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Gangliosides and Inflammatory Bowel Disease

by

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ABSTRACT

Inflammatory bowel disease (IBD) involves chronic inflammation of all or part of the digestive tract. IBD primarily includes ulcerative colitis and Crohn disease. Gangliosides are negatively charged glycosphingolipids that consist of a hydrophobic ceramide and a hydrophilic oligosaccharide chain bearing one or more sialic acid. Both animal and human cell studies suggest that gangliosides have anti-inflammatory effects by decreasing tumor necrosis factor-α (TNF-α), prostaglandin E2 (PGE2) and leukotriene B4 (LTB4). The objective of this study was to evaluate Lipopolysaccharide (LPS) stimulated and dextran sulfate sodium (DSS) induced inflammation of Caco-2 cells as a model of IBD and to investigate the effect of ganglioside supplementation.

Ganglioside was extracted from milk powder and used as the ganglioside supplement in cell cultures. Intestinal integrity was examined by electron microscopy. Epithelial barrier function was examined by measuring trans epithelial electric resistance (TEER). HBD-2, IL-23, NF-kB and sPLA2 (secretory phospholipase A2) was determined by ELISA methods. apoB 48 was measured by western blot.

Both LPS and DSS treatment result in higher HBD-2 level, activation of NF-kB P65 and secretion of sPLA2 in the basolateral medium, while decreased intestinal barrier function ($P \leq 0.05$).

In LPS induced IBD Caco-2 model, the research demonstrates that ganglioside incubation decreases pro-inflammatory HBD-2 and IL-23 levels ($P \leq$

0.05) by improving barrier function and inhibiting NF-kB activation (P≤ 0.05) and independent of sPLA2 level. Ganglioside also increases the secretion of apoB 48 after LPS stimulation (P≤ 0.05).

This research demonstrates that in DSS induced IBD Caco-2 model ganglioside incubation decreases the secretion of sPLA2 in the basolateral medium after DSS treatment (P≤ 0.05). Ganglioside incubation protected the integrity of cellular phospholipids, including phosphatidylethanolamine (PE) and phosphatidylinositol (PI).

In summary, the present study indicates that both LPS and DSS induced Caco-2 cells are useful models to study IBD. Ganglioside incubation in cell culture may have promising potential beneficial effects on IBD by decreasing inflammatory signaling and improving intestinal integrity and epithelial barrier function. The anti-inflammatory effect observed is related to sPLA2 and NF-kB activation depends on the pathogenesis of IBD.

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List of Abbreviations

ASA	aminosalicylates
AZA	azathioprine
BMI	body mass index
CD	crohn's disease
FOS	fructooligosaccharide
GalCer	galactosylceramide (GalCer)
GALT	gut-associated lymphoid tissue
GBF	germinated barley foodstuff
GI	gastrointestinal
GLC	gas liquid chromatograph
Glyco-SL	glycosphingolipid
GOS	galactooligosaccharide
HBD-2	human β -defensin 2
IBD	inflammatory bowl disease
IC	indeterminate colitis
IL-23	interleukin-23
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MDP	muramyl dipeptide

MDR1	multidrug resistance protein 1
6-MP	6-mercaptopurine
MPO	myeloperoxidase
NF-kB	nuclear factor-kB
NOD	nucleotide-binding oligomerization domain
PAF	platelet activating factor
PAMPs	pathogen-associated molecular patterns
PGN	peptidoglycan
PRR	pathogen recognition receptors
PPARγ	peroxisome proliferators activated receptor-γ
PUFAs	polyunsaturated fatty acids
SCFA	short chain fatty acids
SGA	subjective global assessment
sPLA2	secretory phospholipase A2
SM	sphingomyelin
SMase	sphingomyelinase
TEER	trans-epithelial resistance
TGF	transforming growth factor
TLC	thin layer chromatography
TLR	toll-like receptor
Th17	T helper 17
TNF-α	tumor necrosis factor α
UC	ulcerative colitis

CHAPTER 1 THESIS OUTLINE AND LITERATURE REVIEW

1.0 Outline of Thesis

Chapter 1 provides a review of the literature on the reseach focus of this thesis Inflammatory Bowel Disease (IBD) and ganglioside. The rationale for undertaking research in the area of ganglioside supplementation on IBD Caco-2 cells culture model will be discussed in Chapter 2 along with thesis research objectives, aims and hypotheses. The benefits of ganglioside supplementation and underlying mechanism affecting the LPS-induced IBD Caco-2 cells culture model are illustrated in Chapter 3. In Chapter 4, the DSS-induced IBD Caco-2 cells culture model is used to further determine the anti-inflammatory effectS of ganglioside. In Chapter 5, the ganglioside composition of Caco-2 cells is determined after ganglioside supplementation to investigate the absorption and mechanism of ganglioside in a model of IBD. Finally, Chapter 6 summarizes the significant thesis findings and suggests potential future directions in ganglioside research.

IBD

1.1 Definitions of IBD, CD and UC.

Inflammatory bowl disease (IBD) is a chronic, relapsing disorder caused by multifactorial conditions in a genetically predisposed host [1], and is represented by a group of uncontrolled inflammatory disorders affecting the mucosa of small intestine or colon [2]. Crohn's disease and ulcerative colitis are the most common inflammatory bowel diseases. IBD affect men and women, children and young adults, and all ethnic groups, and involves virtually all bodily systems in addition to the gastrointestinal tract. Although the etiopathogenesis of IBD has not been clearly elucidated, it is thought to involve a complex interplay among genetic, environmental, microbial, and immune factors [3]. Ulcerative colitis and Crohn’s disease have emerged as major biomedical problems. IBD is

considered to be two separate conditions with distinguishing clinical, endoscopic, and histological findings, but IBDes have overlapping features and may even represent several different diseases with similar characteristics [4].

Crohn's disease was originally called regional ileitis [5], was named after the American physician Burril B. Crohn who published a clinical description of the disease [5]. In 1932, Crohn, Ginzburg and Oppenheimer were the first to publish the modern description of the symptomatology, pathology and clinical features of what has come to be called Crohn's disease to recognize the remarkable degree of accuracy in the published findings, although they are not the first to observe or describe Crohn's disease [5]. In their description of 14 cases, Crohn and colleagues described a chronic disease of young adults that presented with fever, diarrhea, and dull to cramp-like right lower quadrant abdominal pain, together with anemia and constitutional symptoms [5]. In fact, Crohn's disease was first seen by a German surgeon Wilhelm Fabry (aka Guilhelmus Fabricius Hildanus) in 1623 [6]. Crohn's disease today differs somewhat from its presentation in 1932. The Canadian Society of Intestine Research defines Crohn's disease as a chronic inflammatory disease of the digestive system that may affect any area of the gastrointestinal tract from the mouth to the anus. Various parts of the bowel may be affected by Crohn's disease either in continuity or as separate areas. It frequently affects the terminal ileum (the end of the small intestine), especially the section that joins with the large intestine or colon, which may also often be affected. The inflammation involves the full thickness of the bowel wall and consists of swelling, dilated blood vessels, and loss of fluid into the tissues [7].

