ANTI-INFLAMMATORY ROLE OF CHROMOFUNGIN, A CHROMOGRANIN A–DERIVED PEPTIDE, IN THE CONTEXT OF COLITIS

BY

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THESIS ABSTRACT

Background: Inflammatory Bowel Disease (IBD) involves primarily two disorders: Crohn's Disease (CD) and Ulcerative Colitis (UC). Uninhibited inflammation of intestinal mucosa, which can affect any part of the gastrointestinal tract, is the hallmark of IBD. UC is characterized by a decreased level of chromofungin (CHR: Chormogranin-A 47–66) and a dysregulation of $CD11c^+$ cells. A lot of therapies have been developed but most of them are not long-lasting or effective. Hence, new treatment needs to be developed. In this study, we aim to investigate the association between CHR and $CD11c^+$ cells related markers in patients with active UC and underlay the effect of CHR treatment on $CD11c^+$ cell functions in dextran sulfate sodium (DSS)-induced colitis. **Methods:** mRNA levels of CHR (*CHGA Exon-IV*) and its association with DCs markers (interleukin [IL]-23, IL-12p40, CD86, CD11C, CRR7, CD74, IL-12A) were quantified in colonic biopsies of patients and healthy individuals. Colitis was induced in C57BL/6 mice (7–8 weeks) by 5% dextran sulfate sodium (DSS) (wt./vol.) for 5 days. Intra-rectal administration of CHR (2.5 mg/kg/day), which lasted for 6 days. Disease activity index (DAI), macroscopic and microscopic scores were assessed. Colonic levels of pro-inflammatory cytokines and chemokines was done using ELISA and RT-qPCR. RT-qPCR was also performed on cells from mesenteric lymph nodes to check $CD11c^+$ cells related markers. *In-vitro*, splenic $CD11c^+$ cells, isolated from naïve and colitic mice, cultured with/without CHR (10^{-6} M) and surface markers were quantified using flow cytometry. RT^2 PCR was done for colon and splenic $CD11c^+$ cells to have a mechanistic insight. Confirmatory experiments were done using $CD11c^+$ BMDCs and stimulating them with NF-κ B inhibitor/activator along with lipopolysaccharide (LPS) and/or CHR. **Results:** In patients with active UC, CHR level is reduced and showed a negative relationship with DCs markers; *IL23* (r= -0.3725), *IL12p40* (r= -0.3706), *CD11C* (r= - 0.4647), *CRR7* (r= -0.3709), *IL12A* (r = -0.4253),

CD86 (r= -0.6765), and CD74 (r= -0.3647). *In-vivo*, CHR treatment reduced the severity of the colitis and is associated with a significant decrease in colonic levels of mRNA expression of CD11c, IL-12p40, CD80, CD40, IL-6 and CD86 in mice. Protein levels of IL12p40, IL-6, IL-23 and IL-1β showed similar results. Expression of the genes: CD11c, IL-6, CD86, CD80 and IL-12p40 was also down regulated in MLN of CHR+DSS group as compared to DSS+PBS group. *In-vitro*, CHR treatment reduced CD40 and CD80 expression in splenic $CD11c^+$ cells isolated from colitic mice when compared with PBS-treated group. RT^2-PCR suggested that CHR is down regulating NF-κ B pathway and hence reducing inflammation, which was confirmed with *In-vitro* experiment using bone marrow derived $CD11c^+$ dendritic cells (BMDCs) and NF-κ B pharmacological blocker/activator. IL-6 and IL12p40 were significantly reduced with CHR administration as analyzed by ELISA and reduction in the surface co-stimulatory molecules (CD80 and CD86) was also observed with flow cytometry. **Conclusion:** In patients, *CHGA Exon-IV* levels were significantly downregulated and the pro-inflammatory markers were markedly high. In mice experiments, CHR regulates the development of colitis *via* modulation of the markers of $CD11c^+$ cells locally in colon and systemically in MLN and spleen.

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DEDICATION

Dedicated to my mother, father and brother, who have believed in me since time immemorial. To my wife, Sugandhi, who has given me immense and endless support throughout.

To all the IBD patients who have suffered a lot throughout this disease.

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ABBRE*VIA*TIONS

6-MP	6- Mercaptopurine
ADA	Adalimumab
ANOVA	Analysis Of Variance
APC	Annual Percentage Change
APCs	Antigen Presenting Cells
AZA	Azathioprine
BMDCS	Bone Marrow Derived Dendritic Cells
CD	Crohn's Disease
CgDPs	Chromogranin A Derived Peptides
CHGA	Chromogranin A
CHR	Chromofungin
cLP	Colonic Lamina Propria
CNS	Central Nervous System
CSA	Cyclosporine
DAI	Disease Activity Index
DC	Dendritic Cell
ELISA	Enzyme Linked Immunosorbent Assay

FACS	Fluorescence Assisted Cell Sorting
FBS	Fetal Bovine Serum
GI	Gastro Intestinal
GM-CSF	Granulocyte Macrophage – Colony Stimulating Factor
GWAS	Genome Wide Association Studies
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
iES	Intestinal Epithelial Cells
IFN	Interferon
IFX	Infliximab
IHD	Ischaemic Heart Disease
IL	Interleukin
iLFs	Intestinal Lymph Follicles
ir	Intra Rectal
IRAK	IL1 Receptor Associated Kinase
KO	Knock Out
LP	Lamina Propria
LPS	Lipopolysaccharide

LRR	Leucine Rich Region
MHC	Major Histocompatibility Factor
MLN	Mesenteric Lymph Node
MTX	Methotrexate
MyD88	Myeloid Differentiation Primary Response Gene 88
nAChRs	Nicotinic Acetylcholine Receptors
NF-κ B	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NK	Natural Killer
NOD	Nucleotide Oligomerization domain
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffer Saline
PP	Peyer's Patches
PRR	Pattern Recognition Receptors
RBC	Red Blood Cells
RORγ	Nuclear Hormone Receptor Retinoic Acid Receptor-related Orphan Receptor Gamma
SEM	Standard error of Mean
SCFA	Short Chain Fatty Acids

SNP	Single Nucleotide Polymorphism
STAT-1	Signal Transduction and Transcription Activating Factor-1
TB	Tuberculosis
TLR	Toll Like Receptors
TNBS	Trinitrobenzene Sulphonic Acid
TNF	Tumour Necrosis Factor
TRAF6	TNF Receptor Associated Factor 6
UC	Ulcerative Colitis
VEGF	Vascular Endothelial Growth Factor
WPI	Whey Protein Isolate

CHAPTER ONE

INTRODUCTION

1.1. Overview

Inflammatory Bowel Disease (IBD) is a combination of disorders, which cause various regions of gastrointestinal (GI) tract to become ulcerated and inflamed.[1] The immune system plays an unusual response in each of the two main types of IBD: Ulcerative Colitis (UC) and Crohn's Disease (CD).[2] Till date the exact mechanism behind IBD is still unknown but is believed that environmental factors lead to abnormal mucosal immune response leading to dysregulated immune response in the intestinal lining.[3-5] Past epidemiological studies have indicated an increase in the incidence IBD both in adults and children.[6] Europe had the highest prevalence of IBD in 2011 where 505/100,000 had CD and 319/100,000 had UC.[7] The risk of IBD is also increasing in the developing countries where there is enhanced industrialization.[8, 9] Various studies have reported a significant increase in the incidence of IBD over the latter part of 20^{th} century whereas there are other reports which show a stability or decrease in the IBD incidence in some geographical regions[10, 11].

The disease is in progress worldwide but is still considered as a Western Disease and is most common in North America.[12, 13] Although various drugs are available to suppress the symptoms but still the exact etiology is unknown and therefore no curative treatment is available for IBD.[14, 15] Hence, more extensive research is required to find new effective drugs.

1.2 Literature Review

1.2.1 Inflammatory Bowel Disease

1.2.1.1 Definition of IBD

IBD occurs due to an abnormal homoeostasis of the immune system at the gut mucosal barrier [1, 15]. It is an inflammatory disorder of the GI tract with chronic and relapsing symptoms. Based on laboratory tests, clinical manifestations, radiological studies, histologic and endoscopic studies, IBD is divided into CD and UC [1]. The basic difference between these two is that in UC only regions of the colon and rectum are inflamed, whereas, in CD the inflammation can be anywhere from the mouth to anus [16-18].

1.2.1.2 Crohn's Disease and Ulcerative Colitis

Ulcerative Colitis: its most consistent feature is the prevalence of mucus and blood with stool with lower abdominal cramping, which get even intense when the bowel movements are passed [19]. Generally, 95% of UC patients' rectum shows hemorrhage, ulceration, edema limited to mucosa. Mucosal glands damage, crypts abscesses, mucosal layer thinning and inflammation by monocytes and neutrophils are amongst the various morphological changes seen in UC patients [19]. The gross picture of UC shows edema, diffuse erythema and several inflammatory polyps in the left and transverse colon and in the rectum. (Fig 1.1)[20]. Sometime the first signs of UC are misinterpreted as irritable bowel syndrome (IBS), which shows an absence of blood along with mucus during diarrhea. UC is diagnosed at an early stage than CD because of the presence gastrointestinal symptoms like gross blood in the stools which alerts the person [21]. The pain during the disease depends on the location of the inflammation and the extent of colonic involvement.

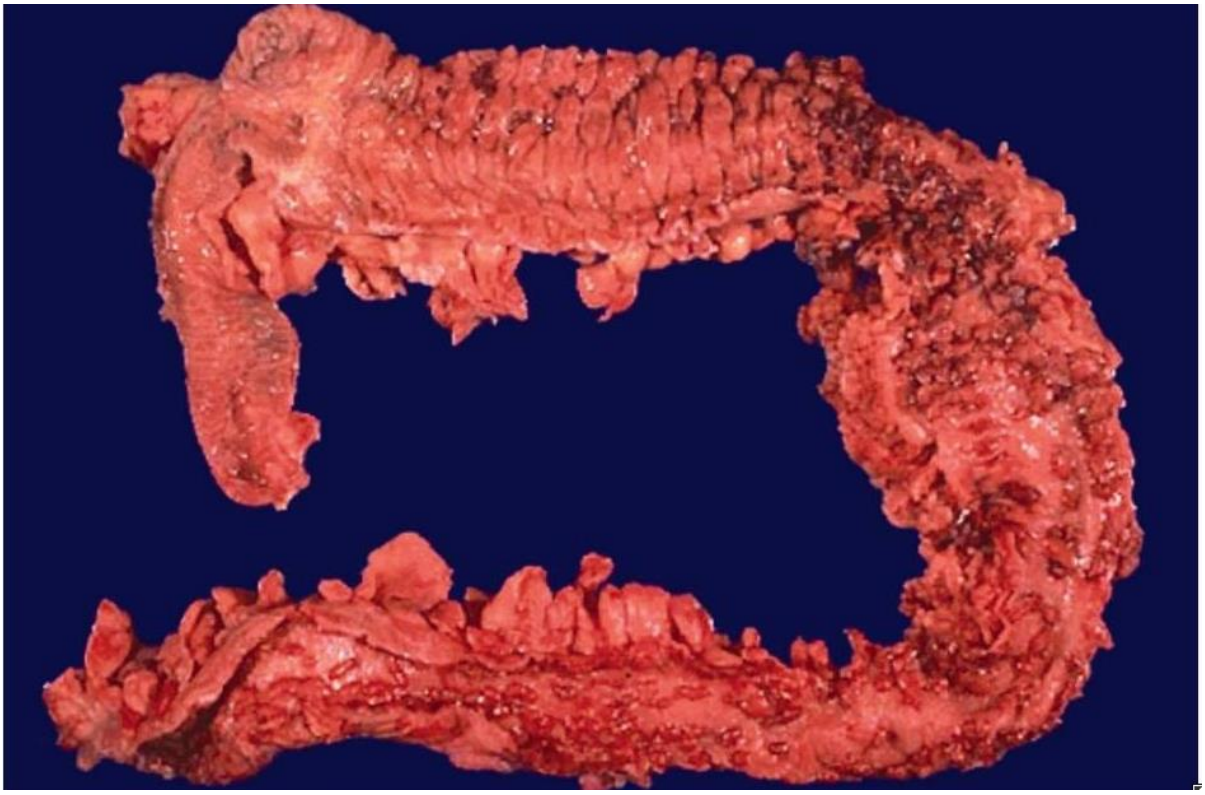


Fig 1.1 Gross Photograph of Ulcerative Colitis [20]

On the other hand**,** CD is a condition in which the inflammatory response can involve any part of the GI tract from the perianal area to oropharynx [19]. In CD "skip areas" are formed in which the disease's regions are separated by normal areas. Transmural inflammation is often seen, which can extend through to serosa and hence leading to fistula formation [19]. Histologic analysis has shown small ulcers over Peyer's Patch and chronic inflammation which extends to submucosa which in few cases is escorted by formation of granuloma. (Fig 1.1)

In addition to all the GI tract disorders, there are various extra-intestinal complications: fever (seen in nearly 40% of the IBD patients), weight loss, delayed growth, and sexual maturation in children, arthralgias and arthritis, mucocutaneous lesions, ophthalmological complications and a lot more [22-24].

1.2.1.3 Epidemiology of IBD

Before the 21st century, IBD was a disease of the Western world (Europe, North America and, Oceania). After that it bloomed as a global disease with a major effect on the newly industrialized nations of South America, Asia, and Africa where more westernization has been introduced into the societies. On the other hand, the prevalence of IBD continues to increase in Western countries (North America, Europe) in New Zealand and Australia, which leads to an increased burden of the disease in these countries. The rising global burden of IBD demonstrate its worldwide presence. The highest reported values were found in Europe where 505 individuals out of 100,000 are suffering from UC (in Norway) and 322 of every 100,000 individuals are suffering from CD (in Germany) [25]. It has been reported that since 1900 the incidents of IBD are increasing in the newly industrialized nations like Asia, South America, and Africa including Brazil (annual percentage change is +14% for CD) and Taiwan (APC is +4% for CD and +4.8% for UC) [25]. On the other hand, South Korean studies have shown no incidence increase from 2006 to 2012 with annual percentage change of -2.4% for CD (-4.7 to 0.0) and APC for UC being only -2.2% (-4.6 to 0.2) [25]. Whereas, study in one of the districts in Seoul showed an increase in the incidence from 1991 to 2005, with APC for UC +9.5% (2.7 to 16.7) and APC for CD +13.8% (8.7 to 19.0) [25].

The highest frequency of UC and CD is found in Canada compared to the rest of the world [26, 27]. The main sources of data for IBD epidemiology are: the Canadian Community Health survey (conducted nationwide) and the Canadian IBD Epidemiology Database Study (conducted only in 5 provinces).[27] The prevalence of IBD for the year 2000 was estimated to be 0.47% of the population (211/1000 for UC and 279/1000 for CD), which was consistent throughout the country except British Columbia (BC) with low prevalence. Males are less likely to have CD than

females (13 females to 10 males) but there is no such difference for UC [27]. In 2012, prevalence of IBD in Canada was estimated using IBD Epidemiology Database Study taking into consideration the population increase and new IBD patients (with assumption of an increase of 17 IBD cases/100,000) [2]. In 2012 there were nearly 233,000 Canadians suffering from IBD with 104,000 UC and 129,000 CD patients, respectively (total prevalence of 0.67%) [2]. In Canada, the average national incidence rate from 1998 to 2000 was 16.3/100,000 for CD and 12.9/100,000 for Ulcerative Colitis. Overall, an increase of 12,200 IBD patients was diagnosed each year (5700 CD patients and 4500 UC patients) [27].

1.2.1.4 Etiology of IBD

1.2.1.4.1 Genetic Factors

The exact reason behind the cause of IBD is still unknown but it is thought to be a multifactorial disease which involves an aberrant immune response to antigens in genetically predisposed individuals [28]. Genome-wide association studies (GWAS) have shown that genetic factors play a major role in the etiology of IBD [29]. The disease is considered as a multi-gene disorder followed by a non-Mendelian paradigm strongly influences by environmental factors [29]. GWAS have shown 99 different gene loci involved in IBD amongst which CD and UC are associated with 71 and 47 gene loci respectively and 28 gene loci are shared [3]. The IBD associated genes have their major role in regulating intestinal homoeostasis by modifying immune cell migration and barrier function [29].

In humans, NOD2 found on the chromosome 16, was the first gene found to be associated with CD [30, 31]. It is very important to activate toll like receptors (TLR) and nucleotide oligomerization domain (NOD) which help in the activation of immune response against microbial

incursions [32, 33]. Mutation in NOD2 leads to alter immune response *via* change in signalling pathways like NF-kB pathway [34].

Higher polymorphism of IFN-γ (found on human chromosome 12) is found in CD patients [35, 36]. This might be the reason behind the reduced tissue repair and enhanced fibroblasts migration in CD patients [37]. IL-6 is also considered as a factor for IBD (located on human chromosome 5) and it has been seen that higher levels of IL6 are associated with greater susceptibility to IBD [35, 38, 39]. Tumour necrosis factor (TNF)-α, located on the human chromosome 6, acts as a major cytokine in IBD necrosis and its polymorphism has association with IBD susceptibility [35]. In an Iranian cohort, it was seen that single nucleotide polymorphism in TNF- α promoter region had a strong relationship with IBD susceptibility [40]. This polymorphism in the promoter region can also alter the outcome of the cytokine inhibitor treatment as seen in that cohort, with patients with TNF- α resistance demonstrating a higher genotype frequency of 308A and 308GA alleles [41]. However, extensive studies are still required to define the clinical importance of these polymorphism in relation to the outcome of IBD.

1.2.1.4.2 Environmental Factors

Environmental factors play a major role in IBD etiology and it has already been shown that western world has greater incidences and prevalence of the disease and the incidences are also increasing in the developing countries. Due to an unknown reason, a clear increase in the incidence of UC followed by CD has been observed in the following developing countries French West Indies[42], Thailand[43], Lebanon[44], Iran[45], India[46, 47], China[48, 49] and South Korea[50]. Surprisingly, first-generation people from an IBD low incidence county tend to develop IBD when they move to a developed nation with high incidence [12, 51, 52]. Migration studies

have also shown that second-generation immigrants to the Western world are more prone to chronic inflammatory disorders as compared to their parents [12, 51, 52].

Smoking is another factor associated with IBD. According to epidemiological data it was observed that smoking acts as a risk factor for CD and it makes the disease more severe as the host develops resistance against the drug and gets increased flares [16]. It is surprising that smoking has a positive association with UC [16]. The real mechanism behind the modulation of immune response by smoking is not well understood but what is known is that nicotine, the major component of smoke regulates T-cells function through nicotinic acetylcholine receptors (nAChRs) [53]. The main regulatory immune function in UC (i.e. T-helper 2 cells function) is inhibited by nicotine [54]. In UC patients, clinical trials were done nicotine as a therapeutic agent, but modest results were seen suggesting nicotine might not be the only factor regulating UC [55].

Breastfeeding has also been found to be protective in IBD, same as seen in other immune diseases [56-59]. Mechanism behind this is still unknown but it is thought that mother's milk allows immune system to develop faster and mount a better immune response against food antigens and commensal microbiota [58]. Not only this, lactoferrin in the breast milk has good anti-inflammatory and anti-bacterial properties [60].

Protective role of physical exercise has also been found in IBD and it is observed that people with clerical jobs have high IBD prevalence as compared to people who have physically demanding and outdoor held jobs [61]. Mortality rate is also higher in IBD patients who do office desk jobs as compared to construction workers [61]. Even though we know that physical activity is protective but the exact protective mechanism behind this protection is still unknown.

1.2.1.4.3 Gut Microbiome

Next, the most current important component in IBD etiology is the gut microbiota. The human gut is heavily colonized with nearly 10^4 microbes of 1100 species (10 times more than the total number of cells in human body) which help in food digestion [62]. In a recent European cohort study, it was seen that every human has 600,000 microbial genes in their gastrointestinal tract and half of these genes are common with 50% of cohort participants [63]. The colonization of microbiota begins at birth and the child keeps on gathering microbes from surroundings as well as maternal milk.[64, 65] The gut microbiota forms by the age of three [66, 67] and Firmicutes and Bacteroidetes forms the major phyla [68, 69]. Birth mode, diet, age and geographical location determine the person's gut microbiota [66]. People with similar cultural and ethical background and living at the same geographical location tend to have similar gut microbiota [66]. Less microbiota variation is seen in Westernized populations [66]. Diet also plays a major role in gut microbiota composition and it was observed that faecal composition of microbiota of two different groups from the United States and Korea were completely different, this was mainly correlated to difference in the diet, which might change their microbiota composition [70]. As carbohydrate, protein, and fats are the three main nutritional components, which leads to the formation of gut microbiota, it has been seen that fat and carbohydrate-rich diet can shift the gut microbiota [66, 71]. Diet rich in fibre has a crucial role in the prevention of obesity and many bowel disorders [72-75]. The beneficial effects of fibre-rich diet is due to fibre that is not properly fermented and it helps to detoxify, enhance colonic transition, grows biomass, and stimulate fermentation [76]. Gut microbiota has a major role in carbohydrate and protein fermentation in human gut [77]. In a dose-dependent manner, whey protein isolate (WPI) can change murine microbial composition [77]. The results of this finding showed that WPI supplemented mice had higher *Lactobacillaceae* and

significantly low level of *Clostridiaceae* when compared with mice group on high fat diet [78]. On one hand gut microbiota helps the eukaryotic host to break down food nutrients and, on the other hand, it also plays an important role in gut immune system regulation and it is known that SCFAs helps in reduction of pH in the gut and therefore reduces the growth of pathogenic bacteria [78].

1.2.1.4.3.1 Gut Microbiome and Immune System

Several studies have shown the development of the immune system *via* gut microbiota signals. The proof of this was seen when germ-free mice models were analyzed for the development of innate and adaptive immune system and important changes were observed [79]. Changing the gut microbiota composition either *via* microbiota reconstitution or *via* treatment with antibiotics indicates that microbiota has a role in immune homoeostasis [80-84].

The key feature of the innate immune system is to protect the body from infection and maintaining tolerance with the symbiotic gut microbiome. The major component of innate immune system i.e. antigen presenting cells (APCs) and more specifically dendritic cells (DCs) plays a major role in this action [85]. High level of anti-inflammatory cytokine interleukin 10 (IL-10) is released by DCs from Peyer's patches than the DCs from the spleen inducing greater tolerance at the gut level [85]. Macrophages also stay in the proximity of gut microbiota and represent a specific phenotype known as "inflammation anergy," where they stay in an homoeostasis state, even though they stay close to microbiota [86]. It was also observed that macrophages that stay in the intestine do not produce pro-inflammatory cytokines for stimuli like TLR ligands [87]. Several experiments show a direct importance of gut microbiota in the regulation of APCs. In germ-free animals it was seen that the number of DCs was decreased in the intestine but the systemic level of DCs was not affected, and mono colonization of germ-free animals with *E. coli* was enough to

get the intestinal DCs to normal levels [88, 89]. Additionally, it was shown that ATP derived from microbes stimulate specific DCs with CX3CR1 and CD70 on their surface, which helps in Th18 cells differentiation [90]. The largest number of macrophages in the body is seen in the intestine [91]. Even though, the intestinal macrophages were either normal in germ-free mice [89], they are reduced in germ-free pigs [92]. The peritoneal macrophages of germ-free mice have compromised microbicidal activity [93, 94] and the activation markers of macrophages are also reduced like major histocompatibility complex (MHC) II [95].

Conventional natural killer (NK) cells are efficient in detecting and killing modified and infected cells by the production of perforin or interferon (IFN)-γ and lately two different types of NK cells are found in intestinal mucosa with natural cytotoxicity receptor NKp46 [96]. Whereas one type of gut $NKp46^+$ resembles the conventional NK cells, the other type differs as it expresses the nuclear hormone receptor retinoic acid receptor-related orphan receptor gamma in thymus (RORγ t) and IL-22. Experiments have shown that GF mice lack IL22 producing $NKp46^+$ cells suggesting a link between gut microbiota and IL-22^+NKp46^+ cells differentiation [97]. Mast cells, which represent 2-3 % of the lamina propria cells of the gastrointestinal tract, have a vast variety functions including blood flow regulation and coagulation and exchange of electrolytes by intestinal epithelial cells (IEC) [98]. It was seen that GF mice have reduced intestinal mast cells density, but it is increased systemically than wild type mice. Several experimental studies have shown that gut microbiota induces CXCR2 ligands *via* MyD88 from intestinal epithelial cells, which promotes the migration of mast cells to the gut [99].

