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Haitao Xiao

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Therapeutic Effects and the Underlying Mechanisms of Qing-Dai Powder against Experimental Colitis in Mice

Haitao XIAO

A thesis submitted in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

Principal Supervisor: Prof. Zhao-xiang BIAN
Hong Kong Baptist University
March 2015
DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of PhD at Hong Kong Baptist University, and has not been previously included in a thesis, dissertation submitted to this or other institution for a degree, diploma or other qualification.

Signature....................................

Date: March 2015
ABSTRACT

Ulcerative colitis (UC), a subset of inflammatory bowel disease (IBD), is a chronic uncontrolled inflammatory condition of the intestinal mucosa. As its etiology remains unclear, no specific effective treatment is available. Therefore, development of novel strategies for IBD treatment remains a major medical need. Qing-dai Powder (QDP), an ancient herbal medicinal formula, exerted potent therapeutic effect on intractable UC patients; however, evidence-based support is needed. The aims of this study are: i) to delineate the anti-colitis effect of QDP and its underlying mechanisms in murine colitis; 2) to explore the rationality of QDP formula; 3) to investigate the anti-colitis effects of major component(s) or/and active ingredient(s) of QDP and their underlying mechanisms in murine colitis.

In the present study, the therapeutic effect of QDP on UC was investigated on dextran sulfate sodium (DSS)-induced acute murine colitis. Results showed that i) QDP dose-dependently attenuated disease activity index (DAI), colon shortening, histological damage and colonic myeloperoxidase (MPO) activity of DSS-treated mice; ii) QDP significantly decreased the infiltration of immune cells, particularly macrophages and CD4+ T cells, colonic levels of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, and plasma level of chemokine MCP-1. In RAW 264.7 cells, QDP significantly suppressed lipopolysaccharide (LPS)-induced the production of TNF-α and IL-6, and the expression levels of COX-2 and iNOS via inhibiting IкB-α degradation and p65 nuclear translocation; Also, in primary CD4+ T cells, QDP significantly suppressed the differentiation of Th1 and Th17 cells. These findings indicate that the anti-colitis effects of QDP might be associated with inhibition of inflammatory responses of colonic macrophages and CD4+ T cells.
QDP is composed of Qing-dai and Ku-fan. The comparative study of anti-colitis of QDP, Qing-dai and Ku-fan revealed that QDP is a reasonable TCM formula, and Qing-dai is mainly responsible for the anti-colitis effect of QDP and Ku-fan exhibits a weak beneficial effect. Mechanistically, it was found that Qing-dai significantly suppressed Th1 and Th17 responses, characterized as i) suppressing mRNA expression of Th1 cytokine IFN-γ and Th17 cytokine IL-17A, inhibiting the production of Th1 and Th17-related cytokines IFN-γ, IL-17A/F and TNF-α in the colon of DSS-treated mice; ii) restraining the proportions of Th1 and Th17 cells in mesenteric lymph nodes of DSS-treated mice; iii) suppressing the differentiation of Th1 and Th17 cells in vitro.

Indirubin is the principle active component of Qing-dai. It was found that indirubin significantly suppressed the generation of Th17 cells in DSS-treated mice, evidenced by i) suppressing the mRNA expression of IFN-γ, IL-17A, and RORγt, and inhibiting the production of IL-17A/F, TNF-α, IL-1β and IL-6 in the colon of DSS-treated mice; ii) reducing Th17 cells in mesenteric lymph nodes of DSS-treated mice through reducing GSK-3β activity and p-STAT3 expression; iii) suppressing the differentiation of Th17 cells through down-regulating the expression of GSK-3β and p-STAT3 in vitro.

In summary, the present study provides evidence-based support for the clinical use of QDP in the management of UC, and indicates that indirubin is the main active compound of QDP responsible for its anti-colitis effect.
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LIST OF ABBREVIATIONS

ASA  aminosalicylate
ATG16L1 autophagy-related protein 16-1
APCs antigen presenting cells
AZA azathioprine
AICAR amino-imidazole carboxamide transformylase
ATP adenosine triphosphate
AHR aryl hydrocarbon receptor
APS ammonium persulfate
BSA bovine serum albumin
CD crohn’s disease
COX cyclooxygenase
CDKs cyclin-dependent kinases
CMC-Na sodium carboxymethylcellulose
CAI clinical activity index
CARD15 caspase recruitment domain-containing protein 15
DCs dendritic cells
DSS dextran sulfate sodium
DMSO dimethylsulphoxide
DEPC diethyl pyrocarbonate
DTT dithiothreitol
<table>
<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>DAI</td>
<td>disease activity index</td>
</tr>
<tr>
<td>EMSAs</td>
<td>electrophoretic mobility shift assays</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<td>Foxp3</td>
<td>forkhead box P3</td>
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<td>FPGS</td>
<td>folyglutamate synthetase</td>
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<td>5-ASA</td>
<td>5-aminosalicylic acid</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>GWAS</td>
<td>genome-wide association studies</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>glycogen synthase kinase-3 beta</td>
</tr>
<tr>
<td>HGPRT</td>
<td>hypoxanthine guanine phosphoribosyl-transferase</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>IRGM</td>
<td>immunity-related GTPase family M protein</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-gamma</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1beta</td>
</tr>
<tr>
<td>IL-2</td>
<td>interleukin-2</td>
</tr>
<tr>
<td>IL-4</td>
<td>interleukin-4</td>
</tr>
<tr>
<td>IL-5</td>
<td>interleukin-5</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>IL-10</td>
<td>interleukin-10</td>
</tr>
<tr>
<td>IL-17</td>
<td>interleukin-17</td>
</tr>
<tr>
<td>IL-23</td>
<td>interleukin-23</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>JAKs</td>
<td>janus kinases</td>
</tr>
<tr>
<td>LP</td>
<td>lamina propria</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LiCl</td>
<td>lithium chloride</td>
</tr>
<tr>
<td>6-MP</td>
<td>6-mercaptopurine</td>
</tr>
<tr>
<td>6-TIMP</td>
<td>6-thioguanine nucleotides</td>
</tr>
<tr>
<td>6-MMP</td>
<td>6-thiouric acid</td>
</tr>
<tr>
<td>6-TGNs</td>
<td>6-thiouric acid</td>
</tr>
<tr>
<td>MDP</td>
<td>muramyl dipeptide</td>
</tr>
<tr>
<td>MTX</td>
<td>methotrexate</td>
</tr>
<tr>
<td>MTXglu</td>
<td>methotrexate polyglutamate</td>
</tr>
<tr>
<td>MTHFR</td>
<td>methylenetetrahydrofolate reductase</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>MLN</td>
<td>mesenteric lymph nodes</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>NOD2</td>
<td>nucleotide-binding oligomerization domain containing 2</td>
</tr>
<tr>
<td>NKT</td>
<td>natural killer T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-kappa B</td>
</tr>
<tr>
<td>PBMCs</td>
<td>peripheral blood mononucleated cells</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>peroxisome proliferator-activated receptor-gamma</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>QDP</td>
<td>qing-dai power</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative real-time PCR</td>
</tr>
<tr>
<td>ROR-γt</td>
<td>retinoid-acid receptor-related orphan receptor gamma t</td>
</tr>
<tr>
<td>RFC1</td>
<td>folate carrier-1</td>
</tr>
<tr>
<td>RI</td>
<td>recombinant inbred</td>
</tr>
<tr>
<td>STAT1</td>
<td>signal transducer and activator of transcription 1</td>
</tr>
<tr>
<td>STAT3</td>
<td>signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>STAT4</td>
<td>signal transducer and activator of transcription 4</td>
</tr>
<tr>
<td>SASP</td>
<td>sulfasalazine</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
</tbody>
</table>
Tregs  T regulatory cells
Th1  T-helper 1
Th2  T-helper 2
Th17  T-helper 17
TNF-α  tumor necrosis factor-α
TREM-1  triggering receptor expressed on myeloid cells 1
TNBS  trinitrobenzene sulphonic acid
TGF-β  transforming growth factor-β
TPMT  thiopurine S-methyltransferase
TCR  T cell receptor
TCM  traditional Chinese medicine
TEMED  N, N, N', N'-tetramethylethylenediamine
TBST  tris Buffered Saline with Tween 20
TIC  total ion MS chromatogram
UC  ulcerative colitis
UPLC-QTOF-MS  ultra-performance liquid chromatography quadrupole time of flight mass spectrometry
XO  xanthine oxidase
CHAPTER 1

INTRODUCTION
1.1 Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) is an uncontrolled chronic inflammatory intestinal disorder characterized by colonic tissue edema, increased colonic epithelial permeability, and extensive infiltration of leukocytes in the colon. Ulcerative colitis (UC) and Crohn’s disease (CD) are two major subsets of IBD; they have essential similarities, but sometimes it was difficult to distinguish from each other. Generally, UC is characterized by a diffuse mucosal inflammation occurring in the rectum and the lower part of the colon as it rarely affects the entire colon. In contrast, CD is characterized by a patchy transmural inflammation, which often affects the deeper layers of the intestinal wall; it can upset any part of gastrointestinal (GI) tract (Yun et al., 2009). IBD represents a major public health and social problem as it not only affects working ability, social life and quality of life of the sufferers, but also brings a huge economic burden to the community (Nurmi et al., 2013). As the etiology and mechanism of IBD remain unclear and no specific treatment is available, this disease usually becomes chronic with refractory relapses, thus seriously endangering health of patients. Current medical therapies of IBD are mainly in inducing remission and in preventing relapse. Therefore, to develop effective therapeutic strategies still remain a considerable unmet need for IBD therapy. In the following subsections, the incidence, pathogenesis, animal study models and treatment of IBD will be summarized in detail to provide the current understanding of IBD.

1.1.1 Incidence

IBD is a common disease. Statistically, in 2006, the incidence rate of IBD in the world was between 0.6 to 40.5 in 100,000 individuals with a rate of 0.5 to 24.5 in 100, 000 individuals for UC and 0.1 to 16 in 100, 000 individuals for CD (Lakatos, 2006). Traditionally, it considered that IBD was highly prevalent in Europe and North America, but less common in Africa, South America and Asia including China. However, recent epidemiologic studies showed that the prevalence of IBD in Asian countries is progressively increasing year after year. For instance, during 1989 to
2007 in mainland of China, the number of hospitalized UC and CD patients has increased 5-fold in the last five years compared with that of the first five years (Wang et al., 2010). In Hong Kong, the incidence of UC in 2006 showed a six-fold increase compared to that of 1985 (Chow et al., 2009). Such increases probably resulted from the changes of environmental factors and lifestyle due to the rapid shift of socioeconomic statuses and industrialization in the Asian regions (Lin et al., 2013).

1.1.2 Pathogenesis

Although there has substantial investigations on IBD during the past several decades, the pathogenesis of IBD is still not completely understood. It is widely recognized that environmental triggers, genetic factors, microbial exposure and immunoregulatory defects contribute to the pathogenesis of IBD (Hanauer, 2006). Here, we will discuss each of these factors and their roles in the pathogenesis of IBD.

1.1.2.1 Environmental triggers

It is generally accepted that the high prevalence of IBD is found in developed nations while the prevalence of IBD in developing countries is usually low. Some recent epidemiological data showed that the incidence of IBD has been steadily rising in a variety of developing countries such as India and China, as they have become more industrialized (Desai and Gupte, 2005; Zheng et al., 2005). Furthermore, some studies among migrants also demonstrated that the risk of developing IBD increases in individuals when they relocated from areas with low incident rates to areas with high incident rates. This observation particularly appears in first-generation children (Bernstein and Shanahan, 2008; Mikhailov and Furner, 2009). The associations between environmental risk factors such as smoking,
medications, stress, diet, as well as infections and the development of IBD have been reviewed by Molodecky and colleague (Molodecky and Kaplan, 2010) and are discussed in detail.

1.1.2.1.1 Smoking

The data from a meta-analysis study has shown that smoking may increase the severity of CD, but reduce the severity of UC (Molodecky and Kaplan, 2010). The reasons behind these differential effects of smoking may be attributed to the chemicals such as nicotine and carbon monoxide in the tobacco. It has been reported that nicotine attenuates the severity of UC, but not CD, through accelerating mucin synthesis because the mucus layer in the left colon and rectum of UC patients is thinner than that of healthy people, whereas it is significantly thicker in CD patients (Birrenbach and Bocker, 2004; Pullan, 1996). In addition, it appears that nicotine has anti-inflammatory properties because it has been reported that decreased pro-inflammatory cytokines namely IL-1β and IL-8 were observed in the mucosa of UC smokers (Sher et al., 1999). However, such anti-inflammatory effects were not observed in CD patients because smoking could increase the abnormalities occur in the microvasculature of GI tract under a CD condition (Danese, 2007). It is commonly acknowledged that smoking increases the concentration of carbon monoxide in plasma and results in the vasodilation of chronically inflamed microvessels, ischemia, ulceration and fibrosis. Furthermore, smoking might be also associated with the increase of thrombotic potential to damage vessels in active CD patients (Hatoum et al., 2003).

1.1.2.1.2 Non-steroidal anti-inflammatory drugs (NSAIDs)

The use of NSAIDs could induce GI mucosal inflammation and aggravate the state of illness in IBD patients, implying that medications such as NSAIDs is one of
environmental factors to cause IBD (Cabre and Domenech, 2012). Data from prospective trials of NSAIDs such as acetaminophen, diclofenac, and indomethacin in IBD patients with remission demonstrated that one-third of patients had increased levels of fecal calprotectin and symptomatic flares after treatment (Ananthakrishnan, 2013). Furthermore, frequent treatment with high doses of NSAIDs over prolonged periods increased the incidences of CD and UC (Ananthakrishnan et al., 2012). These results showed that the consumption of NSAIDs is closely associated with the frequent and early clinical relapse of quiescent IBD. Subsequently, intensive investigations suggested that the detrimental effects of NSAIDs might result from the inhibition of cyclooxygenases (COX). There are two isoforms of COX in mammalian cells: COX-1 is referred to as a “constitutive isoform” expressed in most tissues whilst COX-2 is primarily present at sites of inflammation responsible for synthesizing prostaglandins (Zidar et al., 2009). In the inflamed colons, COX expressions are up-regulated for the purpose of mucosal repair; however, inhibition of both COX-1 and COX-2 might result in an exacerbation of colonic injury and an impairment of the mucosal repair processes (Guslandi, 2006). Whether selective COX-2 inhibitors are safer than conventional NSAIDs for IBD patients is still debatable, and further randomized and double-blind trials are needed to resolve this issue.

1.1.2.1.3 Dietary factors

Dietary substance has been considered as an important environmental trigger of IBD because many components in food could cause inflammation (Cabre and Domenech, 2012). In Japan, it has been reported that dietary substance such as sugars/sweeteners, sweets, fat and oil, and total fat are high-risk factors to induce CD, and similar correlation was also observed in North America (Yamamoto et al., 2007; Yamamoto et al., 2010). In addition, a Canadian study found that over-consumption of fats including total fats, monounsaturated and saturated fats increased the risk of CD
while over-consumption of carbohydrates decreased such risk (Amre et al., 2007). Similarly, over-consumption of monounsaturated and polyunsaturated fats also increased the risk of UC, but results are sometimes inconsistent (Geerling et al., 2000; Lee et al., 2004). It has been reported that oral administration of saturated and unsaturated acids produced opposite effects since the saturated acids in diet can trigger inflammation whereas the unsaturated acids can ameliorate inflammation (Lee et al., 2004). Due to different effects of dietary substance on the incidence and clinical course of IBD, prospective studies are necessary to further dissect what dietary substance influence the development of IBD.

1.1.2.1.4 Cytomegalovirus infection

Intestinal cytomegalovirus infection is an environmental factor to induce IBD. In the study of Cottone and his colleague, the examination of rectal biopsies of cytomegalovirus among 62 IBD patients with severe intestinal inflammation, 19 of those (30%) were resistant to intravenous steroids and bowel rest, indicating that cytomegalovirus infection might be a common factor contributing to severe refractory colitis (Cottone et al., 2001). Domènech et al. also investigated the role of cytomegalovirus in UC patients with steroid-refractoriness. They collected biopsies from 114 subjects with different conditions of UC and medication. The subjects included those with active UC taking intravenous steroids, UC accompanying steroid-refractoriness, inactive UC taking mesalamine, inactive UC taking azathioprine and healthy controls. The investigation found that the cytomegalovirus disease was highly occurred in UC patients with steroid-refractoriness (Domènech et al., 2008).

1.1.2.1.5 Stress

More than 50% of IBD patients believe that stress is a major triggering factor in
inducing relapse of their diseases (Moser et al., 1995). Previous reports showed that the placebo response rate was as high as 30-40% in therapeutic trials of CD and UC patients, and was not only correlated to objective measures such as endoscopic assessment of mucosal inflammation but also to subjective self-reported symptomatology (Feagan et al., 1996), indicating that psychological state plays an important role in the degree of disease activity (Mawdsley and Rampton, 2006). In addition, studies also well-documented that depression, anxiety and stress are positively associated with the increase of relapse and surgery for IBD patients (Ananthakrishnan, 2015). Similarly, evidence from animal models demonstrated that chronic psychological stress exacerbates IBD through promoting damage of intestinal mucosa since stress can alter immune function (Levenstein et al., 2000; Mawdsley and Rampton, 2006; Molodecky and Kaplan, 2010).

1.1.2.1.6 Others

Several other environmental factors have also been proposed to increase the risk of IBD. It has been reported that ambient air pollution was associated with increased rate of hospitalizations for both CD and UC patients. This could be due to elevated atmospheric particulate matter in air pollution which would result in the increase of circulating polymorphonuclear leukocytes and plasma cytokines such as TNF-α which are known to impair vascular function (Ananthakrishnan, 2015). Vitamin D deficiency is also an environmental factor increasing the risk of IBD. Evidence from human study shows that lower vitamin D was associated with an increase of disease activity and a lower health-related quality of life in IBD patients especially CD patients, as well in murine colitis models (Cantorna et al., 2000; Ulitsky et al., 2011). In addition, the environmental factor Helicobacter pylori infection was negatively associated with IBD development, whereas factors such as family size, birth order, urban environment, and history of appendectomy increased the risk of developing IBD (Molodecky and Kaplan, 2010).
1.1.2.2 Genetic factors

Specific racial backgrounds, high rate of co-morbidity in monozygotic twins and the family gathering phenomena exist in the IBD patients suggest that genetic factors are deeply involved in the pathogenesis of IBD (Zhang and Li, 2014). With technological advances in DNA analysis and sequencing, and the success of genome-wide association studies (GWAS) in IBD, a line of key genes responsible for the susceptibility of IBD have been identified. The most susceptible genes include nucleotide-binding oligomerization domain containing 2 (NOD2)/caspase recruitment domain containing protein 15 (CARD15), autophagy-related protein 16-1 (ATG16L1), immunity-related GTPase family M protein (IRGM) and IL-23 (Van Limbergen et al., 2014).

NOD2 is primarily found in antigen-presenting cells. It encodes the intracellular receptor that recognizes the muramyl dipeptide (MDP), which is a conserved motif of peptidoglycan present in both Gram-positive and Gram-negative bacteria (Ogura et al., 2001a). It has been reported that exposure to MDP leads to the conformational change of NOD2 that promotes the oligomerization of NOD2 through binding to kinase RIP2 with homotypic CARD-CARD interactions (Ogura et al., 2001b). Subsequently, the NOD2-RIP2 complex activates RIP2 kinase and initiates a large number of signal transduction pathways. Specifically, RIP2 activation promotes NF-κB and MAPK activation, resulting in the expression of pro-inflammatory cytokines, chemokines, microbial factors, and induction of adaptive responses (Abbott et al., 2007; Philpott et al., 2014). In addition, NOD2 also regulates histocompatibility complex (MHC) cross presentation, autophagy induction, and resistance to intracellular bacterial infection (Cooney et al., 2010; Homer et al., 2010; Wagner and Cresswell, 2012).

Genetic analyses have also revealed that autophagy-related genes ATG16L1 and IRGM are significantly altered in IBD conditions (Zhang and Li, 2014). It has been
reported that a T300A mutation in ATG16L1 is an essential component for autophagy because the degradation of T300A variant would result in diminishing autophagy (Murthy et al., 2014). Meanwhile, it has also been reported that CD patients who are homozygous for the ATG16L1-T300A risk allele show impaired clearance of pathosymbionts in ileal inflammation (Sadaghian Sadabad et al., 2014). In addition, hypomorphic mutation of ATG16 in mice showed markedly abnormal paneth cell morphology, and subsequently developed a CD-like phenotype in the presence of murine norovirus, an intact gut flora and an environmental stressor such as DSS to disrupt the epithelial barrier (Parkes, 2012). IRGM, located on chromosome 5q33.1, which belongs to the p47 immunity-related GTPase family was also found to be involved in autophagy (RiouxF et al., 2007; Zhang and Li, 2014). It has been reported that reduction of IRGM expression by small interfering RNA impairs both measles virus (MeV), Hepatitis C virus (HCV) and human immunodeficiency virus-1 (HIV-1)-induced autophagy and viral particle production. This indicates that IRGM is an important target of RNA viruses-induced autophagy (Grégoire et al., 2011). In addition, IRGM deficient mice are more susceptible to infections of M. tuberculosis and its macrophages present reduced phagocytic ability and the mice demonstrated IRGM also plays an important role in the elimination of pathogens upon inflammation (Kim et al., 2012).

IL-23R gene has also been identified to be closely associated with the pathogenesis of IBD. It is well known that IL-23R gene encodes IL-23 receptor, which participates in the differentiation of Th17 cells, and activation of IL-23/Th17 pathway is involved in the pathogenesis of IBD. Except for IL-23R, other genes such as IL-12B, JAK2, and STAT3 have also been identified as regulators of the IL-23/Th17 signaling pathway in both UC and CD conditions (Zhang and Li, 2014).
1.1.2.3 Microbial dysbiosis

Dysbiosis can facilitate the growth of enteric pathogens in the gut, and thus is closely associated with the development of IBD (De Hertogh et al., 2012; Samanta et al., 2012). A number of studies revealed that the population and species of gut flora in colons of IBD animals and patients differed significantly from their healthy counterparts (Lupp et al., 2007). Such a difference not only reflected at a significant reduction in the biodiversity and stability of beneficial faecal microbes, but also at an altered quantity in some phyla of bacteria related to IBD (Andoh et al., 2011; Joossens et al., 2011). It has been well documented that a decrease of Bacteroidetes and an increase of Proteobacteria in the colon are closely associated with colonic inflammation in IBD patients (Lupp et al., 2007). In addition, Bacteroides fragilis and Faecalibacterium prausnitzii, the two major beneficial gut microbes in normal human subjects, were found significantly reduced in IBD patients (Sokol et al., 2009; Takaishi et al., 2008). Further, some studies showed that deficiency of F. prausnitzii appears to be a cause of ileal CD, because, F. prausnitzii can generate butyrate from unabsorbed fiber to attenuate IBD (Friswell et al., 2010; Qiu et al., 2013). Recently, the success of therapies correcting dysbiosis, including fecal microbiota transplantation and probiotics ingestion, also confirmed that microbial dysbiosis does play an important role in the development of IBD. Fecal microbiota transplantation is a therapy in which fecal material of a healthy donor is introduced into the gut of IBD patients (Matsuoka and Kanai, 2015). A randomized study to compare the efficacy of fecal microbiota transplantation and antibiotics in treatment of recurrent Clostridium difficile infection showed that the resolution of C. difficile-associated diarrhea was 81% in the fecal microbiota transplantation group but only 31% in the antibiotics group (van Nood et al., 2013). Similarly, results from a phase I trial involving 10 mild-to-moderate pediatric UC patients showed fecal microbiota transplantation had a high rate of clinical response (79%) within 1 week without serious adverse events (Kunde et al., 2013). Probiotics are live organisms such as bacteria and yeast those mainly live in the intestines are also believed to
enhance human health. For instance, VSL3 is a freeze-dried probiotic mixture containing eight different lactic acid bacteria namely *Lactobacillus acidophilus*, *L. bulgaricus*, *L. casei*, *L. plantarum*, *Streptococcus thermophilus*, *Bifidobacterium breve*, *B. infantis*, and *B. longum*. Double-blinded placebo-controlled trials showed that treatment of VSL3 alone or combined with conventional therapy were more efficacious in inducing remission of UC patients than that of the placebo group (Matsuoka and Kanai, 2015).

**1.1.2.4 Immune responses**

The immune system has two parts-“innate” and “adaptive”. The innate immune system is the first line of defense; it comprises monocytes/macrophages, dendritic cells (DCs), neutrophils, eosinophils, basophils, and natural killer T (NKT) cells. These immune cells work together to provide an immediate protective response against infections. For instance, these cells initiate phagocytosis; they also initiate the adaptive immune response (Murphy et al., 2012). The adaptive immune system is mainly composed of lymphocytes (T and B cells), which are highly specific and able to confer longlasting immunity (memory) (Murphy et al., 2012; Zhang and Li, 2014). It is commonly considered that macrophages and DCs are mainly responsible for the innate immune responses while CD4+ T cells, including Th1, Th2, Th17 and T regulatory (Treg) cells are mainly responsible for the adaptive immune responses (Davies and Abreu, 2015; Zanello et al., 2014). Available evidence suggest that the dysfunction of both innate and adaptive immune systems contributes to the aberrant intestinal inflammatory responses in IBD patients (Zhang and Li, 2014).

**1.1.2.4.1 Macrophages and dendritic cells**

As mentioned above, macrophages and DCs are the essential effector cells of the innate immune system. In the GI tract, macrophages and DCs residing in the lamina
propria (LP) are the sentinels and first responders to pathogens and other biological contaminants. Evidence from various experimental models of mucosal inflammation resembling human IBD have shown that the dysfunction of intestinal macrophages and DCs is the key element in the pathogenesis of IBD. In human IBD, excessive accumulation of macrophages are found in the inflammatory lesions, and these macrophages display enhanced expression of co-stimulatory molecules such as CD80 and CD86, and macrophage-activating receptors including CD40, TLRs, TREM-1 and CD14 (Steinbach and Plevy, 2014). Besides, enhanced expression of mature DC markers such as CD83, S-100 and CD40 have also been observed in IBD patients (Hart et al., 2005; Steinbach and Plevy, 2014). Moreover, macrophages isolated from IBD patients are capable of producing more pro-inflammatory cytokines, for instance, IL-12 and IL-23, in response to bacterial challenge, but with a lesser content of anti-inflammatory cytokines such as IL-10. DCs isolated from UC patients have also been found to produce more pro-inflammatory cytokines including IL-12, IL-6, IL-8 and TNF-α compared to those isolated from healthy controls (Steinbach and Plevy, 2014). These findings demonstrate that the phenotypic patterns of macrophages and DCs are altered in intestinal inflammation. It has been reported that, in mouse models of colitis, selective depletion of subsets of macrophages and DCs have different effects in the development of IBD. Studies of Ghia and Niess report that the general depletion of macrophages or DCs significantly inhibits the development of colitis in mice with DSS-induced colitis (Ghia et al., 2008; Niess, 2008). Contrary to these findings, Qualls et al. reported that depletion of macrophages and DCs in LP before the induction of DSS colitis actually exacerbates the severity of IBD (Qualls et al., 2006; Qualls et al., 2009). In addition, it has also been reported that polarization of macrophages into a M2 phenotype or increase of CD103+ DCs in LP can alleviate the severity of DSS-induced colitis (Steinbach and Plevy, 2014). These controversial results imply that the different subsets of macrophages and DCs play diverging roles in the development of IBD and varied the degree of severity of colitis in the animals. Thus, defining the functions and phenotypes of macrophages and DCs in LP of IBD sufferers may be an aid in the
development of novel strategies for IBD treatment.

