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EFFECT OF CYCLOPHILIN "A" AND CYCLOSPORINE "A" ON DSS-INDUCED IBD IN SYNDECAN-1 KNOCK OUT MICE IN PRESENCE AND ABSENCE OF PROBIOTICS

by
ROAA ABBAS LEZEIK

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EFFECT OF CYCLOPHILIN "A" AND CYCLOSPORINE "A" ON DSS-INDUCED IBD IN SYNDECAN-1 DEFICIENT MICE IN PRESENCE AND ABSENCE OF PROBIOTICS

by

ROAA ABBAS LEZEIK

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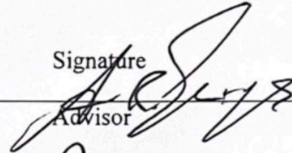
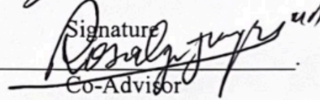


Signature

Dr. Abdo Jurjus, Professor
Anatomy, Cell Biology and Physiology

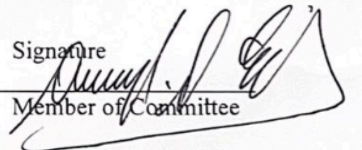
Advisor



Signature

Dr. Rosalyn Jurjus
Anatomy, Cell Biology and Physiology

Co-Advisor

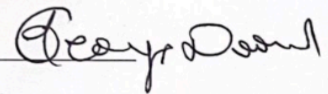


Signature

Dr. Assaad Eid, Professor
Anatomy, Cell Biology and Physiology

Member of Committee

Signature



Dr. Georges Daoud, Associate Professor
Anatomy, Cell Biology and Physiology

Member of Committee

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ABSTRACT
OF THE THESIS OF

Roaa Abbas Lezeik for Master of Science
Major: Physiology

Title: Effect of Cyclophilin “A” and Cyclosporine “A” on DSS-Induced IBD in Syndecan-1 Deficient Mice in Presence and Absence of Probiotics

Introduction: Inflammatory Bowel Disease (IBD) is a chronic relapsing autoimmune inflammatory disease that results from the interplay between genetic and environmental factors. Cyclosporine “A” (CysA) has been described for its potency in IBD therapy, however it has been known for its serious adverse effects such as infections and nephrotoxicity. It forms a complex with intracellular cyclophilin (CypA), thus suppressing the activation of T-cells. On the other hand, extracellular cyclophilin “A”, has been known for its proinflammatory action through its binding to its cell surface receptor CD147.The signaling of Cyp-CD147 interaction remains unknown and requires further investigations. In addition, several studies have demonstrated the importance of syndecan-1 in controlling inflammation. They have shown the exacerbated status of inflammation in the colons of DSS-induced syndecan-1 deficient mice. Regarding probiotics, studies have illustrated their effective role in ameliorating intestinal inflammation in DSS-induced colitis models.

Hypothesis and aims: Based on the literature, we hypothesized first that cyclosporine “A” would reduce inflammation in sdc-1 deficient mice, second, the formation of cyclosporine-cyclophilin complex would further decrease the severity of IBD and third, probiotics would have a positive effect in ameliorating inflammation. Consequently, the aim of this study is to assess the effect of cyclosporine “A”, cyclophilin “A”, (cyclosporine+cyclophilin) complex and probiotics on the expression of proinflammatory markers (IL-6. CD147), pAKT signaling pathway activation and T-cells (CD3) in DSS-induced IBD in sdc-1deficient mice.

Materials and Methods: A total of 42 adult sdc-1 mice were used. IBD was induced by administering 1.5% of DSS, where each cycle of DSS consisted of 7 days of DSS followed by two weeks drinking water. Cyclosporine was intraperitoneally injected to mice, 200 ug every other day for 2 weeks. Cyclophilin was intraperitoneally injected as well, 25ug/kg/day for 1 week. Additionally, a daily dose of 10^8 CFU per animal of probiotics (ProbioLife) was administered for 2 weeks. All the treatments started at day 7 of DSS induction. On the third week, six animals of each group were sacrificed and intestinal biopsies were frozen for protein extraction and fixed in 10% formalin for routine light microscopy. Histological scoring was calculated for each group. In addition, western blot determination of IL-6, CD147, CD3 and pAKT was performed.

Results: This study demonstrated that DSS-treated group showed a significant colon inflammation with more than 50% loss of goblet cells. However, both (DSS+Cyp) and

(DSS+Cys) separately treated groups revealed a mild inflammation with an improvement in the mucosal architecture and a 25% loss of goblet cells. Whereas, the DSS+(Cyp-Cys) complex group exhibited a severe inflammation showing a detrimental effect on the colonic histology with a complete loss of goblet cells and severe inflammation in the intestinal mucosa and submucosa. The addition of probiotics to DSS+(Cyp-Cys) complex group displayed a slight reduction in inflammation where about 25% of goblet cells were restored. On the other hand, a notable reduction in inflammation occurred when mice were treated with DSS and probiotics in the absence of (Cyp-Cys) complex where about 75% of mucus secreting cells were restored. Furthermore, (IL-6, CD147, pAKT, and CD3) were assessed in each group showing similar results correlating with the histological analysis.

Conclusion: In light of these results, cyclosporine is a potent therapeutic agent for IBD in DSS-induced sdc-1 deficient mice and cyclophilin “A” might have a therapeutic role as well in ameliorating inflammation. However, the (Cyp-Cys) complex has a worsened effect on the colons of these mice. On the other hand, probiotics have worked more effectively in the absence of the complex in relieving inflammation. The mechanism by which syndecan-1 mice responded to the induced IBD remains to be elucidated.

Keywords: IBD, DSS, cyclophilin “A”, cyclosporine “A”, (cyclophilin-cyclosporine) complex, probiotics.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS 1

ABSTRACT 2

ILLUSTRATIONS 7

TABLES 8

ABBREVIATIONS 9

INTRODUCTION 11

A. General Characteristics of IBD: 11

B. Epidemiology and Risk Factors: 13

1. Genetics: 14
2. Stress: 16
3. Nutrition: 17
4. Medications: 18
5. Ecological and Socioeconomic Factors: 18

C. Pathophysiology of IBD: 18

1. Microbial Factors: 18
2. Immunological Factors: 21
3. Oxidative Stress in IBD: 27

D. Animal Models of IBD: 28

E. DSS Model of Colitis: 31

F. Syndecan-1 knock-out Animal: 34

1. Characteristics of Syndecan-1 and its Role in Health: 34

2. Role of Syndecan-1 in Disease: 35
3. Role of Syndecan-1 in IBD: 36

G. Management of IBD: 38

H. Cyclosporine: 39

1. Definition of Cyclosporine "A" and its Role in Health 39
2. Role of Cyclosporine "A" in IBD: 40

I. Cyclophilin: 41

1. Definition of Cyclophilin: 41
2. Role of Cyclophilin in Health: 42
3. Role of Cyclophilin in Diseases and IBD: 43

J. CD147: 46

1. Definition of CD147 and its Role in Health: 46
2. Role of CD147 in inflammation and IBD: 46

K. Syndecan-1/Cyclophilin/CD147 interaction: 47

L. Probiotics: 48

1. Definition of Probiotics: 48
2. Mechanisms of Action of Probiotics: 49
3. Effectiveness of Probiotics in Clinical Cases: 50
4. Effectiveness of Probiotics in Animal Models of Colitis: 50

M. Cluster of differentiation 3 (CD3): 52

1. Definition of (CD3): 52
2. (CD3) in Diseases and Inflammation: 52

N. "Phosphorylated Protein Kinase B" (pAKT) in Health and Diseases: 53

OBJECTIVES AND HYPOTHESIS OF THE STUDY 56

MATERIALS AND METHODS 58

A. Experimental Procedure: 58

1. Animal Housing 58
2. Induction of IBD and Treatments 58
3. Groups 59

B. Histology 60

C. Western Blot 61

D. Statistical Analysis: 62

RESULTS 63

A. Histology 63

1. Assessment of Histological Alterations by (H&E): 63
2. Assessment of mucus secreting cells, Goblet cell, by (PAS): 66
3. Histological Scoring Analysis: 68

B. Analysis of molecular parameters 71

1. Interleukin-6 Expression (IL-6) 71
2. Emmprin Expression (CD147) 73
3. pAKT Expression: 75
4. CD3 Expression: 77

DISCUSSION 80

LIMITATIONS OF THE STUDY 95

ILLUSTRATIONS

Figure

1. Treatments administered to the syndecan-1 knockout mice. 59
2. The representative photographs of hematoxylin and eosin staining (magnification x40).. 65
3. The representative photographs of Periodic acid-Schiff staining (magnifications to the left x40 and to the right x100). 68
4. A bar graph showing the histological scoring in all treated groups.................... 70
5. Protein Expression levels of the pro-inflammatory marker Interleukin-6 (IL-6) in syndecan-1 null mice. 72
6. Protein Expression levels of the pro-inflammation marker CD147 in syndecan-1 null mice. 74
7. Protein Expression levels of pAKT in syndecan-1 null mice. 76
8. Protein Expression levels of CD3 in syndecan-1 null mice................................ 78

TABLES

Table

1. Cyclophilin's Role in Diseases.. 44

2. Scoring system used to calculate the histological alterations in dextran sulphate sodium (DSS)-induced colitis. [446] 61

ABBREVIATIONS

>	Greater than
<	Less than
-	Minus
+	Plus
/	Per
%	Percent
ANOVA	Analysis of Variance
AKT.	Protein Kinase B
AMP	Antimicrobial peptides
AUB	American University of Beirut
bFGF	basic fibroblast growth factor
CAT	Catalase
CD	Crohn's Disease
CD3	Cluster of differentiation 3
CD147	EMMPRIN
CysA	Cyclosporine A
CypA	Cyclophilin A
(Cyp-Cys)	Cyclophilin-Cyclosporine
DNBS	Dinitrobenzene sulfonic acid
DSS	Dextran sulfate sodium
GM-CSF	Granulocyte-Macrophage colony-stimulating factor
GPX	Glutathione Peroxidase
GWAS	Genome-wide association studies
H&E	Hematoxylin and Eosin
HFD	High Fat Diet
HSPG	Heparan sulfate proteoglycan
IACAC	Institutional Animal Care and Use Committee
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
IECs	Intestinal epithelial cells
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate Lymphoid cells
ITAMs	Intercellular immunoreceptor tyrosine-based activation motifs
JAK2	Janus Kinase 2
JNF	c-Jun N-terminal kinase
LCN-2	Lipocalin 2
MAPK	Mitogen-activated Protein Kinase
MCFAs	Medium-chain-lengthy fatty acids
MDP	Muramyl dipeptide
MNP	Mononuclear phagocytes
MPO	Myeloperoxidase
NFAT	Transcription factor nuclear factor of activated T cells

NF-κB	Nuclear factor κB
NKT	Natural killer T
NOD2	Nucleotide binding oligomerization domain 2
NOS	Nitric oxide Synthase
NSAIDs	Non-steroidal anti-inflammatory drugs
PAMPs	Pathogen-associated molecular pattern
PAS	Periodic Acid Schiff
PDK1	3-Phosphoinositide-dependent kinase 1
PI3K	Phosphoinositide 3-kinases
PIP2	Phosphatidyl inositol biphosphate
PMN	Polymorphonuclear leukocytes
PRRs	Pattern recognition receptor
PUFA	Polyunsaturated fatty acids
RIPK2	Receptor-interacting serine/threonine protein kinase 2
SCFA	Short-chain fatty acids
SCID	Severe Combined Immune Deficiency
Sdc-1	Syndecan-1
SLE	Systemic Lupus Erythematosus
SNP	Single nucleotide polymorphism
SOD	Superoxide Dismutase
STAT3	Signal transducer and activator of transcription 3
TAMs	Tumor associated macrophages
TGF	Transforming Growth factor
Th	T helper
TL1A	TNF-like molecule 1
TLR	Toll like receptor
TNBS	2,4,6-triinitrobenzene sulfonic acid
TNF-α	Tumor necrosis factor α
Treg	Regulatory T
UC	Ulcerative Colitis
VCAM	Vascular cell adhesion molecule

CHAPTER I

INTRODUCTION

A. General Characteristics of IBD

IBD's constitute a panel of inflammatory autoimmune diseases ranging from Ulcerative Colitis (UC) to Chron's Disease (CD) and others. The basic underlying mechanism of the IBD is inflammation. Upon external aggressions, organism's immune system generates a rapid response as a way to defend itself against aggressors such as bacteria and pathogens; this response is called: "Inflammation" [1]. Thus, inflammation is a crucial process to restore homeostasis and maintain a normal physiological status in the body. Normally, the gastrointestinal tract is responsible for several physiological functions such as absorbing and metabolizing food as well as secreting ions and waste products. As such, the alimentary canal is mainly exposed to different sources of antigens found in diets in addition to various commensal bacteria. The mucosal epithelial cells not only play a vital role as a physical barrier, but also secrete different antimicrobial defense molecules like cathelicidins, defensins, and bacteriostatic proteins like lipocalin 2 (LCN-2) in addition to mucins and cytokines [2-4]. Moreover, the mucosal immune defense depends on different immune cells located in the lamina propria or in different organized structures such as mesenteric lymph nodes and Peyer's patches [5]. Both genetic and environmental factors interplay in this response. When intestinal homeostasis is disrupted, an influx of neutrophils and macrophages, proteolytic enzymes and free radicals are consequently produced [6, 7] , thus causing intestinal inflammation that could be acute or chronic, such as Inflammatory Bowel Disease (IBD).

In brief, IBD is a chronic relapsing disorder that is mainly characterized by the presence of uncontrolled intestinal inflammation and epithelial injury in the gastrointestinal tract, and it includes two main chronic pathological entities: Crohn's disease (CD) and Ulcerative colitis (UC)[8-12].The most common symptoms of IBD include episodes of abdominal pain, fatigue, prolonged diarrhea, bloody stools, severe rectal bleeding, weight loss, and fever [6, 13-15]. The pathogenesis of IBD is known to be multifactorial where a complex of genetic and non-genetic factors plays a vital role in the development and progression of the disease. The non-genetic factors involve epithelial barrier defects, environmental factors, dysregulated immune responses, and disturbance of the gut microbiota [15-21].

CD is mainly characterized by its transmural inflammation that may occur in a discontinuous pattern in any part of the gastrointestinal tract; starting from the mouth and ending in the anus. However, it is mostly localized in the terminal ileum, cecum, perianal area, and colon. Histologically, it is associated with the presence of thickened submucosa, fissuring ulcerations, and granulomas. In addition, colonoscopy have shown the presence of skip lesions, cobblestoning, and strictures in CD. On the other hand, inflammation in UC mainly involves the rectum and sigmoid colon, and it may affect the entire colon (pancolitis) or a part of it in a continuous pattern. Such inflammation is superficial and is mainly confined to the submucosa and mucosa only, where cryptitis and crypt abscesses are present as typical histological findings in UC. In addition, colonoscopy has shown the presence of pseudopolyps and continuous inflammatory areas [13, 22-29].Furthermore, Hanauer et al. showed that 10% of IBD patients are classified as indeterminant colitis patients where their disease symptoms are not typical for either UC or CD, but they have

the capability to develop one of these diseases later on as the disease progresses with time [12].

B. Epidemiology and Risk Factors

Based on a systematic review, it has been shown that the Western world has witnessed an increase in the incidence of Ulcerative colitis and Crohn's disease since the middle of the twentieth century including, Europe, North America, New Zealand, and Australia [30]. Studies showed that about 0.4% of European and North Americans live with the disease [31, 32], and that the incidence rate of IBD is mainly 2.5 million in Europe and 1.5 million in North America [32]. In contrast, this incidence is relatively low in the developing countries at that time including South America, Asia, and the Middle East [33-38]. However, at the turn of the twenty first century, IBD's incidence rate started to rise in those countries, yet it remained lower than that in the industrialized countries [30]. Shivashankar R, et al. have reported that different geographical regions affect the incidence of the disease [39]. For instance, Western Europe has the highest incidence rate, whereas countries adjacent to the Mediterranean have the lowest ones [40, 41]. Burisch J, et al. have pointed out that not only the incidence of IBD is rising worldwide, but also its prevalence too, especially in the developing countries such as South America and Asia [42]. In addition, Ponder A, et al. have noted that individuals are more prone to develop IBD when it comes to migrating to countries that have high prevalence of the disease [43]. Moreover, studies have proven that the development of IBD is not restricted to a certain age, however the majority of affected patients is between 15 and 29 years [44].Further studies have reported that 25% of the cases are diagnosed in children or adolescents[45, 46].

1. Genetics

The exact etiology of IBD remains to be unknown and not fully elucidated, although studies have shown that IBD is a result of a plethora of genetic and non-genetic interactions; involving the microbiome, the environment, and the immune system that plays a vital role in initiating an excessive inflammatory response against host microbiome in genetically susceptible individuals [9, 47, 48] .Over 240 non-overlapping genetic risk loci have been found by genome-wide association studies (GWAS), next-generation sequencing studies, and other analyses, whereby around 30 genetic loci were shared by Crohn's disease and ulcerative colitis[49-51]. CD and UC are both polygenic disorders. Moreover, 12 genome-wide scans have revealed susceptibility areas on 12 chromosomes. No single locus has been consistently detected in all genome scans, which is consistent with the genetic variability of inflammatory bowel disease. The areas on chromosomes 16, 12, 6, 14, 5, 19, 1, and 3 have been redesignated "IBD1–9", according to their original reporting date. More genes have been recently discovered using positional cloning techniques and fine mapping of susceptibility regions revealed from complete genome scans [52]. Interestingly, two comprehensive meta-analyses have reported that the most credible evidence for a relation to inflammatory bowel disease across all populations and disorders is found on chromosome 6 (IBD3), which encodes the major histocompatibility complex[53-55].Over 50% of IBD-related genes have been linked to other autoimmune diseases, yet exerting different effects [56].Although UC and CD have different clinical features, approximately 70% of IBD-related genes are shared between both diseases[57].Studies have reported the existence of several loci containing genes that overlap between ulcerative colitis and primary sclerosing cholangitis (PSC) such as MST1, IL2, CARD9, and REL [58]. Such an overlap is very important in

identifying UC patients that have the risk to develop other diseases such as PSC. Similarly, a set of several genes are shared between Crohn's disease and Mycobacterium leprae infection such as NOD2, C13orf 31, and LRRK2 [58].

It should be noted that the analysis of the genes involved in IBD have paved the way to the understanding of several pathways involved in maintaining intestinal homeostasis, such as cell migration, immune regulation, epithelial barrier function, innate mucosal defense, autophagy, and adaptive immunity [27, 59-61].Interestingly, several studies have confirmed that relatives of IBD patients have a higher chance of acquiring IBD than the general population, bolstering the theory that the disease's pathophysiology is strongly linked to genetic factors [62]. It has been shown that 12% of the affected persons have a family history of the disease [63-65].Moreover, polymorphisms in IBD susceptibility genes affect the gut microbiota and disrupt key host-microbe interactions, raising the likelihood of IBD development. These genes could be used as genetic biomarkers to identify people who are more likely to develop IBD, allowing them to be treated before the appearance of symptoms. Despite the fact that GWAS has identified several strongly linked susceptibility genes, they only account for about 25% of estimated heritability [66].

Several single nucleotide polymorphisms (SNPs) have been found in IBD patients in genes such as (TNFSF15) which encodes for TL1A (TNF-like molecule 1A) and is implicated in T cell response [24, 67, 68]. Other variant genes code different molecules which are involved in the differentiation and regulation of T cells such as IFNG, IL12B, IL2, IL21 [67]. It has been proven that the mutations in IL12B gene which normally encodes for the p40 subunit of IL-12 and IL-23 have an association with IBD and other

immunological diseases. Also, variants in IL-10 have been involved in IBD [69].Other genes play a major role in B cell response and are associated with IBD also such as IRF5 and IL7R [67]. Moreover, there are some IBD susceptibility genes that are implicated in the endoplasmic reticulum (ER) stress pathway and are IBD-related such as XBP1 and ODL3 [70, 71].