Along with the innate immune system, microbiota also has a strong influence on the adaptive immune system. One of the important components of adaptive immune system is the $CD4^+$ T cells, where in the intestine, they are majorly located in the lamina propria (LP). Under

the influence of various immune responses $CD4^+$ T cells differentiate into: T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) or regulatory T cells (Tregs), which are divided based on cytokines they produce, and the transcription factors expressed (Fig1.2). Regulation of all these subtypes is really crucial in the health of a person and a dysregulated T cell responses can lead to autoimmune diseases or various allergic reactions [100].

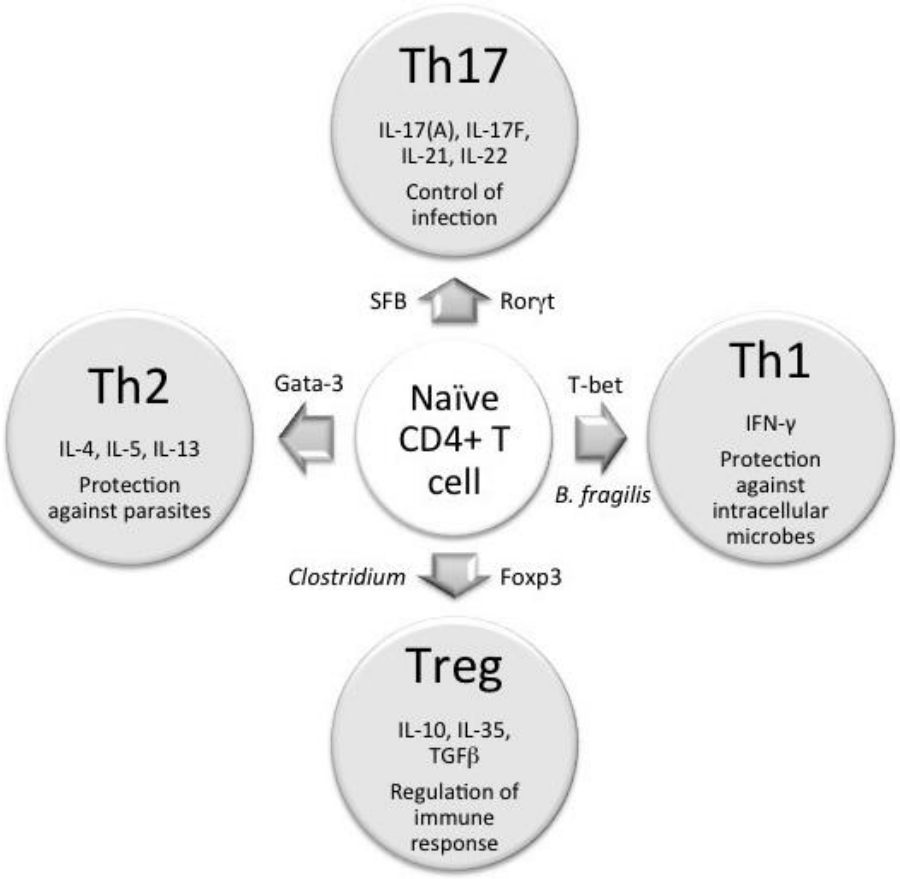


Figure 1.2. $CD4^+$ differentiation is induced by commensal bacteria. The four major T cell types are shown above. The transcription factor induction is unique to each lineage. Different types of lineage secrete different types of cytokines. Bacteria inducing specific type of T cell differentiation is shown in the figure [100].

As the gut microbiome has a role in the development of $CD4^+$ T cells, significant reduction in the level of these cells is observed in GF mice [101]. Similar effect is also seen systemically as

an absence of lymphocytes zones in the spleen and mesenteric lymph nodes (MLN) in GF mice are seen [84]. Th1/Th2 imbalance is seen in the GF mice and additionally it has been observed that Th2 responses are favoured in GF mice [84]. Experiments have shown that specific bacteria can enhance the induction of certain T cell responses such as induction of lamina propria Th17 cells being favoured by segmented filamentous bacteria [83, 102]. As IL-17 is an important pro-inflammatory cytokine, it would be of great interest to see if any changes in the intestinal bacteria can induce Th17 cells. The scenario is more important in humans and recently it was seen that reads from 0.31% of mouse segmented filamentous bacteria were found in the data set of metagenome sequences of human gut which indicated that human Th17 cells can be induced by bacteria [103, 104].

The number and effectiveness of intestinal $CD8^+$ T cells are also affected by the gut microbiota. It was observed that the in GF mice, $CD8^+$ T cells are reduced and are less effective indicating a role of the microbiota in $CD8^+$ T cells development [105-107]. These defects can be due to impaired clonal expansion of intra-epithelial $CD8^+$ T cells. Even though, gut microbiota is not responsible for shaping systemic $CD8^+$ T cells repertoire, it plays an important role in modulating various peripheral immune cells like plasmacytoid DCs, marginal zone B-cells and invariant NKT cells [108-110].

Another type of immune cells called gamma delta ($\gamma\delta$) T cells are popularly known as the connecting link between the adaptive and innate immunity. The percentage of these cells is very high amongst the intestinal intra-epithelial lymphocytes (50%) as compared to its percentage in spleen or lymph nodes (1-5%) [111]. The pool size of these cells is not much affected in the absence of commensal bacteria, but it was seen that the cytotoxicity of these T cells reduces in GF mice, which shows an important role of microbiota in the nurturing of $\gamma\delta$ T cells.

1.2.2 Role of Immune System in IBD

The abnormality in the gut immune system begins with the infiltration of huge amount of pro-inflammatory cytokines like IL-6, IFN-γ, IL-1β and TNF-α. It is also followed by damage to the intestinal epithelium which leads to abnormal mucus production and variances in tissue repair and huge infiltration of immune cells in the intestine [112, 113].

Innate and adaptive immune system are two major wings of intestinal immune system [114]. The innate immune system comprises barrier maintenance function, antimicrobial peptide release, regulation of acidic pH in the stomach to limit microbial growth and the innate immune cells like NK cells neutrophils, macrophages and DCs [112, 113]. On the other hand, the adaptive immune cells (T and B cells) get activated within days of intestinal inflammation. Various classes of adaptive immune cells (Th1, Th2, Th17 and Tregs) gets activated according to their specificity [112]. The intestinal immune system is tightly regulated, and dysregulation of this immune system causes IBD.

1.2.2.1 Innate Immune Responses in IBD

1.2.2.1.1 Epithelial Barriers and Peptides

Innate immune system comprises our first line of defence against foreign antigens and it differs from the adaptive immune responses as it is not specific and does have an immune memory. The major cells of innate immune system are the macrophages, DCs and the intestinal epithelial cells and they sense the microbiota in the intestine and react to the pathogen associated molecular patterns (PAMPs), which initiates the inflammatory response against the invaded microbes [115]. This initiation of the innate immune response leads to the activation of adaptive immune response *via* the professional antigen presenting cells called DCs, which activate T cells [115].

The intestinal epithelium is covered by mucus layer, which serves as the first barrier for the food antigens and intestinal bacteria. Polymerization of the gel forming mucins leads to mucus formation and it is organized in an outer loose and an inner firm layer. These mucins are secreted by the goblet cells and as they have a capacity to bind to water, they expand in the lumen. The outer mucous layer has commensal bacterial in it and is more permeable, whereas the inner layer is firm and sterile. *MUC2*$^{-/-}$ mice lacking mucous layer has been seen to develop colitis and they have an enhanced risk of colitis as the bacteria stays in direct contact with the intestinal epithelium [116, 117]. In comparison to these results to humans, no relationship between the *MUC2* gene mutation and mucosal barrier disruption has been observed, but in the inflamed ileum of the CD patients *MUC1* mRNA expression was found reduced. Moreover, *MUC3*, 4 and 5B mRNA expression were also reduced in the non-inflamed regions of the gut of CD patients as compared to the controls [118].

α–defensins and β–defensins form the major bactericidal agents which also forms a barrier in addition to all other physical barriers. These α–defensins are produced by Paneth cells and β–defensins by most of the epithelial cells. All these anti-bacterial agents are produced on a regular basis but specific recognition of bacterial components is also done by pattern recognition receptors (PRRs), which are expressed in both intra- and extracellular epithelial cells [119]. It has been observed that CD patients have a defective expression of antimicrobial peptide [120]. α-defensins, derived from Paneth cells (HD5 and HD6), have reduced expression in the ileum of CD patients [121]. Moreover, it was observed in ileum, CD patients show strongest genetic association with NOD2 polymorphisms, which are expressed on Paneth cells. Additionally, it was also suggested that the antimicrobial peptide defect has association with NOD2 related risk of the CD,

which was made clear from the fact that the patients with *NOD2* frame shift susceptibility variants have decreased expression of *HD5* and *HD6* [122].

1.2.2.1.2 Microbiota Sensing

The PRRs recognize PAMPs on microbial antigens and microbes and all PRRs are present on innate immune cells like DCs, macrophages, epithelial cells and myofibrolasts. The PRRs include transmembrane as well as intracytoplasmic receptors like toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). An effective innate response against pathogens relies on the activation of PRR and leads to production of pro-inflammatory cytokines through NF-κ B activation. This also leads to greater and effective cross-talk between innate and adaptive immune system by the promotion of maturation of APCs for enhanced antigen presentation and therefore activation of T cells [115].

The TLR family identifies PAMPs extracellularly or in lysosomes and endosomes. After the stimulation of TLR various cascades are activated which includes myeloid differentiation primary response gene 88 (MyD88)-dependent and MyD88-independent like TNF receptor associated factor 6 (TRAF6) and IL1 receptor associated kinase (IRAK) [123]. On the other hand, NLR members (NOD1 and NOD2) are stimulated by various bacterial peptidoglycan components. TRAF2 and TRAF5 are activated by NOD1, while RIPK2 is activated *via* NOD2. Inflammasome formation is also seen in other cases which leads to the activation of caspase and IL-1 secretion [124, 125].

The first gene that was found to be associated with enhanced risk of CD was *NOD2* [30, 126] and until date 3 unusual single nucleotide polymorphisms (SNPs) have been found in *NOD2* and its strong susceptibility is also seen in relation to ileal CD patients. The toughest association

of *NOD2* is seen in IBD with an odds ratio of 17.1 in homozygotes or compound heterozygotes and 2.4 in heterozygote individuals [127]. African-American or Asian populations do not show any association, but the association is very prominent in European heritage [128] [129]. The exact role of *NOD2* mutation is still unknown but there are evidences which suggests that reduction in NF-κB activation was seen due to loss of function mutation in *NOD2* [130]. This reduced NF-κB activation might lead to less antibacterial agent production and hence easy invasion for the pathogens [122]. Enhanced Th-1 responses and inflammatory pathway activation could be another consequence of loss of function mutation in *NOD2* [131]. IL-10, which acts as an anti-inflammatory cytokine, was also found to be inhibited in *NOD2 3020insC* variant and hence compromised immune regulation [132].

1.2.2.1.3 Innate Immune Cells: Macrophages

Macrophages forms one amongst the major and important immune cells in the gut [133]. They are highly crucial in maintaining the barrier integrity in the intestine. Monocytes differentiate to macrophages when they move from the blood to the intestine and this movement is induced by IL-8 and TGF-β [134]. Macrophages account for nearly 20% leukocytes in the intestine. These macrophages residing in the tissues are present right under the intestinal epithelium and they capture the invading microbes and help maintain the gut homoeostasis, without the activation of lymphocytes. Because of their enhanced phagocytic activity, they also help in clearing the cellular debris and cells that are dying [133]. The structure and function of these intestinal macrophages are also different from the macrophages in rest of the body. There is a reduced expression of co-stimulatory molecules like Fc receptors of IgA and IgG, integrins and complement receptors [135]. On the other hand, they have an enhanced phagocytic activity, release IL-10 (anti-inflammatory cytokine) and TLR stimulation is not effective to activate these macrophages [136]. This

phenomenon is called inflammation anergy [86] and it is believed to be facilitated by TGF-β by the blocking of NF-κ B pathways and a down regulation of MyD88. FOXP3 iTregs plays an important role in the maintenance of gut homoeostasis and they are induced and maintained by IL-10 which is released by the resident macrophages [87, 133]. As a result of these unique properties, they are called M2 macrophages or alternatively activated macrophages [133]. Any disruption in the balance of these M2 macrophages and classically activated macrophages results in the inflammation.

The second class of macrophages i.e. M1 macrophages are classically activated and are also called tissue infiltrating macrophages [137, 138]. Intestinal mucosa is infiltrated by many $CD68^{+}$ macrophages in IBD and the characteristic of these macrophages differ in CD and UC. In CD patients, the mesenteric fat and the muscular layer is penetrated by these M1 macrophages. Intermediate monocytes ($CD14^{hi}CD16^{+}$) show an increase, and the classical monocytes ($CD14^{hi}CD16^{-}$) show a decrease in the blood of CD patients [139, 140]. What was concluded in the *In-vitro* studies is that, in response to C-C chemokine ligand, the peripheral monocytes are attracted toward the intestinal mucosa [140]. The infiltrating macrophages attract more monocytes *via* chemokine signalling [141, 142], and hence through NF-κB pathway activation more pro-inflammatory cytokines are expressed like IL-8, IL-6, TNF-α, IL-1β and IFN-γ. It is quite interesting to know that the inflammation milieu from the inflamed intestinal mucosa can favour any monocyte cell lineage towards M1 macrophages. In IBD, a release of inflammatory cytokines is seen in the gut which includes IL-6, TNF-α, and IL-1β and this inflammatory response might be due to the leaky gut barrier [143]. Additionally, due to impaired TGF-β signalling during IBD, the formation of anergic macrophages and the inflammation development is hampered [133]. Hence, considering all these factors the role of macrophages is significant in IBD.

1.2.2.1.4 Innate Immune Cells: Dendritic Cells

The mucosal immune system is highly monitored and is very tight and defects in it is assumed to be the reason behind IBD (UC and CD) [18, 144]. T cells that are located in the intestine plays an important role in the gut adaptive immunity and these cells are activated by DCs. The reactivity of T cells is regulated by the initial activation by the DCs and hence DCs plays an important role in the initiation of adaptive immune response. At this initial stage of infection, the DCs can either regulate the inflammation and maintain the tolerance or they can initiate innate and/or adaptive immune response [145-147]. The DCs are either located in the mucosal tissues or they stay circulating in the lymph tissues and the blood [148]. In the mucosal tissue, the effector site is located at the lamina propria of the intestine. Mesenteric lymph nodes (MLNs), Peyer's patches (PP), intestinal lymph follicles, (iLFs) and DC-aggregate forms the sites where induction of inflammation takes place (Table 1.1). The plasticity of DCs can be seen as, depending on the microenvironment, they can be divided into $B220^{+}CD11c^{low}$ (plasmacytoid DCs) or $CD8\alpha^{+}CD11b^{-}$, $CD4^{+}CD11b^{+}$, $CD4^{-}CD11b^{+}$ (conventional DCs) [149, 150]. The DCs, which are located in the lamina propria of small and large intestine, constantly surveys the food antigens and the commensal bacteria [151]. After the DCs have captured and recognized the foreign antigen, they move ahead toward the draining lymph node to generate an immune response which could be either innate or adaptive or both [152].

Table 1.1 Anatomic compartments of gut associated lymphoid tissue (GALT)

Compartment		Gut Segment		
Inductive/Effector sites	Structure	Small Intestine		Large Intestine
		Upper[1]	Lower[2]	
Effector Sites	Lamina Propria	+++	+++	+++
	Intraepithelial Lymphocyte	+++	+++	+++
Inductive Sites	Peyer's Patch	+	+++	-
	Intestinal Lymph Follicle	+	+++	-
	Intestinal Lymph Aggregate	+	++	++
	Mesenteric Lymph Node	-	-	-

1 Distribution in Duodenum and Jejunum
2 Distribution in Ileum
"+++": Very Frequent, "++": Frequent, "+": Randomly, "- ": Not Present [153]

Looking into the lamina propria of the small intestine, only 2-5% plasmacytoid DCs are located there and the conventional DCs that reside there have the phenotype $CD4^{-}CD8\alpha^{-}$ [154]. Two types of mucosal DCs are found, one that have integrin αE chain CD103 [155, 156] and the other that express CX3CR1 (fractalkine/CX3CL1) [154]. The $Ly\text{-}6C^{high}CCR2^{high}$ monocytes differentiate into $CD103^{+}$ DCs and monocytes which are $Ly\text{-}6C^{low}CCR2^{low}$ gives rise to $CX3CR1^{+}CD11b^{+}$ DCs. [157]. The homing of T cells in tissues of the small intestine is regulated by $CD103^{+}$ DCs by inducing $\alpha_4\beta_7$ integrin and CCR9 expression on CD4 and CD8 T cells [155, 158]. The $CD103^{+}$ DCs also have a role in inducing differentiation of T_{regs} when there is no signalling from the exogenous cytokines.

The activation and proliferation of T cells in the colonic lamina propria (cLP) of the large intestine is induced by $CD4^-CD8^-$ DCs, which capture and process the antigen [159]. In cLP the population of either pDCs or $CD8^+$ DCs is very scarce [159]. Colon biopsies from the CD patients were analyzed for the population of DCs present and it was seen that $CD3^-CD14^-CD16^-CD19^-CD34^-$ DCs formed the major population and they were TLR4 and TLR2 low. The human and mouse DCs derived from the spleen or blood were found to be more responsive to TLR ligands from microbes than the DCs derived from the mucosa [159]. DCs show outstanding plasticity which is determined by the microenvironment where they are present, the type of antigen or the state of activation of DC [150, 160].

Peyer's patches serve as an inductive site for the priming of DCs and this takes place in the sub-epithelial dome region under the follicle associated epithelium. At this location, the delivery of luminal antigens is done by specialized epithelial cells called M cells [161]. $CD8\alpha^-CD11b^-B220^-$ DCs and $CD8\alpha^-CD11b^+B220^-$ DCs populate the sub-epithelial dome regions [162, 163]. The follicle associated epithelium expresses high amounts of chemokines like CXCL16, CCL9 and CCL20 which makes a specific micro-environment for the selective migration of DCs [164, 165].

In the mechanism of activation of adaptive immunity, MLN plays a crucial role as the priming of T cells by DCs takes place here. Sampling of all the antigens from the lumen takes place by the DCs from PP, lamina propria, iLF and the clusters of DCs at intestinal villi base. Lymph serves as the pathway for the transport of sampled antigens by DCs to the MLNs for T cell priming and activation of adaptive immunity [166]. The MLN has DCs originated from lamina propria and PP and it also has some blood born DCs [148]. The phenotype of the DCs from blood and lamina propria is different and can be distinguished by flow cytometry as they show different

surface expression markers [167, 168]. The lamina propria DCs are guided by chemokine CCR7 to move towards the MLN [168], whereas the blood born enter *via* peripheral node adressin (PNAd) and mucosal addressin cellular adhesion molecule (MAdCAM-1) [169]. The tolerance against the self-antigens is induced by the intestinal epithelial cells apoptotic bodies which undergo phagocytosis and are then transported to MLN [170]. The depletion of the commensal bacteria is continuously carried out to the MLN by the DCs from lamina propria [171], which potentate by DC stimulation with TLR-7 and TLR-8 agonists [172]. At this point, antibody production is induced forming IgA to act on the commensal bacteria and prevent systemic infections because of them [173].

The DCs plays a major role in maintaining the tolerance against the commensal bacteria and food antigens in the gut and this is the biggest challenge that the gastrointestinal immune system faces. The DCs behave as "soldiers" of mucosal immune system, which surveys the luminal antigens and generates an immune response against pathogens [174]. Experiments have shown that this balance that DCs maintain with the commensal bacteria if disrupts, it leads to IBD [18] and various animal models have shown the immense importance of mucosal DCs in IBD. Even though, a lot is known about the DCs but still for specific DCs the functions still need to be discovered in future. This will lead to a greater insight into the mechanism of IBD and hence we can find new ways for limiting the mucosal inflammation. In the patients with IBD and in the animal models studied, it was seen that the DCs accumulate at the site of inflammation. The co-stimulatory marker (CD40) and PRR TLR 4 and TLR 2 was found high in the DCs derived from the CD patients [175]. Additionally, enhanced number of MDC8+ monocytes, which acts a s a source of TNF-α, were found in IBD patients and treatment with anti TNF-α antibodies lead to reduced DC activation in CD patients [176, 177]. In IBD patients, enhanced IL-18 and IL-12

cytokines are seen which enhances Th1 production, and all this promoted by $CD83^{-}$ $CD80^{+}DCSIGN^{+}$ DCs [178]. The level of $CD86^{+}CD40^{+}$ DCs is also enhanced in the lamina propria and peripheral blood of CD and UC patients and increased abilities to stimulate immune responses has been seen in the ex-vivo generated DCs from the IBD patient's peripheral blood monocytes [179-181]. The production of IL-23 and IL-12 is seen in the DCs which are extracted from the inflamed colon of mice or from the terminal ileum [182, 183]. IL-10 KO mice, which otherwise develop colitis impulsively, stays protected when IL12p19 is given. In another study using *Helicobacter hepaticus* infection, it was seen that the IL-23 derived from DCs have a prominent role in gut pathology [184, 185]. DC like types, which express CD11c and F4/80, release the pro-inflammatory cytokine IL-23 and it results in the formation of granulomas in CD patients [186]. The GWAS have also been done to see the link between CD and UC and a subunit of IL-23 came out to be common in both the diseases, suggesting the importance of IL-23 in IBD pathogenesis [187, 188]. Experiments have shown that IL-23 released by these $CD11c^{+}$ cells only helps in the maintenance and development of Th17 cells, which produce IL-17, but not in the initial step of their formation [189]. When the DCs were pulsed with fecal extracts and then co-cultured with naïve T–cells culture, in the presence of both Th1 and Th17 cytokines, it was seen that the Th17 cells generated were more pathogenic [190]. Depletion of $CD11c^{+}$ DCs with delayed type response in transgenic animal system by diphtheria toxin, results in suppression of colitis and DSS mice model [191]. From all the experiments, it can be concluded that DCs and their pro-inflammatory downstream signalling lead to long-term activation of T cells leading to IBD.

The role of $CD11c^+$ DCs is very important in the progression of the disease. A table representing the different types of $CD11c^+$ DCs is presented below:

Table 1.2 Dendritic Cells Involved in the Progression of IBD

Dendritic Cells		Function	References
Ancestry	Phenotype		
Conventional DCs	$CD4^-CD8^-CD11b^+CD11c^+$	Expression of chemokines like CXCR1 Allowing the movement of T cells into intestine *via* chemokines and integrins	[154-156, 192-194]
Plasmacytoid DCs	$CD4^-CD8^+CD11b^-CD11c^+$	Priming the response of CD8 T cells	[194, 195]
	$B220^+PDCA1^+CD11c^+$	Production of anti-viral molecules: Type I interferons	[194, 195]
	$CD4^+CD8^-CD11b^+CD11c^+$	Priming the response of CD4 T cells	10[151]

1.2.2.2 Adaptive Immune Responses in IBD

In contrast to the innate immunity, adaptive immunity has the ability to keep the immune memory and has specialization in any giving specific immune responses against antigens. Naïve T cell can be differentiated into Th1, Th2, Th17, and Treg cells based on the specific signals [114].

The Th1 response is regulated by the IFN-γ secreted by the APCs, which acts on the signal transduction and transcription activating factor 1 (STAT-1) to stimulate Th1 cells differentiation. Various pro-inflammatory cytokines are released by the Th1 cells upon activation like: IL2, IL-1, IL-6, IL-12, IFN-γ, and a lot more. In a mouse model of CD, the levels of TH1 cells and IFN-γ were found significantly high in spleen and in the intestinal mucosa [196]. Similar Th1 elevation was also found in CD patients, where Th1 cytokines like TNF, IL-18, and IL-12 were found high. NF-kB signalling enhances Th1 production and the signalling is promoted by IL-18 [197].

The secretion of IL-4 by the APCs activates STAT-6 on the surface of Th cells. STAT-6 initiates the process of formation of Th2 cells by activating specific transcription factor GATA-3, which activates downstream signals. The cytokine secreted by Th2 cells includes IL-13, IL-5 and IL-4. In UC patients, the levels of IL-5 and IL-13 were found upregulated in the regions of gut inflammation [198]. According to literature, UC is a result of combined effect of both Th1 and Th2 cells. The early phase of UC is accompanied by the Th1 cells, while the late phase by Th2 cells. Hence, the contradiction stays that which response close and further research is required to answer this question [199].