1.1.2.4.2 Th1 and Th2 cells

Naïve T-cells (Th0) cells can be differentiated into Th1, Th2, or other subsets of T cells after being activated. Th1 cells are characterized by an increased production of IL-2, IFN-γ and TNF-α whilst Th2 cells are characterized by an increased production of IL-4, IL-5 and IL-13. It is generally accepted that CD is mediated by Th1 cell response whereas UC is mediated by Th2 cell response. This concept has been well presented in the parallel colitis models induced by trinitrobenzene sulphonic acid (TNBS) and by oxazolone. The former produces a dense transmural inflammation as seen in CD with a Th1 response, while the latter produces a relatively superficial inflammation similar to UC with a Th2 response.

The notion of Th1 mediates an inflammatory response in CD began in the early 1990s based on the finding that T cells isolated from inflamed tissues produced high amount of Th1 cytokine IFN-γ while the amount of Th2 cytokine IL-4 was less than basal level, and DCs isolated from LP of CD patients produced much more IL-12, which accelerated the differentiation of naïve T-cells into Th1 cells (Fuss, 2008). Furthermore, electrophoretic mobility shift assays (EMSAs) showed that T cells from CD patients, but not UC patients, had elevated expression of Th1 cytokine IFN-γ and transcription factor STAT4 (Neurath et al., 2002b). In addition, administration of anti-IL-12(p40 chain) antibody or other Th1-inhibiting factors inhibited the development of colitis in mice with TNBS-induced colitis (Fuss, 2008). Collectively, it is generally accepted that CD is a Th1-mediated mucosal inflammation. Conversely, UC is considered as a Th2-mediated mucosal inflammation. Previous findings have demonstrated that, when compared to CD patients, UC patients produced increased amounts of Th2 cytokines IL-4, IL-5 and IL-13, administration of anti-IL-4 antibody significantly ameliorated the severity of
1.1.2.4.3 Th17 cells

Th17 cells were firstly identified in patients with cutaneous inflammation in 1998 (Teunissen et al., 1998). Similar to Th1 and Th2, Th17 cells are also a subset of effector CD4+ T cells, characterized by the expression of the transcription factor retinoid-acid receptor-related orphan receptor gamma t (RORγt), and the selective production of IL-17 cytokines, particularly IL-17A and IL-17F. However, they also produce a variety of additional cytokines including IL-9, IL-21, IL-22, IL-26, and CCL20 (Fouser et al., 2008).

Th17 cells are mainly localized in mucosal tissues such as gut, skin, lung and the female reproductive tract. Functionally, Th17 cells help the host defend against pathogens, such as bacteria and fungi (Blaschitz and Raffatellu, 2010). Upon activation, they secrete a variety of cytokines to facilitate neutrophil mobilization, induce pro-inflammatory mediators from other cells, and promote the expression of antimicrobial peptides such as regenerating islet-derived protein 3-gamma (Reg3γ) (Galvez, 2014). Th17 cells are involved in a number of inflammatory diseases and autoimmune responses, such as rheumatoid arthritis, IBD, asthma, multiple sclerosis, and psoriasis (Bedoya et al., 2013). A series of clinical studies have demonstrated that Th17 cells play an important role in the pathogenesis of IBD. Fujino et al. compared the accumulation of Th17 cells between IBD patients and healthy controls, and found that Th17 cells were profoundly increased in the inflamed gut of IBD patients (Fujino et al., 2003). In line with this, several studies have also shown that RNA transcripts of Th17-type cytokines were upregulated in the inflamed mucosa of IBD patients (Holtta et al., 2008; Kobayashi et al., 2008; Nielsen et al., 2003; Seiderer et al., 2008). In addition, the study in UC patients also showed that the levels of IL-17 secreted by peripheral blood mononucleated cells (PBMCs)
positively correlated to the severity of UC patients (Raza and Shata, 2012). Besides IL-17, other Th17 cytokines such as IL-22, IL-26 and IL-21, were also increased in the inflamed gut of IBD patients, which in turn exacerbated the inflammation by promoting Th1 and Th17 responses (Bogaert et al., 2010). Moreover, GWAS and candidate gene studies showed that Th17-related genes, such as signal transducer and activator of transcription 3 (STAT3) or IL-23R, are associated with the pathogenesis of IBD (Thompson and Lees, 2011).

To verify the results from clinics, a succession of studies in various animal models of IBD were conducted. It has been reported that transgenic mice ubiquitously expressing IL-23p19 spontaneously developed chronic enterocolitis (Wiekowski et al., 2001); administration of IL-23 exacerbated the colonic injury of IL-10-deficient mice (Yen et al., 2006). Also, SCID mice that received IL-17A-producing Th17 cells from C3Bir mice spontaneously developed severe colitis (Elson et al., 2007). In addition, it was also reported that IL-17F-deficient mice showed less severe IBD symptoms than wild-type animals receiving DSS- or TNBS treatment (Ito et al., 2008; Zhang et al., 2006). These results showed that Th17-type cytokines play pathogenic roles in intestinal inflammation. Conversely, some studies also reported that Th17-type cytokines exert some tissue-protective effects in the gut. In mice with DSS-induced colitis, neutralization of IL-17A, either by antibody treatment or by genetic ablation, actually aggravated the intestine epithelial barrier dysfunction, increased permeability to luminal agents and worsened the intestinal inflammation (Garrido-Mesa et al., 2011; Kinugasa et al., 2000; Yang et al., 2008). In addition, some studies showed that IL-22-deficient mice developed severe intestinal barrier dysfunction when compared to wild-type mice (Sugimoto et al., 2008; Zenewicz et al., 2008). These results suggest that IL-17A and IL-22 might facilitate intestinal barrier integrity. Collectively, Th17 cytokines have differential effects on the pathogenesis of IBD, and most probably, the overall effect of these cytokines depends on their relative abundance.
As Th17 cells have been suggested to play crucial roles in the development of intestinal inflammation, targeting Th17 cells has been proposed as a feasible therapeutic strategy for treating intestinal inflammatory conditions. Accordingly, blocking the differentiation of Th17 cells, neutralizing Th17-type cytokines and inhibiting specific transcription factors required for Th17 cell function are considered as effective ways to suppress Th17-mediated inflammation. It has been reported that monoclonal antibodies for neutralizing IL-12/23p40 are effectively to treat murine colitis (Becker et al., 2006; Yen et al., 2006). Recently, ustekinumab, the human monoclonal antibody that targets IL-12 and IL-23, has been also shown to have potent therapeutic effects in moderate-to-severe CD patients, particularly in the patients unresponsive to anti-TNF therapy (Sandborn et al., 2012). On the other hand, RORγt and STAT3, the specific transcription factors required for Th17 cell polarization and function, are also considered as plausible pharmacological targets. For instance, pioglitazone, a peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist, effectively inhibiting human and murine Th17 differentiation via reducing RORγt expression, can use to treat IBD (Klotz et al., 2009). Similarly, halofuginone, a coccidiostat used in veterinary medicine, significantly inhibits the differentiation of Th17 cells through decreasing STAT3 expression and exhibits potential to reduce the severity of IBD (Carlson et al., 2014). Although there is still much to be learned about the role of Th17 cells during intestinal inflammation, targeting Th17 cells is already showing promising results in the treatment of IBD.

### 1.1.2.4.4 Treg cells

It is well known that the dysfunction of the adaptive immune system in the pathogenesis of IBD is associated with excessive activation of effector T cells or impaired function of regulatory T cells (Tregs). Thus, it is reasonable to speculate that Tregs are also the key players in development of IBD. Different to Th17 cells, there is still no final conclusion on the change in the number of Tregs in peripheral
blood and mucosa of IBD patients, but some studies revealed that there are significant changes in the function of Tregs in IBD patients. For instance, Hovhannisyan et al found that a population of Treg cells with high expression level of Foxp3+ and high capability of producing IL-17 produce large amounts of IFN-γ and other inflammatory cytokines in the colonic mucosa of CD patients. Moreover, this specific population of Treg cells shared similar phenotypic characteristics with both Th17 and regular Treg cells, and possessed potent suppressor activity in vitro (Hovhannisyan et al., 2011). Kryczek et al also reported that CD4+ Foxp3+ IL-17+ Tregs from the colonic mucosa of UC patients could produce proinflammatory cytokines and suppress effector T cell function (Kryczek et al., 2011). This specific phenotype of Treg cells in IBD patients might be accounted for the inflammatory milieu promoting the conversion of Tregs into effector T cells.

Although the understanding of the role of Tregs in the pathogenesis of human IBD is limited, data from murine models of colitis have been well documented the important roles of Tregs in maintaining intestinal homeostasis. One of murine models to demonstrate the role of Tregs was the T cell transfer model of colitis. In this model, transfer of CD45RBhi naïve T cells in immunodeficient mice results in colitis, whilst concomitant transfer of CD45RBlo T cells prevents the development of colitis. These different effects may be attributed to CD45RBlo T cells is a fraction of Tregs and transfer of these cells inhibits the progression of colitis (Mottet et al., 2003; Read et al., 2000).

Since Tregs play a requisite role in control of intestinal inflammation, to target Tregs was also explored as a strategy to treat IBD. It has been reported that treatment with aminosalicylates or glucocorticoids increased levels of CD4+CD45R0+CD25+ T cells in the peripheral blood of UC patients (Takahashi et al., 2006). As well, treatment with infliximab (an anti-TNF-α antibody) also elevates the numbers of Tregs in peripheral blood and intestinal LP of IBD patients (Li et al., 2010; Ricciardelli et al., 2008). However, these treatments have broad effects and incur increased risk for
infection.

1.1.3 Animal models

In order to understand the abovementioned etiological factors, a number of animal models resembling the human intestinal inflammation have been developed for scientific research. In general, these animal models can be categorized as spontaneous models, chemically induced models, adoptively transferred models and genetically modified models. Although the above models cannot completely represent the complexity of IBD in humans nor can they substitute for clinical studies, they provide reproducible in vivo systems for us to study topics that are otherwise impractical, such as analysis of pathophysiological mechanisms and efficacy tests of new drugs/therapeutic candidates. In the following subsections, the characteristics of different models are discussed in detail.

1.1.3.1 Spontaneous models

1.1.3.1.1 C3H/HeJ Bir mouse model

C3H/HeJ Bir mice are a subtype of C3H/HeJ mice, developed by the Jackson Laboratory, Maine, USA. It has been reported that C3H/HeJ Bir mice spontaneously develop acute and chronic types of lesions and ulcerations in the cecum and on the right side of the proximal colon mucosa at about 3 weeks of age. Subsequent studies have demonstrated that immune responses initiated by the CD4+ T cells to antigens of the intestinal flora, rather than food antigens, are the important factors in the development of colitis in these mice (Wirtz and Neurath, 2000). Mahler and his colleagues have compared genes susceptible to DSS-induced colitis in different inbred mouse strains, and found that C3H/HeJ Bir and its parent strain are highly DSS-susceptible (Mahler et al., 1998).
1.1.3.1.2 CC011/Unc mouse model

CC011/Unc is a strain generated from a murine recombinant inbred (RI) panel. These mice display chronic proliferative colitis with pathologic features resembling human IBD symptoms, such as dramatic epithelial hyperplasia associated with shedding of goblet cells, winding of glandular structures, ulceration and mucosal nodularity. Colitis in CC011/Unc mice is a good spontaneous model to emulate human UC. This model is origin of genetic susceptibility to colonic inflammation and infection with pathogens (Rogala et al., 2014).

1.1.3.1.3 Cotton-top tamarin model

The cotton-top tamarin (Saguinus oedipus) is an endangered New World primate living in the northern Colombian forests. It has been reported that idiopathic ulcerative colitis frequently occurs among cotton-top tamarins, and its features are very similar to the clinical appearance and histopathological changes of human UC. Further studies revealed that environmental factors are important components in the pathogenesis of cotton-top tamarin colitis; meanwhile genetic factors synergistically contribute to the occurrence of colitis in these animals. As the evolutionary origin of the cotton-top tamarin is highly similar to humans, cotton-top tamarin colitis has been widely used for the study of immunopathogenesis, risk factors and therapies of IBD (Wirtz and Neurath, 2000).

1.1.3.2 Chemical-induced models

1.1.3.2.1 DSS-induced colitis

DSS-induced colitis model was firstly established by Okayasu et al to study the role of leukocytes in the pathogenesis of acute colitis (Wirtz and Neurath, 2000). This colitis model is established by feeding mice with 1-5% DSS drinking water for
several days, which resulted in extensive mucosal inflammation similar to human UC. Symptoms of DSS-induced colitis include body weight loss, diarrhea, bleeding, ulceration and mucosal damage (Solomon et al., 2010). Administration of DSS drinking water induces both acute and chronic colitis in the animals. For chronic colitis, multiple cycles of DSS treatment is needed. The degree of severity of DSS-induced colitis varies according to several factors: DSS concentration (generally 2-5% DSS for acute colitis and multiple cycles of 1-5% DSS for chronic colitis), duration (usually 3-9 days for acute colitis, 2-3 cycles with 5-7 days for each cycle for chronic colitis), the molecular weight, manufacturer and batch of DSS, animal strains (Balb/c mice are highly susceptible), gender of the animals (hormonal effects are often lower in male mice), and the environment of husbandry (Low et al., 2013). Previous studies have demonstrated that DSS does not induce intestinal inflammation directly; rather, it is toxic to the gut epithelial cells of the basal crypts. With the destruction of the mucosal barrier, luminal antigens are allowed to enter the lamina propria and submucosal compartment, thereby triggering inflammation (Wirtz et al., 2007). DSS-induced acute colitis leads to a progressive disruption of colonic crypts, macrophages and CD4+ T cells prominent in the inflammatory areas at the basal portions of the lamina propria. These CD4+ T cells produce increased IFN-γ and IL-4, which suggests DSS-induced colitis is a mixture of responses from Th1 and Th2 cells (Dieleman et al., 1998). Recently, Th17 cells are also found to play a pathogenic role in the development of DSS-induced colitis (Singh et al., 2013). DSS-induced colitis is very simple and reproducible, and commonly used for studying the pathogenesis of UC, as well as screening of potential therapeutic interventions.

1.1.3.2.2 TNBS-induced colitis

TNBS-induced colitis was reported by Neurath et al in 1995. In this model, the hapten reagent of TNBS is dissolved in ethanolic solution and administered to the
animals by colocolysis. This approach is applicable to mice, rats and rabbits. For a successful induction, the use of ethanol is necessary as it disrupts the epithelial layer and exposes the lamina propria and submucosal compartment to luminal bacteria and other antigens. TNBS-induced colitis is characterized by a transmural granulomatous inflammation associated with diarrhea, rectal prolapse, weight loss, and colonic wall thickening, which are similar to the features of CD in humans (Wirtz and Neurath, 2000). In addition, many histopathological changes in TNBS-induced colitis including elevated colonic levels of TNF-α and IL-12 are similar to those observed in CD patients (Qiu et al., 1999). Studies have demonstrated that the chronic stage of TNBS-induced colitis is related to the activation of the mucosal immune system and increase of lymphocyte infiltration, especially CD4+ T cells in the lamina propria (Neurath et al., 1995). It is worth noting that the genetic background of animals and the microflora present in the husbandry facilities can dramatically affect the severity of TNBS-induced colitis (Solomon et al., 2010). Bouma G reported that the susceptibility of TNBS-induced colitis greatly varies in different mouse strains; SJL and BALB/c mice are most susceptible whereas C57Bl/6 and C57Bl/10 mice are more resistant. The susceptibility of TNBS-induced colitis in SJL/J mice has been shown to be related to the high IL-12 response after LPS stimulation (Qiu et al., 1999). Recently, te Velde and colleagues revealed that a limited number of genes, about 21, are either up- or down-regulated in the TNBS colitis model versus 387 genes in the DSS-induced colitis and 582 genes in the CD45RBhigh transfer mouse model. Among the well-known genes for changing transcriptional activity in IBD, two genes named Mmp14 and Timp1 are up-regulated in TNBS-induced colitis (te Velde et al., 2007). TNBS-induced colitis is regarded as a Th1-mediated immune response and is widely used for the study of many aspects of intestinal inflammation, particularly patterns of cytokine secretion and cell adhesion.
1.1.3.2.3 Oxazolone-induced colitis

Like TNBS-induced colitis, oxazolone-induced colitis is also a hapten-based colitis model, which is associated with a Th2-polarized response giving rise to a sharply increased production of IL-4 and IL-5 (Low et al., 2013). This model is induced by administering an ethanol enema of oxazolone. The induction results in severe colitis with dramatic body weight loss, severe diarrhea and loss of goblet cells. Oxazolone-induced colitis is only limited to the distal colon and manifests as relatively superficial ulceration. It has been reported that this model incurs a high mortality rate as half of treated mice died due to the wasting syndrome (Wirtz et al., 2007). According to the literature, oxazolone-induced colitis has often been performed on SJL/J and C57Bl/6 mice. It is worth noting that, for inducing the oxazolone colitis in C57Bl/6 mice, a presensitizing treatment is required since this strain is resistant to haptenating agents (Kawada et al., 2007; Low et al., 2013). For presensitization, subcutaneous injection of oxazolone can be chosen (Kawada et al., 2007). Given its strong resemblance to the histological features and inflammatory distribution of human UC, oxazolone-induced colitis had also been widely utilized for pathological and therapeutic analyses of UC.

1.1.3.2.4 Acetic acid-induced colitis

Acetic acid is an alternative reagent used to induce acute colitis in addition to DSS/TNBS/Oxazolone. This model is inexpensive, repeatable, and often executed in rats. Acetic acid-induced colitis is a diffuse colitis, characterized by serious ulceration and crypt abnormalities of the distal colon (Low et al., 2013). Sometimes, ulceration may extend from the mucosa to the lamina propria. The severity of acetic acid-induced colitis mainly correlates to the dose and the time of exposure to acetic acid. In this model, typically, 4% acetic acid is used as enteroclysis and a 15-30-second exposure of acetic acid to the rectum is chosen. It is worth noting that the concentration of the acetic acid enema should not be too high because high
concentration easily causes intestinal perforations (Low et al., 2013; Wirtz et al., 2007). As acetic acid-induced colitis manifests mucosal injury, increased permeability and acute mucosal/transmural inflammation, it is a very valuable model for the study of early events of inflammation after mucosal injury and the processes of wound healing (Wirtz et al., 2007).

1.1.3.3 CD45RB<sup>High</sup> transfer model

Since T cells play important roles in the pathogenesis of chronic intestinal inflammation, the CD45RB<sup>High</sup> transfer model is usually regarded as an ideal model to study the immunological mechanisms associated with induction, perpetuation, and/or regulation of colonic inflammation (Ostanin et al., 2009). This model is established by transferring CD4<sup>+</sup>CD45RB<sup>high</sup> T cells from healthy wild-type mice to the recipient mice, such as SCID or RAG1/2<sup>−/−</sup> mice that lack T and B cells. This induces a wasting syndrome with intestinal inflammation (Wirtz and Neurath, 2007). The phenomenon of intestinal inflammation generally starts at 5–10 weeks after the adoptive transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells, and the microflora of animal husbandry facilities is a dependent variable (Ostanin et al., 2009; Wirtz and Neurath, 2007). Histopathologically, CD45RB<sup>High</sup> transfer model is characterized by a transmural inflammation associated with epithelial cell hyperplasia, leukocyte infiltration, crypt abscesses, and loss of epithelial cells. The clinical manifestations such as weight loss, diarrhea and fecal bleeding in this model vary, and greatly depend on the mouse strains of the donors and recipients. This model is commonly used to investigate the role of Tregs in inhibiting the onset and/or perpetuation of intestinal and colonic inflammation.

1.1.3.4 Transgenic mouse models
1.1.3.4.1 IL-10 knockout mouse model

Interleukin-10 (IL-10) is an anti-inflammatory cytokine with multiple effects, which mainly functions to restrict and finally terminate immune responses (Rennick and Fort, 2000). Mice deficient in IL-10 spontaneously develop intestinal inflammation, which is characterized by transmural lesions in the small and large intestines at 2–4 months of age (Ostanin et al., 2009; Wirtz and Neurath, 2007). Histopathological study of changes in the colons of IL-10 knockout mice show many characteristics similar to those of the IBD patients, such as infiltration of lymphocytes, activation of macrophages and presence of neutrophils (Wirtz and Neurath, 2000). It has been reported that the generation of IFN-γ-producing Th1 cells plays a crucial role in the pathogenesis of enterocolitis in those mutants, and can be reversed by administration of antibodies of IL-12 or recombinant IL-10 (Ostanin et al., 2009; Wirtz and Neurath, 2007). Further, some studies have shown that enteric organisms play decisive roles in triggering the development of colitis as no improvement in colitic parameters or immune responses in the gut were found in IL-10 deficient mice bred germ-free. Additionally, evidence from B cell-deficient IL-10 knockout mice showed that colitis is not dependent on B cells, and highlighted that T cell-derived IL-10 plays a key role in the regulation of mucosal T cell responses (Ostanin et al., 2009; Rennick and Fort, 2000; Wirtz and Neurath, 2007). IL-10 deficient model is a well-established Th1-mediated transmural colitis, which is widely used to screen potential therapeutic interventions. However, the onset and severity of disease varies, and the duration for disease development might require several months in some cases.

1.1.3.4.2 TCR-α knockout mouse model

T cell receptor (TCR) is a molecule found on the surface of T cells; it is responsible for recognizing antigens bound to MHC molecules (Goldrath and Bevan, 1999). It has been reported that mice deficient in T cell receptor alpha gene (TCR-α−/−) spontaneously develop chronic colitis, usually starting at 6-8 weeks of age. Generally,
about 60% of mice develop chronic colitis by 16-20 weeks of age (Bhan et al., 2000). Like IL-10 knockout mice, colitis in TCR mutant mice also shares features with human UC, in that aberrant number of Th2-type CD4+ TCRαβ+ T cells are found and they are the main source of IL-4. Administration of IL-4 antibodies to TCR-α−/− mice and TCR-α/IL-4 double knockout mice significantly ameliorate the intestinal inflammation (Wirtz and Neurath, 2000). Beside T cells, further studies have shown that B cells may exert important regulatory action in the pathogenesis of this model because B cell-deficient TCR-α−/− mice develop a more severe colitis. For germ-free TCR-α−/− mice, enteric bacteria appears to be the key factor in their colitis because these knockout mice did not develop colitis and because appendectomy at an early age delayed the onset of colitis (Bhan et al., 2000; Wirtz and Neurath, 2000, 2007). In contrast with IL-10 knockout mice, the TCR-α knockout mice model is typically associated with Th2-mediated immune response and a useful tool for studying the pathogenesis of human IBD.

1.1.4 Current therapies

With current understanding of the pathogenesis of IBS and intensive drug testing, dozens of therapeutic approaches have been developed. Currently, the drugs used in clinical practice are aminosalicylates, corticosteroids, immunomodulatory agents, chimeric monoclonal antibodies, and antibiotics. These drugs reduce symptoms but often provide little beneficial therapeutic effects, and many have unwanted side-effects. Moreover, they may also have a low-tolerance to patients in long-term treatments (Curkovic et al., 2013; McLean and Cross, 2014; Talley et al., 2011). Given the high propensity for chemical drugs to have adverse effects, more and more IBD patients also seek help from traditional medicines especially Traditional Chinese Medicine (TCM). In the following subsections, different classes of the therapies for IBD are discussed in detail.
1.1.4.1 Aminosalicylates

The family of aminosalicylate is the first drug used to treat UC in clinics, which was discovered by Nanna Svartz. In the early twentieth century, Nanna Svartz combined sulfonamides, a kind of antibacterial drug, with salicylate, a common type of anti-inflammatory drug, for treatment of rheumatoid arthritis. She found that the combined product, sulfasalazine (SASP), a derivative of 5-aminosalicylic acid (5-ASA) possessing a sulfapyridine moiety with an azo bond, does improve the course of rheumatoid arthritis (Talley et al., 2011). Subsequently, SASP was widely used in clinics to treat rheumatoid arthritis. In the early 1940s, Nanna Svartz noted that rheumatoid patients with coincidental UC unexpectedly exhibited a reduction in the severity of their bowel symptoms (Nielsen and Munck, 2007; Talley et al., 2011). This observation inspired her to explore whether SASPs are effective in UC treatment. Her subsequent studies revealed that SASP can be split by azoreductase, a species of colonic bacteria, and this splitting exposes the effective moiety of 5-ASA. This discovery led to the development of different aminosalicylates (sulfasalazine, olsalazine, balsalazide and mesalazine) and different formulations of 5-ASA (oral, rectal, pH-independent continuous release, pH-dependent release, multimatrix technology) for UC patients. However, the efficacy of aminosalicylates on CD remains uncertain. After reviewing relevant publications in the Cochrane database, Lim and Hanauer report that only SASP shows modest efficacy for the treatment of active CD while other aminosalicylates show little benefit for CD treatment (Lim and Hanauer, 2010). At present, the precise action mechanism of aminosalicylate remains unclear, but it is believed that 5-ASA acts locally on intestinal epithelial cells of the intestinal mucosa (Rousseaux et al., 2005). Their anti-inflammatory effects appear to target several intracellular pathways in regulating the intestinal inflammation, including the tumor necrosis factor-α/nuclear factor-κB (TNF-α/NF-κB), transforming growth factor-β (TGF-β), Wnt/β-catenin, peroxisome proliferator-activated receptor-γ (PPAR-γ), and epidermal growth factor (EGF) signaling pathways (Nielsen and Munck, 2007).
As 5-ASA is rapidly absorbed in the small intestine, only a small amount of 5-ASA can reach the colon. Thus, multiple daily dosages or many pills are required. This often results in low compliance for patients in long-term treatment. In order to maximize the concentrations of 5-ASA in the colon, pharmaceutical manufacturers have developed a series of 5-ASA preparations such as pills with pH-dependent release of 5-ASA, 5-ASA enteric-coated capsules, etc. Among the mainstream therapies of IBD, these drugs are relatively safe; nonetheless, serious adverse effects such as interstitial nephritis, pancreatitis, pneumonitis, pericarditis, and hepatitis have been observed (Talley et al., 2011). Compared to 5-ASA, SASP is better tolerated, but gives rise to more side effects. SASP incurs an adverse effect rate ranging from 10 to 45%, depending on its dosage (Escher et al., 2003). The most frequent adverse effects of SASP are relatively mild and include headache, nausea, epigastric pain or rash; the most serious adverse effects are including impairment of fertility in male and blood dyscrasias (Curkovic et al., 2013).