Ulcerative colitis was first described by the British physician Sir Samuel Wilks in 1859 [8]. Crohn's & Colitis Foundation of America defines that, Ulcerative colitis is a chronic (ongoing) disease of the colon, or large intestine. The disease is marked by inflammation and ulceration of the colon mucosa, or innermost lining. Because the inflammation makes the colon empty frequently, symptoms typically include diarrhea (sometimes bloody) and often crampy abdominal pain. The inflammation usually begins in the rectum and lower colon,

but it may also involve the entire colon. When ulcerative colitis affects only the lowest part of the colon -- the rectum -- it is called ulcerative proctitis. If the disease affects only the left side of the colon, it is called limited or distal colitis. If it involves the entire colon, it is termed pancolitis [9].

While Crohn's disease (CD) involves primarily the small intestine and most specifically the ileum, ulcerative colitis (UC) is a disease of the colon (2). Ulcerative colitis differs from Crohn's disease. Crohn's can affect any area of the gastrointestinal (GI) tract, including the small intestine and colon. Ulcerative colitis affects only the colon. The inflammation involves the entire rectum and extends up the colon in a continuous manner. There are no areas of normal intestine between the areas of diseased intestine. In contrast, such so-called "skip" areas may occur in Crohn's disease. Ulcerative colitis affects only the innermost lining of the colon, whereas Crohn's disease can affect the entire thickness of the bowel wall [9].

Although UC and CD are generally accepted as clinically distinct conditions with distinguishing clinical, anatomical, and histological findings, a diagnostic gold standard remains elusive [10]. In fact, about 10% of patients have indeterminate features between UC and CD that cannot be clearly categorized [3]. There is no established definition for this subset of patients; their condition is simply termed ‘‘indeterminate colitis’’ until the diagnostic features of UC or CD become apparent later on [3].

1.2 Montreal and Vienna Classification of Crohn's Disease (CD) and Ulcerative Colitis (UC)

In recent years, the discovery of a series of genetic and serological markers has led investigators to readdress the complex issues involved in the classification of inflammatory bowel diseases. Most recent attention has focused on CD. From the clinician's perspective, accurate classification of these diseases would have potential benefits with respect to patient counselling, assessing

disease prognosis, and particularly with choosing the most appropriate therapy for each disease subtype [11].

An international working team that issued its report in Rome in 1991 proposed a classification scheme in which CD is divided into numerous subgroups based upon anatomical distribution, operative history and predominant clinical behaviour, including inflammatory and fistulizing or stenotic. Subsequently, the international working group, commissioned for the 1998 World Congress of Gastroenterology in Vienna group classified CD according to three critical phenotypic characteristics: age at diagnosis (A), disease location (L) and clinical behaviour (B). These classification systems have since been applied to numerous clinical trials and studies of the pathogenesis and natural history of CD, as the system is very practical for clinical use [12]. In 2003, a Working Party of investigators with an interest in the issues involved in disease subclassification was formed, with the objective of summarising recent developments in disease classification and examining the practicability of developing an integrated clinical, molecular, and serological classification of inflammatory bowel disease [11]. The results of the Working Party were reported at the 2005 Montreal World Congress of Gastroenterology. As summarized in table 1-1, The Montreal revision of the Vienna classification has not changed the three predominant parameters of CD: age at diagnosis, location, and behaviour, but modification of these of categories has been made [11].

Table 1-1 Vienna and Montreal classification for Crohn's disease [11]

	Vienna	Montreal
Age at diagnosis	A1 below 40 y	A1 below 16 y
	A2 above 40 y	A2 between 17 and 40 y
		A3 above 40 y
Location	L1 ileal	L1 ileal
	L2 colonic	L2 colonic
	L3 ileocolonic	L3 ileocolonic
	L4 upper	L4 isolated upper disease*
Behaviour	B1 non-stricturing, non- penetrating	B1 non-stricturing, non-penetrating
	B2 stricturing	B2 stricturing
	B3 penetrating	B3 penetrating
		p perianal disease modifier†

*L4 is a modifier that can be added to L1–L3 when concomitant upper gastrointestinal disease is present.

† ''p'' is added to B1–B3 when concomitant perianal disease is present.

In contrast with Crohn's disease, neither the Rome nor the Vienna Working Parties addressed subclassification of ulcerative colitis. The Montreal classification of disease extent of ulcerative colitis allows extent to be defined into three subgroups (table 1-2) [11]. As summarized in table 1-3, The Working Party has suggested the classification of severity of relapse into four disease activity/severity categories [11].

Table 1-2 Montreal classification of extent of ulcerative colitis (UC) [11]

Extent		**Anatomy**
E1	Ulcerative proctitis	Involvement limited to the rectum (that is porimal extend of inflammation) distal to the rectosigmoid junction
E2	Left sided UC (distal UC)	Involvement limited to a proportion of the colorectum distal to the splenic flexure
E3	Extensive UC (pancolitis)	Involvement extends proximal to the splenic flexure

Table 1-3 Montreal classification of severity of ulcerative colitis (UC) [11]

Severity		Definition
S0	Clinical remission	Asymptomatic
S1	Mild UC	Passage of four or fewer stools/day (with or without blood), absence of any systemic illness, and normal inflammatory markers (ESR)
S2	Moderate UC	Passage of more than four stools per day but with minimal signs of systemic toxicity
S3	Severe UC	Passage of at least six bloody stools daily, pulse rate of at least 37.5°C, haemoglobin of less than 10.5 g/100 ml and ESR of at least 30 mm/h

ESR, erythrocyte sedimentation rate.

The Montreal classification also readdressed the appropriate use of the term indeterminate colitis (IC) in clinical and research settings. The Montreal Working Party has recommended that the term ‘‘indeterminate colitis’’ should be reserved only for those cases where colectomy has been performed and pathologists are unable to make a definitive diagnosis of either Crohn’s disease or ulcerative colitis after full examination [12].

1. 3. Epidemiology of IBD

The highest incidence rates and prevalence of UC and CD have been reported from northern Europe, the UK, and North America, where the rates are beginning to stabilize [13]. IBD affects approximately 1.5 million people in the United States and more than 2 million in Europe [14]. In Canada, nearly 200,000 Canadian men and women suffer from IBD. The incidence continues to rise in southern Europe, Asia and most developing countries. This number is almost equally divided between CD and UC. IBD tends to be a disease of young adulthood, with those suffering from ulcerative colitis being diagnosed up to 10 years later than those with Crohn's disease [15, 16]. There may be a second "peak" later in life, but this has been debated. There are slightly more women affected with Crohn's disease than men, whereas in ulcerative colitis, there is no sex differences in incidence. CD can occur at any age, but the diagnosis is often

made between the teenage years and early adulthood with a smaller incidence occurring between the fifth and seventh decades of life. The disorder appears to affect women and men equally; however, some studies show a higher incidence in females than in males [15]. CD appears to involve certain ethnic groups more than others. For instance, it is more common in Jewish than in non-Jewish persons and in Caucations more than African Americans [14]. Reports of "familial" IBD appeared in the 1960s and subsequently increased, indicating a genetic relationship in IBD [17]. Familial distributions of IBD involved first-degree relatives (parent, child, or siblings) more often than second-degree or third degree relatives (aunts, uncles, nieces, and nephews) in accord with a polygenic inheritance [17].