Th1 and Th2 responses always stay in dynamic equilibrium under ideal conditions. They regulate each other by specific set of cytokines. Th2 cells proliferation is inhibited by the secretion of IFN-γ from Th1 cells**,** while the Th1 cells are inhibited by IL-4, IL-10, and IL-13 from the Th2

cells. This immune equilibrium is disturbed during various autoimmune and inflammatory disorders and plays a crucial role in the development of IBD [200]. The pro-inflammatory cytokines released by the Th1 cells like IL-8, IL-6, IL-2, and IL-1 participate in cell-mediated immunity and the anti-inflammatory cytokines released by the Th2 cells like IL-13, IL-10, and IL-4 plays a role in the humoral immunity. Hence, the combination of these cytokines maintains the balance of the immune system and decides the dominance of the specific type of immune response. Thus, finding the key cytokines involved in the IBD can be explored to find new treatments for IBD [201].

Th17 also plays a crucial role in the pathogenesis of IBD. The pro-inflammatory cytokine IL-23 activates STAT-3 by acting on the IL-23 receptor on Th cells. This action is also accompanied by other cytokines like IL-6, IL-21, or TGF-β. The transcription factor STAT-3 enters the RORγt and promotes the differentiation of Th cells towards Th17. Once activated Th17 cells secrete IL-21, IL-22, and IL-17 which activates NF-κ B signalling, and further enhances the inflammation by activation downstream pro-inflammatory cytokines. Significant elevated levels of Th17 cells and IL-17 was found in the bowel mucosa of IBD patients [202]. UC and CD patients also have significant higher levels of serum IL-17 [203]. Additionally, IBD patients have also increased mRNA levels of IL-17A and IL-17F [204], which further confirms the importance of Th17 cells in the pathogenesis of IBD.

Another class of T cells called Treg cells helps in the suppression of inflammation. It makes direct contact with Th1, Th2, and Th17 cells and releases anti-inflammatory cytokines IL10 and TGF-β to inhibit their action. The specificity of Tregs is seen by the presence of surface transcription factor Foxp3. Autoimmune colitis is induced if the mice when injected with naïve T cells without CD4+ and CD25+ Treg cells [205]. On the contrary, CD4+ and CD25+ Tregs if

injected to mice model of IBD pathological injuries, they move to the spleen and help in regulating the immune system and treating IBD [206]. The reduction in the levels of Tregs is also found to be associated with IBD pathogenesis and the dropped levels of Tregs have been observed even in the IBD patients [207].

The balance between Th17 cells and Treg cells is important to maintain the immune balance in the gut. This balance gets disturbed when there is increase in the number of Th17 cells and abnormal decrease in the levels of Treg cells and these cause intestinal mucosa damage. Lower concentrations of TGF-β and increased levels of IL-6 promote the differentiation of T cells into Th17 cells and decreases the formation of Treg cells [208]. Even though Treg cells repair the mucosal lining, but they transform into pro-inflammatory Th17 cells under the influence of IL-23 and/or IL-6 [209]. The reversible action of the conversion of Th17 cells into Treg cells in not reported yet and is hence irreversible [115]. Hence, finding the way to maintain the balance between Th17 and Treg cells leads the way to new therapeutic strategies for IBD.

Both the innate and adaptive immunity are linked to each other, as abnormal innate responses lead to imbalanced adaptive immune system and distort the barrier functions of the intestine leading to IBD.

1.2.3 Chromogranin A (CHGA)-Overview

The luminal contents in the intestine are separated from the underlying tissue through the barrier created by the intestinal epithelium. Specific type of cells called entero-endocrine cells (EECs) forms the major category of cells that help recognize the luminal contents and maintain equilibrium by producing many pro-hormones and hormones to regulate the intestinal immunity and motility [210]. The hormones released from the EECs help to maintain gut immune system,

regulates the motility and blood flow *via* its interaction with the central nervous system (CNS) through the gut-brain/brain-gut axis [211-213]. The pathophysiology of IBD is highly influenced by the interaction between enteric nervous system and the immune system. This EECs release various bioactive peptides and secretory molecules which influence endocrine, paracrine, neurocrine and autocrine regulatory effects while interacting with a variety of food nutrients, microbiota and non-dietary antigens [212, 214].

The most prominent protein, which is found locally in the secretory granules of EECs in the GI tract is Chromogranin-A (CHGA) [215]. CHGA plays the role as a precursor of several peptides that perform a wide variety of functions in the human body by affecting the cardiovascular, endocrine, neuroendocrine and immune systems [215]. CHGA is also highly expressed by the neuroendocrine cells [215]. For CHGA to function as a regulatory peptide, it is necessary that CHGA and its derived peptides exist in the extracellular space [212]. It has also been shown that the alteration in the levels of CHGA has a relation with the change in intestinal epithelial homoeostasis in the context of intestinal inflammation [28]. The potential role of CHGA in maintaining the gut inflammation by controlling the innate and adaptive immune response [216] has been suggested in context to the observation that there is an increase in levels of CHGA and EECs in IBD patients and animal models of colitis [28, 216-218].

CHGA, expressed by several neoplastic and normal cells, is seen in abundance in the secretory cells of immune, nervous, and endocrine systems. It is chemically a soluble protein and acidic in nature and is also heated stable [215]. In the adrenal medulla and EECs, it is stored and released from the storage granules along with catecholamines [215, 219-221]. The human *CHGA* gene resides on chromosome 14q32.12, has 7 introns and 8 exons and 12.192 bp long. From the transcript containing 2014 bases, a 42-52 kDa protein of 439 amine acids is encoded [222-224].

CHGA undergoes the process of proteolytic cleavage using pro-hormone convertases at its ten dibasic sides to produce many CHGA-derived peptides (CgDPs)[219, 220]. CHGA processes occur in a tissue-specific manner and also occurs after its release from the neuroendocrine cells[215, 220] [225]. The regulation of CHGA processing is important for the maintenance of the homoeostasis of the GI tract and balancing physiological conditions of the gastrointestinal mucosa.

1.2.3.1 Role of CHGA in Intestinal Homeostasis

1.2.3.1.1 Extracellular Role of CHGA

The largest population of the endocrine cells in the GI tract is of EC cells [226], and the release and circulation of CHGA from these cells are regulated by adrenal medulla and neuroendocrine cells [215] [227, 228]. Where, it is proved that CHGA has a role in maintaining gut homoeostasis, further research is much needed to know its immunological function. CHGA also modulates the peptide secretion from the pituitary gland [229]. Over the past few years various experiments have shown strong relationship between IBD development and CNS which is mediated through the gut-brain axis [230]. The above facts is stirringly favoured by other experiments showing that neuronal apoptosis is promoted by CHGA by TNF-a and other neurotoxic agents [231].

The process of formation of new blood vessels i.e. angiogenesis is a highly regulated process maintained by several anti and pro-angiogenic factors [232] and the dysregulation of this process leads to various disorders, which includes IBD [232]. In IBD patients, various functional and morphological evidences have been found showing the influence of angiogenesis on gut inflammation [233]. Various evidences suggest that CHGA regulates angiogenesis [234] *via* influencing various factors like vascular endothelial growth factor (VEGF) [235], basic fibroblast

growth factor [236] and hypoxia-inducible factor-1 [234]. Additionally, the regulation of angiogenesis by CHGA is done *via* the proteolysis of mechanism induced by thrombin [237]. Therefore, there is high possibility of CHGA maintaining the intestinal homoeostasis and paving the path for anti-angiogenesis therapies.

Human data suggests that the *CHGA* gene variations are correlated with the autonomic functions, which also describes the association between plasma CHGA levels and release of catecholamine [238]. Specifically, to check the role of CHGA in various disease models, knockout (KO) models of *Chga* have been developed [239]. Increased levels of catecholamines were seen in the *Chga* KO mice [27]. One study shows changes in adrenal chromaffin granules and increased blood pressure, while the other study shows normal *via*bility, no abnormality on the development and normal chromaffin granules in the adrenal gland [239]. Even though, more research is needed to fully explore the role of CHGA, but the current knowledge depicts the importance of CHGA derived peptides in regulating gut function.

1.2.3.1.2 Intracellular Role of CHGA

CHGA has a high-binding capacity and low affinity for Ca^{2+} due to which its role is very important in intracellular calcium homoeostasis [240]. Also, CHGA helps in the activation of the calcium channels which thus increases the exchange of free and bound calcium in the secretory granules and cytoplasm as well [215, 241]. During IBD, the patient's colon has continuous and abnormal motility and it is also followed by reduced contractions [242]. Possibility it can be explained by changes in the smooth muscle contractions that can cause colon dysmotility and changes within the enteric nervous system [243]. Ca^{2+} release from both the extracellular and intracellular sources is crucial for the intestinal inflammation [244]. Reduced or altered Ca^{2+} release or influx plays a crucial role in the gut dysmotility during intestinal inflammation [244-

246]. As Ca^{2+} signalling cascade activates various pro-inflammatory pathways, hence regulating Ca^{2+} signalling may contribute in the reduction of gut inflammation [247, 248].

Neuropeptides and hormones are packed in the neuroendocrine cell secretory granules and are stored there for their regulated release [249, 250]. After the exocytosis of these granules, CHGA inhibits their degradation by the formation of dense core granules [239, 251]. Moreover, CHGA also promotes secretory granule formation *via* enhancing the bud formation in trans-Golgi membranes [239, 251].

1.2.3.2 CHGA Derived Peptides and their Functions in Intestinal Mucosa

CHGA gives rise to various bioactive peptides *via* its cleavage at various dibasic sites. Specifically, at N- and C-terminal regions, it undergoes extracellular and intracellular proteolytic cleavage [225]. Many bioactive peptides are generated by pro-protein convertases like chromofungin, vasostatin-I, vasostatin-II. pancreastatin, catestatin, parastatin, serpinin, WE14, chromostatin, and chromacin and all these peptides helps to shape the immune and the neuroendocrine system in one way or the other [252, 253]. All the peptides work in different ways to regulate the gut immune system, where some have similar functions and others have opposite, some have antagonistic effect and others act in a synergistic way [254]. Getting a deep insight into the levels and functions of these peptides will help find potential therapies for IBD patients.

1.2.3.2.1 Chromofungin (CHR)

CHR ($CHGA_{47-66}$) is a N-terminal CHGA derived peptide, from vasostatin-I ($CHGA_{1-76}$), which as whole is known for its anti-fungal and anti-microbial activity. It has a amphipathic helical structure on its C-terminal (Arg^{53}–Leu^{66}) and the N-terminal has more hydrophobic character [255]. It enters the cell membrane *via* its interaction with lecithin monolayer and calmodulin and

the presence of Ca^{2+} plays a significant role in the interaction [256, 257]. When tested for antifungal activity, CHR was found the most effective agent amongst the various peptides derived from vasostatin-I. At a concentration of 50 μM, it completely inhibited the growth of *C.neoforman*, *C. albicans*, and *C. tropicalis* [255]. These antimicrobial peptides are very crucial for maintaining the intestinal barrier integrity and immune function and a variation in the levels of these peptides is seen during IBD in the inflamed mucosa [258, 259].

1.2.3.2.1.1 Role of Chromofungin in Gut and Immune Response

As the major regulation of gut immune system is done by the gut microbiota, and as CHR behaves as an antimicrobial agent, it could play a significant role in pathophysiology of gut inflammation. Besides the antimicrobial properties, CHR also stimulates neutrophils, which are major responders of innate immune system [260, 261]. Moreover, it also promotes the cross-talk between immune and the endocrine systems [219]. As CHR has a role in Ca^{2+} metabolism, recent study has shown that when polymorphonuclear (PMN) neutrophils releases CHR, which is amphipathic and cationic, they worsen the Ca^{2+} influx and hence stimulate the release of several immune-regulatory molecules [256, 257, 262]. Additionally, *In-vitro* experiments from mice macrophages have shown that CHR enhances M2 macrophage activity in response to IL-13/IL-4 stimuli, reduces M1 macrophage activity when stimulated with LPS and even regulates the chemotactic activity [263, 264]. Similar mice studies with CHR have shown that it modulates epithelial cells and macrophage plasticity and hence reduces DSS-induced colitis severity [213, 263, 265] and the studies show that this suppression is due to the inhibition of NF-kB pathway, which reduces the inflammation and enhances the tissue repair [263, 264]. On the other hand, looking at the data from the colon biopsies from UC patients, it was observed that CHR was significantly reduced in the patients as compared to the healthy controls. Moreover, these reduced

levels of CHR were found positively correlated with the intestinal epithelial cells and M2 macrophage markers and in a negatively association with the M1 macrophages and NF-kB activation [263, 264].

CHR is found not only regulating the immune response, but it can also act as a potent antinociceptive agent at a lower dose and it does so by acting on the sensory neurotransmitters [266]. The leukocyte migration, adhesion and infiltration are facilitated by intestinal ischemia, which also plays a crucial role in IBD pathophysiology [267]. In a similar way, various abnormalities like red blood cells extravasation, necrosis and hypertrophy of endothelial cells and tissue edema is a characteristic of vascular endothelium in CD patients [268]. Interestingly, IBD patients have increased chances of ischaemic heart disease (IHD), and they also have an enhanced inflammatory response [269]. In mice studies, it was found that that CHR activate pro-survival kinases and decrease lactate dehydrogenase activity and infarct size and hence affecting the heart performance and in the end also guards against ischemia/reperfusion (I/R) injury. Hence, it explains CHR as a post-conditioning agent [270].

As CHR has a relationship with gut microbiota, pro-survival kinases and neurotransmitters, it has great potential of being as a therapeutic agent in the treatment of IBD.

1.2.3.2.2 Other CHGA-Derived Peptides

Vasostatins (VS): VS consists of two N-terminal CHGA derived peptides, VS-I ($CHGA_{1–76}$) and VS-II ($CHGA_{1–113}$) [271]. VS is involved in a wide range of functions like vasodilation, cell adhesion regulation, antimicrobial effects and inhibits the secretion of parathyroid hormone [215]. Intra-granular processing of the VS is crucial for its immune functions and nearly half of the VS secreted undergoes intra-granular processing [225]. The major event in the pathogenesis of IBD

involves the enhanced permeability of intestinal epithelium and hence increased infiltration of pro-inflammatory cytokines and their inhibition behaves as the central treatment in IBD pathogenesis [272]. Mice studies have shown that VS-1 reduces the permeability induced by IFN-γ- and TNF-α and also decreased the release of IL-8 from the epithelial cells stimulated with LPS [273]. It is also helpful in reducing the symptoms of DSS-induced colitis, when given orally [274, 275].

Pancreastatin (PST): PST is a dysglycemic hormone, which is expressed from Exon-VII of CHGA [276]. In 1986, it was isolated from porcine pancreas [277] and it is also the first recognized chromogranin derived peptide [278]. Both in IBD patients and in a mouse model of colitis, the energy metabolism is very important for the immune response and its reprogramming is necessary for the inflammatory effect [279]. The global immunity by the effective expansion of CD4+ T cells and effector T cells is maintained by the glucose transporters [280] and PST inhibits the secretion of insulin and alters glucose homoeostasis, which shows a direct dysglycemic effect of PST [281]. Additionally, PST stimulates vagus nerve and inhibits the release of parathyroid hormone. It inhibits gastric and pancreatic exocrine secretion and hence modulates the functions of the gastrointestinal tract [282, 283].

Catestatin (CST): CST is found on exon-VII of *CHGA* (rat $CHGA_{367–387,}$ human $CHGA_{352–372}$, bovine $CHGA_{344–364}$,) and is highly conserved sequence of 21 amino acids [283]. CST helps the endothelial cells to proliferate and migrate and also plays a role in the smooth muscle cells chemotaxis stimulation [284]. In IBD patients, human catestatin has been shown to play a crucial role in controlling the intestinal inflammation and it does so by the inhibition of signal transducer and activator of transcription (STAT)-3, which inhibits the production of pro-inflammatory cytokines [285]. In mice, the severity of experimental CD [286] or UC [274, 275] caused by DNBS or DSS is reduced by the upregulated mouse CST signalling by reducing the activation of immune

cells and inflammatory mediators [285]. Additionally, CST suppresses the intestinal inflammation ad release of pro-inflammatory cytokines by reducing the activity of classically activated macrophages and without distressing alternatively activated macrophages [287, 288].

Miscellaneous CgDPs: There are several other CHGA-derived peptides, but more investigation is needed to know the exact function of each of them. Most of the peptides released from CHGA further gets processed to give rise to additional bioactive peptides. For eg. CHR is a VS derived peptide and is a more potent antifungal agent than VS. In the nutshell, there are several other CgDPs like prochromacin, chromacins, parastatin, serpinin, WE-14 and GE-5, whose functional abilities are yet to be identified fully.

1.2.3.2.3 CHGA Relevance in IBD Patients

The CHGA levels are found elevated in the colon biopsies of IBD patients [217, 218, 285, 289-293]. The EECs, from where the CHGA is releases, shows a change in the number during intestinal inflammation [289, 294]. During intestinal inflammation, there are changes in the intestinal motility, reduced feeding and anorexia and it found to be associated with EEC hyperplasia. In the large intestine, it is also followed by the increase in the concentration of CHGA-immunoreactive cells both in UC [295] and IBS [296] patients. Hence, the CHGA acts as a biomarker for the intestinal disorder [297]. Moreover, the EECs react with the gut microbiota metabolites though their functional TLRs [298, 299]. Thus, CHGA acts as a biomarker for looking into the disease activity in IBD patients. The CHGA level goes high in the patients with UC or UC in comparison to the healthy individuals [217]. Correlation is observed between CHGA expression and TNF-α in the disease activity and its extent. The plasma CHGA levels were also found to be identical between UC and CD patients [217].

Human studies have also shown that the level of CHGA in the serum was found significantly high IBD patients in comparison to the health individuals [289]. Moreover, a down regulation of CHGA levels was seen after the biological treatment was done. Significant down regulation in CHGA levels was also seen in the rheumatoid arthritis patients, who were on anti-TNF therapy for four weeks [300]. Elevated *CHGA* gene mRNA expression was observed in the IBD patients who were treated with steroids [301]. For the patients, who were on remission, CHGA values were upregulated when they were treated with thiopurines and steroids [289, 290].

Hence, CHGA plays a vital role in the intestinal inflammation. On the other hand, it is equally important in the diagnosis and/or checking the drug response in neurological and cardiovascular diseases, hypertension and neuro-endocrine tumours [215].

1.2.3.2.4 Gut Microbiota and its Relevance to CHGA

Gut microbiota plays a central role in maintaining the immunity of the gut and they release a whole variety of vitamins, enzymes and several immune modulators [302]. One of the central defects which results in gut inflammation is due to the miscommunication between mucosal system and gut microbiome [303, 304]. Several new experiments showed that many barrier integrity functions are maintained by the interaction between gut microbiota and pathways that involve EECs [299, 305]. As we know, several CHGA derived peptides like CHR, CST, and VS show an antimicrobial effect [256, 257, 287, 306-309], it is highly likely that these peptides regulate the gut inflammation in co-ordination with gut microbiota.

CHGA was found as the marker for the human gut microbiota diversity and composition in a metagenomics study [310]. The study revealed a strong correlation between microbiota diversity, composition, and function and CHGA. By using 16S RNA sequencing, this data was

confirmed in an independent group for 19 people. The levels of CHGA were associated positively with type and frequency of stool, triglycerides blood levels and fecal calprotectin levels. It was found negatively correlated with the functional richness and diversity of the gut microbiota [310]. Additionally, CHGA was found to have a link with 61 microbiota species, which forms nearly half of the microbiome species found in the gut. *Methanobrevibacter smithii* was found to have the strongest association with CHGA, and these bacteria help in digestion process by digestion of polysaccharides [311]. In a nutshell, the gut microbiome and CHGA are found to be closely related to each other and targeting CHGA can improve microbiome related disease severity.

1.2.4 The Role of Nuclear Factor Kappa-B in IBD

The nuclear transcription factor kappaB (NF-kB) was found as one of the most important factors contributing in the mucosal immune system and it is markedly activated in IBD patients. NF-kB consists of five members named p65, p50, p52, RelB and c-Rel. The DNA binding, nuclear localization and the dimerization is regulated by its N-terminal conserved region [312-314]. Small molecules like IκBε, IκBβ or IκBα behave as inhibitors and keep the NF-kB dimers inactivated in the state of equilibrium [315]. The activation of NF-kB is stimulated in the gut *via* various bacterial components like LPS or other pro-inflammatory cytokines like IL-1 and TNF-a, which further induces the inflammatory cascade [316].

In IBD, excessive production of pro-inflammatory cytokines is observed in the gut, which is majorly releases by the epithelial cells, macrophages, and lymphocytes and NF-kB plays a crucial role in maintaining the inflammation through these cells. A significant increase levels of NF-kB p65 has been observed in the gut epithelial cells and macrophages from colonic biopsies isolated from IBD patients [317]. Interestingly, when analyzed for p65 subunit using immunofluorescence, a positive correlation between the activated p65 and the severity of IBD was

found [318]. Not only macrophages and epithelial cells but the fibroblasts in the mucosal immune system play a significant role in the NF-kB, related gut immunity. In IBD patients, macrophages which have enhanced expression of NF-kB, also show greater ability to secrete the pro-inflammatory cytokines like IL-6, IL-1 and, TNF-a [317].

Early studies showed that the blocking of NF-kB by giving p65 antisense oligonucleotide locally can treat the TNBS-induced colitis in mice [317]. Many drugs which inhibit NF-kB activity like methotrexate, corticosteroids and anti-TNF-a antibody have been established [319-323]. The corticosteroids suppress NF-kB activation by increasing the activity of IkBa [319, 320, 324]. In parallel to these results, glucocorticoid-treated patients show reduced levels of NF-kB p65 as compared to untreated patients in the colonic epithelial, endothelial and mononuclear cells [320]. Even though, various NF-kB blocking drugs are available, but their mechanism of action of inhibition is indirect and selective inhibitors targeting NF-kB directly are still in need [317]. Considering the fact that NF-kB markedly regulate the inflammation in the gut during IBD, therapeutic targeting of NF-kB represents a hopeful therapeutic treatment of IBD.

1.2.5 Treatments of IBD

In the last decade, many approaches have been followed to develop new and effective drugs in the field of IBD, with the main motive to develop drugs that can maintain a remission stage [17]. The current drugs available for IBD are divided into 5 categories, which are described below.

1.2.5.1 Anti-inflammatory Drugs

Anti-inflammatory drugs like mesalazine or some corticosteroids are the most widely used drugs in IBD. Mesalazine has been found to be an active functional group of sulfasizine which is a combination of sulfapyridine and 5-aminosalicylic acid. Its mode of action is not well determined

but it is believed to play a role in inflammation by affecting its chemical modulators like leukotrienes and prostaglandins [325]. In UC patients, its use has been supported by several studies but they considered it as non-supportive in CD patients yet [17]. Corticosteroids play a significant role in blocking the early development of inflammation by preventing vasodilation, permeability of vascular and neutrophil infiltration. They also inhibit deposition of collage and activation of fibroblasts. They can also block the production of NF-kB by inhibiting the inflammatory cytokine production, therefore, interfering with the inflammatory process. Budesonide, prednisolone and methylprednisolone are the most commonly used CSs. Current data indicate towards a non-efficacity of mesalazine in CD patients. CSs are effective in some UC patients in activating remission and CD patients as well but also have severe side effects, but it depends on specific classification of the patients.

1.2.5.2 Immunosuppressive Agents

Six-mercaptopurine (6-MP) and azathioprine (AZA) are used for the treatment of both UC and CD. 6-MP and AZA work by inhibiting lymphocyte proliferation by getting incorporated into the cellular nucleotides and hence suppressing T cell and NK cell function [326, 327]. In some cases, treatments can induce, hypersensitivity reactions, fever, pneumonia, hepatitis, and arthritis and pancreatitis, which can occur in nearly 4% of the treated patients [328].

Methotrexate (MTX) is used a popular immunosuppressive drug for CD patients and there is no convincing data to support its use in UC patients [329]. Its mode of action is *via* the inhibition of DNA synthesis and increasing adenosine and some report demonstrated an antagonist effect on folic acid. It also acts by suppressing T-cell function and by inhibiting IL-1 production [330]. MTX is believed to be safe and nausea might occur with its use, which can easily be prevented by folate [329].