1.1.4.2 Glucocorticosteroids

Glucocorticosteroids are the standard therapy for both UC and CD as they result in rapid remission of disease activity (Ford et al., 2011). Due to their adverse effects, however, longstanding treatment with glucocorticosteroids is often avoided. Usually, they are used to treat patients with moderate-to-severe UC when aminosalicylates, such as SASP or mesalamine, are ineffective (Prantera and Marconi, 2013). Mechanistically, the effects of glucocorticosteroids are mainly associated with inhibition of recruitment and proliferation of lymphocytes and monocytes/macrophages, migration of neutrophils to the inflamed sites, and production of proinflammatory cytokines, such as IFN-γ, IL-4, and IL-6. Possibly, the inhibition of the NF-κB signaling pathway is responsible for their broad inhibitory functions (Katz, 2004). Accompanied with their efficiency in reducing
immunologic and inflammatory reactions, their side effects are aware of, and
depended on the dosage and the duration of treatment (Hanauer, 2002; Prantera and
Marconi, 2013). The milder side effects of glucocorticosteroids commonly include
increased appetite, weight gain, sudden mood swings, muscle weakness, and easy
bruising, while the severe side effects generally include hypertension, diabetes,
osteoporosis, as well as a detrimental effect on the hypothalamic-pituitary-adrenal
axis (Hanauer, 2002). Administration of glucocorticosteroids over a short period (i.e.,
1 month or less) can induce negative changes such as fluid and electrolyte
imbalances, acid-base disturbances, and metabolic abnormalities. Long-term
glucocorticosteroid therapy (i.e., over a period of years) mainly results in fat
redistribution, ecchymoses, and abdominal striae. In addition, long-term exposure to
glucocorticosteroids increases the risk of opportunistic infections due to their
suppressive effects on the immune system (Hanauer, 2002; Navarro and Hanauer,
2003).

1.1.4.3 Immunomodulatory agents

The representative drugs of this class are azathioprine (AZA) and methotrexate
(MTX).

1.1.4.3.1 Azathioprine (AZA)

AZA is a thiopurine and it is the pro-drug of 6-mercaptopurine (6-MP). In clinics,
about 10-20% of patients with moderate-to-severe UC easily became
steroid-dependent after taking steroids (e.g., glucocorticosteroids) for one year
(Taba Taba Vakili et al., 2012). AZA and 6-MP are the preferred drugs for treating
steroid-dependent or steroid-refractory UC patients, and they are usually used as
steroid sparing agents for long-term management of UC (Stocco et al., 2014; Taba
Taba Vakili et al., 2012). Routinely, AZA is orally administered at a daily dosage of
2.5 mg/kg; 16-50% of administered drug is absorbed in the gut. Subsequently, absorbed AZA is split into 6-MP and methyl-nitrothioimidazole through non-enzymatic degradation. 6-MP is an active metabolite, which is catalyzed into nucleotide 6-thiopurine monophosphate (6-TIMP) by hypoxanthine guanine phosphoribosyl-transferase (HGPRT). Next, 6-TIMP is further converted into active thioguanine mono-, di- and tri-phosphates [6-thioguanine nucleotides (6-TGNs)]. The active 6-TGNs are purine antagonists that interfere with DNA and RNA synthesis, thereby inhibiting the proliferation of T and B lymphocytes. Alternatively, 6-MP can be also converted to 6-thiouric acid (6-TU) by xanthine oxidase (XO) or 6-methylmercaptopurine (6-MMP) by thiopurine S-methyltransferase (TPMT), to undermine the potency of AZA through decreasing the amount of active 6-TGN (Fig. 1.1) (Stocco et al., 2014; Thomas and Lodhia, 2014).

![Fig. 1.1 Simplified scheme of AZA metabolism (Modified based on Pierik M, et al. (World J Gastroenterol. 2006, 12(23):3657-67).]

Not surprisingly, these agents are not free from adverse effects. The cytotoxic effects of AZA and 6-MP are mainly sourced from the incorporation of active 6-TGNs with DNA processing enzymes so that the de novo purine synthesis is inhibited. The major adverse events include leucopenia, pancreatitis, infection and malignancy.
whereas minor negative effects are rash, nausea, fever, arthralgias, malaise and diarrhea (Stocco et al., 2014; Taba Taba Vakili et al., 2012; Thomas and Lodhia, 2014).

1.1.4.3.2 Methotrexate (MTX)

MTX was firstly used as a therapeutic drug for rheumatoid arthritis; however, in randomized controlled trials of weaning CD patients from steroids, MTX helps induce remission in 39% of patients and maintains remission in 65% of CD patients. Thus, it is often considered as an alternative to azathioprine (AZA) (Pierik et al., 2006). In clinics, MTX is commonly administered intramuscularly or subcutaneously. Mechanically, MTX reduces the formation of folate carrier-1 (RFC1), and is subsequently converted into methotrexate polyglutamate (MTXglu) by folylglutamate synthetase (FPGS) (Cronstein, 2005). Then, MTXglu blocks purine synthesis via inhibiting the activity of amino-imidazole carboxamide (AICAR) transformylase. MTX also inhibits the enzyme 5, 10-methylenetetrahydrofolate reductase (MTHR) that catalyzes the conversion of homocystein to methionine (Fig. 1.2) (Stocco et al., 2014).
The dominant action mechanism of MTX is to inhibit the proliferation of T and B lymphocytes through suppressing the synthesis of MTHR and DNA; however, this inhibitory mechanism causes a lot of side effects such as bone marrow suppression, mucositis, gastro-intestinal toxicity and liver function abnormalities (Shea et al., 2014).

**Fig. 1.2** Inhibitory effects of methotrexate (MTX) on lymphocytes. (Modified based on Pierik M, et al. (World J Gastroenterol. 2006, 12(23):3657-67)). DHFA, dihydrofolate reductase; DHFA; 5,10-CH\textsubscript{2}-THF, 5,10-methenyl tetrahydrofolate; 5,10-CH\textsubscript{2}-THF, 5,10-methylene tetrahydrofolate; Met, methionine; Hcy, homocysteine.

1.1.4.4 Biologic therapy

As the understanding of pathogenetic pathways in IBD advances, 'key players' in the inflammatory pathways have been investigated as therapeutic targets, and several novel therapeutic agents for IBD have been developed. These include anti-TNF-α,
anti-integrin, and anti-adhesion molecule agents (Arora and Shen, 2014). Among these biologic therapeutic drugs, anti-TNF-α agents have been the most extensively studied. Three of these—namely, infliximab, adalimumab, and golimumab—have been approved by the U. S. Food and Drug Administration (FDA) for the treatment of UC patients. These agents mainly target the inhibition of TNF-α activity to reduce mucosal inflammation. The representative drug in this class is infliximab, the first biological agent approved by FDA for UC therapy in 1989 (Dassopoulos and Sninsky, 2012). Infliximab is administered via subcutaneous injection, and it is effective for UC patients at a dosage of 5 mg/kg. However, it is also effective for CD patients. Similar to other therapies, the exact action mechanism of infliximab is not fully known. By one account, infliximab blocks soluble TNF-α; by another account, it induces apoptosis of activated T lymphocytes and monocytes (Shen et al., 2005). Clinically, most IBD patients respond to infliximab treatment within 2-6 weeks of initiation. However, in one trial, 25% of IBD patients did not response to this treatment, while another 20-30% patients had an incomplete response (Stocco et al., 2014). Anti-integrin agents are another class of biologic therapeutic drugs, which are focused on inhibition of integrins to decrease the migration of leukocytes, thus decreasing the inflammatory response (Arora and Shen, 2014). Natalizumab was the first drug to be developed in this class, which was shown to be effective in the treatment of CD (Lowenberg and D'Haens, 2013). In addition, other biological agents such as the monoclonal antibody against MAdCAM-1 (PF-547659) and anti-adhesion molecule (Alicaforsen), are currently under development (Arora and Shen, 2014). Although the randomized, controlled trials have demonstrated a relatively safe profile compared to conventional drugs used for IBD treatment in clinics, a few patients do experience several significant side effects, including infection from tuberculosis and hepatitis B virus, increased risk of malignancies, acute and delayed infusion reactions, production of autoantibodies, and lupus-like syndrome (Arora and Shen, 2014; Sandborn and Hanauer, 2002).
1.1.4.5 Traditional herbal medicines (THM)

In contrast to conventional drugs which are synthetic pharmaceuticals, the ingredients of THM are natural, sourced from animal, vegetable and mineral materials. THM are perceived as being less toxic and safer than conventional drugs for long-term consumption. In recent years, an increasing number of IBD patients are trying THM. A study has shown that nearly 50% of patients with IBD have used some form of complementary or alternative medicine (CAM) during the course of their disease course (Bensoussan et al., 2006). Among CAM, vitamin supplements and THM are the most commonly used, accounting for up to 65% and 58% of CAM users, respectively (Hilsden et al., 2003; Ng et al., 2013).

The most noteworthy is that Traditional Chinese Medicine (TCM) is appreciated by IBD patients, as they have been used for centuries in the Chinese population, and their efficacy, safety and tolerability have already reached acceptable standards. For centuries, there are hundreds of time-tested formulas available for IBD, particularly for UC such as Si-Jun-Zi Tang, Sheng-Lin-Bai-Zhu San, Li-Zhong Tang, Zheng-Ren-Yang-Zang Tang, Bai-Tou-Weng Tang, Tong-Xie-Ya Fang, Bu-Zhong-Yi-Qi Tang, Yi-Gong Tang, and Ge-Gen-Qin-Lian Tang, etc (Zhang et al., 2013). The clinical studies about the successful use of these herbs are recorded in the many medical journals and books. Previously, two systematic reviews have concluded the efficacy and safety of TCM for IBD patients. Their results revealed that overwhelming majority of published clinical data of TCM for IBD treatment were quite rough, which were insufficient to prove their effectiveness due to demerits in design of methodology, restraint of control groups, sample size and quality assurance of herbs in clinical trials, etc (Ng et al., 2013; Zhang et al., 2013). Although the number of well-designed randomized controlled trials of TCM for the treatment of IBD is limited, the current data shows encouraging results that TCM (e.g., Andrographis paniculata extract, Xi-Lei San, and Artemisia absinthium ) are effective to treat UC or CD superior to the placebo in inducing remission (Ng et al.,
Larger controlled clinical studies are needed for evaluating the efficacy and safety of TCM for IBD treatment.

1.1.5 Treatment: Future prospects

IBD represents a chronic inflammation in gut. Until we find a cure, IBD patients are required to take drugs to control this condition for a long period of time, even a lifetime. Therefore, drugs with minimal systemic absorption, maximal drug levels in intestinal wall and good tolerance for use in a long-term are desired. Compared to existing small drug molecules, 5-aminosalicylates are relatively safer; however, their low bioavailability in the colon is still a big problem. Although the pharmaceutical manufacturers have developed a few preparations such as pills with pH-dependent release of 5-ASA and 5-ASA enteric-coated capsules to maximize the concentrations of 5-ASA in the colon, these preparations are expensive and impose a huge financial burden both on individuals and the society. Accordingly, the development of new 5-aminosalicylates preparations with low cost and high bioavailability in the colon is still needed. Recently, anti-TNF-α agents are used clinically as novel medications for UC patients. Although these agents are quite effective and reasonably safe, one-third of individuals still do not respond to them and they are quite expensive (Bernstein, 2015). Therefore, defining the phenotypic, genetic, or biomarker predictors of responders or non-responders would save money/reduce the cost. Meanwhile, CAMs such as HTM, especially TCM, are popular among IBD patients. Although these herbs have been used by humans for centuries, their uses are commonly based on their longstanding experience of Chinese people without evidence-based analysis. While numerous TCM have been proved effective for curing or relieving the symptoms of IBD, but there is no exact answer to the patients when they wonder how these herbs work and what substances in these herbs work. More clinical studies to verify the efficacy and safety of herbs and biochemical analyses to illuminate their active components and action mechanisms will not only provide answers to patients’
questions but will also illuminate important mechanisms for refining therapeutic effects, maximizing production, minimizing costs and overall helping people.

1.2 Qing-Dai Powder (QDP)

1.2.1 Introduction

Qing-Dai Powder (QDP) is an ancient herbal medicine formula, which was first recorded in "YI-FANG-LEI-JU (醫方類聚)" , a collection of classical medical formulas used during the Tang to Ming Dynasty of China, compiled by Jin Li-meng and his colleagues in 1443 (Cheng, 2006; Zhang and Liang, 2012, 2013). QDP comprises Qing-dai (also known as indigo naturalis) and Ku-fan (also known as dried alum). Traditionally, QDP was used to treat hematemesis (吐血) and hemorrhinia (衄血) (Cheng, 2006). Qing-dai, the principal ingredient of the formula QDP, originates from Baphicacanthus cusia (Nees) Bremek or Indigofera tinctoria L. or Isatis indigotica Fort or Polygonum tinctoria Ait or Strobilanthes formosanus Moore (Chiang et al., 2013). Qing-dai has been used for the purposes of heat-clearing and detoxifying the body, cooling the blood and dissolving ecchymoses (清热解毒，凉血消斑) (Chanayath et al., 2002). Ku-fan, the subordinate ingredient of the formula, is the mineral alum and used to eliminate phlegm, dry dampness, arrest diarrhea, stop bleeding, remove toxicity and kill parasites (消痰，燥湿，止泻，止血，解毒，杀虫) (Commission, 2010).

1.2.2 Chemical components

The QDP formula comprises Qing-dai and Ku-fan. Qing-dai contains 10% organic compounds and 90% inorganic compounds (Liu, 2013). Up to now, about 11 organic compounds have already been isolated and identified from Qing-dai. Their structures are shown in Table 1.1 (Li, 1987; Wang, 2004). The inorganic constituents of
Qing-dai are composed of 70% of calcium carbonate, 10% of silicon dioxide and trace amounts of iron carbonate and aluminum carbonate (Chen et al., 2005). Unlike Qing-dai, Ku-fan is a mineral; it comprises aluminium potassium sulfate (Chen et al., 1996).

**Table 1.1** Structures of compounds isolated from Qing-dai

<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular Formula</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indigo</td>
<td>C_{16}H_{10}N_2O_2</td>
<td><img src="image" alt="Indigo structure" /></td>
</tr>
<tr>
<td>Indirubin</td>
<td>C_{16}H_{10}N_2O_2</td>
<td><img src="image" alt="Indirubin structure" /></td>
</tr>
<tr>
<td>Indican</td>
<td>C_{14}H_{17}NO_6</td>
<td><img src="image" alt="Indican structure" /></td>
</tr>
<tr>
<td>Isatan B</td>
<td>C_{14}H_{13}NO_6</td>
<td><img src="image" alt="Isatan B structure" /></td>
</tr>
<tr>
<td>Tryptanthren</td>
<td>C_{15}H_{8}N_2O_2</td>
<td><img src="image" alt="Tryptanthren structure" /></td>
</tr>
</tbody>
</table>
Isatin $\text{C}_8\text{H}_5\text{NO}_2$

Isoindigo $\text{C}_{16}\text{H}_{10}\text{N}_2\text{O}_2$

Qingdainone $\text{C}_{23}\text{H}_{13}\text{N}_3\text{O}_2$

N-phenyl-2-napht hylamine $\text{C}_{16}\text{H}_{13}\text{N}$

1-Dotriacontanol $\text{C}_{32}\text{H}_{66}\text{O}$

n-nonacosane $\text{C}_{29}\text{H}_{60}$

Daucosterol $\text{C}_{35}\text{H}_{60}\text{O}_6$
1.2.3 Application for IBD

QDP and its modified formulas have been widely used to treat colitis in clinics (Du and Liu, 1998; Xie et al., 2013). In our research group, QDP has been prescribed to UC patients who failed to respond to treatment with 5-ASA, prednisolone or infliximab, with the normal dosage 7.5 g/day (packaged in capsules, twice a day for two weeks). The retrospective observational study showed that QDP profoundly improved clinical symptoms of UC patients with no obvious adverse side effects. Zou et al reported that oral administration of formula containing QDP significantly ameliorated the infiltration of inflammatory cells in TNBS-induced colitis in rats (Zou et al., 2006). Yuan et al reported that Qing-dai enemas exhibited significant clinical efficacy in the treatment of chronic hemorrhagic radiation proctitis (Yuan et al., 2009). Suzuki et al also reported that Qing-dai at 2 g/day for 4 months significantly decreased the clinical activity index (CAI) score and the endoscopic Matts grade of intractable UC patients (Suzuki et al., 2013). In addition, Gao et al. reported that oral administration of Ku-fan-dominated formula Hou-shi-hei Powder, with Ku fan as major component, significantly ameliorated the clinical symptoms of UC patients such as diarrhea and bleeding, and the total effective rate was up to 96.15% (Gao, 1993).
1.3 Indirubin

1.3.1 Introduction

Indirubin, a red-colored bisindole alkaloid, is a major active compound of Qing-dai. It is poorly soluble in water, slightly soluble in alcohol, but completely soluble in ethyl acetate, acetone, chloroform and diethyl ether. Nevertheless, it is poorly absorbed in the small intestine (Chen et al., 2012). Oral consumption of indirubin often results in unfavorable pharmacokinetic properties in animals and humans (Vougogiannopoulou and Skaltsounis, 2012). Indirubin can induce cell cycle arrest at the G1/S and/or G2/M checkpoints followed by apoptosis, and potently inhibit the proliferation of human tumor cells (Xiao et al., 2002). In China, indirubin tablets were successfully approved by the China State Food and Drug Administration (CFDA) in 2010 as a medicament to treat chronic myelogenous leukemia in clinics (http://app1.sfda.gov.cn/datasearch/face3/base.jsp).

1.3.2 Pharmacological activities

Indirubin exerts biological activities against cancer, neurodegeneration, and protozoan infections, and these effects are closely associated with its ability to inhibit protein kinases including cyclin-dependent kinases (CDKs) and glycogen synthase kinase-3 beta (GSK-3β).

1.3.2.1 Inhibition of CDKs

The inhibitory effect of indirubin on CDKs gives rise to anti-cancer activities. It is generally accepted that there are four phases in the cell cycle: the S-, M-, G1- and G2 phases. S-phase is a period for DNA replication; M-phase is the phase for mitosis in which the chromosomes are separated over two new nuclei. These two phases are separated by two so called “Gap” phases, G1 and G2, in which the cell prepares for
the upcoming events of S and M, respectively. During the division process, cyclins are synthesized and bind to CDKs to form a cyclin-CDK complex (Coqueret, 2002; Tenderenda, 2005). This complex usually acts as a signal to direct the cell to pass into the next cell cycle phase (Fig. 1.3). Different Cyclin-CDK complexes are involved in the regulation of different cell cycle transitions: Cyclin-D-CDK4/6 for G1 progression, Cyclin-E-CDK2 for the G1-S transition, Cyclin-A-CDK2 for S-phase progression, and Cyclin-A/B-CDK1 for entry into M-phase (Bindels et al., 2002). Many tumor cells have a deregulated cell cycle and lack appropriate checkpoint controls. Therefore, agents targeting the cell cycle arrests may be promising therapeutic interventions for tumors (Vougogiannopoulou and Skaltsounis, 2012). Accumulating data showed that indirubin is a promising inhibitor of cyclin-dependent kinases CDK1/cyclin B (IC$_{50}$ = 10 µM), CDK2/cyclin A (IC$_{50}$ = 2.2 µM), CDK2/cyclin E (IC$_{50}$ = 7.5 µM), CDK4/cyclin D1 (IC$_{50}$ = 12 µM) and CDK5/p35 (IC$_{50}$ = 5.5 µM) (Hoessel et al., 1999). The topology of the interaction between indirubin and CDKs shows that indirubin binds to the ATP binding sites of the CDKs with high affinity (Eisenbrand et al., 2004). Experimental studies confirmed that indirubin causes cell cycle arrest at G1/S or G2/M in a wide spectrum of human tumor cells, such as HBL-100, MCF-7, CCL-39, PC-12, L1210, K-562, and HL-60 (Eisenbrand et al., 2004).
1.3.2.2 Inhibition of GSK-3β

GSK-3 is a cytoplasmic serine/threonine protein kinase that mediates the addition of phosphate molecules onto the serine and threonine amino acid residues. It is involved in a number of signaling pathways important for cell proliferation, stem cell renewal, apoptosis and development. Malfunction of this kinase is known to be involved in the pathogenesis of a wide range of diseases in humans, such as nervous system disorders, diabetes, bone malformation, inflammation, cancer and heart failure (Takahashi-Yanaga, 2013) (Fig. 1.4). In mammals, GSK-3 has two isoforms, GSK-3α and GSK-3β.
GSK-3 is abundant in brain cells and neurons, and is involved in the abnormal phosphorylation of Tau protein in Alzheimer’s disease (AD). Aggregation of Tau is responsible for the formation of neurofibrillary tangles (NFTs) and β-amyloid deposition observed in AD (Boutajangout et al., 2011; Kramer et al., 2012). It has been reported that indirubin is a potent inhibitor of GSK-3β with IC$_{50} = 600$ nM (Leclerc et al., 2001). Interaction of indirubin or its derivatives with GSK-3β modulated multiple physiological pathways of neurodegenerative diseases. In vivo and in vitro studies have shown that indirubin and its derivatives have neuroprotective potential to reduce LPS-induced neuroinflammation, β-amyloid-associated neuropathology, tau phosphorylation and total tau levels in neurons, which were associated with GSK-3β inhibition (Eisenbrand et al., 2004; Vougogiannopoulou and Skaltsounis, 2012). Inhibition of GSK-3β could also affect the progression of Wnt. Wnt is a signal transduction pathway involved in embryonic development, stem cell fate in adults, neuronal development, and neuroprotection.
GSK-3β had been found to phosphorylate several components of the Wnt signaling pathway, such as β-catenin (Vougogiannopoulou and Skaltsounis, 2012). Several studies have shown that indirubin and its derivatives exerted multiple beneficial effects in the modulation of osteoporosis and obesity, including inhibition of adipocyte differentiation, inhibition of osteoblast differentiation, down-regulation of abdominal fat and net increment in trabecular bone density. Furthermore, inhibition of GSK-3β by indirubin and its derivatives was capable of modulating the expression of factors associated with hypoxia, ischemia and apoptosis in serum-deprived conditions through regulating phosphatidylinositol 3-kinase/Akt (PI3K/Akt) signaling pathways (Choi et al., 2014; Eisenbrand et al., 2004; Schnitzer et al., 2005; Vougogiannopoulou and Skaltsounis, 2012).

1.3.2.3 Others

Apart from the aforementioned functions, indirubin and its derivatives also target Aryl hydrocarbon receptor (AHR) (Adachi et al., 2001; Benson and Shepherd, 2011), Fms-like tyrosine kinase 3 (FLT3) (Choi et al., 2010), Janus kinases (JAKs) (Nam et al., 2013) and STAT3 (Blazevic et al., 2013; Nam et al., 2013) in regulating multiple pathological processes of different diseases. In addition, indirubin possessed potent antifungal activity in treatment of dermatophytosis (Ponnusamy et al., 2010).

1.4 Hypothesis

As the efficacy and relative safety of traditional Chinese medicines are commonly rationalized based on their longstanding use in majority of clinical cases without evidence-based analysis, their therapeutic potentials should be further validated in pre-clinical studies before being used in patients. QDP, an ancient herbal medicinal formula, exerted potential therapeutic effect in intractable UC patients in our TCM practice. However, the precise mechanisms behind its anti-colitis effect remain
unknown. According to the formulation of QDP, Qing-dai (indigo naturalis) is the major herbal component. Previous study showed that indirubin 3'-monoxime, the derivative of active compound indirubin isolated from Qing-dai, significantly inhibited LPS-induced macrophage activation (Kim and Park, 2012). Meanwhile, it is well known that indigubin, the major components of Qing-dai, is a potent GSK-3β inhibitor (Meijer et al., 2003). Studies showed that GSK-3β is a master regulator of TLR-mediated chronic intestinal inflammation, and treatment of GSK-3β inhibitors could significantly reduce TNBS-induced acute colitis in rats (Hofmann et al., 2010; Whittle et al., 2006). As well, GSK-3β is also an early determinant in the differentiation of pathogenic Th17 cells (Beurel et al., 2011).

Based on the above knowledge and clues, we hypothesized that:
1) QDP can promote the recovery of murine colitis, and the underlying mechanisms are associated with suppressing the activation of macrophages and differentiation of CD4+ T cells, in particular the Th17 cells;
2) The herb of Qing-dai (indigo naturalis) is mainly responsible for the anti-colitis effect of QDP;
3) Qing-dai can attenuate murine colitis by suppressing Th1 and Th17 inflammatory responses;
4) Indirubin, the major compound of Qing-dai can suppress the differentiation of Th17 cells through influencing the GSK-3β signaling pathway in murine colitis.

1.5 Objectives

The objectives of the current study are as follows:
1) To assess the anti-colitis effect of QDP and investigate its underlying mechanism in murine colitis;
2) To verify whether QDP is a reasonable TCM formula;
3) To investigate the anti-colitis effect of Qing-dai and its underlying mechanism in
murine colitis;

4) To investigate the anti-colitis effect of indirubin and its underlying mechanism in murine colitis.
CHAPTER 2

MATERIALS AND METHODS
2.1 Materials

2.1.1 Animals

Seven to 8-week-old male C57BL/6 mice weighing about 20–24 g were purchased from the Laboratory Animal Services Center, The Chinese University of Hong Kong. The animals were fed with a rodent standard diet with free access to water, and were kept in rooms maintained at 22 ± 1 °C with a 12 h light/dark cycle following international recommendations. All experimental protocols were approved by The Animal Ethics Committees of Hong Kong Baptist University, in accordance with the “Institutional Guidelines and Animal Ordinance” (Department of Health, Hong Kong Special Administrative Region).

2.1.2 RAW 246.7 cells

Mouse RAW 264.7 macrophage cells were purchased from American Type Culture Collection.