Furthermore, a strong association between IBD (CD and UC) and IL23R has been revealed by researchers [54, 72]. Of note, IL23R is a gene which encodes the receptor of a pro-inflammatory cytokine IL-23 which is mainly involved in the production of Th17 cells. In addition, it has been shown that through modulating IL-23R recycling and cytokine production by macrophages, Arg381Gln, an uncommon variant at a highly conserved amino acid polymorphism, has a protective influence in individuals with Crohn's or ulcerative colitis[54, 73] .It should be noted that Th-17/IL23 pathway constitutes a crucial role in the pathogenesis of IBD, and that several gene loci are implicated in CD and UC such as IL23R, IL-12B, JAK2, and STAT3[74, 75]. New studies on human have demonstrated the clinical advantage of IL-23 and anti-IL-23R antibodies in the management of IBD. Besides, recent studies have shown that epigenetic modification which is the methylation of DNA and non-coding RNAs has an indispensable role in the initiation and course of the disease[76-79].

2. *Stress*

It has been evidently confirmed that psychological stress is one of the important risk factors that may increase or initiate the likelihood of UC and CD [80, 81]. A study has demonstrated that people living in low stress conditions are less susceptible to developing

IBD [82].

3. Nutrition

It was reported that people in the western world have been exposed to bad food habits, and this is mainly due to the increased variability, availability, affordability, and processing of food [83, 84] which have consequently lead to changes in people's diet, causing an impaired function and reduced diversity of gut microbial composition, as well as deteriorating their mucosal immunity [85-88]. An evident study has shown that a typical Western diet fed to the mice has altered the composition of gut microbiota and has affected the texture of colonic mucus, thus showing some characteristics of IBD [88, 89]. Recent data has highlighted the crucial role of nutrition in modifying various epigenetic mechanisms that may further lead to the development of IBD [90].

Moreover, multiple studies have demonstrated the relation between a "High Fat Diet" (HFD) and the onset of IBD. In addition, it has been proven that different types of polyunsaturated fatty acids (PUFA) have different effects on the pathogenesis of the disease. For instance, individuals consuming omega-3 (PUFA) are less prone to IBD than those consuming omega-6 (PUFA). This is because omega-3 has an anti-inflammatory role whereas omega-6 acts as pro-inflammatory [91]. More studies have shown that protein intake may act as a risk factor for IBD. There are mainly two types of proteins; animal-based and plant-based proteins. Animal-based proteins involve more saturated fats than the plant-based one. Hou JK et al. have reported that IBD has developed only in people consuming animal-based proteins and not plant-based [92]. Furthermore, Jantchou P et.al have shown that individuals consuming fish which is mainly an animal-based protein, have a higher risk of IBD [93].

4. Medications

Evidently, many drugs have been implicated in the development of IBD. They include non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, oral contraceptives, antibiotics, and hormonal replacement therapy [94-99]. Many studies have pointed out the relationship between the use of antibiotics and the risk of IBD [100].

5. Ecological and Socioeconomic Factors

Among the ecological and epidemiological factors, air pollution has been recognized as a risk factor for the development of UC and CD. Tan WC et al. have shown that there's a positive association between elevated level of air pollution and the increase of circulating polymorphonuclear leukocytes and plasma cytokines [101, 102]. Moreover, the rise of IBD's incidence in developing countries have been associated with several socioeconomic changes influencing hygiene [103, 104].

C. Pathophysiology of IBD

1. Microbial Factors

It is estimated that the intestinal microbiome consists of a plethora of microbial organisms that inhabit the gut in a way to form a strong symbiotic relationship between microbes and the entire immune system. This relationship is based on supplying key nutrients to the host, modulating energy metabolism, providing host defense, and developing the immune system. This consequently leads to the establishment of host-microbiome homeostasis [105-113]. Normally, gut microbiota promotes the production of short-chain fatty acids (SCFAs) which constitute butyrate, propionate, and acetate playing an indispensable role in maintaining a healthy mucosa and producing anti-

inflammatory cytokines [114, 115]. It is proved that most bacterial colonies reside in the colon, and that in most healthy individuals, 99% of the bacteria are divided into four major groups: Firmicutes, Bacteriodetes, Actinobacteria and Proteobacteria showing that Firmicutes and Bacteriodetes represent 90% of the total microbiota [116-119]. Interestingly, the strains of Lactobacilli which belong to the phylum Firmicutes, have played a pivotal role in inhibiting the growth of many Gram-negative pathogenic bacteria, thus maintaining a healthy state of the mucosa.

Gut microorganisms are normally recognized by pattern recognition receptors (PRRs involving NOD-like, Toll-like receptors, C-type lectin receptors, and RIG-like receptors) of pathogen-associated molecular patterns (PAMPs), which are found in epithelial and immune cells [120-122], thus stimulating the innate immune system, activating NF-κB and inflammasome, and producing proinflammatory cytokines to maintain intestinal homeostasis [123-126].Under normal conditions, several mechanisms including epithelial and immune cell molecules such as RegIIIγ, IgA, and defensins regulate the microbiota which regulates subsequently the activation of the immune response favoring the induction of distinct T cell subsets [127].Well, the intestinal homeostasis is maintained unless a disturbance such as illness, antibiotic exposure, or alteration in diet occurs, thus creating a dysbiosis status [128, 129].

When a disruption in the microbial diversity, composition, and function occurs, intestinal homeostasis is no more maintained, and inappropriate immune activation takes place, leading to “Gut Dysbiosis” [130-132].It has been shown that gut dysbiosis is an indispensable factor leading to IBD pathogenicity; fortifying the notion that IBD may result from the dysregulation that occurs in the intestinal immune system, contributing further to a pathogenic immune response [133, 134]. Studies have shown that a reduction

in the species that produce butyrate is present in IBD patients, knowing that butyrate is a SCFA which is essential for maintaining intestinal homeostasis [135-137]. In a study, 132 IBD patients were recruited to assess their microbial activity during the progression of the disease. A gut dysbiosis was detected during the course of the disease; the microbial composition was altered, and the microbial transcription was impaired [138]. Additionally, it has been demonstrated that intestinal microbiota plays an essential role in most animal models of colitis [126].Similarly, further studies have established the changes that occur in the gut microbial composition in both UC and CD as a result of dysbiosis [139].

Patients with active disease have shown several changes concerning bacterial diversity, abundance, and composition compared to healthy individuals [140]. Evidently, an increase in mucolytic and pathogenic bacteria have been validated in IBD patients, contributing to the mucosal barrier's degradation [139, 141-143].Other studies have reported that these patients display a reduction in Firmicutes and increase in Proteobacteria such as Bilophila, Enterobacteriaceae, and specific members of Bacteroidetes, showing the occurrence of temporal instability in the dominant taxa in Ulcerative Colitis and Crohn's diseases [132, 135, 144-149].

In addition, other studies have reported that CD patients display a reduction in Bifidobacterium adolescentis*,* Dialister invisus, Faecalibacterium prausnitzii, in addition to Clostridium cluster XIVa [150-152] .Eventually, the differences in the microbial diversity have been detected within the same individual between inflamed and non-inflamed tissues. For instance, CD patients have demonstrated a reduced overall bacterial diversity at inflamed regions compared to the non-inflamed ones [153].Furthermore, UC patients have an increased overall diversity of invasive bacteria localized in the colonic

mucus layer, such as “Fusobacteria” relative to healthy individuals [141, 154]. In addition, colonic mucosal erosion in mice have been evidently revealed post rectal enema involving human isolates of Fusobacterium varium [155].

2. *Immunological Factors*

It has been documented that the mechanisms of both innate and adaptive immunity play a major role in controlling the state of low-grade inflammation in the healthy intestinal mucosa [156]. First, innate immune system is known as the first body defense against foreign antigens where it provides protection in the first minutes to hours after the occurrence of an infection. It is mainly constituted of macrophages, neutrophils, natural killer cells (NKTs), endothelial cells, and mucosal epithelial cells. It is characterized by its nonspecific response where macrophages engulf and digest invading microorganisms, thus paving the way for the adaptive immune system to complete its function in the protection process. However, the adaptive immune system is known for its specificity against invaders, and is composed of both humoral and cellular immune responses involving the activation and proliferation of both B- and T- lymphocytes respectively. In contrary to the innate, the adaptive immune system requires days and not hours to be activated, having an advantage in creating memory B- and T- cells. Of note, the complete abolition of infecting microbes is mainly based on the specific role of the adaptive system. It has been shown that Toll-like receptors (TLRs) play a crucial role in the innate system where they recognize and bind a plethora of viral, fungal, and bacterial antigens invading the host. However, the induction of a defective TLR signaling may influence the host in a deleterious way, leading to a detrimental immune response, and consequently to the pathogenesis of many inflammatory diseases such as IBD. Regarding IBD, it has been

reported that macrophages and intestinal epithelial cells of IBD patients have an increased expression of both TLR-2 and TLR-4. Similarly, it has been demonstrated that the loss of TLR-4 expression in an animal model of IBD has afforded a protection against colitis, thus TLRs play a vital role in the initiation of inflammatory cascade [157].

Moreover, IBD pathogenesis, as mentioned above, involves the participation of immune system in addition to the non-immune system which is mainly composed of endothelial cells, nerves, mesenchymal and epithelial cells. As mentioned before, the immune responses are of two types; humoral and cell-mediated. The humoral immune response activates B-lymphocytes to eventually secrete antibodies, whereas the cell-mediated immune response stimulates effector T-lymphocytes, macrophages, and neutrophils. It is known that antigen presenting cells such as dendritic cells, macrophages, or intestinal epithelial cells, bind to the foreign antigen, process, and then present it on their surface to CD4+ T- helper cells. Such mechanism takes place in the lamina propria (intestinal epithelial cells and dendritic cells) or in mesenteric lymph nodes (dendritic cells) where T-lymphocytes are activated and differentiated as Th-1, Th-2, Th-3 or T-regulatory-1 (Treg) lymphocytes. It has evidently been shown that Th-1 cells produce IL-2 and interferon gamma IFNγ, thus shaping the cell-mediated immunity. On the other hand, Th-2 cells induce the production of antibodies and the secretion of IL-4, IL-5, IL-10 and other cytokines, thus supporting the humoral immunity. Knowing that IFNγ is produced by Th-1 lymphocytes, it has been shown that IFNγ stimulates the activation of macrophages, thus stimulating the production of further cytokines such as IL-12 and tumor necrosis factor (TNF). IL-12 further activates Th-1 differentiation of T-cells. However, IL-4 produced by T-cells evokes Th-2 differentiation. Additionally, Th-2 and T-reg1 cells both produce IL-10, and Th-3 cells produce transforming growth factor

(TGF). Interestingly, it has been proven that IL-10 and TGF have a vital role in downregulating inflammation. Moreover, the role of Treg-1 cells, Th-3, CD4+ CD25+ T-cells, and B-cells has been demonstrated in the immunoregulation mechanism, specifically in reinforcing the connection between innate and adaptive immune systems. Not only immune cells affect an immune response, but also non-immune cells play a vital role in modulating it. They involve the constituents of the intestinal extracellular matrix, for instance, matrix metalloproteinases, adhesion molecules, fibroblasts, intestinal epithelial cells, and granulocytes. Such intercalating network has a great impact on inflammatory areas, contributing to a less inflammatory state or maybe to complete healing [158]. Furthermore, neurons have been known for their critical role in modulating immune responses; they induce the production of histamine from mast cells and produce substance P, thus leading to an increased intestinal vascular permeability. In contrast, fibroblasts have been known to produce IL-11 and other cytokines. Now, regarding adhesion molecules, it has been shown that selectin and integrin families, and immunoglobulin (Ig) supergene family such as (intercellular adhesion molecule ICAM) are all expressed on the surface of leukocytes or endothelial cells, thus recruiting leukocytes into the mucosa, and leading to their adherence in addition to the transendothelial emigration [159, 160].

Regarding cytokines produced during episodes of inflammation and specifically IBD, it has been reported that IL-6 plays a major role in the pathogenesis of the disease [161, 162].It has been shown that IL-6 is implicated in the pathogenesis of UC and colorectal cancer too [163]. Moreover, the administration of monoclonal antibodies in DSS-induced mice models of colitis has evidently contributed to the neutralization and decrease in IL-6 and TNF-α levels. This shows that IL-6 plays a pro-inflammatory role

during inflammation. However, it has been reported that the phase of inflammation in addition to the target cell have both a pivotal role in specifying the pleotropic effects of IL-6. For instance, when inflammation is initiated, IL-6 stimulates the mesenchymal and epithelial cells to recruit macrophages and polymorphonuclear leukocytes (PMNs) and initiate wound healing [164].Well, the blockade of IL-6 has been shown to prevent T-cell expansion and decrease Th-cell driven intestinal inflammation [165, 166].It has been proven that the signaling of IL-6 on intestinal epithelial cells (IECs) provokes YAP/notch signaling pathway which fosters the differentiation of absorptive epithelium leading to further stimulation of repair pathways [167].Moreover, a study has demonstrated that IL-6 stimulates STAT3 signaling in IECs, thus promoting barrier function and mucus secretion [168].

Furthermore, a plethora of cytokines other than IL-6 have been involved in the pathogenesis of IBD; such as IL-1 family cytokines (IL-1b and IL-18). The loss of IL-1b and IL-18 expression due to the deletion of the inflammasome component caspase-1 has consequently ameliorated DSS-induced colitis in mice models [169].

Additionally, IL-8 has been known as a chemoattractant that targets neutrophils which migrate from peripheral blood into inflamed areas. A study has reported an increase in IL-8 expression in UC patients compared to normal [170].

Regarding IL-9, it has been shown that Th9/IL-9 pathway plays a pro-inflammatory role in IBD and in UC specifically. Its presence mainly aggravates the course of intestinal inflammation, and the administration of IL-9 antibody has been shown to ameliorate oxazolone-induced murine colitis [171].

In contrast, the presence of IL-10 has a protective role against inflammation. A study has reported that T-cells which lack IL-10 receptor are more prone to developing colitis

[172]. Similarly, the absence of IL-21R aggravates the case of DSS-induced colitis [173].A study has demonstrated that UC patients have increased expression of IL-33 and its receptor ST2 [174]. Regarding IL-23 signaling, it has been shown that IL-23 in activated Th17 cells induces JAK2 and STAT3 signaling contributing to the production of IL-17 and IL-22 [175].

Interestingly, the overexpression of STAT3 can induce the differentiation and proliferation of Th17 cells. Thus, the absence of STAT3 can absolutely prevent the differentiation of naïve T-cells into Th17 cells [176]. Well, in a DSS model of colitis, the contrasting roles of IL-17A and IL-17F have been demonstrated. An excessive inflammation and deterioration of intestinal epithelium has been reported after the administration of IL-17A antibody [177, 178].Whereas, lacking IL-17F in DSS-induced models of colitis has consequently contributed to the improvement of intestinal inflammation [179].

It has been proven that Toll-like receptors (TLRs) have specific sensing mechanisms in the gut microbiome which normally control the immune responses and maintain intestinal homeostasis [180]. Normally, when TLR2 is stimulated, it induces the association with the adaptor protein myeloid differentiation primary response protein 88 (MyD88) and recruits further proteins leading finally to the activation of NF-κB [8]. However, studies have shown that an alteration in TLR2/6 signaling leads to colitis [181, 182]. Shmuel-Galia L, et al have proven that the inhibition of TLR2 in this pathway has lead consequently to the amelioration of colitis in DSS-treated mice [183]. Interestingly, the differentiation of more pathogenic Th1 and Th17 cells is caused by the upregulation of TLR6 expression and thus leading to colitis in mice [184].

NOD2 (nucleotide binding oligomerization domain 2) pathway has been considered to be one of the important pathways involved in IBD. It has been reported that NOD2 gene was strongly associated with IBD [50, 185-187]. Normally, NOD2 senses muramyl dipeptide (MDP) and activates NF-κB via receptor- interacting serine/threonine-protein kinase 2 (RIPK2) which associates with the inflammasomes to induce the secretion of IL-1β [188, 189]. It has been demonstrated that NOD2 deficiency in mice contributed to modified TLR signaling and production of high levels of pro-inflammatory cytokines by macrophages leading to inactivation of NF-κB and subsequently promoting injury [190].

Besides, IL-22/IL-22R pathway has been implicated in the pathogenesis of IBD. The stimulation of mononuclear phagocytes (MNPs) by commensal microbiota has triggered the secretion of several interleukins such as IL-1b, IL-6, and IL-23 leading to the activation of innate lymphoid cells (ILC3s) inducing IL-22 production [191]. It has been shown that STAT3 and MAPK pathways are both activated as IL-22 binds to its receptor, contributing to the production of IL-10, mucus, and antimicrobial peptides (AMPs). In addition, IL-22 has been shown to have an anti-apoptotic role, thus enhancing the proliferation of the damaged intestinal epithelium via the increase of claudin-2 expression [192]. In this context, a study has proven that colitis is exacerbated in mice models after the blockade of IL-22/IL-22R pathway; this shows that IL-22 pathway has a protective role against the development of colitis [192, 193].

Type1 Interferon (IFN-1) mainly promotes intestinal epithelial integrity through the activation of STAT1 and STAT2 signaling pathways [194]. However, mutations in type1 Interferon receptor gene (IFNAR1) occur, contributing to IBD pathogenesis [195]. In addition, it has been demonstrated that tumor necrosis factor-α (TNF-α) plays a vital role in IBD through triggering the upregulation of IL-1β, IL-33, and IL-6 levels [196, 197].In

contrast, transforming growth factor- β (TGF-β) has been shown to suppress the development of T-cell mediated colitis [198].

Hence, the strong connections during inflammatory episodes between immune cells which produce chemokines and cytokines and non-immune cells such as fibroblasts and epithelial cells as well as adhesion molecules lead to an enhanced production of cytokines, neuropeptides, growth factors, and antibodies. In addition to oxygen and nitrogen reactive species which all participate in the pathophysiology of IBD [199].

3. Oxidative Stress in IBD

The production of ROS has been always controlled by the production of an antioxidant system that involves a plethora of enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPXs), and their antioxidant substrates (glutathione and α-tocopherol) [200]. In addition to other antioxidant compounds such as β-carotene, vitamin C, bilirubin, zinc, selenium, copper, and urate [201].

One of the well-known antioxidants is "Superoxide dismutase" (SOD) which has been expressed in three different isomer forms (SOD1, SOD2, and SOD3) in IBD patients. It has been shown that its activity is correlated with the course of the disease [202]. The expression of SOD2 has shown a significant increase in IBD, while a downregulation of SOD3 has been detected especially in intestinal epithelial cells (IECs). Well, regarding SOD1, no effect has been reported [203].Moreover, several studies have demonstrated contradictory results regarding SOD's expression in IBD animal models of colitis. For instance, in studies on UC induced by intrarectal acetic acid, an increased SOD level has been detected [204]. Whereas, other studies on UC induced by TNBS have reported a reduced SOD activity [205-207]. This implies that the level of oxidative stress

has increased, leading further to the oxidation of proteins and membranes' lipid peroxidation, therefore damaging cells [208].

On the other hand, "Catalase enzyme" (CAT) is found in peroxisomes and is chemically responsible for reducing hydrogen peroxide into water and oxygen [209]. Clinical studies have proven that CD and colorectal cancer patients have both downregulated CAT levels [210, 211].Also, a decreased expression of CAT in genetically modified mice has been linked to a decreased occurrence of colitis and colon cancer too [212, 213].

