HOPX EXPRESSION IN IMMUNE REGULATORY B CELL REGULATE INTESTINAL INFLAMMATION

By

NALAYINI THAYAPARANA, M.Sc., B.Sc

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Master of Science

McMaster University

MASTER OF SCIENCES (2012) **McMaster University**
(Medical Sciences) **Hamilton, Ontario**

TITLE: HOPX EXPRESSION IN IMMUNE REGULATORY B CELL REGULATE INTESTINAL INFLAMMATION

AUTHOR: Nalayini Thayaparana

M.Sc (Applied Microbiology) (University of Madras, India), 2009

SUPERVISOR: **Dr. Pingchang Yang**, MD, PhD, Professor, Department of Pathology and Molecular Medicine, McMaster University

SUPERVISORY COMMITTEE: **Dr. Damu Tang,** MD, PhD, Professor, Department of Pathology and Molecular Medicine Infectious Disease Division, Centre for Gene Therapeutics, McMaster University

Dr. Paul Forsythe, MBBS, PhD, Associate Professor, Department of Pathology and Molecular Medicine, McMaster University

NUMBER OF PAGES: 99

ABSTRACT

Inflammatory bowel disease (IBD) is chronic, relapsing, intestinal inflammation characterized by periods of remission and exacerbation. Pathogenesis of IBD is a complex process and the exact aetiology is unclear yet. Studies have provided evidences that IBD is a result of a genetic predisposition that leads to a mucosal immune regulatory cell defect, barrier defects, and susceptibility to environmental triggers like luminal bacteria and specific antigens. Innate and adaptive immune responses to commensal bacteria are balanced by the multiple regulatory cells like T regs and B regs. Monoclonal-antibody-based therapies have been identified for the treatment of IBD but, they have side effects and the efficacy is not stable. Hence, great expectations lie towards finding a successful cure for IBD.

Recent studies have proved the involvement of several different genes in the pathogenesis of IBD and there expressions were highly reduced in IBD. Homeodomain only protein-Hopx is a nuclear protein required to modulate heart growth and function. Expression of Hopx has been reported in various types of normal tissues but not in malignant tissues. Recent study on Hopx revealed that Hopx is needed for the function of regulatory T cells induced by DCs. However the correlation between Hopx gene and IBD remains unanswered. In this study, we examined the role of Hopx expressing regulatory B cell in IBD.

The aim of this study is to investigate whether the expression of Hopx in regulatory B cell play a key role in suppressing colitis in murine model.

The expression of Hopx was studied using cellular and molecular techniques including flowcytometry, immunohistochemistry, western blotting and real time RT-PCR.

As a first step, we investigated the Hopx expression in colitis and control murine model. After we found the possible involvement of Hopx gene in regulating intestinal inflammation, we further our study to investigate whether Hopx is expressed by regulatory B cell and function together to inhibit intestinal inflammation. After establishing Hopx and B regs association, we used probiotics to modulate Hopx expression in regulatory B cells in order to prevent/reduce intestinal inflammation. Finally we elucidate the importance of Hopx expression by injecting neutralizing anti-Hopx antibody to block Hopx.

Together, studies on human intestinal tissue and murine model revealed that Hopx expression is suppressed during inflammation condition. Probiotic administration helps to increase Hopx expressing Breg cell thereby, prevent IBD. Hopx deficient group expressed high frequency of proinflammatory cytokines and reduced immune regulatory cells. This particular study proposes that the down regulation of Hopx contributes to the development of intestinal inflammation.

ACKNOWLEDGEMENT:

First, I would like to thank my supervisor, Dr. Pingchang Yang for his continuous support and encouragement. This research would not have been successful without his guidance. In addition, I would like to thank my committee members Dr. Damu Tang and Dr. Paul Forsythe for their valuable advice and expertise research related questions. Next, I would like to thank my encouraging co-workers Dr. Xiao Chen, Dr. Zhiqiang Liu, Natasha, Hong, Tahrin and Ben. I am thankful to my dear friend Liz Garside for all her timely help and support throughout the Masters program. Finally, I would like to thank all my family members and kanna for their love and encouragement.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

APC	Antigen Presenting Cells
Breg	B regulatory cells
CD	Crohn's Disease
FoxP3	Forkhead box P3
Hopx	Homeodomain protein X
IBD	Inflammatory Bowel Disease
IFN-g	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
NFκB	Nuclear Factor kappa B
PRR	Pattern Recognition Receptor
Th	T helper
TGF-β	Transforming Growth Factor Beta
TNF-α	Tumor Necrosis Factor-alpha
Treg	T regulatory cells
UC	Ulcerative Colitis

CHAPTER 1: INTRODUCTION

1.1 Intestinal Immune System

The mucosal surface of the intestinal tract is considered as the body's largest organ of host defence[1]. The mucosal surface area is ~ 400m^2 which is much larger than the entire surface area ~ 1.8m^2 of the skin[2,3]. It acts as a home for trillions of microbes and at the same time it is also an important entry site for pathogens because of its large surface area[4]. Important function of intestinal immune system is protection against infectious agents without affecting the normal microbiota apart from other major functions like digestion, absorption and assimilation of nutrients[5,6]. The large area of gastrointestinal tract demand a large complement of immune cells including both innate and adaptive cells which are disperse widely on the mucosal surfaces[7]. It is estimated that ~ 60% of the total lymphocytes are connected with the small intestinal epithelium. Intestinal immune system is a complex network[7,8].

A detailed understanding of the intestinal immune system and the factors that stimulate mucosal immune responses such as food components, gut microbiota, intestinal immune cells, Toll-like receptors and cytokines are important to elucidate a better therapeutic way to treat intestinal inflammation and to maintain the mucosal homeostasis[9].

1.2 Epithelial Barrier and Pattern Recognition Receptors

Intestinal mucosa is covered by single layered epithelia and which is not impenetrable[2,10]. There is a cross talk between lumen and lamina propria using multiple antigen sampling strategies[2,10]. The specialised epithelial phenotype M cells and specialized DC- extends their dendrites between tight junctions of intestinal epithelial cells and sample luminal

contents[10]. Lamina propria is the layer below the mucosal barrier and enriched with secretory IgA producing plasma cells, Tcells, stromal cells and antigen presenting cells like DC and macrophages[10,11]. The secretion of cytokines like Thymic stromal lymphopoietin- TSLP, TGF and IL-10 as the result of the basal recognition of commensal bacteria by intestinal epithelial cells, influence the production of proinflammatory cytokines by intestinal resistant DC and macrophages[10,11]. The production of cytokines like B-cell-activating factor-BAFF, a proliferation inducing ligand-APRIL, secretory leukocyte peptidase-SLPI and prostaglandin E_2-PGE2 also regulate the functions of intestinal resistant lymphocytes and APCs[10,11]. Activation of DC results in the maximum production of IgA which plays an important role in intestinal homeostasis[10].

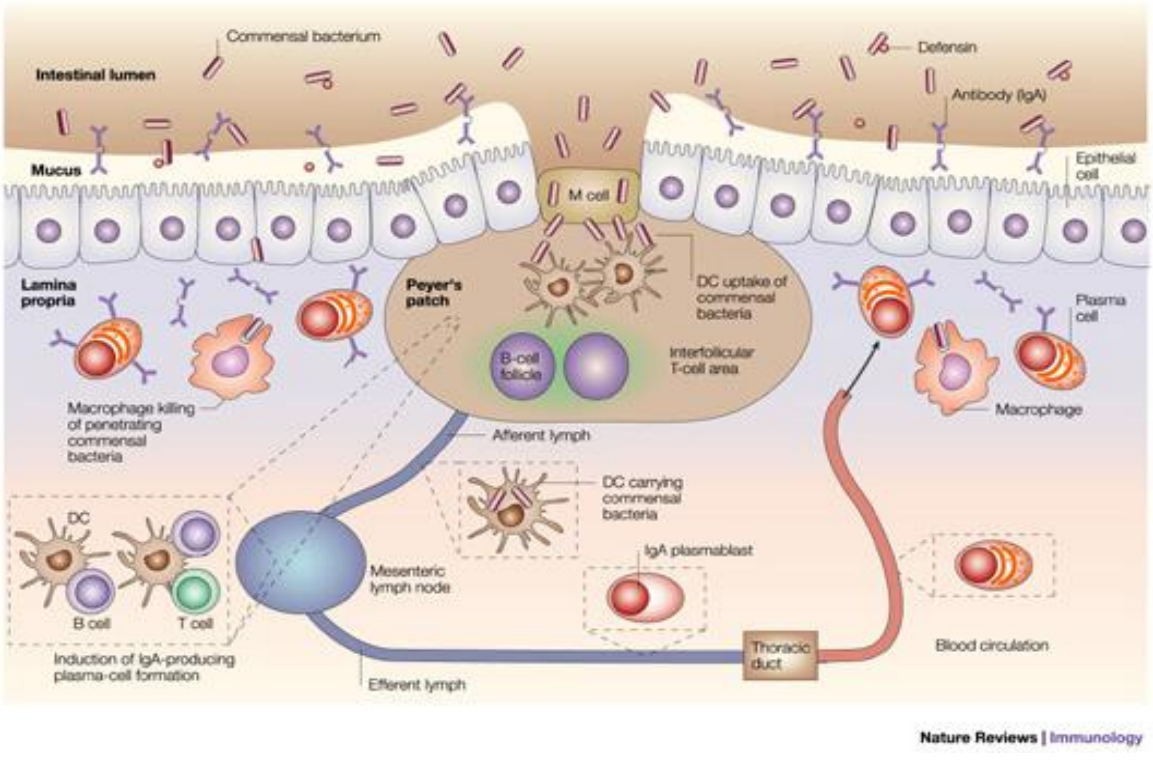


Figure 1: Intestinal Immune System[10]

The Toll like receptors-TLRs are crucial component and their activation by commensal bacteria plays an essential role in innate immune system and thereby maintaining intestinal homeostasis. [9]. The pattern recognition receptors (PRRs) are endogenous molecules present on the surface of innate leukocytes like phagocytes, macrophages, mast cells, eosinophils, basophils, natural killer cells[9,10]. PRRs recognize the pathogen-associated molecular patterns (PAMPs) such as LPS, flagellin, lipopeptides, RNA and DNA expressed on the surface of pathogens[10]. TLRs are transmembrane signaling molecule and are primary family members of early sentinels PRRs[9,10]. TLRs identify nature of pathogen and initiate innate immune responses and helps as a link to the T cell-mediated adaptive response which leads to the expression of cytokine genes. Most of the TLRs are surface expressed[9,10,11]. Extracellular TLRs include- TLR2 (lipoproteins), TLR4 (lipopolysaccharide) TLR5 (flagellin)[10,12]. Pathogen which enters the host cell through phagocytosis/endocytosis recognized by the subset of TLRs located in endosomal compartment[9,10]. Intracellular Toll like receptors target a variety of microbial ligands-, TLR3 (double stranded RNA), TLR7 and TLR 8(single-stranded RNA), TLR9 unmethylated CpG motifs in DNA)[9,10,12].

Antigen presenting cells migrate to lymph system and then to draining lymph nodes once they phagocytosed the pathogen[9,13]. Lymph nodes play a key role in maintaining immune system and connecting place of antigen-presenting cells and lymphocytes[11,14]. When antigens are recognized by APC cells they are processed, spliced in to peptides in the cystol and re-expressed on MHC molecule[11,15]. There are two classes of MHC (class I and II) where MHC class I helps to stimulate T lymphocytes (CTLs) and MHC class II helps

to stimulate T helper cells (Ths)[11,15]. When the antigens are expressed on MHC class I molecule, CTL are activated to destroy the antigen, the antigens re-expressed on MHC class II, Th cells are activated to initiate immune-regulatory effects[12,13,14]. Interleukin 12 (IL-12) activates Tcells to become TH1cells, which will attack intracellular pathogens[13,15]. IL-23, differentiate T cell in to Th17 helper cells, which attack extracellular bacteria. IL-4, differentiate T cell into Th2 cells which help B cell in the antibody production[11,13,15]. TGF-β and IL-10, helps the formation of regulatory T cells (Treg and Tr1) that dampen responses[11,13]. Dendritic cells are the pacemaker of the immune system and help to control intestinal inflammation and maintain immune tolerance[11,14]. Dendritic cells can activate both memory and naive T cells and act as a messenger between innate and adaptive immunity[11,14].

1.3 Innate and adaptive immunity

Immune system is composed of innate (non-specific) and adaptive (specific) immunity which work together to protect against wide variety of infectious and damaging agents[9]. Innate immunity provides first layer of defence once the pathogen enters the host[9,14,15]. Adaptive immunity acts as a second layer of defence which provides specific immune response to pathogen that evades the innate response[9,15]. The adaptive response is mediated by two major types of lymphocytes B cell and T cell ($CD4^+$ and $CD8^+$)[16]. Innate immune system is important for controlling early stages of infection and last only for few hours or days as they express finite number of germline encoded receptors and not somatic recombination receptors to sense pathogens and tissue damage[16,17]. Hence they

are not subjected to stimulation and clonal expansion, they reacts in similar way after repeated exposure as they do not develop immunological memory[17].

Adaptive immune cells which have not encountered the antigen yet, express delayed response to infected cells (processed and carried to draining lymph node by antigen presenting cell)[16,18]. Once activated these cells will traffic to the infected site to clear the pathogen infected cells[18]. This immune response is retained as immunological memory and resides for long term in lymphoid and non-lymphoid tissue as they express somatically rearranged receptors[17,18]. Adaptive immunity depends on T cell and B cell as the somatic rearrangement of antigen receptor genes are expressed only by T cell and B cell[18]. Memory lymphocytes protect host from subsequent encounters with same antigen by antigen stimulation and clonal expansion[17,19]. The two primary features of immunological memory following antigen exposure are (1) antigen specificity and (2) amplified response[17,20]. They have the ability to adapt its defenses against future infectious agent by the presence of recombination proteins necessary to rearrange V (D) J genetic elements to distinguish antigen-sensing receptor[19,10,21].

1.4 Inflammatory Bowel Disease

The inflammatory bowel disease (IBD) is a chronic, relapsing, immunologically mediated disorder of the gastrointestinal tract and characterized with periods of remission and exacerbation[22,23]. The affected area becomes inflamed (red and swollen) causing abdominal pain, diarrhea, and gastrointestinal bleeding as the result of the immune reaction of the body against its own intestinal tissue[22,23,24]. The exact aetiology is

unknown but studies have provided evidences that IBD is a result of a genetic predisposition that leads to a mucosal immune regulatory cell defect, barrier defects, and susceptibility to environmental triggers, including luminal bacteria and specific antigens[23,24].

The two major phenotypes of inflammatory bowel diseases (IBD) are ulcerative colitis (UC) and Crohn's disease (CD)[22,23,24]. Crohn's disease is an idiopathic inflammatory bowel disease that occurs from the mouth to the anus, although the terminal ileum is most common site of disease[22,24]. The most common clinical manifestation of ulcerative colitis is an inflammation of the colon[22,23,24]. Both ulcerative colitis and Crohn's disease cause inflammation and they have similar clinical features or overlapping symptoms[22,24]. Loss of epithelial barrier function, lymphocytes infiltration, over expression of pro-inflammatory mediators in different effector T lymphocytes subsets-Th1, Th2, Th17 are the common altered immune reactions of IBD[22,24].

1.5 Pathophysiology of IBD

Research over the last couple of years has led to great advances in scientific discovery and has expanded our understanding of inflammatory bowel diseases (IBDs) and their underlying pathophysiological mechanisms[22,23,24]. The four basic tenets of the pathophysiology basis of IBD are: 1) genetics, 2) immune dysregulation, 3) barrier dysfunction and 4) the role of the microbial flora[24].

1.5.1 Genetics

Genome-wide association studies (GWAS) have revealed that more than 50 genetic association with IBD (CD and UC). The first gene-NOD2, related to Crohn's disease was

discovered in 2001[22,24,25,26]. CARD15/NOD2 gene is located on chromosome16[22,25,26]. It is an intracellular pattern recognition receptor and mediates innate immune responses by recognizing bacterial peptidoglycan[25,26]. Activation of NOD1, NOD2 and Ipaf leads to the activation of NF-kB or caspase and thereby, increase the production of proinflammatory cytokines[25,26]. NOD2 gene plays a key role in immune system and the gene mutation results in early onset of disease like strictures, abscesses and fistulas in IBD patients. [22,25,26,27]. NOD2 frame- shift mutation appears frequent in Crohn's ileitis than in Ulcerative colitis[25]. Loss of interaction between NOD2 and MDP (muramyl dipeptide) result in the inability to control bacterial infection leading to uncontrolled inflammation which contributes to the pathophysiology of CD[22,25,26,27].

MDR-1 is the candidate gene involved in the pathogenesis of IBD[22,28]. MDR-1 encodes the membrane transport protein P-glycoprotein (pgp-170) which is highly expressed in intestinal epithelial surface[28].MDR-1 provides protection against xenobiotics and bacterial products[22,28]. It is located on chromosome7q and function as an AT-dependent efflux transporter pump[22]. Absence of MDR-1 spontaneously results in colitis (UC) in humans[22,28,29]. Mutation of MDR-1gene (MDR1 C3435T genotypes) was identified in IBD[22,28,29].

OCTN (organic cation transporter) is another gene plays a role in IBD[30]. OCTN1 is an integral protein of plasma membrane help to transport sodium ions and ergothioneine-antioxidant in to cell[22,30]. Polymorphisms in OCTN gene-OCTN1 (SLC22A4) and OCTN2 (SLC22A5) represent disease causing mutation in IBD5 locus[31]. Defect in these genes associated with CD and UC[22,30,31].

Recent studies proved that DLG5 (disks large homolog 5) gene-located on chromosome 10, polymorphism is associated with CD[22,32]. DLG5 helps in maintaining epithelial cell integrity[22,32]. Genetic variation in DLG5 leads to the development of IBD[22,32].

1.5.2 Immune dysregulation

The hypointestinal mucosa is constantly exposed to milieu of commensal bacteria and foreign antigen[22]. The key function of intestinal mucosal barrier is providing balanced immune responses[33,34]. The single layer of epithelia cells interspersed with goblet cells-produce mucus, Paneth cells-produce anti microbial peptide and M-cells forms the main barrier between outside and inside body[35]. The tolerogenic reaction towards the foreign antigens is provided by the both innate and adaptive immune cells like- T cells, B cells, dendritic cells, macrophages, eosinophils present in the lamina Propria[33,34,35]. Mucosal homeostasis is a balance between immune regulatory cells and effector cells-Th1, Th2, and Th17[22,33]. An inappropriately robust intestinal immune response results in dysregulated relationship with professional antigen-presenting cells and an increased differentiation of proinflammatory cells[34,35]. An increase in effector cell population with excessive inflammatory response or decreased in regulatory cells result in mucosal inflammation[22,34]. Gut associated lymphoid tissue (GALT) includes Peyer's patches (PP), lymphoid follicles (ILF), cryptopatches (CP) and MLN - is the largest lymphoid organ and its dysfunction is also predisposing to IBD[22,34,35].

1.5.3 Barrier Dysfunction

The intestinal epithelial cells act as a critical mediator between external environment and mucosal immune system[22,36]. They cover the gastrointestinal tract and are held together by tight junctions at the apical and basal poles which prevent the passive flow of

microbes and macromolecules[36,37]. Epithelial cells produce cytokines and chemokines when they encounter the pathogen[36,37]. During mucosal inflammation proinflammatory cytokines are released and which induce increased permeability to pathogens which result in IBD[36,37]. Electrolyte transport is controlled by the epithelial cells and disturbed in electrolyte transport leads to diarrhea[36,37]. Diarrhea is the initial symptom in 50% flares in CD and 100% in UC[22,36,37]. Neutrophil influx into the mucosa is one of the early signs of intestinal inflammation[36,37]. During intestinal inflammation, transepithelial migration of neutrophil act as a first line of defence[36,37]. Intestinal microvascular endothelial cells also play an important role in IBD[22,36,37]. Increased binding of leucocytes to human microvascular endothelial cells is observed in IBD[22,36,37]. Growth factors help to improve and protect gut barrier[36,37]. Intestinal mucosal damage and defective barrier repair are the outcomes of imbalance of inflammatory mediators and hormones[36,37]. Studies proved that there are nearly 30 different peptide growth factors like epidermal growth factor, fibroblast growth factor helps in the mucosal integrity[36,37].

1.5.4 The role of the microbial flora

Our gastrointestinal tract is a home for more than 500 bacterial and other less described microbes[22,39]. These microbes play a key role in digestion, providing nutrient and protecting immune system[39]. Intestinal bacteria weigh up to 1kg and they produce several nutritional factors like vitamin K and B, folate and short- chain fatty acids[39,40]. From the by-product of bacterial fermentation ~ 10% of an individual's daily energy is obtained. Thus gastrointestinal microbiota plays a critical role in normal immune function and maintenance[39,40].

Most interesting and best evidence to prove that gut microbiota plays an important role in intestinal bowel disease came from animal model studies. -Induced, spontaneous or genetically engineered, chronic colonic inflammation in the presence of enteric bacteria is initiated and perpetuated in several animal models[22,39,41]. IBD is prevented drastically reduced or prevented in germ-free (sterile)[24,40]. Recent studies also proved that the loss of the transcriptional factor T-bet (regulates the differentiation and function of immune cell) also promote the bacterial community to become colitogenic[24,39,41]. From these recent studies on IBD, its getting clearer that pathogenesis of IBD depends on the interaction between four major factors mentioned above- microbial, genetic, immune and environmental[22-41].

1.6 Etiology of IBD

As discussed above, specific reason for the cause of IBD is still remains unclear and further investigation is required[42-44]. One of the important cause for IBD is dysregulated immune response directed against the intestinal microbiota and results in a disruption of the intestinal microbiota equilibrium[37,42-44]. The etiology of IBD can be conceptualized as an aberrant immune response to gut microbiota triggered following an environmental insult in a genetically susceptible individual[39-44]. Genes involved in IBD plays an important role in maintaining intestinal mucosal barrier and regulating innate and adaptive immunity[22-41]. It is clear that the microbiome plays a critical role the development of IBD[39-41].

1.7 Epidemiology of Inflammatory Bowel Disease

The second half of the twentieth century shows that 2.2 million persons in Europe, 1.4 million persons North America and other countries adopt a Western lifestyle suffer from IBD[45,46.]. Genetic and environmental factors like cigarette smoking, appendectomy are also involved in IBD[45-49].

IBD occurs in patients belongs to the age groups of 15 and 25 years and shows no significant difference between men and women[42-44,47]. Clinical features observed in adults and children with IBD may be similar[42,43,47]. Depending on the part of intestinal tract involved symptoms in IBD may range from mild to severe[42-48]. Common symptoms are abdominal cramps and pain, bloody diarrhea, vomiting, rectal bleeding, loss of appetite, weight loss, anemia, perfuse bleeding from the ulcers, perforation of the bowel, malignancy etc[42-48]. Children may develop incomparable complications like growth failure and delayed puberty[47,48]. The reported cases on IBD both Crohn's disease and ulcerative colitis shows a steady increase[42-49]. Hence understanding the mechanism of IBD is a very active area of research.

1.8 Background of Hopx

Homeodomain only protein X –Hopx is a newly identified gene encodes an atypical homeodomain containing protein[50,51]. This small, divergent homeodomain protein is also referred as HOD, HOP, CAMEO, LAGY, NECCI, SMP31 or OB1. HOPX-a (NM 139212.2), HOPX-b (NM 139211.2) and HOPX-g (NM 032495.4), are the three splice variants of Hopx gene which encode the same protein[50-53]. Transcription repression is mediated by putative homeodomain motif which acts as an adapter protein[50].