Cyclosporine (CSA) is used for the treatment of UC. CSA inhibits the antigen induced secretion of lymphokines by binding to the calcium calmodulin-dependent protein phosphatase calcineurin [331], leading to a decrease of pro-inflammatory lymphokines. Studies have shown that using CSA during the acute phase of the disease can induce remission and delay or avoid colectomy [332].

1.2.5.3 Antibiotic Treatment

Several varieties of antibiotics have been tested to treat CD [333]. The current antibiotics which are in use include: ornidazole, metronidazole, tobramycin, cotrimoxazole and clarithromycin. Metronidazole is used since 1970 but it failed to show its efficacy as it showed no difference between the placebo and treatment group [334]. However, its effectiveness was observed in patients who were resistant to sulfasalazine [335, 336]. Moreover, it has been described as effective when given in combination with ciprofloxacin [335, 336]. Omidazole, which has similar antimicrobial and chemical properties to metronidazole was effective in lowering the Crohn's Disease Activity Index (CDAI) in 4 weeks of treatment. It reduced factors like abdominal pain, recurrence rate and Serum C levels. Another antibiotic used in CD patients is Ciprofloxacin, which, has also shown efficacy in down regulating CDAI and is believed to have great effect in patients whose colon has inflammation [333]. Clarithromycin is another broad-spectrum antibiotic which penetrates through the macrophage and helps in treatment against IBD, although in a clinical study when administered for three months it only showed efficiency in the first month, which suggested that bacterial resistance against the antibiotic reduces its effect [337]. A lot of research is still required to understand the role of antibiotics and their mechanism of action. Another limitation of using antibiotics is the development of resistance against the bacteria and development of a second infection in CD patients. Ciprofloxacin which is effective against

Yersinia enterocolitica still causes infection with *Y. enterocolitica* in ciprofloxacin treated CD 297 patients [333].

1.2.5.4 Biological Agents

Infliximab (IFX) is one of the biological agents. It's an immunoglobulin G1 type chimeric antibody which binds to free and membrane bound TNF-α and in turn activates the T-cell cytotoxicity and activates cell clearance [284]. In both UC and CD, IFX has a positive effect in mucosal healing. Adalimumab (ADA), which is a human monoclonal antibody has been found to be effective in IFX non-responders, corticosteroids resistant UC patients and minor to severe CD patients [17, 338]. ADA is injected subcutaneously and provides remission stage [17, 339]. There are side effects of the use of anti TNF-α biologics, like autoimmunity and infections which occur due their immunosuppressant profiles [340]. Therefore, before administering these biological agents, a test for tuberculosis (TB) should be done on the patients as the treatment can activate dormant TB [341].

1.2.5.5 Probiotics

Probiotics are non-pathogenic microbes that help counteract the imbalance of microbiota in IBD. Yeast like *Saccharomyces boulardii* or lactic acid bacteria like *Lactobacillus* and *Bifidobacterium* spp are currently used in the treatment of IBD. [17, 342]. Probiotics play an important role in improving the gastrointestinal health by avoiding pathogenic microbial colonization, decreasing pH of the intestine or by influencing the immune response. For example, the lymphocyte content and the plasma levels of pro-inflammatory cytokines are significantly reduced in UC patients after administration of *Lactobacillus paracasei* [342]. *S. boulardii* plays a role in IBD by interrupting NF-kb production and reducing the production of pro-inflammatory

cytokines. *S. boulardii* releases a protease that cleaves *Clostridioides difficile* toxin and stops its bacterial overgrowth. Also, the mitogen activated protein kinases ERK1/2 and P38 activity is modulated by *S. boulardii* secreted protein [343]. When administered in UC patients, probiotics have shown efficient results. Like other treatments probiotics have their limitation as well, as no beneficial effects have been seen in CD patients. Up to today, there is not enough data regarding the dosage, patient group or beneficiary probiotic strain [17].

1.2.6 Animal Models for IBD Study

The recent progresses made to understand the immune functions during intestinal inflammation are due to the development of several animal models of colitis [344]. Even though, we know that the experimental models are not effective enough to mimic the level of complexity of the disease that occurs in humans, they are still valuable tools to study the immune mechanisms which are very hard to define in humans. To study IBD, several models are available, but the chemically-induced models are preferred due to their ease of induction and quick onset of disease and a good control [344].

1.2.6.1 Dextran Sulfate Sodium (DSS) Model of Colitis

DSS model of experimental colitis described in 1990 by Okayasu et al., is the most widely used model of colitis and it is induced by giving DSS in the drinking water [345]. The molecular weight of the DSS and the sanitary status of the facility can determine the severity of colitis and an acute or chronic model can be established by altering the duration and concentration of DSS administration [346]. Even though, the exact mechanism behind the DSS induced colitis is still unknown, it has been demonstrated a reduced epithelial proliferation and an increase of apoptosis that can lead to a defect of the epithelium integrity. In homeostatic condition, the luminal microbes

are prevented from entering through the intestinal mucosa *via* an effective epithelial integrity, but if the epithelial integrity is lost, the immune system is activated leading to an enhanced infiltration of immune cells and an increase of pro-inflammatory cytokines [347, 348]. Additionally, DSS can also have an impact on the microbiota and can induce a microbial dysbiosis [349].

1.2.6.2 Dinitrobenzene Sulfonic Acid (DNBS) Model of Colitis

The DNBS model of colitis is induced by the administration of DNBS intra-rectally with ethanol in varying concentrations [350]. Here, the role of ethanol is to damage the epithelium and allow the DNBS penetration to induce inflammation. DNBS, which acts as a hapten, enhances the immunogenicity of the luminal contents, which disturbs the intestinal homoeostasis and induce inflammation. Weight loss, intestinal thickening and bloody diarrhea are its major characteristics, but the symptoms vary on the basis of the amount and exposure of DNBS and the type of animal. DNBS is a less expensive method of inducing colitis in comparison to DSS, but greater skills are required to induce the colitis.

1.2.6.3 Other Models of Colitis

Trinitobenzene sulfonic acid (TNBS) is another widely used chemical agent to induce colitis and it is used for inducing both acute and chronic colitis. It is used in various animal groups to induce colitis like rodents [351], rabbits [352], guinea pigs and non-human primates. Just like DNBS, TNBs is also administered with ethanol [353]. C57BL/6 mice are usually resistant to TNBS colitis whereas BALB/C mice are more prone to TNBS-induced gut inflammation, hence the importance of genetic background also needs to be considered before inducing TNBS colitis [353]. Moreover, due the instability of the tri-nitrile residue, TNBS is almost not used anymore.

Oxazolone is another chemical agent which can induce colitis. It induces a Th2 type immune response by acting as a hapten itself [354]. It leads to diarrhea, ulcers and body weight loss in murine model of colitis. It is a perfect model to induce acute gut inflammation as its administration leads to an active tissue injury [354, 355]

There are several other agents used for the induction of colitis like azoxymethane, which induces inflammation and leads to colorectal cancer [356, 357]. Acetic acid is another agent used for colitis induction. Although there are changes observed in the NF-kB signalling, however, the immediate tissue injury was found not due to any immune response [358]. Due to the complication in various models of colitis, DSS-induced colitis is the preferred model, because of its ease of induction and lower cost and a steady reproducibility of the results, therefore, in the study presented within this thesis, I decided to use that model.

CHAPTER 2

RATIONAL, HYPOTHESIS AND AIMS

2.1 Rational

IBD, which is mainly divided into UC and CD shows chronic gut inflammation. The leading factors to its symptoms include imbalanced gut immune interaction, dysregulation of EECs, genetic susceptibility, dysregulated immune responses [28] [37] [35]. CHGA, found in the secretory vesicles of EECs, gives rise to several bioactive peptides, which regulates various endocrine, cardiovascular and immune responses [215]. CHR, derived from CHGA, is a short active peptide, and is known to have antimicrobial and immune regulatory functions [255]. CHR has also been described as a post-conditioning agent against I/R injury damage through activation of pro-survival kinases. A recent study also revealed that CHR can reduce the DSS-induced experimental colitis through the regulation of macrophages [213]. In parallel, $CD11c^+$ cells play a major role in the regulation of experimental colitis *via* the modulation of surface co-stimulatory molecules like CD40, CD80 and CD86 [359]. Currently, there is a gap of knowledge in understanding the influence of CHR on $CD11c^+$ cells during the development of experimental colitis.

2.2 Hypothesis

We hypothesized that the intrarectal administration of CHR reduces the disease severity in the experimental model of DSS-induced experimental colitis by targeting the major antigen presenting cells i.e. $CD11c^+$ cells.

2.3 Aims

Three aims have been developed to address our hypothesis.

1. To investigate the relationship between *CHGA (Exon-IV)* and CD11c+ cells, related markers in colonic biopsies from the UC patients.

2. To investigate the effect of CHR in an experimental model of DSS-induced colitis in mice.

3. To investigate mechanism responsible for the reduction of $CD11c^{+}$ cells-related bio markers.

CHAPTER 3

3. Material and methods

3.1 Human Samples

The University of Manitoba research Ethics Board [HS14878 (E)] approved the study. The collection of the biopsies was done from the persons undergoing colonoscopy with known IBD or without IBD and with their consent. A total of four biopsies were taken from the inflamed sites, which were next to the tissues taken for histopathology assessment in patients with active UC (n=10) and inactive (mild) UC (n=9). Form healthy subjects (n=10), biopsies were taken from comparable uninflamed sites. The information of the healthy individuals and UC patients are described in Table 3.1. These biopsies were used instantly for further RNA extraction and gene expression analysis. Confirmed active UC endoscopic analysis was used as the addition criteria. Patients were ≥ 18 years and they were not having any immune suppressive therapy and there was no treatment received by eight out of ten UC patients. Those patients who were taking thiopurines and anti-TNF therapy were not included. One patient was on 5-amino salicylates and he was having it 8 weeks before he was included in the study, whereas another patient was getting methotrexate treatment. Every associated IBD therapy like corticosteroids, 5-amino salicylates and methotrexate were allowed in the subjects where UC was not active.

Table 3.1 Healthy Individuals Description

Status	Birth Year	Endoscopic Results	Histology
Healthy	1954	Normal	Normal
Healthy	1958	Normal	Normal
Healthy	1959	Normal	Normal
Healthy	1959	Normal	Normal
Healthy	1959	Normal	Normal
Healthy	1964	Normal	Normal
Healthy	1968	Normal	Normal
Healthy	1981	Normal	Normal
Healthy	1986	Normal	Normal
Healthy	1989	Normal	Normal

Table 3.2 Patients with Ulcerative Colitis (UC)

Status	Birth Year	Endoscopic Results	Histology
UC	1956	Severely Active Colitis	Active Colitis
UC	1962	Active Colitis	Active Colitis
UC	1970	Active Colitis	Active Colitis
UC	1975	Severely Active Colitis	Severely Active Colitis
UC	1979	Active Colitis	Active Colitis
UC	1979	Active Colitis with Ulcers	Severely Active Colitis
UC	1981	Severely Active Colitis	Active Colitis
UC	1990	Severely Active Colitis	Severely Active Colitis
UC	1993	Severely Active Colitis	Severely Active Colitis
UC	1996	Active Colitis	Active Colitis

3.2 Animals

All the experiments were conducted under the protocol # 15-010, approved by University of Manitoba Ethics Committee. Male C57BL/6 mice (6 to 8 weeks old) with body weight between 20 to 25 g were purchased from Charles River, Sherbrooke, Canada. All these animals were kept in the pathogen-free barrier facility maintained by animal care facility at the University of Manitoba.

3.3 Peptide Used

The supply of the peptide was obtained from Pepmic Co., Suzhou, China. Mass spectrometry and reverse-phase-high-performance liquid chromatography was used for the processing of the peptide. CHR peptide resembles to CHR ($ChgA_{47–66}$: RILSILRHQNLLKELQDLAL) [255, 360-363]. Mass spectrometry and reversed-phase high-performance liquid chromatography was used to characterize and purify the peptide. The sequence specificity has already been confirmed using the scrambled CHR peptide (sCHR, $ChgA_{47–66}$: RARDHQQENKILLLSLILLL) [263]. Based on previously published data in relation to the intra-rectal injection of the peptide, the effective dose of CHR was adjusted to 2.5 mg/kg/day [285, 364]. 1% phosphate buffer saline (PBS) (Invitrogen®, NY, USA) was given intra-rectally to the control group of mice [263]. The injection of both CHR and PBS was given one day before the DSS induced colitis and lasted for 5 days.

3.4 DSS-Induced Experimental Colitis

Injection of 1% PBS or CHR was given in the colon one day before the induction of colitis and lasted for five days. DSS (molecular weight, 40 kDa: MP Biomedicals, Soho, OH, USA) was added to normal drinking water at a concentration of 5% (wt/vol) for five days till the mice are sacrificed [345]. Only 6 to 8-week-old mice were used for the experiment. Every 2-days DSS was

freshly dissolved in the drinking water. Time-matching of the controls were done with mice receiving normal drinking water only. The average consumption of DSS was noted per cage each day.

3.5 Severity of DSS Colitis: Disease Activity Index (DAI)

Disease activity index scores have been correlated since long with the pathological findings in a DSS induced model of IBD [365]. It is an altogether score of weight loss, rectal bleeding and stool consistency. The scores were defined as follows: 0, no loss; 1, 5%–10%; 2, 10%–15%; 3, 15%–20%; and 4, 20% weight loss; stool: 0, normal; 2, loose stool; and 4, diarrhea; and bleeding: 0, no blood; 2, presence of blood; and 4, gross blood. Blood was assessed using the Hemoccult II test (Beckman Coulter, Oakville, Canada). DAI was scored for all 6 days from days 0 to 5 during the DSS treatment.

3.6 Severity of DSS Colitis: Macroscopic Scores

After the sacrifice of mice, the abdominal cavity was cut wide open and colon was located. Removal and longitudinal opening of the colon was done, and the macroscopic damage was assessed on the whole colon section. Four parameters were used for the assessment of severity: rectal prolapse, rectal bleeding, colonic bleeding and stool consistency (Table 3.3). The previously described scoring system was used for the macroscopic scored for DSS-induced colitis [365].

Table 3.3: Macroscopic Score

Score	Rectal Bleeding	Rectal Prolapse	Stool Consistency	Blood
0	None	None	Normal	Normal
1	Red	Signs of Prolapse	Soft	Red
2	Dark Red	Clear Prolapse	Very Soft	Dark Red
3	Gross Bleeding	Extensive Prolapse	Diarrhea	Black

3.7 Colonic Histology

Colon sections isolated from the colonic flexure were first formalin fixed and then paraffin embedded. Three mm sections of these segments were hematoxylin and eosin (H & E) (Sigma, Mississauga, Canada) stained. Further, based on already published scoring system, depletion of goblet cells, distortion in the architecture, inflammatory cell infiltration and edema/ulceration, colonic damage was scored [365].

Table 3.4 Colonic Histology scoring

Inflammatory Cell Infiltrates		Epithelial Changes	Mucosal Architecture	Score
Severity	Extent			
Minimal	Mucosa	Minimal hyperplasia	Normal	1
Mild	Mucosa and submucosa	Mild hyperplasia, minimal goblet cell loss	Normal	2
Moderate	Mucosa and submucosa, sometimes transmural	Moderate hyperplasia, mild goblet cell loss	Normal	3
Marked	Mucosa and submucosa, often transmural	Marked hyperplasia with moderate to marked goblet cell loss	Ulcerations, crypt loss	4

3.8 Protein Quantification

Enzyme-linked immunosorbent assays (ELISAs) were used for the protein estimation from full thickness tissue homogenates and/or supernatants from the cell cultures. Protease inhibitors (Sigma, Mississauga, Canada) were dissolved in 700 μ l of Tris HCl to mechanically homogenize colonic samples. Centrifugation was done for 30 min and the supernatants were stored at -80º C until the assay is done [285]. Bradford protein assay (BioRad CA, USA) was used to quantify the total protein concentration in the colonic tissue samples.

To quantify the levels of IL-6, IL-23, IL-1β, and IL-12p40 (R & D Systems, Inc., Minneapolis, USA). Sandwich ELISA was used for the detection of all these proteins. The antigen specific antibody was first coated on the microtiter plate which was kept overnight at 4º C. The primary antibody was then blocked using blocking solution the following day and kept at 37º C for 2 hours. The plate is then washed 4 times and the samples and standards were added at various dilutions and then kept overnight at 4º C. The unbound or the non-specific proteins were then removed using multiple washing. Detection of the bound antigen-antibody complexes was performed by secondary antibodies (detection antibody), followed by enzyme substrate incubation. The amount of the calorimetric signal produced was proportional to the amount of enzyme conjugate present. The quantification was done using microplate reader (BioTek Instruments, Winooski, VT, USA). BioTek Gen 5 software was used for the calculation of sample concentration, blanks, dilution factor and standard curve. The determination of linearity was done by diluting the samples serially. A correlation coefficient of >0.99 and a slope of 1.03 was obtained from the line.

3.9 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

3.9.1 RNA Extraction and Synthesis of cDNA

The extraction of total RNA from the Colonic tissues/cells from the culture was done using TRIzol™ Plus RNA Purification Kit (Life Technologies, NY, USA), according to the instructions by the manufacturer. NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the quality and quantity of the extracted RNA at an absorbance of 260 nm and 280 nm. An absorption ratio (A260/A290) of greater than 1.8 was observed in all the samples. According to the manufacturer's instruction, 1μg of RNA from each sample was treated with RQ1 RNase-Free DNase® (Promega Corporation, Madison, WI, USA) to remove any contamination from genomic DNA.

According to the manufacturer's instructions, SuperScript VILO cDNA Master Mix (Invitrogen, Grand Island, NY, USA) was used for performing reverse transcription in an Eppendorf Thermo cycler at 25º C for 10 min, followed by 42º C for 60 min and 85º C for 5 min. all the samples were cooled to 4º C and then the cDNA sample were stored at -20 º C for RT-qPCR analysis.

3.9.2 Primers

NCBI BLAST (http://blst.ncbi.nlm.nih.gov/Blast.cgi) was used to design the primers from nucleotide sequences and melting temperature (Tm) of 58-62º C, GC content of 45-55%, lengths 18-22 bp and amplicon size between 75-175 bp was sued as the selection criteria. Only those genes have been used as a reference gene which have been already used as endogenous controls in the studies already done. All the primers were ordered from Life Technologies with their certificate of analysis. Table 3.5 and 3.6 shows the primer characterization of both reference and target genes.

Tables:

Table 3.5: Human primers sequences.

Gene name	Forward (5'-3')	Reverse (3'-5')
INFg	GCCTATCTGTCACCATCTCATC	CCTCTGGCTGCTGGTATTTAT
CCR7	GTTCAAATCTCAGGTCCCTCTC	TACCCTGGTGATCCTCATCTTA
CD74	CCCAAGGAAGAGCCAATGT	CATGGCCCTGAAAGCTGATA
Il12p40	ACCAGAGCAGTGAGGTCTTA	CTCCTTTGTGACAGGTGTACTG
CD86	CAGACCACATTCCTTGGATCA	CCGCTTCTTCTTCTTCCATTTC
TBP	CCCGAAACGCCGAATATAATCC	AATCAGTGCCGTGGTTCGTG
CD11C	ACTCAGATCGGCTCCTACTT	TCGGGTCTGCTCGTAGTAAT
IL12A	ATTCCAGAGAGACCTCTTTCATAAC	CCACCTGGTACATCTTCAAGTC
IL23A	CAGGTCACTATTCAATGGGATGC	GCAGTTCTTAATTGCTGCTTGG

Table 3.6: Mouse primers sequences.

Gene	Forward (5'-3')	Reverse (3'-5')
IL6	CCTGAACCTTCCAAAGATGGC	TTCACCAGGCAAGTCTCCTCA
CD86	TTACGGAAGCACCCACGATG	ACTACCAGCTCACTCAGGCT
CD11c	CCAAGACATCGTGTTCCTGATT	ACAGCTTTAACAAAGTCCAGCA
Eef2	TGTCAGTCATCGCCCATGTG	CATCCTTGCGAGTGTCAGTGA
CD80	TCGGCGCAGTAATAACAGTC	GTTTCTCTGCTTGCCTCATTTC
CD40	GTCACACAGGAGGATGGTAAAG	AAAGCAGTTCCAGGGTTCAG
IL12p40	TGGTTTGCCATCGTTTTGCTG	ACAGGTGAGGTTCACTGTTTCT
CCR7	TGGCTCTCCTTGTCATTTTCCA	CTTGAAGCACACCGACTCGTA
IFNg	CTCTTCCTCATGGCTGTTTCT	TTCTTCCACATCTATGCCACTT

3.9.3 Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

All the reactions were performed in a final volume of 20 μl. The reaction was performed according to the manufacturer's instructions, using SYBR green master mix (Life Technologies) in a Roche light Cycler 96 Real Time System. The conditions used for the reaction were: 95º C for 10 min, followed by 40 cycles at 95º C for 15 seconds and one cycle at 60º C for 60 seconds. Duplicates of at RT-qPCRs were run and the average standard de*via*tion within duplicates of all the samples studied was 0.25 cycles.

3.10 DCs isolation from Bone Marrow: Bone Marrow Derived Dendritic Cells (BMDCs)

The 6-8 weeks old mice were sacrificed by cervical dislocation and then using 75% alcohol it was disinfected. Using sterile conditions, the femur and tibia were removed and soaked in RPMI -1640 medium (Invitrogen, USA) and supplemented with 10% fetus bovine serum (FBS)). To extract the cells out of the bone marrow, both the ends of the bone were cut, and a 1 ml syringe was used to flush the bone with sterile RPMI-1640 into a sterile Petri dish. The cell suspension was then collected and centrifuged for 5 min at 1000 rpm. The supernatant was discarded and then pellets resuspended in red blood cells (RBC) ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA; Life Technologies) for the lysis of RBC. After the second centrifugation the pellet was resuspended in RPMI-1640 and counted [366]. The purity of the cells was then verified by flow cytometry.

3.11 Granulocyte Macrophage Induced Colony Stimulating Factor (GM-CSF) induced Culture of BMDCs

Complete RPMI 1640 medium (containing 10% heat-inactivated FBS, 25 mg/ml^{-1} gentamicin, 2 mM L-glutamine) was used for the cell culture. The cells were suspended at a concentration of 1×10^6 cells/ml in culture plates and were stimulated with 20 ng/ml of GM-CSF. The cell culture was then put in an incubator at 37º C with 5% CO_2. The culture media, supplemented with GM-CSF, was replaced on 3rd and 6th day. On the 8th day, the semi-suspended cells were collected with gentle pipetting and the loosely attached cells were scraped using a cell scraper. These mature cells can then be further used for further stimulation experiments [366], [367].

3.12 CD11c$^+$ Cell Isolation from Spleen

After the induction of DSS colitis and sacrifice of the mice on day 5, the spleen was collected and digested in 2 mg/ml Collagenase D (Roche Diagnostics, Meylan, France) dissolved in RPMI 1640 at 37° C for 30 min with intermittent shaking after every 10 min. To stop the reaction and disrupt the DC-T cell complexes, the cell suspension was given 5mM EDTA (Sigma) during the last 5 min. The homogenate was then passed through a 70-μm cell strainer (VWR, Mississauga, ON, Canada) and then washed with RPMI-1640 at 1200 rpm for 5 min. Lysis of RBC was done after that using ACK lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA; Life Technologies). According to the manufacturer's instructions, sorting of CD11c$^+$ cells from the splenocytes was done using EasySep™ Mouse CD11c Positive selection kit (Stemcell Technologies ™). The cells were then collected and counted and used for the further stimulation experiment.

3.13 $CD11c^+$ Cell Stimulation

The $CD11c^+$ cells (1×10^6 per well) isolated from either spleen or derived from bone marrow were cultured in complete RPMI 1640 medium (Life Technologies) containing 10% heat-inactivated FBS, 25 mg/ml^{-1} gentamicin and 2 mM L-glutamine in 12-well plates with 10^{-6} M of CHR for 12 hours and later stimulated with 100 ng/ml of lipopolysaccharide (LPS) for 24 hours. The cell culture was then centrifuged, and the supernatant was collected for protein estimation by ELISA and the cell pellet was used for RT-qPCR and flow cytometric analysis. The determine the intracellular pathway, in another experiment using pharmaceutical blocker/activator, betulinic acid (NF-κB activator, 10 μM; Sigma) or BAY 11-7082 (NF-κB inhibitor, 10 μM; Sigma) were added to the culture medium for 24 hours.