IBD also has a higher incidence among smokers than nonsmokers [14]. Smoking is associated with an earlier age at diagnosis and a higher likelihood of requiring immunomodulator therapy or surgery for Crohn's disease. Individuals who smoke and have a diagnosis of CD are at higher risk of a complicated clinical course and increased frequency of exacerbations. Conversely, smoking is associated with a later diagnosis of ulcerative colitis, and with a better clinical course overall. Current smokers have a decreased risk for UC, whereas ex-smokers have 70% likelihood of developing UC. There are numerous studies confirming this unusual finding, but this effect that has yet to be satisfactorily explained [15]. It is not clear whether smoking prevents ulcerative colitis from developing or keeps disease that has already developed from becoming clinically apparent.

1. 4. Pathogenesis of IBD

Although the exact aetiology of IBD remains unclear, it is thought that inflammation results from an inappropriate chronic activation of the innate and adaptive mucosal immune systems in a genetically susceptible host [18]. Various environmental and host factors (e.g. genetic-, epithelial-, immune and non-immune) play a major role in the pathogenesis of IBD [19].

1. 4.1 IBD Genetics

The epidemiological data on IBD suggest a strong genetic contribution to disease pathogenesis, and a familial inheritance pattern that does not follow simple Mendelian models [20]. Because of this, IBD has been classified as a complex disease. In contrast to single-gene disorders, where the causal relationship between mutation and disease is clear, complex diseases result from the interaction of multiple genetic and non-genetic factors [21]. The role of host genetic regulation of the innate immune response in the pathogenesis of CD has been brought to sharp focus by identification of NOD2 (CARD15) mutations [19].

1. 4.1.1 Family aggregation studies in IBD

There are several lines of evidence that suggest that genetic predisposition is an important factor in the aetiology of both diseases. Familial aggregation of IBD was first reported in the 1930s [22]. A positive family history is found in 5–20% of patients with IBD and first degree family members of IBD patients are at a 10 to 15-fold increased risk to develop IBD themselves, somewhat more in CD than in UC [23]. Although family clustering could possibly be due to shared familial exposure to an environmental risk factor, no evidence for an increased risk of CD to spouses or adopted family members could be found [24, 25]. The most convincing evidence for genetic predisposition comes from twin studies, which showed increased concordance rates for both UC and CD in monozygotic twins as compared to non-identical twins. This effect appeared to be more pronounced in CD than in UC: the concordance for CD in monozygotic twins is 35% compared with 7% in dizygotics, with the equivalent in UC being 11 versus 3% [23]. The markedly increased concordance rates in identical twins can only be explained by a genetic component underlying IBD, especially CD.

In 80% of families with IBD, affected individuals are concordant for disease type, with all of the affected individuals having Crohn's disease or, all of

the affected individuals having ulcerative colitis. The remaining multiply affected families are ‘mixed’ [20]. These findings imply that a subset of genes associated with IBD will be common to both Crohn's disease and ulcerative colitis, and others would be found only in one of the two diseases.

1.4.1.2 Genetic approaches to study susceptibility genes in IBD

Until very recently two approaches were used in the search for genes involved in the pathogenesis of IBD. The ‘candidate gene approach’ tests for associations between disease and genes that are involved in the regulation of the inflammatory response [23]. In general, differences in allelic frequencies are compared between patients and ethnically matched unrelated healthy controls. An association between the disorder and a specific marker suggests a causal relationship or, alternatively, linkage disequilibrium.

The second approach is the ‘genetic linkage study’, which does not rely on a hypothesis driven approach to identity a functional candidate gene, but use of genome wide screening in multiple affected IBD pedigrees for the purpose of identifying chromosomal regions, loci, that are shared in excess of statistical expectation in affected family members. If there is significantly increased co-inheritance of polymorphic markers in the affected relatives in a given region, this region is considered to be linked to the disease. Once linkage is established the association studies of candidate genes in this region are used to identify the specific disease gene. These studies led to the discovery of a number of susceptibility regions, according to the date of discovery number named IBD 1–9 [23].

In recent years a new technique evolved which has accelerated genetic research considerably. With the completion of the human genome sequence it became possible to perform ‘genome-wide association studies’ (GWAS), providing systematic assessment of the contribution of common variation to disease pathogenesis. This approach has had an unprecedented impact on our knowledge of the genetics of many autoimmune diseases, including systemic diabetes mellitus, lupus erythematosus, rheumatoid arthtritis and

multiple sclerosis, bringing to light many unexpected candidate genes and biological pathways. GWAS involves genotyping with several hundred thousand of markers and compare allele frequencies between patients and healthy controls. The studies have resulted in the identification of many novel loci for Crohn's disease as well as ulcerative colitis, have highlighted the importance of the innate immune system and implicated new pathogenic pathways such as autophagy [23].

1. 4.1.3 Genes involved in IBD

The NOD2 gene on chromosome 16 q (IBD1) was the first susceptibility gene identified for IBD [26, 27]. The product of this gene is expressed in many cells, including monocytes, dendritic cells, Paneth cells and intestinal epithelial cells and contains an apoptosis related CARD domain [28-30]. The NOD2 gene codes for the NOD2 protein, which is a pattern recognition receptor that senses bacterial breakdown product of peptidoglycans muramyl dipeptide (MDP) [31-33].

Since then, genome wide association searches have found multiple other loci contributing to the risk of developing CD (Table 1-4) [34]. Currently, at least 71 loci show unequivocal association with CD risk, 2 show highly probable association, and another 8 show likely association [35], [36]. Other searches have found additional loci [37]. Some CD loci also influence the risk for ulcerative colitis [38]. Identified gene products include NOD2, XBP1, ATG16L1, IRGM, and IL23R.

Interleukin-23 signaling is mediated through the engagement of heterodimeric interleukin-23 (comprising p19 and p40 subunits) with its heterodimeric receptor (comprising interleukin-23R and interleukin-12RB1) [39]. The engagement activates the JAK-STAT (Janus-associated kinase–signal transducers and activators of transcription) signaling pathway, which regulates the transcription of several genes. The importance of interleukin-23 signaling in mediating inflammation has been shown in animal models [39].Moreover, reports of highly significant genetic associations between IL23R and inflammatory bowel disease, psoriasis, and ankylosing spondylitis indicate that inflammatory bowel

disease shares genetic associations with certain other autoimmune diseases [39]. Interleukin-23, secreted by macrophages and dendritic cells, may contribute to Th17 proliferation, survival, or both [40]. Interleukin-23 also contributes to intestinal inflammation through Th17-independent pathways [41]. Levels of interleukin-23 and Th17 cytokines are elevated in the colonic mucosa in both Crohn's disease and ulcerative colitis [39].