In addition, "Glutathione peroxidase" (GPX) is another antioxidant which has mainly three different isoforms. Whereby, the polymorphisms occurring in GPX1 and GPX2 levels have resulted in intestinal inflammation and oxidative stress, in addition to the appearance of IBD symptoms in mice models [214]. Other studies have demonstrated the significant association between increased GPX2 levels and colitis [215, 216]. In mice models of colitis treated with DSS, the plasma glutathione peroxidase (E-GPx) has been increased by more than 60% compared to the control, showing that GPX is implicated in the pathogenesis of the disease [217].

D. Animal Models of IBD

The multifactorial nature of IBD has emphasized the need to develop a vast number of animal models that served as relevant tools for a better understanding of the disease pathogenesis and the underlying mechanisms involved in its initiation and progression. Researchers attempted to induce the disease (in its acute and chronic forms) in those models in several ways, thus allowing them to express various defects mimicking its

different aspects [57, 218]. Such models have provided pivotal insights into histopathological and morphological modifications occurring in the intestinal tract during the course of the disease [219]. However, it has been shown that none of these models has provided a sufficient clinical and histopathological representation of IBD, yet they have elucidated a detailed understanding of its pathogenesis [220, 221]. Importantly, researches focused on those models in research to use them for preclinical studies of drug development [220]. These models have been categorized into chemically induced colitis, spontaneous colitis, adoptive transfer, and genetically modified models [172, 218, 219, 222-224]. The chemically induced models were the first used by Kirsner and Elchlepp in 1957 when they induced colitis in a rabbit model by irritating its rectum with a diluted formalin solution, and then localizing its crystalline egg albumin within its colon.[225] After several years, chemically induced murine models of colitis were developed; the dextran sulfate sodium (DSS) and the 2,4,6-trinitrobenzene sulfonic acid (TNBS) models. In the following years, more than 60 different types of acute and chronic models of intestinal inflammation were generated [226]. In 1981, spontaneous colitis was induced in the cotton –topped tamarin animal which used to live in Colombia. Nine years later, Dr. Powrie and other researchers induced experimental colitis in immunodeficient mice using an adoptive T-cell transfer system; they transferred lymphocytes in lymphopenic mice [227]. Interestingly, this model system has significantly lead to a deeper understanding of the vital role of regulatory T-cells and the regulation of the mucosal immunity [228, 229]. Cell-transfer models include CD45RB-^{high}, CD62L-^{high}, and Hsp60 CD8^{+} T cells [230]. In 1990, colitis was developed in genetically engineered modified rats that carry (HLA-B27) gene, a specific human gene [231]. In 1993, three different types of knockout (KO) mice which are interleukin-2 (IL-2) KO, interleukin-10 (IL-10)

KO, and T-cell receptor (TCR) KO mice also developed spontaneous colitis [232]. Additionally, IBD was developed in transgenic (Tg) models where a specific gene has to be selected, overexpressed, and introduced into such models leading to intestinal inflammation. More than 40 different types of genetically engineered mouse strains in addition to other strains possessing congenital gene mutations develop spontaneous colitis and/or ileitis. Genetically manipulated models include STAT3 KO, TGFβ-1 KO, Caspase-8 KO, XBP-1 KO, Keratin-8 KO, WASP KO, NEMO KO, STAT4 Tg, IL-7 Tg, Gp39 Tg, and many others [230]. Moreover, it has been experimentally shown that the mating of two different inbred strains of mice having different genetic backgrounds caused spontaneous models of intestinal inflammation, and such models represent congenic models of IBD [226]. Congenic models, the most relevant to human conditions and diseases, are characterized by their complexity and similarity to human immunopathogensis, thus they are poorly understood by researchers [233]. They include C3H/HeJBir, SAMP1/Yit, and cotton-top tamarin models [230]. However, most of the researchers tended to use the chemically induced models instead due to the direct procedures they require, in addition to the rapid onset of inflammation they develop consequently [222, 234].

Compared to genetically modified models, chemically induced models are better in avoiding different developmental abnormalities that can be the result of a certain genetic defect that takes place in genetically engineered models. Additionally, they have an advantage over the cell-transfer system which requires the use of immunodeficient mice to be successful. However, chemical induction is applied in immunocompetent mice which is much easier in performing experiments. Furthermore, researchers have to carefully select the model taking into consideration the standardized experimental design

being used because such factors are very critical in affecting the extent of the disease development. It should be noted that such models are easily used in the field of IBD research. Specifically, chemically induced models serve as pivotal tools in mimicking some of the histopathological and pathophysiological aspects of the disease, thus paving the way to important therapeutic interventions and strategies to treat IBD. Although these models have provided great insights into the disease pathogenesis, still none of them have fully elucidated its complexity and heterogeneity in humans [235]. There are several chemically induced models including dextran sulfate sodium (DSS), 2,4,6-trinitrobenzene sulfonic acid (TNBS), dinitrobenzene sulfonic acid (DNBS), acetic acid, oxazolone, carrageenan, peptidoglycan polysaccharide, immune complex, indomethacin, and cyclosporine A [218]. However, the most commonly used IBD models are the DSS and TNBS as models of colitis [230].

E. DSS Model of Colitis

The DSS IBD model, adopted in this research, is one of the most commonly used chemically induced models of IBD due to several reasons. First of all, DSS is a water-soluble polysaccharide that is made up of dextran and sulfated anhydro-glucose unit [236], carrying a highly negative charge due to its sulfated groups. Its molecular weight ranges from 5 to 1400 kDa. It has been shown that supplementing the drinking water of murine models with DSS (40-50 kDa) leads subsequently to murine colitis [237]. Additionally, Kitajima et al. reported that a mild colitis is caused by a low molecular weight DSS (5 kDa) while a high molecular weight (500 KDa) doesn't contribute to colonic injury [238]. Due to unknown reasons, the huge pathology of the disease induced by DSS has been limited to the large intestine, specifically the distal colon [239]. Laroui

et al. have suggested that DSS-induced colitis is associated with the formation of nano-lipocomplexes with medium-chain-lengthy fatty acids (MCFAs) in the colon [240]. It has been shown that the oral administration of DSS via drinking water in mice had disastrous effects on their gut epithelial cells and the integrity of their intestinal mucosal barrier [241]. It mainly contributes to epithelial injury in the gut; exposing the mucosal and submucosal immune cells to foreign antigens and promoting a severe inflammatory response [235]. Consequently, an upregulation in the levels of nitric oxide, inducible nitric oxide synthase (iNOS) and chemokines has been noticed [242, 243]. Yan et al have evidently reported the increase in the levels of proinflammatory cytokines after the administration of DSS also [244]. The assessment of different inflammatory mediators has played an important role in understanding the pathology of DSS-induced colitis; it included TNF-α, IL-1β, IL-6, IL-17, IL-10, TGF-β, mucin, TLR2/4 gene expression, and MPO activity [219, 222]. In addition, several changes in the expression of the tight junctions have been reported one day post treatment with DSS [245].

Several researchers have used the DSS model of murine colitis to study the association between colonic inflammation and Gall-R expression. The results of their experiment have evidently demonstrated an induction of a progressive and severe colitis whose severity has been associated with the activation of NF-κB and a further increase in Gall-R expression. Researchers assumed that Gall-R expression might be a vital component in the excessive fluid secretions detected in IBD [246, 247]. In 2000, Soriano et al. used this model to study the involvement of several adhesion molecules (CAMs) considering their important role in the pathogenesis of human IBD (UC and CD), specifically in regulating the recruitment of leukocytes during inflammation. They have studied three different endothelial CAMs; the vascular cell adhesion molecule 1 (VCAM-

1), intercellular adhesion molecule 1 (ICAM-1), and mucosal addressing cell adhesion molecule 1 (Mad-CAM-1) which have been implicated in various acute and chronic inflammatory diseases [248].

Although the experimental protocol of DSS-induced colitis is easy, several factors affect its efficiency in animal models, involving concentration of DSS (usually 1% to 5%), molecular weight of DSS, duration and frequency of treatment (acute or chronic), strain (BALB/c and C3H/HeJ mice strains are more susceptible to the disease) and gender (males show increased susceptibility) of the animal model, and the microbial environment of the animals (if it is pathogenic or germ-free) [237, 249]. Interestingly, studies have highlighted the effect of enteric bacteria on acute colitis; it has been validated that the enteric bacteria suppress the development of the disease. Researchers kept mice in germ-free conditions and after a while, they detected a massive intestinal bleeding in those mice as a result of DSS administration contributing to the development of lethal colitis [250].

Several acute histological modifications occur at the level of the colon after a short exposure to a relatively high dose of DSS. They include mucin depletion, epithelial erosion and necrosis contributing to the disappearance of epithelial cells. Araki et al. have proven that an acute phase of DSS is mainly characterized by the presence of a leaky epithelial barrier caused by a decreased proliferation of the epithelium in addition to an increased level of apoptosis [251]. It should be noted that during inflammation, neutrophils migrate to the inflamed site, thus forming cryptitis and crypt abscesses. Although those are considered to be important histological aspects of human IBD, they are rarely reported in DSS-induced colitis [246]. Several clinical symptoms have been observed when inducing acute colitis such as bloody stools, weight loss, diarrhea,

hunched back, and death [239]. However, a continuous administration of relatively low dose in a cyclic manner causes chronic lesions. A chronic phase of DSS-induced colitis is mainly characterized by an infiltration of mononuclear leukocytes, disarray in the crypt architecture; where the gap between the muscularis mucosa and crypt bases is widened, in addition to the formation of deep mucosal lymphocytosis and transmural inflammation [222].

Interestingly, the DSS model is characterized by its simplicity, controllability, reproducibility and rapidity in inducing inflammation [239]. Further, it has been used in screening potential therapeutic substances. Studies have elucidated that the induction of strong acute colitis by DSS in lymphopenic immunodeficient mice (SCID and Rag-/-) appeared to be linked to the innate immune cells and not adaptive ones [252, 253]. Thus, acute models have offered insights only into innate immunity being more relevant in studies that address the physiology of acute flares, wound healing, and resolution of acute inflammation. Thus, providing limited information about the pathogenesis of IBD. Whereas, chronic models have been implicated in studying the adaptive immunity which seems to be an advantage in studying the complications of chronic diseases such as neoplasia and tissue fibrosis [235].

F. Syndecan-1 knock-out Animal

1. Characteristics of Syndecan-1 and its Role in Health:

Syndecan-1 or (CD138) is a member of the heparan sulfate (HS) proteoglygan family which is mainly expressed on the glycocalyx of epithelial cells, endothelial cells, and plasma cells [254, 255]. It consists of three different domains; a long variable ectodomain

, a single transmembrane domain, and a short cytoplasmic domain [254]. SDC-1 mainly modulates different proteolytic activities [256], and plays a crucial role in a plethora of biological processes involving cell proliferation, differentiation and redifferentiation [257]. In addition, it acts as a co-receptor for different tyrosine kinase receptors [258, 259]. For instance, it modulates and stimulates the activity of the complex of basic fibroblast growth factor (bFGF) and the FGF receptor, thus leading to keratinocyte proliferation and consequently to improved wound healing [260]. In addition to its role in integrin activity [261] and migration [262], it has been implicated in development, tissue repair, tumor progression and inflammatory responses. [255] J. Angsana has proven that sdc-1 expression on macrophages enhances macrophage's motility in human and murine models and is significantly associated with an anti-inflammatory M2 polarization [263]. Sdc-1 has been involved in maintaining the function of the mucosa barrier. It seems to activate Stat3 leading to the restoration of the tight junctions' dysfunction in the cell, thus maintaining the epithelial integrity [264]. The extracellular domain of SDC-1can be shed from the cell surface and released into extracellular fluids by the action of distinct metalloproteinases, cytokines, chemotactic factors, as well as oxidative stress [265].

2. *Role of Syndecan-1 in Disease*

It has been shown that patients suffering from multiple myeloma, acute graft-versus-host disease, diabetic nephropathy, acute coronary syndrome, cardiogenic shock, acute myocardial infarction, cardiac fibrosis, and severe sepsis, in addition to patients undergoing dialysis or major vascular surgery have increased levels of soluble sdc-1 [265-275]. Additionally, it has been demonstrated that an elevated expression of sdc-1

has been mainly implicated in invasive growth and development of distinct tumor entities involving breast cancer and myeloma cancer [276-280]. Moreover, a clinical study has proven that colorectal carcinoma patients have significant high epithelial sdc-1 levels which is mainly associated with tumor size [281]. A further study highlighted the association between serum sdc-1 levels and Systemic Lupus Erythematosus (SLE), showing that this level is significantly upregulated in SLE patients with active disease compared to individuals with inactive one [282]. Knowing that sdc-1 plays a pivotal role in migration, it has been shown that the gene knockout and overexpression of sdc-1 both lead to a reduced migration in a wound healing process[260, 283]. H Salminen-Mankonen et al. have proven that sdc-1 expression is upregulated in the knee joints of Dell mice during the early stages of articular cartilage degeneration, and that sdc-1 positive cells have been localized in chondrocytes that are close to degenerated areas [284]. G. Diab et al. has demonstrated the association between sdc-1 expression and Rhematoid Arthritis (RA) as well, showing that after a six-week of antirheumatic treatment, serum levels of sdc-1 have been reduced reflecting the reduction in sdc-1 shedding from glycocalyx [285]. Furthermore, it has been reported that sdc-1 deficiency leads to skin inflammation in experimentally induced psoriasisform dermatitis mouse models and other models leading to exaggerated airway hyperresponsiveness [286, 287].

3. *Role of Syndecan-1 in IBD*

It has been shown that sdc-1 plays a crucial role in intestinal inflammation. Patients with chronic IBD have shown downregulated levels of sdc-1 in their colons [288, 289]. Specifically, patients with ulcerative colitis (UC) have increased sdc-1 shedding from their cell surface, leading to the activation of several inflammatory factors and higher

circulation of neutrophils. Further results in that study have shown that cell-surface anchored sdc-1 inhibits the secretion of pro-inflammatory cytokines due to its suppressed ectodomain shedding, causing amelioration of intestinal inflammation and neutrophil transmigration [290].

It has been demonstrated that loss of sdc-1 contributes to an increase in epithelial permeability causing protein-losing enteropathy which is similar to the case of IBD where a defect in the intestinal epithelial barrier takes place [291]. It has been evidently revealed that inflammation disrupts the normal healing process in IBD, and this is maybe due to the loss of sdc-1, leading to a decreased rate of healing. Well, patients with ulcerated mucosa have actually shown a lack in sdc-1 expression and a decreased rate of tissue repair, consequently leading to a state of persistent chronic inflammation [291-293]. Another study has demonstrated the correlation between the release of TNF-α and IL-1β by lamina propria mononuclear cells in active IBD and degree of mucosal inflammation [294]. The stimulation of HT29 and T84 colonic epithelial cells with TNF-α and IL-1β leads to sdc-1 shedding, and thus downregulation of the epithelial sdc-1 expression [292]. Additionally, it has been reported that patients with CD have reduced sdc-1 levels which is mainly caused by upregulated TNF-α levels [292]. Importantly, several murine models of IBD have similarly shown reduced levels of sdc-1 [295-297]. For instance, Floer M. et al have proven that syndecan-1- null mice have delayed and exaggerated recruitment of leukocytes, impaired mucosal repair, and worsening of DSS colitis symptoms with high lethality [298]. Another study has revealed that a DSS-induced model of colitis is characterized by an increased syndecan-1 shedding which leads to a reduced expression of anchored sdc-1 in the colonic mucosa. This proves explicitly the correlation between sdc-1 shedding process, decreased mucosal sdc-1 expression, and sustained inflammation

[299].

G. Management of IBD

Most available treatment protocols for IBD aim to maintain patients in remission, ameliorate the symptoms of the disease, reduce its severity, and prevent surgeries [300]. It is crucial to study the symptoms of patients very well, and to specify the type of the disease before giving them a certain treatment. Specifically, treatment for IBD is mainly based on pharmaceutical drugs and self-care [301]. “Aminosalicylates” were the first to be used to treat IBD. It involves two different drugs, “Salazopyrin” which is the oldest one in this category, and “Mesalazine” which is the main aminosalicylate used in IBD management nowadays [302]. It has been proven that the effectiveness of aminosalicylates depends on the dose administered to patients [303]. Moreover, corticosteroids mainly “Hydrocortisone” and “Prednisolone” have been commonly used for treatment. Studies have shown that corticosteroids can be used either alone or in combination with “Mesalamine” which might be better and more effective [304].

Knowing that TNF-α is one of the proinflammatory factors produced during inflammation, several anti-TNF agents such as “Infliximab”, “Adalimumab”, and “Golimumab” have been produced as classic IBD drugs. The combination of “Infliximab” and “Azathioprine” has been shown to be efficient in maintaining remission in both CD and UC patients [305-307]. However, “Vedolizumab”, an α4β7 integrin blocker, is transcribed for those who do not respond to anti-TNF therapy [308, 309].

In addition, classical immunosuppressive drugs such as Azathioprine, Methotrexate, and Cyclosporine “A” have been used in IBD therapy. Azathioprine has been shown to

have a positive effect in both UC and CD cases while the effect of "Methotrexate" has been limited only to CD cases. Interestingly, Cyclosporine "A" has efficacy in only UC cases [310-313]. It is very essential in patients who did not respond to corticosteroid treatment, for that it might prevent them from surgery. However, after one year of cyclosporine treatment, it has been proven that most of the patients might require surgery [314]. Cyclosporine "A" and Methotrexate have been shown to inhibit the production of proinflammatory cytokines and stimulate apoptosis [315-318].

H. Cyclosporine

1. Definition of Cyclosporine "A" and its Role in Health

Cyclosporine "A" is a large hydrophobic, cyclic, organic molecule that was extracted from the soil fungus *Tolypocladium inflatum Gams* in 1971 [319, 320]. After organ transplantation, it acts as an immunosuppressant to prevent rejection. In addition, it has a pivotal role in ameliorating chronic inflammatory diseases [321]. Cyclosporine downregulates the activation of T-lymphocyte by blocking the production of IL-2 through inhibition of the calcineurin pathway [322-324]. Its mechanism of action starts when it binds to cyclophilin, an intracellular binding protein for CysA, forming a (Cyp-Cys) complex. The formed complex binds to calcineurin, a protein phospahatase that has a vital role in T-lymphocyte activation, and prevents the dephosphorylation of the nuclear factor of activated T-lymphocytes (NFAT). Thus, the cytosolic component of NFAT can't enter the nucleus, and NFAT complex is not formed. Thereby, the production of IL-2 and its receptor are further inhibited [325]. Not only IL-2 is inhibited when the complex binds to calcineurin since calcineurin is a regulatory factor that leads to the transcription of several cytokine genes such as IL-3, IL-4, TNF-α, granulocyte-macrophage colony-

stimulating factor (GM-CSF), and interferon-γ (IFN-γ). Knowing that calcineurin controls the phosphorylation of nitric oxide synthase (NOS), and knowing that CysA prevents the calcineurin pathway, it has been shown that CysA reduces the production of nitric oxide, thus affording an anti-inflammatory activity [326].

In addition, it has been proven that CysA inhibits the activation of c-Jun N-terminal kinase (JNK) and p38 signaling pathways [316]. Interestingly, it contributes to the apoptosis of CD4+ T-lymphocytes [327] and prevents the activation of antigen presenting cells, specifically B-lymphocytes [324]. Evident studies have shown that it plays a critical role in impeding granulocyte infiltration, thus leading to an increased vascular permeability [326].Consequently, CysA inhibits NF-κB and reduces the expression of cellular adhesion molecules, contributing further to a decreased adhesion contact, transendothelial migration, and neutrophils' infiltration [328]. Moreover, it has a pivotal role in the blockade of chemotactic migration and inhibition of superoxide and IL-8 production in a dose-dependent manner [319].