Initially, Hopx gene is identified as an important gene required for the cardiac growth and development[50-53]. Hopx gene inactivation leads to embryonic heart failure and lethality[50-53]. Homeodomain protein lacks certain conserved residues required for DNA binding[50-52]. Hopx which is incapable of sequence specific DNA binding functions by interacting with serum response factor- SRF and by recruiting histone deacetylase (HDAC)[50-53]. The transcription of SRF-dependent cardiomyocyte specific genes is affected by this kind of interaction[50-56].

Expression of Hopx is ubiquitous in variety of normal tissues[50-56]. Hopx is widely expressed in heart, brain, placenta, lung, skeletal and smooth muscles, uterus, urinary bladder, kidney and spleen[50-57]. Loss of Hopx expression in diverse types of malignant tissues-lung cancer, esophageal cancer, squamous cell carcinoma and uterine endometrial cancer demonstrated the importance of Hopx as a tumor suppressor gene and role in immune regulation[50-57].

Hopx Gene: Hopx, Gene Bank accession number NT 022853

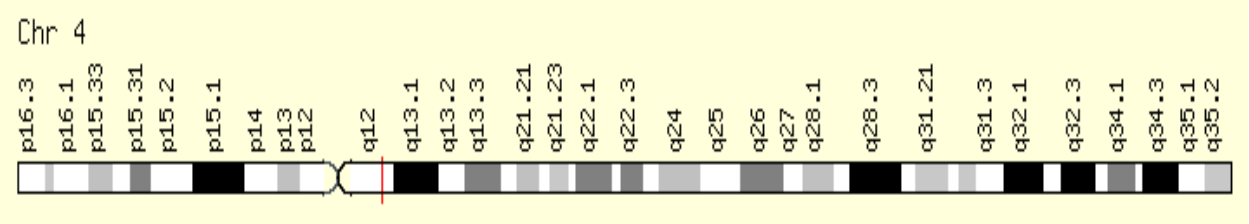


Figure 2: Hopx is located on chromosome 4q12[57]

Chen et al demonstrated the unique function of Hopx gene that it participate in multiple steps in the pathway of heart development-Hopx expression initiates in cardiogenesis, continues in cardiomyocytes throughout embryonic and postnatal development[51]. Hopx

functions directly by downstream of NKx2.5 and NKx 2.1[50,51]. A partial penetrant embryonic lethal phenotype with the severe cardiac defects development involving the myocardium was observed as the result of homologous recombination inactivation of Hopx[51-55]. This unusual function of Hopx was demonstrated in murine model[51-55]. CM Trivedi et al demonstrated the unexpected function of Hopx in embryonic heart development[52,53]. He actually found that Gata4 dependent cell cycle genes are regulated by the interaction of Hopx and Hdac2[54]. Hypertrophy was observed as the result of Hopx over expression[50-56].

In addition, Hopx expression loss was reported in several types of neoplasm[51-62]. Hopx expression was not found in various kinds of malignant tissues like choriocarcinoma, lung cancer, head and neck squamous carcinoma, esophageal squamous cell carcinoma and uterine endometrial cancer[51-62]. Yamaguchi et al reported that malignant conversion of placental trophoblasts associated with the suppressed Hopx expression[63,64]. Chen Y and colleagues studied the role of Hopx in lung cancer[64]. Hopx transfected in to lung cancer cell line H2170 suppressed the tumor formation in murine model. This study revealed that expression of Hopx inhibits lung cancer[64]. F Lamaire and colleagues reported that Hopx expression is strongly reduced in hypopharyngeal carcinoma[65]. Yamaguchi et al have reported the defective Hopx expression in human uterine endometrial cancer (HEC)[66]. He also demonstrated that in HEC- the promoter region of Hopx was densely methylated. In vivo analysis of HEC by forced expression of Hopx resulted in partial block in cell proliferation whereas, Hopx knockdown resulted in accelerated proliferation[66]. Thus this study demonstrated that HEC development was the result of transcription silencing of

Hopx results from hypermethylation[66]. Ookia et al studied the Hopx promoter methylation in gastric cancer[66,67]. Hopx-β promoter region harbors CPG islands among the three spliced transcript variants[67]. He reported that Hopx-β methylation quantitative assessment as a potential marker to detect tumor aggressiveness[67]. Moreover, studies in esophageal squamous cell carcinoma showed that Hopx promoter DNA is hypermethylated suggesting that Hopx methylation can be used as a marker to detect tumorigenicity[68]. However Albrecht I and colleagues demonstrated that adoptive transfer of Hopx deficient mice Th1 cells, fails to induce chronic inflammation in transfer-induced colitis and arthritis murine models[69]. He found that Hopx selectively expressed in TH1 cells and not in Th2, Th17 or Treg cells of Th-cell lineage[69].

Moreover D. Hawiger and colleagues studied the key function of Hopx in immune regulation. They proved that the Hopx expression is required for the function of iTreg induced by DC[70]. DC is a powerful antigen presenting cell needed for the induction of iTreg[70]. The iTreg induced by DC play a significant role in maintaining peripheral tolerance[70]. Activity of transcription factor AP-1complex is inhibited by Hopx sufficient iTreg and thereby other Tcells are inhibited[70]. This study proved that Hopx is significant for the function of Treg induced by DCs and maintains the steady state of T cell unresponsiveness[70]. More recently, N Takeda and colleagues demonstrated that expression of Hopx can be a specific marker of +4 cells[71]. He proved a unique functionality of Hopx in intestinal stem cells. Two kinds of intestinal stem cell – actively cycling and slow/quiescent cell population have been reported so far[71,72]. Lgr5 expressing crypt base columnar-CBCs and SOX9EGFP belongs to actively cycling. The

+4 cells which reside above the CBCs and Paneth cells are considered as slow cycling cell population. They give rise to all mature epithelium including goblet cells, neuroendocrine cells, Paneth cells and epithelial absorptive cells. They demonstrated that Hopx expressing +4 cells gives rise to Lgr5 and Lgr5 give rise to Hopx expressing +4 cells[71,72]. Hence they proved a bidirectional lineage relationship between the two kinds of intestinal stem cells-Hopx and Lgr5[71,72].

1.8.1 Impact of Hopx Expression

Expression of Hopx plays a significant role in normal development of tissues including heart and skeletal muscle[51-56]. Hopx modulate cardiac growth and development[51-56]. Through the negative regulation Hopx maintains a balance between cell proliferation and differentiation. Expression of Hopx acts as a tumor suppressor gene. Hopx can be used as a marker to detect gastric cancer and various other kinds of cancers through its promoter methylation[67]. Hopx expression regulate the function of iTreg induces by DC and regulate immune tolerance[70]. Expression of Hopx can be used as a marker to detect +4 niches of intestinal epithelial cells[71].

1.9 B cell

The immune system is highly dynamic and diverse. It helps to protect an individual from different kinds of pathogenic encounters[73]. Lymphocytes-white blood cells originate in the bone marrow and migrate to different parts of lymphatic system like lymph nodes, spleen and thymus[73]. T cells and B cells are the important types of lymphatic cell. Lymphatic cells have antigen specific receptors on its cell surface which recognize specific pathogen/foreign substances. Pathogenic encounter will result in the activation of lymphocytes-T cell and B cell. Lymphocytes activation will lead to cell mediated immunity[74]. B cells play an important role in the immune responses against pathogenic encounter[71-74]. B cell finds the specific antigen with the help of its receptor BCR(B cell receptor) which leads to the activation of B cell[71-74]. B cell can process the specific pathogenic antigen and present it to MHC class II molecules. It result in recruiting CD4+Tcells help leading to B cell proliferation and differentiation[73,74].

B cells can mainly differentiate in to two main classes 1) extra follicular plasma blasts – important for antibody production and early protection of immune receptors 2) activated B cell enter the germinal centre and differentiate in to plasma cells – plasma cells secrete high affinity antibody and also forms memory B cells[71-74]. Memory B cells provide long term protection from pathogen antigen / from their secondary encounter. Recent studies on B cell reveal that, through various mechanisms B cell can rapidly respond to antigenic stimulation[71-74].

1.9.1 B regulatory cell in the pathogenesis of IBD

For a long time B cells are considered mainly as antibody producing cells and play a role in pathophysiology of allergic diseases by the production of antibodies[76]. Generation and maintenance of mature B cells were not clearly understood compare to T cells[71-76]. However, recent investigations proved the new aspects of B cells[76,77]. B cells can act like antigen-presenting cells[71-76]. It can produce different kinds of cytokines like IL-6, IL-10, TNF-a[76,77]. B cell possesses functionally distinct subset capable to play pathogenic and regulatory function like T cells[71-76]. Recent studies proved that the regulatory B cells function by the production of regulatory cytokine IL-10, growth factor TGF[76,77]. CD 40 is required for the activation of B regs[77]. CD40 and TGF required for the maturation of B cell into antibody producing cells[77]. However, growth factors are considered as the mediators for several autoimmune diseases like rheumatoid arthritis and systemic lupus erythematosus[77-81].

CD19 is an important component of B cell receptor[77-82]. Sato S et al demonstrated CD19 mutation is associated with immunodeficiency like B-lymphomagenesis[82,83]. Shinichi and colleagues using CD19 deficient murine model demonstrated the crucial and important role of CD19 in BCR activation and antigen presentation[82-84]. However further investigation is required to find B regs development by BCR activation[82,83]. Presence of $CD19^{+}$ B regs have been demonstrated in both human and murine model[84]. Matsushit et al studied the regulatory function of $CD19^{hi}CD1d^{hi}CD25^{hi}$B cell[85,86,87]. Paul et al., demonstrated the regulatory mechanism of $CD19^{+}CD24^{hi}$CD38 B cell in autoimmune disease[87]. Recent study demonstrated the presence of Foxp3 expressing CD19+CD5+

regulatory B cells[82]. However, there is no single or multiple markers so far available for Breg identification[84]. B reg receive full activation signals- CD40, BCR, CD80-86, when antigenic epitopes are available from damaged tissues[88,89]. Thus, IL10 production is strongly enhanced and stabilized[84,88,89]. IL-10 is an immune regulatory cytokine and has a variety of pleiotropic functions like restraining inflammation, differentiation and maintenance of regulatory T cell (Treg)[84,87]. Importance of IL-10 is primarily studied in autoimmune diseases SLE and multiple sclerosis (MS)[84,91,92]. B regs inhibit exacerbated immune reaction and maintain immune tolerance via IL-10 production. It is robustly proved in murine models of autoimmune diseases[84,91,92].

Simon and colleagues studied the existence of an immunoregulatory B cells which are capable of recovering experimental autoimmune encephalomyelitis (EAE) and plays a major role in immune regulation[93]. Atsushi et al demonstrated the GALT associated B cell which was characterised by the upregulation of CD1d expression and enhanced production of IL-10 participate in the suppression of intestinal inflammation in colitis model[94]. Claudia et al and colleagues studied the new aspect of B cell subset- IL-10 producing B cell in controlling T cell differentiation and autoimmune disorders and thus proving that this subset play a immunoregulatory role in arthritis model[95,96]. In IBD, the chronic inflammation is mediated by Th1 response and leads to Crohn's disease (CD)[97]. Initially it was believed that the inflammation is regulated by regulatory T cells[76-79]. But using murine models including TCR_knockout (KO), B cell-deficient TCR_double KO and different kinds of genetically engineered models current studies proved that B cells can regulate inflammation in colitis[76,97,98]. Regulatory B cells also reduce harmful

immune responses by interacting with pathogenic T cells are capable to inhibit Th1, Th2, and Th17 responses[84,99,100]. In addition, B regs helps to maintains Foxp3 expression and thereby maintaining Treg population[76,99,100].

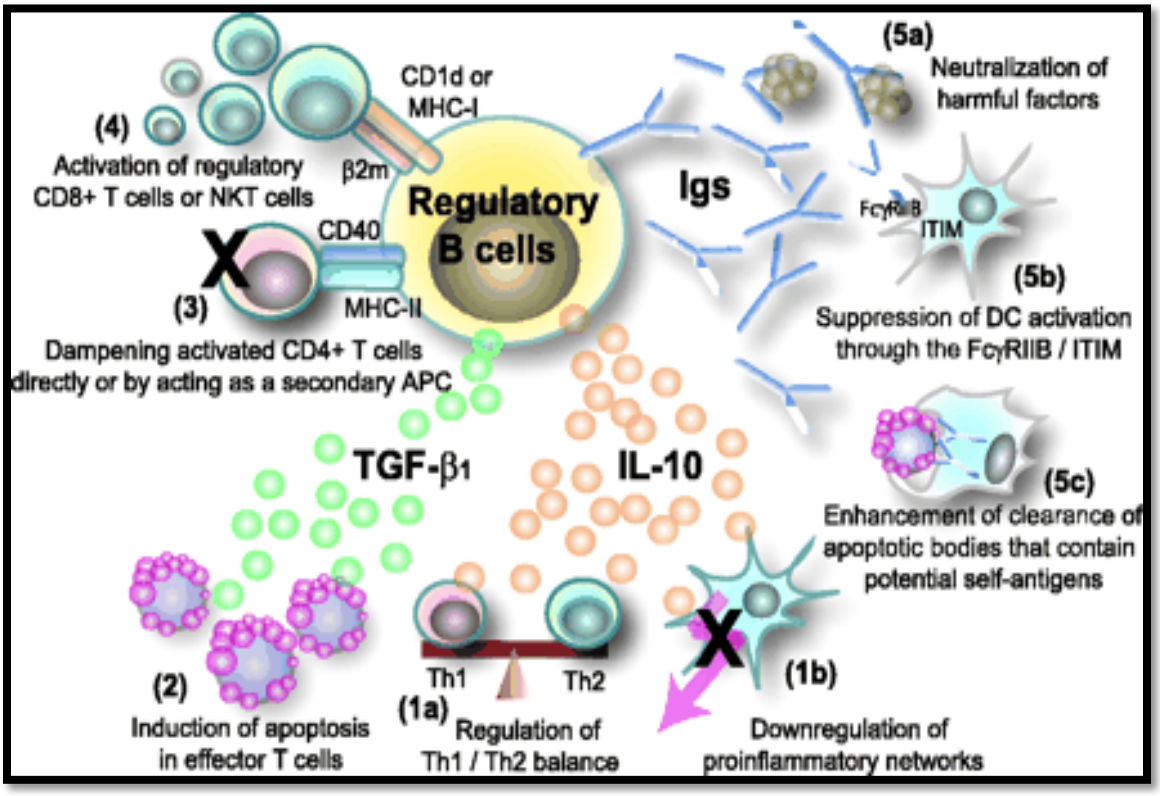


Figure 3: Regulatory mechanism of B cells in immune responses[76]

Together, these studies indicate that regulatory B cells are capable of inhibiting inflammation and induce immune tolerance like T cells. Hence, B regulatory cells are placed under the spotlight as important immune regulators.

1.10 Probiotics in Inflammatory Bowel Disease

The importance of bacteria in sustaining inflammation in IBD is supported by the clinical experience that antibiotics reduce disease activity[101]. Inflammatory bowel disease may be

prevented or treated using probiotics without affecting the normal flora in the intestine[101,102]. Probiotics have recently become a topic of significant focus in basic and clinical investigation[103,104]. Most common microorganisms in the human gastrointestinal tract are members of the genera Lactobacillus and Bifidobacterium[101]. Bacteria in these two genera resist gastric acid, bile salts and pancreatic enzymes, adhere to intestinal mucosa and readily colonize the intestinal tract[105]. They are important components of the gastrointestinal tract and are harmless[101,104,105]. Lactic acid bacteria have been demonstrated to inhibit the in vitro growth of enteric pathogens including Salmonella typhimurium, Staphylococcus aureus, Escherichia coli, Clostridium perfringens and Clostridium difficile[106]. These two strains are commonly used in dairy and pharmaceutical probiotic preparations considering their beneficial properties[101-106]. Research has proved significant reductions in pain, bloating, bowel movement difficulty, and composite symptom score vs placebo and a lactobacillus species[107]. In a large follow-up study, reduction in pain and global relief of IBS symptoms were significantly greater in the Bifidobacterium infantis-treated group compared with placebo[107].

Their beneficial properties include maintenance of intestinal homeostasis by competitive exclusion of pathogenesis[101-106]. They produce of antimicrobial compounds promotion of gut barrier function and immune modulation[102-106]. Probiotics modulate the balance of pro and anti inflammatory cytokine production and also inhibit the production of proinflammatory cytokine[106].

Probiotics induce dendritic cells to produce of anti-inflammatory cytokines like IL-10 which suppress the TH1 response[104]. Probiotics protect gastrointestinal disorders in many different mechanisms[104,106]. 1) Probiotics produce different kinds of antibacterial substances like bacteriocins and acid- organic acids, hydrogen peroxide and bacteriocins. These substances acts against both gram-positive and gram-negative bacteria and also helps to reduce viable cells and reduce bacterial metabolism or toxin production[101,106]. 2) Probiotics have the ability to adhere to the epithelial cells by lectin-like adhesion and proteinaceous cell surface components and thereby, prevent bacterial adhesion to the intestinal epithelial surfaces[101,106]. Blockade of carbohydrate binding sites for pathogen on intestinal epithelial cells plays a key role in intestinal microbial environment[101,106]. 3) Probiotics compete with pathogenic microorganisms to utilize the nutrients and thereby prevent pathogens from not consuming it[101,106]. 4) Probiotics (S.boulardii) helps in the degradation of toxin receptors (C.difficle) on the intestinal mucosa[101,106]. 5) Probiotics protect against intestinal disease by the stimulation specific and non specific immunity[101,106].

Gastrointestinal infections remain a major clinical problem due to increase incidence of antibiotic-resistant microbial pathogens. Probiotics are an attractive alternative treatment of antibiotics since the chances of for developing bacterial resistance are rare as they provide protection against pathogens by multiple mechanisms[101]. Recent finding proved the increased usage of probiotics among 50% patients with IBD[101-106].

CHAPTER 2: Study Plan and Hypothesis

2.1 Objective

Many evidence shows gut micro flora plays an important role in initiating and maintaining the mucosal inflammatory responses in IBD. Recent studies have proved the involvement of several genes in regulating intestinal inflammation. To elucidate regulatory mechanism behind the intestinal immune cells is a prerequisite to find specific protocols that could be used to prevent intestinal inflammation from future generation. This research is designed to investigate the role of Hopx in the regulation of the intestinal inflammation.

The outcome/findings of this study has the potential to further understand the regulating mechanism of Hopx expressing B regs in order to inhibit the intestinal inflammation and to provide the novel information for a broad array of studies in intestinal immune cells.

2.2 Central Hypothesis

Researches on Hopx have proved that the Hopx expression is reduced in a number of human diseases. Recent investigation demonstrated that the Hopx play an important role in colorectal cancer. IBD can be suppressed or prevented by immune regulatory cells and studies have proved that Hopx is required for the function of Treg cells. This research is designed to explore the role of Hopx-expressing B regulatory cells in suppression of intestinal Inflammation.

Successful finding of Hopx expression in human intestine- normal and IBD patient's sample consisting of both UC and CD revealed that Hopx expression is higher in normal intestine compare to IBD patient's intestine. Hopx expression was suppressed under inflammatory condition.

Based on our preliminary finding, we hypothesize that Hopx expression plays a significant role in regulating intestinal inflammation.

2.3 Project Aims

To elucidate our central hypothesis, we designed three specific and scientifically linked Aims

- Aim 1: To investigate the Hopx expression in TNBS colitis mice model.
- Aim2: To investigate the administration of probiotic in intestinal immune inflammation as well as modulates Hopx expression in intestinal B regs.
- Aim 3: To neutralize Hopx expression in order to prove that Hopx expressing B regs plays a key role in maintaining mucosal immunity.

CHAPTE 3: Materials and Methods

3.1 Reagents

Crystal violet dimethyl sulfoxide (DMSO), ethidium bromide, EDTA, DTT, Triton X-100, Tween 20, dipotassium hydrogen orthophosphate (K_2HPO_4), sodium dihydrogen orthophosphate (NaH_2PO_4), disodium hydrogen orthophosphate (Na_2HPO_4), DNase, Blenzyme, Percoll Solution, Peptidoglycan, Hematoxylin, Eosin, protease inhibitor cocktail were purchased from Sigma, Oakville, ON.

Agarose, bovine serum albumin (BSA), Tris, glycine, sodium dodecyl sulphate (SDS), sodium citrate, sodium chloride (NaCl), O.C.T compound embedding medium, TEMED were purchased from Bioshop Burlington, ON.

Trypsin-EDTA, Trinitrobenzene Sulphonate (TNBS), Penicillin-Streptomycin, RPMI-1640 media, DEPC water, Dulbecco's Modified Eagle's Medium (DMEM), and Fetal Bovine Serum (FBS) were purchased from Invitrogen, Carlsbad, CA.

30% Acrylamide/Bis solution and 20% SDS solution were purchased from Bio-Rad, Mississauga, ON.

Glycerol, methanol, chloroform and isopropyl alcohol were purchased from Caledon Laboratories, Georgetown, ON

Reagent Alcohol, Trizol, xylene, and acetone were purchased from Fisher Scientific, Ottawa, ON.

3.2 Antibodies

Hopx anti-rabbit antibody, FoxP3 mouse monoclonal antibody, CD19 anti mouse monoclonal antibody, beta actin mouse monoclonal IgG, anti-mouse HRP, anti-rabbit

HRP, propidium iodide/DAPI were purchased from Santa Cruz Biotechnology, Inc, USA, R&D systems Inc. Rest of the antibodies were purchased from eBioscience, Inc,USA.

3.3 Human Samples

Healthy human sample- used as control was obtained from Huazhong Science and Technology University, Tongji Medical University, China. IBD patients sample were obtained from Zhengzhou University First Affiliated Hospital Gastroenterology Department, China.

3.4 Animal Model

BALB/C mice were purchased from Charles River Laboratories International, Inc. through McMaster University and housed in specific pathogen-free vivarium at St.Joseph animal facility. Mice were monitored for one week prior to the experiment. Experiments were contacted in 5-6 weeks old mice.

3.4.1 Experimental Model: Classic TNBS Model

Colitis studies were developed in 5-6-wk-old male BALB/C mice. The experiments were contacted with a 7 days protocol. All animal models procedures and protocols were contacted in accordance to McMaster University AUP. Animals were monitored for one week prior to any experimental models and they were fed according to the standard laboratory procedure. Mice were allowed to fast overnight before the induction of colitis. For induction of colitis, TNBS in 50% ethanol (0.5-2.5 mg of TNBS in 50% ethanol) was administered per rectum to lightly anesthetized mice using isoflurane. Nearly 4 cm of catheter/soft tube was inserted proximal to the anal verge and 100 µl of TNBS/ethanol was slowly injected to the colon and the mouse will be held in a vertical position for 30

seconds. The body weight, water and food intake, and the presence of diarrhea (as detected by perianal fur soiling), was recorded daily throughout the experiment. Mice were sacrificed looking at the end point. Occurrence inflammation in the animals was evaluated by observing several parameters like- weight changes, mortality, and microscopic appearance of the intestine and evaluation of isolated colonic mononuclear cell cytokine production. Comparator groups were matched for age and sex typically as littermates. Male mice were preferentially used.