The genetic variants that have been found to confer Crohn's disease risk point to the importance of innate immunity, autophagy and phagocytosis in Crohn's disease pathogenesis [42]. In particular, a number of genes associated with Crohn's disease (IL23R, PTPN2) are also associated with other autoimmune disorders, suggesting that a subset of Crohn's disease patients share common triggers with these conditions. In addition, multiple disease-associated intergenic segments have been identified and replicated in genome-wide association studies [42]. These intergenic regions implicate new genes and pathways—possibly including genes that are expressed within these regions and others that are remotely regulated to modify the disease phenotype. Further understanding of regulatory elements within non-coding genomic regions and gene–gene interactions will lead to a better understanding of the underlying mechanisms that cause disease [42].

1.4.2 Immune function

The intestinal immune system is in a constant state of activation, and there is substantial evidence that loss of the ability to control activation (inflammation) is an important pathogenic mechanism in IBD. A major current working hypothesis defines Crohn’s disease as a dysregulated immune response towards components of the intestinal flora, leading to chronic intestinal inflammation [18]. The causes for this inappropriate response can be attributed to (a combination of) defects in the epithelial barrier, the innate immune response or the adaptive immune response [43].

Table 1-4 Genetic loci with identified or candidate genes associated with the risk of developing Crohn's disease [34].

Locus	Gene/candidate	Function
Loci with confirmed or with highly probable contribution to CD risk		
16q12	NOD2/CARD15	MDP sensor (recognition of bacteria)
2q37	ATG16L1	Autophagy
5q33	IRGM	Initiates autophagy of intracellular bacteria
22q12	XBP1	ER stress response
18p11	PTPN2	Cell signaling (tyrosine kinase), growth factor stimulation
12q12	MUC19, LRRK2	Mucus protein, unknown (Parkinson's disease, autophagy)
5p13	PTGER4 (EP4)	Prostaglandin receptor
9q32	TNFSF15	Induces endothelial cell apoptosis
10q21	ZNF365	Unknown (zinc-finger protein)
1p13	PTPN22	Cell signaling (tyrosine kinase) associates with CBL
1q23	ITLN1	Galactose-binding lectin (recognition of bacteria)
6p22	CDKAL1	Unknown (regulation of a cyclin-dependent kinase)
6q27	CCR6	Chemokine receptor
9p24	JAK2	Cell signaling (tyrosine kinase), cytokine stimulation
11q13	C11orf30	Unknown (oncogene)
17q21	ORMDL3	Unknown (also associated with asthma risk)
17q21	STAT3	Cell signaling, cytokine stimulation
21q22	ICOSLG	ICOS ligand (costimulation)
Loci with confirmed or with highly probable contribution to CD and UC risk		
1p31	IL23R	IL23 receptor
5q33	IL12B (IL12p40)	Component of IL12 and IL23

Locus	Gene/candidate	Function
6p21	MHC genes	Epitope selection
10q24	Nkx2-3	Regulates epithelial cell and lymphocyte development
3p21	MST1	Cytokine, macrophage stimulatory protein
Loci with likely contribution to CD risk		
2p23	GCKR	Cell signaling (e.g. Wnt signaling)
2p16	PUS10	Unknown (tRNA pseudouridine synthesis)
17q12	CCL2, CCL7	C–C chemokines, macrophage recruitment
6p25	LYRM4	Unknown (protein folding)
6p25	SLC22A23	Organic ion transporter
2q11	IL18RAP	IL18 receptor component

The gut associated lymphoid tissue contains a complex population of immune cells that balance the requirement for immune tolerance of luminal microbiota with the need to defend against pathogens, the excessive entry of luminal microbiota, or both [39]. The hallmark of active inflammatory bowel disease is a pronounced infiltration into the lamina propria of innate immune cells (neutrophils, macrophages, dendritic cells, and natural killer T cells) and adaptive immune cells (B cells and T cells). Increased numbers and activation of these cells in the intestinal mucosa resulted in elevated concentrations of tumor necrosis factor α(TNF-α), interleukin-1β, interferon-γ, and cytokines of the interleukin-23–Th17 pathway [39].

The innate immune system is composed of cells, which are the first to contact invading microorganisms, and include antigen presenting dendritic cells, macrophages and natural killer cells. This arm of the immune response recognises bacteria mainly via pathogen associated molecular patterns (PAMPs). Such recognition allows the immune cells to respond to a wide array of microorganisms using a restricted number of receptors. A major family of PAMP receptors is the membrane associated Toll-like receptor (TLR) family, which bind to different

bacterial products and transduce an inflammatory signal to the cells [44]. Following the activation of the innate immune system, dendritic cells activate T cells, which are part of the adapted immune system, leading to differentiation of naïve T cells in effector and regulatory T cells. In experimental models of colitis inflammation appears to be the result of both excessive effector T cell function and deficient regulatory T cell function [45, 46].

Following activation, T cells respond by secreting soluble mediators, including pro- and anti-inflammatory cytokines. These molecules serve to activate or suppress the immune response and it is postulated that the balance between pro-inflammatory and anti-inflammatory/regulatory cytokines plays an important role in the pathogenesis of IBD. Certain helper T cells (Th1, Th2, and Th17) and regulatory T cells (e.g., forkhead box P3 [Foxp3+]Treg), which are subgroups of CD4+ T cells, secrete characteristic types of cytokines. Balance of these subgroups is needed to maintain intestinal immune homeostasis [39]. Effector subgroups (Th1, Th2, and Th17 cells) are critical for defenses against pathogens and excessive entry of luminal microbiota, but expansion and overactivity of these cells relative to the regulatory CD4+ T cells can facilitate to intestinal inflammation. Studies of inflammatory bowel disease in mice and humans implicate dysregulation of intestinal CD4+ T-cell subgroups in the pathogenesis of these diseases. In Crohn's disease, for example, there is increased production in the intestinal mucosa of the Th17 cytokine interleukin-17 and the Th1 cytokines interferon-γ and TNF-α [39]. In ulcerative colitis, by contrast, there is usually an increase in interleukin-17 and Th2 cytokines [39]. The interleukin-23 pathway is central to the function of Th17 cells. Polymorphic variants of multiple genes involved in this pathway and in Th17 cell function have been associated with both Crohn's disease and ulcerative colitis [39].

The role of B cells in inflammatory bowel disease has not been as extensively studied as that of T cells. Intestinal B cells produce IgA antibodies, which contribute to immune protection without provoking inflammation. In animal models of colitis, both anti-inflammatory and proinflammatory roles of B cells have been described [39].

Most encounters with mucosal and intraluminal antigens do not induce effector responses, but, rather result in tolerance (‘oral tolerance’) which is the induction of specific T cells [47]. The pathogenesis of these diseases includes a disruptive interaction between the immune system and gut luminal factors, it has been proposed that IBD is caused by defective T-cell mediated regulation. Animal studies have identified a CD4+ CD25+ cell population which expresses the FOXP3 transcription factor [48]. This regulatory T cell population (Tregs) is thought to suppress the activation of effector T cells at the level of the antigen-presenting cell and produce IL10 and transforming growth factor (TGF) β. In addition to this ‘naturally occurring’ Tregs, there are also ‘adaptive’ Tregs, the so-called Tr1 and Th3 cells [43]. Regulatory T cells appear to be key players of immune regulation, and they have important functions in regulating unwanted inflammatory responses towards self-antigens, and the antigens of endogenous intestinal bacteria. IBD patients do not seem to have a primary defect in regulatory cells, but the regulatory capacity of these cells is apparently insufficient to down regulate inflammation.