2.1.3 Reagents and assay kits

Reagents and assay kits used in the study are summarized and listed in Table 2.1

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Brand</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACK lysis buffer</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Ammonium persulfate (APS)</td>
<td>USB</td>
</tr>
<tr>
<td>Anti-biotin micro beads</td>
<td>Miltenyi</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>USB</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>Sigma</td>
</tr>
<tr>
<td>Collagenase D</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
CD4\(^+\) CD62L\(^+\) T cell isolation kit II
DSS (molecular weight: 36000 to 50000)
Dimethylsulphoxide (DMSO)
Diethyl pyrocarbonate (DEPC)
Disodium phosphate (Na\(_2\)HPO\(_4\) · 7H\(_2\)O)
DNase I
DTT
Dispase II
Eosin
Ethanol
Ethylendiaminetetraacetic acid (EDTA)
ECL kit
4,6-diamidino-2-4-phenylindole (DAPI)
Anti-fade solution
Fetal bovine serum (FBS)
Glycine
GolgiPlug
Hexadecyltrimethylammonium bromide
Hematoxylin
Hydrogen peroxide
Heparin
Hydrochloric acid (HCl)
HBSS
Hemoccult sensa slides
Iscove’s modified dulbecco’s medium
Indirubin
Ionomycin
IFN-\(\gamma\) ELISA kit

Milenyi
MP Biologicals
Sigma
Sigma
Merck
Invitrogen
Thermo Scientific
Invitrogen
Sigma
BDH Laboratory
USB
Amersham
Vector
Gibco
USB
BD Biosciences
Sigma
Sigma
Sigma
BDH Laboratory
Gibco
Beckman Coulter
Sigma
Nanjing Zelang Medical Technological Co. Ltd
BD Biosciences
eBioscience
IL-17 ELISA kit: eBioscience
Isopropanol: Merck
IL-1β ELISA kit: eBioscience
IL-6 ELISA kit: eBioscience
LS midi column: Miltenyi
Lysis buffer: Cell Signaling
Lipopolysaccharide (LPS) (Escherichia coli Serotype 055:B5): Sigma
L-glutamine: Invitrogen
LiCl: Sigma
Methanol: RCI LabScan
N, N, N', N'-tetramethylethylenediamine( TEMED): Amersham Bioscience
Neutral gum: Sigma
Nonessential amino acids: Life Technologies
Non-fat dry milk powder: Bio-Rad
o-dianisidine dihydrochloride: Sigma
Paraformaldehyde (PFA): Sigma-Aldrich
Potassium chloride (KCl): USB
Potassium dihydrogen phosphate (KH₂PO₄): Merck
Proteinase inhibitor (PI): Roche
Proteinase K: Invitrogen
PVDF membrane: Millipore
Penicillin: Invitrogen
PMA: Sigma
PrimeScript RT master mix: TaKaRa Bio Inc
Sulfasalazine (SASP): Sigma
Sodium carboxymethylcellulose: Sigma
<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>CMC-Na</td>
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<tr>
<td>Sodium chloride (NaCl)</td>
<td>Merck</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>USB</td>
</tr>
<tr>
<td>Sodium hydroxide (NaOH)</td>
<td>USB</td>
</tr>
<tr>
<td>Sodium phosphate, dibasic (Na₂HPO₄·7H₂O)</td>
<td>Merck</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Gibco</td>
</tr>
<tr>
<td>Tris-Base</td>
<td>USB</td>
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<tr>
<td>Tris-HCl</td>
<td>Sigma</td>
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<td>Triton X-100</td>
<td>USB</td>
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<td>TRIZOL</td>
<td>Ambion</td>
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<tr>
<td>Tween-20</td>
<td>USB</td>
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<td>TNF-α ELISA kit</td>
<td>eBioscience</td>
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<tr>
<td>[³H] Thymidine</td>
<td>Perkin Elmer</td>
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<tr>
<td>3-(4,5-dimethylthiazol-2yl-)-2,5-diphenyltetrazolium bromide</td>
<td>Sigma</td>
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<tr>
<td>TGF-β ELISA kit</td>
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<tr>
<td>Vectastain ABC kit</td>
<td>Vector</td>
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<td>X-ray film</td>
<td>Fujifilm</td>
</tr>
<tr>
<td>Xylene</td>
<td>RCI LabScan</td>
</tr>
</tbody>
</table>

2.1.4 Antibodies

The antibodies used in the study are summarized and listed in **Table 2.2 and 2.3**.
### Table 2.2 List of primary antibodies used in the study

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Application</th>
<th>Brand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse CD11b-Cy5.5 PerCP</td>
<td>FC</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Anti-mouse F4/80-FITC</td>
<td>FC</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Anti-mouse CD4-FITC</td>
<td>FC</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Anti-mouse IL-17A- Cy5.5 PerCP</td>
<td>FC</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Anti-mouse F4/80-FITC</td>
<td>FC</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Anti-mouse CD45-eFlour 450</td>
<td>FC</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Anti-mouse CD3e</td>
<td>C</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Anti-mouse CD28</td>
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<td>eBioscience</td>
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<tr>
<td>Anti-mouse IL-2</td>
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<td>BioLegend</td>
</tr>
<tr>
<td>Anti-mouse IFN-γ</td>
<td>C</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Anti-mouse IL-4</td>
<td>C</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Anti-rabbit IκB-α</td>
<td>WB</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Anti-rabbit NF-κB(p65)</td>
<td>IF</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>Anti-rabbit p-p38</td>
<td>WB</td>
<td>Cell signaling</td>
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<tr>
<td>Anti-rabbit p-ERK</td>
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<tr>
<td>Anti-rabbit p-JNK</td>
<td>WB</td>
<td>Cell signaling</td>
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<tr>
<td>Anti-rabbit p-STAT1</td>
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</tr>
<tr>
<td>Anti-rabbit p-STAT3</td>
<td>WB</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>Anti-rabbit STAT3</td>
<td>WB</td>
<td>Cell signaling</td>
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<tr>
<td>Anti-rabbit p-GSK-3β(s9)</td>
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<tr>
<td>Anti-rabbit GSK-3β</td>
<td>WB</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>Anti-rabbit COX-2</td>
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<td>Cell signaling</td>
</tr>
<tr>
<td>Anti-rabbit iNOS</td>
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<td>Cell signaling</td>
</tr>
<tr>
<td>Anti-mouse Actin</td>
<td>WB</td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>

Note: FC, flow cytometry; C, primary cell culture; IF, immunofluorescence; WB, Western blot.
### Table 2.3 List of secondary antibodies used in the study

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Conjugate</th>
<th>Reactivity</th>
<th>Application</th>
<th>Brand or Supplier</th>
</tr>
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<td>HRP</td>
<td>Anti-rabbit</td>
<td>WB</td>
<td>Cell signaling</td>
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<tr>
<td>IgG</td>
<td>HRP</td>
<td>Anti-mouse</td>
<td>WB</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>IgG (H+L)</td>
<td>Alexa Fluor 488</td>
<td>Anti-mouse</td>
<td>IF</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>IgG (H+L)</td>
<td>Alexa Fluor 568</td>
<td>Anti-mouse</td>
<td>IF</td>
<td>Molecular Probes</td>
</tr>
</tbody>
</table>

Note: IF; immunofluorescence; WB, Western blot.

2.1.5 Primers

The primers (mouse) used in the study are summarized and listed in Table 2.4.

### Table 2.4 List of primers used in the study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>forward 5’-TGT CCA CCT TCC AGC AGA TGT-3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’-AGC TCA GTA ACA GTC CGC CTA GA-3’</td>
</tr>
<tr>
<td>RORγt</td>
<td>forward 5’-GGA GCT CTG CCA GAA TGA GC-3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’-CAA GCC TCG AAA CAG CTC CAC-3’</td>
</tr>
<tr>
<td>T-bet</td>
<td>forward 5’-AGC AAG GAC GGC GAA TGT T-3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’-GGG TGG ACA TAT AAG CGG TTC T-3’</td>
</tr>
<tr>
<td>IL-17A</td>
<td>forward 5’-TCG AGA AGA TGC TGG TGG GT-3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’-CTC TGT TTA GGC TGC CTG GC-3’</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>forward 5’-TGA GTA TCG CCA AGT TTG AGG TCA-3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’-CGG CAA CAG CTG GTG GAC-3’</td>
</tr>
<tr>
<td>IL-1β</td>
<td>forward 5’-TTG ACG GAC CCC AAA AGA TG-3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’-AGA AGG TGC TCA TGT CCT CA-3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>forward 5’-ATG AGC ACA GAA AGC ATG ATC-3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’-TAC AGG CTT GTC ACT CGA ATT-3’</td>
</tr>
</tbody>
</table>
2.2 Phytochemical analysis

2.2.1 Preparation of QDP

The Qing-Dai Powder (QDP) prescription (7.5 g) contains 5.0 g of Qing-dai (as known as indigo naturalis) and 2.5 g of Ku-fan (as known as dried alum). These two Chinese medicinal materials were obtained from the clinics of School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China. Qing-dai was derived from the herbs of *Strobilanthes cusia* (Nees) Kuntze and prepared by Fujian Jian-Qing-dai Co. Ltd. (福建建青黛有限公司). The authentication and quality control of these two materials were performed based on the references and requirements of the Chinese Pharmacopoeia (Commission, 1991, 2011; You et al., 2011). The Voucher specimens (voucher numbers: TCM-0110-Q01, TCM-0110-Q02) were stored in the Research Laboratory, School of Chinese Medicine, Hong Kong Baptist University, Hong Kong. QDP was prepared by mixing Qing-dai and Ku-fan in a fixed proportion as described earlier, powdered to homogeneous size in a mill, and sieved through a 120-mesh filter. For HPLC-UV determination or cellular treatment, QDP and Qing-dai were accurately weighed, and ultrasonically extracted with DMSO. Finally, the extracted solution was filtered through a syringe filter and stored at 4 °C until use.

2.2.2 UPLC-QTOF-MS analysis

Components in QDP and Qing-dai were identified by UPLC-QTOF-MS.
Chromatographic separation was performed using an Agilent 1290 Infinity UPLC system (Santa Clara, CA, USA), equipped with a binary solvent delivery system and a standard auto-sampler. A 100 mm × 2.1 mm ACQUITY BEH C\textsubscript{18} 1.7 μm column (Waters Corp., Milford, USA) was used to separate the components of QDP. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. A linear gradient was optimized as follows (flow rate, 0.40 mL/min): 0-2.5 min, 2-5% B; 2.5-10 min, 5-35% B; 10-20 min, 35-75% B; 20-23 min, 75-100% B; 23-26 min, 100% B; 26-26.1 min, 100-2% B, 26.1-30 min, 2% B. The injection volume was 2 μL and the column temperature was maintained at 40 °C in each run. Mass spectrometry was performed using an Agilent 6540 ultra-high definition (UHD) QTOF mass spectrometer (Santa Clara, CA, USA), equipped with a Jet Stream electrospray ionization (ESI) source. Parameters for the Jet Stream technology are set with the superheated nitrogen sheath gas temperature at 350 °C and with a flow rate at 10 L/min. ESI conditions were set as follows: negative ion mode, capillary 4500 V, nebulizer 30 psi, drying gas 8 L/min, gas temperature 300 °C, nozzle voltage 300 V, skimmer voltage 65 V; octapole RF peak 600 V, fragmentor 175 V. Mass spectra were recorded across the range \( m/z \) 100-1700 with accurate mass measurement of all mass peaks. A sprayer with a reference solution was used for continuous calibration in negative ion mode with reference masses at \( m/z \) 112.9856 and 966.0007. The full-scan and MS/MS data was processed with Agilent Mass Hunter Workstation software (version B.02.00).

2.3 Methods used for animal studies

2.3.1 Induction of colitis

DSS was dissolved in distilled water at a concentration of 2.0 % (w/v). Experimental colitis was induced by giving mice drinking DSS solution \textit{ad libitum} for 5 consecutive days as previously described by Wirtz (Wirtz et al., 2007). The first day of DSS treatment and the day of treatment completed were designated as Day 0 and Day 5,
respectively. Distilled water without DSS was given to mice in the normal group during the same period. Mice were marked and monitored daily for body weight. On day 6, body weight, stool consistency and gross bleeding of each DSS-treated mouse were assessed and mice showing body loss, diarrhea and bleeding were selected for the investigation. All colitic mice were then randomly divided into different groups for subsequent experiments.

2.3.2 Evaluation of disease activity index

Body weight, stool consistency and stool blood were recorded daily. Disease activity index (DAI) was determined by combining scores of (i) body weight loss, (ii) stool consistency and (iii) stool blood as previously described (Xiao et al., 2013). Each score was determined according to the parameters outlined in Table 2.5. Body weight loss was calculated as the percentage difference between the original body weight (day 0) and the body weight on any particular day. Blood in feces was detected with the hemoccult sensa slides according to the manufacturer’s protocols (Beckman Coulter, Inc, USA). At the end of the experiment, mice were killed and the colon was dissected from each mouse, and length between the ileo-cecal junction and anal verge was measured.

Table 2.5 Scoring System to Calculate the Disease Activity Index (DAI)

<table>
<thead>
<tr>
<th>Score</th>
<th>Weight loss</th>
<th>Stool consistency</th>
<th>Visible blood in feces</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>normal</td>
<td>none</td>
</tr>
<tr>
<td>1</td>
<td>1-5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6-10%</td>
<td>loose</td>
<td>Slight bleeding</td>
</tr>
<tr>
<td>3</td>
<td>11-15%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>&gt;15%</td>
<td>diarrhea</td>
<td>Gross bleeding</td>
</tr>
</tbody>
</table>

2.3.3 Histological Analysis

The colons were opened longitudinally, gently washed with ice-cold phosphate
buffered saline (PBS), fixed in 4% paraformaldehyde overnight and embedded in paraffin. The paraffin blocks were sectioned at a thickness of 5 μm with a microtome (Leica). The colonic sections were subjected to H&E staining. Briefly, the sections were deparaffinized, rehydrated, and stained with hematoxylin for 5 min at room temperature. After rinsing with running tap water for 15 min, the sections were further stained with Eosin for 2 min and rinsed for 10 min. The slides were dehydrated with a series of graded ethanol, cleared with xylene, and then coverslipped with mounting medium. The tissue damage was analyzed and scored in a blind fashion by an experienced pathologist as described previously (Xiao et al., 2013). The scoring system was shown in Table 2.6.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severity of inflammation</td>
<td>0</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>mild</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>moderate</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>severe</td>
</tr>
<tr>
<td>Extent of inflammation</td>
<td>0</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>mucosa</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mucosa and submucosa</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>transmural</td>
</tr>
<tr>
<td>Crypt damage</td>
<td>0</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1/3 damaged</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2/3 damaged</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Crypt lost, surface epithelium present</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Crypt lost, surface epithelium lost</td>
</tr>
</tbody>
</table>

*Scores were calculated by adding the score for the three parameters giving a maximum score of 10.
2.3.4 MPO activity assay

Myeloperoxidase (MPO) is an enzyme mainly released by neutrophils, and its activity is directly associated with the severity of inflammation in a given tissue. In this study, MPO activity was measured as described in our previous study (Xiao et al., 2013). Briefly, the colonic tissues kept at -80 °C were weighed and homogenized in 0.5% hexadecyltrimethylammonium bromide solution at 100 mg tissue/mL. The homogenates were centrifuged at 19 000 × g at 4 °C for 15 min. Aliquots of 80 μL supernatant were mixed with 120 μL potassium phosphate buffer (50 mmol, pH 6.0) with 0.0005% o-dianisidine dihydrochloride and 0.1% hydrogen peroxide. Changes in optical density were measured at 460 nm at room temperature. MPO activity was calculated as the rate of optical density changes over a period of two minutes. The amount of protein in each sample was measured using the Bradford method (Bradford, 1976), and bovine serum albumin was used for constructing the standard curve. The results of MPO activity were normalized and quantified as units/mg protein.

2.3.5 Measurement of cytokines and chemokines

Cytokines secreted from cells and in colon tissues were assayed by using commercially available enzyme linked immunosorbent assay (ELISA) kits. For measuring the cytokines secreted from cells, the cell culture supernatants were collected and centrifuged. For measuring the cytokines in colonic tissues, colonic samples were weighed and homogenized in phosphate buffer containing 0.05% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 IU aprotinin A. The homogenates were centrifuged at 16, 000 × g at 4 °C for 15 min and supernatants were collected for the determination of levels of cytokines according to the manufacturer’s protocols. The amount of protein in each sample was measured using the Bradford method (Bradford, 1976), and bovine serum albumin was used for constructing the standard curve. The levels of cytokines were normalized to the protein concentration of the samples and expressed as pg/mg. The
levels of chemokines in serum were also assayed by using commercially available ELISA kits according to the manufacturer’s protocols.

2.3.6 Immunohistochemical analysis

Colonic tissues were fixed in 4% buffered paraformaldehyde, embedded in paraffin and sectioned into 5 μm slices. Sectioned samples were deparaffinized in xylene, rehydrated in a series of graded alcohol, and subjected to antigen rehabilitation. Antigens were retrieved after incubation with protease K solution (1: 100) for 20 min whereas endogenous peroxidase was quenched with 3.0% hydrogen peroxide in methanol for 30 min. Sections were further blocked with 3% bovine serum albumin in PBS, exposed to 0.5% Triton X-100 for 1 h for reducing nonspecific antibody binding and incubated with appropriate primary antibodies at 4°C overnight. The sections were washed with PBS three times, incubated with biotinylated anti-rabbit immunoglobulins, followed by peroxidase-labeled streptavidin, and added 3, 3’-diaminobenzidine chromogen substrate to make them visible according to the protocol of The LSAB kit (LSAB-DAKO, Copenhagen, Denmark). Sections were then washed with PBS and counter-stained with hematoxylin. After dehydration with a series of increasingly concentrated ethanols, sections were mounted with neutral gum. Five random fields at 400 × magnifications in each sectioned sample were counted for the presence of macrophage by a researcher blinded to the treatment; the number of macrophages per μm² of mucosa was quantified using the Image NIH image software (National Institutes of Health, Bethesda, Maryland, USA).

2.3.7 Analysis of colonic macrophages and CD4+ T cells in colon tissues

Colonic lamina propria cells were isolated as previously described with slight modifications (Chen X et al., 2013; Weigmann et al., 2007). Briefly, the colons were removed immediately from the mice after sacrifice. Subsequently, the colonic tissues
were immersed in DMEM medium containing 100 U/mL of penicillin and 100 μg/mL of streptomycin, dissected longitudinally and cut into 0.5-1 cm pieces. Then, intestinal epithelial cells were dissociated by incubation with Ca\(^{2+}\) and Mg\(^{2+}\)-free HBSS containing 5 mM EDTA and 1 mM DTT for 20 min at 37 °C twice. After complete washing with PBS, colonic tissues were then incubated with digestion buffer (RPMI 1640 containing 3 mg/mL dispase II, 0.5 mg/mL collagenase D, and 0.5 mg/mL DNase I) for 30 min at 37 °C twice. Finally, the cells were labeled with anti-CD11b and anti-F4/80 antibodies for macrophages and anti-CD4 antibody for CD4 T cells, and then analyzed using flow cytometry. Appropriate isotype-matched IgGs were used as negative controls.

2.3.8 Western blot assay

Colonic tissues and mesenteric lymph nodes were homogenized in ice-cold RIPA lysis buffer [(1% Triton, 0.1% SDS, 0.5% deoxycholate, 1 mM EDTA, 20 mM Tris (pH 7.4), 150 mM NaCl, 10 mM NaF, 1 mM Na\(_3\)VO\(_4\), 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] containing 1% (v/v) protease inhibitor and 10% (v/v) phosphatase inhibitor. The homogenates were centrifuged at 16,000 × g at 4 °C for 15 min, and the supernatants were collected and stored in aliquots at −80 °C until use. Protein concentrations were quantified by Bradford method (Bradford, 1976). Samples were diluted to a final concentration of 2 mg/mL with SDS-loading buffer and boiled for 5 min. Fifty micrograms of protein from each sample was separated by 10-12% SDS-PAGE and transferred to PVDF membranes. Gels were also loaded with colored molecular weight markers to assess electrophoretic transfer and biotinylated protein ladder marker to estimate the molecular weights of bands of interest. The membranes were blocked with 5% nonfat milk in TBST (pH 8.0) for 1.5 h and then incubated overnight at 4 °C with suitably diluted primary antibodies. The expression of β-actin was used to show equal protein loading. After extensive washing with TBST (3×10 min), the
membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibodies in TBST with 5% nonfat milk for 1 h at room temperature. Membranes were rewashed (TBST, 3×10 min). The blots were detected using the enhanced chemiluminescence (ECL) reaction. Quantification of protein bands was achieved by densitometric analysis using Image-Pro Plus® software (Media Cybernetics, Inc. USA).

2.3.9 Quantitative real-time PCR analysis

Colonic tissues (0.05g) were homogenized in 1ml TRIZOL reagent in a 1.5 ml tube. After incubation at room temperature for 10 minutes, 0.2 ml chloroform per 1ml TRIZOL was added in the tube and shocked vigorously for 30 seconds. Afterwards, the mixture was incubated in room temperature for 5 minutes and subsequently centrifuged at 11,000 rpm for 15 minutes at 4°C. The upper aqueous layer was carefully transferred into a new tube and RNA was precipitated with LiCl solution (final concentration: 3 M). RNA pellet was added 1 ml 75% ethanol and chilled at -80 °C overnight. Then, the tube with RNA sample was subjected to centrifugation at 9,500 rpm for 5 minutes at 4°C. The RNA pellet was dried by air at room temperature for 5 minutes, and subsequently dissolved into DEPC-treated water. The concentration of RNA was determined by NanoDrop 1000.

The cDNA was synthesized using PrimeScript RT Master Mix (TaKaRa Bio Inc., Dalian, China). Briefly, 500 ng of total RNA was firstly mixed with 2 μl 5× PrimeScript RT Master Mix and added RNase-free water to a final volume of 10 μl. After gently mixing the reaction solution, the reverse transcription was incubated at 37 °C for 15 min. The reverse transcriptase reaction was terminated by heating at 85 °C for 5 sec.

Quantitative real-time PCR (qPCR) was performed using the ABI 7500 Real-Time
PCR System (Applied Biosystems, Foster, CA, USA) with SYBR Green Master Mix. Relative quantification of mRNA expression of the gene of interest was normalized to β-actin control and analyzed using the Delta Delta Ct (2-ΔΔCT) method. The qPCR reaction was prepared with the optimized concentration of cDNA and other reagents as listed below (Table 2.7). PCR amplification program was shown in Table 2.8.

Table 2.7 Composition of qualitative real-time PCR

<table>
<thead>
<tr>
<th>Composition</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer (5 mM)</td>
<td>2 μl</td>
</tr>
<tr>
<td>Reverse primer (5 mM)</td>
<td>2 μl</td>
</tr>
<tr>
<td>cDNA</td>
<td>1 μl</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>5 μl</td>
</tr>
<tr>
<td>2 × Fast SYBR Green Master Mix</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

Table 2.8 Program of quantitative real-time PCR

<table>
<thead>
<tr>
<th>Routine</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>95 ℃</td>
<td>10 min</td>
</tr>
<tr>
<td>Step 2</td>
<td>95 ℃</td>
<td>15 sec</td>
</tr>
<tr>
<td>(40 cycles)</td>
<td>60 ℃</td>
<td>60 sec</td>
</tr>
<tr>
<td></td>
<td>95 ℃</td>
<td>15 sec</td>
</tr>
<tr>
<td>Dissociation</td>
<td>60 ℃</td>
<td>20 sec</td>
</tr>
<tr>
<td></td>
<td>95 ℃</td>
<td>15 sec</td>
</tr>
</tbody>
</table>

2.3.10 Analysis of Th1, Th17 and Tregs cells in mesenteric lymph nodes

T lymphocytes of mesenteric lymph nodes from C57BL/6 mice were gently disrupted with sterile plunger and passed through a 40-μm nylon cell strainer (BD Biosciences, San Jose, CA, USA) with PBS containing 4% HI-FBS. After centrifugation at 1200 rpm in room temperature for 5 min, 1× ACK lysing buffer was
added for the removal of erythrocytes. Next, lymphocytes were washed with 1× PBS and re-suspended in RPMI 1640 medium supplemented with 10% HI-FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Afterwards, the T lymphocytes were seeded in 96-well round bottom plates and re-stimulated with phorbol myristate acetate (PMA, 50 ng/mL) and ionomycin (500 ng/mL) in the presence of GolgiPlug (BD Biosciences, San Jose, CA, USA) for 5 h, and cells were then collected for surface staining and intracellular staining. For the surface staining, the cells were transferred into the FACS tubes. After centrifugation at 1200 rpm for 5 minutes, supernatant was discarded. FcR portions were blocked with 50 μl 3% mouse serum. Appropriate amounts of surface antibodies were added. After gently mixing, the cells were incubated for 30 minutes at 4 °C in dark. Next, the cells were washed with FACS buffer twice. To the live cells, 200 μl FACS buffer/well were added and acquisition was performed using FACS. For intracellular staining, cells were fixed in 100 μl 1× Fix/Perm buffer and incubated at room temperature for 30 minutes or 4 °C overnight. Subsequently, the cells were rinsed with 1× Perm buffer twice. 50 μl of 3% mouse serum was added to block FcR and 50 μl Perm buffer containing anti-Foxp3 antibody was loaded. After gentle mixing, the cells were incubated for 30 minutes at 4 °C in dark and then washed with 1× Perm buffer twice. Finally, the cells were re-suspended in 200 μl Perm buffer and the acquisition was performed on FACS.

2.4 Methods used for cell studies

2.4.1 RAW 264.7 cells culture

Mouse RAW 264.7 macrophage cells were maintained in RPMI 1640 medium (Gibco/Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS; Sigma, St. Louis, MO, USA), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco/BRL, Grand Island, NY, USA) at 37 °C in a 5% CO2 incubator. All experiments were carried out after cells had reached the log phase of growth.
2.4.2 MTT Assay for RAW 264.7 cells Viability

RAW 264.7 cells were seeded at a density of $1 \times 10^4$ cells per well in a 96-well plate. After 24 h incubation, the cells were pretreated with various concentrations of QDP for 1 h, followed by the stimulation of LPS (1 μg/mL), and incubated for 20 h at 37 °C in the 5% CO$_2$ incubator. After treatment, cells were reacted with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (0.5 mg/mL, 3 h). Media was aspirated and formazan crystals formed in the wells were dissolved by the addition of 200 μL DMSO. Absorbance was measured at 570 nm using an ELISA microplate reader (Tecan, USA). Cell viability was defined relative to untreated control cells.

$$\text{Viability (\% control)} = \frac{\text{Absorbance of treated sample}}{\text{Absorbance of control}} \times 100$$

2.4.3 Measurement of cytokines produced from RAW 264.7 cells

Secretion of cytokines from RAW 264.7 cells was measured using commercially available ELISA kits. Briefly, $2 \times 10^5$ cells/ml was seeded in a 12-well plate. Raw 264.7 cells were pretreated with indicated concentrations of QDP for 2 h, followed by the stimulation of LPS (1 μg/mL). After the 24-h incubation, culture supernatants were collected. Levels of pro-inflammatory cytokines were quantified by ELISA.