2. *Role of Cyclosporine "A" in IBD*

It has been demonstrated that cyclosporine A is an effective treatment in reducing colitis [329]. Since the mid-1980s, it has been used as a substituent for corticosteroid therapy in UC patients [304]. Additionally, it has been reported that cyclosporine therapy plays a pivotal role in fistula closure in 44% of CD patients [330]. It is known that cyclophilin is an endogenous protein secreted by activating macrophages and has a pro-inflammatory effect. When CysA binds to cyclophilin, it neutralizes its chemotactic properties, thus inhibiting intestinal inflammation [319]. Several studies have reported an

increase in the expression of several cytokines such as TNF-α, IL-4 and IL-17 in IBD patients and IL-10 in UC patients [196, 331, 332]. However, a significant decrease in their expression has been detected in IBD groups treated with CysA [315]. It has been demonstrated that cyclosporine A is an effective treatment in reducing colitis [329]. Sumit Sharma et al. has proven that TNBS induced colitis in New Zealand rabbits is ameliorated after using a targeted delivery system of cyclosporine, and that the expressions of TNF-α, IL-6, IL-10 are downregulated in treated animals [333]. Additionally, in a DSS-induced model of colitis, cyclosporine has ameliorated body weight loss, epithelial apoptosis, and colonic mucosal destruction[334]. Similarly, it has particularly prevented the presence of ulcerations and lymphocytes' infiltration in treated animals. In addition, it has avoided colon shortening which is usually detected as an inflammatory marker, and has decreased the production of pro-inflammatory cytokines [335]. In another study, Holger Sann et al. has shown that systemic and colonic anti-DSS effects are produced after the oral treatment of cyclosporine A in a dose-dependent manner [335]. However, serious adverse effects have been evaluated in IBD patients taking intravenous CysA (4 mg/kg/day) followed by oral CysA (8 mg/kg/day). They involve renal insufficiency, hypertension, hypomagnesemia, paresthesias, seizures, anaphylaxis, infections, and death [336]. Along this line, research has been active towards finding ways to decrease these side effects of CysA; coupling it with Cyp is one option to work on.

I. Cyclophilin

1. Definition of Cyclophilin

Cyclophilins (Cyps) are ubiquitous proteins which are well conserved and present

in both prokaryotes and eukaryotes [337]. The peptide bonds of Cyps are isomerized from trans form to cis form at specific proline residues, and this isomerization is mainly catalyzed by the peptidyl prolyl isomerase activity which facilitates protein folding [338]. Cyps in human are intracellular and extracellular, and they consist of 16 family members having different structures [337]. Among them, CypA is the most abundant member that was first purified from bovine thymocytes and accounts for ~ 0.1-0.6% of the total cytosolic proteins. It has been shown to be the primary cytosolic binding protein of cyclosporine A [339-341]. Macrophages, vascular smooth muscle cells, endothelial cells, and fibroblast-like synoviocytes secrete CyPA, and it plays a role in both autocrine and paracrine signaling pathways [342-345].

2. *Role of Cyclophilin in Health*

CypA stimulates the intercellular communication and is characterized by its chemoattractant influence on inflammatory cells which consequently aggravates oxidative stress and inflammation [337]. It has been proven that extracellular Cyp is a ligand for the surface receptor CD147, and that the binding of Cyp to CD147 evidently contributes to its chemotactic activity [346]. It should be noted that the presence of heparan sulfate proteoglycans (HSPGs) is very important in the signaling activity of CypA because they act as primary binding sites for CypA on their targets. If HSPGs are removed from the cell surface of target cells (neutrophils), the signaling responses to cyclophilins will be accordingly abolished, and therefore the chemotaxis and adhesion of T cells and neutrophils will be eradicated [346, 347]. Moreover, it has been shown that CypA is implicated in protein folding, trafficking, and assembly, in addition to its role in the activation of T cells and cell signaling [337, 348]. Interestingly, it is related to

molecular chaperones because of its enzymatic properties and cellular localization, in addition to its critical role in protein folding [349].

3. Role of Cyclophilin in Diseases and IBD

It has been reported that CypA is involved in many key processes which underlie different human diseases [337]. Growing body of evidence has shown that an upregulated level of extracellular CypA is present in patients with inflammatory responses such as asthma [350], sepsis [351], and coronary artery disease [352]. The table below shows the involvement of cyclophilin in different pathologies.

Table 1: Cyclophilin's Role in Diseases.

Cardiovascular Disease	Angiotensin II- induced Abdominal aortic aneurysm (AAA) was prevented by the deletion of CypA in mice.	[353]
	CypA induces ROS production and proliferation of cardiac fibroblasts, thus causing cardiac myocyte hypertrophy.	[354]
	Patients with myocardial infarction and unstable angina have significant high serum CypA concentration compared to patients with stable angina.	[355]
Rheumatoid Arthritis	CypA is the major constituent in macrophages of the synovial lining layer in RA.	[356]
	In RA, the destruction of cartilage and bone are mainly caused by CyPA-CD147 interaction which upregulates MMP-9 expression and causes the adhesion of macrophages to the extracellular matrix.	[357]
Diabetes	Plasma levels of CypA in diabetic patients are much higher than that in healthy individuals.	[358]
Asthma	It was found that asthmatic mice have an elevated level of extracellular CypA in their airways, and that the airway epithelial mucin is reduced after anti-CD147 treatment.	[359]
Sepsis	The expression of CypA is elevated in a mouse model, specifically in the liver after sepsis.	[360]
Periodontitis	Inflamed gingival tissues has an elevated cyclophilin expression compared to healthy tissues.	[361]

CypA plays a role in migration of dendritic cells [362] ,activation of ERK1/2 MAPK pathway, and NF-κB phosphorylation, therefore causing proliferation of macrophages [363]. It has been further demonstrated that the stimulation of cyclophilin leads to the activation of AKT and NF-κB pathways, and subsequently to the upregulation of antiapoptotic protein Bcl-2 levels in endothelial cells [364]. Similarly, the binding of cyclophilin to the cell surface receptor CD147 has been shown to increase both ERK and AKT signaling [340, 346, 365]. Thus, the main signaling pathways that are implicated in CypA/CD147 interactions are ERK1/2, AKT, MAPK, and NF-κB pathways. Furthermore, the association between elevated CypA levels and cancer has been evidently illustrated [364, 366, 367]. Moreover, CypA plays a pivotal role in stimulating monocytes to produce matrix metalloproteinases MMP-9 and MMP-2 in inflammatory diseases [368]. The increase of MMP-9 has been interestingly reported in both inflammatory bowel diseases UC and CD [369]. Evident studies have shown that the reduction of inflammation and intestinal mucosal damage in DSS-induced ulcerative colitis mice is mainly caused by the absence of MMP-9 expression [370]. More studies have highlighted the role of tissue inhibitor of matrix metalloproteinase (TIMP-1) in IBD, showing that TIMP-1 level is elevated in IBD patients [371, 372]. It has been evidently shown that the expression of TIMP-1/MMP-9 is regulated by the high expression of serum CypA in IBD by the activation of ERK1/2 which contributes to the development of IBD, specifically ulcerative colitis.[357, 373, 374]. A study has reported a significant increase of CypA levels in the colonic tissue [375], serum [376], and lymphocytes of UC patients [376] showing that CypA has a crucial proinflammatory role in IBD.

J. CD147

1. Definition of CD147 and its Role in Health

CD147, known as ECM metalloproteinase inducer or "EMMPRIN", is a 50-60 kDa transmembrane glycoprotein which is highly glycosylated. It is made up of two extracellular immunoglobulin domains: a transmembrane domain and a cytoplasmic domain containing 39 amino acids [377]. It is mainly expressed on macrophages, endothelial cells, and human peripheral blood cells, in addition to cultured cells. Interestingly, it is implicated in a plethora of cellular processes such as signal transmission, cell adhesion, neural functions, and reproduction.

Additionally, its role has been illustrated in inflammation, production of matrix metalloproteinases, cancer development, and HIV infections [377, 378]. Moreover, EMMPRIN has also participated in transport of calcium [379] , chaperone functions, [380] development of blood brain barrier [381] , and neutrophil chemotaxis. [346] Accumulating evidence has shown that CD147 may bind to integrins. In a study, the colocalization CD147 with β1 integrins has been illustrated in adhesive cellular areas [382]. Well, as mentioned before CD147 has been implicated in several signaling pathways such as: MAPK p38, NF-κB, ERK1/2, and PI3K pathways [383-385].

2. Role of CD147 in inflammation and IBD

It has been proven that CD147 plays an essential role in modulating inflammation and immune responses [386]. Experimental models of human diseases such as rheumatoid arthritis [387], multiple sclerosis [388], asthmatic lung inflammation [359], and myocardial ischemia/reperfusion injury [389] have demonstrated a reduction in

inflammation and disease severity when targeting CD147. Regarding IBD, an elevated expression of CD147 has been detected in the intestinal mucosa of IBD patients. Similarly, serum CD147 has been also elevated in addition to disease activity (DAI) in these patients. This shows that CD147 is a pivotal proinflammatory biomarker for IBD [390].

K. Syndecan-1/Cyclophilin/CD147 interaction

Rachel Pakula et al. and her colleagues have investigated the role of Syndecan-1/Cyclophilin “B”/CD147 association in the activation of p44/42 mitogen activated protein kinases and further stimulation of cell adhesion and chemotaxis [391].Their previous investigations showed that cyclophilin plays an essential role in chemotaxis and integrin-mediated adhesion of T lymphocytes. They have interestingly shown that such processes involve the collaboration with two types of binding sites, CD147 and cell surface Heparan sulfate (HS). In this study, they have demonstrated that only syndecan-1 among other syndecans has a physical association with CD147. They have proven that the addition of antibodies to syndecan-1 or the knockout of syndecan-1 gene have both contributed to a reduction in the Cyp-induced p44/42 activation, and thus a reduction in the migration and adhesion of T cells. Knowing that cyclophilin evokes different responses by a specific mechanism which involves the prolyl isomerization of CD147, findings have shown that syndecan-1 serves as a crucial coreceptor for cyclophilin, and it acts in collaboration with CD147, thus stimulating the activation of p44/42 MAPK pathway, leading to adhesion and migration of T cells. In addition, the pretreatment of T cells with a MAPK pathway inhibitor has consequently inhibited ERK activation, and thus inhibiting Cyp-mediated cell adhesion to fibronectin. Similarly, the addition of

antibodies to CD147 has contributed to the same previous result showing that CD147 has a pivotal role in the signaling pathways triggered by Cyp. This has significantly shown that Cyp evokes ERK activation through CD147 and cell adhesion by a process that involves the functional activity of cell surface heparan sulfate proteoglycan "HSPG" (syndecan-1). Furthermore, it has been hypothesized in this study that a physical interaction between CD147 and HSPG occurs, leading to the formation of an active complex at the cellular membrane of T lymphocytes. Specifically, the association between syndecan-1 and CD147 has been demonstrated despite the absence of Cyp. Well, the presence of Cyp has subsequently contributed to the stabilization or even increase in the association between HSPG and CD147, thus forming an active ternary complex with CD147 at the membrane of T cells. The disturbances in CD147/syndecan-1 complex which occur when adding antibodies to either CD147 or syndecan-1 have shown to neutralize Cyp-mediated ERK activation, thus decreasing the adhesion of responsive T-cells to fibronectin. Knowing that CD147, syndecan-1, and cyclophilin are all incorporated in the inflammatory responses, data in this study have suggested that the interaction between the three subunits may have a pivotal role in the pathogenesis of several inflammatory diseases including IBD.

L. Probiotics

1. Definition of Probiotics

Probiotics are active nonpathogenic living microorganisms which confer positive effects to the host when consumed in sufficient doses [392]. Their efficient role has evidently been demonstrated in the treatment of several human diseases [393-395]. It has been shown that probiotics have specifically beneficial effects in treating inflammatory

diseases such as arthritis [396],ulcerative colitis [397, 398],and experimental colitis [399-401].

2. Mechanisms of Action of Probiotics

No doubt that the effect of probiotics mimics that of the intact microbiota during intestinal homeostasis. Their mechanism of action first of all depends on their strain and involve the assembly of antibacterial components such as bacteriocins, lactic acid, and hydroperoxides. Second, they play a crucial role on the intestinal epithelial surface where they have the ability to block completely the epithelial binding sites, thus preventing bacteria from binding. Third, they fortify the integrity of the mucosal barrier by upregulating the tight junction molecules. Also, they have the ability to destroy toxin receptors and modify pH, thus creating a more acidic milieu which is unfavorable for the survival of proinflammatory bacteria. Finally, they tend to compete for indispensable nutrients that are needed for the survival of the host [132, 402-404]. Moreover, probiotics are very well known for their pivotal role in regulating immunity and assisting in the defense mechanism of the immune system. Actually, they are involved in the activation of Toll-like receptors and differentiation of T-helper cells, and the production of mucosal antibodies (IgA). Furthermore, they play a vital role in a plethora of mechanisms. They stimulate phagocytosis and natural killer (NK) activity and provoke apoptosis of T cells. They also decrease proinflammatory cytokines (TGF-α, IFN-γ), and increase the secretion of anti-inflammatory cytokines (TGF-β,IL-10) [405-407].

3. *Effectiveness of Probiotics in Clinical Cases*

Interestingly, the effect of probiotics in the reduction of inflammation has been established in various clinical studies. For instance, Li et al. has revealed the essential role of Lactobacillus acidophilus, Streptococcus, and Bifidobacterium bifidum in the downregulation of the proinflammatory cytokine IL-1β and upregulation of IL-10 which is a well-known anti-inflammatory cytokine [408]. Additionally, Chen et al. has highlighted the important role of Escherichia coli Nissle 1917 and VSL#3 which is mainly a food product that involves (L. paracasei, L. plantarum, L. acidophilus, L. delbrueckii, B. longum, B. breve, B. infantis, and Streptococcus thermophilus) in having a positive impact on UC patients [409]. Moreover, it has been revealed that the supplementation of Bifidobacterium-fermented milk (BFM) causes a reduction in the concentration of a molecule which is involved in colitis remission (Luminal Butyrate), showing an improvement in the function of colorectal mucosa [410].

4. *Effectiveness of Probiotics in Animal Models of Colitis*

A study has demonstrated the effect of a seven-days pretreatment with L. plantarum DSM 9843, Bifidobacterium sp. 3B1 and B. infantis DSM 15158 in a DSS-model of colitis. Accordingly, a reduction in both disease activity and bacterial translocation have been evidently revealed in rats [411]. Another study by McCarthy et al. has shown the effect of Lactobacillus salivarius and Bifidobacterium infantis strains in the IL-$10^{-/-}$ model where a significant reduction in the pro-inflammatory cytokines TNF-α, IL-12, IFN-γ has been detected, consequently leading to a reduction in the mucosal inflammation [412]. Similarly. Madsen et al. has validated the effect of VSL#3 in the IL-$10^{-/-}$ mice

model where a significant reduction in the pro-inflammatory cytokines (TNF-α and IFN-γ), colitis severity, and histological scores have been detected as well [413].

Moreover, Fujiwara et al. has shown the effect of Bifidobacterium longum in a DSS-mouse model of colitis where a reduction in colonic shortening and disease severity have been revealed [414]. Another study has shown that the supplementation with other strains of Bifidobacterium such as B. animalis subsp. lactis BB12 has caused a reduction at the level of colon length and colon's histology, in addition to the decrease in TNF-α levels and in the apoptosis in intestinal epithelial cells [415]. Interestingly, it has been proven that probiotics have a pivotal role in supporting and intensifying gut-tight junctions, in addition to affecting the proportions of T cell subpopulations. It has been shown that the mixture of Bifidobacterium, Lactobacillus acidophilus, and Enterococcus in a DSS-model of colitis has subsequently contributed to a reduction in the total number of T cells in the colon and peripheral blood, an increase in the number of T reg cells, and strengthening in the gut-tight junctions [416]. In other models of colitis such as TNBS models, it has been demonstrated that the oral treatment with Bifidobacterium bifidum has reduced the inflammatory state, histological scores, and macroscopic damage, and it has avoided weight loss of the animals [417, 418]. However, no beneficial impact has been detected at the levels of histological scores, weight changes, and gut permeability in TNBS rat models of colitis after the supplementation of Lactobacillus plantarum species 299 in contrast to other studies and reports. This may be to several factors including the used dose, the severity of the disease, the animal model of colitis (mice or rats),and the probiotics' strains used which may have different properties affecting their mechanisms of action [419].

M. Cluster of differentiation 3 (CD3)

1. Definition of (CD3)

Cluster of differentiation 3 or CD3 is a cell surface multimeric protein complex, identified as a T3 complex. It mainly constitutes of four different subunits; epsilon, gamma, delta and zeta, where they dimerize and form three distinct pairs. These subunits are chains of integral membrane glycoproteins that have a non-covalent association with the T cell receptor (TCR). They are necessary for TCR cell surface expression and signaling transduction as well [420-422]. It has been shown that the expression of CD3 has initially taken place in the cytoplasm of the developing T cells. As the T cell maturation process progresses, the cytoplasmic CD3 is subsequently abolished, and CD3 antigen is only presented on the cell surface [421]. At the pathophysiological level, the binding of antigen peptides leads to the stimulation of TCR, thus evoking the phosphorylation of (ITAMS) which are intracellular immunoreceptor tyrosine-based activation motifs found in the CD3ζ subunits [422].

2. (CD3) in Diseases and Inflammation

CD3 antigen has evidently been demonstrated as an ultimate immunohistochemical T cell marker in tissue sections to distinguish between normal T cells and T cell neoplasms such as lymphomas and leukemias [423, 424]. Additionally, CD3 has been interestingly shown as a marker for various diseases such as collagenous colitis [425],lymphocytic colitis [426],and coeliac disease [427]. Moreover, Takeuchi et al. has proven the association between the deficiency in the CD3 ζ polypeptide chain and the

development of Systemic Lupus Erythematosus (SLE), an auto-immune disease [428]. Furthermore, deficiencies in CD3 have been linked with the development of “Severe Combined Immune Deficiency” (SCID) where T cells are either defective in their function or production [429, 430]. It has been also demonstrated that the addition of antibodies specific to CD3 in animals has consequently stimulated tolerance to allografts [431].

In a murine model of “Pneumocystis Pneumonia”, it has been proven that anti-CD3 antibody treated to mice has evidently reduced inflammation after one week, and improved their health consequently compared to mice receiving control antibody [432]. Another study has revealed the association between the incubation of intestinal tissues from IBD patients with “Otelixizumab”, an anti-CD3 antibody, and the reduced production of pro-inflammatory markers (IL-17A, IFN-γ). In contrast, it has been shown that an increased production of anti-inflammatory cytokine IL-10 has been detected post-treatment with anti-CD3. This indicates that CD3 is an inflammatory marker that is upregulated during inflammatory diseases, including “IBD” [433].

N. “Phosphorylated Protein Kinase B” (pAKT) in Health and Diseases

The PI3K/Akt pathway is one of the crucial pathways that control several mechanisms such as cell proliferation, differentiation, and survival by inhibiting other apoptotic processes. It has been evidently shown to be activated in various types of cancers [434-438]. Phosphatidyl inositol bisphosphate (PIP2) is converted to phosphatidyl inositol trisphosphate (PIP3) by the action of PI3K enzyme. Subsequently, the formed (PIP3) translocates Akt to the plasma membrane where its phosphorylation

process takes place by the action of 3-Phosphoinositide-dependent kinase1 (PDK1) enzyme [439]. Thus, the activated phosphorylated form of Akt (pAKT) is formed which further modulates the function of a plethora of substrates incorporated in the regulation of cell growth and survival.