3.14 Flow Cytometric Analysis

Sorted $CD11c^+$ cells (from the spleen of C57BL/6 mice) treated for 12 hrs with CHR (10^{-6} M/ml) cells were used for flow cytometric analysis. 2×10^{-6} cells were distributed into each fluorescence assisted cell sorting (FACS) tube. Before the beginning of staining, the Fc receptors were blocked for 20 minutes by using Fc blocker. The cells were then washed with FACS buffer (2% FCS in PBS) and the surface was stained with fluorescently labelled rat anti-mouse antibodies such as: anti-CD4, anti-CD4, anti-CD40 and anti-CD80 (all from BD Biosciences). The cells were stained for 15 minutes, washed and re-suspended in FACS buffer for acquisition. The cells were assessed using a BD FACS Canto II instrument (BD Biosciences, San Diego, CA). Before acquiring the samples, appropriate compensation controls were used for multicolor staining and to

adjust the voltage for each fluorochrome. The analysis was done using FlowJo software (BD Biosciences) [368].

3.15 Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). Unpaired Mann-Whitney U test was applied to compare between two groups. To compare between more than two groups, One-Way ANOVA followed by a post-hoc test was used. The analyze DAI repeated measure Two-Way ANOVA followed by a post-hoc test was used. To study the association between different markers studied Spearman's correlation test was used. Th statistical two tail significance level was determined at $p<0.05$. GraphPad Prism software (version 6; GraphPad Software, Inc, La Jolla, CA, USA) was used to compute the statistics.

AIM 1: To investigate the relationship between *CHGA (Exon-IV)* and CD11c+ cells, related markers in colonic biopsies from the UC patients.

CHAPTER 4

Results

4.1 Regulation of *Exon-IV* and Surface and Functional Markers Related to CD11c$^+$ Cells in colonic biopsies of active UC patients.

Colon biopsies form UC patients were analysed using RT-qPCR to find the relationship between *CHGA-Exon-IV* and CD11c+ cells related markers. the A significant reduction ($P<0.0001$) in the levels of *Exon-IV* was detected in active UC patients (n=10) as compared to healthy controls (Figure 4.1 a). Furthermore, a significant up regulation of surface markers related to CD11c$^+$ cells was detected (Figure 4.1 b-e): CD11c (P=0.0002), CD86 (P=0.0002), CCR7 (P=0.0001) and CD74 (P=0.0030). We also demonstrated a marked enhancement in the levels of functional markers (Figure 4.2 a-d): IL12A (P=0.0005), IL-23A (P=0.0250), IL-12p40 (P=0.0006) and IFNγ (P=0.0148).

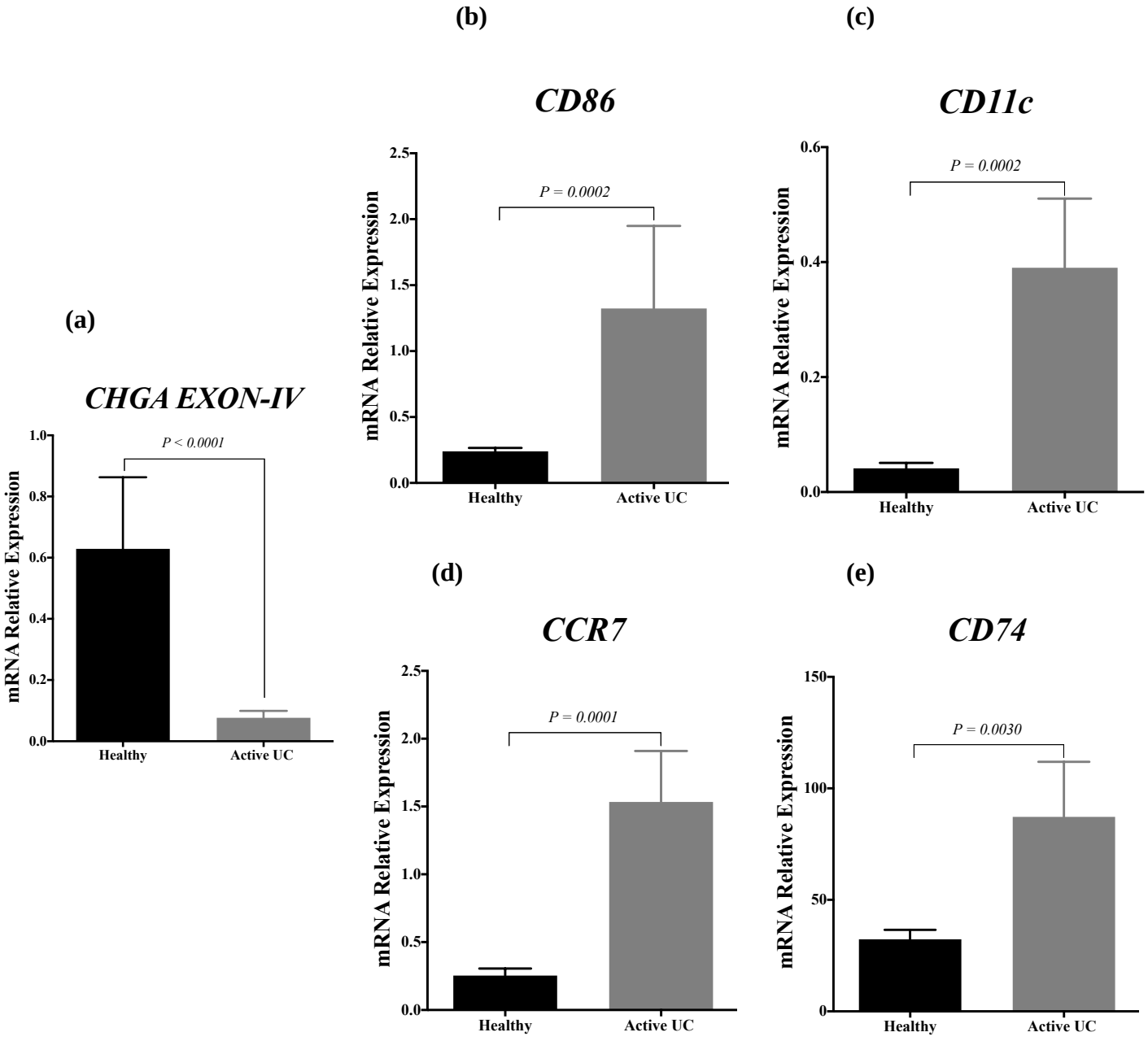


Figure 4.1. *CHGA Exon-IV* is down regulated in active UC patients and the CD11c^{+} cells-related surface markers are significantly upregulated in UC patients. Levels of mRNA (a) from healthy individuals (n=10) and active UC patients (n=10). Relative expression of mRNA (b-e) of CD86, CD11c, CCR7 and CD74 from the colonic biopsies extracted from active UC patients and healthy controls. Unpaired Two-tailed Mann-Whitney U and Spearman's correlation testes were used, data represent mean ± SEM. The significance level was adjusted at 0.05.

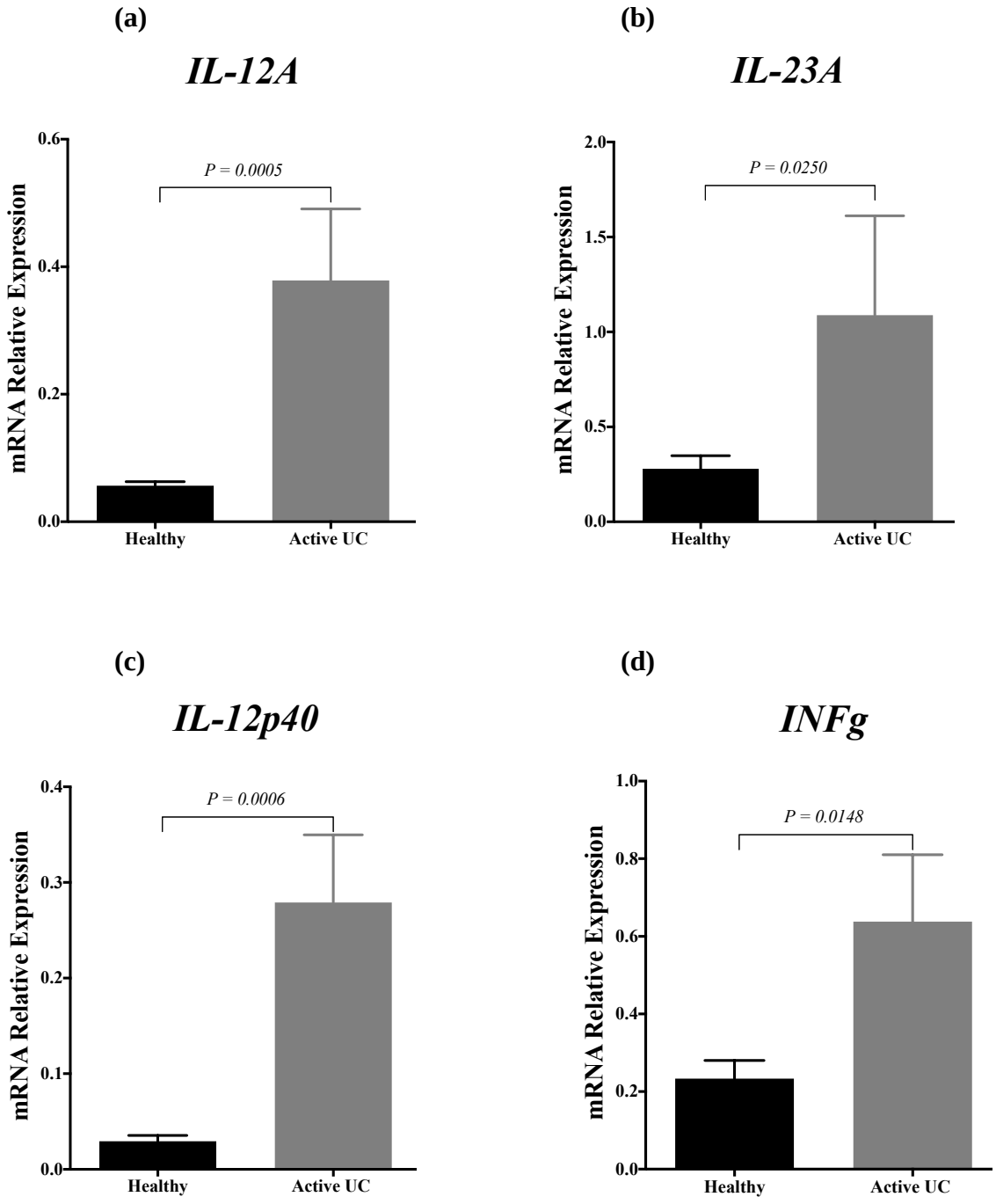


Figure 4.2. CD11c^{+} cells-related functional markers are upregulated in active UC patients. Relative mRNA expression of functional markers (a-d) IL-12a, IL-23a, IL-12p40 and interferon gamma (IFNg) in healthy individuals and UC patients (n=10). Unpaired Two-tailed Mann-Whitney U and Spearman's correlation testes were used, and the data represent mean ± SEM. The significance level was adjusted at 0.05.

4.2 Correlation Between *Exon-IV* and Various Pro-Inflammatory Functional and Surface Markers of CD11c^{+} Cells in colonic Biopsies of Active UC Patients.

To demonstrate the existence of a relationship between *Exon-IV* and various surface and functional markers in UC patients, a correlation analysis was performed. *Exon-IV* showed a moderate to strong negative correlation with CD11c^{+}cells related surface markers (Figure 4.3 a-c) such as CD86 (r= -0.6765, P=0.0051), CD11c (r= -0.4647, P=0.0710) and a weak correlation with the chemokine receptor CCR7 (r= -0.3709, P= 0.1512). Further analysis of functional markers demonstrated a nonsignificant weak to very-weak negative correlation (Figure 4.4 a-d): IL12A (r= -0.3725, P= 0.1415), IL-12p40 (r= -0.3706, P=0.1583), IL-12A (r=0. -4253, P=0.1036) and interferon gamma (IFNg) (r= -0.2706, P=0.3097).

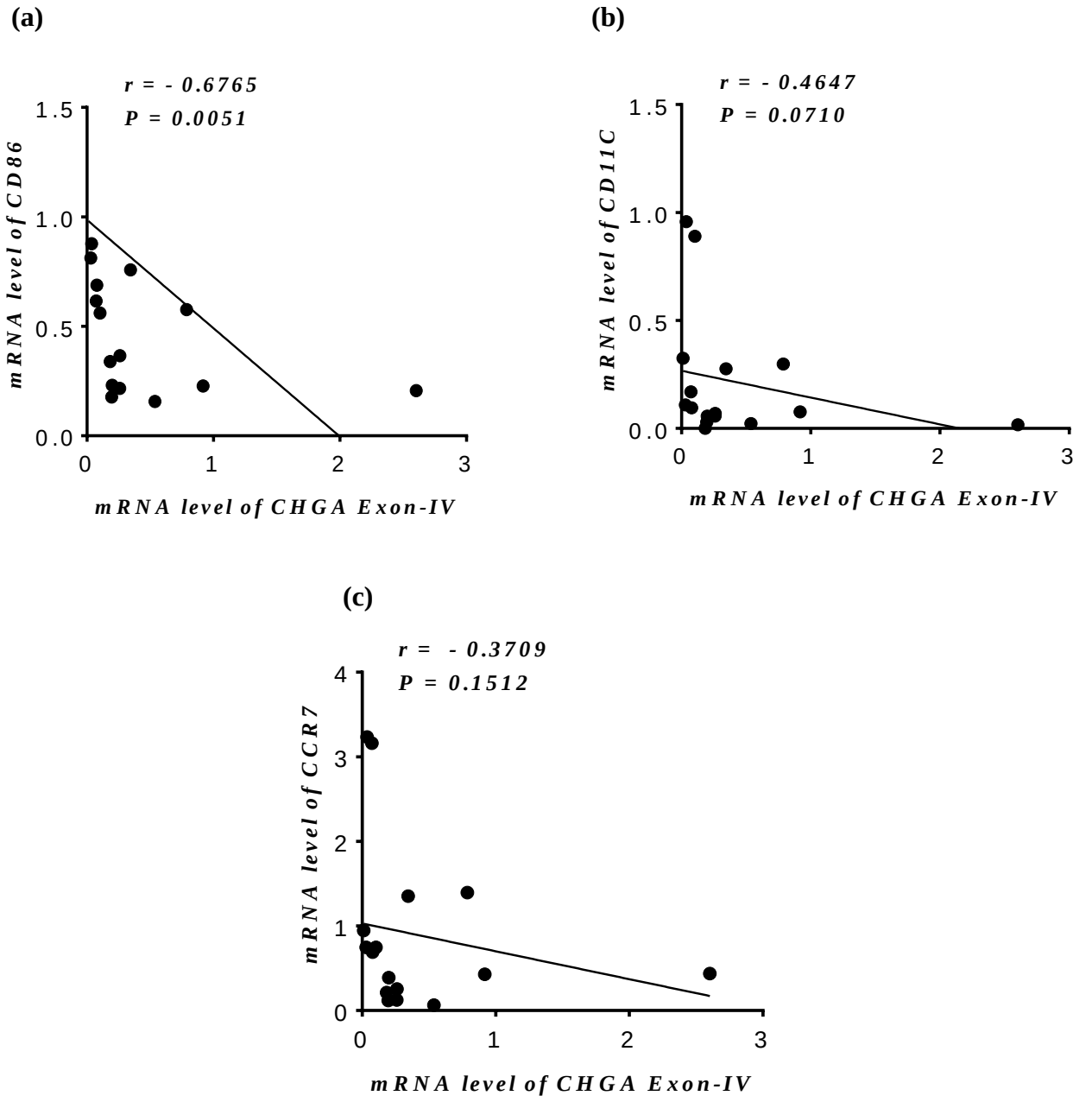


Figure 4.3 Surface co-stimulatory markers and chemokines shows a negative correlation with *CHGA Exon-IV* in active UC patients. Correlation analysis of *CHGA Exon-IV* mRNA from colon biopsies of active UC patients (n=10) with (a) CD86, (b) CD11c and (c) chemokine receptor 7 (CCR7). Unpaired Two-tailed Mann-Whitney U and Spearman's correlation testes were used, and the data represent mean ± SEM. The significance level was adjusted at 0.05.

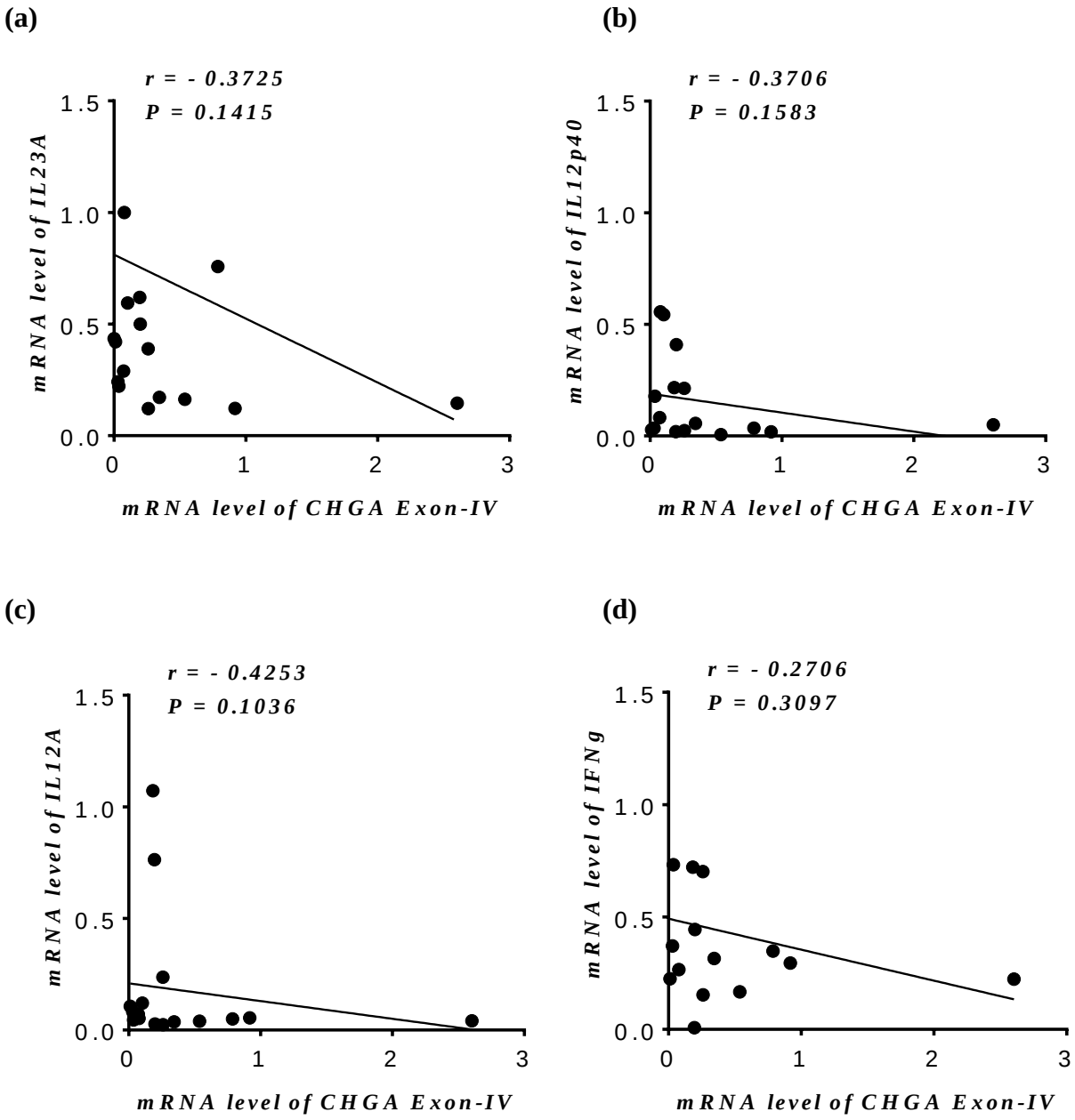


Figure 4.4 Functional markers of CD11c^{+} cells are non-significantly negatively correlated with *CHGA Exon-IV* in active UC patients. Correlation analysis of *CHGA Exon-IV* mRNA from colon biopsies of active UC patients (n=10) with (a) IL-12A, (b) IL12p40, (c) IL12A and interferon gamma (IFNg). Unpaired Two-tailed Mann-Whitney U and Spearman's correlation testes were used, and the data represent mean ± SEM. The significance level was adjusted at 0.05.

AIM2: To investigate the effect of CHR in an experimental model of DSS-induced colitis in mice.

4.3 In Colitic Mice, CHR treatment Decreases Colonic inflammatory macro- and microscopic scores

Next, we investigated the translational applicability to a mouse model of the results that we obtained from the human data. As reported previously [213], first we confirm the anti-inflammatory effect of CHR. CHR (2.5 mg/kg/day) peptide were given for 6 days intrarectally (i.r.) to mice (Fig 4.5 a) and resulted in a marked reduction in the external DAI (Fig 4.5 b). We did not see any difference in the DAI between the DSS+PBS and DSS+CHR group at the beginning but, after day 2, changes were observed and a 3-fold reduction in DAI within the treatment group.

On day 6, when the mice were sacrificed, rectal prolapse, rectal bleeding, colonic bleeding and diarrhea were quantified. CHR treatment decreased significantly (P=0.002) the macroscopic scores in DSS+CHR in comparison to DSS+PBS group (Fig 4.5 c).

Moreover, H and E tissue staining of the mice colonic sections showed a notable reduction in the infiltration of the immune cells into the colon (Fig 4.5 d).

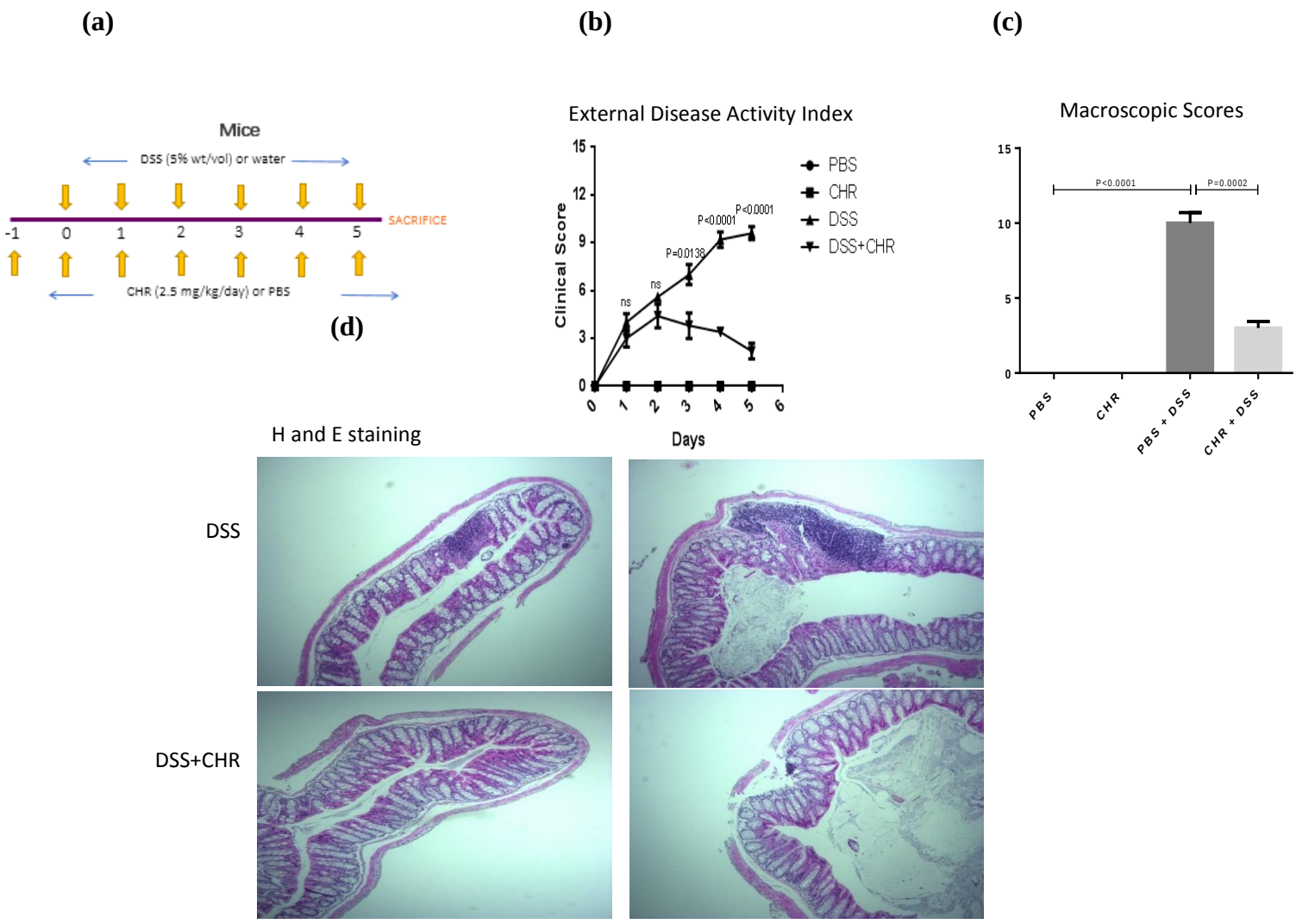


Figure 4.5 Treatment with Chromofungin (CHR): CHGA 47-66 decreases DSS-induced experimental colitis. Colitis was induced with 5% DSS in drinking water to C57BL/6 male mice and the control group of mice received regular drinking water. CHR (2.5mg/kg/da.y, i.r) was given as a preventive treatment or phosphate buffer saline (PBS 1%) starting from one day before the induction of colitis till the sixth day when the mice were sacrificed. (a) Experimental design, (b) external disease activity index recorded over the period of five days, (c) macroscopic scores, and (d) colonic sections, hematoxylin and eosin stained (H&E) (100× magnification) after sacrifice of mice after DSS and CHR administration. Unpaired Two-tailed Mann-Whitney U and Two-way repeated measures or One-way ANOVA followed by multiple comparison tests were used to analyze the data. Each value represents the mean ± SEM, n=6 mice/group. Each experiment was repeated at least three times.