2.4.4 Immunofluorescence Analysis of NF-κB translocation in RAW 264.7 cells

Cells were cultured directly on glass cover slips in 35-mm dishes for 24 h, pretreated with QDP for 1 h and stimulated with 1 μg/mL of LPS for 30 min. Next, cells were fixed with 4.0% paraformaldehyde, treated with 0.2% triton X-100, and blocked
with 3% BSA. Afterwards, cells were incubated with a polyclonal rabbit antibody (1:100) against NF-κB p65 overnight. After extensive washing with PBS, cells were incubated with a secondary FITC-conjugated donkey anti-rabbit IgG antibody (1:100) for 1 h at room temperature. Nuclei were stained with DAPI solution (1 μg/mL), and the cells were analyzed by fluorescence microscope (Carl Zeiss, Baden-Württemberg, Germany).

2.4.5 Preparation of mouse CD4⁺ T cells

Spleens and/or mesenteric lymph nodes were harvested from male C57BL/6 mice, and the tissues were gently disrupted with sterile plunger and filtered through a 40-μm nylon cell strainer (BD Biosciences) with PBS containing 4% HI-FBS. Afterwards, the filtrate was centrifuged at 1200 rpm for 5 minutes, washed with MACS buffer once and centrifuged again. Supernatant was discarded. Next, the cell pellet was re-suspended in MACS buffer and diluted to a concentration of 10⁷ cells per 90 μL of buffer, and then 10 μL of CD4 (L3T4) MicroBeads was added to the cell suspension. After mixing well and incubation for 15 minutes at 4 °C in dark, the cells were washed with MACS buffer once and centrifuged at 1200 rpm for 5 minutes. Cell pellets were subsequently re-suspended in 500 μL of buffer for magnetic separation. The total cell number in this suspension should not exceed a maximum of 10⁸ cells. The magnetic separation was performed according to the manufacturer’s instruction. In brief, the MS column was firstly placed in the magnetic separator and rinsed with appropriate amount of buffer. After the column had been activated, cell suspension was loaded to it. The column was washed three times with appropriate amount of buffer when the column reservoir is empty. Unbounded cells were washed away as flow-through. Finally, the column was removed from the magnetic separator, and bounded cells were flushed out. Cells were collected in a new eppendorf tube and subjected to centrifugation at 1200 rpm for 5 minutes. Supernatant was discarded. The isolated CD4⁺T cells were washed
with 1× PBS and re-suspended in RPMI 1640 medium supplemented with 10% HI-FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin for further experiments.

2.4.6 Sorting of mouse naïve CD4 T cells

2.4.6.1 Preparation of mouse naïve CD4 T cells by cell sorting

Mouse CD4+ T cells were prepared as described in section 2.4.5. Subsequently, the isolated CD4+ cells were labeled with anti-CD4, anti-CD25 and anti-CD45 antibodies and sorted into naïve CD4+CD25+CD45RBhigh T cells. After centrifugation at 1200 rpm for 5 minutes, supernatant was discarded. The isolated CD4+ T cells were washed with 1× PBS and re-suspended in RPMI 1640 medium supplemented with 10% HI-FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin for further experiment.

2.4.6.2 Preparation of mouse naïve CD4 T cells by CD4+CD62L+ T Cell Isolation Kit II

The lymphocyte suspension was firstly prepared as described in section 2.4.5. Next, the pellet of lymphocytes was re-suspended in 400 μL of MACS buffer per 10⁸ cells. After washing with MACS buffer once, and 100 μL of CD4+ T Cell Biotin-Antibody Cocktail II was added to the cell suspension and incubated for 10 minutes at 4 °C in dark. Afterwards, 300 μL of MACS buffer and Anti-Biotin MicroBeads at 200 μL/10⁸ cells were added to the cell suspension and well mixed. After incubation for 15 minutes at 4 °C in dark, the cells were rinsed with MACS buffer once and the pellet was re-suspended in 500 μL of buffer for depleting the non-CD4+ T cells. The non-CD4+ T cells were washed away after filtering with the LS columns. Principally, with the aid of the magnetic separator, CD4+ T cells were tightly held when bounded
to the beads of the LS columns while the non-CD4+ T cells were rinsed into the flow-through. Subsequently, the effluent was centrifuged at 1200 rpm for 5 minutes and supernatant was discarded. The cell pellet was re-suspended in 800 µL of buffer. Next, 200 µL of CD62L (L-selectin) MicroBeads was added to the cell suspension and the mixture was incubated for 15 minutes at 4 °C in dark. After washing with MACS buffer once, the pellet was re-suspended in 500 µL of buffer for CD4+CD62L+ T cells isolation, which was performed with the same steps mentioned in the CD4+ T cells isolation section and described in 2.4.5. After centrifugation at 1200 rpm for 5 minutes, supernatant was discarded. The isolated CD4 T cells were washed with 1× PBS and re-suspended in RPMI 1640 medium supplemented with 10% HI-FBS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin for further experiment.

2.4.7 Proliferation of CD4+ T cells

Purified CD4+ T cells were counted and diluted to the density of 2 × 10^6 cells/mL in RPML-1640 medium. 1 × 10^5 CD4+ T cells were seeded and stimulated with radiated APCs (2 × 10^5) and anti-CD3e Ab for 72 h in the presence or absence of QDP/Qing-dai/indirubin. At the last 6 hours of incubation, 1 μci/ml [3H]-thymidine was added to the culture. The level of CD4+ T cells proliferation was evaluated by the detection of incorporated thymidine in the cells, and the thymidine activity was taken using the cell harvester (Tomtec®) and liquid scintillation counter (Trilux beta counter, Perkin Elmer).

2.4.8 Differentiation of CD4+ T cells

Purified naïve CD4+ T cells or CD4+ T cells were counted and diluted to the density of 1 × 10^6 cells/mL in RPML-1640 medium. Cells were seeded in a 96-well plate at 1 × 10^5 cells/well and were stimulated with plate-bound anti-CD3e Ab (5 µg/ml), soluble
anti-CD28 Ab (2 µg/ml) and different polarized conditions, in the presence of
indicated concentrations of QDP/Qing-dai/indirubin for 72 h at 37 °C. For the
generation of iTreg cells, CD4⁺ T cells were cultured with TGF-β (2 ng/mL). For the
generation of Th17 cells, CD4⁺ T cells were cultured with IL-6 (100 ng/mL), TGF-β
(2 ng/mL), anti-IFN-γ (5 µg/mL), anti-IL-2 Ab (5 µg/mL), anti-IL-12 Ab(5 µg/mL)
and anti-IL-4 Ab (2 µg/mL) simultaneously. Iscove’s modified Dulbecco’s medium
(IMDM, Sigma-Aldrich) was used in Th17 polarizing cultures, and RPMI-1640
(Lonza Bio Whittaker, Walkersville, MD) was used in all other cultures. The media
were supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT)
containing 2 mM glutamine, 100 IU/ml penicillin, and 100µg/ml streptomycin, 10
mM HEPES, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids and 50 mM
2-ME

2.4.9 Intracellular cytokine staining

Polarized naive CD4⁺ T cells or CD4⁺ T cells were firstly re-stimulated with phorbol
myristate acetate (PMA, 50 ng/ml) and ionomycin (500 ng/ml) in the presence of
GolgiPlug (BD Biosciences) for 5 h and then stained with anti-CD4 and anti-IFN-γ
or anti-IL-17A or anti-Foxp3 antibodies for 45 min after the blocking of FcR.
Acquisition was performed using FACS and data analysis was conducted using the
FlowJo software (Tree Star Inc., Ashland, OR, USA).

2.4.10 Western blot analysis

The cells were harvested, rinsed with PBS, and lysed with ice-cold lysis buffer
containing 1% (v/v) protease inhibitor and 10% (v/v) phosphatase inhibitor. After
incubation for 30 min on ice, the cells were centrifuged at 16, 000 g at 4 °C for 15
min. Supernatants were collected for the determination of target proteins. Protein
concentrations were quantified by Bradford method (Bradford, 1976). The rests of
the procedures for western blot analysis were as the same as described in 2.3.8.

2.5 Statistical analysis

The data are presented as mean value ± standard deviation. Statistical significances were evaluated using one-way ANOVA, followed by Duncan's multiple range tests. GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA) was used for all calculations, and $p < 0.05$ was considered statistically significant.
CHAPTER 3

QDP PROMOTES RECOVERY OF COLITIS AND
INHIBITS INFLAMMATORY RESPONSES OF
COLONIC MACROPHAGES AND CD4 T CELLS IN
MICE WITH DSS-INDUCED COLITIS
3.1 Introduction

As the etiology of IBD remains unclear and no specific effective treatment is available, this disease becomes chronic with refractory relapses; thus, IBD is seriously undermining the quality of life and endangering the health of an ever increasing number of sufferers. Current medical therapies for UC mainly focus on inducing remission and preventing relapse and to develop new strategies for the treatment of this debilitating disease is in an urgent need. As mentioned in Chapter 1, Qing-Dai Power (QDP) is an ancient herbal medicinal formula described in “Yi Fang Lei Ju”. Our observational study showed that QDP profoundly improved clinical symptoms of UC patients with no obvious adverse side effects. However, the precise mechanisms behind its anti-colitis effects remain unknown. Therefore, the aim of this study is to investigate the anti-colitis effect of QDP and its underlying mechanisms in experimental colitis.

It is well known that mucosal accumulation of leukocytes is a characteristic feature of IBD, and the activation of macrophages and T cells have been regarded as crucial factors in its pathogenesis (Zhang and Li, 2014). In the healthy gut, resident macrophages phagocytose and eliminate enteric bacteria that breach the intestinal epithelial barrier (Sheikh and Plevy, 2010). The DCs constantly survey the microenvironment and constitutively induce the differentiation of naive T cells into effector T cells and Treg cells (Farache et al., 2013). Treg cells antagonize the effects of effector T cells for keeping a homeostatic status of the gut, thus inflammatory disorder is prevented. In the gut of IBD patients, bacteria breached the intestinal epithelial barrier, and effector T cells in the neighboring area secrete cytokines and chemokines to recruit immune cells such as neutrophils and macrophage to phagocytose the invading pathogens. In this process, a large number of monocytes were recruited from bloodstream and matured into macrophages under the stimulation of inflammatory mediators surrounding the inflamed context of the GI. These monocyte-derived macrophages are inflammatory macrophages, which facilitate naïve
T cells to be differentiated into effector T cells for specific immune functions. Meanwhile, these macrophages also produce chemokines such as monocyte chemoattractant protein-1 (MCP-1/CCL-2) to recruit more monocytes and to facilitate the maturation of inflammatory macrophages; thereby the excessive production of pro-inflammatory cytokines sustained the inflammatory condition. However, resident macrophage has no such effect (Bain et al., 2013). On the other hand, DCs are often considered as the major antigen presenting cells (APCs). They inspect the inbreak of pathogens and intestinal inflammation, and present antigens to the naïve CD4⁺ T cells for their differentiation into effector T cells, thereby initiating dysregulated immune responses. Among effector T cells, Th1 cells primarily secrete cytokines such as IFN-γ and TNF-α. Th2 cells primarily secrete IL-4, IL-5 and IL-13 while Th17 cells are those primarily secrete IL-17A, IL-17F and IL-22. The polarization of T cells to different types of cytokine-secreting cells is principally regulated by particular transcription factors namely T-bet in Th1 cells, GATA-3 in Th2 cells and ROR-γt in Th17 cells. Upon initiation of dysregulated mucosal immune response, excessive proinflammatory cytokine production from effector T cells result in tissue injury and clinical symptoms which are the characteristic of IBD (Blumberg, 2009; Stephani et al., 2011).

In the formula of QDP, Qing-dai (also known as indigo naturalis) is the major herbal component. Previous studies showed that active compounds indirubin and tryptanthrin isolated from Qing-dai exhibited significant anti-inflammatory activities on DSS-induced colitis, the mechanism of these compounds might be associated with the inhibited production of IL-2, IFN-γ and TNF-α, and increased Foxp3⁺ expression on the CD4⁺CD25⁺ Treg cells, etc (Hao et al., 2011; Micallef et al., 2002). In addition, a study also showed that indirubin 3'-monoxime, a derivative of the active compound indirubin isolated from Qing-dai, significantly inhibited LPS-induced macrophage activation (Kim and Park, 2012). Based on above knowledge and clues, we hypothesize that QDP exerts its anti-colitis effects somehow through regulation of macrophages and CD4⁺ T cells. Therefore, in present
study, we aim to investigate the therapeutic effect of QDP on DSS-induced acute colitis in mice and explore its modulatory effects on macrophages activation and CD4⁺ T cell differentiation.

3.2 Experimental design

The therapeutic effect of QDP against experimental colitis was conducted *in vivo*. As described in Chapter 2, the colitis was induced by 2% DSS. The colitic mice were randomly divided into five groups with 9-10 mice per group, and designated as DSS model group, sulfasalazine (SASP) (as a reference positive drug)-treated group and QDP low, medium and high dose-treated groups in this study. One negative control group received drinking water without DSS. The dosages of QDP in mice were designed according to the human dosage of QDP used in clinics, and given as 0.77, 1.54 and 3.08 g/kg for the low-, medium- and high-dose treatment respectively. SASP was given at 200 mg/kg body weight according to references (Kim et al., 2010; Singh et al., 2009). SASP and QDP were reconstituted in 0.5% sodium carboxymethylcellulose (CMC-Na) and orally administered to mice for 7 consecutive days. The vehicle-treated control group and DSS model group animals received the same volume of the vehicle (0.5% CMC-Na). During the experiments, body weight, severity of diarrhea and fecal bleeding were recorded daily. After the 7-day treatment, the mice were sacrificed for colon sample collection. The whole colons of mice in each group were collected and divided into four parts: the proximal part of anus was fixed and embedded in paraffin for H&E staining, and the other three parts were collected for mRNA expression, MPO activity and cytokines production analysis. A flow diagram of the study is shown in Fig. 3.1A. In order to explore the effects of QDP on the changes of macrophages and CD4 T cells in the colon of DSS-treated mice, another set of experiment was set up to study the changes of immune cells in colonic lamina propria, as shown in Fig. 3.1B.
Fig. 3.1A

Flow diagram of experiment to study the therapeutic effect of QDP
Macrophage-like cell line RAW 264.7 was used in in vitro study. RAW 264.7 cells were seeded in 96 or 12-well plates and stimulated with 1 μg/mL LPS for 24 hour in the presence or absence of QDP. The effects of QDP on cell viability were evaluated by means of the MTT assay; levels of various kinds of pro-inflammatory cytokines were measured using enzyme-linked immunosorbent assay (ELISA), and the inflammatory markers in macrophage activation were evaluated by Western blot analysis. A flow diagram of the study is shown in Fig. 3.1C. Likewise, as for in vitro studies of CD4⁺ T cells, murine primary CD4⁺ T cells were isolated using L3T4 microbeads. Primary CD4⁺ T cells were seeded in 96 wells plates and stimulated with plate-bound anti-mouse CD3e antibody in the presence or absence of QDP. The proliferation of CD4⁺ T cells was determined using [³H] thymidine incorporation assay, and the differentiation of CD4⁺ T cells were performed using different Th polarization culture conditions and detected using flow cytometry. A flow diagram of the study is shown in Fig. 3.1D.
Fig. 3.1C
Flow diagram for the study of macrophages in vitro.

Fig. 3.1D
Flow diagram for the study of CD4$^+$ T cells in vitro.
3.3 Results

3.3.1 QDP ameliorated the severity of DSS-induced colitis

As shown in Fig. 3.2A and B, starting from day 5, DSS resulted in a rapid loss of body weight and serious clinical disease symptoms (diarrhea and occult fecal blood) in C57BL/6J mice, and such phenomena had lasted for 3-4 days. After that, mice began to recover. Treatments with SASP and QDP clearly improved body weight recovery and ameliorated clinical disease symptoms of colitis. Compared to the DSS model group, oral administration of QDP at dose of 3.08 g/Kg significantly improved body weight recovery from day 10 to day 12 ($p < 0.05$). Oral administration of QDP (1.54 g/Kg) or SASP (200 mg/Kg) significantly improved body weight recovery from day 11 to day 12 ($p < 0.05$). In the case of disease activity index (DAI), treatment with QDP at dose of 1.54 g/Kg significantly suppressed DAI score from day 11 ($p < 0.05$), and exerted an effect comparable to SASP at dose of 200 mg/Kg, and QDP at dose of 3.08 g/Kg led to a higher DAI score than SASP from day 8 to 12 ($p < 0.05$). In addition, DSS-induced colitis is associated with a remarkable decrease in colon length. Results here showed that treatment with QDP significantly prevented colon shortening in a dose-dependent manner, namely $7.25 \pm 0.38$ cm, $7.28 \pm 0.22$ cm and $7.49 \pm 0.55$ cm versus $6.70 \pm 0.29$ cm in colitic mice ($p < 0.05$) (Fig. 3.2C).
Fig. 3.2 Effects of QDP on change of body weight (A), disease activity index (B) and colon length (C) of mice with DSS-induced colitis. Colitis was induced in all groups except the control group. QDP and SASP were administered to mice from day 6 to day 12. The change
in body weight was taken as the difference between the body weight before induction of colitis and that immediately before sacrifice on day 13. Disease activity index was determined by combining scores of (i) body weight loss, (ii) stool consistency and (iii) stool blood. On day 13, mice were sacrificed, and colon length was measured. Data are expressed as means ± SEM (n = 7-9). ### p < 0.001, compared with control group; † p < 0.05, ‡ p < 0.01 and *** p < 0.001, compared with DSS model group.

### 3.3.2 QDP decreased colonic tissue damage and reduced colonic MPO activity of DSS-treated mice

As shown in Fig. 3.3, 2% DSS drinking water caused extensive colonic tissue damage, including inflammatory cell infiltration, lesion formation and crypt destruction. Mice receiving QDP treatment showed less marked colonic damage. Scores of histological changes are displayed in Fig. 3.3G. The score was significantly higher in the DSS model group than that in the vehicle-treated control group. Treatment with QDP clearly reduced inflammation, mucosal and crypt damage in the colon, in particular to those mice treated with medium and high doses of QDP; thus their scores were lower than that of the DSS model group. Consistent with the histological scores, colonic MPO activity was greatly increased in the DSS model group, whereas the MPO activities in the QDP-treated groups were significantly suppressed in a dose-dependent manner, as shown in Fig. 3.3H.
Fig. 3.3. Effects of QDP on histopathological changes and MPO activity in colon of mice with DSS-induced colitis (A-F. Representative images of H & E staining; A. control, B. DSS model, C. SASP, D. 0.77 g/Kg QDP, E. 1.54 g/Kg QDP, F. 3.08 g/Kg QDP (magnification, 100×), G. histological score, H. MPO activity). Colitis was induced in all groups except control group. QDP and SASP were administered to mice from day 6 to day 12. On day 13, mice were sacrificed, and colonic tissue damage was evaluated by histopathological analysis (H & E staining). MPO activity in colonic homogenate was determined. Data are expressed as means ± SEM (n = 7-9). ### p < 0.001, compared with control group; *p < 0.05, **p < 0.01 and ***p < 0.001, compared with DSS model group.
3.3.3 QDP suppressed the production of colonic pro-inflammatory cytokines and serum MCP-1 in DSS-treated mice

A large body of evidence implicated that colonic inflammation resulted from aberrant activation of intestinal immune cells led to excessive secretion of pro-inflammatory cytokines. The suppressive effects of QDP on the production of pro-inflammatory cytokines in the colon of DSS-treated mice were investigated using enzyme immunoassays. The data in Fig. 3.4A-C showed that the production of TNF-α, IL-1β, and IL-6 drastically increased in mice with colitis in comparison to those in the vehicle-treated control group. Treatments with QDP and SASP significantly inhibited the levels of TNF-α, IL-1β, and IL-6 in the colon of DSS-treated mice in dose-dependent manners. MCP-1 is a key chemokine that has been implicated in macrophage recruitment in various inflammatory diseases including IBD (Takada et al., 2010). As shown in Fig. 3.4D, oral administration of 2% DSS drinking water resulted in a sharp increase of MCP-1 in the serum of DSS-treated mice, and treatments with QDP greatly reduced serum MCP-1 level in a dose-dependent manner, 1.60 ± 0.47, 1.55 ± 0.36, 0.92 ± 0.33 ng/mL versus 2.01 ± 0.63 ng/mL in colitic mice (p < 0.05 and p < 0.01).
Fig. 3.4. Effects of QDP on production of colonic pro-inflammatory cytokines and serum MCP-1 level in DSS-treated mice (A. colonic TNF-α; B. colonic IL-6; C. colonic IL-1β; D. serum MCP-1). QDP and SASP were administered to mice from day 6 to day 12. On day 13, mice were sacrificed, various cytokines in colonic homogenates and MCP-1 levels in serum were determined by means of ELISA. Data are expressed as means ± SEM (n = 7-9). ##p < 0.01, ###p < 0.001, compared with control group; *p < 0.05 and **p < 0.01, compared with DSS model group.

3.3.4 QDP decreased the infiltration of macrophages in the colon of DSS-treated mice

The infiltration of macrophages in colonic tissue was analyzed by means of immunohistochemical study with macrophage marker F4/80. As shown in Fig. 3.5A, the number of macrophages in the colons of model group mice was significantly higher than that in the vehicle-treated control group (p < 0.01). Compared with the model group, the macrophages infiltrated in the colons of QDP-treated mice were dramatically fewer (p < 0.05, p < 0.01 and p < 0.001). To confirm this result, we also
examined the changes of macrophage population in the colon lamina propria of DSS-treated mice by means of flow cytometry. Similar to the results in immunohistochemical study, the number of macrophages was markedly increased after DSS treatment, and QDP at 1.54 g/Kg significantly decreased the recruitment of macrophages in colon lamina propria of the DSS-treated mice ($p < 0.05$), as shown in Fig. 3.5B.
Fig. 3.5. Effects of QDP on macrophage infiltration in colons of DSS-treated mice [A. Immunohistochmical (IHC) analysis of colonic macrophages using F4/80 marker (a-f representative images (a, Control, b, DSS model, c, SASP, d, 0.77 g/Kg QDP, e, 1.54 g/Kg QDP, f, 3.08 g/Kg QDP), g. Summary of colonic macrophage numbers in the colon of mice with different treatments); B. flow cytometric analysis of macrophages in the colon lamina propria (LP) (a, Control, b, DSS model, e, 1.54 g/Kg QDP, d. Summary of percentage of macrophages in total colonic cells of lamina propria of mice with different treatment)]. Colitis was induced in all groups except control group. QDP and SASP were administered to mice from day 6 to day 12. On day 13, mice were sacrificed. The colon section was evaluated by IHC analysis with F4/80 marker (n = 7-9). For flow cytometric analysis, the population of macrophage in lamina propria mononuclear cells from whole colonic tissue...
was determined using CD11b and F4/80 markers (n = 5-6). Data are expressed as mean ± SEM. *** p < 0.001, compared with control group; * p < 0.05, compared with DSS model group.

3.3.5 QDP suppressed the production of TNF-α and IL-6 and expression of iNOS and COX-2 in LPS-induced RAW 264.7 cells

Previous results showed that QDP could suppress the production of colonic pro-inflammatory cytokines TNF-α, IL-1β and IL-6. It is well known that macrophages are a major source of these cytokines. We therefore evaluated the anti-inflammatory effect of QDP on LPS-stimulated RAW 264.7 cells (murine macrophage cell line). As shown in Fig 3.6B, treatment with LPS resulted in significantly increased production of pro-inflammatory cytokines (TNF-α, 2.0-fold; IL-6, 14.3-fold) from the RAW 264.7 cells, while QDP at a concentration of 1 μg/mL significantly inhibited LPS-induced production of TNF-α and IL-6. The activities of iNOS and COX-2 are important as they are respectively the immediate modulators of NO and PGE₂, which are the inflammatory mediators produced by macrophages with essential roles in the initiation and progression of IBD. Therefore, we also investigated the inhibitory effects of QDP on the expression of iNOS and COX-2 by means of Western blot analysis. As shown in Fig. 3.6C, protein levels of iNOS and COX-2 were outstandingly upregulated in response to LPS, and treatment with QDP resulted in dose-dependent inhibitions of iNOS and COX-2 protein expression.
Fig. 3.6. Effects of QDP on the production of TNF-α and IL-6 and expression of iNOS and COX-2 in LPS-induced RAW 264.7 cells. (A) RAW 264.7 cells were treated with the indicated concentrations of QDP for 24 h, and cell viability was determined as described using MTT method. For examination of anti-inflammatory effect of QDP, cells were treated with QDP for 1 h, followed by continuous incubation with LPS (1 μg/mL) for 24 h. (B) Concentrations of TNF-α and IL-6 in culture medium were monitored by ELISA. (C) Cells were lysed and expression of iNOS and COX-2 were determined using Western blot. Data represent the means ± SEM (n = 3). ###p < 0.001, compared with LPS treated alone; **p < 0.01, ***p < 0.001, compared with no LPS treated control.
3.3.6 QDP reduced IκB-α degradation and NF-κB (p65) nuclear translocation in LPS-induced RAW 264.7 cells

It is well known that most pro-inflammatory cytokine are transcriptionally regulated by nuclear factor κB (NF-κB), which is sequestered into an inactive cytoplasmic complex by binding to an inhibitory κB protein, IκB. Alternatively, the degradation of IκB-α indicates NF-κB activation. We therefore firstly examined the effects of QDP on LPS-induced IκB-α degradation using Western blot analysis. As shown in Fig. 3.7A, the LPS-induced degradation of IκB-α was dramatically inhibited by QDP in a dose-dependent manner. Further, we also investigated the effect of QDP on LPS-induced NF-κB (p65) nuclear translocation using immunofluorescence staining. The results showed that NF-κB (p65) was normally sequestered in the cytoplasm whilst LPS induced NF-κB (p65) accumulation in the nucleus; however, pre-treatment with QDP abolished the LPS-induced NF-κB (p65) translocation into the nucleus (Fig. 3.7B).

Fig. 3.7 Effects of QDP on IκB α degradation and p65 nuclear translocation in LPS-induced RAW 264.7 cells. (A) Cells were pretreated with the indicated concentrations of QDP for 1 h
and incubated with LPS (1μg/mL) for another 30 min. And then, cells were lysed and IκB α expression was determined by Western blot. (B) Cells were pretreated with 1 μg/mL QDP for 1 h prior to stimulation with LPS (1μg/mL) for 1 h. The nuclear localization of NF-κB (p65) was determined using fluorescence microscopy after staining with DAPI, anti-NF-κB (p65), and FITC-labeled anti-rabbit IgG antibody. NF-κB (p65) sequestered in the cytoplasm were indicated by yellow arrows and NF-κB (p65) translocated into nuclear were indicated by blue arrows). Data represent the means ± SEM. (n=3). ###p < 0.001, significantly different compared with control group; *p < 0.05, significantly different compared with LPS-treated group. The images shown are the representatives of three independent experiments.