3.4.1.1 Control/Naive group

The experiment was contacted with seven days protocol like experimental model. Control group was treated with 50% ethanol (100% ethanol in 50% distilled water). Administration of ethanol was contacted in similar way of experimental model. The body weight, water and food intake, and the presence of diarrhea (as detected by perianal fur soiling), was recorded daily throughout the experiment. Mice were sacrificed at day 7 monitoring the end point carefully and same parameters were considered to evaluate the inflammation.

3.4.1.2 Probiotic Group

Probiotics were obtained from Huazhong Science and Technology University, Tongji Medical University, China. Bifidobacterium was cultures according to the standard bacterial culture laboratory protocol in MRS broth. Bifidobacteria (anaerobic bacteria) - cultured at 37ºC for 24 hours/ until it reach the log growth phase. Densitometer DEN1 was used to measure bacterial concentration. The standard formula was used calculated the bacterial concentration. Standard curve: Y=300X (10^6 CFU/ml).

Then the bacterial culture was centrifuged immediately at 1200rpm for 10 minutes and washed twice with cold PBS. For the probiotic administration, they were diluted to get the desired number of bacteria per gavage. The body weight, water and food intake, and the presence of diarrhea (as detected by perianal fur soiling), was recorded daily throughout the experiment. Mice were sacrificed at day 7 monitoring the end point carefully and same parameters were considered to evaluate the inflammation.

3.4.1.3 Probiotic + TNBS Group

This model is performed in a similar way of probiotic group. For the probiotic + TNBS administration, they were diluted to get the desired number of bacteria per gavage (200-300ul) and then treated in the same way.

3.5 Isolation of Lamina Propria Mononuclear Cells

LPMC were isolated from freshly obtained intestine as soon as the mouse was sacrificed. Mice were sacrificed and the intestinal sample was collected in a sterile petridish containing ice cold sterile phosphate buffered saline (PBS, 1.36 M NaCl, 14.7 mM KH2PO4, 80 mM Na2HPO4, 26.8 mM KCL, pH 7.4). It is transferred carefully to the biological safety hood. LPMC procedure was carried out in a sterile condition to avoid contamination by using modification method described by Van der Heijden and Stok. As the first step, cut the intestine to ~ 4cm long with the help of sterile scissor and then cut open the intestine and get rid of the stools completely. Then, cut the intestine into ~1cm long pieces and incubate in predigesting solutions EDTA (0.37 mg/ml), DTT (0.145 mg/ml) at 37°C for 10 minutes in a thermal shaker in 60rpm speed. As next step, the predigested intestine was cleaned from mucous layers and the intestine was cut in to ~0.5-

1mm. The tissues are digested further in RPMI 1640 media containing blenzyme-collagenase D (400 U/ml) and DNase (0.01 mg/ml) in a shaking incubator (60rpm) at 37°C for 10 minutes. The digested intestinal pieces were strained in to a 50ml conical tube using 50um thickness strainer and centrifuged (1200rpm) in a room temperature for 10 minutes. The pellet (LPMC) was washed twice and re-suspended in 10 ml of 40% percoll solution. Then it was carefully laid over the top of a 5ml of 80% percoll very slowly without affecting the liquid surface using transfer pipette. This dual layered percoll was gradient centrifuged in room temperature for 20 minutes using 2400rpm. After the centrifugation, LPMC is obtained as a thin layer of buffy coat in between the two layers. The layer was carefully removed using transfer pipette and washed with media(centrifuge at 1200rpm for 10 minutes) to remove the excess percoll. Finally, the obtained LPMC cells were cultured in a cell culture flask containing RPMI 1640 media (10% FBS and 1% Penicillin-Streptomycin) at 37ºC incubator.

Inflammatory Score and Mononuclear Cell Count

Hematoxylin and Eosin Staining: A small section of the intestinal sample was used for H&E staining. After sacrificing mice, a small section of the intestine was taken and cleaned from faeces, collected in cassettes and transferred to 4% formalin container. Then the samples were processed (embedded on paraffin) by pathologists- pathology laboratory located in St.Joseph hospital.

Sample sectioning: Microtome was used to cut the sample, and the sections were mounted on pre-baked slides. The slides were kept for 24-48 hours before they used for staining.

H & E staining protocol: The paraffin from the sections were de-waxed and dehydrated in xylene and ethanol (100%, 90%, and 70%) for 5 minutes each. Then the sample slide was incubated in distilled water for 5 minutes and stained with hemotoxylin solution for 30-60 seconds. Then the slides were washed in running warm water for 3 minutes. The sections were then dehydrated in 90% ethanol for 3 minutes and counterstained with eosin for 30-60 seconds. The slides were dehydrated in ethanol and cleared in xylene for 5 minutes each. Then the sections were air dried in room temperature and mounted using DPX- mounting media.

These H&E slides were observed under light microscope and used to take pictures to count mononuclear cells and to perform histological scoring.

3.6 Inflammatory Score

Pictures were taken from Hematoxylin and Eosin stained slides using light microscope. From each mouse intestinal section, 20 pictures were taken. Inflammatory scoring were performed using the chart below.

Parameter	Score	Condition
Epithelium and glands	0	Normal
	1	Focal destruction of epithelial surface and/or glands
	2	Zonal destruction of epithelial surface and/or zonal crypt loss
	3	Diffuse mucosal ulceration involving sub-mucosa and/or diffuse crypt loss
Inflammatory cells infiltration	0	Absence of infiltrate
	1	Sub-epithelial and in the lamina propria
	2	Infiltrate reaches the muscular mucosa
	3	Severe and diffuse infiltrate reaching the submucosa and/or involving the muscolaris propria
Edema	0	Absent
	1	Focal
	2	Zonal and/or moderately diffuse
	3	Extensive and severe

Table 1: Inflammatory Score Marking Indicator for IBD[108] and Natasha N Thesis 2012.

3.7 Mononuclear Cell Count

Pictures were taken from Hematoxylin and Eosin stained slides using light microscope. From each mouse intestinal section, 20 pictures were taken using 20X magnification. Mononuclear cells were counted and adjusted to the camera magnification to determine the number of cells per mm^2.

Myeloperoxidase Test (MPO): After sacrificing mice, a small portion was obtained to detect MPO activity. The intestinal sample was homogenised in 50mM potassium buffer

pH 6.0 with 0.5% Hexadecyltrimethylammonium bromide(HTAB) using homogeniser. Then the homogenised suspension has to go through freeze-thaw cycle for 3 times. From this suspension 0.1 ml was mixed with 2.9 ml of 50 mM potassium phosphate buffer pH 6 with 0.167 mg/ml 0-dianisidine dihydrochloride and 0.0005% H_2O_2 in 96 well plate. The samples were read at 450nm absorbance within one minute. One unit MPO is equal to degradation of 1mM H_20_2.

3.8 Immunohistochemistry

Immunohistochemistry staining was performed either by using Cryostat Section or Paraffin Section.

Cryostat Section: The intestinal sample was cleaned and the stool was removed with the help of sterile forceps. The sample was placed inside the OCT vial and frozen in -80ºC. The frozen samples were used to cut cryostat sections for staining. OCT was removed completely from section slides prior to staining using acetone (20 minutes)/ 4% formalin for one hour in room temperature. Then it was washed for three times (5 minutes incubation with PBS prior to each wash) with PBS to avoid any interference with antigen binding capacity.

Paraffin Section: After sacrificing mice, a small section of the intestine was taken and cleaned from faeces, collected in cassettes and transferred to 4% formalin container. Then the samples were processed (embedded on paraffin) by pathologists- pathology laboratory located in St.Joseph hospital. Microtome was used to cut paraffin embedded sample and used for staining. The paraffin wax was removed prior to staining technique. Th paraffin

wax was de-waxed using xylene (5 minutes) and dehydrated using ethanol (100%, 90%, and 70%) for 5 minutes each. Then the sample were rehydrates using distilled water (5 minutes) and washed in PBS for 5 minutes followed by steam fixing for 5 minutes. Steaming is used to retrieve the antigen attached to the antibodies epitopes.

3.8.1 Staining procedure

The immunohistochemistry staining was contacted in a humid chamber. OCT was removed from slides as mentioned above, and then it was treated with bovine serum albumin (1% BSA) for 20 minutes to avoid non-specific bindings. The sections were stained using primary antibody, Hopx primary antibody (Santa Cruz Biotechnology, USA) for overnight in 4ºC. After the primary antibody incubation, the slides were washed three times (each wash for five minutes) using 1X PBS. Then it was incubated with secondary antibody for 1-2 hours in room temperature and was washed again using 1X PBS for three times (each was for 5 minutes). Then the slides were air dried in room temperature and mounted using antifade mounting media. Stained sections of the tissue were examined for positive cells using fluorescence microscope. Minimum twenty pictures were taken choosing random numbers.

Antibody dilution: Different dilutions of primary antibody (Hopx) were checked and 1:100 was preferentially chosen as a best dilution. Hopx antibody 1:100 dilution and secondary antibody anti-rabbit FITC 1:200 dilution in 1X BSA was used for staining. For regulatory B cell staining, CD19 was used as primary antibody (1:100 dilution in 1%BSA) and anti-mouse PE (1:200 dilution in 1% BSA) was used a secondary antibody.

Negative Staining-Isotype IgG: Isotype staining was used as a negative control for immunohistochemistry staining. Isotype IgG was used as primary antibody (1:100 dilution in 1%BSA) and anti-rabbit FITC (1:200 dilution in 1% BSA) was used a secondary antibody.

3.9 Protein analysis

Extraction of protein: The intestinal sample was cleaned and the stool was removed with the help of sterile forceps. The tissue sample was homogenised using lysis buffer and protein inhibitor (cocktail) in the ratio of 1:9(lysis buffer- 180 microlitre; cocktail- 20 microlitre).Then incubated in 4ºC for 30 minutes. Mix well in between incubation time. After 30 minutes of incubation, centrifuge for 20 minutes at 4ºC (14,000 rpm speed). Supernatant was pipette out in to a new vial (protein) and the pellet-debris was discarded to maintain the protein purity. The purified protein sample was checked for purity and concentration using spectrophotometer and good quality protein was used to perform respective experiments.

Western Blotting: Steps Involved: Gel Preparation, Electrophoresis, Membrane transfer, Blocking and Film processing.

Gel Preparation: Glass slides were cleaned using 70% ethanol. Gels were prepared according to required concentration. Electrophoresis: Samples protein was diluted in ddH_20 until it reached the concentration of 100ug/ml, then the sample buffer and reducing agent were added according to the company recommended ratio. Prepared samples were then boiled at 100ºC for 5 minutes on a dry plate. Electrophoresis chamber was filled

with running buffer and the samples were loaded carefully in to the wells using the long pipette tips. Samples were separated according to their molecular weight in the 12% SDS-polyacrylamide gel at 130V. Once the electrophoresis was done, power codes were disconnected (positive and negative charges). After electrophoresis, proteins were transformed to membrane.

Membrane transfer: Sandwich was prepared using sponge, filter papers, membrane and gel. The proteins were transferred to the membrane using transfer buffer. Transfer was performed at 4ºC for ~90 minutes (30V). After complete transfer of protein from the gel to the membrane, transfer was stopped. Blocking: Then the membrane was blocked with 5% skim milk (2gm in 20ml 1XTBST) on a shaker (60rpm speed) for one hour at room temperature to prevent non-specific binding. After one hour incubation, the membrane was incubated with primary Hopx antibody on a shaker at 4º C for overnight. Then the membrane was washed three times with 1X TBST for three times (15 minutes each wash). Then the membrane was re-incubated with secondary antibody for one hour in room temperature. After the incubation, the membrane was washed again with 1X TBST for three times (15 minutes each wash). Finally, ECL solution (solution A and solution B) was used for one minute prior to film processing. Film processing: Kodak exposure cassette was used to expose the film.

For Hopx antibodies, Hopx anti rabbit was used as the primary antibody with 1:2000 dilution ratio and anti-rabbit HRP was used as the secondary antibody with 1:7500 dilution ratio. For beta actin antibodies, anti-mouse beta actin was used as the primary

antibody with 1:1000 dilution ratios and anti-mouse HRP was used as the secondary antibody with 1:16000 dilution ratio. All antibodies were diluted in 5% skim milk in TBST solution.

3.10 RNA isolation and Real Time PCR

Extraction of RNA: The intestinal sample was cleaned and the stool was removed with the help of sterile forceps. The tissue sample was homogenised until no tissue was visible using 1 ml of Trizol to lyse the cell, mix well and then vortex vigorously for few minutes and then incubated for 15 minutes in 4ºC. After the incubation, the solution was centrifuged for 1200 rpm for 10 minutes. Then the supernatant was transferred to a new sterile eppendorf tube and 200 ul of chloroform was added. Vortex for few minutes and then incubated at 4ºC for 15 minutes (mix well in-between the incubation time). After the incubation time, it was centrifuged at 1200 RPM for 15 minutes at 4C. Three layers were visible after centrifugation and the RNA was found on the top layer with an aqueous phase. Transfer supernatant (clear solution) to the new eppendorf tube followed by adding 500 ul of isopropyl to the clear supernatant. Incubate for 10 minutes at room temperature and then centrifuge for 10 minutes at 4ºC (1200rpm speed).Remove the supernatant. The pellet (RNA) was washed with freshly prepared 1ml of 75% ethanol twice and then dried for 5 minutes. The pellet was resuspended in 50ul of DEPC water and mixed well. The purity and concentration of RNA was checked using spectrophotometer.

RT-PCR/ Real-Time PCR: All RNA had to be reversed transcriptase into cDNA by using a RT kit. Once cDNA was obtained from RNA, it was diluted in DEPC water to reach

approximately the same concentration. Then PCR mix and primers were then added to the cDNA samples.

3.10.1 **Primers**

HOPX Forward 5'- GAT GAT GTG GGA ACC GTC TT-3'

HOPX Reverse 5'- CGT TCT CAT TCA ACC ACC AG-3'

CD19 Forward 5'-CTG ACG TCT GAA GCA TTC CA-3'

CD19 Reverse 5'- ACC AGT CAA CAC CCT TCC TG-3'

TNF-a Forward 5'- AGG AGG GAG AAC AGA AAC TC -3'

TNF-a Reverse 5'- AAT GAG AAG AGG CTG AGA CA -3'

INF-g Forward 5'- GGC CAT CAG CAA CAA CAT AA-3'

INF-g Reverse 5'- TGA GCT CAT TGA ATG CTT GG-3'

IL4 Forward 5'- TGA CGG CAC AGA GCT ATT GA-3'

IL-4 Reverse 5'- GGT GTT CTT CGT TGC TGT GA-3'

Beta Actin Forward 5'- GCT ACA GCT TCA CCA CCA CAG-3'

Beta Actin Reverse 5'- GGT CTT TAC GGA TGT CAA CGT C-3'

3.11 Flow cytometry

Cells were collected from culture flasks by gentle tapping at the bottom of the flask for non-detachable cells or by using trypsin for detached cells. Then the cells were then washed in PBS twice and centrifuged at 4^{o}C with 1200 rpm for 5 minutes. Then suspended the cells in 200 ul FA buffer, and then antibodies were then added to the cell suspension followed by 60 minutes incubation in 4^{o}C and kept from direct light. After incubation, the cells suspension were then centrifuged at 4^{o}C with 1200 rpm for 5 minutes and washed with PBS twice. For primary antibodies (unconjugated), secondary antibodies can be added at this stage. Incubation for secondary antibodies usually takes the same time and temperature as the primary antibodies. After the incubation time is done, the cells suspension were also washed twice with PBS and re-suspended in FA buffer and ready to be analyzed. For later use, these cells can also be re-suspended in 4% para-formaldehyde solution and can be kept in a 4^{o}C fridge for 1 week.

3.12 Statistical Analysis

All data are presented as mean ± standard error (SE). Statistical analysis was carried out using Graph Pad Prism for Windows, version 5.04. Two-tailed Student's *t*-test was used to analyze data differences between experimental and control murine models.

CHAPTER 4: RESULTS

4.1 Hopx expression in Intestinal Bowel Disease

As the first step we want to investigate whether the expression of Hopx regulates intestinal inflammation. We investigated the Hopx expression in human intestine before we begin our studies in murine model. Expression of Hopx in both healthy human intestinal samples and IBD patient intestinal samples were examined to study whether there is a significant difference between the two groups. Several paraffin sections of biopsy samples obtained from different IBD patients consists both UC and CD and non-IBD human sample were randomly chosen for the evaluation Hopx expression.

Immunohistochemistry analysis of human sample revealed that the Hopx expression is lesser or reduced in IBD patient's sample compare to normal human sample (Figure 4). The result indicate that the Hopx expression play a role in regulating intestinal inflammation.

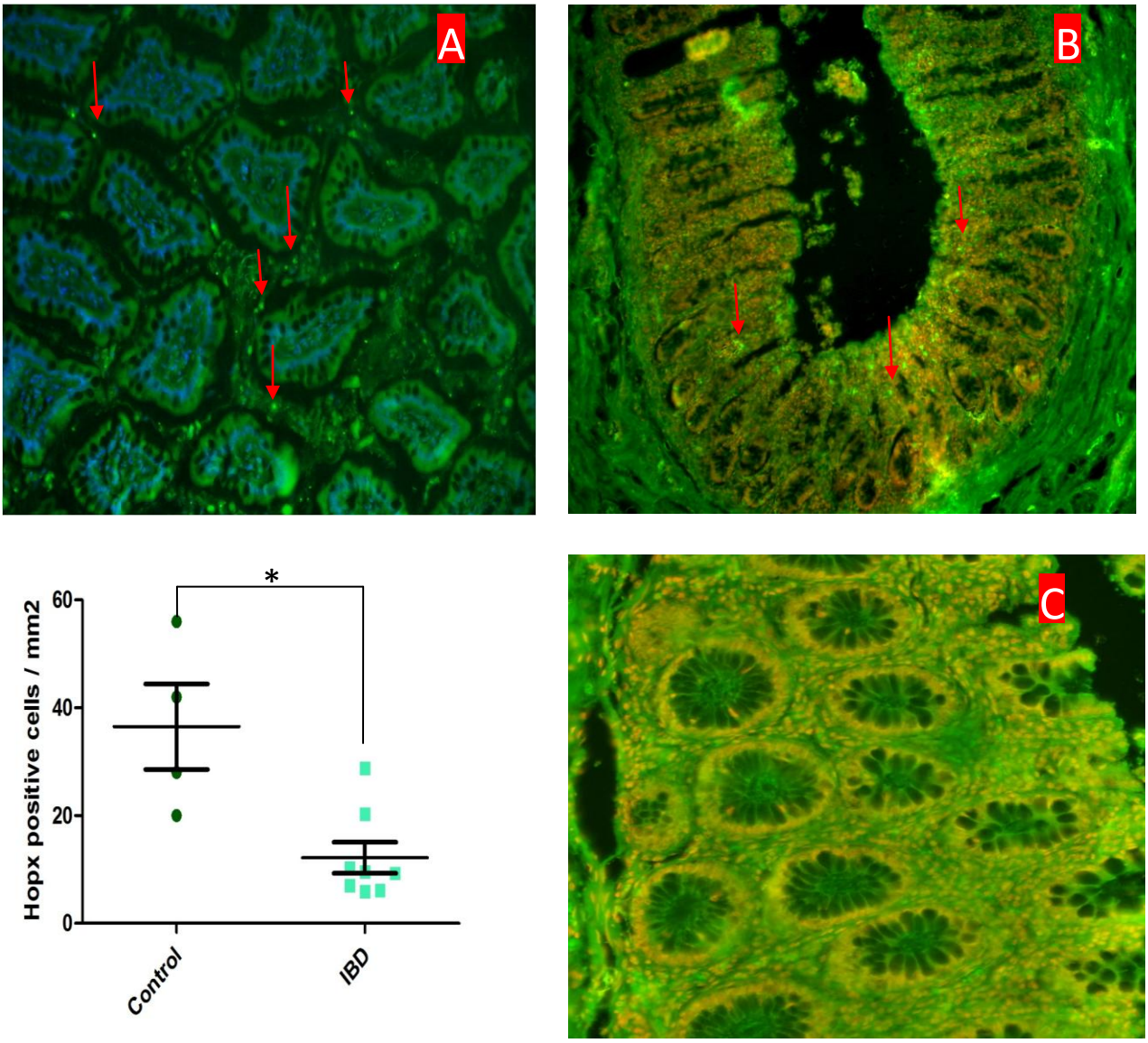


Figure 4: Immunohistochemistry staining and positive cells counting results

Using Hopx antibody (green) and nuclear staining with DAPI/PI (blue/red) showed significant reduction of Hopx expression in human IBD samples. Isotype staining was used as a negative control. (A) Non-inflamed intestine, (B) inflamed intestine, (C) Isotype IgG. Mean ± SEM (n=3-8sample per group). The original magnification of the images A, C-200X and image B was 100X. Arrows pointing positive cells. Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

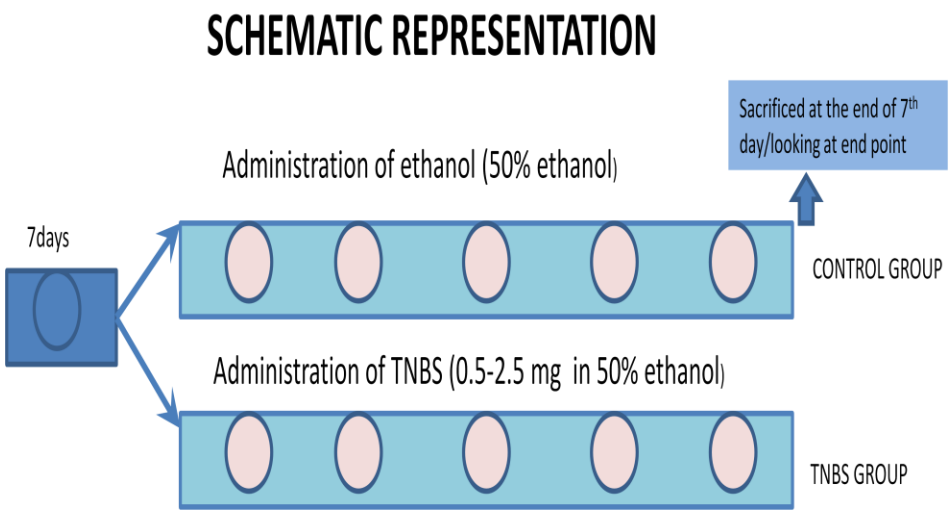
4.1.1 Hopx expression in Murine Intestine

After discovering the Hopx expression in human intestinal tissue, experimental colitis model was used to study the role of Hopx in IBD. The widely accepted TNBS was preferably used to induce colitis in murine model.

To confirm that the TNBS-induced colitis model demonstrate the various pathophysiological aspects of human IBD, several parameters were considered. We used the histological score to study the severity of the inflammation(Fig 8). In order to support this data, and also to find the characteristic features of IBD in chemically induced murine model, we looked at other parameters like weight loss (Fig 6), morphological appearance of the intestine, mononuclear cell counts (Fig 9), and MPO(Fig10). Intestinal sample collected from TNBS-induced colitis model was used for demonstrating the severity of inflammation. All the datas were compared to control group. Studies were contacted in both cellular and molecular levels

Once we confirm the severity of inflammation in murine model was similar to the characteristic features of colitis in human IBD, we moved further to explore the expression of Hopx in intestinal inflammation.