4.4 In Colitic Mice, CHR treatment Decreases Colonic $CD11c^+$ Cells-Related Pro-Inflammatory cytokines.

As the external disease activity index and the macro- and microscopic scores do not give us a precise information of the pro-inflammatory regulators implicated, we detected the concentration of pro-inflammatory cytokines related to $CD11c^+$ cells in the colon.

Analysis of four important pro-inflammatory cytokines released principally by $CD11c^+$ cells was performed in context to colitis, using an ELISA technique. A significant increase in the levels of IL12p40, IL-6, IL-23 and IL-1β (Fig 4.6 a-d) was detected in the DSS group when compared with the PBS control group ($P<0.0001$). A 6 days CHR treatment decreased all cytokines ($P = 0.05$). (Fig. 4.6 a-d.)

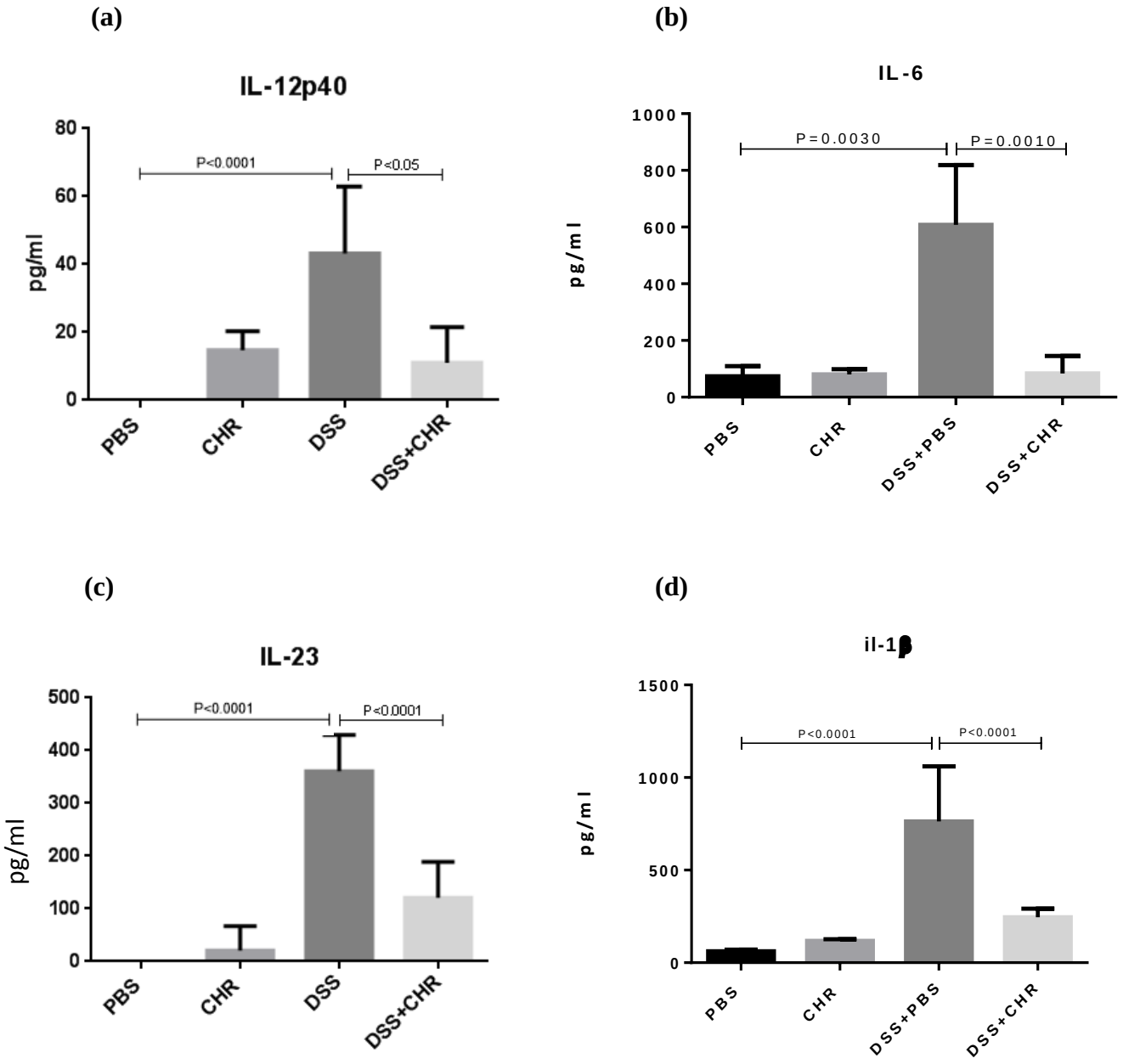


Figure 4.6 CHR decreases the protein levels of CD11c^{+} cells-related colonic pro-inflammatory cytokines. Colitis was induced with 5% DSS in drinking water to C57BL/6 male mice and the control group of mice received regular drinking water. CHR (2.5mg/kg/day, i.r.) was given as a preventive treatment or phosphate buffer saline (PBS 1%) starting from one day before the induction of colitis till the sixth day when the mice were sacrificed. (a) IL-12p40, (b) IL-6, (c) IL-23 and (d) IL1β. One – way ANOVA followed by multiple comparison tests were used to analyze the data. Each value represents the mean ± SEM, n=6 mice/group. Each experiment was repeated at least three times.

4.5 In Colitic Mice, CHR treatment Decreases Colonic CD11c^{+} Cells-Related Surface and Functional Markers

Next, we confirmed these data at the gene expression level using a Q-RT-PCR technique. mRNA expression was detected for four surface co-stimulatory marker and four markers cytokines CD11c^{+}-related cells. Significant changes were observed in all the surface markers like CD11c (Fig 4.7 a), where levels of normalized mRNA expression were markedly reduced (P = 0.0273) in CHR administered colitic mice when compared with the control group. Identical results were found in other surfaces co-stimulatory markers: CD40 (Fig 4.7 b) (P = 0.0237), CD86 (Fig 3.7 c) (P = 0.0296) and CD80 (Fig. 4.7 d) (P = 0.0069).

Similar results were observed when mRNA expression of cytokine markers was detected: IL12p40 (Fig 4.8 a) (P = 0.0076), IL-6 (Fig 3.8 b) (P = 0.0048). However, no significant changes were observed in the levels of IL- and IL-1β.

Alternatively, we studied chemokine receptor 7 (CCR7) (Fig 4.7 c) and demonstrated a significant decrease (P = 0.0114), however, only strong trend was observed for IFN-g (Fig. 4.7 d).

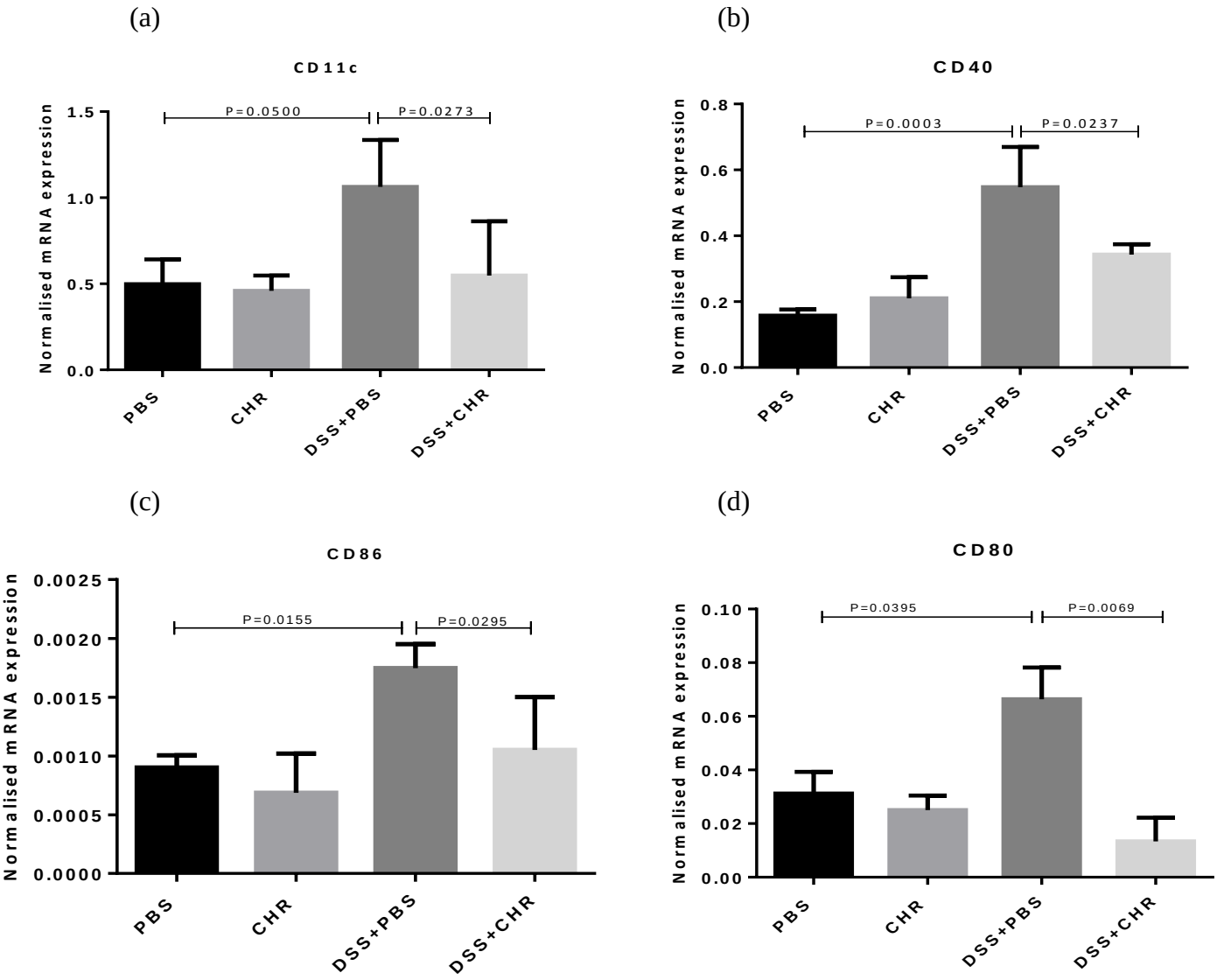


Figure 4.7 CHR reduced the mRNA expression level of CD11c$^+$ cell-related markers. Colitis was induced with 5% DSS in drinking water to C57BL/6 male mice and the control group of mice received regular drinking water. CHR (intra-rectal) (2.5mg/kg/day) was given as a preventive treatment or phosphate buffer saline (PBS 1%) starting from one day before the induction of colitis till the sixth day when the mice were sacrificed. mRNA levels of (a) CD11c, co-stimulatory molecules (b) CD40, (c) CD86 and (d) CD80. One – way ANOVA followed by multiple comparison tests were used to analyze the data. Each value represents the mean ± SEM, n=6 mice/group. Each experiment was repeated at least three times.

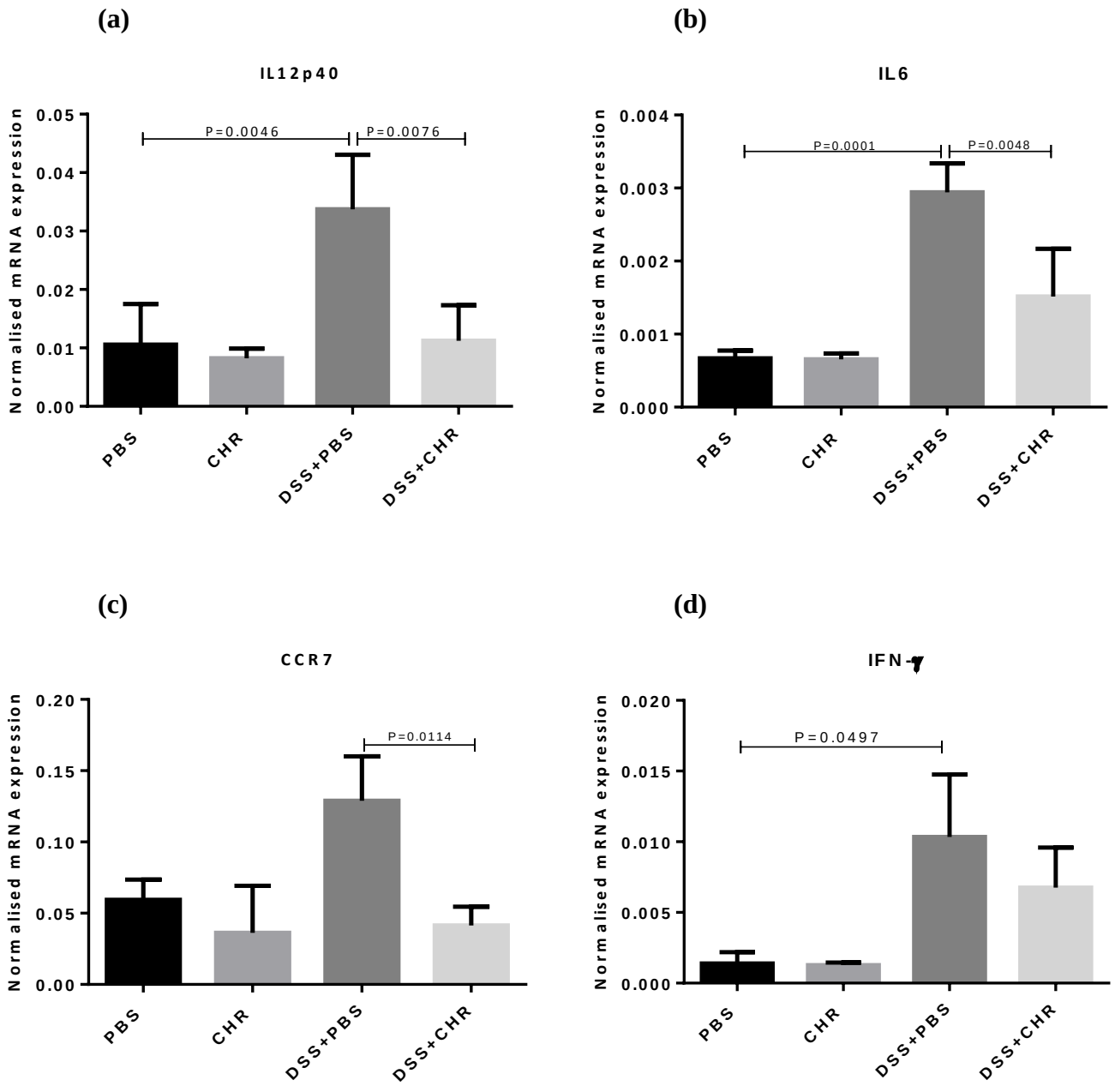


Figure 4.8 CHR reduced the mRNA expression levels of CD11c$^+$ cells-related cytokines. Colitis was induced with 5% DSS in drinking water to C57BL/6 male mice and the control group of mice received regular drinking water. CHR (intra-rectal) (2.5mg/kg/day) was given as a preventive treatment or phosphate buffer saline (PBS 1%) starting from one day before the induction of colitis till the sixth day when the mice were sacrificed. Normalized mRNA expression of functional markers (a) IL-12p40, (b) IL-6, (c) chemokine receptor 7 (CCR7) and (d) interferon gamma (IFN-γ). One – way ANOVA followed by multiple comparison tests were used to analyze the data. Each value represents the mean ± SEM, n=6 mice/group. Each experiment was repeated at least three times.

4.6 In Colitic Mice, CHR treatment Decreases MLN CD11c^{+} Cells-Related Surface and Functional Markers

As we came to know that CHR is modulating the colonic local inflammation both at the protein and at the genetic level, we decided to study if the effect would have been visible within the lymph node site.

The intra-rectal administration of CHR in colitic mice significantly influenced the pro-inflammatory markers in the MLN. CHR decreased the relative mRNA expression of CD11c marker ($P=0.0010$) (Fig 4.9 a) and other co-stimulatory markers like CD80 ($P<0.0001$) (Fig 3.9 b) and CD86 ($P=0.0168$) (Fig 4.9 c), in colitic mice when compared with the mice in which no treatment was given. No significant changes were observed for CD 40 (Fig 4.9 d).

CHR also influenced the functional markers, where IL-6 and IL12-40 (Fig 4.10 a, b) were significantly ($P<0.0001$) decreased in DSS+CHR group when compared with DSS+PBS group. Although, no marked difference was seen in the levels of IL-23 and IL-1β.

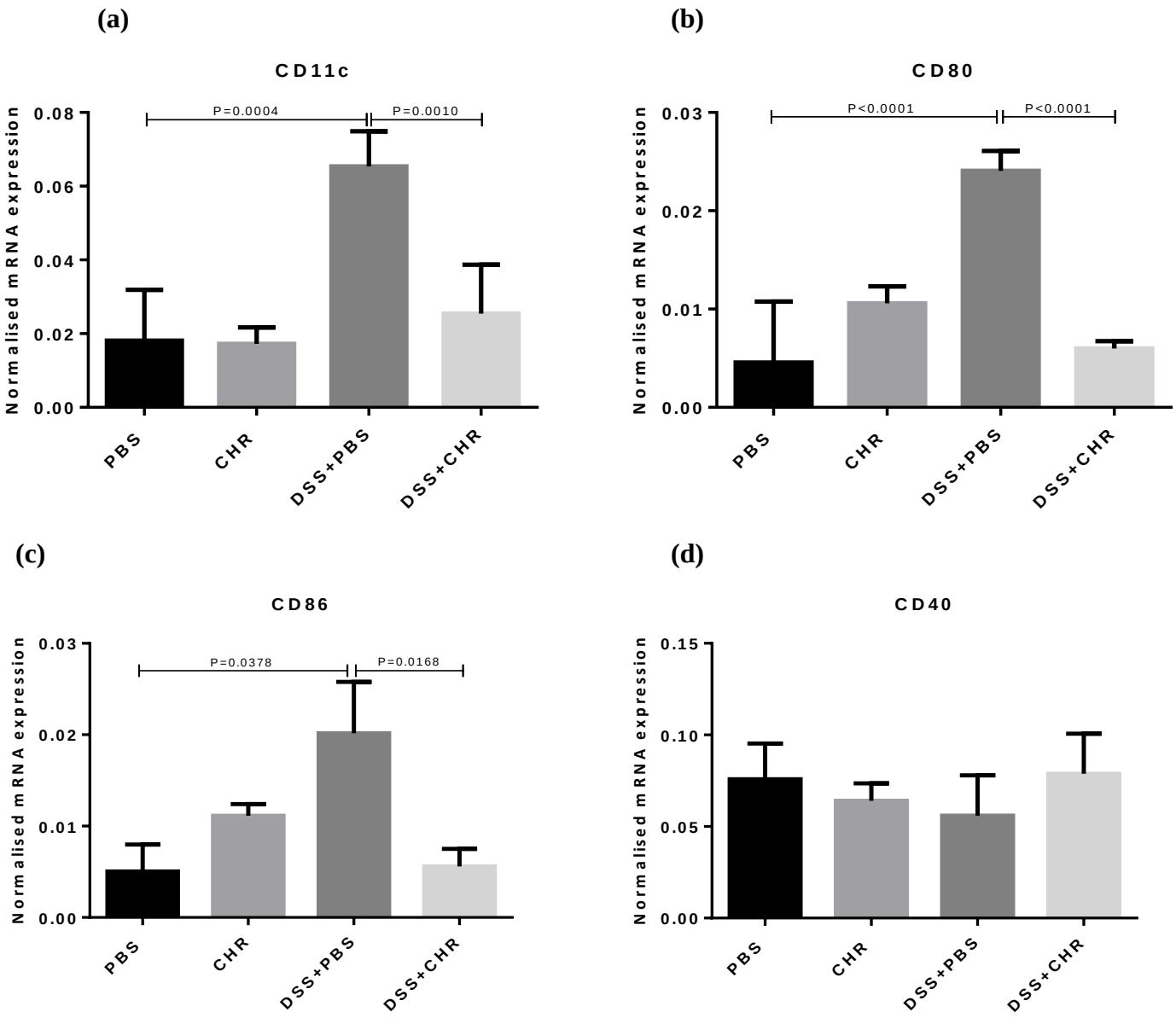


Figure 4.9 CHR reduces the level of MLN CD11c and surface co-stimulatory markers (CD80 and CD86) during colitis development. Colitis was induced with 5% DSS in drinking water to C57BL/6 male mice and the control group of mice received regular drinking water. CHR (intra-rectal) (2.5mg/kg/day) was given as a preventive treatment or phosphate buffer saline (PBS 1%) starting from one day before the induction of colitis till the sixth day when the mice were sacrificed. mRNA levels of (a) CD11c, (b) CD80, (c) CD86 and (d) CD40 from mesenteric lymph nodes. One – way ANOVA followed by multiple comparison tests were used to analyze the data. Each value represents the mean ± SEM, n=6 mice/group. Each experiment was repeated at least three times.