### 3.3.7 QDP decreased the proportion of CD4⁺ T cells and mRNA expression of IFN-γ, IL-17A and RORγt in the colon of DSS-treated mice

The percentage of CD4⁺ T cells in the colon lamina propria of DSS-treated mice was examined by flow cytometric analysis. As shown in Fig. 3.8A, the population of CD4⁺ T cells in the colon was significantly increased after DSS treatment (p < 0.001). However, such an increase was dramatically suppressed by QDP at 1.54 g/Kg (p < 0.05). Th1 and Th17 are effector CD4⁺ T cells to mediate DSS-induced acute intestinal inflammation. We subsequently examined the effects of QDP on the mRNA expression of their specific cytokines and transcription factor in the colon of DSS-treated mice. As shown in Fig. 3.8B, the mRNA expression of IFN-γ, IL-17A and RORγt was remarkably induced by DSS. In contrast, the increased mRNA expression of RORγt after DSS treatment was significantly suppressed by QDP treatment. The increased mRNA expression of IFN-γ and IL-17A were also suppressed but no statistically significant differences were observed.
Fig. 3.8 Effects of QDP on the percentage of CD4\(^+\) T cells and mRNA expression of IFN-γ, IL-17A and RORγt in the colon of DSS-treated mice. **A.** The percentage of CD4\(^+\) T cells in total colonic cells of lamina propria (LP) of DSS-treated mice; **B.** mRNA expression of IFN-γ, IL-17A and RORγt in the colon of DSS-treated mice. Colitis was induced in all groups except control group. QDP and SASP were administered to mice from day 6 to day 12. On day 13, mice were sacrificed. The mRNA expression of IFN-γ, IL-17A and RORγt in the colon were performed using RT-PCR. For flow cytometric analysis, the population of CD4\(^+\) T cells in lamina propria mononuclear cells from whole colonic tissue was determined using CD4 marker (n = 5-6). Data are expressed as mean ± SEM. ### \( p < 0.001 \), compared with control group; *\( p < 0.05 \), compared with DSS model group.

### 3.3.8 QDP inhibited the differentiation of Th1 and Th17 cells *in vitro*

The above results showed that QDP suppressed the population of CD4\(^+\) T cells and the mRNA expression of IFN-γ, IL-17A and RORγt. We therefore further examined the effects of QDP on proliferation and differentiation of CD4\(^+\) T cells. As shown in
Fig. 3.9A, QDP did not significantly alter the proliferation rate of CD4+ T cells. However, QDP significantly inhibited the differentiation of Th1 and Th17 cells ($p < 0.05$ and $p < 0.01$) though no significant effect on the differentiation of Treg was observed (Fig. 3.9B and C).

Fig. 3.9 Effects of QDP on the proliferation and differentiation of CD4+ T cells in vitro. (A) CD4+ T cells isolated from C57BL/6 mice were incubated for 72 h at 37 °C in the presence or absence of different concentrations of QDP under stimulation of CD3e Ab. Cell proliferation was measured by [3H] thymidine assay. (B-C) CD4+ T cells from C57BL/6 mice were stimulated with CD3e/CD28 Abs and incubated under Th1-, Th17-, iTreg-polarizing conditions in the presence or absence of QDP (500 μg/ml) for 3 days. The phenotypes of Th1, Th17 and iTreg cells were analyzed by flow cytometry. All data are presented as means ± SEM. *$p < 0.05$, compared without QDP treated group.

3.4 Discussion

In the present study, we investigated the therapeutic effect of QDP on DSS-induced
acute colitis in mice and examined the regulatory effects of QDP on macrophages and CD4⁺ T cells. We found that orally administration of QDP profoundly attenuated the severity of DSS-induced colitis in mice, which was evidenced by reduced clinical manifestations such as colon shortening, histological damages and colonic myeloperoxidase activity. We showed that this effect was associated with suppression of colonic macrophages activation and CD4⁺ T cell differentiation.

To elucidate the pathogenic and molecular biological mechanisms of human IBD, several animal models of intestinal inflammation including acetic acid-, TNBS- and DSS-induced colitis have been developed. Among these models, DSS-induced colitis mouse model mimics a number of symptoms and histopathological characteristics of UC found in humans, including diarrhea, rectal bleeding, crypt destruction, infiltration with granulocytes, edema and ulceration of colon tissue (Moodley et al., 2013). This model is very simple and reproducible, and commonly used in the study of pathogenesis of UC and the screening of potential therapeutic interventions. By using this model, we found that QDP significantly ameliorated clinical symptoms including body weight loss, stool consistency, and bleeding of DSS-treated mice. Furthermore, results from macroscopic and microscopic examinations showed that QDP significantly prevented colon shortening and colon tissue damage induced by DSS. Myeloperoxidase (MPO) enzyme was measured as a marker of neutrophil influx into the tissue (Xiao et al., 2013). Consistent with histological changes, oral administration of QDP profoundly attenuated the colonic MPO activity of DSS-treated mice in a dose-dependent manner. Collectively, our results clearly demonstrated that QDP reduced DSS-induced intestinal inflammation.

IBD is thought to be mediated by aberrant immune responses. Macrophages are the typical effector cells to orchestrate innate and adaptive immune host responses. Their roles in the pathogenesis of IBD have been well documented (Steinbach and Plevy, 2014). In the inflamed gut, macrophages secrete excessive proinflammatory cytokines to drive the initiation of innate immune response and the consequential
adaptive immune response, which result in tissue damage (Steinbach and Plevy, 2014; Yoshino et al., 2010). In the present study, we analyzed the infiltration of macrophages in the colon by immunohistochemical and flow cytometric studies. The results showed that 2% DSS resulted in a sharp increase of macrophages in the colon tissues, and the oral administration of QDP significantly suppressed macrophage accumulation. A series of studies has shown that the accumulated macrophages in the inflamed colon were recruited and differentiated from Ly6Chi monocytes in the bloodstream, which were different from resident intestinal macrophages and they presented a pro-inflammatory phenotype. The recruitment of monocytes to inflamed tissues was mediated by MCP-1 through binding to hemokine receptor CCR2 expressed on circulating monocytes (Bain et al., 2013). Accordingly, we examined MCP-1 levels in the serum of treated mice, and observed that 2% DSS treatment profoundly increased serum MCP-1 levels and QDP treatment significantly reversed this increase. Previous study demonstrated that accumulated colonic macrophages were activated under the context of gut inflammation, resulting in excessive secretion of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, to exacerbate IBD (Bain et al., 2013). Our results showed that QDP significantly suppressed the production of TNF-α, IL-1β, and IL-6 in colons of DSS-treated mice. In RAW 264.7 cells, we also observed that QDP significantly decreased the LPS-induced TNF-α and IL-6 production and expression of iNOS and COX-2 via inhibiting IκB-α degradation and p65 translocation. Taken together, these findings indicated that QDP suppressed the recruitment and activation of colonic macrophages.

CD4 T cells are key elements of adaptive immune system, as they secrete a variety of cytokines to facilitate, suppress or regulate immune responses. CD4^+ T cells are known to secrete cytokines such as TNF-α and IL-6. The deregulated adaptive immune system is regarded as the main contributor to the development of IBD since it may lead to an increase of effector T cells or an increase of ineffective Treg cells. The imbalanced ratio of effector T cells to Treg cells probably results in an excessive
production of pro-inflammatory cytokines for the initiation of IBD (Wallace et al., 2014). In the present study, we observed that the population of CD4$^+$ T cells was significantly increased in the colon lamina propria after DSS treatment, and the oral administration of QDP significantly suppressed the population of CD4$^+$ T cells. Previous studies indicated that DSS-induced colitis was characterized by the Th1/Th17 responses in acute inflammation and the Th2/Th17 responses in chronic inflammation (Alex et al., 2009). Accordingly, we examined the effects of QDP on the mRNA expression of Th1/Th17 specific cytokines and transcription factor in the colon of DSS-treated mice, and observed that QDP treatment clearly suppressed the mRNA expression of IFN-γ, IL-17A and RORγt. We also observed that QDP significantly inhibited Th1 and Th17 cell differentiation in vitro. These findings revealed that QDP exerts its beneficial effect probably associated with suppression of Th1/Th17 cells.
Fig. 3.10 Proposed actions of QDP on DSS-induce colitis in mice (QDP exerts its beneficial effects are associated with 1) suppressing recruitment and activation of colonic macrophages, and 2) inhibiting the differentiation of Th1 and Th17 cells.)

3.5 Summary

In summary, the present study demonstrated that QDP possesses potent anti-colitis effect. The beneficial effect of QDP may be partially due to its ability to suppress the inflammatory responses of colonic macrophages and CD4+ T cells. These findings represent evidence-based support for the clinical use of QDP in the management of UC.
CHAPTER 4

COMPARATIVE STUDY OF CHEMICAL COMPONENTS AND ANTI-COLITIS EFFECTS OF QDP AND ITS MEDICINAL MATERIALS
4.1 Introduction

Qing-dai powder (QDP) is composed of Qing-dai (also known as indigo naturalis) and Ku-fan (also known as dried alum). Qing-Dai is the major component of QDP and possesses potent anti-inflammatory, anti-cancer, anti-psoriatic and anti-bacterial activities (Chiang et al., 2013; Ginzinger et al., 2012; Lin et al., 2009; Lin et al., 2014). In China, Qing-dai had been traditionally used as an antipyretic, an antiphlogistic, and as a hemostatic remedy (Suzuki et al., 2013). Qing-dai enemas have been used for ulcerative proctitis and are currently described in Chinese medical guidelines of Branch of Spleen-stomach disease, China Association of Traditional Chinese Medicine for the treatment of UC (Yuan et al., 2009). Likewise, most recently, a retrospective observational study reported that Qing-dai can significantly improve the clinical symptoms and endoscopic scores in UC patients who failed to respond to conventional medications (Suzuki et al., 2013). Ku-fan is another component of QDP and possesses extensive functions to eliminate phlegm, dry dampness, arrest diarrhea, stop bleeding, remove toxicity and kill parasites. In the TCM practice, Ku-fan had been traditionally used as an external remedy to treat various scabies, but sometimes it can also be orally taken in combination with other medicines for the treatment of digestive diseases, such as upper GI haemorrhage, colitis and fatty liver, etc. For example, Gao has reported that oral administration of Ku-fan-predominant formula Hou-shi-hei Powder with lactobacillin table exerts a high response rate to chronic UC patents, as 71.79% of 78 patients resolved the clinical symptoms of their colitis such as diarrhea and bleeding completely (Gao, 1993).

According to the principle of TCM, TCM formulas are usually formed with multiple medicinal materials. In a formula, some medicinal materials are aimed to treat the main disease while the others are used to enhance therapeutic effects or diminish the adverse effects of the main drugs through different biological pathways (Wang et al., 2008). Results in Chapter 3 demonstrated that QDP possesses potent anti-colitis
effects via suppressing the inflammatory responses of macrophages and CD4⁺ T cells. However, it is unclear whether these two medicinal materials in QDP exert synergic anti-colitis effects? If not, which herb is mainly responsible for the anti-colitis effects of QDP? Therefore, in this study, we compared the chemical composition and anti-colitis effects of QDP and its medicinal materials.

4.2 Experimental design

Firstly, the composition of QDP and its medicinal material Qing-dai were identified using UPLC-QTOF-MS, and their difference in chemical composition was compared. A flow diagram of the study is shown in Fig. 4.1A. Secondly, the anti-colitis effects of QDP and its medicinal materials Qing-dai and Ku-fan were assessed and compared against DSS-induced colitis in mice. Five groups of 8 mice were used here. The colitic mice were randomly divided into 4 groups, with 8 mice in each group, and treated with 5% CMC-Na solution (Group 1), QDP (1.54 g/kg/day, Group 2), Qing-dai (1.02 g/Kg, Group 3) and Ku-fan (0.51 g/Kg, Group 4) respectively; mice in the control (Group 5, n = 8) were treated with 5% CMC-Na solution. The dosage of QDP was set up according to the amount used in clinics, and 1.54 g/kg/day was determined to be equivalent to the clinical dosage (7.5 g/60 kg//day). The dosages of Qing-dai and Ku-fan were set up as 1.02 and 0.51 g/kg/day respectively according to their proportions in QDP. A flow diagram of the study is shown in Fig. 4.1B.
Fig. 4.1A
Study design for phytochemical analysis of QDP and its medicinal materials in Chapter 4.

Fig. 4.1B
Study design for anti-colitis effects of QDP and its medicinal materials in Chapter 4.
4.3 Results

4.3.1 Identification of major components in QDP and Qing-dai by UPLC-QTOF-MS

After optimization of chromatographic and MS condition, the major constitutes of QDP and Qing-dai were well separated and detected within 30 min under the positive ESI-MS ion mode. The total ion MS chromatogram (TIC) of QDP is very high similarity to that of Qing-dai except for the peaks at tR 15-20 min (As shown in Fig. 4.2). When comparing the retention time (tR), mass fragment ions (m/z) and UV absorption characteristics (lambda max) with those chemical compounds described in the literature and Dictionary of Natural Product, 27 chemical component signals in QDP were identified, and 23 peaks in Qing-dai were identified (As shown in Table 4.1). Among the identified compounds, four compounds namely indigo, indirubin, sitosterol and 10H-indolo [3,2-b] quinoline-11-carboxylic acid amide were identified as the major components of Qing-dai and QDP.
Fig. 4.2A Elementary particle flow graph (BPI) chromatogram monitored in positive ion mode for QDP.

Fig. 4.2B Elementary particle flow graph (BPI) chromatogram monitored in positive ion mode for Qing-dai.
Table 4.1A. Main identified compounds in QDP.

<table>
<thead>
<tr>
<th>Peak</th>
<th>tR(min)</th>
<th>Assigned identity</th>
<th>Molecular formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.999</td>
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<td>2</td>
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<td>Deoxyvascione</td>
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<td>6</td>
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- 100 -
### Table 4.1B. Main identified compounds in Qing-dai.

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<th>Peak</th>
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<td>26.831</td>
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<td>C₂₀H₄₈O</td>
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4.3.2 Anti-colitis effects of QDP and its medicinal materials Qing-dai and Ku-fan

4.3.2.1 Effects of QDP and its medicinal materials on the severity of DSS-treated mice

The therapeutic efficacy of QDP and its medicinal materials, Qing-dai and Ku-fan on experimental colitis were determined in mice with DSS-induced colitis. As shown in Fig. 4.3A, oral administration of 2% DSS drinking water resulted in a high mortality, in which about 25% of mice in DSS model group were dead. Treatments with QDP and Qing-dai greatly reduced the mortality of DSS-treated mice, but this beneficial effect was not observed in the Ku-fan group. DSS also resulted in a rapid loss of body weight and serious clinical disease symptoms (diarrhea and occult fecal blood) in the mice from day 5 onwards. Mice began to recover 3 to 4 days after the 5-day DSS treatment. Treatment with QDP and Qing-dai greatly improved body weight recovery and ameliorated the disease activity index (DAI) of DSS-treated mice, but Ku-fan treatment only exhibited a slight effect (As shown in Fig. 4.3B and 4.3C). In addition, DSS-induced model of colitis is associated with a remarkable decrease in colon length. Results here showed that treatment with QDP and its medicinal materials prevented the shortening of colon length. Particularly, both QDP and Qing-dai exhibited potent comparable effects (p < 0.05) (As shown in Fig. 4.3D).
Fig. 4.3 Effects of QDP and its medicinal materials on mortality (A), body weight change (B), disease activity index (C) and colon length (D) of mice with DSS-induced colitis. Colitis was induced in all groups except control group. QDP and its medicinal materials were administered to mice from day 6 to day 12. The change in body weight was taken as the difference between the body weight before induction of colitis and that immediately before sacrifice on day 13. Disease activity index was determined by combining scores of (i) body weight loss, (ii) stool consistency and (iii) stool blood. On day 13, the mice were sacrificed, and the colon length was measured. Data are expressed as mean ± SEM (n = 5-8). ### $p < 0.001$, compared with control group; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, compared with DSS model group.

4.3.2.2 Effects of QDP and its medicinal materials on histological changes of colon tissues in DSS-treated mice

Quantification of histological changes in the colon of colitic mice provides an alternative, more sensitive measure of intestinal injury and repair. As shown in Fig.
A-E, tissue sections from colitic mice exhibited typical inflammatory changes in colonic architecture, including mucosal ulceration, crypt damage, edema, and cell infiltration in mucosal tissue. Treatments with QDP and its medicinal materials clearly decreased inflammatory cell infiltration, crypt damage, and mucosal ulceration in the colon of colitic mice, thus, the QDP and Qing-dai groups had lower injury and inflammation scores (Fig. 4.4F).

Fig. 4.4. Effects of QDP and its medicinal materials on histopathological changes in the colon of mice with DSS-induced colitis (A-E. Representative image of H & E staining (A. control; B. DSS model; C. QDP; D. Qing-dai; E. Ku-fan.) (magnification, ×10). F. histological score). Colitis was induced in all groups except control group. QDP and its
medicinal materials were administered to mice from day 6 to day 12. On day 13, the mice were sacrificed, and colonic tissue damage was evaluated by histopathological analysis (H & E staining). Data are expressed as mean ± SEM (n = 5-8). ### p < 0.001, compared with control group; *** p < 0.001, compared with DSS model group.

### 4.3.2.3 Effects of QDP and its medicinal materials on colonic MPO activity of DSS-treated mice

Consistent with histological changes, the colonic MPO activity was greatly increased in the DSS model group. Treatment with QDP and its medicinal materials suppressed colonic MPO activity in DSS-treated mice. The QDP and Qing-dai groups showed significant differences when comparing with that of DSS group (As shown in Fig. 4.5).

![Fig. 4.5](image)

**Fig. 4.5** Effects of QDP and its medicinal materials on MPO activity in colon of mice with DSS-induced colitis. Colitis was induced in all groups except control group. QDP and its medicinal materials were administered to mice from day 6 to day 12. On day 13, the mice were sacrificed, and MPO activity was determined in colon homogenates. Data are expressed as mean ± SEM (n = 5-8). ### p < 0.001, compared with control group; ** p < 0.01, compared with DSS model group.
4.3.2.4 Qing-dai and Ku-fan in QDP exhibited an additivity to mitigate DSS-induced colitis in mice

To determine whether the anti-inflammatory effects of Qing-dai and Ku-fan in QDP are synergistic, Jin’s formula was used for the quantitative analysis of their relationships. According to the formula, the incidence of synergism, additivity or antagonism of two independent medicines was calculated as follows:

\[ q = \frac{E(a+b)}{Ea + Eb - EaxEb} \]

--where \( q \) is the incidence of synergism, additivity or antagonism between A and B, \( E(a+b) \) is the inhibitory rate of A combined with B, and \( Ea \) and \( Eb \) are the inhibitory rates of A and B, respectively. Their relationships are determined as: 1) synergism, \( q > 1.15 \); additivity, \( 0.85 < q < 1.15 \); antagonism, \( q < 0.85 \) (Dai, 1998; Sun et al., 2014). Accordingly, the relationships between Qing-dai and Ku-fan in the aspect of DAI score, MPO activity, colon length, and pathological score were determined (As shown in Table 4.2). The results showed that the relationship between Qing-dai and Ku-fan in QDP is additivity, but not synergism, in mitigating the DSS-induced colitis in mice.

**Table 4.2** Potential synergistic effect of Qing-dai and Ku-fan against DSS-induced colitis

<table>
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<th>Pathological index</th>
<th>( q )</th>
</tr>
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<td>DAI score*</td>
<td>0.864</td>
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<tr>
<td>Colon length</td>
<td>0.947</td>
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<tr>
<td>MPO activity</td>
<td>0.889</td>
</tr>
<tr>
<td>Pathological score</td>
<td>0.863</td>
</tr>
</tbody>
</table>

*Note: The DAI score was calculated from data of day12.*
4.4 Discussion

The present study reveals that Qing-dai and Ku-fan additively ameliorated the severity of colitis in DSS-induced mice, and Qing-dai is mainly responsible for the anti-colitis effect of QDP.

QDP comprises two medicinal materials, Qing-dai and Ku-fan. As shown in Figure 4.2, 10H-indolo [3, 2-b] quinoline-11-carboxylic acid amide, indigo, indican, indirubin and sitosterol are the major compounds of Qing-dai. Previous studies showed that indirubin exhibited significant anti-inflammatory activities on DSS-induced colitis via inhibiting the production of IFN-γ and TNF-α, and increasing Foxp3⁺ expression on CD4⁺CD25⁺ Treg cells (Hao et al., 2011; He et al., 2010). In addition, indirubin 3'-monoxime, the active derivate of indirubin, significantly inhibited LPS-induced macrophage activation and suppressed malignant lymphoid cell proliferation (Chebel et al., 2009; Kim and Park, 2012). Moreover, study also reported that sitosterol can significantly suppress the production of proinflammatory cytokines TNF-α, IL-1β, and IL-6, as well as protein expression of COX-2 in the colon of TNBS-induced colitic mice through inhibiting NF-κB signaling pathway (Lee et al., 2012). In addition, the components of Qing-dai such as isatin, tryptanthren, and quinazolinone derivatives were reported with great therapeutic potential for experimental colitis (Brain et al., 2005; Micallef et al., 2002; Socca et al., 2014). Except for organic constituents, calcium carbonate is the major inorganic substance of Qing-dai as it occupies 70% of its total inorganic constituents (Wang, 2004). Previous study has reported that long-term daily oral administration of calcium carbonate substantially rehabilitated either the rate or distribution of proliferating cells in the colon crypts of UC patients (Bostick et al., 1997). Collectively, these findings indicate that the compounds abovementioned in Qing-dai play important roles against colitis.

Ku-fan, derived from alunite, is an inorganic constituent of QDP. Structurally, it
appears as an uneven crystal surface, looks like a loose spongy and possesses a strong absorption capacity of water or other solvents (Chen et al., 1996). It is well known that aluminum salts such as aluminum potassium sulfate have been widely used in allergy therapies for many decades and are assumed to be safe with few reported side-effects (Exley, 2014). It has been shown that aluminum exposure suppressed rat splenic T and B lymphocyte proliferation, and inhibited production of IL-2, IL-6 and TNF-α in vitro (She et al., 2012). In addition, the study of Mori et al also shown that aluminum hydroxide and aluminum salts such as aluminium phosphate inhibited the secretion of the Th1 polarizing cytokine IL-12 from DCs, and selectively inhibited the expression of the IL-12p35 subunit in DCs through activation of PI3 kinase signaling pathway (Mori et al., 2012). In vivo, study in healthy volunteers showed that there was no obvious immune change except for a slightly smaller CD8+CD45RO+ population was observed after the ingestion of total 590 mg aluminum hydroxide three times daily for 6 weeks (Graske et al., 2000). In GI disease, aluminum hydroxide and gaoline (contains aluminum silicate) were commonly used as anti-diarrheal agents (McClung et al., 1980; Pichaipat et al., 1989). However, recent study has revealed that aluminum enhanced inflammation and decreased mucosal healing in experimental colitis in mice, as impaired intestinal barrier function, granuloma formation and elevated LPS-induced production of inflammatory cytokines expression were obtained in the epithelial cells of aluminum-treated individuals (Pineton de Chambrun et al., 2014). However, our results showed that Ku-fan treatment did not show obvious beneficial or deleterious effect in DSS-induced colitis in mice except for a slight higher mortality when compared to DSS model group. It seems that Ku-fan possesses bidirectional actions. On one hand, it works as an adsorbent, likes gaoline and muscovite (Chen et al., 2009; Gardiner et al., 1993), for mitigating intestinal inflammation; on the other hand, it injures intestinal epithelial cells and aggravates intestinal inflammation. Nevertheless, the action mechanism of Ku-fan in intestinal inflammation needs further investigation.

According to the principles of drug interaction of TCM, one herb can interact with
the other at six conditions: mutual reinforcement (相须), mutual assistance (相使), mutual detoxification (相杀), mutual restraint (相畏), mutual antagonism (相恶), mutual incompatibility (相反). Mutual reinforcement and mutual assistance are synergistic interactions. These two conditions are illustrated as two herbs with similar functions are used together resulting in an effect that is more than or equal to the sum of the effects of two interacting drugs, respectively. Mutual detoxification, mutual restraint, mutual antagonism and mutual incompatibility are antagonistic interactions, which are interpreted as one herb minimizes the toxicity of the second herb or suppresses the effects of its interacting herbs with different intensities (Luo, 2012). Generally, a multi-herb formula is comprised by four parts including Jun (君), Chen (臣), Zuo (佐) and Shi (使). The Jun is the herbs that treat the main cause or primary symptoms of a disease. The Chen is the herbs that serve to enhance or broaden the effects of Jun. The Zuo and Shi are herbs that to counteract the toxic or side effects or promote the absorption of all components of these herbs (Qiu, 2007).

In QDP, Qing-dai is the Jun herb with the efficacy of clearing heat, counteracting the toxicity and cooling blood (清热解毒，益气凉血), which mainly focuses on removing the pathogenetic factors of the disease; Ku-fan is the Chen with the efficacy of drying dampness and arresting diarrhea (收敛固涩), which mainly ameliorates the symptoms of the disease (Chanayath et al., 2002; Liu, 2009). Qing-dai and Ku-fan work together, which will result in an increased therapeutic effect to treat colitis in comparison to the effect of these two herbs used individually. In the present study, results showed that Qing-dai and Ku-fan additively ameliorated the severity of colitis in DSS-induced mice, which is consistent with the condition of mutual assistance in the principles of drug interaction of TCM.

4.5 Summary

Taken together, our data demonstrate that QDP is a reasonable TCM formula, and reveal Qing-dai is mainly responsible for the anti-colitis effect of QDP and Ku-fan is
secondary to be beneficial to the anti-colitis effect of QDP. It is worth noting that the safety of QDP should be further assessed strictly since the neurotoxicity of aluminum in Ku-fan is widely in complaint (Kumar and Gill, 2014), and its impairment in intestinal barrier function is also in declare (Pineton de Chambrun et al., 2014), although in the present study Ku-fan did not present any obvious deleterious effect on DSS-induced colitis in mice.
CHAPTER 5

QING-DAI ATTENUATES DSS-INDUCED COLITIS
THROUGH INHIBITING TH1 AND TH17 RESPONSES
5.1 Introduction

Although the precise mechanisms of IBD remain not to be fully understood, it is well known that the aberrant adaptive immune system is attributable to the pathogenesis of IBD (Geremia et al., 2014). Excessive activation of effector T cells (Teffs) or impaired function of regulatory T cells (Tregs) were found in the IBD patients, resulting in a disturbed balance between effector T cells and Tregs (Kaser et al., 2010; Zenewicz et al., 2009). It has been repeatedly reported that the elevated levels of IFN-γ expression was detected in the colons of both active UC and CD patients (Dong et al., 2013; Olsen et al., 2009) and Th1 responses was proposed to play a key role of in the inflammation of IBD (Zenewicz et al., 2009). Recently, Th17 cells were also identified as an important pathogenic factor in the development of IBD (Gu et al., 2013) and massive infiltration of Th17 cells and elevated levels of Th17-related cytokines were detected in inflamed intestine (Monteleone et al., 2011b). Pharmacologically targeting Th1 and Th17 cells has been shown to ameliorate intestinal inflammatory conditions in IBD patients (Monteleone et al., 2011a; Neurath et al., 2002a).