It has been demonstrated that Akt signaling controls the differentiation of Th1 cells [440, 441]. Further studies have proven that the activation of PI3K/Akt/mTOR pathway in the lymphocytes of murine models has displayed symptoms of systemic autoimmunity, showing the critical role of this pathway and its involvement in autoimmune disorders [442]. Moreover, it has been shown in one of the studies that "Beauvericin" ameliorates experimental colitis in mice through the inhibition of activated T lymphocytes by downregulating the PI3K/Akt signaling pathway. Hence, this pathway is upregulated in mice inflammatory bowel disease[443]. Another study has shown that its activation is also involved in human IBD as well as tumor invasion in the Piroxicam / IL-10−/− mouse model. The use of LY294002, a mast cell and tumor-associated macrophages (TAMs) inhibitor, has targeted the PI3K/Akt pathway, consequently impeding the progression of colitis. This indicates that the development of colitis and progression to cancer depend on the activity of stromal PI3K [444]. A study has revealed that fibrinogen stimulates vascular permeability through Akt activation and consequent microfilament depolymerization. A significant increase in the expression of phosphorylated Akt (pAkt) has been shown in the colons of DSS and TNBS-induced mice models of colitis [445]. Therefore, PI3K/Akt pathway is a therapeutic target for IBD.

In summary, Inflammatory Bowel disease or (IBD) is a complex multifactorial disease that is amplified by various genetic and environmental factors which disrupt the

immune microbiome axis and promotes intestinal inflammation. One of the used drugs that leads to the amelioration of the disease is “cyclosporine”, an immunosuppressant that prevents organ transplant rejection and forms a complex with its binding protein “cyclophilin”. Additionally, several studies have highlighted the positive role of probiotics in restoring the healthy mucosal state inside the gut while reducing inflammation. Moreover, the role of syndecan-1 has been explained in the previous sections showing that it is an essential cell membrane proteoglycan that participates in a plethora of biological processes such as adhesion, migration, and plays a vital role in inflammation. Besides, it has been shown that Syndecan-1/Cyclophilin /CD147 pathway has a pivotal role in the healing process during the onset of inflammation (IBD), but it has been rarely investigated in other research studies. Several inflammatory markers such as (IL-6, CD147, CD3 and pAKT) have been briefly discussed concerning their role in inflammation, specifically IBD.

CHAPTER II

OBJECTIVES AND HYPOTHESIS OF THE STUDY

Several studies have shown that cyclosporine “A” plays a role in the therapy of IBD in both clinical and animal models. However, as mentioned previously, various systemic adverse effects have been reported in treated patients and animals. Moreover, studies have highlighted the prominent role of Syndecan-1/Cyclophilin “A”/CD147 interactions in inflammation and their possible role in the pathogenesis of IBD. Furthermore, it is hypothesized that the combination of CypA and CysA might decrease the side effects of CysA, particularly in the presence of probiotics since research studies have evidently revealed the positive effect of probiotics on the course of IBD in reducing colonic edema, macroscopic damage, histological alterations, and clinical features in both human and animal models. Based on the literature, we hypothesized that:

- Cyclosporine “A” would reduce inflammation in syndecan-1 knock out mice IBD models due to its proven immunosuppressive therapeutic effects in IBD models.
- The cyclosporine “A” and cyclophilin A would have an added value to improve the healing process in IBD with limited side effects.
- Probiotics would have a positive effect on IBD in syndecan-1 knock outs in ameliorating inflammation and speeding up the healing process.

Accordingly, our project was based on three objectives:

- To evaluate the effect of cyclosporine “A” on the expression of several inflammatory markers (CD147, IL-6), CD3 and pAKT in syndecan-1 knock out mice.

- To assess the anti-inflammatory activity of cyclosporine “A” in the presence of extracellular cyclophilin “A” in Syndecan-1 knock out mice with DSS induced IBD.
- To explore the effect of probiotics on IBD alone and in presence of (Cyp-Cys) complex in Syndecan-1 knock out mice.

CHAPTER III

MATERIALS AND METHODS

A. Experimental Procedure

1. Animal Housing

A total of 42 adult Syndecan-1 mice were used. Approval of the experimental protocol was obtained from the Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut (AUB), Lebanon.

2. Induction of IBD and Treatments

DSS-induced IBD is a well-established model commonly used in experimental colitis studies. Optimized concentration of the pro-inflammatory agent Dextran Sodium Sulfate (DSS-Sigma-Aldrich,42867-100G) 1.5% was prepared in autoclaved water and administered to animals in their drinking water. Each DSS cycle consisted of 7 days of DSS followed by 2 weeks normal drinking water. The syndecan-1 null mice were divided into seven groups and treated as follows:

Cyclophilin A (Human recombinant expressed in E-coli) from Sigma - Aldrich (C3805-1MG) was injected intraperitoneally (IP) at a dose of 25µg /Kg/Day for one week starting day 7 of DSS administration. Similarly, Cyclosporine A (Novartis, SPE31) was administered by IP injections at a concentration of 200ug every other day for 2 weeks starting day 7 of DSS treatment. In addition, probiotics (P) used was a mixture of 7 strains of lactic acid-producing bacteria: *lactobacillus rhamnosus, saccharomyces boulardii, bifidobacterium breve, bifidobacterium lactis, lactobacillus acidophilus, lactobacillus plantarum and lactobacillus reuteri.* One capsule of (P) was dissolved in 1.75 L of

autoclaved tap water to reach a daily dose of 10^8 CFU per animal given for 2 weeks starting day 7 of DSS treatment.

3. Groups

- Not treated mice
- DSS only
- DSS+cyclophilin
- DSS +cyclosporine
- DSS+cyclophilin+cyclopsporine
- DSS+cyclophilin+cyclopsporine+probiotics
- DSS+probiotics

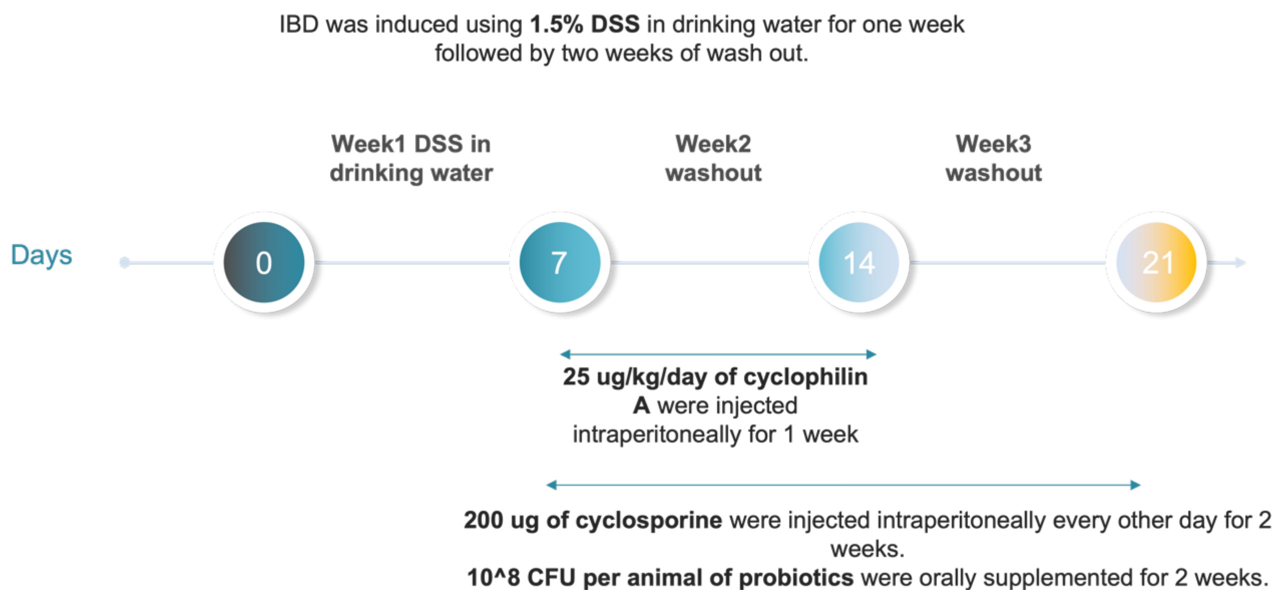


Figure 1: Treatments administered to the syndecan-1 knockout mice.

B. Histology

For the microscopic analysis, the proximal and distal sigmoid colon tissues were removed and fixed in 4% formaldehyde solution for 48 hours. Then formaldehyde fixed tissues were dehydrated using a series of alcohol concentrations. They were embedded with paraffin wax, cut into 5 µm sections, and stained with hematoxylin and eosin (H&E) to investigate the histopathological changes that might be detected at the level of the intestinal layers. In addition, Periodic Acid Schif (PAS) stain was also used to examine mucus and goblet cells. The slides were finally scanned using a “Zeiss” Microscope. The histological score of each animal within each group was assessed and calculated based on a histological scoring system shown in table (2) [446].

Table 2: Scoring system used to calculate the histological alterations in dextran sulphate sodium (DSS)-induced colitis. [446]

C. Western Blot

Histological Scoring System for DSS-Induced Colitis

Feature	**Score**	**Description**
Severity of inflammation	0	None
	1	Mild
	2	Moderate
	3	Severe
Extent of inflammation	0	None
	1	Mucosa
	2	Mucosa and submucosa
	3	Transmural
Crypt damage	0	None
	1	1/3 damages
	2	2/3 damaged
	3	Crypts lost, surface and epithelium present
	4	Crypt and surface epithelium lost

Western blotting analysis was performed according to standard protocols. Briefly, 100 mg of colon tissue homogenized with Laemli lysis buffer, centrifuged at 10,000 rpm for 10 min at 4ºC, supernatant was collected and kept in aliquots. Protein concentration was determined by the Lowry method using the DC™ Protein Assay Kit (#5000111). For gel loading, protein samples were boiled with loading buffer for 5 min at 95ºC and separated in SDS PAGE. Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratory, CA, USA). Tricolor Broad Protein Ladder (3.5-245 kDa) was used from Abcam (K00059-0250). The blots were then blocked with 5% BSA in Tris-buffered saline for 1 hour at room temperature, and then incubated overnight at 4°C at a dilution of 1:500 with their respective primary antibodies. Antibodies against IL-

6 (anti-mouse SC-57315), CD147 (anti-mouse SC-46700), CD3 (anti-mouse 20047) and pAKT (anti-mouse SC-57315) were purchased from Santa Cruz Biotechnology. Then, the primary antibodies were detected using a secondary antibody (horseradish peroxidase-conjugated anti-mouse; Abcam 97046 at a dilution of 3:40000), in which membranes were incubated for 1 hour at room temperature. The membranes were rinsed with TBST before and after the incubation with the secondary antibody. Finally, the immunoprecipitated protein bands were detected with ChemiDoc MP Imaging System-Biorad.

D. Statistical Analysis

Data are presented as mean± standard error (SE) (n=6 per group). Statistical analysis is performed using GraphPad Prism and results are analyzed by One -way analysis of variance (ANOVA) to detect the difference among the treatments. P-value < 0.05 is considered significant and is indicated by (*).

CHAPTER IV
RESULTS

A. Histology

1. Assessment of Histological Alterations by (H&E)

As shown in (Fig.2, a), the negative control group (no DSS) has a morphologically normal colon showing vertically oriented crypts lined by columnar epithelium. All intestinal layers are preserved and the leukocyte infiltration is in the normal range. In contrast, the DSS group suffered a severe localized inflammation extending from submucosa to mucosa layers with the presence of a marked edema between musculosa and submucosa. A massive increase of immune cell infiltrate and a complete loss of epithelial architecture are both observed. However, the remaining portions of the section are characterized by semi-preserved crypts with a low leukocyte infiltration; a spotted inflammation characteristic of Crohn's disease (Fig.2, b).

However, mice treated with (DSS+Cyp) and (DSS+Cys) exhibited a significant improvement in colonic histology showing a mild inflammation compared to the group treated with DSS only Fig. 2 (c, d). Only a one-third damage of the crypts is present with a very low level of edema between musculosa and submucosa. The overall architecture is almost preserved and a low level of leukocyte infiltration localized in the mucosa and submucosa layers with a little more in the DSS+Cys group where Peyer's patches are active.

In contrast to the (DSS+Cyp) and (DSS+Cys) separately treated groups, the treatment with (Cyp-Cys) complex along with DSS causes a detrimental effect on the tissue architecture where two–third of the crypts are lost in addition to the intensive

leukocyte infiltration spreading throughout the mucosa and submucosa. The thickening of the mucosa and submucosa layers is significant, as well as the notable areas of complete epithelial denudation (Fig. 2, e).

However, a slight reduction in the severity of inflammation is clearly observed after the addition of probiotics to (Cyp-Cys) along with DSS (Fig. 2, f). The inflammation is still invading the mucosa and submucosa layers and even between musculosa and submucosa compared to the group treated with DSS+(Cyp-Cys) complex. The heavy infiltrates are mainly localized between the musculosa and submucosa layers and also in the mucosa. The crypts are partially preserved, only one-third is distorted with very active Peyer's patches.

On the other hand, group treated with (DSS+probiotics) only, represented in (Fig. 2, g), showed a marked reduction in inflammation in the absence of (Cyp-Cys) complex. About one-third of the crypts is inflamed while the remaining crypts are preserved. A heavy leukocyte infiltration is present, probably at Peyer's patches. However, the remaining parts of the tissue (3/4) reflect a normal morphological image with very low levels of infiltrates.

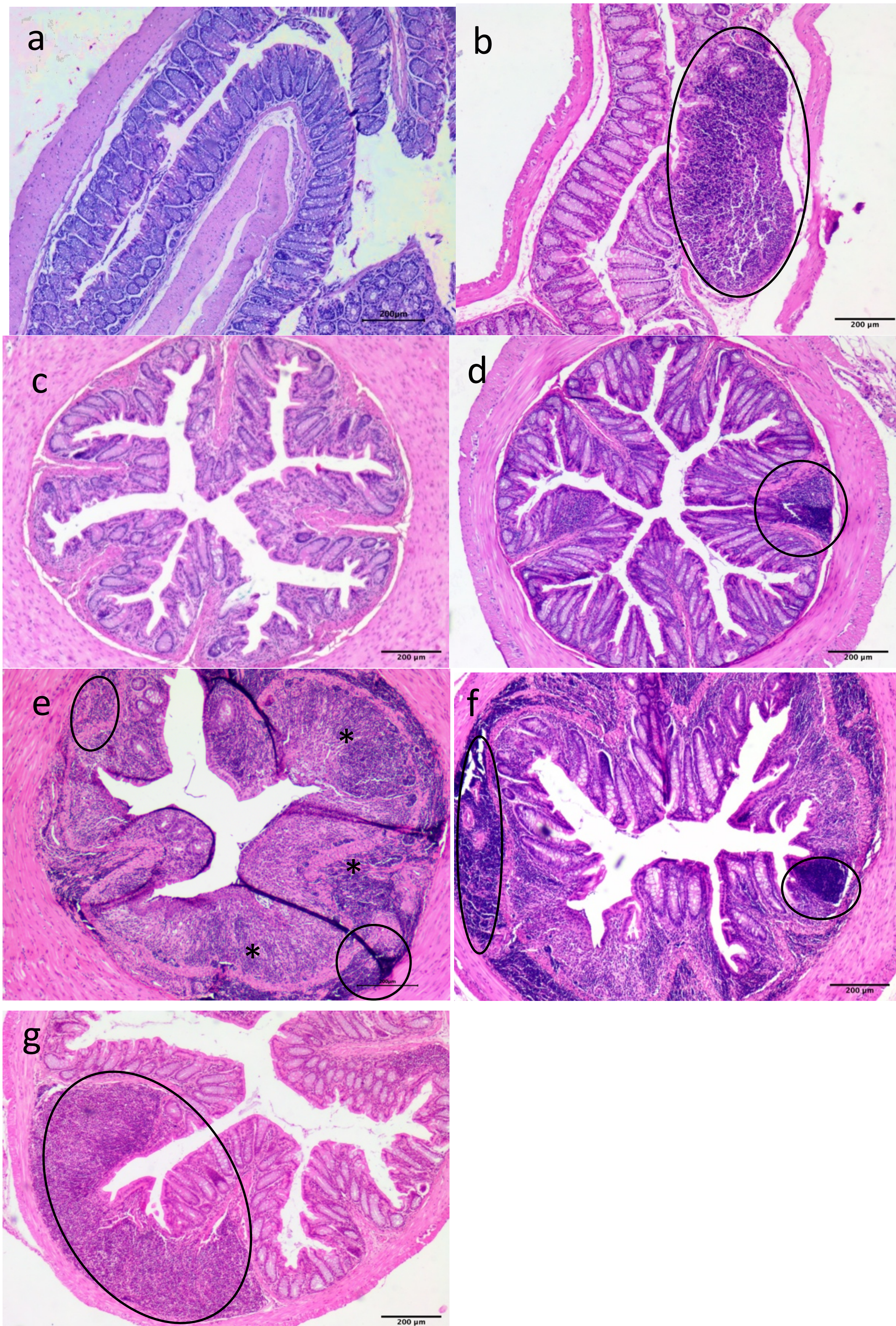


Figure 2: The representative photographs of hematoxylin and eosin staining (magnification x40).

(a) Negative control group (no DSS) showing normal colon morphology with normal crypt architecture; **(b) DSS-treated group** showing a marked localized inflammation invading mucosa and submucosa and characterized by edema, massive increase of leukocyte infiltration. However semi-preserved crypts are present throughout the remaining areas with low infiltration. **(c) DSS+Cyp treated group** showing a mild inflammation with an improvement in the colonic histology, preservation of overall architecture, and low leukocyte infiltration. **(d) DSS+Cys treated group** showing a mild inflammation with only a one-third crypt damage and very active Peyer's patches. **(e) DSS+(Cyp-Cys) complex treated group** showing a detrimental destruction of the complete colonic morphology and crypt architecture with an intensive leukocyte infiltration. **(f) DSS+(Cyp-Cys) complex+ probiotics treated group** showing a moderate inflammation extending from submucosa to mucosa with a one-third damage of the crypts. A heavy leukocyte infiltration is restricted to the space between musculosa and submucosa in addition to mucosa. **(g) DSS+probiotics group** showing a marked reduction in inflammation where one-third of the crypts is distorted. However, a massive leukocyte infiltration is concentrated at Peyer's patches while the remaining areas reflect a normal morphology with a low infiltration.