- Aim 1: To investigate the Hopx expression in TNBS colitis mice model.



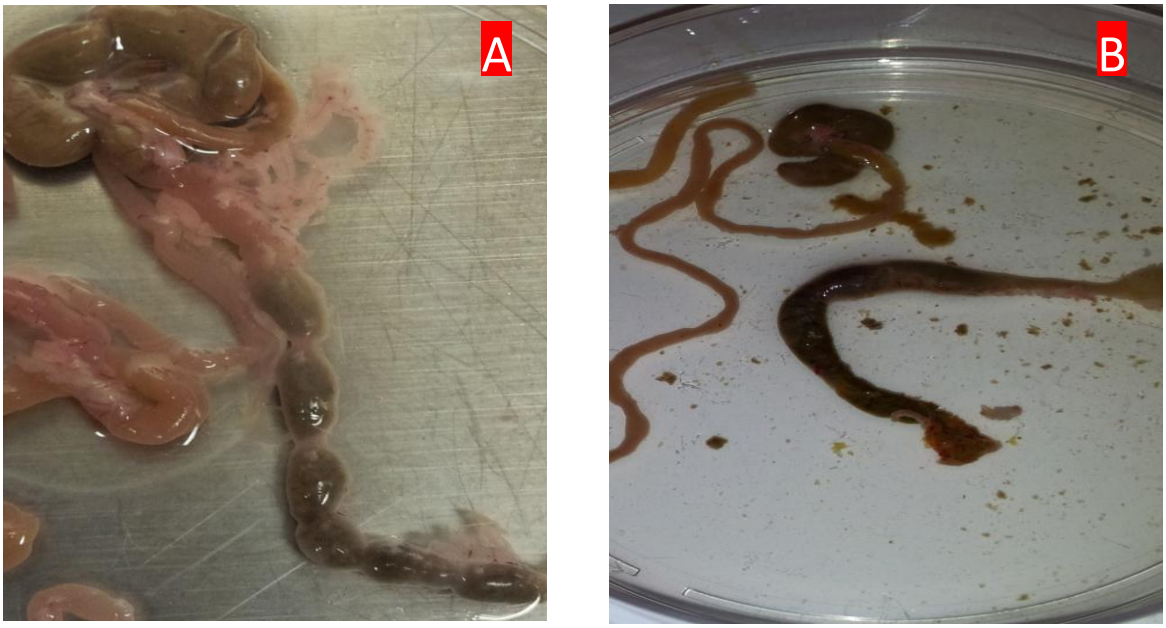


Figure 5: Macroscopic examination of the intestines.

For induction of colitis, TNBS in 50% ethanol (0.5-2.5 mg of TNBS in 50% ethanol) was administered to the experimental group. Control group treated with 50% ethanol. (A) Control mice colon (B) TNBS mice colon.

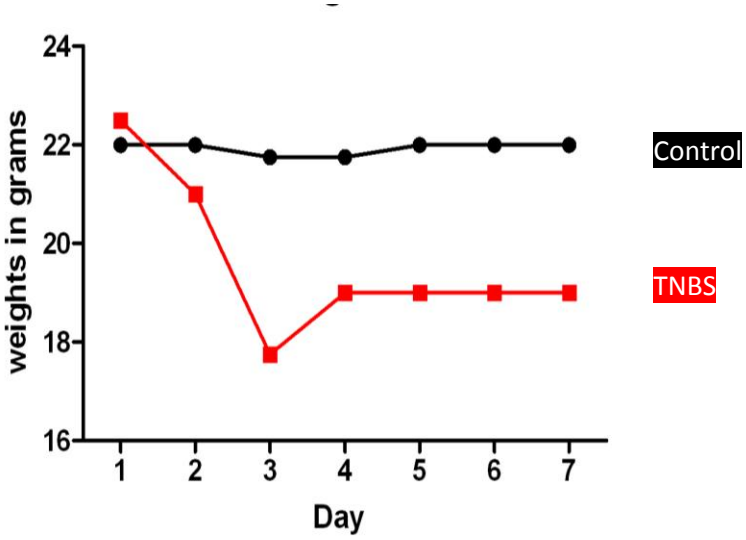


Figure 6: Mice weight chart.

Weight loss in colitis experimental model and control group. Mice were injected on day 1 and sacrificed looking at the end point (n=5 mice per group).

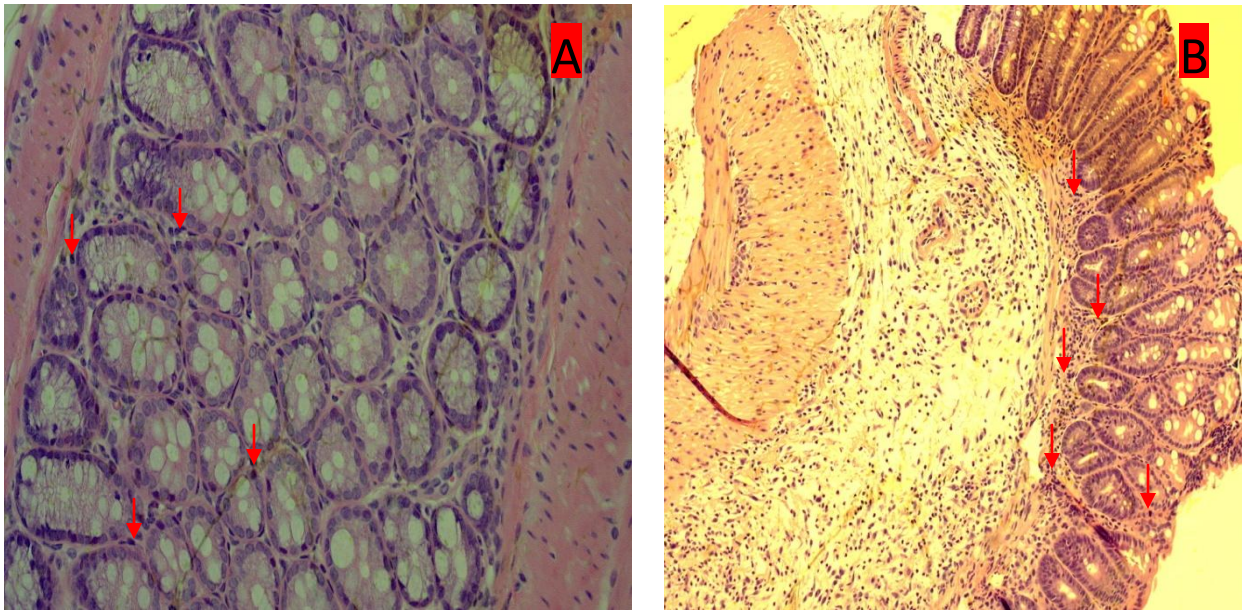


Figure 7: Representative H &E staining showing histology.

The presence of lymphocyte infiltration, elongation and distortion of crypts and many infected cells evidence the intestinal inflammation in test group (TNBS) and not in control group (ethanol).(A) control intestine (B) TNBS induced colitis intestine. Magnification=200X (A), 100X(B). Arrows pointing mononuclear cells.

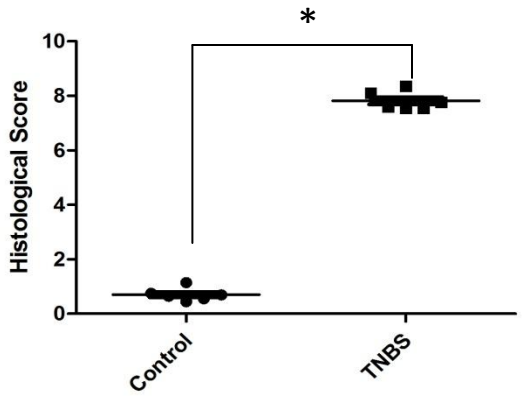


Figure 8: Inflammatory score.

Inflammatory scoring for mouse intestinal tissue stained with H&E stain. Inflammatory score was significantly higher in TNBS colitis compare to control group. Mean ± SEM (n=5-6 sample per group). Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

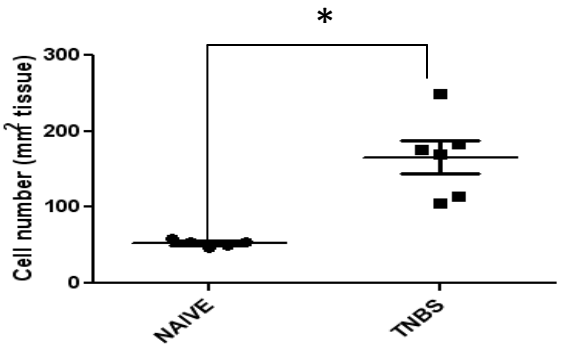


Figure 9: Mononuclear cell count.

Number of cells counted per mm^2 slides from mouse intestinal tissue stained with H&E stain. Mononuclear cells were significantly higher in TNBS colitis compare to control group. Mean ± SEM (n=5-6 sample per group). Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

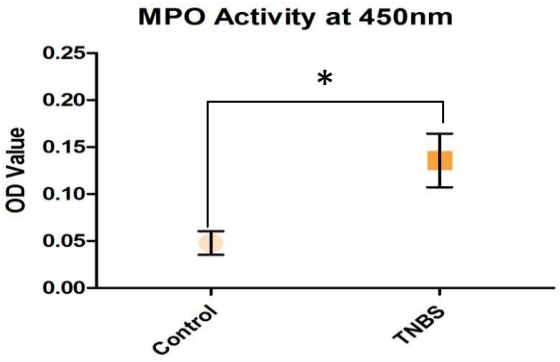


Figure 10: Comparison of MPO activity read at 450nm.

Mean ± SEM (n=5-6 sample per group). Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

These datas confirmed the severity of inflammation in colitis induced by TNBS and the murine models had the similar to the characteristic features of colitis in human IBD. Hence, we studied the expression of Hopx in TNBS and in control group. Several experimental techniques such as immunohistochemistry analysis, Flowcytometry (Fig11), western blotting (Fig 14) and real-time PCR (Fig 13) were performed to illustrate the role of Hopx in colitis.

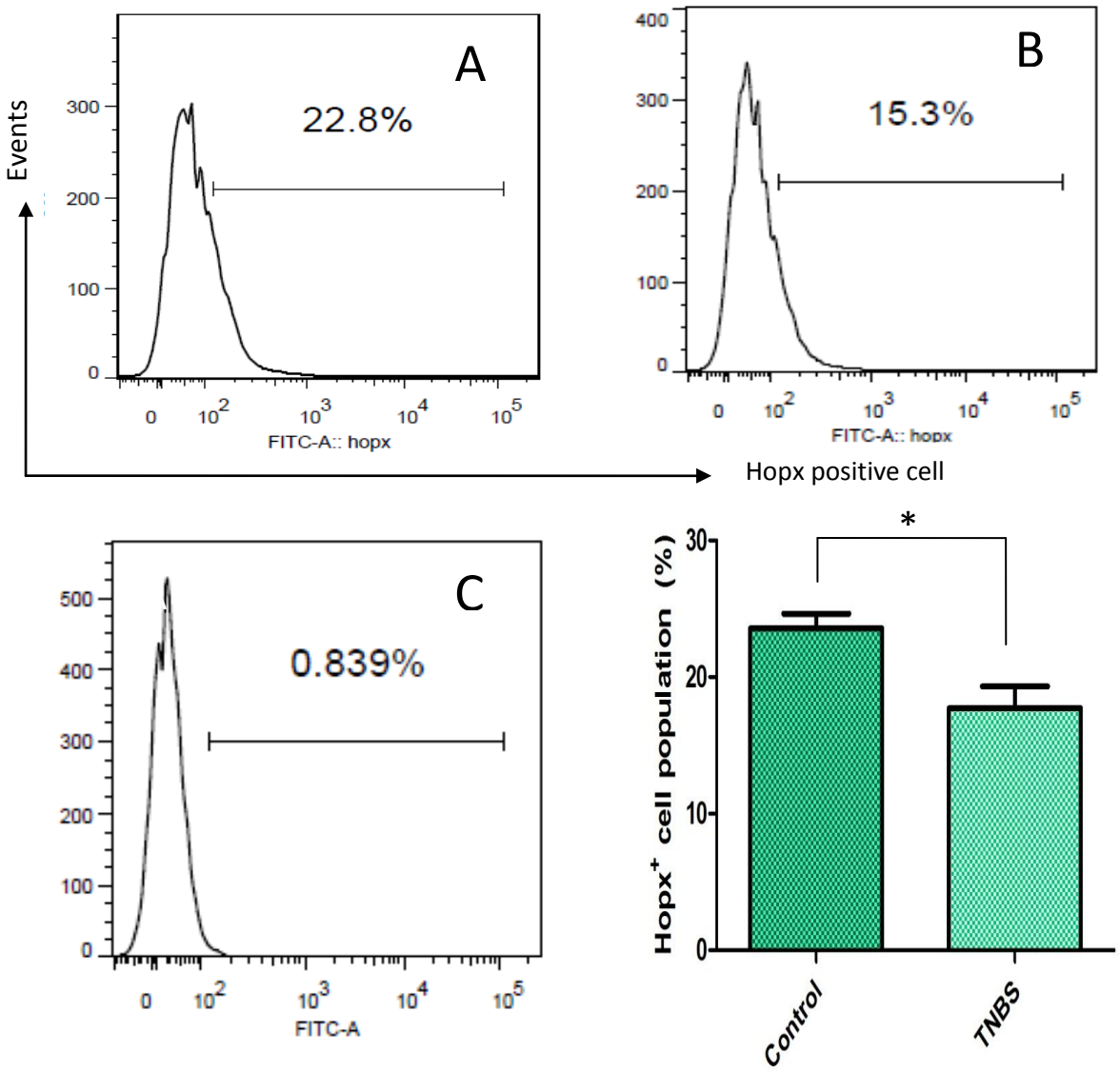


Figure 11: Flowcytometry Analysis of Hopx^{+} cells

Flow cytometric profiles of intestinal LPMC cells from control and TNBS mice stained as indicated. (A) Control LPMC Hopx+ cell population (%), (B) TNBS LPMC Hopx+ cell population (%), (C) Isotype IgG. Mean ± SEM (n=5sample per group). Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

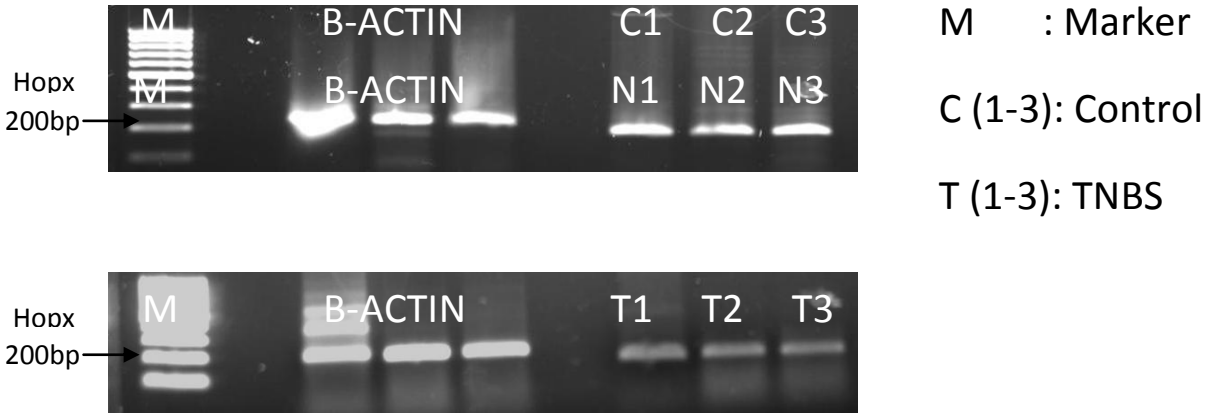


Figure 12: Agarose gel electrophoresis of RT-PCR products.

β-Actin was used as loading control. Bright bands were observed in control group compare to TNBS group.

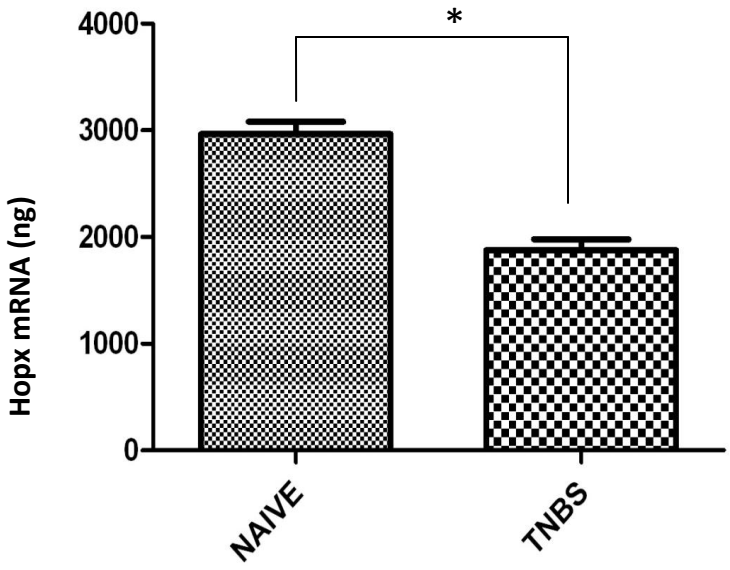


Figure 13: Real Time PCR Analysis-Hopx mRNA

Real Time PCR analysis of RNA extracts from intestinal tissue of control and TNBS mice. Mean ± SEM (n=5sample per group). Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

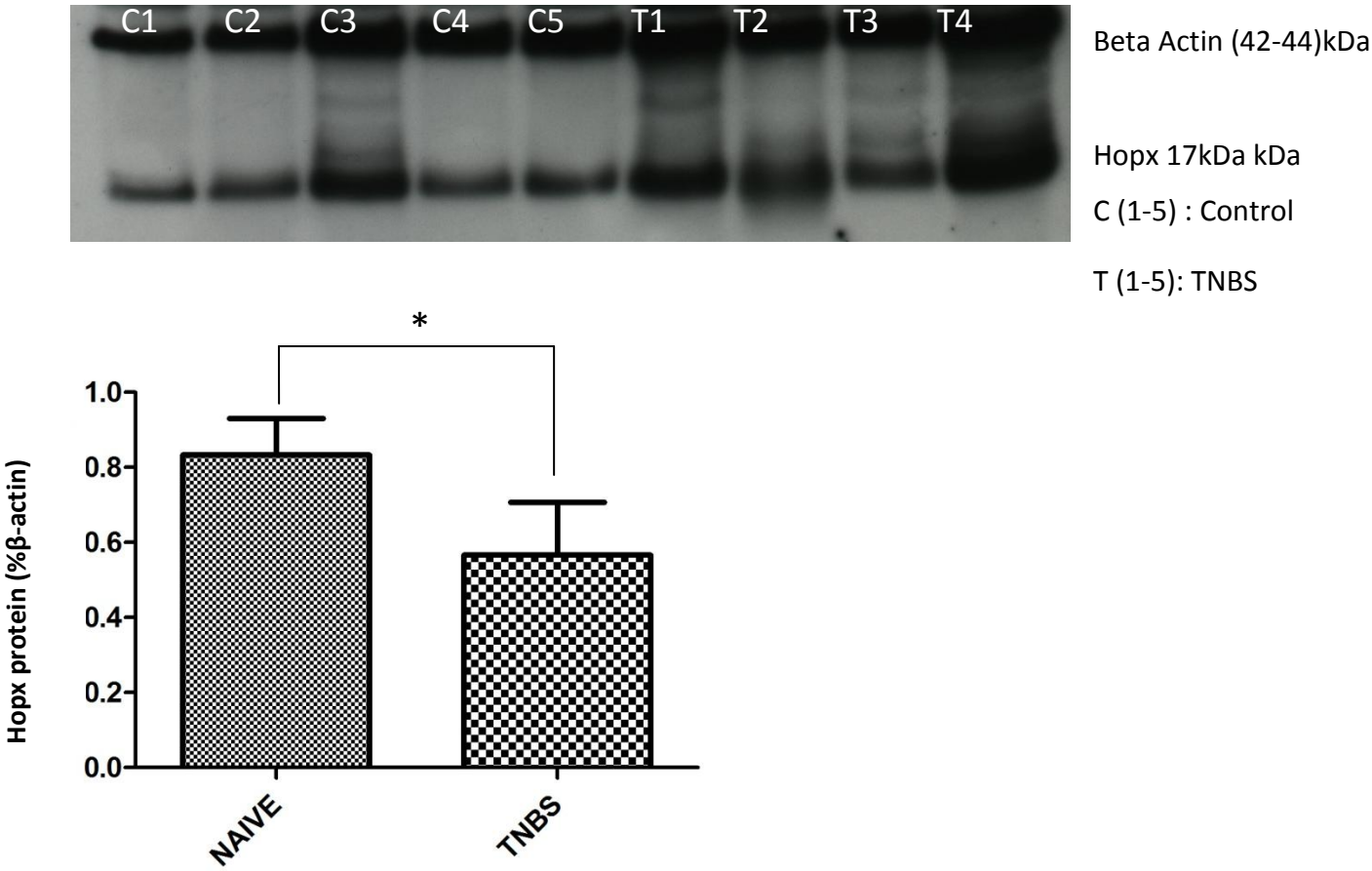


Figure 14: Western blot Analysis

Western blot analyses of Proteins extracted and purified from control and TNBS intestinal tissue. Hopx expression was significantly reduced in protein extracts from TNBS colitis mice compare to control mice. Mean ± SEM (n=5sample per group). Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

4.2 Hopx expression in Colitis

Based on the interesting findings in human intestinal sample, we designed mice models for further investigation of Hopx. Widely accepted classic TNBS experimental group was compared to control/naïve group to elucidate the role of Hopx expression in Colitis. Several parameters were considered to prove that the TNBS model mimics human intestinal bowel disease. Study using mouse model supported the preliminary data collected from human sample. The immunohistochemistry and flowcytometry datas from mouse model proved that Hopx expression is reduced /suppressed under inflammatory condition. In addition we found that Hopx- mRNA and Hopx protein expression were reduced under inflammatory condition. Thus importance of Hopx and its role to prevent/inhibit inflammation has been successfully proved from murine model.

As the next step, we want to study the Hopx expression in regulatory B cells. We investigate the Hopx expressing regulatory B cell using the same techniques. Hopx expressing Breg cells were significantly reduced under the inflammatory condition.

These findings indicate that the Hopx expressing Breg plays a critical role in regulating intestinal inflammation.