1. 4.3 Permeability

The single layered intestinal epithelium is a physical and immunological barrier that prevents direct contact of the intestinal mucosa with the luminal microbiota. A compromised intestinal barrier may play a crucial role in the development of IBD, by allowing the entry of luminal antigens and microorganisms into the mucosa and initiating overwhelming inflammatory responses [49]. A common feature of gut inflammation is increased epithelial permeability, both paracellular (i.e., the pathway between adjacent cells) and transcellular (i.e., material crosses apical and basolateral plasmalemmas) permeability [50]. One of the distinct features of Crohn’s disease is impaired intestinal epithelial function, characterized by increased permeability (altered barrier function) and ion secretion, often resulting in a luminally directed driving force for water movement causing diarrhea. Interestingly, in otherwise clinically asymptomatic CD patients, increased intestinal epithelial permeability preceding

clinical relapse has been observed suggesting that a barrier defect may be an early event in disease reoccurrence. Since first degree relatives of IBD patients, without evidence of disease, also are reported to have abnormal intestinal permeability, it has been proposed that increased intestinal permeability may be a primary etiologic factor in IBD [51, 52]. However, it has also been shown that the inflammatory process itself leads to increased intestinal permeability [53]. Thus at present it is not clear if the changes in barrier integrity observed in IBD are particularly involved in the early events of IBD pathogenesis or are rather a secondary phenomenon [54, 55].

1. 4.4 Interaction between genetics, immune function and permeability

The first proposed mechanism in IBD involves the inappropriate regulation of the innate immune response at the level of the intestinal mucosa. The innate immune system is the first line of defense against resident luminal microflora and invading pathogens, and can respond to a wide variety of microorganisms. The innate immune system has evolved to monitor the resident microflora and relay danger signals in response to infection by invasive organisms. This response is mediated through the recognition by specific pathogen recognition receptors (PRR) of microbial components, known as pathogen-associated molecular patterns (PAMPs). The PRRs include the members of the Toll-like receptor (TLR) family, which are predominantly cell surface receptors, and the cytosolic Caterpillar-(CARD)/NOD intracellular receptors [56, 57]. The binding of PAMP ligands to specific PRRs leads to the activation of several intracellular signaling pathways, which include the nuclear factor-kB (NF-kB) and mitogen-activated protein kinase (MAPK) pathways for TLRs and predominantly the NFκB pathway for CARD/NOD receptors. These pathways in turn lead to the activation of transcriptional programs resulting in the broad-spectrum non-specific killing mechanisms of innate immunity. These mechanisms include synthesis of reactive oxygen species, activation of the complement protein system, secretion of chemokine and cytokines for chemotaxis of phagocytotic macrophages, and secretion of antimicrobial proteins by Paneth cells [21]. Paneth cells are specialized epithelial cells located at the base of small intestinal crypts,

which monitor the intestinal lumen and are considered important mediators of mucosal innate immune defense. They contribute to host defense and maintenance of the gastrointestinal barrier through the luminal secretion of a number of antibacterial peptides (defensins, lysozyme and secretory phospholipase A2), which protect nearby intestinal stem cells and control microbial density [21]. Recent studies in experimental models estimate that close to 40 different genes (including known TLR family members) may play a role in innate immune response to bacterial invasion, many of which have yet to be identified [58].

The second proposed mechanism for the development of IBD involves the increased permeability across the mucosal epithelial barrier due to loss of structural integrity and/or abnormal transepithelial transport. While innate immunity is a key mediator of mucosal immune defense, the epithelial mucosa is the first line of physical defense against invading microorganisms. The intestinal epithelium represents a unique challenge for the organism, as it must balance the need for an extensive surface area permitting effective absorption of nutrients, with the need of defending against the intrusions of microorganisms which colonize the gastrointestinal (GI) tract [59, 60]. Unlike other epithelia, the intestinal mucosa is composed of a single layer of polarized intestinal epithelial cells which protects against direct contact of enteric antigens, bacteria or other pathogens with the underlying gut-associated lymphoid tissue (GALT). The integrity of the epithelium is maintained mostly through a combination of intercellular adhesion structures and specialized junctions, which also define cellular polarity. In addition, the presence of mucins and trefoil peptides, the rapid turnover of epithelial cells, and the peristaltic movement of the GI tract all help to protect against colonization and invasion of the intestinal mucosa by pathogens [60]. The role of increased epithelial permeability across the gut epithelial barrier (leaky gut) has gained increasing support in IBD pathogenesis, particularly as this epithelium represents an interface for genetic and environmental influences [21]. Evidence of a genetic contribution to increased intestinal permeability has long been suspected in IBD patients and their unaffected relatives [21]. Interestingly, some of the loci identified through linkage and association analysis have been

suggested to be involved in epithelial integrity, differentiation and transepithelial transport, although a causal link of these to modulation of permeability remains speculative at this time [61, 62].

The third proposed mechanism for the development of IBD involves the deregulation of the adaptive immune system stemming from an imbalance between regulatory and effector-cell immune responses to luminal antigens or other antigen (e.g. self-antigens). In contrast to innate immunity, adaptive immunity generates a slow and more targeted response involving antigen-specific recognition and immune memory. The GALT represents the largest part of the body's immune system, and given the large surface area of the mucosal epithelium, the immune system encounters more antigens in the gut than any other location in the body. In addition, since most of the antigens encountered by the mucosal immune system are derived from food proteins and commensal bacteria, the immune system must remain relatively unresponsive to avoid responses to harmless antigens and maintain epithelial integrity. It has been proposed that tolerance to these luminal antigens, also known as oral tolerance, occurs through a state of active cellular suppression or clonal anergy of immune reactive cells induced by specialized regulatory T cells [63]. The proposed role of adaptive immunity in IBD is derived from genetic studies indicating a central role of the MHC in the development of IBD and also from in vivo observations in IBD patients of abnormal patterns of cytokine production and immune cell responses (excessive Th1 response in CD and Th2 response in UC), of modulations in regulatory T and B cell functions, and of antibodies to luminal antigens [64].

1. 5. Animal Models of IBD

Much of the recent progress in the understanding of mucosal immunity has been achieved by the study of new experimental animal models of intestinal inflammation [65, 66]. Although these models do not represent the complexity of human disease and do not replace studies with patient material, they are valuable tools for studying many important disease aspects that are difficult to address in humans, such as the pathophysiological mechanisms in early phases of colitis and

the effect of emerging therapeutic strategies. The clinical appearance of human IBD is heterogeneous, a fact that is also reflected by the steadily increasing number of transgenic or gene targeted mouse strains displaying IBD like intestinal alterations. Most of these models are based either on chemical induction, immune cell transfer or gene targeting, only in some models disease occurs without any exogenous manipulation. Mouse models are broadly categorized into 3 categories according to the defect in mucosal immunity that is believed to be most important for the onset of the disease (Table 1-5) [42]: (1) defects in epithelial integrity/permeability, (2) defects in innate immune cells and (3) defects in cells of the adaptive immune system.