2. Assessment of mucus secreting cells, Goblet cell, by (PAS)

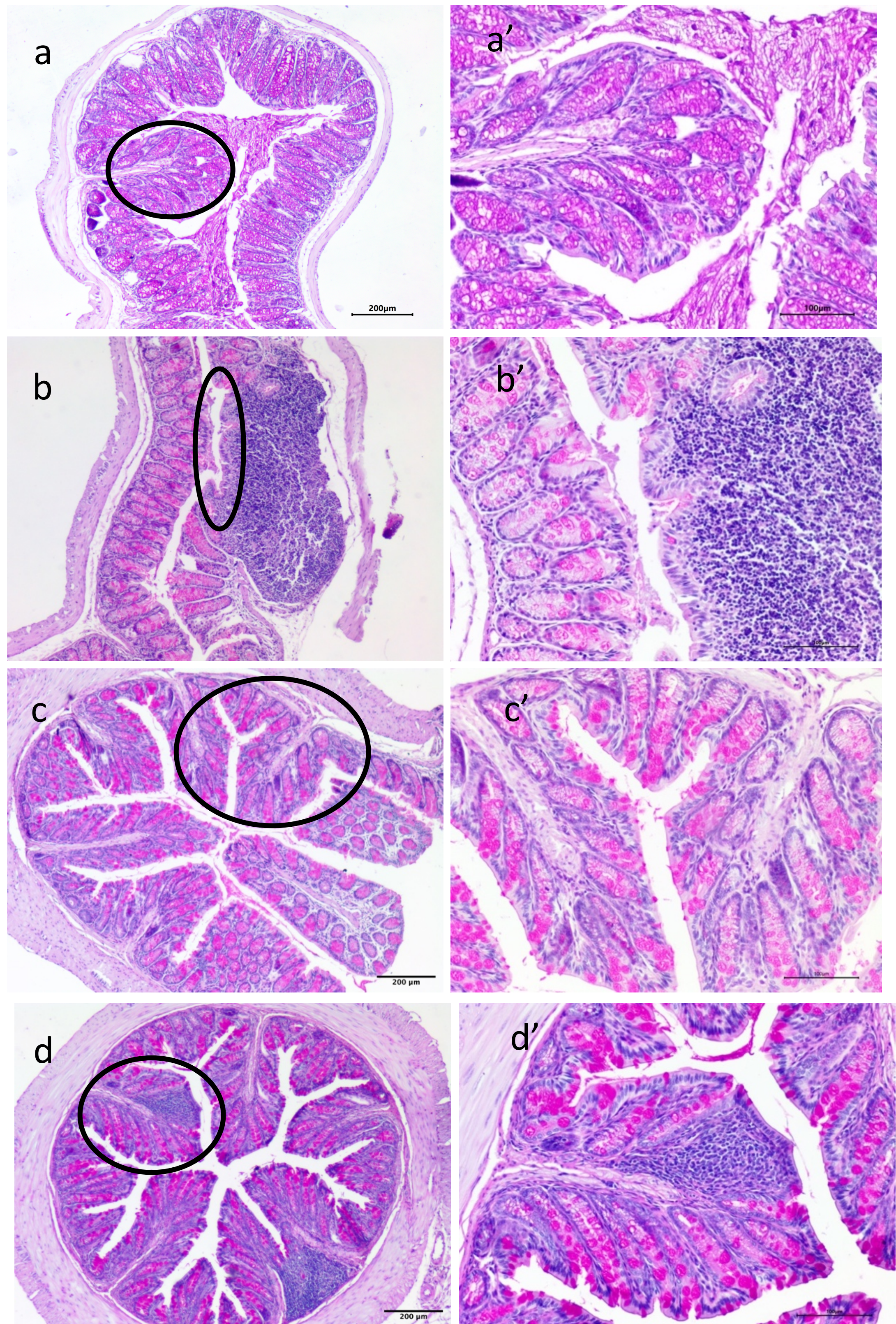
The use of PAS is to demonstrate mucopolysaccharide moieties, in particular, in the goblet cells.

The negative control group shows a normal tissue morphology where a vast amount of goblet cells resides throughout the mucosa layer (Fig. 3, a). However, a loss of more than 50% of goblet cells is detected in the DSS-treated group (Fig. 3, b).

In contrast, in the group treated with (DSS+Cyp), more than 75% of goblet cells have been present throughout the tissue, and less than 25% have been only lost (Fig. 3, d). Similarly, same effect has been revealed in the group treated with (DSS+Cys) where only 25% of goblet cells has been lost (Fig. 3, e).

On the other hand, the group treated with DSS+(Cyp-Cys) complex in (Fig. 3, f) presented a complete loss of goblet cells while the group treated with DSS+(Cyp-Cys) complex+probiotics has retained 25% of goblet cells and about 75% loss has been detected (Fig. 3, g). In contrary, the treatment of (DSS+probiotics) only, in the absence

of the complex, has evidently improved the colonic histopathological state in retaining 75% of goblet cells (Fig.3, h).



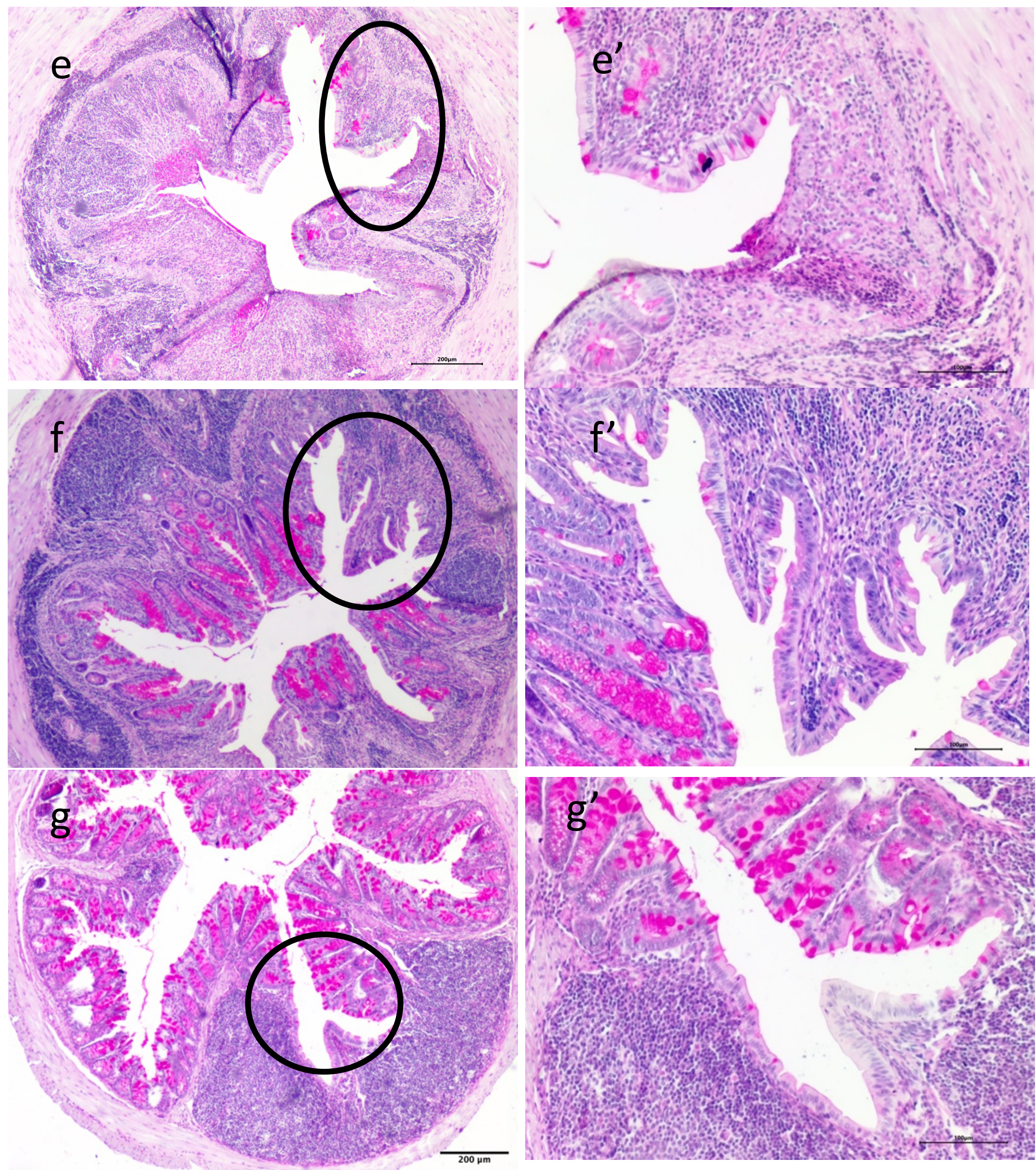


Figure 3: The representative photographs of Periodic acid-Schiff staining (magnifications to the left x40 and to the right x100).

(a, a') Negative control group (no DSS) showing a vast amount of goblet cells (no loss). **(b, b') DSS-treated group** showing a 50% loss reflecting moderate inflammation. **(c, c') DSS+Cyp and (d, d') DSS+Cys treated groups** showing both a mild inflammation with 25% loss of goblet cells. **(e, e') DSS+(Cyp-Cys) complex treated group** showing a complete loss of goblet cells due to severe inflammation. **(f, f') DSS+(Cyp-Cys) complex+ probiotics treated group** showing a 75% loss. **(g, g') DSS+probiotics treated group** showing a reduction in inflammation and loss of only 25% of goblet cells.

3. Histological Scoring Analysis:

The comparison of the tissue damage scores based on the histological scoring table [446] has revealed that colons from the negative control group (no DSS) have the lowest histological damage score (0.55/10) among all groups. In contrast, the DSS group

shows a significantly high histological score (3.11/10) compared to the negative control group.

Regarding the groups (DSS+Cyp) and (DSS+Cys), it is obvious that both treatments have same positive ameliorating effect on the colonic tissue reflected by their low damaging score (1.33/10) which is significantly less than the DSS-treated group (3.11/10) and higher than the negative control (0.55/10) showing no significance.

However, the histological score has remarkably increased to (3.8/10) in the DSS+(Cyp-Cys) complex group compared to the negative control (0.55/10), (DSS+Cyp), and (DSS+Cys) (both 1.33/10), but showing no significance with the DSS group (3.11/10).

After the addition of probiotics to the DSS+(Cyp-Cys) complex group, a slight reduction in the histological scoring has been demonstrated indicating a score of (3.3/10) compared to the DSS+(Cyp-Cys) complex (3.8/10). However, a marked reduction has been reported in the (DSS+probiotics) compared to the DSS+(Cyp-Cys) complex and DSS+(Cyp-Cys) complex+probiotics groups showing a score of (2.2/10).

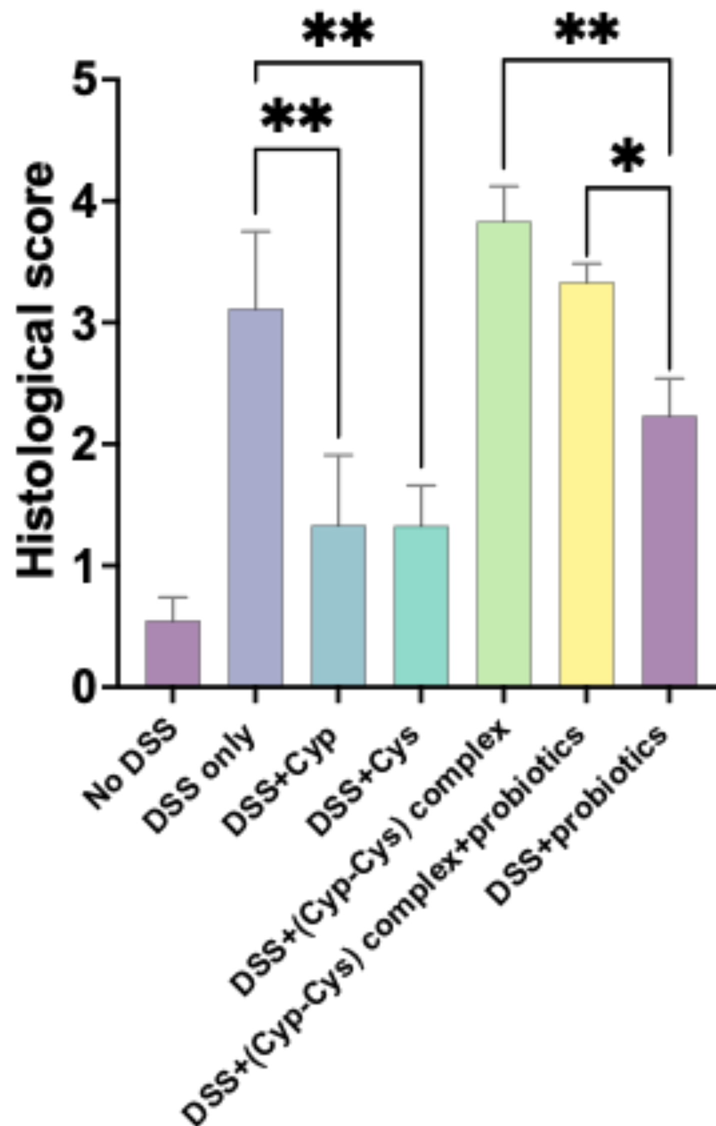


Figure 4: A bar graph showing the histological scoring in all treated groups.

(a) Negative control group (no DSS) showing a score of (0.55/10). **(b) DSS group** showing a score of (3.11/10). **(c) DSS+Cyp and (d) DSS+Cys groups** have the same histological score (1.33/10). **(e) DSS+(Cyp-Cys) complex group** showing a high score of (3.8/10). **(f) DSS+(Cyp-Cys) complex+probiotics group** showing a score of (3.3/10). **(g) DSS+probiotics group** showing a score of (2.2/10). Data is expressed as mean ± SD. Statistical significance is determined by one-way ANOVA. P-value < 0.05 is considered significant and is indicated by (*).

B. Analysis of molecular parameters

1. *Interleukin-6 Expression (IL-6)*

As shown in Fig.5, the protein level of IL-6 in the negative control group has been (0.14), the lowest significant level among all groups except (DSS+Cyp) and (DSS+Cys), whereby the values are slightly higher than in the control but significantly lower than the DSS alone (0.79) where this decrease has failed to reach significance. The notable increase revealed in the DSS group for IL-6 expression has level has remarkably decreased to (0.25) and (0.24) in the groups treated with (DSS+Cyp) and (DSS+Cys), respectively. On the other hand, a sharp increase has been evidently demonstrated in the group treated with DSS+(Cyp-Cys) complex to reach (1.27) exacerbating the inflammation.

A very slight decrease from (1.27) to (1.19) has occurred after the addition of probiotics to the DSS+(Cyp-Cys) complex group. In contrast, a notable drop has been shown in the (DSS+probiotics) group reaching (0.54). It's obvious that the expression in DSS group is significantly less than DSS+(Cyp+Cys) complex and DSS+(Cyp-Cys) complex +probiotics, but more than that in (DSS+probiotics) which is importantly higher than that in (DSS+Cyp) and (DSS+Cys) groups. In contrary, the expression in (DSS+Cyp) group (0.25) is approximately the same as that in the (DSS+Cys) (0.24).

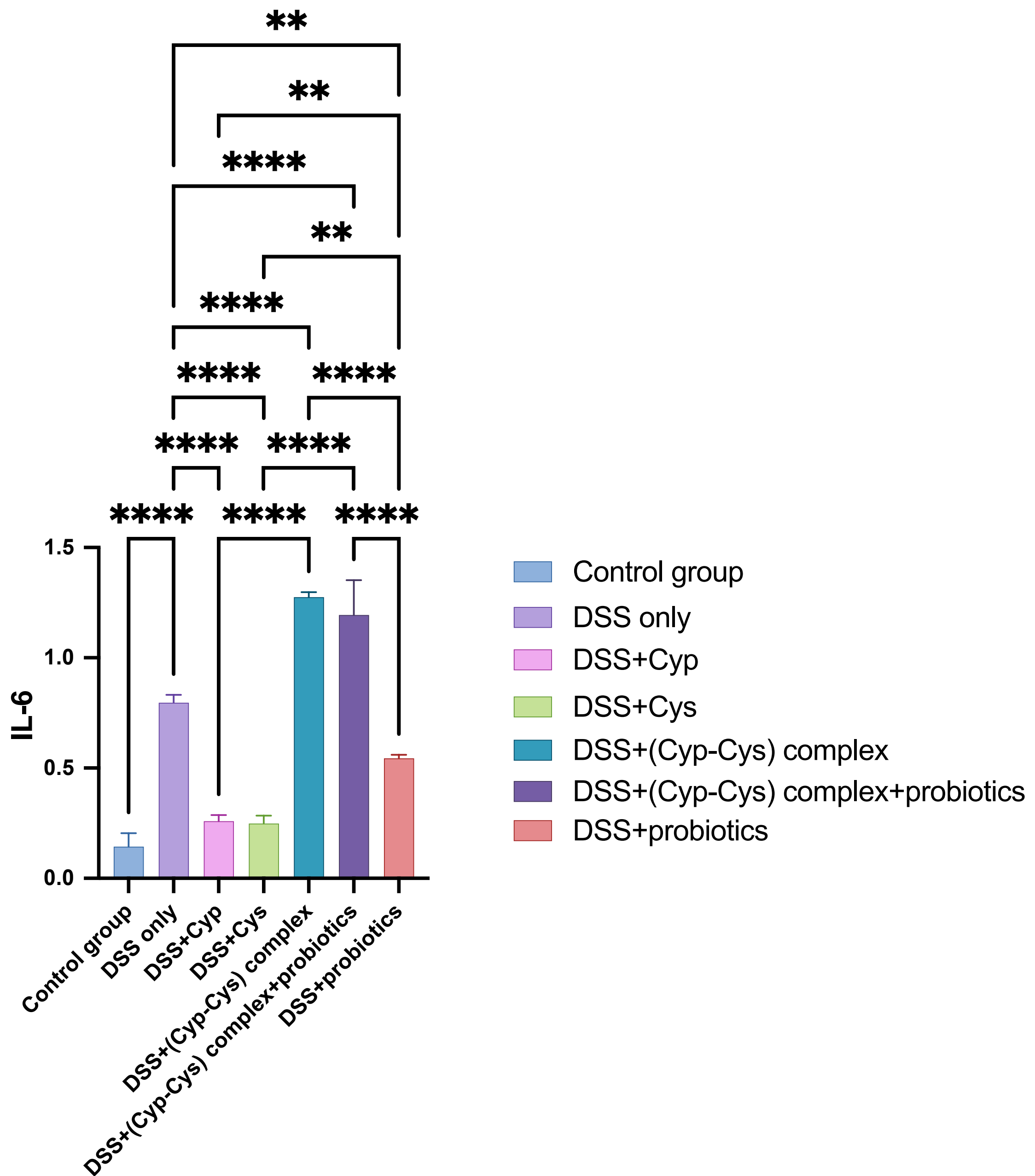


Figure 5: Protein Expression levels of the pro-inflammatory marker Interleukin-6 (IL-6) in syndecan-1 null mice.

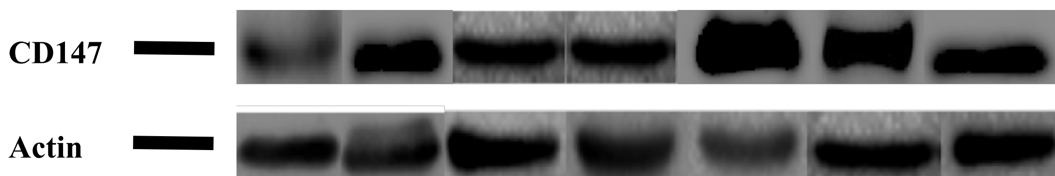
Upper: Representative figure for Western blot reflecting the protein expression of IL-6 normalized by the levels of housekeeping protein "Actin". Lower: Bar graph reflecting the quantification of protein expression of IL-6/Actin. Data is expressed as mean ± SD. Statistical significance was determined by one-way ANOVA. P-value < 0.05 is considered significant and is indicated by (*).

2. *Emmprin Expression (CD147)*

As shown in Fig.6, the protein level of CD147 in the negative control group has been (0.23), the lowest significant level among all groups except (DSS+Cyp) and (DSS+Cys) where this decrease has failed to reach significance between the 2 groups. On the other hand, the significant increase has been shown in the DSS only group where CD147 expression has reached (0.58). However, a notable drop from (0.58) to (0.3 and 0.26) has taken place in the (DSS+Cyp) and (DSS+Cys) groups respectively. Whereas, a remarkable increase has been demonstrated in the DSS+(Cyp-Cys) complex group reporting a value of (0.99).

On the other hand, CD147 level has tended to decrease from (0.99) to (0.81) after the addition of probiotics to DSS+(Cyp-Cys) complex treatments. In addition, a significant reduction has been detected in the (DSS+probiotics) group; decreasing from (0.81) to (0.47). It's noteworthy that the expression in the DSS group is significantly lower than that in DSS+(Cyp-Cys) complex and DSS+(Cyp-Cys) complex +probiotics groups, but slightly higher than that in (DSS+probiotics) group.

No significance has been reported between (DSS+probiotics) group and (DSS+Cyp) & (DSS+Cys) groups which have both revealed approximate values of CD147 expression (0.3 and 0.26).