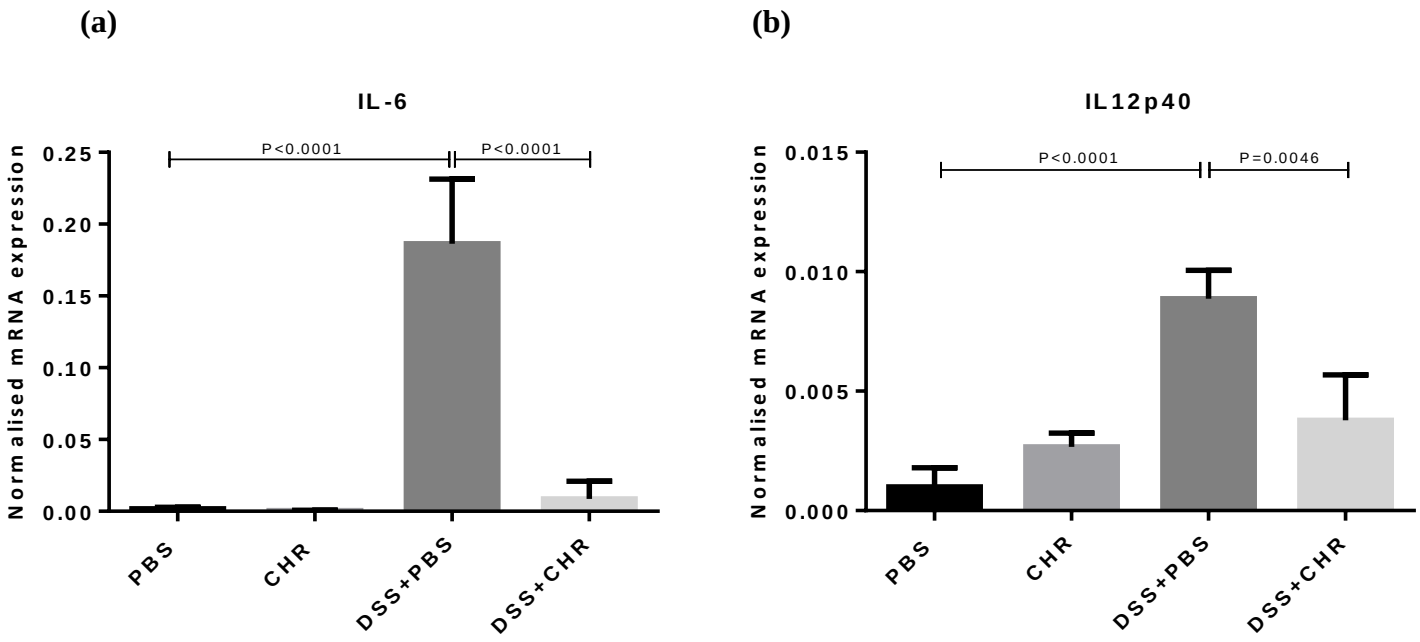


Figure 4.10 CHR decreases the mRNA level of functional markers during the development of colitis in the mesenteric lymph node. Colitis was induced with 5% DSS in drinking water to C57BL/6 male mice and the control group of mice received regular drinking water. CHR (intra-rectal) (2.5mg/kg/day) was given as a preventive treatment or phosphate buffer saline (PBS 1%) starting from one day before the induction of colitis till the sixth day when the mice were sacrificed. mRNA levels of (a) IL-6 and (b) IL-12p40 from mesenteric lymph nodes. One – way ANOVA followed by multiple comparison tests were used to analyze the data. Each value represents the mean ± SEM, n=6 mice/group. Each experiment was repeated at least three times.

4.7 In Colitic Mice, CHR treatment Decreases Splenic $CD11c^+$ Cells-Related Surface and Functional Markers

As the effect of CHR was seen both locally and at the MLN, we decided to detect the presence of that in the spleen.

In colitic conditions, CHR treatment significantly decreased the levels of the surface markers: CD11c ($P=0.0059$) (Fig 4.11 a), CD80 ($P=0.0261$) (Fig 4.11 b) and CD86 ($P<0.0001$) (Fig 4.11 c).

As seen in the colon and within the mesenteric lymph node, the markers were also significantly down regulated in the presence of CHR in the spleen: IL-6 ($P=0.0168$) (Fig 4.11 d) and IL12p40 ($P=0.0069$) (Fig. 4.11 e).

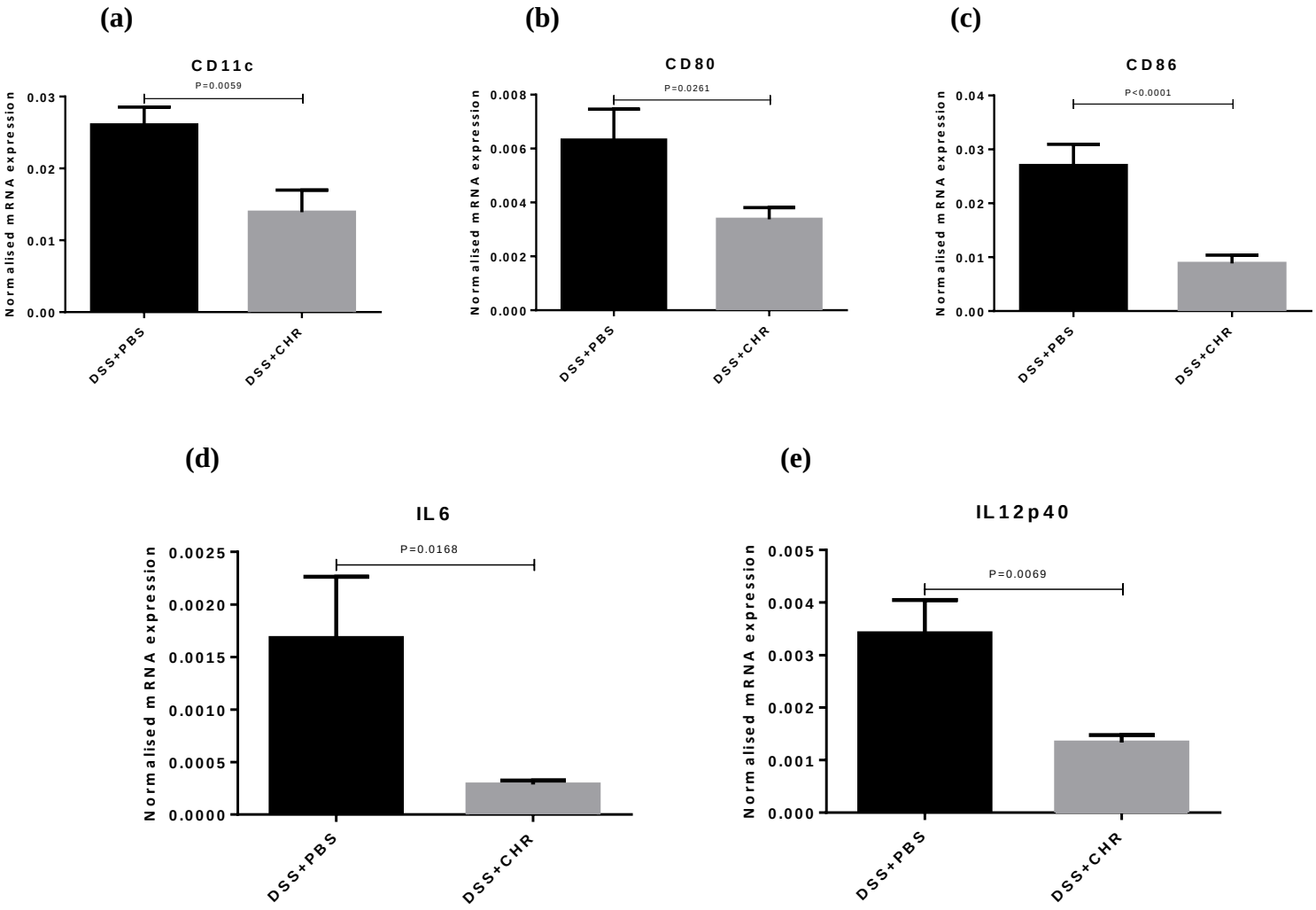


Figure 4.11 CHR decreases functional markers during the development of colitis in the spleen. Colitis was induced with 5% DSS in drinking water to C57BL/6 male mice and the control group of mice received regular drinking water. CHR (intra-rectal) (2.5mg/kg/day) was given as a preventive treatment or phosphate buffer saline (PBS 1%) starting from one day before the induction of colitis till the sixth day when the mice were sacrificed. mRNA expression levels in the spleen of the surface markers: (a) CD11c, (b) CD80 and (c) CD86 and functional markers: (d) IL-6 and (e) IL-12p40. One – way ANOVA followed by multiple comparison tests were used to analyze the data. Each value represents the mean ± SEM, n=6 mice/group. Each experiment was repeated at least three times.

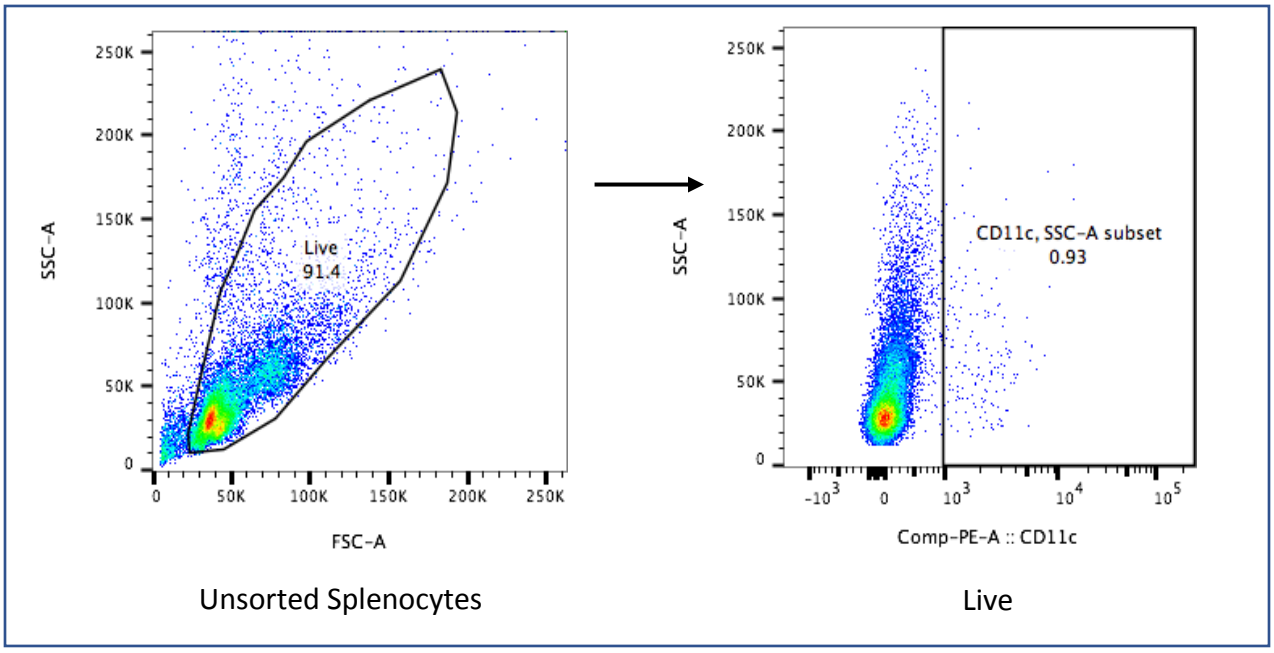
4.8 In Colitic Mice, CHR treatment Decreases $CD11c^+$ Isolated Cells Surface Markers

To have a deeper insight into the surface co-stimulatory markers activation or suppression in the $CD11c^+$ cells, we used flow cytometry. *In-vitro* CHR treatment significantly reduced the level of surface co-stimulatory markers in the *in-vivo* activated $CD11c^+$ splenic cells from control and DSS group of mice.

Spleenocytes were extracted by flowcytometry (91.4%) (Fig 4.12 a), but the population of the specific $CD11c^+$ cells were scarce (0.93%) (Fig. 4.12 b) but the magnetically sorted $CD11c^+$ cells from the spleen had a better population (80.3%) (Fig 4.12 d), which was further used for the treatment with the peptide (10^{-6} M/ml).

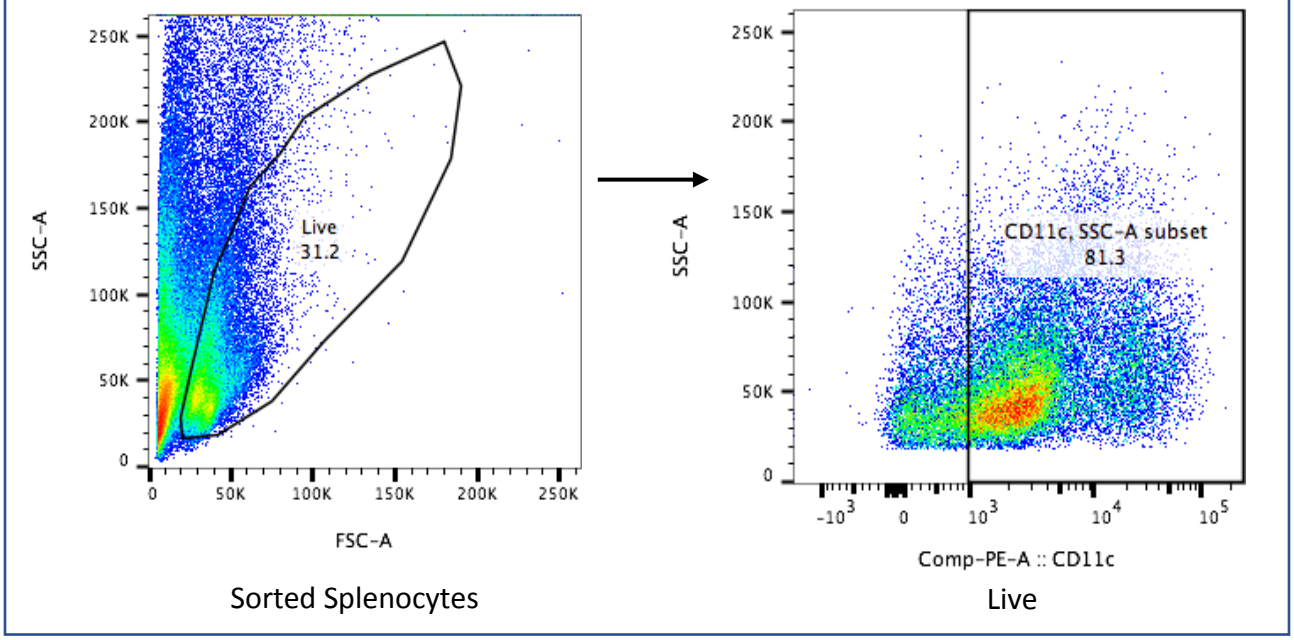
Significant changes were observed in the surface co-stimulatory markers after the culture with CHR. CD4 levels were reduced in the DSS+CHR group when compared with DSS group ($P=0.0315$) (Fig 4.12 e). In parallel, levels of CD80 was also regulated after CHR administration ($P=0.0045$) (Fig 4.12 f). The levels of CD86 and MHC-II were also analysed but the changes were not significant.

(a) **(b)**



Unsorted Splenocytes Live

(c) **(d)**



Sorted Splenocytes Live

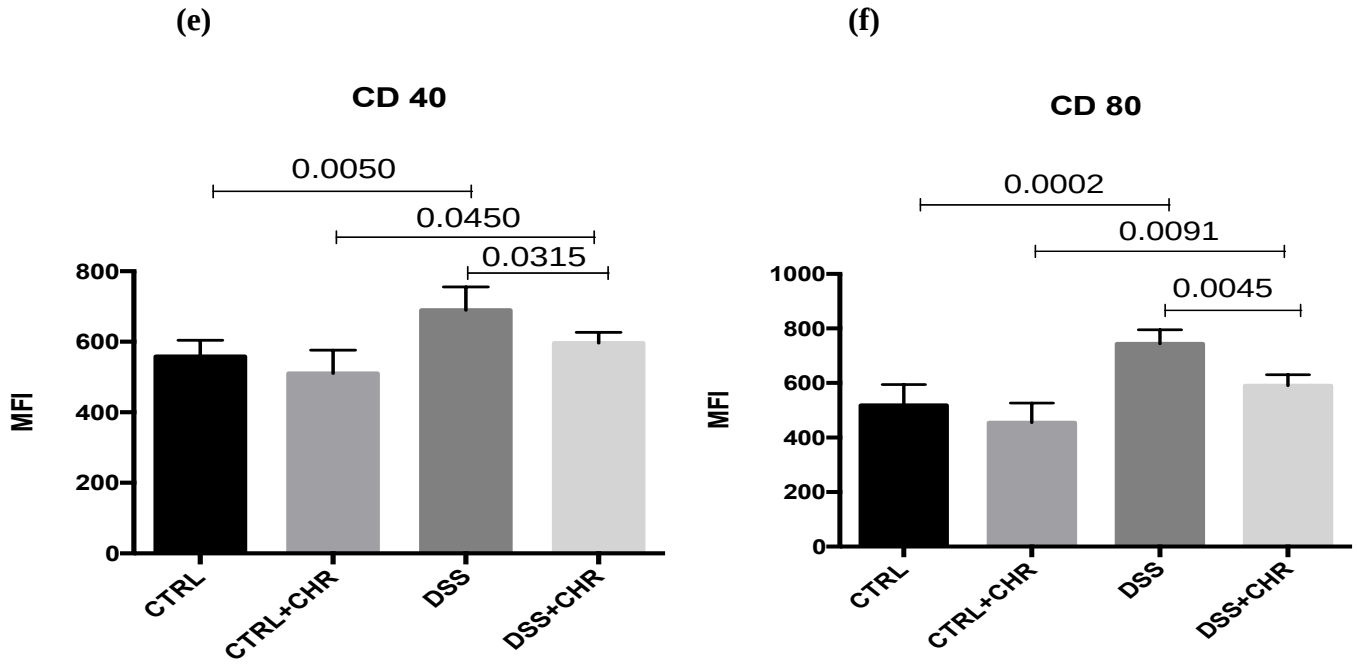


Figure 4.12 *In-vitro* CHR administration reduced the surface co-stimulatory markers on *in-vivo* activated splenic CD11c$^+$ cells. Colitis was induced with 5% DSS in drinking water to C57BL/6 male mice and the control group of mice received regular drinking water. CHR (*In-vitro*) (10^{-6} M/ml cell culture) was given to the sorted CD11c$^+$ splenic cells from colitic and control mice for 12 hours. (a-b) Unsorted splenocytes with lower CD11c$^+$ cells percentage and (c-d) magnetically sorted CD11c$^+$ cells before stimulation with CHR. Median Fluorescence Intensity (MFI) of CD11c$^+$ cells after CHR treatment with the following markers: (e) CD40 and (f) CD80. FlowJo was used to analyze the data followed by one – way ANOVA and by multiple comparison tests were used to analyze the data. Each value represents the mean ± SEM, n=6 mice/group. Each experiment was repeated at least three times.

AIM 3: To investigate mechanism responsible for the reduction of $CD11c^+$ cells-related bio markers.

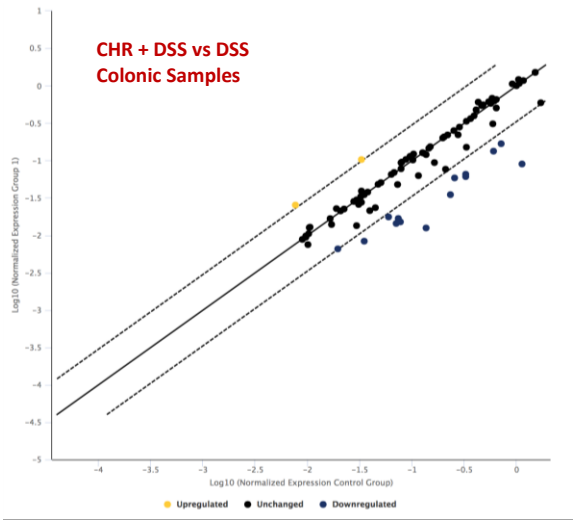
4.9 In Colitic Mice, CHR Treatment Demonstrates a Significant Down Regulation of NF-κB in Colon and in isolated Splenic $CD11c^+$ Cells

To unveil the mechanism behind the regulation of the surface and functional markers studied, an RT^2 – PCR array analysis of 96 $CD11c^+$ cells-related markers was conducted.

Colonic samples showed a significant down regulation of several pro-inflammatory markers and pathways and an up regulation of anti-inflammatory markers after treatment with CHR. The NF-kB signalling pathway was down regulated 11.02 times, and the surface co-stimulatory markers that we studied i.e. CD80 and CD86 were also reduced by 4.21 and 4.43-fold respectively. Anti-inflammatory markers like IL-10 and TGF-β were upregulated 3.29 and 3.12 times after administration of CHR (Fig 4.13 a-b).

In parallel, $CD11c^+$ cells extracted from the spleen of colitic mice showed many pro-inflammatory markers down regulated which were similar to the makers detected within the colon (Fig 4.13 c-d). NF-κB was decreased by 20.01-fold and CD80 by 3.8-fold, and CD86 by 4.0-fold.

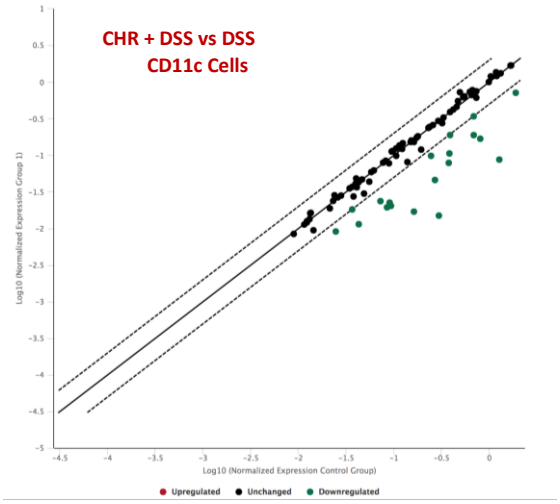
(a)



(b)

Gene Symbol	Fold Regulation	p-value
Ccl3	-5.35	0.024186054
Ccl4	-5	0.058817497
Cd74	-4.23	0.025356502
Cd80	-4.21	0.038795102
Cd86	-4.43	0.033942053
Nfkb1	-11.01	0.018674655
Fas	-3	0.061148417
Il12a	-3.41	0.046198902
Il16	-4.53	0.03263877
Itgam	-12.7	0.013344543
Mif	-6.65	0.021840425
Rag1	-4.37	0.037534798
Tnf	-5.17	0.029682772
Tnfsf11	-4.9	0.04830788
Il10	3.29	0.011756002
Tgfb1	3.12	0.206663188

(c)



(d)

Gene Symbol	Fold Regulation
Ccl2	-2.04
Ccl3	-4.83
Ccl4	-3.61
Cd74	-4.82
Cd80	-3.8
Cd86	-4
Csf1r	-9.68
Fas	-2.73
Ifng	-2.06
Il12a	-3.1
Il16	-3.67
Il2	-2.02
Itgam	-14.72
Mif	-5.95
Rag1	-2.52
Nfkb1	-20.01
Tap2	-2.65
Tnf	-4.64
Tnfsf11	-4.41

Common marker with colon

Markers of interest

Figure 4.13 RT^2-PCR data demonstrated an up regulation of several marker including the NF-kB in colon and in CD11c$^+$ splenic cells.

3.10 CHR Decreases Pro-Inflammatory Markers when Bone Marrow-Derived DCs are Treated with LPS and NF-κB Activator/Blocker.

As indicated by the RT^2-PCR array and our previous data, we confirmed the potential implication of these two pathways. A pharmacological study using NF-κB blocker and/or activator along with LPS and CHR on bone marrow-derived $CD11c^+$ BMDCs was conducted.

In control conditions, treatment with NF-κB activator increased significantly the protein levels of IL-6 ($P<0.0001$) and IL-12p40 ($P<0.0001$) and pre-treatment with CHR decreased the effect of the NF-κB activator on the level of the two markers. In parallel, LPS treatment increased significantly the level of IL-6 ($P<0.0001$) and IL-12p40 ($P<0.0001$) and pre-treatment with CHR decreased the effect of LPS on the level of the two markers. Moreover, CHR treatment blocked the effect induced by the co-treatment LPS and NF-κB activator. NF-κB blocker demonstrated the same effect by decreasing IL-6 ($P<0.0001$) and IL-12p40 ($P<0.0001$) release in LPS-stimulated BMDCs (Fig 4.14 a-b). Whereas, no significant changes were observed in the cytokine levels of IL-23 and IL-1β (Fig 4.14 c-d).

RT-qPCR was used to analyze the surface co-stimulatory markers and it was observed that CD40 ($P=0.0045$), CD80 ($P=0.0093$) and CD86 (0.0084) were significantly reduced in $CD11c^+$ BMDCs under the influence of CHR (Fig 4.15 a-c). CHR also significantly down regulated the combined effect of LPS+NF-κB stimulator in CD40 ($P=0.0005$), CD80 ($P=0.0143$) and CD86 ($P=0.0018$) markers. NF-kB blocker also resulted in a significant down regulation of these markers i.e. CD40 ($P<0.0001$), CD80 ($P=0.0011$) and CD86 ($P<0.0001$) in LPS stimulated $CD11c^+$ BMDCs (Fig 4.15 a-c).