Key lessons learned from IBD models include: (a) a compromised epithelial layer is sufficient to result in intestinal inflammation; (b) T cells have been implicated in numerous models presumably promoting an inappropriately activated autoreactive effector T-cell population; (c) a variety of other haematopoietic cells have been shown to be able to mediate or regulate intestinal inflammation; (d) many studies have elucidated the roles of the different cytokines at play in the different models of colitis—chemoattractant cytokines may have a unique role in IBD pathogenesis; and (e) although no specific pathogen has been isolated from the intestinal flora of spontaneous colitis models, the resident enteric flora seems necessary for colitis induction [42].

Table 1-5 Mouse models of colitis with altered barrier, innate or adaptive immune responses [42]

Model	Known defects
Multidrug-resistant 1α-/- (also known as ABCB1-/-)	Altered epithelial barrier
Gαi2-/- (GNAI2-/-)	Defective epithelial barrier; defective regulatory B cells
Macrophage-PMN Stat3-/-	Increased response to lipopolysaccharide (LPS); resistance to IL-10 regulation
Bone marrow Stat3-/-	Increased response to LPS; impairment of innate immune function
A20-/- (TNFAIP3-/-)	Increased response to LPS
Il10 and Il10Rb-/-	Lack of Trl (Tr1; Treg cells) activity; lack of TGF-β signalling
NF-κB (Nfkb1-/-, Rela-/-)	Increased IL-2 production
TGFb1-/-, TGFbR2-/-	Decreased numbers of regulatory T cells
SAMP1/Yit mutant mice	Epithelial cell defects; expanded B-cell population; increased numbers of activated T cells
Il2-/- and Il2Ra-/-	Decreased numbers of CD4+ CD25+ T cells
TNFaΔARE-/- (ARE, AU-rich elements)	Increased TNF-α production
CD4+, CD45RBhigh transfer	Decreased numbers of regulatory T cells
TCRa-/- (T-cell antigen receptor mutant)	Loss of a regulatory B-cell function
WASP-/- (WAS-/-)	Regulatory T cells
CD40L transgenic mice	Increased numbers of activated T cells
Smad3-/-	Decreased numbers of regulatory T cells
Epithelial cell specific deletion of NEMO	Barrier function/innate immunity
Dextran sulphate sodium	Direct damage to epithelial barrier
Dextran sulphate sodium/Tff3-/-	Goblet cell dysfunction; impaired epithelial repair
Dextran sulphate sodium/Ptger4-/-	Altered epithelial barrier
Muc2-/-	Barrier function/mucus defect
N-cadherin mutant	Barrier function

1.6. Nutrition Deficiencies – Methods of Assessment

1.6.1 Nutrition deficiency in IBD

IBD is frequently associated with malnutrition. The pattern and severity of malnutrition in IBD depends on the duration, activity and extent of the disease. Up to 75% of patients with an active phase of IBD suffer from weight loss and hypoalbuminemia (Table 1-6) [67]. Anaemia and vitamin deficiencies (specifically vitamin D and vitamin B12) as well as trace element deficiencies are also described. Malnutrition with loss of muscle mass and body fat is more frequent in Crohn's disease than in ulcerative colitis and is usually restricted to the active phase. In quiescent disease malnutrition is uncommon and restricted to patients with short bowel, high output fistulas or a severe stenosis reducing food intake [67].

The aetiology of malnutrition is partly due to anorexia because of food associated symptoms, e.g. pain, and partly due to increased intestinal losses (albumin and blood). Increased energy expenditure in the active phase may only play a smaller role in the development of malnutrition [67]. If IBD develops in childhood, growth retardation can result in up to 40% of the patients. Almost 90% of children have a slowing of growth rate before the diagnosis of IBD is made. Eventually, 7–30% of children with Crohn's disease will remain below the 5th percentile of their growth curve. Table 1-6 shows a summary of nutritional deficiencies in IBD [67]. In quiescent IBD energy and substrate metabolism is normal or shows the typical changes of a shortage of energy intake with a decrease in carbohydrate and an increase in fat oxidation. During the active phase patients develop a mixed picture of an inflammatory reaction and malnutrition with slight increases in energy expenditure and a relative increase in fat oxidation and decrease in carbohydrate oxidation. These changes are, however, non-specific and quickly reversible when patients are given nutritional support.

1. 6.2 Methods of Assessment

Determine nutritional status requires a nutritional history, physical exam, objective laboratory parameters and clinical judgment. Subjective global assessment (SGA) developed originally for use in oncology patients, is a useful tool for screening an IBD patient [68]. Using SGA, patients are categorized into one of three stages: well nourished, moderately malnourished or severely malnourished. SGA takes into account history of weight change, food intake, gastrointestinal (GI) symptoms and functional capacity. SGA, coupled with physical exam, provides clinicians with an indication of the patient's nutrition risk and need for intervention. SGA has been shown to provide reproducible results with greater than 80% agreement [69].

Further evaluation of nutrition risk can involve the use of body mass index (BMI). However, one measured weight cannot provide a thorough picture of risk. In addition, a normal appearing BMI does not necessarily correlate with an adequately nourished patient. One has to establish if the weight has significantly changed, over what period of time and if weight loss was intentional or not. A very low BMI or significant change in BMI requires more immediate nutrition intervention [68].

Clinicians often use serum albumin as a marker of nutritional status. However, in the case of a hospitalized or sick patient, a low albumin reflects an acute or chronic inflammatory process such as infection, trauma or cancer. The IBD patient often falls into this category.

Table 1-6 Nutritional deficiencies in IBD [67].

Nutritional deficiencies	Crohn's disease frequency (%)	Ulcerative colitis frequency (%)
Weight loss	65–75	18–62
Hypoalbuminaemia	25–80	25–50
Intestinal protein loss	75	+
Negative nitrogen balance	69	+
Anaemia	60–80	66
Iron deficiency	39	81
Vitamin B_{12} deficiency	48	5
Folic acid deficiency	54	36
Calcium deficiency	13	+
Magnesium deficiency	14–33	+
Potassium deficiency	6–20	+
Vitamin A deficiency	11	Not reported
Vitamin B_1 deficiency	+	Not reported
Vitamin C deficiency	+	Not reported
Vitamin D deficiency	75	+
Vitamin K deficiency	+	Not reported
Zinc deficiency	+	+
Cu deficiency	+	+
Metabolic bone disease	+	+

1.7. Nutrition Deficiencies – Pathophysiology

1.7.1 Causes of malnutrition in IBD

From the earliest descriptions of the disease, IBD, especially CD, has been traditionally associated with serious nutritional deficiency. The origins of malnutrition in CD are multifactorial, but dietary restrictions (due to intolerance of diet or therapeutic fasting) are the most important. Also included are: the increase in energy requirements, the malabsorption of nutrients in the case of extensive intestinal involvement, gastrointestinal losses and the interaction between nutrients and drugs [70]. Furthermore, the underlying inflammatory mediators of the physiopathology of IBD [71], such as tumor necrosis factor (TNF)-α , and interleukins-1 and -6 can increase catabolism and lead to anorexia. Table 1-7 provides a summary of the causes of malnutrition in IBD [70].