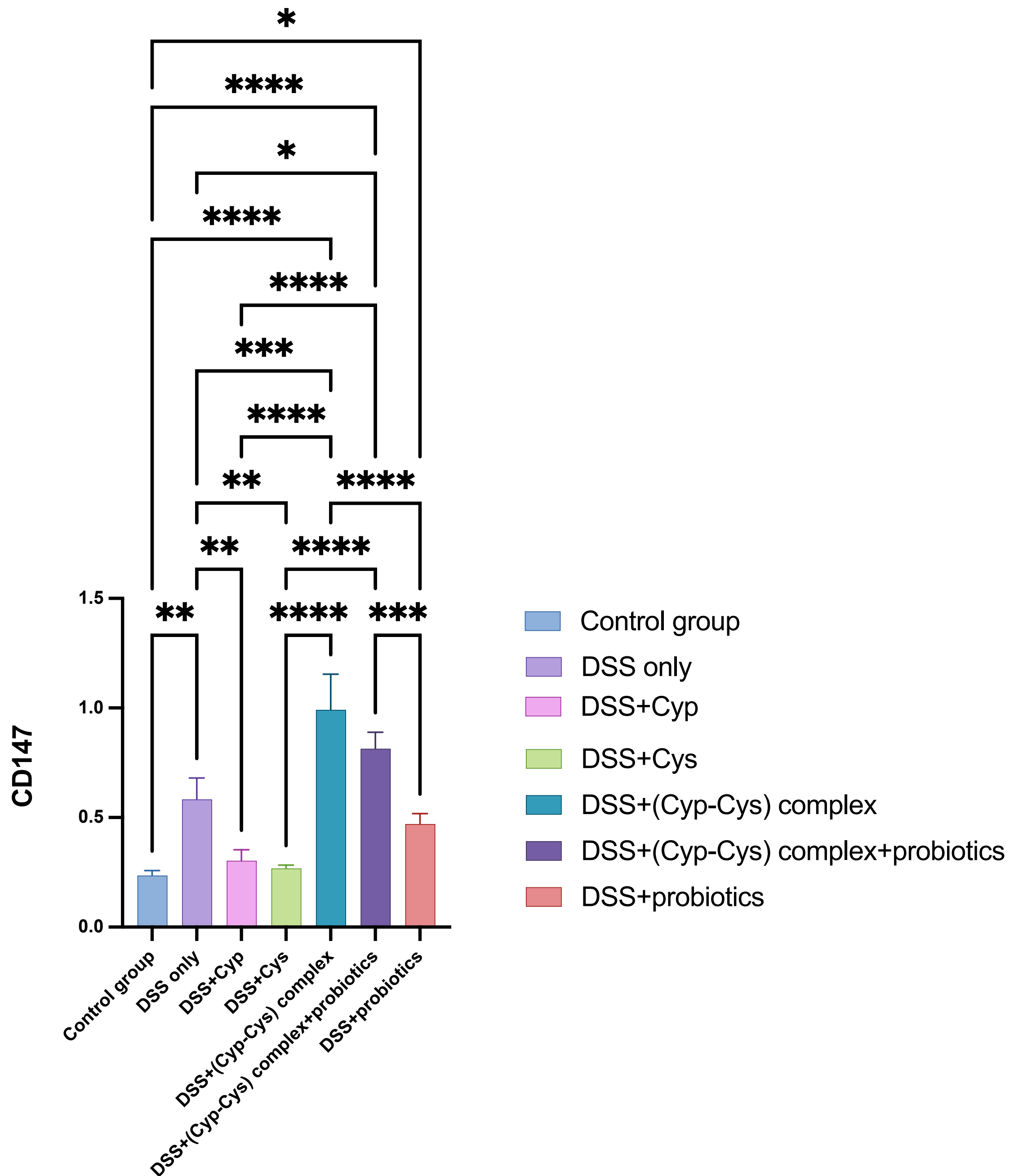


Figure 6: Protein Expression levels of the pro-inflammation marker CD147 in syndecan-1 null mice.

Upper: Representative figure for Western blot reflecting the protein expression of CD147 normalized by the levels of housekeeping protein "Actin". Lower: Bar graph reflecting the quantification of protein expression of CD147/Actin. Data is expressed as mean ± SD. Statistical significance was determined by one-way ANOVA. P-value < 0.05 is considered significant and is indicated by (*).

3. pAKT Expression:

As shown in fig.7, pAKT level in the negative control group has been (0.16), significantly lower than that in the DSS only (0.43), DSS+(Cyp-Cys) complex (0.6), DSS+(Cyp-Cys) complex+probiotics (0.53), and (DSS+probiotics) (0.26) groups. However, it has been slightly higher than the reported expression in (DSS+Cyp) (0.12) and (DSS+Cys) (0.15) groups respectively. An evident increase has been shown in the DSS group reaching (0.43). In contrary, this value has remarkably decreased to (0.12) in the (DSS+Cyp) group, then increased slightly to (0.15) in (DSS+Cys). On the other hand, a notable increase has been demonstrated in the DSS+(Cyp-Cys) complex group reporting a value of (0.6). This value has tended to decrease indicating a value of (0.53) in the DSS+ (Cyp-Cys) complex+probiotics group. Nonetheless, pAKT expression has significantly decreased from (0.53) to (0.26) in the presence of DSS and probiotics only. It is noteworthy that (DSS+probiotics) group shows significance with all groups. Similarly, the DSS group has also significant differences among all groups except DSS+(Cyp-Cys) complex+probiotics group.

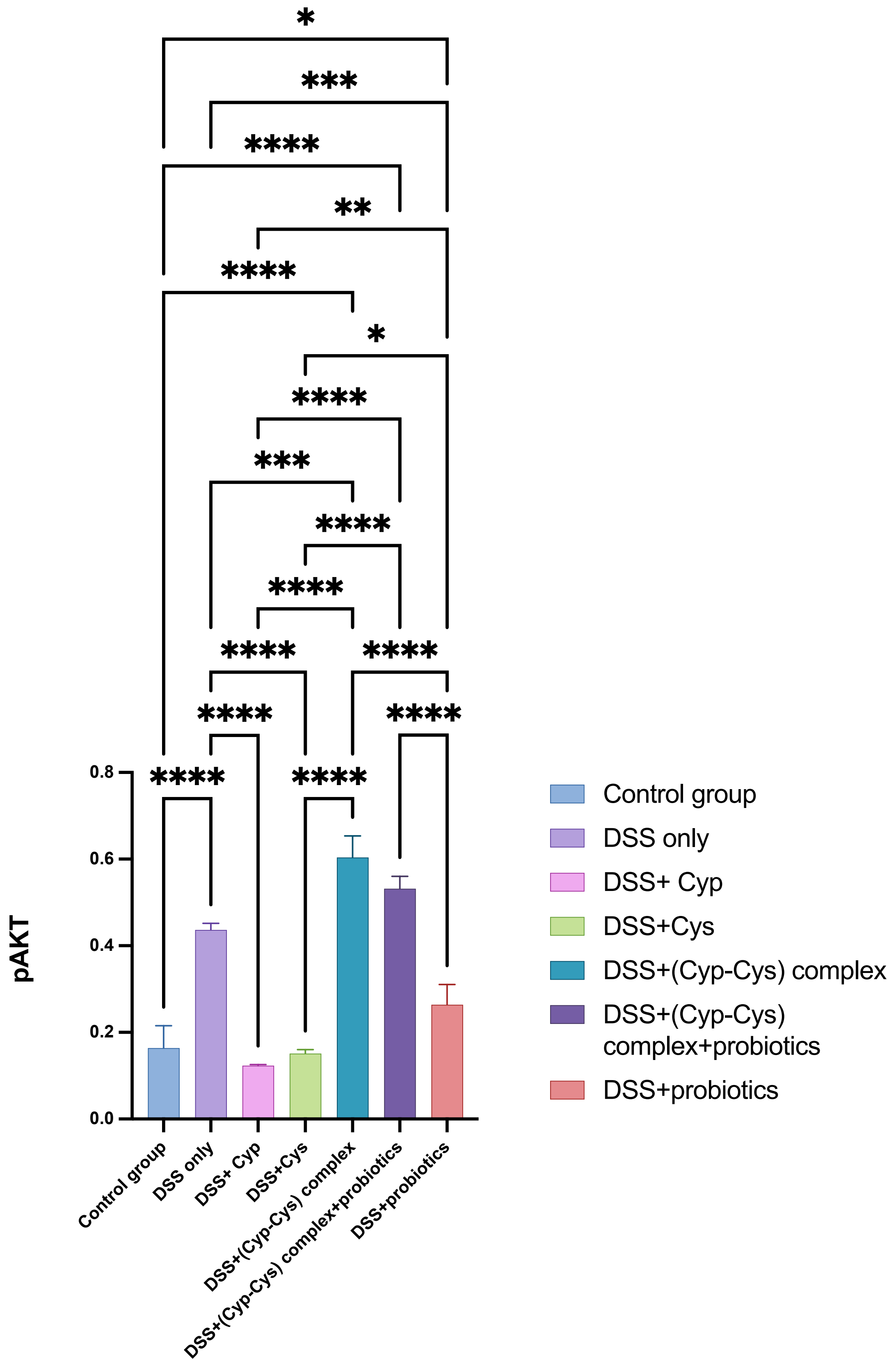


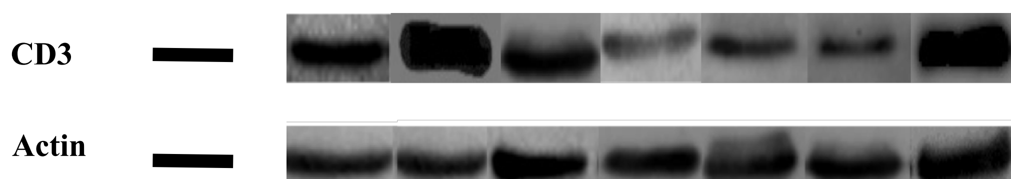
Figure 7: Protein Expression levels of pAKT in syndecan-1 null mice.

Upper: Representative figure for Western blot reflecting the protein expression of pAKT normalized by the levels of housekeeping protein "Actin". Lower: Bar graph reflecting the quantification of protein expression of pAKT/Actin. Data is expressed as mean ± SD. Statistical significance was determined by one-way ANOVA. P-value < 0.05 is considered significant and is indicated by (*).

4. *CD3 Expression:*

As shown in fig.8, the expression of CD3 in the negative control group has been (0.16), significantly higher than that in (DSS+Cys) (0.07) and DSS+(Cyp-Cys) complex +probiotics (0.11) groups. However, this increase has failed to reach significance compared to DSS+(Cyp-Cys) complex group (0.12). CD3 expression in the control group has shown a significant decrease compared to that in the DSS group (0.25) and (DSS+probiotics) (0.21) while showing no significance compared to that in (DSS+Cyp) group (0.2). A sharp increase has been reported in the DSS group reaching (0.25). Nevertheless, this value has evidently decreased to (0.25) in the (DSS+Cyp) group. This significant decrease has sharply continued to reach (0.07) in the (DSS+Cys) group.

In contrast, a notable increase has taken place in the DSS+(Cyp-Cys) complex group reaching (0.12). Yet, a slight decrease has been demonstrated after the addition of probiotics indicating a value of (0.11). On the other hand, this value has significantly increased to (0.21) in the (DSS+probiotics) group (0.25). It's noteworthy that the expression of CD3 in the DSS group has been significantly higher than that in all groups except (DSS+probiotics) where the expression has been lower than that in DSS group with no significance. Obviously, no significant difference has been detected between (DSS+Cyp) and (DSS+probiotics) groups.



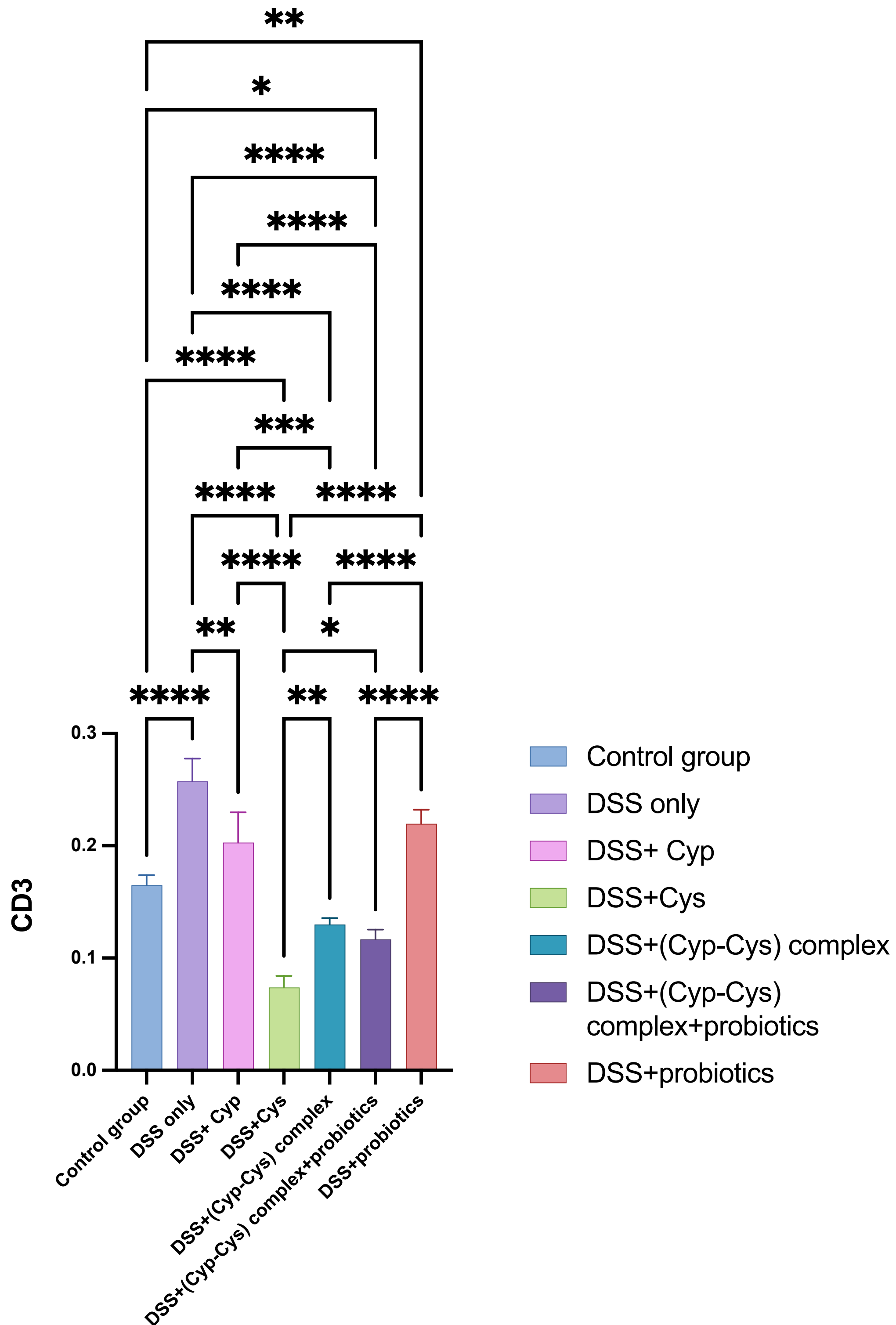


Figure 8: Protein Expression levels of CD3 in syndecan-1 null mice.

Upper: Representative figure for Western blot reflecting the protein expression of CD3 normalized by the levels of housekeeping protein "Actin". Lower: Bar graph reflecting the quantification of protein expression of CD3/Actin. Data is expressed as mean ± SD.

Statistical significance was determined by one-way ANOVA. P-value < 0.05 is considered significant and is indicated by (*).

CHAPTER VI

DISCUSSION

IBD has been described as a chronic multifactor idiopathic disorder that is associated with uncontrolled inflammation and epithelial injury within the gastrointestinal tract [8-12]. Over the years, multiple promising treatment protocols have emerged, including cyclosporine "A", based on clinical data as well as animal experiments. Numerous IBD models have been reported in the literature including chemically-induced colitis models. [218] The best described and most commonly used one is the DSS model [447, 448]. It has been proven to be simple, controllable, reproducible and rapid in inducing inflammation [239].

In this study, DSS successfully induced colitis in syndecan-1 null mice. The DSS-treated group showed a strong localized severe inflammation in the mucosa and submucosa with more than 50% loss of mucus secreting cells, goblet cells. However, both treatments with either (DSS+Cyp) or (DSS+Cys) separately revealed a mild inflammation with an improvement in the mucosal architecture and only about 25% loss of goblet cells. Whereas, the DSS+(Cyp-Cys) complex group exhibited a severe inflammation showing a severe detrimental effect on the colonic histology with a complete loss of goblet cells. Interestingly, the addition of probiotics to DSS+(Cyp-Cys) complex group displayed a partial insignificant reduction in inflammation of the tissues where only 25% of goblet cells were restored. On the other hand, in the group treated with DSS and probiotics in the absence of the (Cyp-Cys) complex, inflammation in the mucosa and submucosa was significantly reduced and 75% of mucus secreting cells were restored. Proinflammatory markers (IL-6, CD147) and pAKT as well as CD3 were assessed in each group and the results correlated very well with the histological analysis data. The marked upregulations

in the proinflammatory markers, increase in Akt phosphorylation and decrease in CD3 expression were illustrated in the (Cyp-Cys) complex DSS-treated mice. On the other hand, probiotics alone downregulated these molecular markers. Adding probiotics to the complex did not improve significantly the outcome.

In this study, we were able to show that syndecan-1 is basically implicated in controlling intestinal inflammation, and that DSS treatment has serious toxic effects on the overall colonic histology leading to a worsened inflammatory episode. This was reflected by a marked severe inflammation invading the mucosa and submucosa layers with high levels of proinflammatory markers as compared to the negative control. This is in accordance with other studies reporting that many factors are linked to the mechanism of DSS-induced colitis, such as the destruction of enteric epithelium barrier, the alteration of normal intestinal flora, the disturbance in the function of macrophages and other immune cells, and the toxic effect on the synthesis of DNA [449, 450]. Besides, several studies have also demonstrated that DSS is toxic in damaging the colonic mucosal architecture and further alternating the mucosal permeability, where numerous intra-luminal antigens penetrate into the mucosa and contribute to cytokine production, thus leading to the onset of inflammatory responses [245, 451]. What did cyclosporine along with other combination do to improve the outcome in these syndecan-1 knock-out mice?

Sdc-1 is the most pivotal heparan sulfate proteoglycan (HSPG) located on the surface of epithelial cells [452, 453]. It has important functions in cell proliferation and repair, as it serves as a vital co-receptor in cytokine signaling pathways [454]. Day et al. [293] were the first to verify that sdc-1 is less expressed in ulcerative colitis patients. Similarly, numerous animal models indicated that the exacerbation of inflammation was mainly due to sdc-1 deficiency [286, 287, 455]. In a DSS-induced model of colitis, an

amplified and continuous recruitment of leukocytes was shown in sdc-1 null mice compared to wild type mice. [298] Other similar studies demonstrated an increased mucosal inflammatory cells infiltration in sdc-1 deficient mice treated with DSS with an increase in the expression of the proinflammatory marker IL-6 [456]. Their results are in agreement with ours. Indeed, we have observed in the DSS group an increased recruitment of inflammatory cells. This increase was coupled with an increase in IL-6, CD147, CD3 and pAKT expressions.

