IL-12/IL-23 is dysfunctional such as in multiple sclerosis. In addition, this strategy may also serve as a research tool in our understanding of the pathogenesis of IBD, as it provides a relative persistent blockade of a pathogenic cytokine rather than the temporary blockage using mAbs or the permanent deletion using the gene knockout method.

Part Six. Literature cited

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