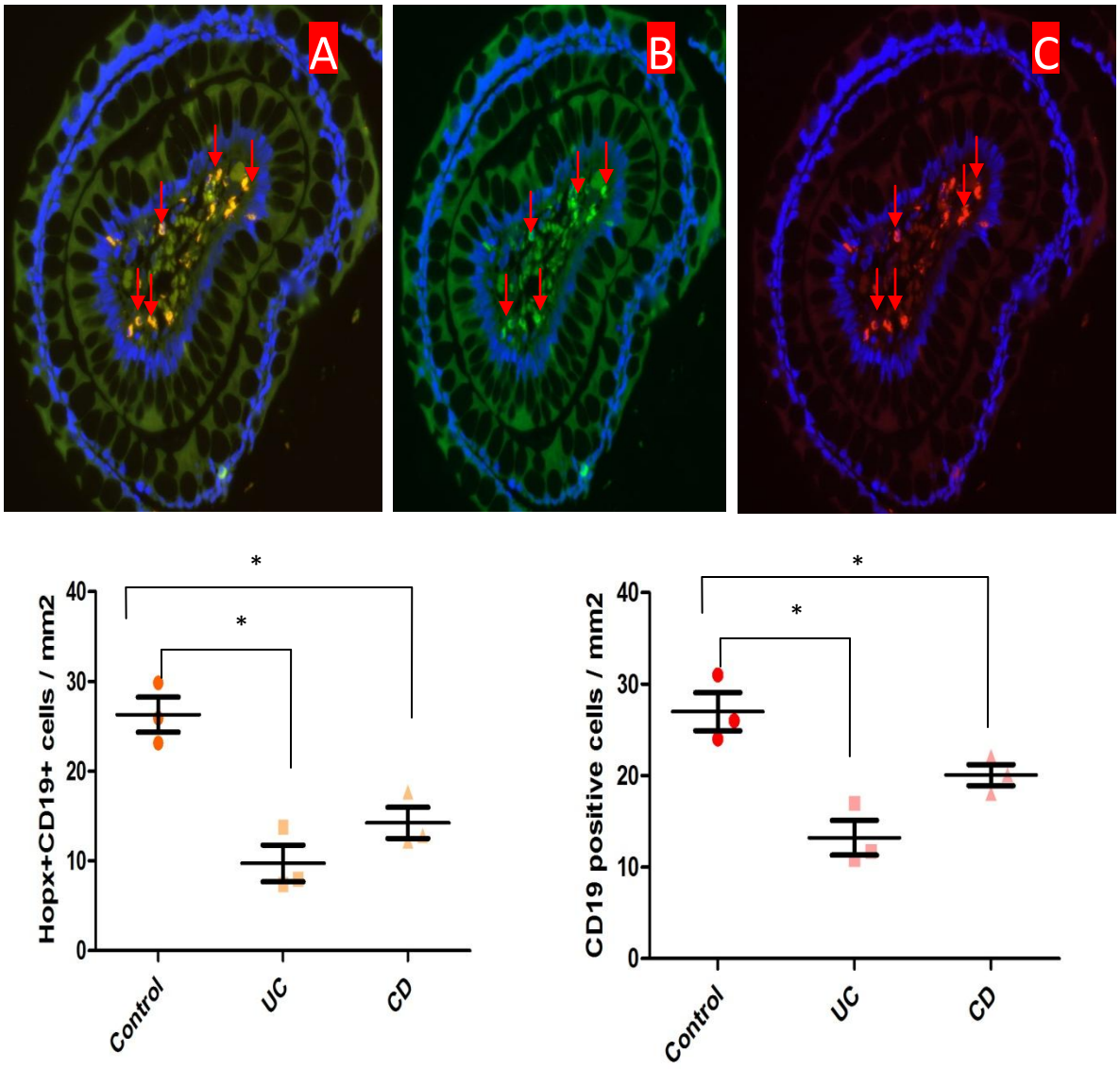


Figure 15: Immunohistochemistry staining and positive cells counting results.

Staining using Hopx antibody (FITC), Breg antibody CD19 (PE) and nuclear staining with DAPI (blue) showed significant reduction of Hopx expressing Breg cells in human IBD samples. (A) Hopx+ Breg+ cells (B) Hopx expression,(C) Breg expression. Mean ± SEM (n=3 samples per group). Magnification = 400X. Arrow pointing positive cells. Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

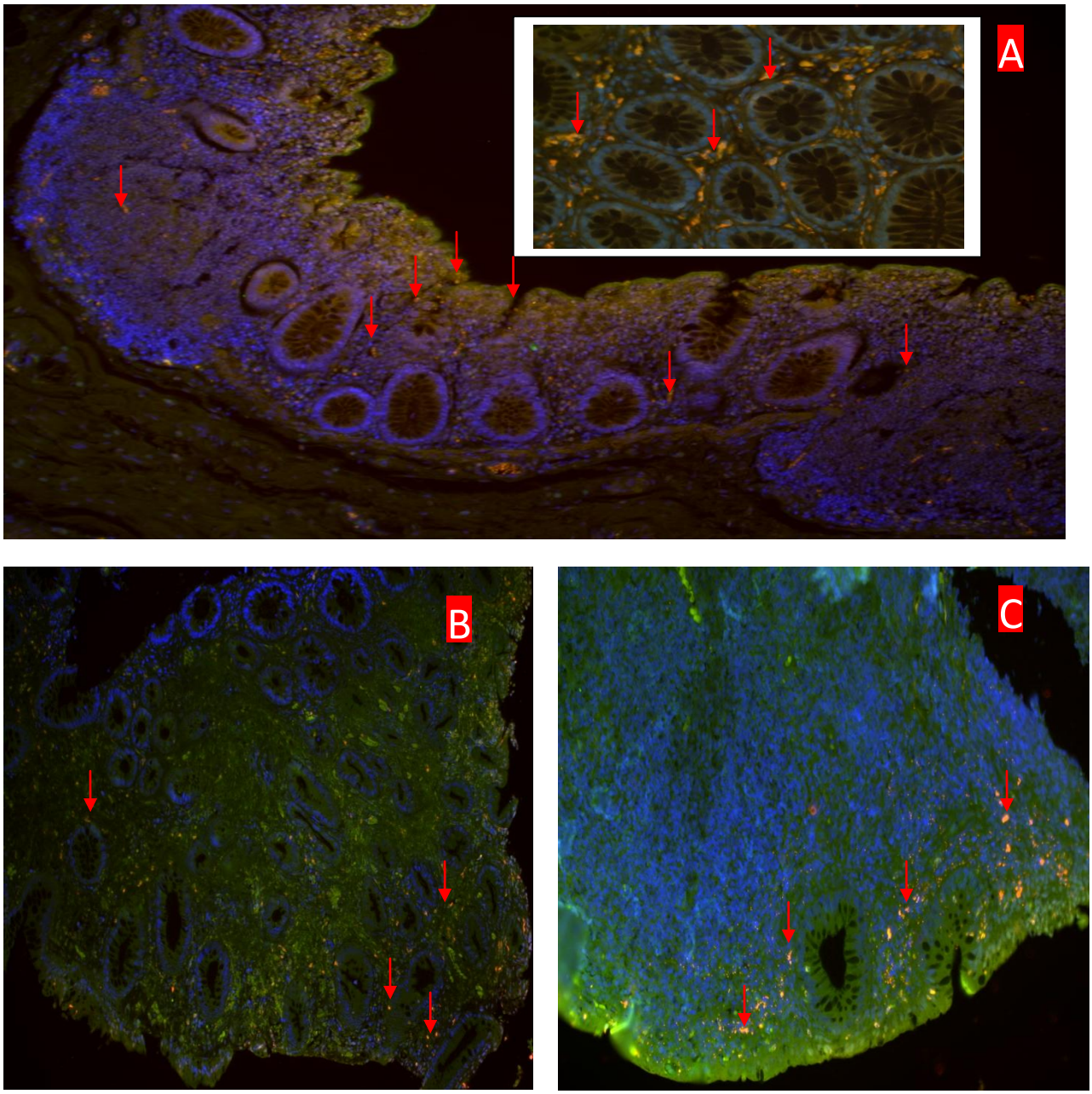
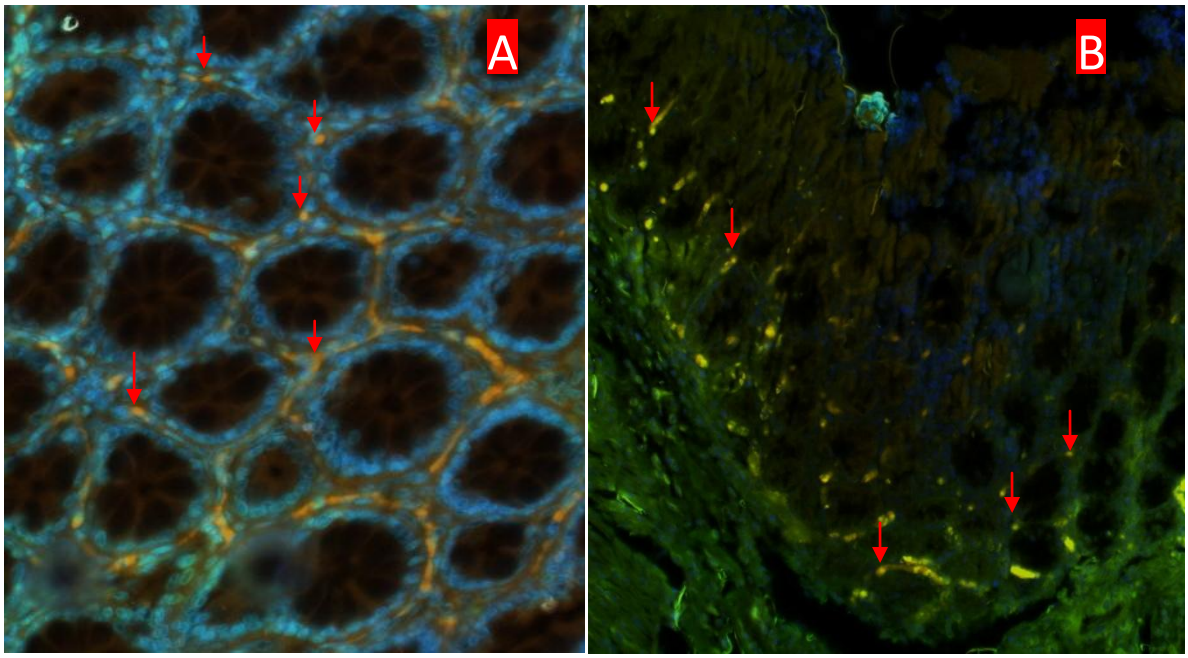


Figure 16: Hopx expression in Intestinal Epithelial Barrier

Staining using Hopx antibody (FITC), Breg antibody CD19 (PE) and nuclear staining with DAPI (blue) showed significant reduction of Hopx expressing Breg cells in human IBD samples. No Hopx expression in biopsies of inflamed mucosa. Hopx expression is located near mucosal barrier and around the healthy cells. (A)Healthy human donor sample,(B) UC patient sample (C) CD patient sample. Magnification of image A=100X, B and C=200X. Arrows indicating positive cells.



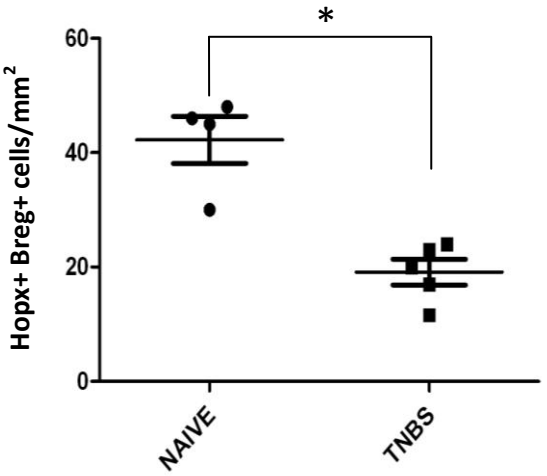


Figure 17: Immunohistochemistry staining and positive cells counting results.

Staining using Hopx antibody (FITC), Breg antibody CD19 (PE) and nuclear staining with DAPI (blue) showed significant reduction of Hopx expressing Breg cells in TNBS induced colitis mice. (A) Control (B) TNBS. (Mean ± SEM (n=3-5sample per group). Magnification of image A=400X, and B=200X. Arrows pointing positive cells. Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

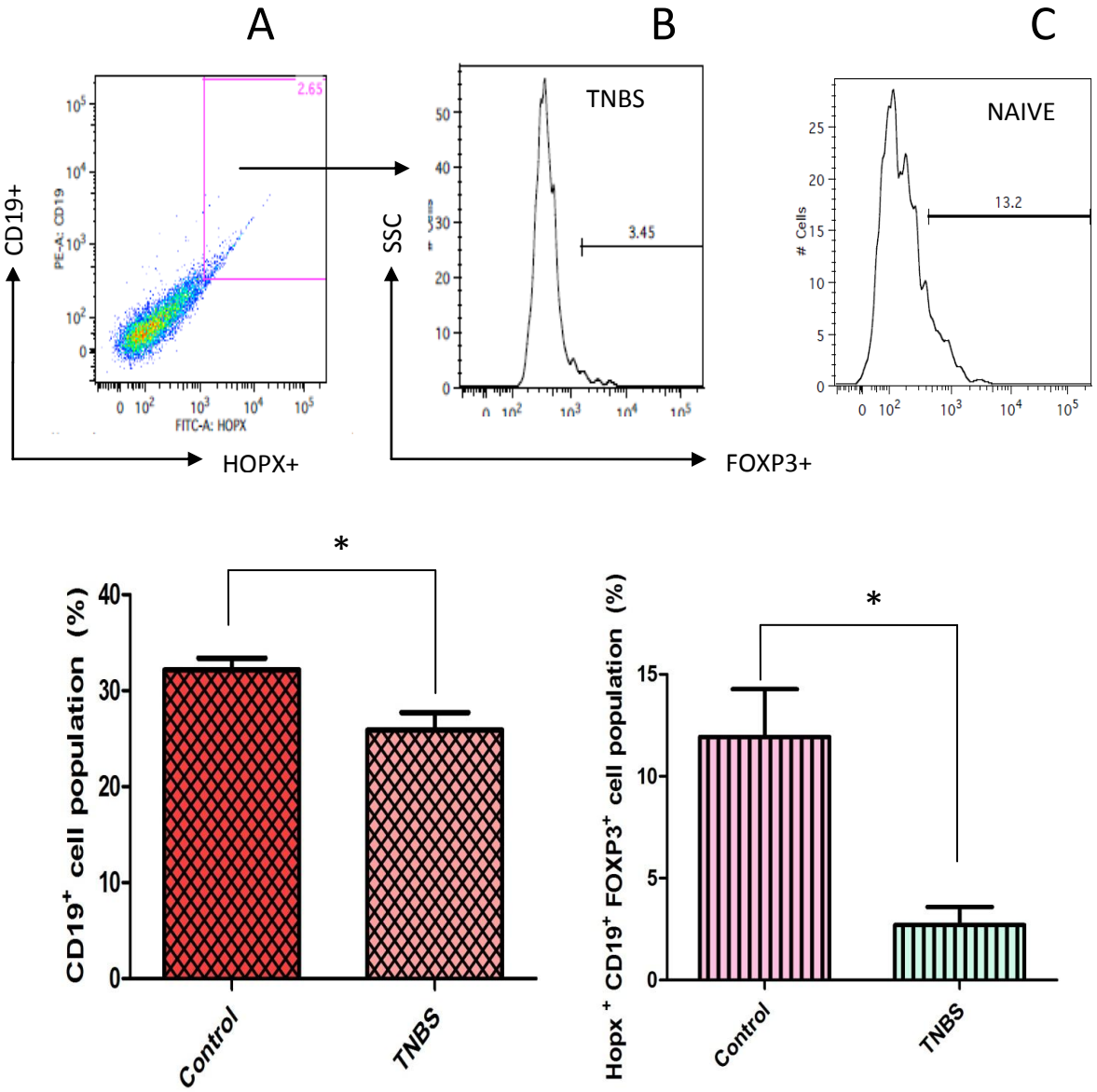


Figure 18: Flowcytometry

Flow cytometric profiles of intestinal LPMC cells from control and TNBS mice stained as indicated. (A) TNBS LPMC Hopx+ CD19+ cell population (%), (B) TNBS LPMC Hopx+CD19+FOXP3+ cell population (C) Control LPMC Hopx+ CD19+FOXP3+ cell population (%). Mean ± SEM (n=5sample per group). Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

Hopx expression in Colitis

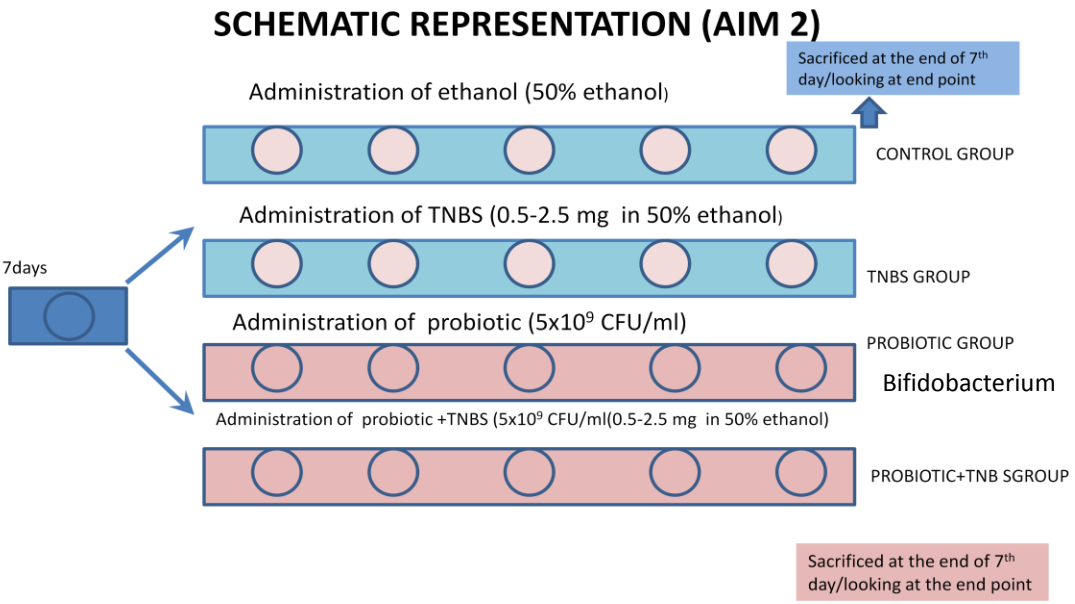
As the next step, we want to investigate the administration of probiotics in intestinal immune inflammation as well as to modulate Hopx expression in intestinal B regulatory cells. Administration of probiotic bacteria being a new therapeutic approach for inflammatory bowel disease in proceeding studies we would like to modulate the Hopx expression in intestinal B regs by the administration of probiotic- Bifidobacterium.

The datas obtained from flowcytometry (Fig 25), immunohistochemistry (Fig24) and western blot (Fig28) revealed that the Hopx expression was modulated by the administration of probiotics-Bifidobacterium. We found significant difference between the probiotics group and control group. And, probiotic+TNBS group demonstrated significant reduction in the inflammation compare to the classic TNBS model and increased expression of Hopx expressing B regs compare to the colitis model.

Together, the results indicate that Hopx gene expression is reduced /suppressed under intestinal inflammatory conditions. In other words, Hopx plays an important role to prevent intestinal inflammation.

Finally, we used the blocking peptide to neutralize the Hopx expression. We performed same experimental models and followed same experimental procedures to demonstrate the significance of Hopx gene in regulating intestinal inflammation. Hopx depleted group demonstrated very severe inflammation compare to the classic TNBS model with significant reduction in the regulatory B cells.

Aim 2: To investigate the administration of probiotic in intestinal immune inflammation as well as modulates Hopx expression in intestinal B regs.



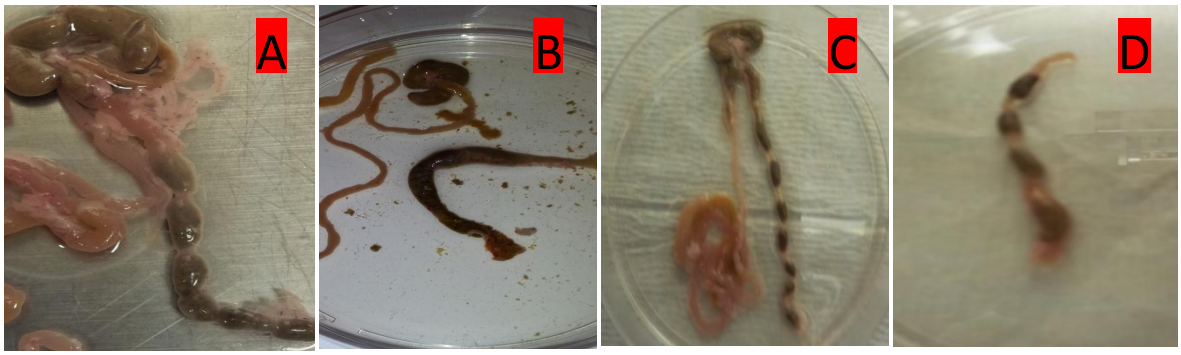


Figure 19: Macroscopic examination of different group intestines.

For induction of colitis, TNBS in 50% ethanol (0.5-2.5 mg of TNBS in 50% ethanol) was administered to the experimental group. Control group treated with 50% ethanol. Probiotic group (Bifidobacterium) was treated with $5x10^9$ CFU/ml and probiotic + TNBS group was treated with $5x10^9$ CFU/ml (0.5-2.5 mg in 50% ethanol). (A) Control mice intestine (B) TNBS mice intestine (C) Probiotic mice intestine (D) Probiotic+TNBS mice colon.

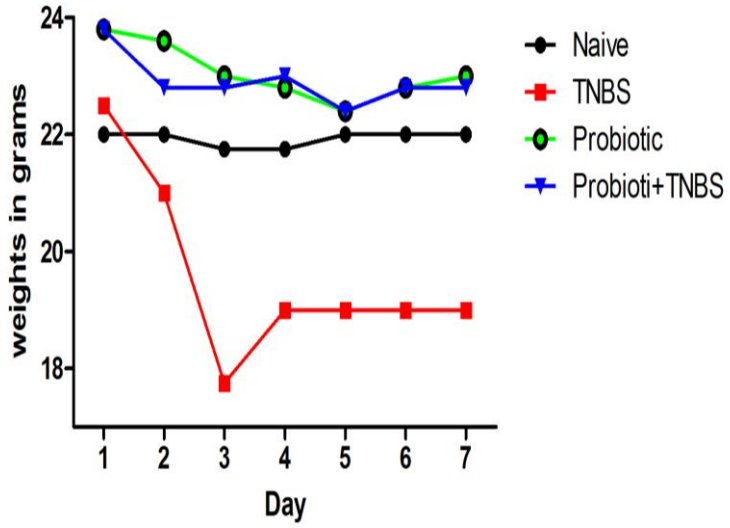


Figure 20: Mice weight chart.

Weight loss in different groups mice models as indicated. TNBS mice and control mice were injected on day 1 and sacrificed looking at the end point. Probiotic mice and Probiotic +TNBS mice were gavage for seven days and sacrificed on day seven (n=5 mice per group).

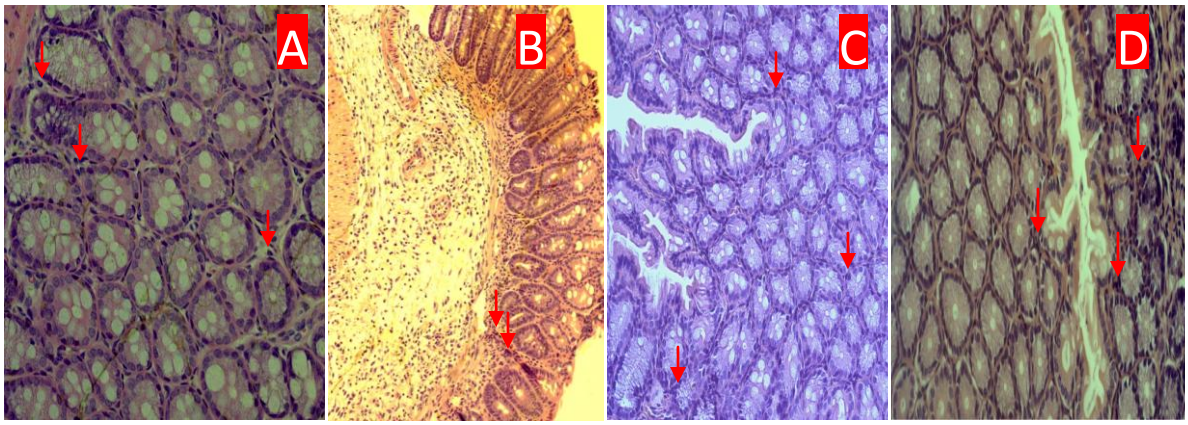


Figure 21: Representative H &E staining showing histology.