In traditional Chinese medicine (TCM), Qing-dai (also known as indigo naturalis) has been widely used for treating various infectious and inflammatory diseases, such as enteritis, carbuncles, eczema, and psoriasis (Chiang et al., 2013; Xiao et al., 2013). Recent clinical studies have demonstrated Qing-dai treatment effectively ameliorated ulcerative proctitis and intractable UC (Suzuki et al., 2013; Yuan et al., 2009). However, the precise mechanisms behind its anti-colitis effects remain unknown. In Chapter 3, the data showed that QDP, a Qing-dai-predominant formula, decreased the frequency of CD4⁺ T cells and the mRNA expression of IFN-γ, IL-17A and RORγt in the colon of DSS-treated mice in vivo, and inhibited Th1 and Th17 differentiation in vitro. In Chapter 4, the data revealed that Qing-dai is mainly responsible for the anti-colitis effect of QDP against DSS-induced colitis. Therefore, we hypothesize that Qing-dai exhibits its anti-colitis effects through suppressing Th1
and Th17 responses. In the present study, the anti-colitis effects and underlying mechanisms of Qing-dai on DSS-induced colitis in mice were investigated.

5.2 Experimental design

The anti-colitis effects of Qing-dai were assessed in mice with DSS-induced colitis. The colitic mice were induced by 2% DSS for 5 days as described in Chapter 2 and randomly divided into 4 groups, with 8 mice in each group, and treated with 5% CMC-Na solution (Group 1), Sulfasalazine (SASP, 200 mg/kg/day, Group 2), Qing-dai (1.0 g/kg/day, Group 3) and Qing-dai (0.5 g/kg/day, Group 4). Mice in the control (Group 5, n =8) were treated with 5% CMC-Na solution only without DSS induction. The dosages of Qing-dai were set up as 0.5 and 1.0 g/kg/day according to the dosage of Qing-dai (1.0 g/kg/day) used in Chapter 4. During the experiments, body weight, diarrhea and bleeding were recorded daily. After 7 days of treatment, mice were sacrificed for colon sample collection. The mesenteric lymph nodes in each mouse were collected for flow cytometric analysis. The whole colon of each mouse was collected and divided into four parts: the proximal part of anus was fixed and embedded in paraffin for H&E staining, and the other three parts were collected for and RT-PCR analysis, MPO activity determination and cytokines production analysis. A flow diagram of the study is shown in Fig. 5.1A.
Fig. 5.1A
Study design for *in vivo* study of Chapter 5.

As for *in vitro* studies, T lymphocytes were dissociated from mesenteric lymph nodes. Further, CD4$^+$ T cells were isolated using L3T4 microbeads whereas naive CD4$^+$ T cells were sorted into CD4$^+$ CD25$^-$/CD45RB$^{high}$ T cells using anti-CD4, anti-CD25 and anti-CD45 antibodies. The proliferation of CD4$^+$ T cells was performed using $[^3]$H] thymidine incorporation assay. The differentiation of CD4$^+$ T cells were induced using different Th polarization culture conditions and analyzed using flow cytometry. A flow diagram of the study is shown in Fig 5.1 B.
Fig. 5.1B
Study design for in vitro study of Chapter 5.

5.3 Results

5.3.1 Qing-dai ameliorated the severity of DSS-induced colitis in mice

Mice orally treated with 2% DSS for 5 consecutive days developed typical symptoms of clinical colitis, including loss of body weight, diarrhea and rectal bleeding. By employing this model, we examined the therapeutic effect of indigo naturalis at two doses, 1 and 0.5 g/kg, on the established colitis. SASP, a well-characterized anti-colitis medicine, was used as a positive control. As shown in Table 5.1, the body weight of the DSS-treated mice was decreased by 5% on day 5 and 17.13% on day 12. The loss of body weight by DSS treatment was partially
reversed by the treatment with SASP or indigo naturalis, with a body weight loss of 12.9% and 12.4% respectively, which were significantly higher than that of DSS treatment alone (both \( p < 0.01 \)). Oral administration of indigo naturalis for 7 days also resulted in a marked reduction of the disease activity index (DAI), a clinical parameter reflecting the severity of weight loss, rectal bleeding and stool consistency, as compared with DSS treatment alone. In addition, the length of colon in DSS-treated mice was 6.86 cm, which was decreased by 16.70% as compared with control mice \( (p < 0.001) \). In contrast, the length of colon in SASP and indigo naturalis (0.5 and 1 g/kg) were 7.44, 7.33 and 7.36 cm, respectively, although still shorter than normal control mice, but significantly longer than DSS treatment alone \( (p < 0.01 \) for SASP and \( p < 0.05 \) for indigo naturalis). Therefore, indigo naturalis has a therapeutic effect on DSS-induced mouse colitis, and its efficacy was comparable to that of SASP.

DSS treatment also caused histopathological changes such as destruction of crypts, loss of epithelial and goblet cells, and infiltration of inflammatory cells, whereas indigo naturalis treatment partially restored the normal architecture of colon (Fig.5.2A and 5.2B). The colonic inflammation, as assessed by MPO activity, was 3.2-fold increase than untreated control mice \( (p<0.001) \), which was markedly reduced by the treatment with SASP and indigo naturalis (1g/kg) by 49.0% and 48.1% respectively (Fig.5.2C).
Table 5.1  Effects of indigo naturalis (QD) on body weight change, DAI score and colon length of DSS-treated mice (n= 6-8)

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight change (%)</th>
<th>DAI score</th>
<th>Colon length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 5</td>
<td>Day 12</td>
<td>Day 5</td>
</tr>
<tr>
<td>Control</td>
<td>100.23±2.14</td>
<td>100.19±1.27</td>
<td>-</td>
</tr>
<tr>
<td>DSS</td>
<td>95.03±1.91***</td>
<td>83.06±3.64###</td>
<td>9.63±0.52###</td>
</tr>
<tr>
<td>QD (1)</td>
<td>94.86±1.28</td>
<td>87.56±0.94**</td>
<td>9.50±0.53</td>
</tr>
<tr>
<td>QD (0.5)</td>
<td>94.77±2.03</td>
<td>85.45±2.37</td>
<td>9.38±0.52</td>
</tr>
<tr>
<td>SASP</td>
<td>94.59±0.93</td>
<td>87.10±4.27**</td>
<td>9.75±0.46</td>
</tr>
</tbody>
</table>

### p < 0.001, compared with control group; * p < 0.05 and ** p < 0.01, compared with DSS model group.
Fig. 5.2. Effects of indigo naturalis (QD) on colon length, colonic damage and MPO activity in DSS-induced colitis in mice. Colitis was induced by orally treated with 2% DSS for 5 days, followed by the treatment of indigo naturalis and SASP for 7 days. On the day of last treatment, mice were sacrificed and colons were harvested. (A) The representative photograph of histopathological changes of colons. The original amplification was 200×. (B) Histological score of colons. (C) Colonic MPO activity. All data are presented as means ± SEM (n = 6-8). ***p < 0.001, compared with control group; *p < 0.05, **p < 0.01 and ***p < 0.001, compared with DSS model group.
5.3.2 Qing-dai suppressed Th1- and Th17-characterized cytokines in the colon of DSS-treated mice

It has been reported that mice with colitis induced by DSS treatment showed increased expression of Th1- and Th17-characterized cytokines, similar to the cytokine profile found in human IBD (Tao et al., 2013). Consistent with this previously report, we observed an 4-5 folds increase of gene expression of IFN-γ and IL-17A, which was completely inhibited by Qing-dai (1 g/kg) treatment ($p < 0.05$ for IFN-γ and $p < 0.01$ for IL-17A, Fig 5.3A). The protein levels of IFN-γ, TNF-α and IL-17A/F in colon tissue were increased by 2-3-fold respectively, and elevated levels of these proinflammatory cytokines were completely inhibited by Qing-dai treatment (all $p < 0.01$) (Fig. 5.3B).

![Fig.5.3](image-url) Effects of Qing-dai (QD) on gene and protein expressions of Th1 and Th17-related cytokines in the colon of mice treated with DSS. Treatment of DSS and Qing-dai was described in Fig 5.2. On the day of last treatment, mice were sacrificed, colon was harvested. (A) Total RNA isolated from colon tissues was assessed by quantitative RT-PCR. Data shown is summary of mRNA levels of IFN-γ and IL-17A (fold increase vs actin). (B) Cytokine levels in homogenated colonic proteins were assessed by ELISA. Data shown is summary of cytokines (pg/ml). All data are presented as means ± SEM (n = 6-8). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, compared with control group; †$p < 0.05$ and ‡$p < 0.01$, compared with DSS model group.
5.3.3 Qing-dai reduced the proportions of Th1 and Th17 cells in the colon of DSS-treated mice

We also examined the effect of Qing-dai on the proportions of INF-γ-expressing and IL-17A-expressing cells in mesenteric lymph nodes, the draining lymph nodes of large intestine. As shown in Fig.5.4, the proportions of INF-γ-expressing and IL-17A-expressing cells in mesenteric lymph nodes were increased greater than 2-fold after DSS treatment. The treatment of Qing-dai completely inhibited the elevated levels of INF-γ ($p < 0.05$) and partially but significantly inhibited IL-17A-expressing cells ($p < 0.05$). In contrast, the proportion of CD4$^+$Foxp3$^+$ cells was not altered by the DSS-treatment and subsequent Qing-dai treatment (Fig. 5.4).

![Graph showing changes in cell proportions](image)

**Fig.5.4.** Effects of Qing-dai (QD) on the proportion of IFN-γ-expressing and
IL-17A-expressing cells in mesenteric lymph nodes of mice with DSS-induced colitis. Treatment of DSS and indigo naturalis was described in Fig 5.2. On the day of last treatment, mice were sacrificed and mesenteric lymph nodes were harvested. Single cell suspension was prepared, and cells were restimulated with PMA and inomycin in the presence of GolgiPlug for 5 hours. Expression of IFN-γ and IL-17A by CD4 cells was analyzed by FACS, gating on CD4+ cells. Data shown are typical flow dot plots. The summary of IFN-γ-expressing, IL-17A-expressing and Foxp3-expressing cells is shown on the right. The data are presented as means ± SEM (n = 6-8). *p < 0.05, ###p < 0.001, compared with control group; *p < 0.05, compared with DSS model group.

5.3.4 Qing-dai suppressed the differentiation of Th1 and Th17 cells in vitro

Whether Qing-dai inhibits the differentiation of Th1 cells and Th17 cells were determined by using an standard in vitro Th differentiation protocol (Zhou et al., 2014). We first determined the general effect of Qing-dai on the activation of T cells in response to TCR stimulation. As shown in Fig. 5.5A, Qing-dai (0-1000 μg/mL) did not inhibit proliferation of anti-CD3/CD28 Abs-stimulated CD4+ T cells, suggesting that at concentration lower than 1 mg/ml, Qing-dai was unlikely to have toxic effect on CD4+ T cells. We there examined the effect of Qing-dai at concentration of 250 and 500 μg/ml on the expression of IFN-γ and IL-17A by Th1- and Th17-polarized cells. As shown in Fig 5.5B, at 500 μg/ml, Qing-dai potently inhibited the proportion of IFN-γ-expressing cells (% of inhibition is 37.1%, p < 0.01), and at both 250 and 500 μg/ml, Qing-dai markedly inhibited the proportion of IL-17A-expressing cells by 34.2% and 42.2%, respectively (p < 0.01, Fig. 5.5B).
**Fig. 5.5.** Effects of Qing-dai (QD) on proliferation of CD4⁺ T cells, and on the differentiation of Th1 and Th17 cells *in vitro.* (A) Summary of proliferation of CD4⁺ cells to TCR stimulation. MACS-sorted CD4⁺ T cells from C57BL/6J mice were stimulated with APCs and anti-CD3 Ab, in the presence or absence of different concentrations of Qing-dai. After incubation for 72 hours, the cell proliferation was measured by [³H] thymidine assay. (B) Expression of IFN-γ and IL-17A. Flow-sorted naïve CD4⁺ T cells from C57BL/6J mice were cultured in Th1- and Th17-polarizing conditions in the presence or absence of different concentrations of Qing-dai for 3 days. After re-stimulation with PMA and inomycin with the presence of GolgiPlug, the expression of IFN-γ and IL-17A was analyzed by flow cytometry. Data shown are typical flow dot plots. The summary of CD4⁺INF-γ⁺ and CD4⁺IL-17A⁺ cells is shown on the right. All data are presented as means ± SEM. ***p < 0.001, compared
with control group; \( p < 0.05 \) and \( **p < 0.01 \), compared with none Qing-dai treated group. The representative data from three separate performances were shown.

### 5.3.5 Qing-dai suppressed the phosphorylation of p38 and ERK, and inhibited the degradation of IκB-α in the colon of DSS-treated mice

NF-κB plays a key role in the development of colitis, and the degradation of IκB-α protein is an essential step for its activation (Yamamoto and Gaynor, 2004). As shown in Fig. 5.6, DSS could significantly cause IκB-α degradation, and Qing-dai (1 g/kg) significantly suppress the degradation of IκB-α in the colon of test mice \( (p < 0.05) \). Mitogen-activated protein kinases (MAPKs) are major components to activate NF-κB, in turn to mediate IBD. We also examined that Qing-dai treatment significantly suppressed the phosphorylation of p38 and ERK in the colon of DSS-treated mice \( (both \ p < 0.05) \). As well, Qing-dai treatment could also repress the phosphorylation of JNK, but the difference was no significance.

![Fig. 5.6](image)

**Fig. 5.6.** Qing-dai (QD) inhibited the activation of MAPK and the degradation of IκB-α in the colon of DSS-treated mice. Colitis was induced by 2% DSS, and then Qing-dai was administered to mice from day 6 to day 12. On day 13, mice were sacrificed. The expression of IκB-α, p-p38, p-ERK, p-JNK and β-actin in the colon tissues was assessed by western blot and the relative densities were analyzed by Image J software. All data are presented as means ± SEM \( (n = 6-8) \). 

\[ * p < 0.05, ** p < 0.01, \text{and} *** p < 0.001, \text{compared with control group;} \]
\*p < 0.05, \**p < 0.01 and \***p < 0.001, compared with DSS model group. Abbreviation: QD(1), QD at dosage of 1 g/kg.

5.4 Discussion

In the present study, we demonstrated that Qing-dai attenuated the severity of DSS-induced colitis in mice, which is evidenced by increase of body weight, reduction of disease activity score, inhibition of colonic shortening, amelioration of colonic lesions and histological signs of damage, suppression of colonic MPO activity. Our data therefore verify indigo naturalis is an effective regimen for the treatment of IBD.

IBD is characterized by the imbalance of pro-inflammatory and anti-inflammatory cells in the gastrointestinal tract. Excessive activation of Teffs such as Th1 and Th17 cells play a key role in the pathogenesis of IBD by instigating inflammatory cascade. Consequently, it was proposed that attenuation of Th1 responses and Th17 responses reduced the severity of IBD (Liu et al., 2014). As a frequently used mouse model of human IBD, DSS-induced colitis shows an elevated levels of Th1 and Th17 cytokines (Tao et al., 2013). By using this model, we found that indigo naturalis potently suppressed the mRNA expression of Th1-cytokine IFN-γ and Th17-cytokine IL-17A, as well as the production of Th1- and Th17-related cytokines, namely IFN-γ, IL-17A/F and TNF-α in protein levels. Moreover, the proportion of both Th1 (CD4+IFN-γ+) and Th17 (CD4+IL-17A+) cells in CD4+ cells present in mesenteric lymph nodes was also markedly reduced after indigo naturalis treatment, however, the proportion of CD4+Foxp3+ cells was not altered. Further, we determined that in vitro differentiation of Th1 and Th17 cells were potently inhibited by indigo naturalis treatment. These observations suggest that the inhibition of Th1 and Th17 responses is likely to contribute to the anti-colitis effect of indigo naturalis.

It is well known that the accumulation of pro-inflammatory cytokines excessively in the gut can activate multiple intracellular signaling cascades including the
mitogen-activated protein kinase (MAPK) signal transduction pathway and NF-κB signaling pathway to trigger and maintain intestinal inflammation (Guo et al., 2014; Liu et al., 2014). The inhibition of Th1 and Th17 differentiation resulting in the decrease of Th1/Th17-related cytokines such as IFN-γ, IL-17A/F and TNF-α might be accounted for the suppression of NF-κB and MAPK pathways by indigo naturalis treatment. For the reasons, on the one hand, NF-κB signaling pathway could be activated by IFN-γ, IL-17A/F and TNF-α (Rimbach et al., 2000; Sønder et al., 2011; Schutze et al., 1995); on the other hand, MAPKs are major components to activate NF-κB, and these pro-inflammatory cytokines could also stimulate the phosphorylation of MAPK (ERK1/2, p38 or JNK) in various different cells (Iyoda et al., 2010; Moron et al., 2013; Mouzaoui et al., 2014). In the present study, the data demonstrated that indigo naturalis treatment significantly suppressed the phosphorylation of p38 and ERK, and degradation of IκB-α in colonic tissue of DSS-treated mice, which confirmed the efficacy of indigo naturalis in regulation of intestinal inflammation.

5.5 Limitation

It is well known that the cytokines stimulating the Janus kinase (Jak) and signal transducer and activator of transcription (STAT) pathways play important roles in regulating the development of helper T cell subsets (Ortmann et al., 2000). For instance, IFN-γ and IL-12 binds to their receptors, which results in the autophosphorylation and activation of Jak2, and in turn activates STAT1 and STAT4 respectively, and then induce the expression of the Th1 transcription factor T-bet to facilitate Th1 differentiation (Horvath, 2004). Similarly, IL-6 binds to its receptors IL-6R, which results in activation of Jak, and in turn phosphorylates and activates STAT3 to control Th17 cell differentiation (Hirahara et al., 2010). In the present study, we demonstrate that Qing-dai treatment attenuates DSS-induced colitis through inhibition Th1 and Th17 cell differentiation. However, the underlying
mechanisms whether Qing-dai inhibits STAT1, STAT3 and STAT4 directly or indirectly to control Th1 and Th17 cell differentiation remains to be clarified.

5.6 Summary

In conclusion, the present study demonstrates that Qing-dai exhibits potent therapeutic effect on DSS-induced colitis, which are associated with suppression of Th1 and Th17 cell responses. These findings support that Qing-dai is an effective regimen for the treatment of IBD.
CHAPTER 6

INDIRUBIN SUPPRESSES TH17 DIFFERENTIATION THROUGH INHIBITION OF GSK-3β SIGNALING IN DSS-INDUCED COLITIS
6.1 Introduction

Glycogen synthase kinase-3 (GSK-3), a multi-tasking serine/threonine kinase, having the GSK-3α and GSK-3β isoforms, functions in a wide range of cellular processes such as cellular proliferation, migration, inflammation and immune responses, glucose regulation, and apoptosis (Forde and Dale, 2007; Jope et al., 2007). Among the diversified functions of GSK-3, a large body of studies had been carried out in the aspect of inflammation. It has been reported that GSK-3 promoted the production of pro-inflammatory cytokines, such as IL-6, IL-1β, and TNF-α, and reduced the production of anti-inflammatory cytokine IL-10 in multiple inflammatory conditions, such as sepsis, arthritis, and multiple sclerosis (Cortes-Vieyra et al., 2012). In colitis, increased GSK-3β activity was observed in TNBS- and DSS-induced models, and inhibition of GSK-3β signaling has been reported to reduce experimental colitis (Hofmann et al., 2010; Whittle et al., 2006). Recently, a study reported that inhibition of GSK-3β could blocked the generation of Th17 cells in vitro and in vivo, indicating that GSK-3β is a critical mediator of Th17 cell generation (Beurel et al., 2011). Therefore, inhibition of GSK-3β activity is a potential target for therapeutic intervention of Th17-mediated diseases.

As described in Chapter 5, we demonstrated that Qing-dai attenuates DSS-induced colitis through inhibiting Th1/Th17 differentiation. However, it is unclear that what are major active compounds in this herbal medicine contributing to these effects? In Chapter 4, we analyzed the major components of Qing-dai using UPLC-QTOF-MS and identified 23 compounds, in which 10H-indolo[3,2-b]quinoline-11-carboxylic acid amide, indigo, indirubin and sitosterol were identified as major compounds. Previous studies reported that indirubin is the major active ingredient of Qing-dai and exerts a wide range of biological activities against cancer, neurodegeneration, and protozoan infections through inhibiting the activities of cyclin-dependent kinase (CDK) or GSK-3β, and activating aryl hydrocarbon receptor (AHR) (Sugihara et al., 2004; Vougogiannopoulou and Skaltsounis, 2012). Study of Leclerc et al reported
that indirubin is a GSK-3β inhibitor and IC$_{50}$ is 600 nM (Leclerc et al., 2001). Therefore, we hypothesized that indirubin can inhibit Th17 cell generation and ameliorate intestinal inflammation through inhibition of GSK-3β signaling. In the present study, effects and underlying mechanisms of indirubin on Th17 cell generation in DSS-induced colitis in mice were investigated.

6.2 Experimental design

Similar to the study described in Chapter 5, the effect of indirubin on Th17 differentiation was also examined using the DSS-induced colitis mouse model. Firstly, the colitic mice were induced by 2% DSS, randomly divided into 4 groups, with 8 mice in each group, and treated with 5% CMC-Na solution (Group 1), lithium chloride (LiCl, 200 mg/kg/day, Group 2), indirubin (50 mg/kg/day, Group 3) and indirubin (100 mg/kg/day, Group 4). The vehicle control group (Group 5, n=6) received drinking water without DSS and treated with 5% CMC-Na solution during the experimental period. Indirubin was orally administered to mice at 50 and 100 mg/kg/day according to our previous experiment. GSK-3β inhibitor, LiCl was selected as a reference positive agent and was used at a dosage of 200 mg/kg/day (i.p) according to the literatures (Hofmann et al., 2010; Uddin et al., 2013). During the experiments, body weight, severity of diarrhea and fecal bleeding were recorded daily. After 7 days of treatment, mice were sacrificed for sample collection. The mesenteric lymph nodes in each mouse were collected for Western blotting and flow cytometric analysis. The whole colon of each mouse was collected and divided into four parts: the proximal part of anus was fixed and embedded in paraffin for H&E staining, and the other three parts were collected for and RT-PCR analysis, MPO activity determination and cytokine production analysis successively. A flow diagram of the study is shown in Fig. 6.1A.
Fig. 6.1A

Study design for *in vivo* study of **Chapter 6**.

As for *in vitro* studies, murine primary CD4$^+$ T cells were isolated using L3T4 microbeads and naïve CD4$^+$ T cells were sorted into CD4$^+$CD25$^+$CD45RBhi T cells using anti-CD4, anti-CD25 and anti-CD45 antibodies or the CD4$^+$CD62L$^+$ T Cell isolation kit II. The proliferation of CD4$^+$ T cells was determined using $[^{3}H]$ thymidine incorporation assay, and naïve CD4$^+$ T cells were differentiated in Th17 polarization culture condition and analyzed by means of flow cytometry. The expression of GSK-3β and p-STAT-3 in Th17 differentiation were detected on immunoblots. A flow diagram of the study is shown in **Fig 6.1 B**.
6.3 Results

6.3.1 Indirubin ameliorated the severity of DSS-induced colitis in mice

DSS-induced colitis is one of the most common chemically induced colitis models, which well resembles the acute phase of human UC. Oral administration of 2% DSS drinking water resulted in a rapid loss of body weight, and serious clinical disease symptoms (diarrhea and occult fecal blood) in C57BL/6J mice. As shown in Table 6.1, the body weight of the DSS-treated mice was decreased by 4.23% on day 5 and 17.76% on day 12. The loss of body weight by DSS treatment was partially reversed by the treatment with LiCl or indirubin (100 mg/kg), with a body weight loss of 13.47% and 14.07% respectively, which were significantly higher than that of DSS.
treatment alone (both $p < 0.05$). As well, oral administration of indirubin at the dosage of 100 mg/Kg for 7 days also significantly suppressed disease activity index (DAI) of DSS-treated mice at day 12 ($p < 0.01$). In addition, the colon length in DSS-treated mice was 7.34 cm, which was decreased by 16.70% as compared with control mice ($p < 0.001$), and oral administration of LiCl and indirubin (50 and 100 mg/kg) greatly reversed this decrease with the length of 8.17, 7.99 and 8.11 cm, respectively ($p < 0.05$ for both LiCl and indirubin at dosage of 100 mg/kg).

DSS-induced colitis led to some typical inflammatory changes, including mucosal ulceration, crypt damage, edema, and cell infiltration in the mucosal tissue. As shown in Fig. 6.2A, indirubin notably attenuated these histopathological manifestations. From the H&E images, we observed that the indirubin-treated mice showed a markedly lower degree of crypt destruction and inflammatory cell infiltration when compared to the control mice. Moreover, the histological scores were substantially decreased in the indirubin-treated groups ($p < 0.05$ and $p < 0.01$, Fig. 6.2B). In agreement with the histopathological changes, colonic MPO activity in the DSS model group was greatly increased, whereas this increased colonic MPO activity was significantly depressed after indirubin treatment (Fig. 6.2F).
## Table 6.2 Effects of Indirubin (Ind) on body weight change, DAI score and colon length of DSS-treated mice (n= 6-8)

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight change (%)</th>
<th>DAI score</th>
<th>Colon length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 5</td>
<td>Day 12</td>
<td>Day 5</td>
</tr>
<tr>
<td>Control</td>
<td>100.69±1.67</td>
<td>101.52±1.14</td>
<td>-</td>
</tr>
<tr>
<td>DSS</td>
<td>95.77±1.89***</td>
<td>82.24±1.79***</td>
<td>8.50±0.71***</td>
</tr>
<tr>
<td>Ind (50 mg/kg)</td>
<td>94.90±2.25</td>
<td>85.02±2.95</td>
<td>8.63±0.52</td>
</tr>
<tr>
<td>Ind (100 mg/Kg)</td>
<td>95.59±1.51</td>
<td>85.93±1.42**</td>
<td>8.36±0.52</td>
</tr>
<tr>
<td>LiCl (200 mg/kg)</td>
<td>96.03±1.73</td>
<td>86.53±4.42</td>
<td>8.50±0.53</td>
</tr>
</tbody>
</table>

***p < 0.001, compared with control group; *p < 0.05 and **p < 0.01, compared with DSS model group.
Fig. 6.2. Effects of indirubin (Ind) on colon length, colonic damage and colonic MPO activity in DSS-treated mice. Colitis was induced by orally treated with 2% DSS for 5 days, followed by the treatment of indirubin and LiCl for 7 days. On the day of last treatment, mice were sacrificed and colon was harvested. (A) The representative photograph of histopathological changes of colons. The original amplification was 200×. (B) Histological score of colons. (C) Colonic MPO activity. All data are presented as means ± SEM (n = 6-8). 