1.8. Nutrition Support

Nutrition therapy plays a fundamental role in the clinical management of all patients with IBD. Its objectives are to correct macro and micronutrient deficiencies in malnourished patients subjects to reverse the physiopathological consequences of such deficiencies and to regulate inflammation [70].

1. 8.1 Enteral Nutrition

Enteral feeding using formulas or liquids should always take preference over parenteral feeding, unless it has been completely contraindicated. If oral feeding were not possible, feeding the patient through a nasogastric or nasoenteric tube should be considered. The value and benefits deriving from its use are directly dependent on the geographical location of the disease, its extent and gravity and enteral feeding is therefore especially indicated for CD patients when the small intestine is affected, while there is no evidence which supports the use of enteral nutrition in the treatment of UC [70].

Table 1-7 Contributing factors to of malnutrition in IBD [70]

Decrease in oral intake	Restrictive diets, therapeutic fasting
	By the disease itself: diarrhea, abdominal pain, nausea and vomiting, etc.
	Alteration in taste: due to drugs, vitamin and mineral deficiencies,
	pro- inflammatory mediators
	Anorexigenous effect of pro-inflammatory cytokines
Gastrointestinal losses	Diarrhea
	Rectorrhagia/hematochezia
	Loss of mucus and electrolytes
	Protein-losing enteropathy
Metabolic disorders	Increase in resting energy expenditure
	Enhanced fat oxidation
Increase in nutritional requirements	Inflammatory states
	Increased basal oxidative metabolism
	Infectious complications
	Post-surgery
Drug interaction	Corticoids and calcium reabsorption
	Corticoids and protein catabolism
	Salazopirine and folates
	Methotrexate and folates
	Cholestyramine and liposoluble vitamins
	Antimicrobials and vitamin K
	Anti-secretors and iron
Poor absorption of nutrients	Reduction of the absorptive surface: intestinal resection,
	enteric fistulas, hypertrophy of the villi
	Blind loops, bacterial overgrowth
	Poor absorption of bile salts in ileitis or resection

Apart from the intake of calories, proteins and micronutrients, enteral nutrition using liquid formulas performs other primary therapeutic functions in CD [72]. In 1973, the therapeutic effect of enteral nutrition exclusively using basic formulas (amino acids with no antigenic capacity) was described for the first time in adults with CD resistant to other therapies [73], as similar remission rates were achieved to corticosteroids [74]. This ability to abate

CD activity in both adults and children extends to efficiency in maintaining remission, allowing delay in the need for surgery or reintervention [70]. Furthermore, it is a safe treatment for which no significant adverse effects have been reported.

With regard to enteral nutrition formulas, no differences were identified between the efficiency of elemental diets and polymeric formulas [75], which leads to the rejection of the previously held idea that a diet lacking in antigenic capacity could restore the altered intestinal immune response. In this respect, the therapeutic effect of enteral nutrition in CD seems to be independent of the form of the nitrogen source used [76]. On the other hand, the fat composition of the enteral diet seems to be more important in terms of its therapeutic effect on CD [77], as this fat composition could be the key factor of the diet's therapeutic action on the disease [78]. Various studies have assessed the efficiency of supplements using n-3 PUFAs in maintaining patients with CD and UC in remission, showing that they might only prove effective for maintaining CD cases in remission, although more extensive studies are required in order to unequivocally establish the utility of these therapies [70]. In any case, these treatments are safe and no side effects have been reported.

The precise mechanism of action through which enteral nutrition operates in CD is not well known, but it has been suggested that it could act by modulating the immune system's mucosa, regulating imbalances in the bacterial flora capable of precipitating inflammation [79, 80], or by modifying the luminal content, thereby altering the expression of certain genes in the epithelium with an effect on the immune system of the mucosa, as well as reducing the exposure of the intestine to antigens (Table 1-8).

1. 8.2 Parenteral Nutrition

Dudrick et al (1969) were the first to suggest that parenteral nutrition (PN) was possibly beneficial to patients with IBD [82]. In fact, PN is of scant therapeutic interest in IBD since diverse studies have shown that intestinal rest is not beneficial to control the disease [83, 84]. Consequently, parenteral nutrition is

not useful for the induction or maintenance of remission in CD, nor do we have any evidence to support its use in UC [85]. It is also very expensive and poses an additional risk due to the use of venous catheters [86]. Its utility is therefore restricted to certain cases involving efforts to close enterocutaneous or other complicated fistulas in patients with fistulizing CD, the treatment of short bowel syndrome following extensive resections for CD, or when enteral feeding is impractical for other reasons [70].

Table 1-8 The mechanism of action of enteral nutrition in CD [81]

Proposed mechanism of action
Improvement of nutritional status
Down regulation of pro-inflammatory cytokines
Anti-inflammatory effects
Promote epithelial healing
Decrease gut permeability
Decrease antigenic load to the gut, bowel rest
Modification of gut flora

1. 9. Bacterial microbiotics

The precise nature of the initiating events involved in Crohn's disease is unknown, although findings in animal models and human studies support the concept that either an inappropriate or exaggerated immune response is responsible for much of the pathogenesis of the disease [87]. This immune response may be driven by exposure to enteric bacteria (commensal or pathogenic) or bacterial products such as lipopolysaccharide (LPS) derived from Gram negative bacterial.

Evidence supporting the hypothesis that intestinal bacteria play a role in the pathogenesis of IBD includes the observation that inflammation and lesions generally occur in intestinal regions with the highest bacterial concentrations (i.e. the ileum and colon) [88], that IBD patients typically have greater numbers of adherent bacteria compared to normal subjects[89], diversion of the fecal stream can prevent intestinal inflammation whilst re-establishment of the flow will lead to recurrence [90, 91], and that antibiotic treatment is often a viable treatment option, particularly in CD[92]. Further evidence arises from animal models, including IL-10−/− mice, which remain healthy when housed in germ-free conditions, but which spontaneously develop colitis upon intestinal colonisation by typical commensal bacteria [93].