The presence of lymphocyte infiltration, elongation and distortion of crypts and many infected cells evidence the intestinal inflammation in test group (TNBS) and not in control group/probiotic group. Probiotic +TNBS treated group shows lesser inflammatory cell compare to TNBS treated. (A) Control colon (B) TNBS colon (C) Probiotic colon (D) Probiotic+TNBS colon. Magnification of image A, D=200X and B,C= 100X. Arrows pointing mononuclear cells.

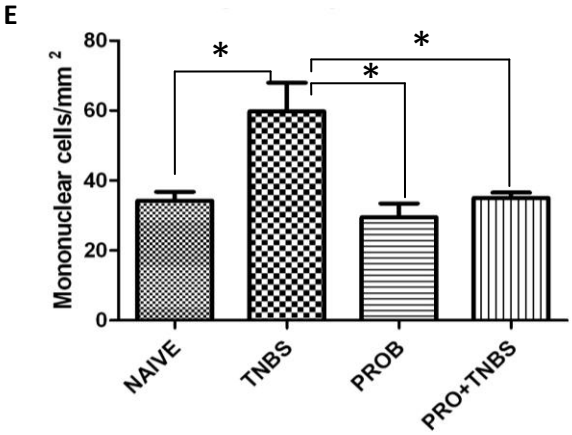


Figure 22: Mononuclear cells count.

Number of cells counted per mm^2 slides from mouse intestinal tissue stained with H&E stain (E). Mononuclear cells were significantly higher in TNBS colitis compare to other groups- control group, probiotic (PROB), and probiotic+TNBS group. Mean ± SEM (n=5-6 mice per group). Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

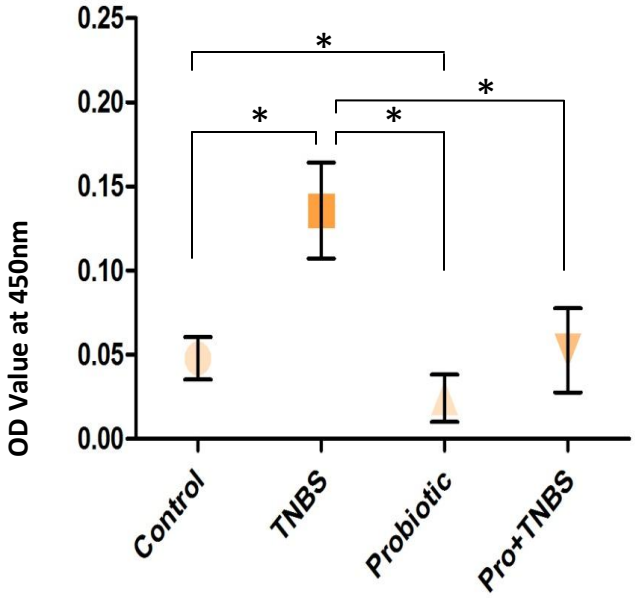


Figure 23: Comparison of MPO activity read at 450nm.

Mean ± SEM (n=5-6 sample per group). MPO was significantly higher in TNBS colitis compare to other groups- control group, probiotic (PROB), and probiotic+TNBS group. Probiotic group (Bifidobacterium) had lesser MPO activity compare to other groups including control group. Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

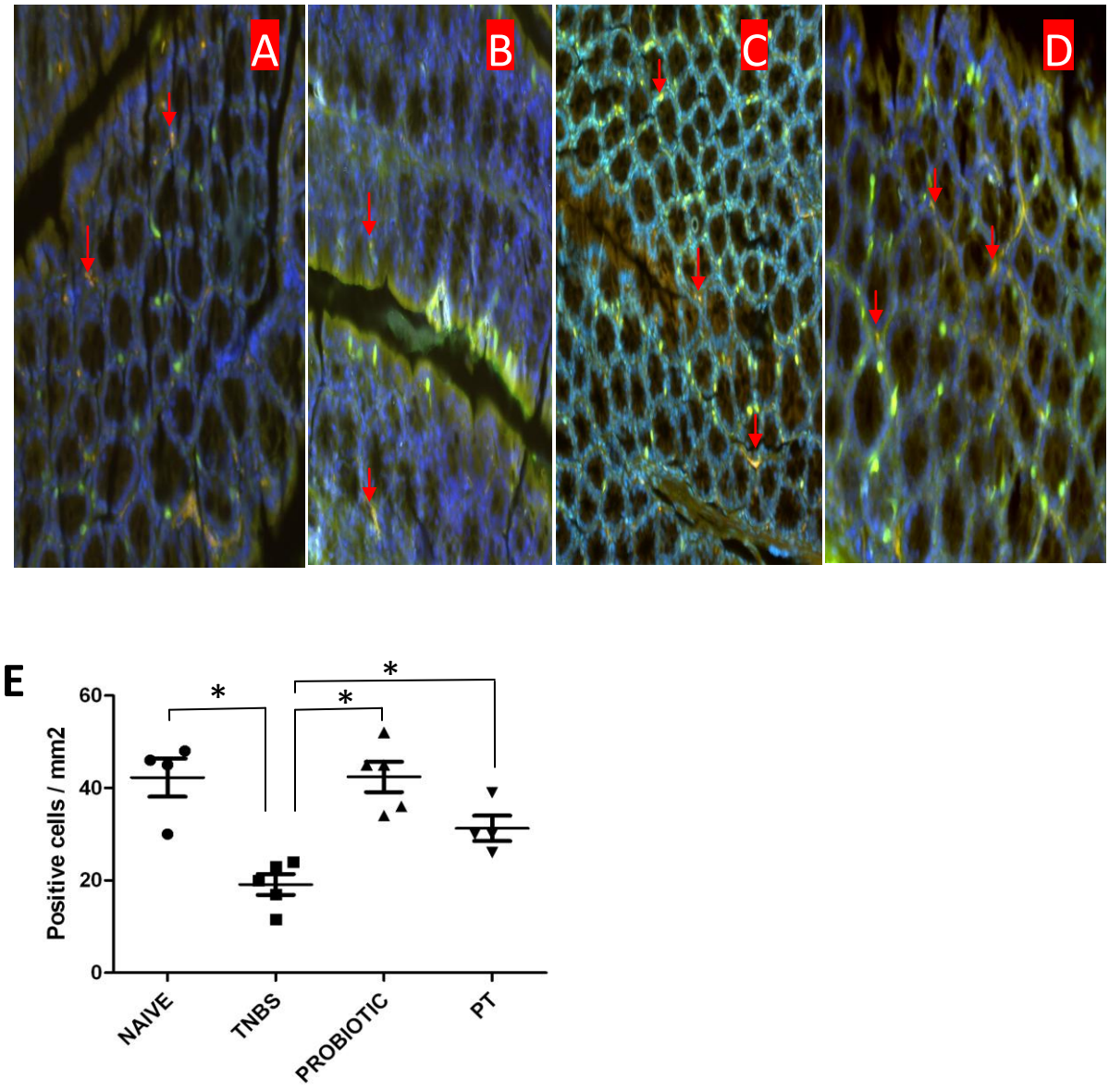


Figure 24: Immunohistochemistry staining and positive cells counting results.

Section of colon was stained using Hopx antibody (FITC), Breg antibody CD19 (PE) and nuclear staining with DAPI (blue). (A) Control (B) TNBS (C) Probiotic and (D) Probiotic + TNBS. E) Significantly reduced Hopx+ Breg+ cell populations in TNBS group compare to other groups like control group, probiotic, and probiotic+TNBS group. (Mean ± SEM (n=5sample per group). The original magnification of the representative images was 200X. Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

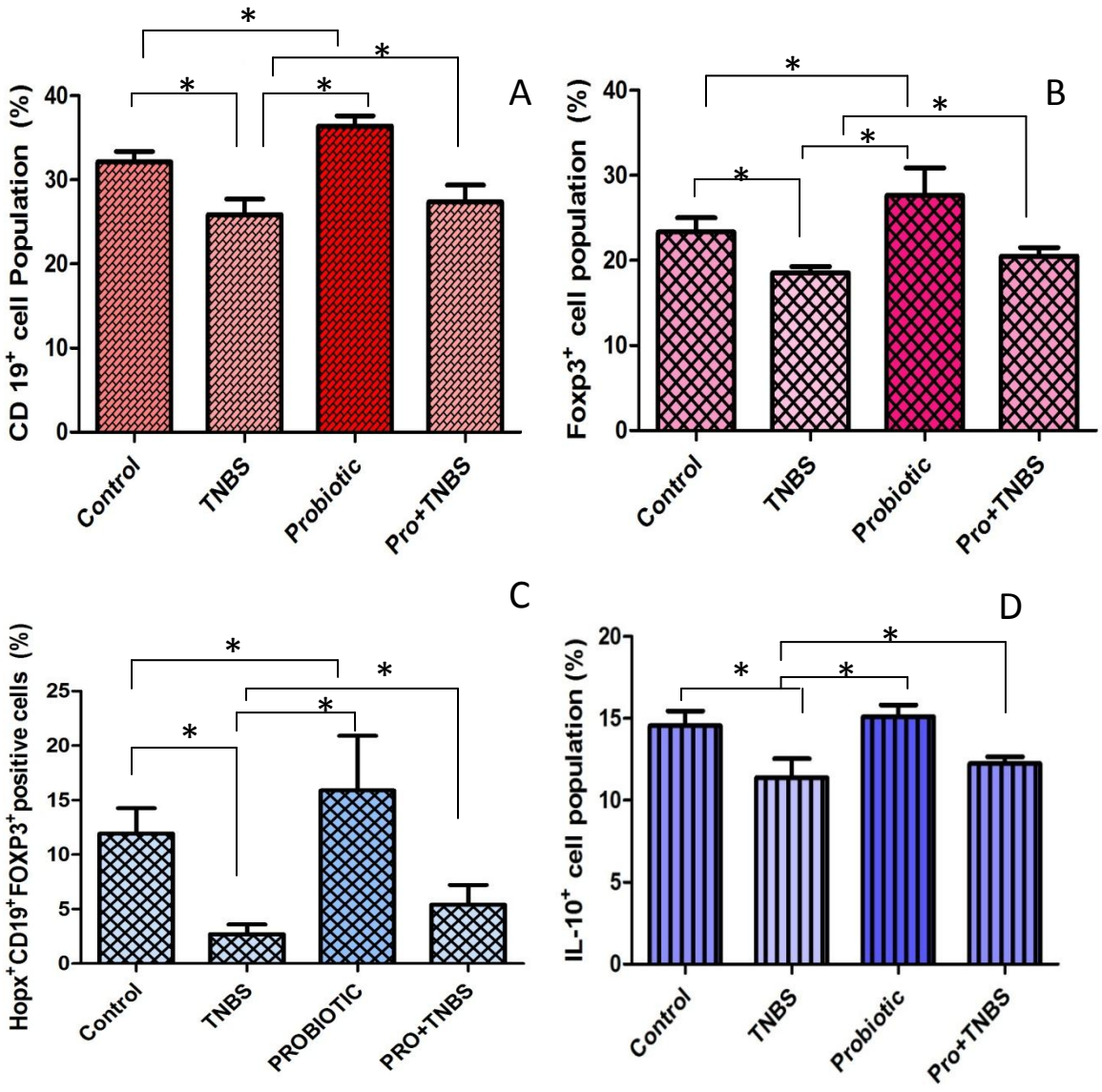


Figure 25: Flowcytometry

Flow cytometric profiles of intestinal LPMC cells from control, TNBS, Probiotic, Probiotic(Pro) +TNBS mice stained as indicated. (A) CD19+ cell population (%), (B) FOXP3+ cell population (C) Hopx+ CD19+FOXP3+ cell population (%) and (D) IL-10+ cell population (%). The frequency of CD19+, FOXP3+, Hopx+ CD19+FOXP3+ cell population was significantly reduced in experimental-TNBS model compare to other groups like control, probiotic and probiotic +TNBS group. Mean ± SEM (n=5sample per group). Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

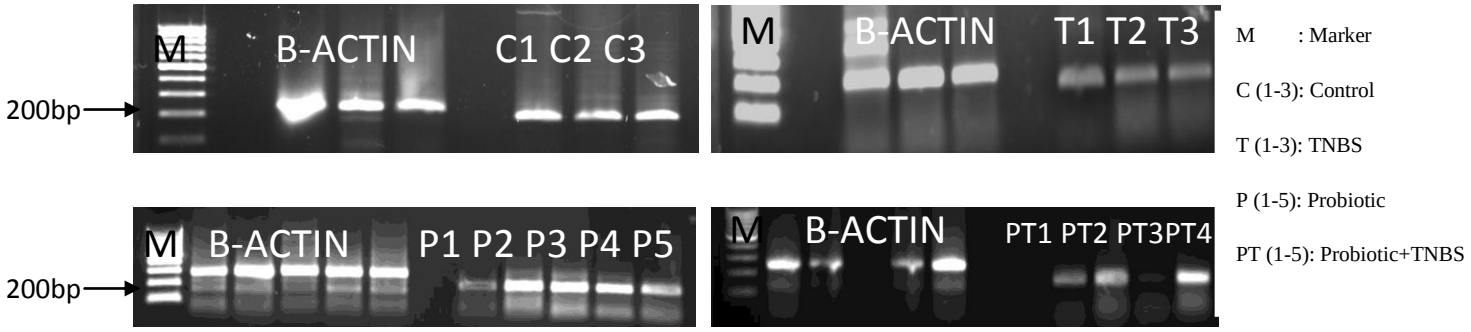


Figure 26: Agarose gel electrophoresis of RT-PCR products.

β- Actin was used as loading control. Bright bands were observed in probiotic group and control group compare to TNBS group (n=3-5 mice per group).

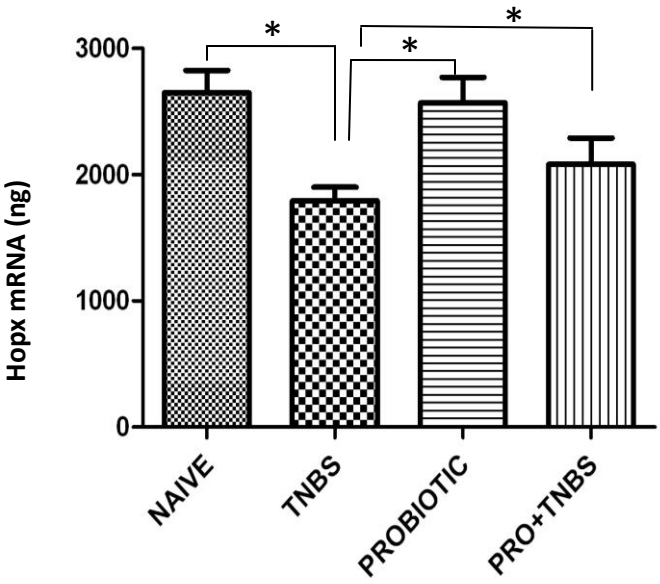


Figure 27: Real Time PCR

Real Time PCR analysis of RNA extracts from intestinal tissue of probiotic, control, TNBS and Probiotic (PRO)+ TNBS mice. Mean ± SEM (n=5sample per group). Expression of Hopx was significantly reduced in experimental group-TNBS, compare to control group, probiotic and probiotic+TNBS group. Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

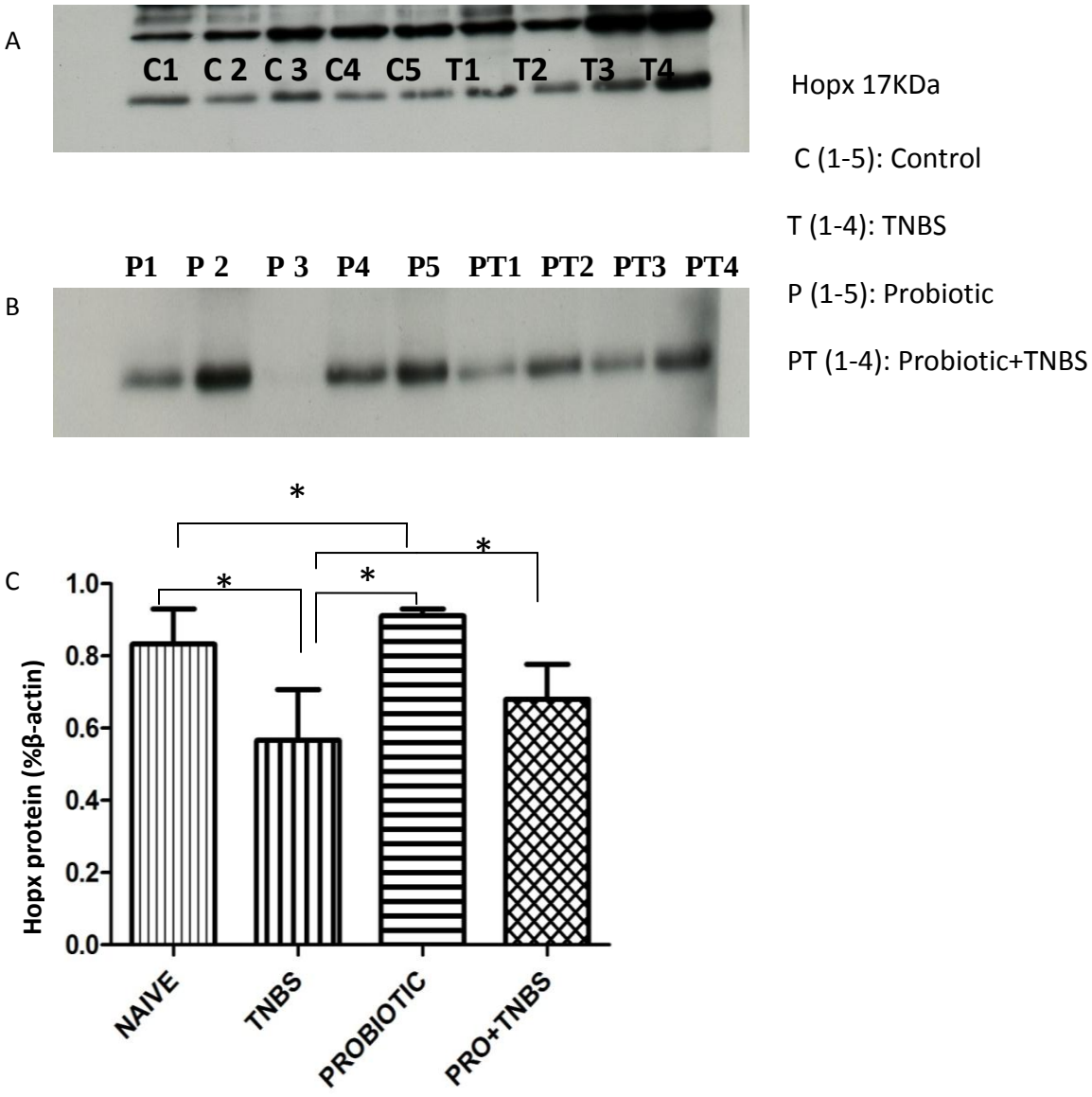
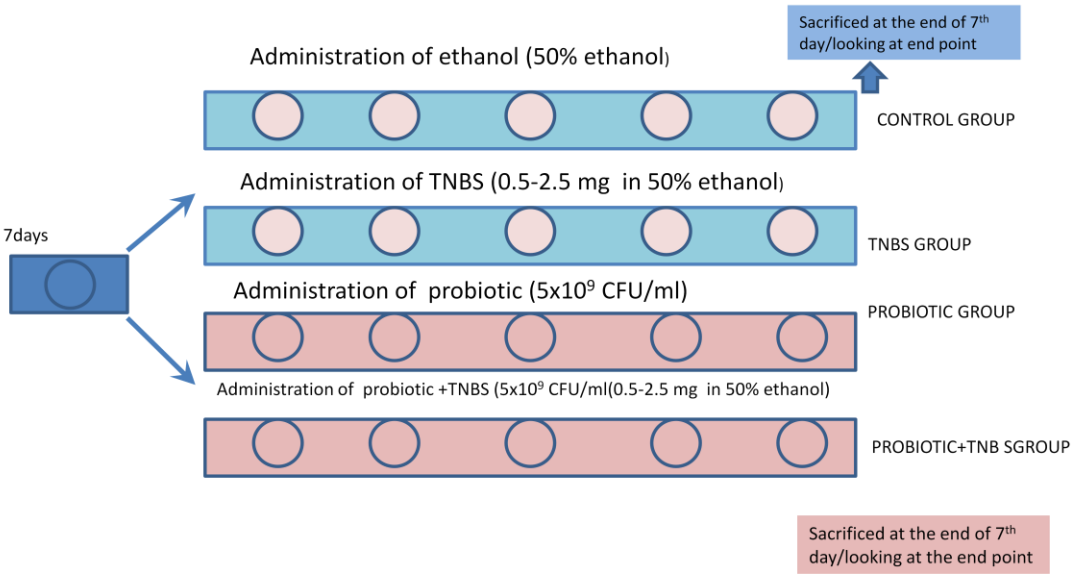


Figure 28: Western blot Analysis

Western blot analyses of Proteins extracted and purified from control and TNBS intestinal tissue. Hopx expression was significantly reduced in protein extracts from TNBS colitis mice compare to probiotic mice. Mean ± SEM (n=5sample per group). Expression of Hopx was significantly reduced in experimental group-TNBS, compare to control group, probiotic and probiotic+TNBS group. The frequency of Hopx protein expression was higher in probiotic compare to control group. Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

- Aim 3: To neutralize Hopx expression in order to prove that Hopx expressing B regs plays a key role in maintaining mucosal immunity.