***p < 0.001, compared with control group; *p < 0.05, **p < 0.01 and ***p < 0.001, compared with DSS model group.
6.3.2 Indirubin suppressed Th17-related cytokines in the colon of DSS-treated mice

As shown in Fig. 6.3, real-time PCR analysis showed that the mRNA expression of Th1/Th17 cytokines and transcription factors such as IFN-γ, IL-17A, T-bet and RORγt, were remarkably induced by DSS; and Th17-related cytokines including TNF-α, IL-1β, IL-6 and IL-23, were also substantially increased. In contrast, these DSS-increased mRNA expressions were significantly decreased upon the indirubin treatment. By means of ELISA, the specific cytokines of Th1 and Th17 cells, IFN-γ and IL-17A/F, and other Th1/Th17-related cytokines including TNF-α, IL-1β and IL-6 in the colon were detected with remarkable increases in the colonic tissues of the DSS-treated mice; and indirubin significantly suppressed the production of pro-inflammatory cytokines IL-17A/F, TNF-α, IL-1β and IL-6 (p < 0.05 and p < 0.01, Fig. 6.4). However, the levels of TGF-β in the colon tissues of DSS-treated mice did not differ significantly from the control or the indirubin treatment groups.
Fig. 6.3. Indirubin regulated mRNA expression of Th1- and Th17- cytokines and transcription factors in the colon of mice with DSS-induced colitis. Colitis was induced by 2% DSS, and then indirubin and LiCl were administered to mice from day 6 to day 12. On day 13, mice were sacrificed. Total RNA isolated from colon tissues was assessed by quantitative RT-PCR (A. IL-17A; B. RORγt; C. IFN-γ; D. T-bet; E. IL-1β; F. IL-6; G. TNF-α; H. IL-23). All data are presented as means ± SEM (n = 6-8). *p < 0.05, **p < 0.01, and ***p < 0.001, compared with control group; *p < 0.05, **p < 0.01 and ***p < 0.001, compared with DSS model group.
Fig. 6.4. Indirubin regulated the production of Th1- and Th17- cytokines in the colon of mice with DSS-induced colitis. Colitis was induced by 2% DSS, and then indirubin and LiCl were administered to mice from day 6 to day 12. On day 13, mice were sacrificed. Cytokine levels in homogenated colonic proteins were assessed by ELISA (A. IL-1β; B. TGF-β; C. TNF-α; D. IL-6; E. IL-17A/F; F. INF-γ). All data are presented as means ± SEM (n = 6-8). *p < 0.05, **p < 0.01, and ***p < 0.001, compared with control group; *p < 0.05, **p < 0.01 and ***p < 0.001, compared with DSS model group.

6.3.4 Indirubin reduced the proportions of Th1 and Th17 cells in mesenteric lymph nodes of DSS-treated mice

Th17 cells display context-dependent plasticity, as they can be differentiated from Tregs or/and acquire functional characteristics of Th1 cells (Muranski and Restifo, 2013). We therefore examined the effects of indirubin on the proportions of Th1, Th17 and Treg cells in mesenteric lymph nodes. As shown in Fig. 6.5, the proportions of Th1 and Th17 cells in mesenteric lymph nodes were remarkably increased after DSS treatment. In contrast, the increased Th17 cells were
significantly decreased after indirubin administration (p<0.05), but the decrease of Th1 cells was not statistically significant. However, the proportion of Tregs (CD4⁺Foxp3⁺) cells was not altered by the DSS challenge or the indirubin treatment.

**Fig. 6.5.** Effects of indirubin on the proportions of Th1 and Th17 cells in mesenteric lymph nodes of mice with DSS-induced colitis. (A) Typical flow dot plots of Th17, Th1 and Tregs in mesenteric lymph nodes. (B) The summary of Th17 cells. (C) The summary of Th1 cells. (D) The summary of Tregs. Colitis was induced by 2% DSS, and then indirubin and LiCl was administered to mice from day 6 to day 12. On day 13, mice were sacrificed. The Th1, Th17 and Tregs cells in mesenteric lymph nodes of mice with DSS colitis were detected by flow cytometry. The data are presented as means ± SEM (n = 6-8). *p < 0.05, **p < 0.001,
compared with control group; *$p < 0.05$ and **$p < 0.01$, compared with DSS model group.

### 6.3.5 Indirubin up-regulated GSK-3β phosphorylation and reduced STAT3 phosphorylation in mesenteric lymph nodes of DSS-treated mice

Previous studies showed that GSK-3β is a critical mediator of Th17 cell generation (Beurel et al., 2011), and its activity is regulated negatively by the phosphorylation of serine 9 (p-GSK-3β (s9)), we therefore examined the effects of indirubin on the expression of GSK-3β and p-GSK-3β (s9) in mesenteric lymph nodes. As shown in Fig. 6.6, the p-GSK-3β(s9) was greatly down-regulated after DSS challenge, but both indirubin and GSK-3β inhibitor LiCl treatment markedly stimulated phosphorylation of GSK-3β. On the contrary, DSS treatment slightly increased GSK-3β expression and this increase was reversed by indirubin or GSK-3β inhibitor LiCl treatment without significant difference. In addition, STAT3 is a critical transcription factor for Th17 differentiation and directly regulates the locus encoding IL-17 (Chen et al., 2007). Previous studies showed that inhibition of GSK-3β suppresses the activation of STAT3 (Cortes-Vieyra et al., 2012). We further examined the effects of indirubin on STAT3 expression in mesenteric lymph nodes. Consistent with the frequency of Th17 cells in mesenteric lymph nodes, both indirubin and GSK-3β inhibitor LiCl treatment significantly inhibited DSS-induced the phosphorylation of STAT3 in mesenteric lymph nodes of mice.
Fig. 6.6. Effects of indirubin on the expression of p-GSK-3β (s9), GSK-3β, p-STAT3, and STAT3 in mesenteric lymph nodes of mice with DSS-induced colitis. (A) The representative photograph of Western blot analysis of p-GSK-3β (s9), GSK-3β, p-STAT3 (y705), and STAT3 in mesenteric lymph nodes. (B) Summary of expression of p-GSK-3β (s9), GSK-3β, p-STAT3 (y705), and STAT3 in mesenteric lymph nodes. Colitis was induced by 2% DSS, and then indirubin and LiCl was administered to mice from day 6 to day 12. On day 13, mice were sacrificed. The expressions of p-GSK-3β (s9), GSK-3β, p-STAT3 (y705), and STAT3 in mesenteric lymph nodes were assessed by western blot and the relative densities were analyzed by Image J software. The data are presented as means ± SEM (n = 6-8). *p < 0.05, **p < 0.01, ***p < 0.001, compared with control group; *p < 0.05 and **p < 0.01, compared with DSS model group.

6.3.6 Indirubin suppressed the differentiation of Th17 cells via influencing the GSK-3β signaling in vitro

To confirm the in vivo observation that indirubin suppressed the frequency of Th17 cells in mesenteric lymph nodes of DSS-treated mice, we examined the effects of indirubin on CD4⁺ T cell proliferation and Th17 cell differentiation in vitro. The
results showed that indirubin at the concentration below 100 μM did not show any significant effect on CD4⁺ T cell proliferation (Fig. 6.7A). At the concentration of 25 μM and 50 μM, however, indirubin significantly inhibited Th17 cell differentiation ($p<0.05$ and $p<0.01$) (Fig. 6.7B). Subsequently, we examined the effects of indirubin on GSK-3β signaling in Th17 cell polarization. We found that both indirubin and GSK-3β inhibitor LiCl treatments significantly suppressed GSK-3β expression and phosphorylation of STAT3 in Th17 cell differentiation (Fig. 6.7C).

Fig. 6.7. Indirubin suppressed Th17 cell differentiation via influencing the GSK-3β signaling in vitro. (A) CD4⁺ T cells from C57BL/6 mice were incubated for 72 h at 37 °C in the presence or absence of indirubin with stimulation of radiated APCs and CD3 Ab. Cell proliferation was measured by [3H] thymidine assay. (B and C) Naïve CD4⁺ T cells from C57BL/6 mice were stimulated with CD3/CD28 Abs and incubated under Th17-polarizing conditions in the presence or absence of indicated concentrations of indirubin and LiCl for 3 days. The phenotypes of Th1 and Th17 cells were analyzed by flow cytometry. (B) Typical flow dot plots of Th17 cell differentiation; (C) Summary of Th17 cells. (D-F). CD4⁺ T cells
from C57BL/6 mice were incubated under Th17-polarizing conditions in the presence or absence of indicated concentrations of indirubin and LiCl with CD3/CD28 stimulation for 3 days. Protein levels were assessed by western blot, and the relative densities were analyzed using the Image J software. (D) The representative photograph of Western blot analysis of GSK-3β and p-STAT3 (y705) in Th17 cells; (E) Summary of expression of GSK-3β in Th17 cells; (F) Summary of expression of p-STAT3 (y705) in Th17 cells. All data are presented as means ± SEM. *p < 0.05, ### p < 0.001, compared with control group; †p < 0.05 and ††p < 0.01, compared without indirubin or LiCl treated group.

**6.4 Discussion**

In the present study, we demonstrated that indirubin attenuated the severity of DSS-induced colitis in mice. Generally, oral administration of indirubin at the doses of 50 and 100 mg/kg significantly attenuated disease activity index score, and inhibited colon shortening. Meanwhile, indirubin administration markedly prevented colonic lesions and histological signs of damage, and reduced colonic MPO activity. These findings showed the potential beneficial effect of indirubin on DSS-induced colitis.

According to the classical immune paradigm of IBD, CD was considered as a Th1-mediated response while UC was a Th2-type-mediated inflammation. However, some studies also revealed that high ex vivo levels of IFN-γ were observed in UC patients (Wallace et al., 2014), indicating Th1 cells participate in the pathogenesis of both CD and UC. Recently, a novel subset of helper T cells, the Th17 cells, was also identified as an important player in the pathogenesis of both CD and UC (Monteleone et al., 2011a). DSS-induced acute colitis is a well-established animal model, which displays a predominant cytokine pattern of Th1/Th17 type closely resembles the human IBD conditions (Tao et al., 2013). In the present study, we demonstrated that indirubin greatly suppressed the mRNA expression of Th1/Th17
cytokines and transcription factors such as IFN-γ, IL-17A and RORγt in the colon of DSS-treated mice, as well as Th17 related cytokines including TNF-α, IL-1β, IL-6 and IL-23. The ELISA assays also revealed that indirubin significantly inhibited the production of Th17-related cytokines such as IL-17A/F, TNF-α, IL-1β and IL-6 in the colon of DSS-treated mice. Moreover, it also reduced Th1-related cytokine IFN-γ in the colon, though significant difference was not achieved when comparison to the DSS-treated mice. IL-17A/F and TNF-α are Th17 secreted cytokines, whereas IL-1β and IL-6 are activators of Th17 cell differentiation. These decreased cytokines in the colon of DSS-treated mice after indirubin treatment prompted us to examine the changes of Th17 cells in mesenteric lymph nodes, and found Th17 (CD4+IL-17A+) cells in mesenteric lymph nodes of DSS-treated mice were significantly decreased after indirubin treatment. This observation implied that decreased the production of colonic IL-17A/F and TNF-α may be attributed to the decreased numbers of Th17 cells in mesenteric lymph nodes to migrate into the colon. Subsequently, we examine the effect of indirubin on the proliferation of CD4+ T cells and the differentiation of Th17 cells to identify whether indirubin can inhibit the generation of Th17 cells directly, and found that indirubin did not influence the proliferation of CD4+ T cells, but significantly suppressed the differentiation of Th17 cells in vitro. Taken together, these data clearly revealed that indirubin attenuates DSS-induced colitis at least partly through inhibiting Th17 differentiation.

GSK-3β is a constitutively active serine/threonine protein kinase that is involved in a large number of cellular functions and is capable of regulating various transcription factors such as NF-κB, STAT1 and STAT3, etc in the proinflammatory immune responses (Cortes-Vieyra et al., 2012). GSK-3β activity is inhibited by phosphorylation of serine 9 residue. Previous studies reported that inhibition of GSK-3β signaling could significantly reduce experimental colitis (Hofmann et al., 2010; Whittle et al., 2006), and in vivo inhibition of GSK-3β in mice could deplete constitutive Th17 cells in intestinal mucosa (Beurel et al., 2011). In the present study, we confirmed that GSK-3β inhibitor LiCl significantly attenuated the severity of
DSS-induced colitis in mice and suppress the generation of Th17 cells *in vivo* and *in vitro*. As indirubin is also considered as a potent GSK-3β inhibitor, we further examined the expression of GSK-3β and p-GSK-3β (s9) in mesenteric lymph nodes of DSS-treated mice. The results showed that the expression of GSK-3β was slightly decreased but the expression of p-GSK-3β(s9) was significantly increased after indirubin treatment, indicating indirubin could decrease the activity of GSK-3β. While GSK-3β specifically promotes Th17 differentiation, STAT3 is a critical transcription factor for Th17 differentiation. We therefore further detected the effects of indirubin on the expression of STAT3 and p-STAT3 in mesenteric lymph nodes and found that indirubin significantly suppressed p-STAT3 expression but not STAT3, which was consistent with previous studies that inhibition of GSK-3β reduced the phosphorylation of STAT3 (Beur el and Jope, 2008; Chang et al., 2013). In addition, the study *in vitro* also found that indirubin greatly inhibited GSK-3β and p-STAT3 expression in the differentiation of Th17 cells. These findings indicated that indirubin suppresses Th17 cells differentiation via influencing GSK-3β signaling.

**6.5 Summary**

In conclusion, the present study demonstrates that indirubin exhibits potent therapeutic effect on DSS-induced colitis, which might be associated with the suppression of Th17 cell differentiation via influencing the GSK-3β signaling. It can be concluded that indirubin is useful in the prevention or treatment of colitis.
CHAPTER 7

CONCLUSION AND PROSPECTS
7.1 Conclusion

The present study has investigated the anti-colitis effect and underlying mechanism of Qing-dai powder (QDP), Qing-dai, and indirubin (the principle component of Qing-dai) in mice with DSS-induced colitis. These findings represent the evidence-based support for the clinical use of QDP in the management of UC, verify the rationality of QDP, and evidence that Qing-dai and indirubin are potent candidates for treating intestinal inflammation.

7.1.1 QDP promoted the recovery of colitis and reduced inflammatory responses of colonic macrophages and CD4\(^+\) T cells in DSS-treated mice

Results demonstrated that QDP treatment dose-dependently ameliorated disease activity index, prevented colon shortening and colonic tissue damage, and reduced colonic MPO activity in the DSS-treated mice, indicating the potential beneficial effect of QDP on DSS-induced colitis. Moreover, we found that the infiltration of immune cells, particularly macrophages and CD4\(^+\) T cells significantly increased in the colon of DSS-treated mice when compared to those of the normal control group. QDP treatment significantly suppressed the infiltration of macrophages and CD4\(^+\) T cells in DSS-treated mice. Remarkable increases of pro-inflammatory cytokines TNF-\(\alpha\), IL-6, and IL-1\(\beta\) were observed in the colon of DSS-treated mice, and these pro-inflammatory cytokines were decreased after QDP treatment. Furthermore, elevated MCP-1 levels were thus observed in the DSS-treated mice. QDP treatment significantly decreased the serum levels of MCP-1 in a dose-dependent manner. Moreover, in RAW 264.7 cells, the cultured murine macrophages, QDP (1 \(\mu\)g/mL) significantly suppressed LPS-induced production of TNF-\(\alpha\) and IL-6 and the expression levels of COX-2 and iNOS via inhibiting the degradation of I\(\kappa\)B-\(\alpha\) and the nuclear translocation of NF-\(\kappa\)B p65. In CD4\(^+\) T cells, QDP also significantly suppressed the differentiation of Th1 and Th17 cells. These data suggest that both
macrophages and CD4$^+$ T cells, particularly Th1 and Th17 cells, play important roles in the development of DSS-induced colitis. Meanwhile, these results also indicate that the anti-colitis effects of QDP on DSS-induced colitis are associated with inhibition of inflammatory responses of colonic macrophages and CD4$^+$ T cells.

7.1.2 Qing-dai is mainly responsible for the anti-colitis effect of QDP, and its beneficial effect is associated with suppression of Th1 and Th17 differentiation

QDP is composed of Qing-dai (also known as indigo naturalis) and Ku-fan (also known as dried alum). In the present study, we comparatively studied the anti-colitis effect of QDP, Qing-dai and Ku-fan. Results showed that Qing-dai is the major herb responsible for the anti-colitis effect of QDP, which significantly attenuated the severity of DSS-induced colitis in mice as evidenced by the attenuated disease activity index score, inhibited colonic shortening, ameliorated colonic lesions and histological signs of damage and reduced colonic MPO activity. However, Ku-fan exhibits a very weak beneficial effect on DSS-induced colitis. These data reveal that QDP is a reasonable TCM formula, and reveal Qing-dai is mainly responsible for the anti-colitis effect of QDP. Subsequently, we investigated the underlying mechanisms of Qing-dai against DSS-induced colitis in mice and found that oral administration of Qing-dai markedly inhibited the development of colitis in DSS-treated mice. DSS treatment increased the expression of IFN-$\gamma$ and IL-17A in both gene and protein levels by 2~5-fold in the colon tissue and increased the proportion of IFN-$\gamma$-expressing and IL-17A-expressing cells in mesenteric lymph nodes, the draining lymph nodes of intestines. The up-regulation of IFN-$\gamma$ and IL-17A levels in DSS-treated mice was completely blocked by Qing-dai, while the proportion of Foxp3$^+$ regulatory T cells remained unchanged. Furthermore, in vitro differentiation of Th1 and Th17 cells was also markedly inhibited by Qing-dai. These findings clearly indicate that Qing-dai had the capacity to inhibit colitic damage by dampening proinflammatory Th1 and Th17 responses, which may be a mechanistic
basis of Qing-dai in the treatment of IBD.

7.1.3 Indirubin suppressed the differentiation of Th17 cells in DSS-induced colitis via influencing the GSK-3β signaling

Indirubin is the principle active ingredient of Qing-dai. Data from this present study showed that oral administration of indirubin at the doses of 50 and 100 mg/kg significantly attenuated the disease activity index scores and colon shortening. Meanwhile, indirubin administration also markedly prevented colonic lesions and histological signs of damage, and reduced colonic MPO activity. Moreover, we found that indirubin greatly suppressed the mRNA expression of Th1/Th17 cytokines and transcription factors such as IFN-γ, IL-17A and RORγt, as well as Th17-related cytokines including TNF-α, IL-1β, IL-6 and IL-23 in the colon of DSS-treated mice. Indirubin also significantly inhibited the production of Th17-related cytokines such as IL-17A/F, TNF-α, IL-1β and IL-6 in the colon of DSS-treated mice. In addition, indirubin significantly reduced Th17 cells and reciprocally reduced Th1 cells in mesenteric lymph nodes. These findings suggest that indirubin suppressed the generation of Th17 cells in mice with DSS-induce colitis.

Indirubin is a potent GSK-3β inhibitor, and GSK-3β activity was significantly elevated in experimental colitis. In the present study, the data showed that indirubin significantly up-regulated GSK-3β phosphorylation and suppressed p-STAT3 expression in mesenteric lymph nodes of the DSS-treated mice. Our In vitro data also showed that both indirubin and GSK-3β inhibitor LiCl significantly suppressed Th17 cell differentiation via suppressing the expression of GSK-3β and p-STAT3. These findings clearly revealed that indirubin suppressed Th17 cell differentiation in DSS-induced colitis via influencing the GSK-3β signaling, and suggested that indirubin is a potent agent for treating colitis.
7.2 Prospects

Based on the findings from this present study, the following investigations are proposed for further exploration.

7.2.1 The underlying mechanisms of Qing-dai and indirubin on macrophages in colitis

It is well known that both innate and adaptive immune responses are involved in the development of IBD (Blumberg, 2009). Macrophages are the essential effector cells of innate immune system responsible for host defense. Evidence from various experimental models of mucosal inflammation showed that activation of macrophages plays a crucial role in the pathogenesis of IBD. For instance, upon macrophage activation, they produced excessive proinflammatory cytokines such as TNF-α, IL-1β and IL-6 in the inflamed sites, which mediate Th17 cell differentiation (Grip et al., 2003). Moreover, in the development of inflammation, the intracellular signaling molecules are dysregulated, such as GSK-3β, which is activated and AHR is dampened (Huai et al., 2014; Wang et al., 2014). It has been reported that GSK-3β promotes NF-κB transcriptional activity (Wang et al., 2014); and AHR inhibits NF-κB transcription activity to reduce IL-1β production in an inflammasome-independent manner (Huai et al., 2014). In the present study, we demonstrated that indirubin dampens the production of IL-6, IL-1β and TNF-α in the colon of DSS-treated mice. It is well known that indirubin, the major component of Qing-dai, is a GSK-3β inhibitor and AHR ligand (Sugihara et al., 2004; Vougogiannopoulou and Skaltsounis, 2012). However, whether Qing-dai or indirubin suppress the production of proinflammatory cytokines such as IL-6, IL-1β and TNF-α in the colon of DSS-treated mice through inhibition of GSK-3β signaling and activation of AHR signaling of macrophages synergistically remains unknown.
Therefore, further studies are needed in the future to verify proposed regulatory mechanisms of Qing-dai and indirubin on macrophages.

7.2.3 The beneficial effects and underlying mechanisms of Qing-dai and indirubin on chronic colitis and colitis-associated colorectal cancer

IBD is a chronic inflammatory disorder in the gastrointestinal tract, which is a well-established risk factor of colorectal cancer. It is well known that the inflammation stems from the imbalance of pro-inflammatory and anti-inflammatory cytokines. Massive effector Th17 cells infiltrated in the mucosa produce excessive amount of Th17 cytokines, such as IL-17A, IL-17F, IL-21 and IL-26, which contribute to the imbalance and mediate inflammatory responses (Singh et al., 2014). It has been reported that elevated levels of Th17-related cytokines IL-17A, IL-17F and IL-26 were detected in the inflamed intestine of IBD patients, and inhibition of Th17 cell generation and Th17-mediated inflammatory responses have been verified to be aid to mitigate intestinal inflammation (Monteleone et al., 2011b). Moreover, elevated levels of Th17 cytokines were also found in human colorectal cancers (CRCs) (De Simone et al., 2013). Numerous animal studies showed that these cytokines facilitated the survival and growth of CRC cells, and Th17 cytokine blockers exerted potent antitumor effects in several CRC models (Chae et al., 2010; De Simone et al., 2013). Accordingly, targeting effector Th17 cells to reduce Th17 cytokine-mediated inflammatory responses might be a potent approach to control intestinal inflammation and prevent colitis-associated colorectal cancer. In the present study, we demonstrated both Qing-dai and indirubin inhibit Th17 cell differentiation. In addition, previous studies also reported that indirubin exerts potent anti-tumor activities in several cancer models (Braig et al., 2013; Nam et al., 2012; Shi and Shen, 2008); however, it has not been reported the effects of indirubin and Qing-dai on colitis-associated colorectal cancer. Considering IBD as a chronic inflammatory condition in patients, which is high associated with the occurrence of
CRCs, it is noteworthy to investigate the beneficial effects of Qing-dai and indirubin on chronic colitis and colitis-associated colorectal cancer and their regulatory mechanism on Th17 cells in chronic inflammation or tumor development.

7.2.3 Development of Qing-dai or indirubin-based pharmaceutic preparation in treatment of IBD

As the etiology of IBD remains unclear and no specific effective treatment is available, the development of new drugs remains an unmet medical need in the treatment of IBD. Qing-dai, a Chinese herbal medicine, is a common used herb presented in many successful TCM formulas for UC treatment; however, there is no scientific evidence to support its anti-colitis effect. In the present study, we demonstrated that Qing-dai and its active component indirubin significantly attenuate the severity of DSS-induced colitis in mice, indicating that Qing-dai and indirubin are potent candidates for IBD treatment. To analyze the component of Qing-dai using UPLC-QTOF-MS, we revealed that indigo, indirubin, sitosterol and 10H-indolo [3,2-b] quinoline-11-carboxylic acid amide are the major components of Qing-dai. Previous and our studies showed that indigo, indirubin, and sitosterol are active anti-colitis components (Kim et al., 2014; Lee et al., 2012), and 10H-indolo [3,2-b] quinoline-11-carboxylic acid amide is a potent anti-tumor compound (Brain et al., 2005). Accordingly, it is reasonable to speculate that the standard extract of Qing-dai included abovementioned four compounds represents a potent remedy, which can be developed as a useful anti-colitis pharmaceutical used in clinics. However, more studies are needed in the future. As well, indirubin is a potent candidate for development of anti-colitis pharmaceutical to be used in clinics. However, it is insoluble in water, which results in unfavorable pharmacokinetic properties in animals and human (Chen et al., 2012). Thus, to increase the bioavailability of indirubin in vivo is a big challenge, which deserves further investigation.


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Published full papers:


7. Hong-Yan Qin, **Hai-Tao Xiao**, Fung-Ping Leung, Zhi-Jun Yang, Justin C. Y. Wu, Joseph J. Y. Sung, Hong-Xi Xu, Xu-Dong Tong, and Zhao-Xiang Bian.


**Paper under review and in preparation**


PATENTS

1. Zhao-xiang Bian, **Hai-tao Xiao**, Shi-lin Chen, Da-jian Yang, Hong-xi Xu, Ai-ping Lu, Albert Sun-Chi Chen. Bioactive Fractions and Compounds from Polygonum genus, their Use in Anti-diarrhea and the Method of Preparation. (CN103705586 A, filed date: 2013/6/21)

2. Zhao-xiang Bian, Hong-yan Qin, **Hai-tao Xiao**, Albert Sun-Chi Chan, Hong-xi Xu, Shi-lin Chen, Da-jian Yang. Method for preventing or treating enterochromaffin cell hyperplasia-related diseases by quercetin administration. (US 2013/0210907, filed date: 2013/2/14).

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