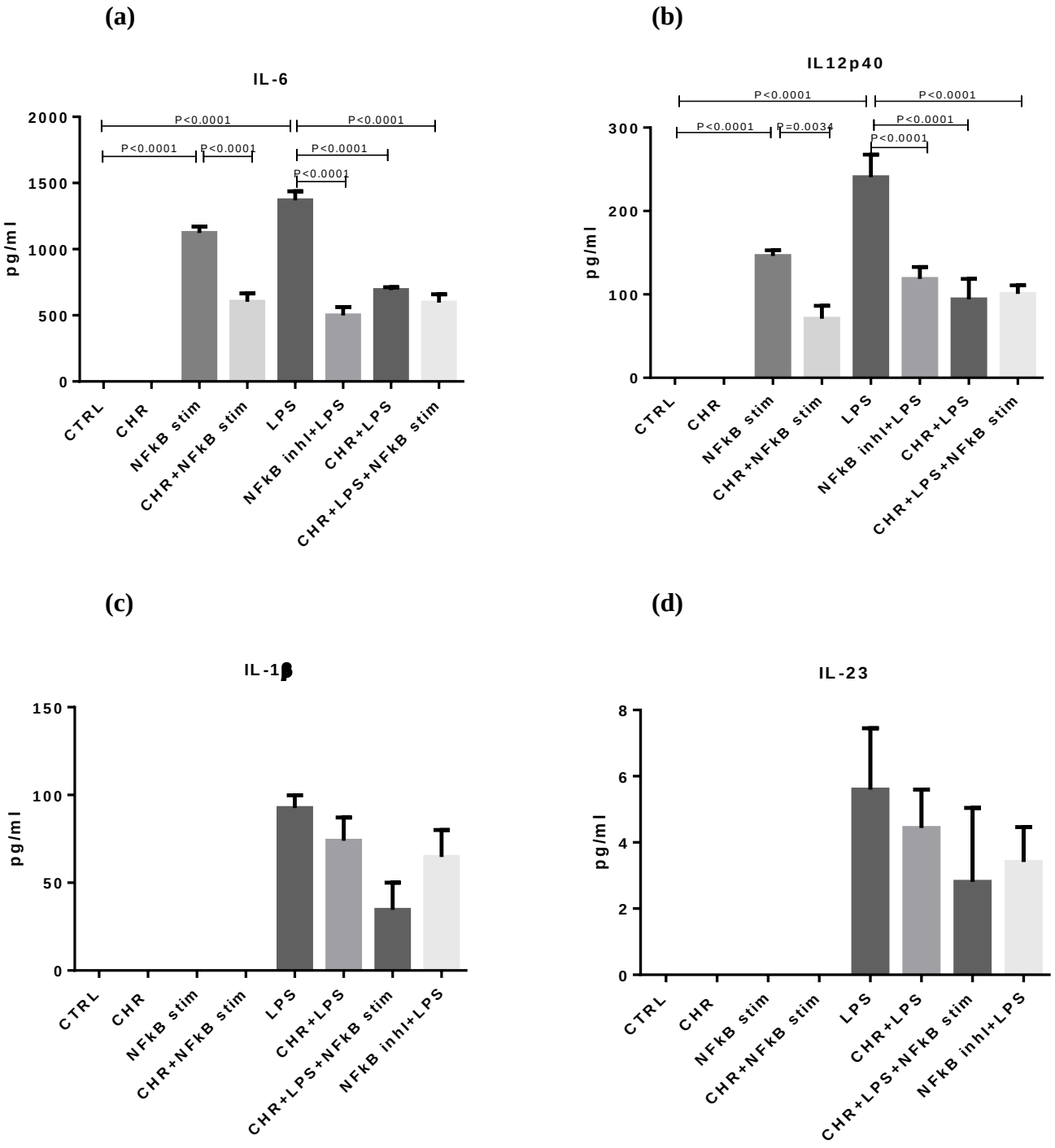


Figure 4.14 *In-vitro*, CHR treatment reduces pro-inflammatory cytokines level in LPS-related bone marrow derived CD11c$^+$ cells. Bone marrow derived CD11c$^+$ cells DCs cells were cultured with GM-CSF for 8 days till they get mature. They were given CHR (10^{-6} M/ml) for 12 hours and then stimulated with LPS (100 ng/ml) with/without NF-κB activator/stimulator (10μM/ml) for 24 hours. The supernatant was collected for cytokine measurement: (a) IL-6, (b) IL-12p40, (c) IL-1β and IL-23. One – way ANOVA followed by multiple comparison tests were used to analyze the data. Each value represents the mean ± SEM, n=6 mice/group. Each experiment was repeated at least three times.

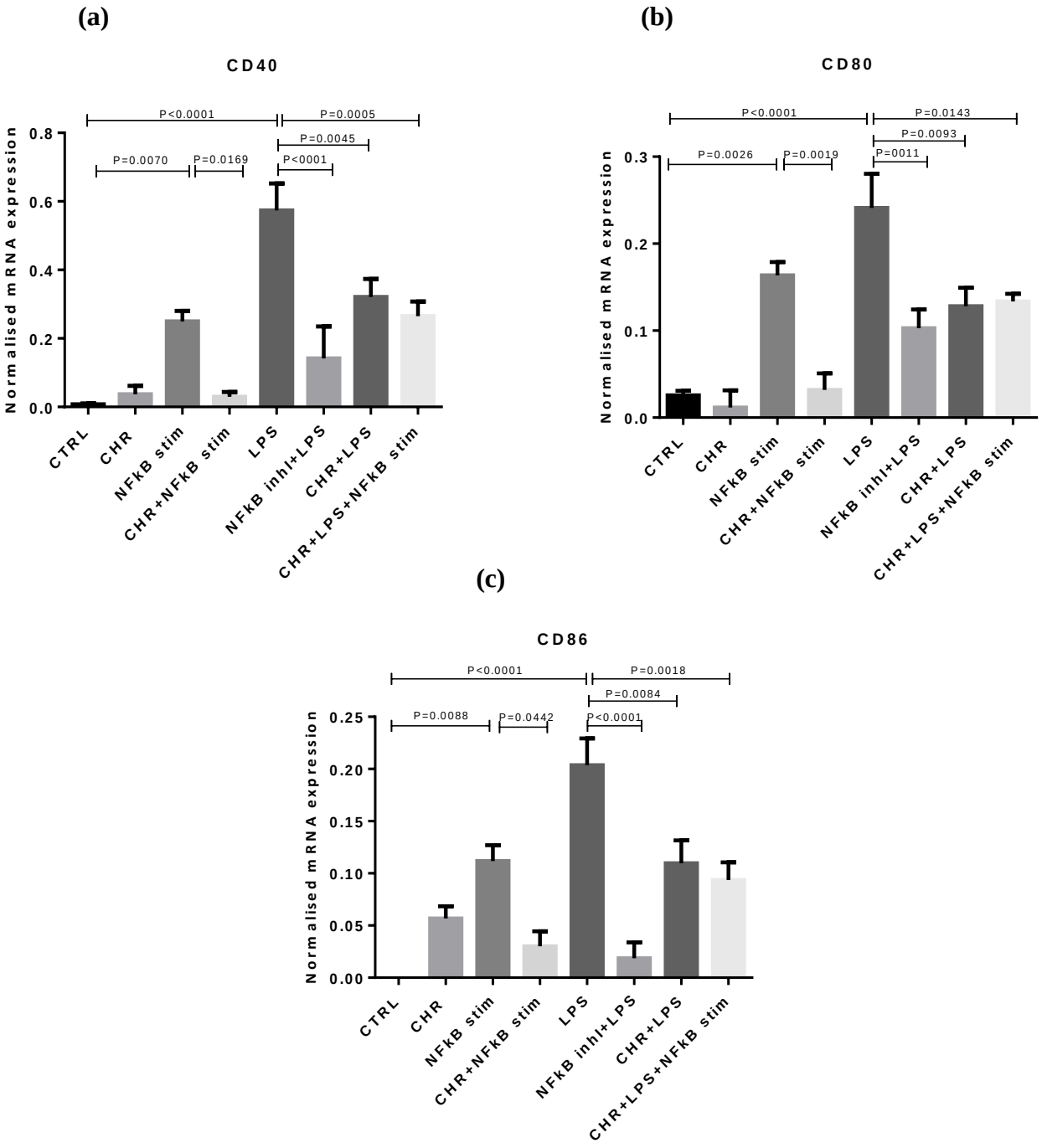


Figure 4.15 ***In-vitro,*** **CHR treatment decreases surface co-stimulatory markers in LPS-treated bone marrow derived CD11c$^+$ cells.** Bone marrow derived CD11c$^+$ cells DCs were cultured with GM-CSF for 8 days till they get mature. They were given CHR (10^{-6} M/ml) for 12 hours and then stimulated with LPS (100 ng/ml) with/without NF-κB activator/stimulator (10μM/ml) for 24 hours. The mRNA expression of the CD11c$^+$ cells is as follows: (a) CD40, (b) CD80 and (c) CD86. One – way ANOVA followed by multiple comparison tests were used to analyze the data. Each value represents the mean ± SEM, n=6 mice/group. Each experiment was repeated at least three times.

CHAPTER 5

Discussion

As described in the literature, IBD is an idiopathic inflammatory gut condition divided into two major subtypes: UC and CD [303, 369]. There are several different reasons involved in the causes of the disease, which includes environmental factors, genetic factors, dysregulated immune response towards commensal bacteria or genetically susceptible individuals [370, 371]. Although treatments and therapeutic strategies are evolving really fast, there is still a lack of efficacy and major long-term side effects induced by the current treatments. These treatments are not optimal and the field of IBD stays in an area where a lot of work needs to be performed [372].

Recently, our lab determined the role and the importance of an antifungal and antimicrobial peptide called *CHR* in active UC patients and in the experimental model of colitis, where the importance of CHR was studied on macrophage's regulation [293, 373]. As macrophages are not the only APCs, we extended our initial study by investigating the regulatory role of CHR on $CD11c^+$ cells, which are the major class of antigen presenting cells that can regulate the initiation and progression of the disease [374]. First, with some limitations, confirmed the regulation of *Exon-IV* in active UC patients [375], by demonstrating a significant lower expression in IBD patients compared with healthy controls. This change was associated with altered mRNA levels of pro-inflammatory surface co-stimulatory and functional markers related to $CD11c^+$ cells. This finding was also associated with a negative correlation of *Exon-IV* with surface and functional markers in active UC patients. To demonstrate the physiological effect of CHR on these markers, we hypothesized that the same effect would be visible using an animal model of colitis. Treatment with exogenous CHR was used to demonstrate this latter statement in an experimental model of

colitis using DSS. We demonstrated a decrease of the inflammatory process. Colonic $CD11c^+$ cells related pro-inflammatory markers were significantly down regulated at both colonic mRNA and protein levels. The effect of CHR was also visible at the level of the MLN. Additionally, splenic levels were also decreased. RT^2-PCR array data demonstrated the existence of a modulation of the NF-κ B pathway after CHR treatment. Moreover, blocking *In-vitro* this pathway in bone marrow derived $CD11c^+$ cells through the use of an NF-κ B blocker had a similar result. Taken together, CHR seems to act as an NF-kB blocker and hence, reduces the activation of major antigen presenting cells, and ultimately would lead to decreased intestinal inflammation. CHR alone had no side effect on the mice or on the cells, indicating a relative safety of CHR at the studied dose.

ECs are well distributed within the intestine. ECs produce CHGA and its derived peptides [28]. The CHGA-derived peptides have different roles in the inhibition and initiation of inflammation [253]. Amongst all those derived peptides, CHR has already been shown to have some antifungal and antimicrobial activity [376, 377], and antimicrobial peptides play a significant role in maintaining the homoeostasis of the gut [378]. To the best of our knowledge, there are no antibodies available for the quantification of CHGA derived peptides except for pancreastatin, but with some limitations. During the quantification of Pancreastatin, false positive results are a serious clinical problem and they can lead to needless further testing, invasive procedures, and patient's anxiety [379]. Therefore, indirectly we checked the levels of the CHR mRNA which is a quantification of *CHGA (Exon-IV)* in human samples. In this thesis, through human sample analysis, we demonstrated that *CHGA (Exon-IV)* is highly down regulated in UC patients showing that the disease is affecting the levels of *CHGA (Exon-IV)*. But this type of technique comes with some major limitation (see paragraph: limitations).

As $CD11c^+$ cells play a role in the pathology of IBD in humans [380, 381], we choose these to investigate the effect of CHR as our model system. In colonic biopsies from UC patients, we demonstrated that CD11c and its related co-stimulatory surface markers (CD86, CCR7 and CD74) were significantly upregulated, leading to potentially to an enhancement of colitis. As an increase level of DCs-related cytokines has been reported in IBD patients [198], we investigated related markers (IL12a, IL-23a, IL-12p40 and IFN-γ) in active UC patients and found them upregulated. Furthermore, in active UC patients, we observed a negative correlation between the *CHGA (Exon-IV)* with some of the CD11c cells-related markers. These alterations can be explained by the previous data which demonstrated changes at the level of the ECs during the inflammatory process [382-385]. Additionally, our data go in parallel with previous studies demonstrating the severe infiltration of immune cells and the increase release of pro-inflammatory cytokines during the inflammatory process [386, 387].

After understanding the correlation between the CHR (*CHGA Exon-IV*) and various CD11c related markers in humans, we tried to translate the importance of our finding to an animal model of experimental colitis. Previously, our lab demonstrated the role of CHR on M1 macrophages and its contribution in the regulation of experimental colitis in mice [373]. Several GWAS studies have shown the importance of certain common cytokines which act as markers of IBD onset. IL-6, IL-12 and IL-23 from the major subset of cytokines implicated during the development of gut inflammation [387]. The $CD11c^+$ cells show an important role in the progression of IBD and the disruption of intestinal DCs or macrophages may contribute to the disruption seen in IBD [380]. Increased levels of $CD11c^{hi}$ cells are found in the colon and in the draining mesenteric lymph node and a marked enhancement is seen in the levels of $CD11c^{hi}HLADR^{int}$ DC from the inflamed draining gut[380]. These $CD11c^+$ cells form the major APCs in the intestinal inflammation[388]

and help in activating and differentiating Th cells and therefore are important in regulating the adaptive immunity. CD80 and CD86, which behaves as the surface co-stimulatory molecules are found to be highly activated in inflamed conditions in $CD11c^+$ cells, and it is known that T-cell activation is blocked by the suppression of CD80 and CD86 [389, 390]. In our model of experimental colitis, administration of CHR leads to a down regulation of the external disease activity index and the macroscopic score along and is associated with the improvement of the colonic structure as seen under H & E staining. Additionally, in colitic conditions, the protein levels of major colonic cytokines like IL-12p40, IL-6, IL-23 and IL-1β were significantly down regulated after CHR treatment. According to our mRNA studies, CHR decreased CD80, CD86 and CD40 expression, which form the surface markers required for the stimulation of the adaptive immune cells. In addition to this, the mRNA levels of IL-12p40, IL-6, CCR7 and IFN-γ were decreased in CHR-treated colitic mice. The results we obtained from our mice model are in agreement with previously published data which shows that IL-23 is required for the release of IL-6 and the activation of T-cells in T-cell mediated experimental colitis [391].

The $CD11c^+$ cells in the MLN play a crucial role in the progression of both inflammatory and non-inflammatory bowel diseases [392]. Human DCs, specifically myeloid (mDCs) $CD11c^+$ $DC\text{-}SIGN^+$, are characterized by the production of a large number of interferons, and further activate the adaptive immune cells [393-395]. Recent studies have shown that $CD11c^+$ $CD103^+$ mDC from the lamina propria induce retinoic acid -dependent Tregs suppression [396-398]. Moreover, the same category of DCs has recently been found to show tolerogenic role in the MLN of humans [399, 400]. At the opposite, plasmacytoid DCs from the MLN of IBD patients have been analyzed for the expression of activation, maturation and homing receptors and CD40, CD80, CD86, CD103 and CCR7, which all were found increased [392]. In our mice study,

we found that CHR could significantly down regulate the MLN CD11c marker and markers like CD80 and CD86 in DSS-induced colitis. In parallel, a down regulation IL-6 and IL-12p40 was also demonstrated after CHR treatment in colitic mice. Taken together, we can conclude that i.r. treatment not only affects locally the immune mucosal response, but also at the level of the ML.

MLs are not the only immune organ affected by the i.r. CHR treatment. Previous studies have shown that the surface co-stimulatory molecules CD40, CD86 and CD80 on the $CD11c^+$ APCs from the spleen are modified during inflammation [401]. Those cells are major components, especially, $CD11c^+$ DCs in the spleen, which can act as early responder cells during the development of an infection [402]. Based on previous literature, our mouse study showed that CHR is decreasing CD11c cells related inflammatory markers in the spleen, and this CD11c marker reduction is accompanied by a down regulation of CD80 and CD86. Furthermore, our mouse experiment demonstrated a reduction of functional markers (IL-6 and IL12-p40) in the spleen after treatment with CHR. To study the specificity of the cells involved, magnetically sorted $CD11c^+$ cells from the colitic and control mice treated with CHR (*In-vitro*) were analyzed by flow cytometry. We demonstrated a reduction in the surface co-stimulatory markers (CD40 and CD80). Assembly of CD80/86 is required to activate APCs and for the release of NF-kB pathway related pro-inflammatory cytokines [403]. To decipher if in our model the NF-kB pathway was implicated we conducted a pharmacological study.

Role of NF-kB signalling is very crucial in murine experimental colitis [404, 405] and human IBD [406]. Several inflammatory cytokines are regulated by the NF-κ B signalling [407], and previous studies have reported that blocking NF-kB in mice could be considered as a potential treatment for preventing gut inflammation [404, 405, 408, 409]. In our mice study, CHR abrogated DSS-induced colitis potentially through the suppression of NF-κB signalling. This was confirmed

by RT^2-PCR where 96 genes related to the $CD11c^+$ cells related markers from colon and splenic $CD11c^+$ cells were analyzed. In colitic mice treated with CHR, NF-kB was down regulated 11.1-fold and CD80 and CD86 were down regulated 4.21 and 4.43-fold respectively. Surprisingly, the anti-inflammatory markers IL-10 and TGF-β were upregulated 3.29 and 3.12 times respectively. Consistent with our colon data, CHR also down regulated NF-κB and co-stimulatory markers in $CD11c^+$ cells from the spleen of CHR treated colitic mice, moreover, NF-kB, CD80 and CD86 were down regulated by 20.01, 3.80 and 4.00-fold respectively. NF-κB regulates the effective antigen presentation by the most APCs i.e. DCs, as NF-κB is required for the activation of HLA class II, CD80, CD86 and CD40 and the pro-inflammatory cytokines like IL-6, IL-12 and TNF-α [410]. . Thus considering the previous literature related to NF-κB and IBD, our results support the concept that CHR can potentially decrease intestinal inflammation by regulating the NF-κB pathway. As NF-κB was down regulated under the impact of CHR, we checked its importance by using NF-κB blocker and activator on $CD11c^+$ BMDCs. According to the previous literature, NF-κB inhibitor (BAY 11-7082) significantly reduces the production of IL23 and IL-12p70 by DCs [368], and the NF-κB activator (betulinic acid) markedly enhances the level of IL-23 and IL-12p70 in CD11c+ cells [368]. In our *In-vitro* study, CHR treatment significantly decreased the levels of IL-6 and IL-12p40 in LPS-stimulated $CD11c^+$ BMDCs form mice. Along with this, the mRNA data in mice also suggested a significant reduction in the expression of CD40, CD80 and CD86 in LPS-stimulated $CD11c^+$ BMDCs.

Limitations exist in our study; due to the strict inclusion and exclusion criteria for the patient selection, the size of the samples was too small, additional biopsies need to be added explaining why for some markers the statistical significance did not reach. Therefore, the level of CHR with CD11c markers needs to be quantified in a bigger group of active UC patients. Moreover, our

study was limited to UC patients, it would be important to demonstrate if we can see the same in CD patients and extrapolate our results to IBD in general. More specifically, due to lack of any specific antibody for CHGA and CHR, we had to continue only with the mRNA study as protein levels couldn't have been analyzed. Proteomics approach should be used for protein quantification in the colon and all correlation analysis should be repeated accordingly. In our mice model, we used the preventive and prophylactic treatment, therefore, further experiments are required to determine the efficacy of CHR as a therapeutic agent, using a chronic or a healing model. Several other factors might lead to the protective effect of CHR, which we have observed in our study. For example, previous studies demonstrated a link between the gut microbiota and experimental colitis and human IBD [411, 412], and there are evidences demonstrating that CHR can have antimicrobial function [255, 377]. Therefore, it is possible the i.r. injection could have affected or induced a microbiota disbalance in favour of beneficial bacteria, further metagenomic analysis are needed to study this aspect.

CHAPTER 6

CONCLUSION, SIGNIFICANCE AND FUTURE DIRECTIONS

6.1 Conclusion

Our experiments confirmed that the level of CHGA (Exon-IV) is significantly down regulated in the colonic samples from active UC patients as compared to controls, describing a relationship between CHGA (Exon-IV) and UC. A negative correlation was also seen in the CHGA (Exon-IV) and $CD11c^+$ cells related surface and functional markers. In our mice study, CHR significantly improved the severity of the colitis as observed by the external DAI and macroscopic scores. Most importantly, i.r. CHR administration reduced the colonic pro-inflammatory surface and functional markers, related to $CD11c^+$ cells, as observed by ELISA and RT-qPCR. In parallel, it also reduced similar $CD11c^+$ cells related markers in MLN. Surprisingly, the systemic levels of $CD11c^+$ cells related markers, as observed in the spleen, were also significantly down regulated. RT^2-PCR showed NF-κB behind the mechanism responsible for the down regulation of inflammation by CHR. *In-vitro* experiment using $CD11c^+$ BMDCs confirmed that suppression of NF-κB pathway with CHR administration. All this data confirmed the role of CHR in regulation of DSS-induced experimental colitis *via* regulation of $CD11c^+$ cells and NF-κB pathway.

6.2 Significance

Since the beginning of my project, my focus was to have a better idea of the pathophysiology of IBD and possibly to develop a novel and effective therapeutic target. Using both the human colonic study and the acute model of UC, this study for the first time depicted the relationship CHGA (Exon-IV)/CHR and $CD11c^+$ cells related markers in humans and CHR and $CD11c^+$ cells related markers in experimental model of colitis in mice. Our preliminary

observations highlight that CHR could be in the future to be used as potential new drugs to suppress the action of APCs and ultimately keep IBD on remission. As it is already known that the lymphatic system and other vital organs are affected with the intestinal inflammation [413], we in this study have shown that CHR reduced the inflammatory markers in the MLN and spleen along with colon, which shows a systemic effect for a drug locally injected. Till date, several immunosuppressive drugs are available but adverse side effects have been demonstrated with them. Therefore, a better and safer treatment are major requirements to treat IBD, and CHR can pave the path for obtaining safer therapeutic strategies for the treatment of IBD.

6.3 Future directions

In our project we studied the role of CHR using an acute model of colitis, however, as mentioned in our introduction, IBD is a chronic disease associated with active and remission phases. More experiments are required to study its role over a longer span, for example in the healing context where the peptide is injected at the end of the acute phase to quantify if the treatment can increase the healing capacity of the mucosa by targeting factors like KLF5 or others. The alternative would be to use the chronic 3-cycles of DSS model that induce a chronic quiescent colitis and to demonstrate if the treatment is administered during the remission can decrease the reactivation of colitis. A setup fitting better the natural history of the disease.

Moreover, CHR is known for its antimicrobial effects and there is possibility that CHR modulates the microbiota. Regulation of microbiota is a current important field of research and its regulation by altering the good or bad bacteria can lead to a modification of the inflammatory process. Therefore, the relationship of CHR with the gut microbiota needs to be delineated.

In our study we injected i.r. the native peptide at a specific dose, but there is no data about the behaviour of it. Pharmacokinetic studies should be performed to define i.e. half-life and optimal dose of CHR to enhance efficacy and to decrease toxicity. Also, kinetic homogeneity describing the predictable relationship between plasm level and level at the site of action. Those are studied that need to be explored.

Because of the limitation of the ELISA (epitope recognized by the Ab, structural modification of CHR) the level of CHR was not studied. They can be used as an indirect indicator but would not confirm the exact presence of the peptide. Thus, mass spectroscopy could be used to have a greater insight to quantify the peptide during the course of inflammation.

Finally, diarrhea or constipation are the two major symptoms of IBD patients as their intestinal and colonic smooth muscle contractions and colonic permeability are altered. Smooth muscle contractions physiology is facilitated by calcium ion influx. As CHR is known to have a relationship with calcium, studies targeting CHR in context of colonic motility through the use of an organ bath technique or the use of calcium imagine are important elements of that aspect. The permeability aspect related to the presence of leaky gut in IBD patients is another point that can be studied using a technique called Ussing chamber or using more basic technique like chromium-EDTA absorption. Up today, there is no data that demonstrate the interaction between CHR and the smooth muscle mechanical activity in the colon.

CHAPTER 7

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