Injection of Hopx Neutralizing antibody



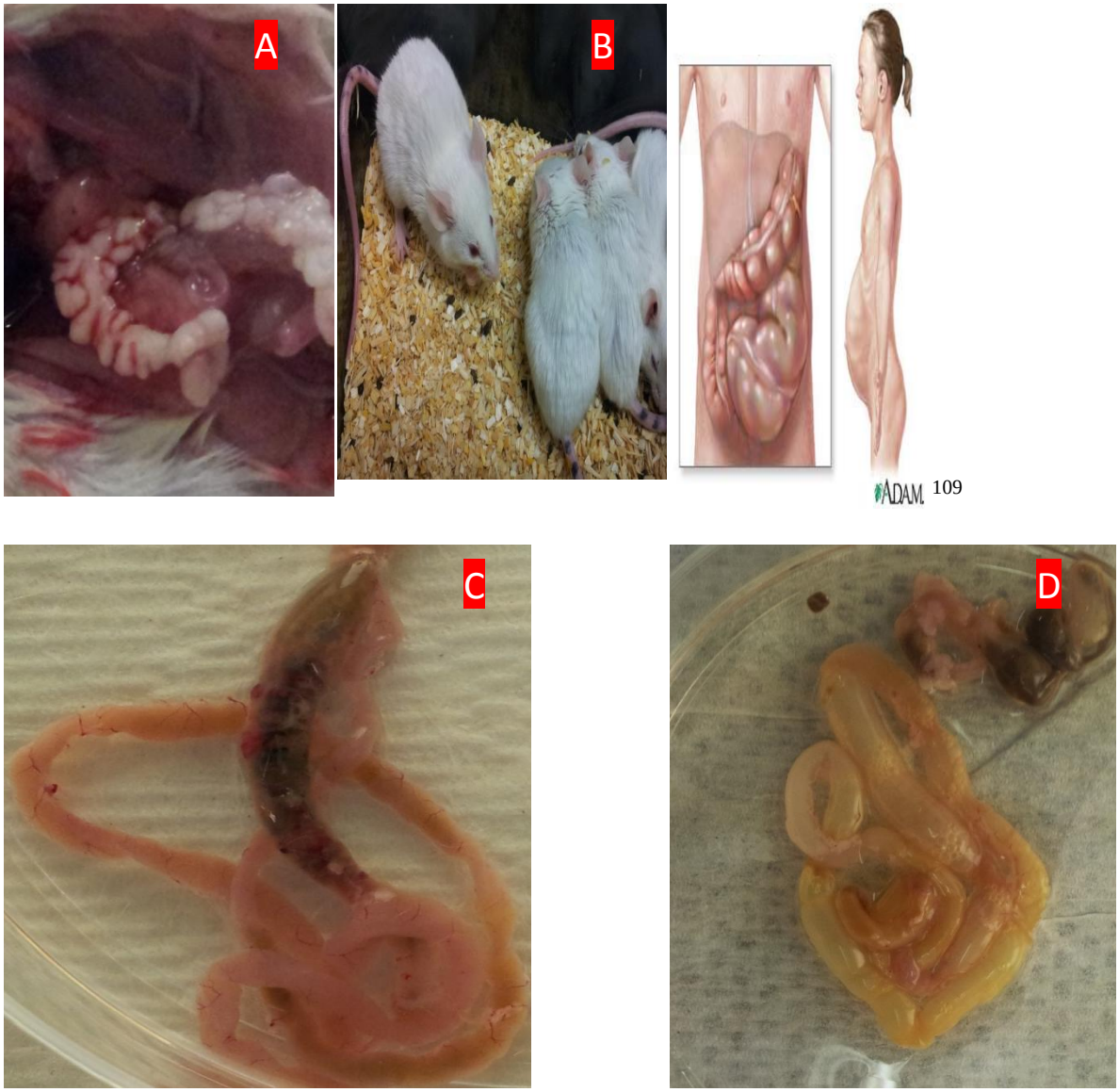


Figure 29: Representative macroscopic pictures of the Hopx blocked groups.

(A)Fat accumulation (B) Bloating in murine model similar to human (C) inflamed colon (D) Bloating of the intestine.

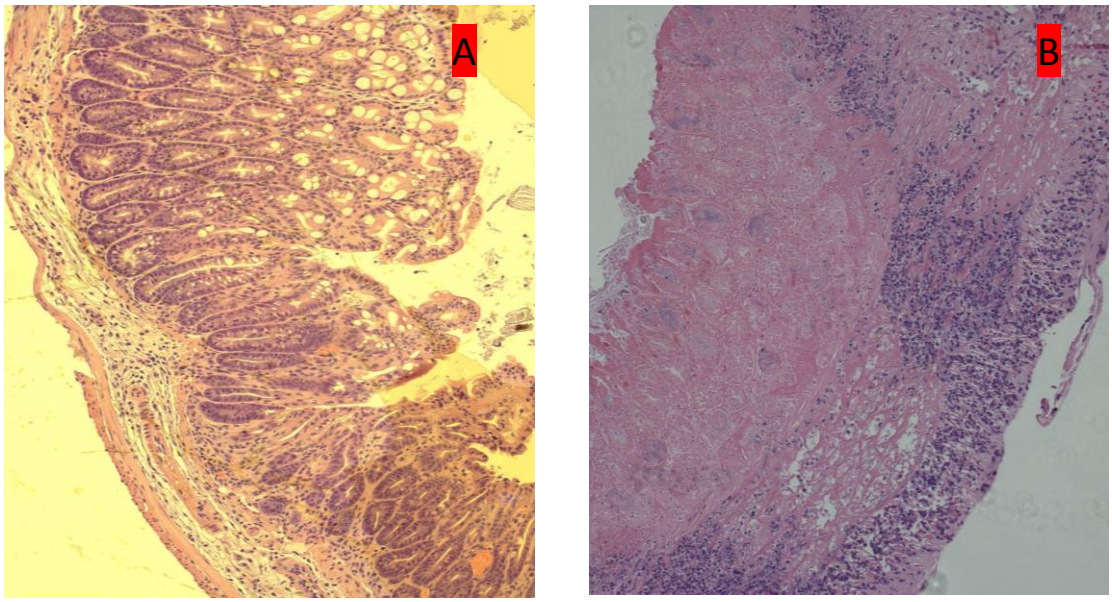


Figure 30: Representative H &E staining showing histology.

(A)Classic TNS model (B) TNBS induced colitis in Hopx depleted group. Very severe inflammation in Hopx-/+ inflamed colon compare to classic TNBS colitis colon. Magnification=100X.

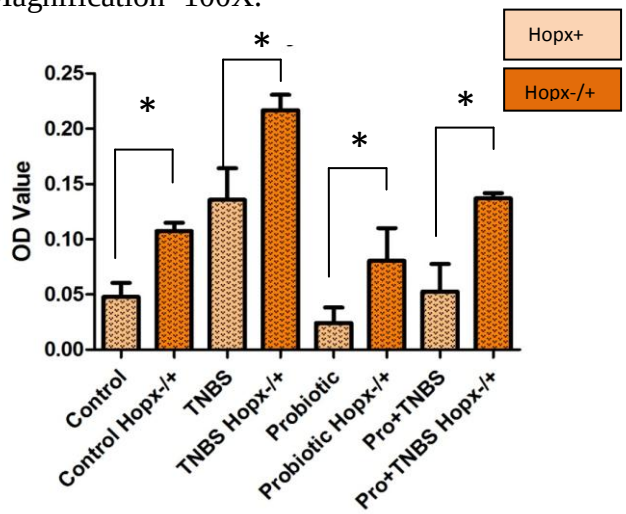


Figure 31: Comparison of MPO activity read at 450nm.

Mean ± SEM (n=5-6 mice per group). Comparison between different murine models. Hopx depleted group expressing high frequency of MPO activity. Mean ± SEM (n=5sample per group). Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

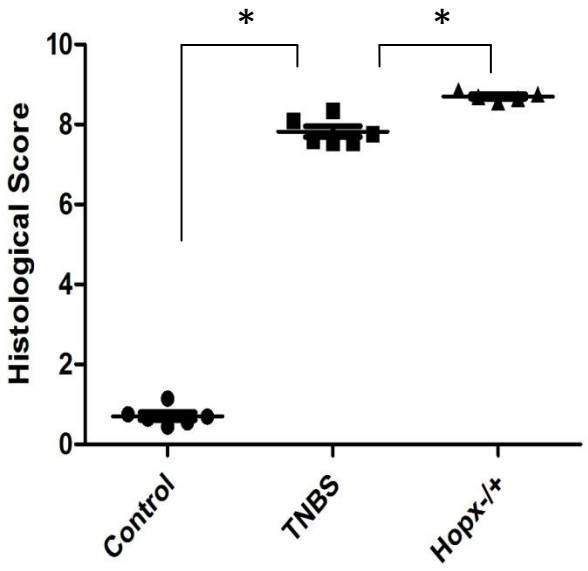


Figure 32: Representative H &E staining showing histology.

Inflammatory scoring for mouse intestinal tissue stained with H&E stain. Inflammatory score was significantly higher in TNBS colitis compare to control group. Hopx-/+ group showing significantly high inflammatory score than the classic TNBS model. Mean ± SEM (n=5-6 sample per group). Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

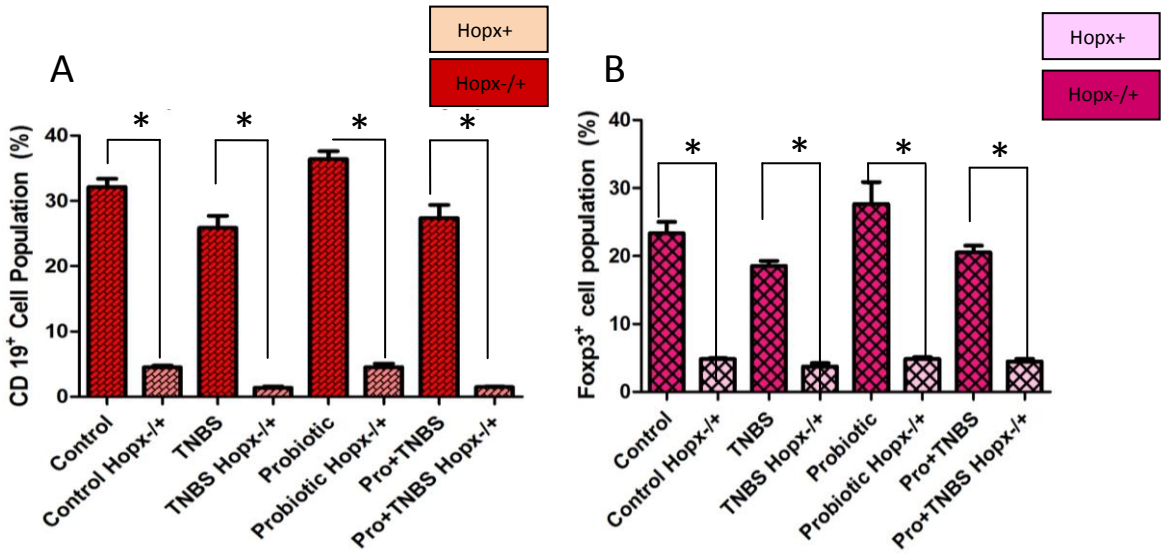


Figure 33: The gut regulatory immune cells (Treg and Breg) are altered in Hopx -/+ mice group.

LPMC cells isolated from respective group intestine are stained with respective markers. (A) The percentage of CD19+ cells population and (B) The percentage of Foxp3+ cells population as determined by Flowcytometry. Mean ± SEM (n=5sample per group). One-way ANOVA test was used for all statistical analyses *P < 0.001.

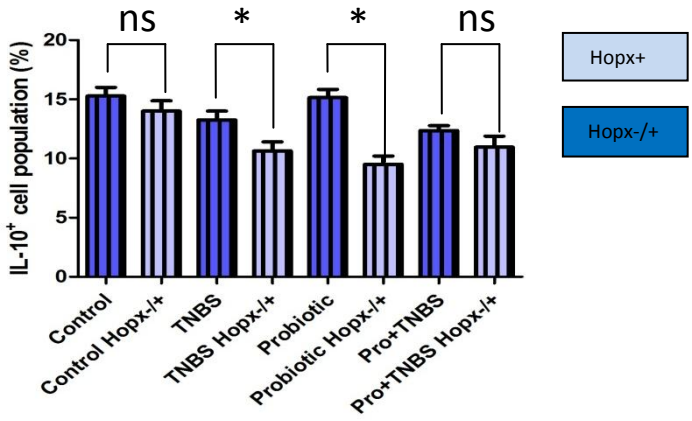


Figure 34: The production of anti- inflammatory cytokine IL-10 suppressed in Hopx depleted group.

LPMC cells isolated from respective group intestine are stained with respective marker. (A) The percentage of IL-10+ cells population as determined by Flowcytometry. Not significant is indicated by 'ns'. Mean ± SEM (n=5sample per group). Mean ± SEM (n=5sample per group). Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

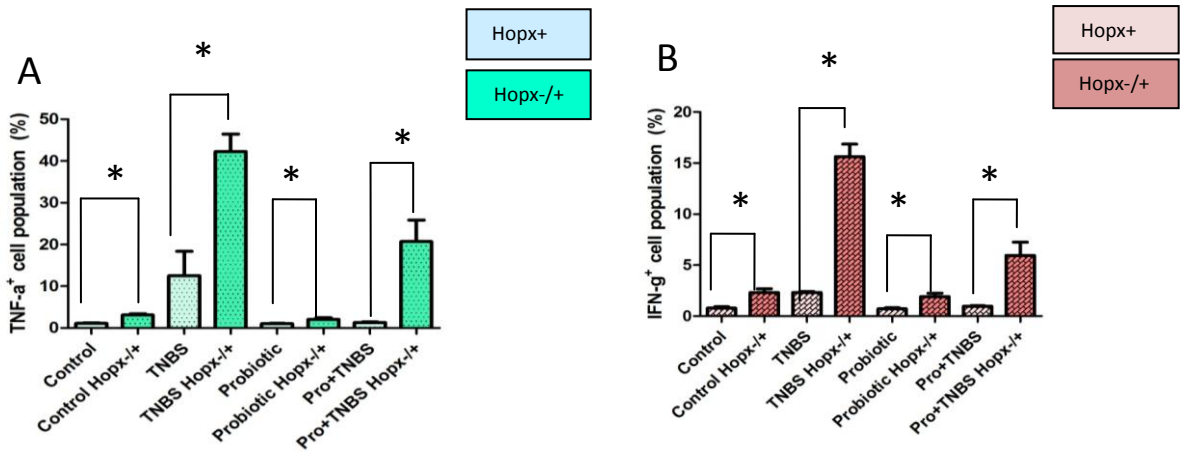


Figure 35: Flowcytometry Analysis

Increased production of proinflammatory cytokines TNF-a, IFN-g in Hop-/+. LPMC cells isolated from respective murine group intestine were stained with respective marker. (A) The percentage of TNF-a+ cells population and,(B) The percentage of IFN-g+ cells population as determined by Flowcytometry. Not significant is indicated by 'ns' Mean ± SEM (n=5sample per group). Two-tailed unpaired student's *t* test was used for all statistical analyses *P < 0.001.

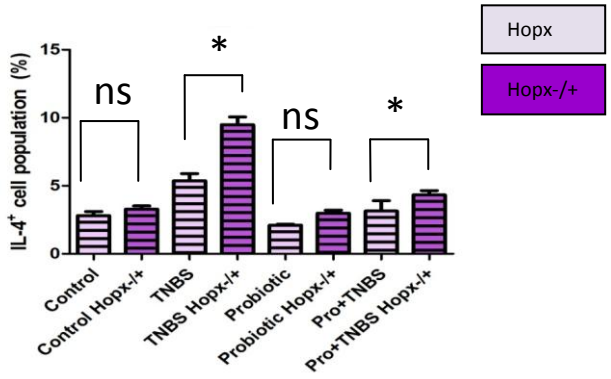


Figure 36: Flowcytometry

Increased production of inflammatory cytokine IL-4 in Hop-/+. LPMC cells isolated from respective murine group intestine were stained with respective marker. The percentage of IL-4+ cells population as determined by Flowcytometry. Not significant is indicated by 'ns'. Mean ± SEM (n=5sample per group). One-way ANOVA test was used for all statistical analyses *P < 0.001.

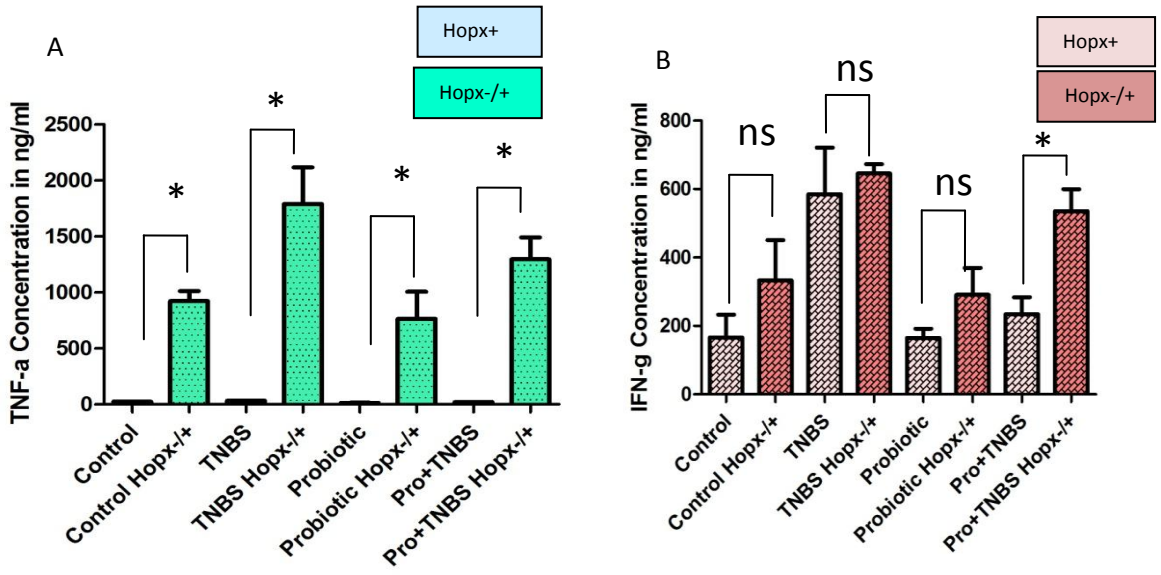


Figure 38. Real Time PCR Analysis

Increased production of proinflammatory cytokines TNF-a, IFN-g in Hop-/+. RNA extracted from respective group murine intestine expressing TH1 response. (A)The concentration of TNF-a, (B)The concentration of IFN-g as determined by Real- time PCR. 'ns'-Not significant. Mean ± SEM (n=5sample per group). One-way ANOVA test was used for all statistical analyses *P < 0.001.

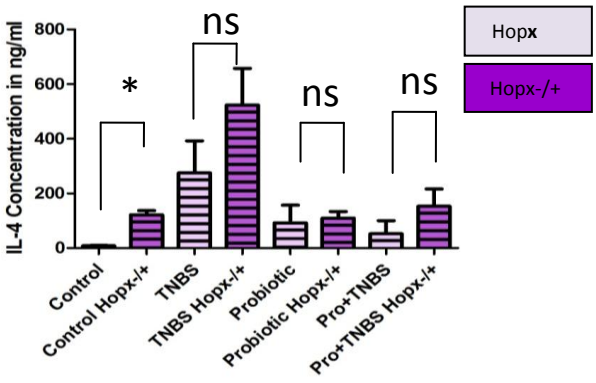


Figure 39. Real-Time PCR Analysis

Increased production of inflammatory cytokine IL-4 in Hop-/+. RNA extracted from respective murine group intestine expressing TH2 response. The concentrations of IL-4 cytokine as determined by Real-time PCR. 'ns'-Not significant. Mean ± SEM (n=5sample per group). One-way ANOVA test was used for all statistical analyses *P < 0.001.

CHAPTER 5: DISCUSSION

Intestinal inflammation is often caused by altered intestinal permeability which results in inflammatory disease like intestinal bowel disease-CD and UC[22-24]. There are several factors contribute to the development of IBD such as microbes, genetic, immunologic and environmental[22-32]. This study proved the involvement of Hopx in intestinal inflammation. Expression of Hopx is remarkably reduced under inflammatory conditions. Hopx expressing regulatory B cell suppress intestinal inflammation and thereby, regulate intestinal homeostasis. Reduced Hopx expression leads to the increased production of proinflammatory cytokines and facilitate the initiation of intestinal inflammation. Hopx expression is ubiquitous in normal tissues. Understanding the role of Hopx in intestinal inflammation is important to elucidate a novel anti-inflammatory therapeutics. This current study suggests that the significant reduction in Hopx leads to the initiation of intestinal inflammation. Hopx plays a critical role in inhibiting intestinal inflammation and maintaining intestinal homeostasis.

5.1 Human IBD and murine colitis model

IBD- both UC and CD which affects the individual throughout the life depicts- chronic, relapsing and remitting inflammatory condition characterized by altered intestinal immune function mediated by lymphocyte infiltration, diarrhea, weight loss, increased production of proinflammatory cytokines such as TNF-α, INF-γ, IL-2 and IL-17[22-41]. Characteristic features of IBD are well represented by the intrarectal administration of

TNBS. Colitis experimental models help us to understand the pathogenic components occur during different phases of colitis like-acute, recovery and chronic phases[110]. At the same time it also enables us to investigate novel genes intimately involved in disease susceptibility. Colitis is induced by the administration of hapten reagent TNBS in ethanol and this novel colitis experimental model invented by Neurath et al in 1995. Balb/C is susceptible strain and studies have proved that altered gene expression in human IBD closely reflected in colitis[110]. In our current study, TNBS-induce colitis murine model expressed severe inflammation with lymphocytes infiltration, mucosal surface destruction, and edema which was similar to IBD characteristic features. In addition, weight loss and MPO supported the severity of intestinal inflammation.

5.2 Hopx expression in human and murine model

Immunohistochemistry was preferentially used to study the pattern of Hopx expression in both human and inflamed colon. Both the normal human and murine colon expressed significantly higher Hopx expression compare to the inflamed colon section obtained from IBD and colitis model. Expression of Hopx mRNA as well as Hopx protein was reduced under inflammatory condition in the similar way. Thus our results indicate that the level of Hopx expression is reduced under inflammatory condition than in normal state which suggests that expression of Hopx is down-regulated in the intestinal inflammation.

5.3 Hopx expressing Breg in intestinal homeostasis

To elucidate how Hopx function under normal conditions is necessary to modulate the Hopx expression to maintain intestinal immune system. Previous study has shown that Hopx is required for the function of Treg[70]. Therefore, in this present study, we investigated the role of Hopx in B regs. Studies indicate that regulatory B cells are capable of inhibiting inflammation and induce immune tolerance like T cells. The regulatory B cell plays a key role in maintain intestinal immune system. B regs suppress the expression of proinflammatory cytokines by interacting with pathogenic T cell. B regs also maintains Foxp3 expression. Breg produce immune regulatory cytokine IL-10, which suppress the expression of proinflammatory cytokines-TH1, TH2 and TH17[73-81]. Regulatory B cells in the adaptive immune system helps to maintain intestinal homeostasis through the production of anti-inflammatory cytokine IL-10[77-81]. Indeed, in our study we found that Hopx expressing B regs were higher in normal colon tissue than in inflamed colon tissue. Hence indicating that Hopx is required for the function of B regs and in turn Hopx expressing B regs modulate intestinal inflammation and maintain intestinal homeostasis.

5.4 Probiotics administration to modulate Hopx expressing Breg cells expression

Gastrointestinal tract is densely colonized by > 70% of microbiota-most heavily populated organ compare to other organs exposed to external environment such as respiratory or genitourinary tract[101]. The balance between the microbiota has to be

maintained to avoid inflammatory condition. Gastrointestinal infections remain a major clinical problem due to increase incidence of antibiotic-resistant microbial pathogens[22-41]. Probiotics are an attractive alternative treatment of antibiotics since the chances of for developing bacterial resistance are rare as they provide protection against pathogens. At the same time they do not harm the normal bacterial flora[101]. As mentioned in he introduction, administration of probiotics have many beneficial properties including maintain the intestinal homeostasis. Studies have proved that probiotics helps to increase the expression of regulatory immune cells[101-106]. O' Mahony and colleagues demonstrated that Bifidobacterium infantis helps to promote regulatory T cells and thereby preventing pathogen induced inflammation[105]. In a recent study, researchers proved that probiotics-Bifidobacterium lactis have immunomodulatory effects and which helps to improve regulatory T cells [101-106]. One common thing often observed in intestinal inflammatory conditions is expression of low level immune regulatory cells. In this study we want to use probiotic as a tool to modulate Hopx expressing B regs, in order to prevent intestinal inflammation. As expected we found that mice treated with probiotics in TNBS remarkably reduced inflammation compare to the TNBS colitis model. Interestingly probiotics administration increased the expression of Hopx expressing Breg cell expression compare to the control group but, we could not find the significant result in real-time PCR. From this study we found that Hopx expressing Breg perform a significant role in controlling intestinal inflammation.