IL-6 is one of the best-characterized proinflammatory cytokines. It has been implicated in several mechanisms such as cell survival, proliferation, differentiation, migration, invasion, metastasis, angiogenesis, metabolism and inflammation [457]. In addition, it has been strongly involved in the pathogenesis of human and experimental models of colitis [163, 458, 459]. Physiologically, IL-6 can induce the activation of three main pathways; the Janus kinase (JAK)-signal transducer and activator of transcription 3 (STAT3) pathway, the Src homology 2 (SH2)-containing protein tyrosine phosphatase-2 (SHP-2)-Ras-Raf-MEK-extracellular signal- regulated kinase (ERK) pathway, and the phosphoinositide 3-kinase (PI3K)-AKT pathway. It has been demonstrated that the activation of these pathways occurs through the engagement of their unique receptor (s) and the common signaling receptor subunit glycoprotein 130 (grp130, CD130 and IL6ST) [457, 460].

IL-6 creates a proinflammatory milieu by inhibiting the differentiation of Treg cells that normally suppress the development of tumor cells. During a chronic intestinal inflammation episode, IL-6 trans-signaling causes an increased resistance of mucosal T-cells against apoptosis [461]. It is noteworthy that CD3 marker is necessary for the

expression of T cell surface receptor (TCR), and that it is located on the surface of all subsets of T- cells [420-422]. This elucidates that the increase of IL-6 in the DSS-treated group caused the activation of T-cells and ultimately the increase in CD3 expression. Moreover, IL-6 stimulates the production of chemokines in endothelial cells (ECs) and plays a vital role in recruiting leukocytes; this indeed agree with our results [462]. Furthermore, IL-6 influences the differentiation of myeloid lineages, involving dendritic cells (DCs) and macrophages, both in vivo and in vitro via STAT3 activation [463]. Accordingly, macrophages and other immune cells are activated upon the binding of IL-6 to its receptor (IL-6Rα) which is expressed on the surfaces of macrophages, monocytes, neutrophils, T and B cells [464]. In addition, during an immune response, activated macrophages amplify the pathogenetic cascade via activating fibroblasts by cytokines such as IL-1 and TNF-α. Other molecules, such as CD147, present on surface of macrophages, play a vital role in this process and may play essential roles in IBD pathogenesis[465]. Interestingly, CD147 is one of the markers expressed on the surface of activated monocytes and macrophages [465-467]. CD147, known as extracellular MMP inducer (EMMPRIN), is an immunoglobulin super-family transmembrane protein that was primarily recognized on the surface of human cancer cells and has been demonstrated to stimulate the production of various MMPs from the activated stromal cells [378, 468, 469]. One of the studies has illustrated the upregulation of CD147 expression in the rheumatoid arthritis synovial membrane leading to elevated expressions of MMPs [470, 471]. In addition, an increased expression of CD147 has been detected in the intestinal mucosa of IBD patients [390]. Evident studies have highlighted the role of CD147 as a high affinity receptor for cyclophilin "A" and have verified its crucial role in contributing to cyclophilin signaling cascade that eventually leads to chemotaxis and

ERK activation [346, 472]. It has also been proven that CypA ,which is one of the immunophilin family of peptidyl- prolyl cis-trans isomerases [473], has a potent chemotactic activity towards leukocytes (neutrophils or eosinophils) [474]. It is supposed that CypA is released into the medium during inflammation, acting as a ligand of CD147, thus stimulating the accumulation of immune inflammatory cells which highly express CD147 in the intestinal wall in case of IBD.

One of the main pathways activated by IL-6 is (PI3K)-AKT pathway. AKT is a downstream target of PI3Ks belonging to the AGC family of protein kinases [475]. PI3K products bind to the pleckstrin homology (PH) domain, thus translocating Akt to the plasma membrane where it is phosphorylated by the action of specific upstream protein kinases such as the phosphoinositide-dependent kinase 1 (PDK1). The binding of PIP3 to PDK1 occurs via the PH domains. The activated PDK1 phosphorylates Akt at Thr308 in its kinase domain. Further phosphorylation of AKT occurs at Ser473 by the action of PDK2 enzyme [476]. When Akt is fully activated (in its phosphorylated form), it moves to the cytoplasm and nucleus to phosphorylate several downstream targets and subsequently regulate cellular functions. It has been proven that phosphorylated Akt is a biomarker for cancer used in cancer therapy [477] ,in addition, it is implicated in IBD pathogenesis where a significant increase in its expression has been reported in DSS-induced model of colitis [445]. More in- depth studies have evidently demonstrated the pivotal role of (PI3K)-AKT signaling for the interaction between tissue infiltrating macrophages, mast cells and epithelial cells in colitis-induced cancer. PI3K has been shown to mediate proliferation and activate Akt and β-catenin in epithelial stem cells leading to crypt architectural disturbances that contribute to colitis [478]. This applies to our study whereby the elevated expression of IL-6 in the DSS-treated sdc-1 deficient mice

stimulated the activation of (PI3K)-Akt pathway, thus leading to an increase in pAKT expression (Fig.s 5,7).

In addition, pAKT can be activated upon the engagement of T cell surface receptor (TCR) [479]. When the antigen is presented to TCR, a dual response is evoked; sodium channels and PI3K signaling pathway are both activated. An influx of calcium ions penetrates into the cells through the activated sodium channels, thus activating the transcription factor nuclear factor of activated T cells (NFAT) and eventually leading to the production of proinflammatory cytokines [480]. Concerning the PI3K pathway, it transmits its signal to multiple pathways including NFκB and AKT pathways resulting in migration, inflammation, and proliferation of cells [481, 482]. Several studies have previously shown that activated Akt affects T cell activation and survival by restraining apoptotic processes [483, 484]. Our results on pAKT expression in the DSS-treated group are consistent with previous studies demonstrating that activated AKT (pAKT) inhibited T-cells apoptosis and contributed to a more activation of T cells, leading to an increase in CD3 expression. In light of other previous studies, our data show that sdc-1 gene has a crucial role in controlling inflammation, and that DSS has toxic effect on sdc-1 null mice reflected by increased expression of proinflammatory markers and disrupted mucosal architecture [245, 298].

In the group treated with DSS and cyclophilin “A”, inflammation was notably reduced as compared to the DSS group. This was reflected by an improvement in the colonic mucosal architecture whereby about 75% of goblet cells were restored, in addition to the significant decrease in the levels of expression of molecular parameters (IL-6, CD147, pAKT and CD3). The value of IL-6 expression (0.25) has been decreased by 3

compared to that in the DSS group (0.79). Concerning CD147, its value has been decreased by 2 (0.3<0.58). Interestingly, 3.5 decrease in the value of pAKT has been reported (0.12<0.43) where only 1.25 decrease has been illustrated for CD3 (0.2<0.25), (Fig.s 5,6,7,8).

The results obtained in the (DSS+Cyp) group opposed that of other studies in the literature. Other studies have established the chemoattractant influence of CypA on inflammatory cells, as mentioned previously, leading to an aggravated course of inflammation [337, 485]. Furthermore, it has been shown that CypA mainly binds to its primary binding site, heparan sulfate proteoglycan (HSPG), on the surface of target cells. All the cyclophilin-dependent signaling responses and chemotactic effects, in addition to the adhesion of neutrophils and T cells responses are subsequently inhibited when HSPGs, sdc-1, are absent [346, 347]. It could be speculated that sdc-1 deficiency caused a partial inhibition of the proinflammatory effects of CypA.

Furthermore, studies have emphasized the role of CD147 as a surface receptor for extracellular cyclophilin [346]. It has been shown that the binding of extracellular Cyp A to CD147 contributes to the activation of CD147, thus increasing Akt phosphorylation and exacerbating inflammation [340, 346]. In contrast, our current findings showed that CypA has a significant ameliorating effect on the colons of sdc-1 deficient mice in presence of DSS. Knowing that our mice are sdc-1 null and based on the literature, we assumed that the extracellular CypA might bind to CD147 on the surface of macrophages in the absence of HSPG (sdc-1) and induce an increased inflammatory response. Yet, our results revealed a significant decrease in the levels of molecules like IL-6, CD147, CD3, and pAKT compared to the DSS group. It is possible that the IL-6 trans-signaling was

masked by the activation of other signaling pathway that contributed to the amelioration of inflammation. Probably, IL-6 was not sufficient to activate macrophages and other inflammatory cells. This explains the low expression of CD147 on the non-activated target cells. Obviously, IL-6 failed to activate the (PI3K)-AKT pathway, and this was reflected by the low expression of pAKT. It is expected that T-cells are less activated due to the inactivation of (PI3K)-Akt pathway; this was revealed by the low expression of CD3 reflecting a low number of T- cells. It is probable that extracellular CypA binds to a distinct receptor (other than Sdc-1 and CD147) on the surface of its target and needs further investigation. It might be involved in one of the pathways which are still not described and that lead to healing.

In the group treated with DSS and cyclosporine "A", the ameliorating effect of cyclosporine "A" has been evidently illustrated on the colonic histology of the sdc-1 deficient mice (Fig.2, d). The overall picture showed a mild inflammation compared to the only DSS group, with a partially restored mucosal architecture and only 25% loss of goblet cells. Notably, histology revealed a high aggregation of leukocyte infiltration concentrated at the Peyer's patches, reflecting the extent of the reaction of the immune cells during the induction process. CD3 expression has dropped as expected from treatment with cyclosporine and the other molecular parameters IL-6, CD147 and pAKT followed a similar pattern.

Multiple studies have proven that cyclosporine "A" is an immunosuppressive drug that is implicated in IBD therapy and reduction of colitis [329]. Its role has been established in stimulating apoptosis and inhibiting the production of proinflammatory cytokines [315, 316]. Physiologically, it complexes with intracellular cyclophilin, hinders

the action of the protein phosphatase, calcineurin, in a calcium-dependent reaction and consequently blocks the entering of cytosolic nuclear factor of activated T cells (NFAT) to the nucleus of the cell, eventually contributing to the inhibition of T-helper cells [486].

Our results showed 3.3 times decrease in the value of IL-6 expression (0.24) compared to that in the DSS group (0.79). Concerning CD147, its value has been reduced by 2.2 (0.26<0.58). Moreover, the value of pAKT has been reported to decrease by 2.8 (0.15<0.43). Notably, as expected,3.6 decrease has been revealed for CD3 (0.07<0.25). However, this group revealed a result similar to that of (DSS+Cyp) group concerning all molecular parameters except CD3, where it showed 3 times decrease with respect to the (DSS+Cyp) treated group. This sharp decrease in CD3 expression is firmly clarified by the mechanism of action of Cys concerning the inhibition of T-helper cells that express on their surfaces CD3 marker.

Further studies have shown that Cys impedes neutrophil adhesion to the venular endothelium, thus restraining leukocyte infiltration [326]. Regarding this point, CysA reduces adhesion, interaction and infiltration of neutrophils by attenuating cell adhesion molecules (E-selectin and vascular cell adhesion molecule 1) and blocking the production of NF-κB, a pivotal transcription factor transcribed during an inflammatory response [328], in particular in IBD. Many studies have reported the effectiveness of CysA in ameliorating inflammation and reducing colitis in DSS mice models, showing an improvement in the mucosal architecture and infiltration of lymphocytes, in addition to the reduction in the expression of proinflammatory cytokines (IL-6) [329, 334]. Our results are in agreement with that of other studies where the expression of IL-6 was reduced in the (DSS+Cys) group compared to the DSS only. This contributed to a reduced

activation of (PI3K)-AKT pathway, subsequently reducing pAKT expression. Consequently, less macrophages and other immune cells expressing CD147 were activated. This justifies the low expression of CD147 in this group. Therefore, our findings are supported by the results of other studies concerning the role of cyclosporine in reducing inflammation in DSS induced colitis models.

Despite the high efficacy of cyclosporine, it has been shown to have serious adverse effects such as infections, nephrotoxicity, hepatotoxicity, hypertension, and anaphylaxis [336]. In an attempt to prevent the development of these side effects, our study was the first of its kind trying to test the combination of cyclosporine and cyclophilin “A” in DSS induced colitis sdc-1 deficient mice. Unfortunately, this combination displayed an exacerbated status of inflammation showing a severe detrimental effect on the overall colonic histology; ranging from complete epithelial erosions and massive leukocyte infiltration to a marked thickening of the mucosa and submucosa layers, in addition to the sharp increase in the levels of the proinflammatory markers (IL-6, CD147) and in the phosphorylation of AKT. However, a drop in CD3 expression was detected. More explorations are needed to explain such data.

Regarding extracellular cyclophilin “A”, studies have reported its potent chemotactic effect on monocytes, leukocytes, and lymphocytes [342, 474]. As mentioned previously, CD147 has been demonstrated as a cell surface receptor for extracellular cyclophilin, and has been implicated in mediating most of its signaling [487]. Yet, the mechanism of CypA- CD147 interaction remains unclear. Interestingly, the direct binding of CypA to CD147 without the involvement of its PPIase activity, has been illustrated to stimulate leukocyte chemotaxis [488]. Moreover, reports have demonstrated a high

expression of CD147 in inflamed tissues [489, 490]. In addition, CypA has been shown to be involved in activating Akt and NF-κB signaling pathways, thus aggravating the status of inflammation [364]. However, the exact role of cyclosporine "A" in this respect needs more clarification.

Concerning cyclosporine "A", it has been proven, as mentioned previously, that it binds to intracellular CypA, inhibits the activity of calcineurin and restrains the translocation of (NFAT) into the nucleus. This ultimately inhibits the activity of T-helper cells and reduces intestinal inflammation [316], as reported by our study.

Our results demonstrated 5.29 times increase in the value of IL-6 (1.27) compared to that in the (DSS+Cys) group (0.24). Concerning CD147, its value has increased by 3.8 (0.26<0.99). Moreover, the value of pAKT has 4 times increased compared to (DSS+Cys) group (0.15<0.6). Only 1.7 times increase has been illustrated for CD3 expression. However, the value of CD3 expression has been 2.08 lower than that in the DSS only group (0.12<0.25). In light of the data discussed above and our findings, we suggest that the aggravation of inflammation in the group treated with DSS+(Cyp-Cys) complex may be caused by the action of the proinflammatory extracellular CypA. The binding of extracellular CypA to the cell surface CD147 has most probably contributed to the activation of CD147 and further increased its expression. Indeed, a notable increase in IL-6 has also been detected during this inflammatory response. It is expected that both CypA and IL-6 activate AKT pathway; this elucidates the significant increase in pAKT expression. On the other hand, the expression of CD3 has been significantly lower than that in the negative control, DSS only and (DSS+Cyp) groups, but significantly higher than that in (DSS+Cys) group. The low CD3 expression reflecting a low number of total

T-cells, is perhaps due to the immunosuppressive effect of Cys that targets the activity of T-helper cells. However, the high levels of IL-6 and CD147 could be reflecting a high number of activated macrophages or other cells having the same receptor, they are probably due to the proinflammatory effect of extracellular cyclophilin “A”.

The effect of (Cyp-Cys) complex treatment in DSS induced sdc-1 deficient mice needs further investigation to clarify the mechanism underlying the interaction between Cys and Cyp in the context of IBD.

After the addition of probiotics to DSS+(Cyp-Cys) complex group, a slight reduction in the severity of inflammation is clearly observed. However, the overall histological picture shows a massive leukocyte infiltration invading the mucosa and also localized between the muscularis mucosa and submucosa layers. Only one-third of the crypts is damaged, yet very active Peyer’s patches are present. Concerning the molecular parameters, an insignificant decrease in the levels of proinflammatory markers (IL-6, CD147), in addition to pAKT and CD3 is revealed compared to the DSS+(Cyp-Cys) complex group. More investigations are needed to unveil the mechanism of action of the complex in the presence of probiotics.

On the other hand, the addition of (DSS+probiotics) in the absence of (Cyp-Cys) complex reveals a marked reduction in inflammation. Histology shows a normal preserved architecture in 75% of the section, yet very active Peyer’s patches are existent. Regarding the molecular parameters, the levels of proinflammatory markers are notably reduced compared to that in DSS+(Cyp-Cys) complex and DSS+(Cyp-Cys) complex probiotics groups. The values of IL-6, CD147 and pAKT expressions in (DSS+probiotics) group have decreased by 2.3, 2.1 and 2.3 times, respectively, compared to that in

DSS+(Cyp-Cys) complex group, however, as probably expected in the absence of Cys, the value of CD3 expression has increased by 1.75. Similarly, the values of these markers have decreased by 2.2, 1.7 and 2, respectively compared to that in DSS+(Cyp-Cys) complex+probiotics group. Yet, the value of CD3 expression has increased by 1.9. In brief, probiotics alone are more efficient in reducing the inflammation in the colon. This result is in line with previous studies which have evidently shown that probiotics stimulate the differentiation of T-helper 1 cells, boost antibody production, promote the activity of both natural killer cells and phagocytic cells, and increase T-cell apoptosis by inhibiting the transcription of NF-κB. In addition, they have a pivotal role in increasing the production of anti-inflammatory cytokines while decreasing that of proinflammatory cytokines [392, 491-493]. Moreover, probiotics prevent apoptosis of intestinal epithelial cells and stimulate the production of proteins that are essential components of tight junctions, thus decreasing the paracellular permeability and restoring the barrier function [494-497]. Furthermore, probiotics produce bacteriocins, thus creating an acidic medium detrimental to pathogenic bacteria, yet favorable to the growth of beneficial microorganisms such as lactobacilli and bifidobacteria [409, 498-500]. Furthermore, in a DSS-induced model of colitis, it has been shown that the supplementation with a mixture containing Bifidobacterium, Lactobacillus acidophilus, and Enterococcus has consequently contributed to a reduction in total T-cells and increase in the number of Treg cells in the colonic tissue and blood, in addition to enhancing the function of tight junctions [416]. In this study, the results of the group treated with DSS+(Cyp-Cys) complex+probiotics demonstrate a slight reduction in CD3 expression compared to DSS+(Cys-Cyp) group while a sharp increase has been observed in the (DSS+probiotics) group where (Cyp-Cys) complex is absent. It is likely that the sharp increase in CD3

expression in (DSS+probiotics) group is due to the increase in the number of T-helper cells or Treg cells in the colons of the sdc-1 deficient mice. However, the presence of (Cyp-Cys) complex in DSS+(Cyp-Cys) complex+probiotics group has been shown to partially decrease the effectiveness of probiotics by immunosuppressing T-cells. The slight reduction in the molecular markers (IL-6, CD147 and pAKT) fosters the same conclusion, that probiotics are less effective in the presence of (Cyp-Cys) complex. However, the mechanism underlying the action of Cys and Cyp in presence of DSS and probiotics need further and deeper investigations. Would it be possible that the complex (Cyp-Cys) could override the effect of probiotics?

On the other hand, the marked reduction in inflammation is clearly noticed in (DSS+probiotics) only, as reflected by the molecular markers (IL-6, CD147 and pAKT), supporting the fact that probiotics decrease the production of anti-inflammatory cytokines and have a pivotal role in relieving inflammation.

Altogether, our findings suggest a therapeutic role for cyclophilin “A” in DSS-induced sdc-1 deficient mice. The presence of distinct receptors for extracellular CypA (other than CD147 and sdc-1) on its target cell merits further exploration. In addition, the potent role of cyclosporine in IBD therapy has been demonstrated due to the marked reduction of inflammation taking place in (DSS+Cys) group. However, the exacerbation of inflammation in the group treated with the complex group (Cyp-Cys) needs further investigation concerning the overlap between Cys and Cyp and the mechanisms underlying them. Moreover, the effectiveness of probiotics has been clearly revealed when treated alone in DSS-induced sdc-1 deficient mice. In contrast, this effectiveness has been partially inhibited in the presence of (Cyp-Cys) complex. Further and deeper

investigations need to be carried out to answer the question on how the complex decreases the function of probiotics.

CHAPTER VII
LIMITATIONS OF THE STUDY

Concerning the limitations of the study, more inflammatory markers and more cells such as mast cells, eosinophils and basophils should be further studied to establish a better understanding of the mechanisms underlying the role of Cyp, Cys and probiotics in IBD.

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