5.5 Importance of Hopx in intestinal Homeostasis

Like mentioned, Hopx is expressed in variety of human healthy tissue and its expression is affected under inflammatory condition[50-56]. This is the first experimental model to directly correlate the role of Hopx in TNBS induced colitis model.

We used the neutralizing antibody to neutralize Hopx expression. Mice deficient of Hopx developed severe inflammation compare to classic TNBS induced colitis model. Hopx deficient mice strengthen the production of proinflammatory cytokines including TNF-α, IFN-γ and IL-4. And there was a reduction in the production of anti-inflammatory cytokine IL-10. Interestingly, IL-10 production was affected in Hopx deficient probiotic group compare to the probiotic group. Which suggest that Hopx is required for the production of anti-inflammatory cytokines. Increased proinflammatory cytokine in Hopx deficient group suggest that Hopx plays a significant role in controlling intestinal inflammation. Together these studies indicate that Hopx plays a key role in controlling intestinal inflammation and it control the production of anti-inflammatory cytokine. In addition it strengthens the production of anti-inflammatory cytokine like IL-10.

Together our findings shed new light on the beneficial effects of Hopx gene expression in regulating intestinal inflammation. For the first time, this study demonstrates the novel feature of Hopx gene in suppressing colitis in murine model.

CHAPTER 6: Summary and Future Direction

Hopx expression in inflamed and non-inflamed human colon was studied. Hopx expression was reduced in inflamed colon compare to the non-inflamed colon. Widely accepted TNBS induced colitis was used as experimental model. Study on murine models revealed that Hopx expression is suppressed under inflammatory condition. Hopx expressing Breg cells were reduced under intestinal inflammation, suggesting Hopx expressing Breg cell plays a critical role in regulating intestinal inflammation. Hopx expression was modulated by the administration of probiotics. Hopx depletion resulted in severe inflammation. Hopx gene suppression leads to the increased expression of proinflammatory cytokines like TNF-α, IFN-γ, IL-4 and suppressed immune regulatory cells like Treg and Breg. The production of anti-inflammatory cytokine IL-10 was affected by Hopx depletion.

Our study clearly demonstrates that the Hopx expression is reduced during intestinal inflammation. But the mechanism involved in the reduction of Hopx should be investigated in future studies. This study implicates the involvement of proinflammatory cytokines in suppressing Hopx gene under inflammatory conditions. The role of proinflammatory cytokines in modulating Hopx expression remains unclear. Hopx knockout murine model can be considered to study the involvement of Hopx in colitis. Hopx expressing cell lines can be used to treat intestinal inflammation. Probiotic strains expressing Hopx gene will be a challenging therapeutic way to prevent intestinal inflammation.

REFERENCES

1. Ouwehand A., Isolauri E., Salminen S., The role of the intestinal microflora for the development of the immune e system in early childhood. European Journal of Nutrition. 2002:1/32-1/37.
2. Neutra MR, Pringault E, Kraehenbuhl J-P. Antigen sampling across epithelial barriers and induction of mucosal immune responses. Annu. Rev. Immunol. 1996.14:275–300.
3. Brandtzaeg P. Mucosal immunity: induction, dissemination, and effector functions. Scand. J. Immunol. 2009. 70:505–15.
4. Sansonetti, P.J. Host-bacteria homeostasis in the healthy and inflamed gut. Curr. Opin. Gastroenterol. 2008.24:435–439
5. Ouwehand A., Isolauri E., Salminen S., The role of the intestinal microflora for the development of the immune e system in early childhood. European Journal of Nutrition. 2002:1/32-1/37.
6. Inna sekirov, Shannov L.Russel, Brett Finlay.: Gut Microbiota in Health and Disease. Physiol Rev 2010,90:859-904.
7. Saurabh Mehandru, MD The Gastrointestinal Tract in HIV-1 Infection: Questions, Answers, and More Questions. Physiscians Research Network, Inc.December 2007.
8. Guy-Grand D, Vassalli P. Gut intraepithelial T lymphocytes. Curr Opin Immunol. Apr 1993;5(2):247-252.
9. SchirbelA., Fiocchi C., Targeting the innate immune system in pediatric inflammatory bowel disease. Expert Review of Gastroenterology and Hepatology. February 2011, 1, 33-41.

10. David Artis, Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut Nature Reviews Immunology 8, 411-420 .June 2008 doi:10.1038/nri2316.
11. Trine H. Mogensen, Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. Clinical microbiology reviews, Apr. 2009, p. 240–273.
12. O'Neill, L. A., and A. G. Bowie. The family of five: TIR-domain containing adaptors in Toll-like receptor signalling. Nat. Rev. Immunol. 2007.7:353–364.
13. Jason D. Marshall., Darren S., Negative regulation of TLR9-mediated IFN-_ induction by a small-molecule, synthetic TLR7 ligand. Journal of Leukocyte Biology. Sep 2007, 497-508.
14. Janine L Coombes., Fiona Powrie., Dendritic cells in intestinal immune regulation. Nature Reviews Immunology, June 2008, 435-446.
15. Kenneth Todar et al., Immune Defense against Microbial Pathogens. Text book of Bacteriology. 2005.
16. Harty JT, Badovinac VP. Shaping and reshaping CD8+T-cell memory. Nat Rev Immunol 2008;8:107–119.
17. Bassing CH, Swat W, Alt FW. The mechanism and regulation of chromosomal V(D)J recombination. Cell. 2002; 109 (Suppl):S45–55.
18. SunJc, et al.,Adaptive immune features of natural killer cells.Nature.2009 Jan 29: 457(7229):557-61
19. Thompson CB. New insights into V(D)J recombination and its role in the evolution of the immune system. Immunity 1995;3:531–539.

20. Janeway CA Jr, Medzhitov R. Innate immune recognition. Annu Rev Immunol. 2002; 20

21. SunJc, et al.,Adaptive immune features of natural killer cells.Nature.2009 Jan 29: 457(7229):557-61:197–216.

22. Torsten Kucharzik, Christian Maaser, Andreas Lu¨gering, Martin Kagnoff,0 Lloyd Mayer,1 Stephan Targan,z and Wolfram DomschkeRecent Understanding of IBD Pathogenesis: Implications for Future Therapies. Inflamm Bowel Dis & Volume 12, Number 11, November 2006

23. Baumgart DC., Sandborn., WJ .Inflammatory bowel disease. Clinical aspects and established and evolving therapies. May 2007, 369, 1641–57.

24. M. Gersemann, J.Wehkamp&E. F.Stange1 Innate immune dysfunction in inflammatory bowel disease 2012 The Association for the Publication of the Journal of InternalMedicine. doi: 10.1111/j.1365-2796.2012.02515.x

25. William A Rowe., Inflammatory Bowel Disease. Gastroenterology, 2004.

26. Hampe J, Grebe J, Nikolaus S, et al. Association of NOD2 (CARD 15) genotype with clinical course of Crohn_s disease: a cohort study. Lancet. 2002; 359: 1661Y1665.

27. Lesage S, Zouali H, Cezard J, et al. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. Am J Hum Genet. 2002;70:845Y857

28. Panwala C, Jones J, Viney JL. A novel model of inflammatory bowel disease: mice deficient for the multiple drug resistance gene, mdr1a,spontaneously develop colitis. J Immunol. 1998;161:5733Y5744.

29. Micha³ Dudarewicz1, Ma³gorzata Barañska1, Mariola Rychlik-Sych1, Radzis³aw Trzciñski2, Adam Dziki2, Jadwiga Skrêtkowicz1C3435T Polymorphism of the ABCB1/MDR1 gene encoding P-glycoprotein in patients with inflammatory bowel disease in a Polish population Pharmacological Reports 2012, 64, 343.350 ISSN 1734-1140.
30. Peltekova V, Wintle R, Rubin L, et al. Functional variants of OCTN cation transporter genes are associated with Crohn disease. Nat Genet. 2004;36:471Y475.
31. Noble CL, Nimmo ER, Drummond H, Ho GT, Tenesa A, Smith L, Anderson N, Arnott ID, Satsangi J. The Contribution of OCTN1/2 Variants Within the IBD5 Locus to Disease Susceptibility and Severity in Crohn's Disease Source Gastrointestinal Unit, Western General Hospital, University of Edinburgh, Edinburgh, Scotland. Gastroenterology Volume 129, Issue 6 , Pages 1854-1864, December 2005.
32. Stoll M, Corneliussen B, Costello C, et al. Genetic variation in DLG5 is associated with inflammatory bowel disease. Nat Genet. 2004;36: 476Y480.
33. Paul Henderson, Johan E. van Limbergen, Ju¨rgen Schwarze, and David C. Wilson. Function of the Intestinal Epithelium and Its Dysregulation in Inflammatory Bowel DiseaseInflamm Bowel Dis 2011;17:382–395
34. Saad Y. Salim, PhD, and Johan D. Soderholm, MD, PhD Importance of Disrupted Intestinal Barrier in Inflammatory Bowel Diseases Inflamm Bowel Dis 2011;17:362–381.
35. Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. Nat Rev Immunol. 2003;3:521Y533.

36. Michael A. McGuckin, PhD, Rajaraman Eri, PhD, Lisa A. Simms, BSc, Timothy H.J. Florin, MD, and Graham Radford-Smith, DPhil. Intestinal Barrier Dysfunction in Inflammatory Bowel Diseases. Inflamm Bowel Dis 2009;15:100 –113

37. Monica Porras, Maria Teresa Martin, Ping-Chang Yang, Jennifer Jury, Mary H. Perdue, and Patri Vergara. Correlation Between Cyclical Epithelial Barrier Dysfunction and Bacterial Translocation in the Relapses of Intestinal Inflammation Inflamm Bowel Dis & Volume 12, Number 9, September 2006

38. Sartor RB. Microbial influences in inflammatory bowel diseases. Gastroenterology2008;134:577–94.

39. Chassaing B, Darfeuille-Michaud A. The commensalmicrobiota and enteropathogens in the pathogenesis of inflammatory bowel diseases.Gastroenterology2011;140:1720–8.

40. Madsen K, Malfair D, Gray D, et al. Interleukin-10 gene-deficient mice develop a primary intestinal permeability defect in response to enteric microflora. Inflamm Bowel Dis. 1999;5:262Y270.

41. D'Haens G, Geboes K, Peeters M, et al. Early lesions of recurrent Crohn's disease caused by infusion of intestinal contents in excluded ileum. Gastroenterology. 1998;114:262Y267.

42. Baumgart DC., Sandborn., WJ .Inflammatory bowel disease. Clinical aspects and established and evolving therapies. May 2007, 369, 1641–57.

43. Loftus EV., Sandborn WJ., Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences. Gastroenterology. May 2004, 1504-1517.

44. Niess JH.,Role of mucosal dendritic cells in inflammatory bowel disease. World J Gastroenterol. Sep 2008, 14(33): 5138-48.

45. Leslie M Higuchi., Athos Bousvaros., Epidemiology and environmental factors in inflammatory bowel disease in children and adolescents October 2010.

46. Natalie A., Molodecky., Increasing Incidence and Prevalence of the Inflammatory Bowel Diseases With Time, Based on Systematic Review. Gastroenterology 2012;142:46–54.

47. Cosnes J., Gower-Rousseauc., Epidemiology and natural history of inflammatory bowel diseases. Gastroenterology. May 2011, 1785-1794.

48. Leslie M Higuchi., Athos Bousvaros., Epidemiology and environmental factors in inflammatory bowel disease in children and adolescents October 2010.

49. Mortaz E., Lazar Z., Cigarette smoke attenuates the production of cytokines by human plasmacytoid dendritic cells and enhances the release of IL-8 in response to TLR-9 stimulation. Respiratory Research. June 2009, 10-47.

50. Tri Chen F, Kook H, Milewski R, Gitler AD, Lu MM, Li J, Nazarian R, Schnepp R, Jen K, Biben C, Runke G, Mackay JP, et al. Hop is an unusual homeobox gene that modulates cardiac development. Cell 2002;110:713–23.

51. Chen F., Kook H., Milewski R., Hop is an unusual homeobox gene that modulates cardiac development. Cell 2002;110:725–35.

52. Shin CH, Liu ZP, Passier R, Zhang CL, Wang DZ, Harris TM, Yamagishi H, Richardson JA, Childs G, Olson EN. Modulation of cardiac growth and development by HOP, an unusual homeodomain protein. Cell 2002;110:725–35.

53. Kook H., Epstein JA., Hopping to the beat. Hop regulation of cardiac gene expression. Trends Cardiovasc. Med. (2004).13 (7): 261–4.

54. Trivedi CM., Zhu W., Hopx and Hdac2 interact to modulate Gata4 acetylation and embryonic cardiac myocyte proliferation. Department of Cell and Developmental Biology. Sep 2010, 450-9.

55. Trivedi CM., Cappola TP., Homeodomain Only Protein X is down-regulated in human heart failure. J Mol Cell Cardiol. 2011 1056-8.

56. Kook H, Yung WW, Simpson RJ, Kee HJ, Shin S, Lowry JA et al. Analysis of the structure and function of the transcriptional coregulator HOP. Biochemistry 45 (2006), 10584–10590.

57. Chen Y, Petersen S, Pacyna-Gengelbach M, Pietas A, Petersen I. Identification of a novel homeobox-containing gene, LAGY, which is downregulated in lung cancer. Oncology 2003;64:450–8.ent.

58. Hopx structure

59. Oshimo Y, Oue N, Mitani Y, Nakayama H, Kitadai Y, Yoshida K et al. Frequent loss of RUNX3 expression by promoter hypermethylation in gastric carcinoma. Pathobiology (2004), 71: 137–143.

60. Oshimo Y, Oue N, Mitani Y, Nakayama H, Kitadai Y, Yoshida K et al. Frequent epigenetic inactivation of RIZ1 by promoter hypermethylation in human gastric carcinoma. Int J Cancer (2004). 110: 212–218.

61. Yamashita K, Kim MS, Park HL, Tokumaru Y, Osada M, Inoue H, Mori M, Sidransky D. HOP/OB1/NECC1 promoter DNA is frequently hypermethylated and involved in tumorigenic ability in esophageal squamous cell carcinoma. Mol Cancer Res 2008;6:31–41.

62. Asanoma K, Matsuda T, Kondo H, Kato K, Kishino T, Niikawa N, Wake N, Kato H. NECC1, a candidate choriocarcinoma suppressor gene that encodes a homeodomain consensus motif. Genomics 2003; 81:15.

63. Shinichiro Yamaguchi., Kazuo Asanoma., Homeobox gene HOPX is epigenetically silenced in human uterine endometrial cancer and suppresses estrogen-stimulated proliferation of cancer cells by inhibiting serum response factor. International Journal of Cancer.Dec 2009, 2577-2588.

64. Chen Y, Pacyna-Gengelbach M, Deutschmann N, Niesporek S, Petersen I. Homeobox gene HOP has a potential tumor suppressive activity in human lung cancer. Int J Cancer (2007), 121:1021–1027.

65. Lemaire F, Millon R, Muller D, Rabouel Y, Bracco L, Abecassis J, Wasylyk B. Loss of HOP tumour suppressor expression in head and neck squamous cell carcinoma. Br J Cancer 2004;91:258–61.

66. Shinichiro Yamaguchi1, Kazuo Asanoma2, Tomoka Takao1, Kiyoko Kato2 and Norio Wake1 Homeobox gene HOPX is epigenetically silenced in human uterine

endometrial cancer and suppresses estrogen-stimulated proliferation of cancer cells by inhibiting serum response factor Int. J. Cancer: 124, 2577–2588 (2009).

67. Ookia., Yamashita K.,Potential utility of HOP homeobox gene promoter methylation as a marker of tumor aggressiveness in gastric cancer. Eur J Cell Biol. June 2010, 3263-75.
68. Ooki A, Yamashita K, Kikuchi S, Sakuramoto S, Katada N, Watanabe M. Phosphatase of regenerating liver-3 as a convergent therapeutic target for lymph node metastasis in esophageal squamous cell carcinoma. Int J Cancer (in press), (2009).
69. Albrecht I., Niesner U., Persistence of effector memory Th1 cells is regulated by Hopx. Eur J Cell Biol. Nov 2010.
70. Daniel Hawiger., YisongY Wan., The transcription cofactor Hopx is required for regulatory T cell function in dendritic cell–mediated peripheral T cell unresponsiveness. Nature Immunology. 2010, 962-968.
71. Norifumi Takeda, Rajan Jain, Matthew R. LeBoeuf, Qiaohong Wang, Min Min Lu, Jonathan A. Epstein, Interconversion Between Intestinal Stem Cell Populations in Distinct Niches .Science vol 334 9 December 2011.
72. Yang JM., Sim SM., Kim HY.,Expression of the homeobox gene, HOPX, is modulated by cell differentiation in human keratinocytes and is involved in the expression of differentiation markers. Eur J Cell Biol. Jul 2010 , 537-46
73. Facundo D. Batista and Naomi E. Harwood. The who, how and where of antigen presentation to B cells nature reviews. Immunology. doi:10.1038/nri2454 Published online 12 December 2008.

74. Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002.

75. von Andrian, U. & Mempel, T. Homing and cellular traffic in lymph nodes. Nature Rev. Immunol. 3, 867–878 (2003).

76. Atsushi Mizoguchi and Atul K. Bhan. A Case for Regulatory B Cells. J Immunol 2006; 176:705-710;

77. Kehry MR. 1996. CD40-mediated signaling in B cells. Balancing cell survival, growth, and death. J. Immunol. 156:2345–48.

78. Rahman A, Isenberg DA. Systemic lupus erythematosus. N. Engl. J. Med. 2008. 358:929–39.

79. Gray M, Miles K, Salter D, Gray D, Savill J. Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. Proc. Natl. Acad. Sci. USA. 2007. 104:14080–85.

80. Carter NA, VasconcellosR, RosserEC, Tulone C,Munoz-SuanoA, et al. Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells. J. Immunol. 2011. 186:5569–79.

81. Edwards JC,Cambridge G. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. Nat. Rev. Immunol. 2006. 6:394–403.

82. Joonyong Noh, Wahn Soo Choi. Presence of Foxp3-expressing CD19(+)CD5(+) B Cells in Human Peripheral Blood Mononuclear Cells: Human CD19(+) CD5(+) Foxp3(+) Regulatory B cell (Breg). Immune network. Nov 2010.

83. Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M, Tedder TF. A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. Immunity 2008. 28:639–50.

84. Claudia Mauri and Anneleen Bosma. Immune Regulatory Function of B Cells. Annu. Rev. Immunol. 2012. 30:221–41.

85. Mauri C. Regulation of immunity and autoimmunity by B cells. Curr. Opin. Immunol. 2010. 22:761–67.

86. Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS, Bhan AK. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. Immunity. 16: 2002.219–30.

87. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, et al. CD19+CD24hiCD38hi. B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic lupus erythematosus patients. Immunity2010. 32:129–40.

88. Carter NA, VasconcellosR,RosserEC, Tulone C,Munoz-SuanoA, et al. Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells. J. Immunol. 2011. 186:5569–79.

89. Mann MK, Maresz K, Shriver LP, Tan Y, Dittel BN. B cell regulation of CD4+CD25+ T regulatory cells and IL-10 via B7 is essential for recovery from experimental autoimmune encephalomyelitis. J. Immunol. 2007. 178:3447–56.

90. Sun JB, Flach CF, Czerkinsky C, Holmgren J. B lymphocytes promote expansion of regulatory T cells in oral tolerance: powerful induction by antigen coupled to cholera toxin B subunit. J. Immunol. 2008.181:8278–87.

91. Duddy M, Niino M, Adatia F, Hebert S, Freedman M, et al. Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. J. Immunol. 2007. 178:6092–99.

92. Correale J, Farez M, Razzitte G. Helminth infections associated with multiple sclerosis induce regulatory B cells. Ann. Neurol. 2008. 64:187–99.

93. Wolf, S. D., B. N. Dittel, F. Hardardottir, and C. A. Janeway, Jr. Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice. J. Exp. Med. 1996.184: 2271–2278.

94. Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS, Bhan AK. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. Immunity. 2002. 16:219–30.

95. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. Nat. Immunol. 2002. 3:944–50.

96. Mauri C, Gray D, Mushtaq N, Londei M. Prevention of arthritis by interleukin 10-producing B cells. J. Exp. Med. 2003.197:489–501.

97. Defendenti et al., B Lymphocyte intestinal homing in inflammatory bowel disease. BMC Immunology 2011 12:71.

98. Mizoguchi, A., E. Mizoguchi, R. N. Smith, F. I. Preffer, and A. K. Bhan. Suppressive role of B cells in chronic colitis of T cell receptor _ mutant mice. J. Exp. Med. 1997. 186: 1749–1756.

99. Gray M, Miles K, Salter D, Gray D, Savill J. Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. Proc. Natl. Acad. Sci. 2007.USA 104:14080–85.

100. Carter NA, VasconcellosR, RosserEC, Tulone C,Mu˜ noz-SuanoA, et al. Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells. J. Immunol. 2011. 186:5569–79.

101. Charles Vanderpool., Fang Yan., Mechanisms of Probiotic Action: Implications for Therapeutic Applications in Inflammatory Bowel Diseases. Inflammatory Bowel Disease. Nov 2008, 1585-1596.

102. SchirbelA., Fiocchi C., Targeting the innate immune system in pediatric inflammatory bowel disease. Expert Review of Gastroenterology and Hepatology. February 2011, 1, 33-41.

103. Kenneth Todar et al., Immune Defense against Microbial Pathogens. Text book of Bacteriology. 2005.

104. Karen Manon Lammers., Patrizia Brigidi., Immunomodulatory effects of probiotic bacteria DNA: IL-1 and IL-10 response in human peripheral blood mononuclear cells. FEMS Immunology and Medical Microbiology. May 2003, 165-172.

105. Yueh-Ting Tsai et al., The immunomodulatory effects of lactic acid bacteria for improving immune functions and benefits. Appl Microbiol Biotechnol. 7 July 2012.

106. Rial D. Rolfe. The Role of Probiotic Cultures in the Control of Gastrointestinal Health. J. Nutr. 130: 396S–402S, 2000.

107. George Aragon, MD, Deborah B. Graham, MD, Marie Borum, MD, EdD, MPH, and David B. Doman, MD, FACP, FACG. Probiotic Therapy for Irritable Bowel Syndrome. Gastroenterology & Hepatology Volume 6, Issue 1 January 2010.

108. Cooney RM, Warren BF, Altman DG, Abreu MT, Travis SP. The Outcome measurement in clinical trials for Ulcerative Colitis: towards standardization. Trials. 2007; 8: 17.

109. The new York Time.com. A.D.A.M.,1997-2008.

110. Kawada M, Arihiro A, Mizoguchi E. Insights from advances in research of chemically induced experimental models of human inflammatory bowel disease. World J Gastroenterol 2007 Nov 14.