Among the commensal intestinal microbes colonizing both IBD patients and experimental animals, anaerobic bacteria, particularly members of the genus Bacteroides, are thought to play an important role in the development of inflammation. In a carrageenan guinea pig model of experimental colitis, germfree animals did not develop colitis until after monoassociation with Bacteroides vulgatus, and furthermore, pretreatment with the antibiotic metronidazole prevented colitis [94]. The degree of cecal inflammation in HLA-B27 transgenic rats correlated with the levels of isolates of Bacteroides and increased anaerobic bacterium/aerobic bacterium and Bacteroides/aerobic bacterium ratios [95] . Microorganisms other than Bacteroides have also been implicated. For example, Helicobacter muridarum has been shown to play an important role in the onset of IBD in SCID mice [96]. An investigation of the rectal mucosa-associated bacterial flora in ulcerative colitis patients showed that both the highest bacterial counts and the highest isolation frequency were observed for B. vulgatus, Bacteroides fragilis, and Bacteroides ovatus, in that order [97]. Altered host response dynamics may also account in part for the development of disease. Several studies have demonstrated that the systemic and local immune responses against gut microflora are distorted in IBD patients. The associated lesions and the immunologic changes indicate a breakdown of mechanisms that maintain oral tolerance to components of the microflora and/or

foodstuffs [98]. The findings supporting the presence of an altered immune status include an exaggerated mucosal antibody response against intestinal bacteria. For example, it has been shown that the serum antibody titer against B. fragilis was elevated in IBD patients and an increased level of serum antibody to the genus Bacteroides was also demonstrated [98]. B. ovatus may be a predominant commensal microbe causing a systemic IgG antibody response in IBD patients and identified a dominant bacterial antigen [99]. Enzymes produced by this bacterium damage intestinal tissue [99] and may compromise the epithelial barrier. This could result in an increase in the antigen load in the submucosal compartment, culminating in an enhanced systemic IgG antibody response to the bacterium within the intestinal lumen. This is without doubt a complex process, probably involving various bacterial phenotypes.

Flagellin, a common antigen present on most motile bacteria in the intestine, was recently identified as a major antigen in CD [100]. Marked reactivity of T cells to flagellin was observed, and flagellin-specific T cells induced colitis when they were adoptively transferred into immunodeficient mice [98]. Whether such antigenic molecules consistently play a directly pathogenic role or even a potentially diagnostic role in intestinal tissue in vivo remains to be conclusively determined, but this is an exciting possibility [98].

It has been proposed that IBD pathogenesis could be traced to a single pathogenic strain of bacteria such as mycobacterium avium subspecies paratuberculosis (MAP), largely due to the similarities between infectious enterocolitis and IBD. A link has been demonstrated between MAP infection and Crohn's disease [101, 102], however, other studies have produced inconclusive results [103] or indicated that MAP is not associated with Crohn's [104]. Other bacterial strains linked to the pathogenesis of IBD include Escherichia coli [105], Mycobacterium kansaii [88], Listeria monocytogenes [88], Pseudomona maltophila [88] and Bacteroides vulgatus [106]. It would seem unlikely that one bacterial strain is responsible for the pathogenesis of IBD, however, there may be a subset of patients for which this is the case. Another hypothesis that is being

investigated is that IBD develops due to an altered response to commensal bacteria, not a pathogenic strain [107].

In the healthy gut, there is a symbiotic relationship between the host and the commensal bacteria in which exposure leads to the down-regulation of inflammatory genes, inhibiting the immune response of the gut to other pathogens [4]. In the case of IBD however, genetically predisposed individuals appear to lose the normal tolerance to commensal bacteria, leading to an elevated inflammatory response. The microbiota then provide a constant stimulus for the host immune system [90, 108].

Toll-like receptors (TLRs) are responsible for microbial recognition, induction of anti-microbial genes and control of the adaptive immune response [109]. TLRs recognise a range of bacterial motifs including lipoproteins (TLR1, 2, 6), double-stranded RNA (TLR3), lipopolysaccharide (TLR4), flagellin (TLR5), single-stranded RNA (TLR7, 8) and CpG DNA (TLR9) [109, 110]. Importantly, TLRs are required to discriminate between pathogenic and commensal bacteria. If tolerance for commensal bacteria is lost, an auto-immune response may be elicited against these non-pathogenic bacteria, leading to increased pro-inflammatory cytokine and chemokine production, as seen in conditions such as IBD [107]. There are a number of mechanisms in place to ensure tolerance including a decreased surface receptor expression to limit recognition, and ligand-induced activation of peroxisome proliferators activated receptor-γ (PPARγ) which uncouple NFκB-dependent target genes [109-111]. TLR4 expression has been shown to be up-regulated in patients with IBD [112]. Polymorphisms of TLR4 leading to gain of function mutations may be responsible for pro-inflammatory responses to normal levels of LPS. Nucleotide-binding oligomerization domain (NOD) proteins function in a similar fashion to TLRs, however, NOD is present intracellularly, in the cytoplasm, as opposed to TLRs, which are located at the cell surface or in vesicles [113]. NOD proteins, NOD1 and 2, recognise peptidoglycan (PGN), a component of bacterial cell walls derived from Gram positive bacteria [113], and are expressed predominantly by antigen-presenting cells and epithelial cells [114]. PGN, however, is also broken

down in endosomes and is thus a source of MDP (muramyl dipeptide), a substance that is sensed by and activates NOD2. Such activation initiates a mechanism of inhibition of PGN-mediated NF-κB activation and thus causes downmodulation of TLR-induced cytokine production [115]. Polymorphisms in NOD1 and 2 (also known as CARD4 and CARD15), are associated with a susceptibility to develop IBD, particularly Crohn's disease in the case of NOD2 [107]. Homozygous mutation in NOD2/CARD15 has been shown to account for 10–15% of patients with CD, and this mutation leads to a decrease in NFκB activation upon stimulation [107].

An additional factor relates to the function of α-defensins, antibacterial peptides produced by Paneth cells at the base of crypts in the terminal ileum (middle inset) [115]. Crohns disease patients with impaired NOD2 function manifest reduced α-defensin production in part because NOD2 in Paneth cells is an inducer of α-defensin production. Thus, it is possible that impaired NOD2 function also leads to increased bacterial density in the crypts of the terminal ileum and thus greater stimulation of a mucosal immune system already set at a higher level of function [115].

1.10. Probiotics

As the microbial environment has been shown to play a role in the development of IBD, targeting of the microbiota presents an option for therapeutic intervention. One potential method to manipulate the intestinal microbiota in an attempt to reduce the inflammatory response is via the administration of probiotics. Probiotics have been described as, “live micro-organisms which, when consumed in adequate quantities, confer a health benefit on the host” [90]. Probiotics have been used in the treatment of a number of inflammatory conditions including arthritis, atopic eczema, pouchitis, radiation-induced and NSAID-induced enteropathy, chemotherapy-induced mucositis, ulcerative colitis, Crohn's disease, antibiotic-induced diarrhoea and experimental colitis [107].

The mode of action of probiotics is complex and not completely understood. There have been a large number of probiotic species identified, most of which have differing mechanisms of action. Further complexity stems from the finding that the mode of action of a given probiotic can differ based on the presence of other probiotics or enteric bacteria in the surrounding environment, and also the disease setting in which the probiotic is used [90, 116]. There are however a number of common mechanisms that are evident in a wide variety of probiotic strains. One such mechanism is adherence to the intestinal mucosal surface which prevents colonisation of pathogenic bacteria [117]. This is a form of competition between the two species. Evidence for this mode of action has been shown in numerous in vitro model systems for example, pre-incubation with Lactobacillus rhamnosus GG (LGG) has been shown to prevent the adherence of B. vulgatus to mouse epithelial IEC-6 cells [116]. A further common mode of action is via stimulation of the intestinal immune system. Probiotics are believed to be involved in the modulation of cytokine levels by inhibiting production of pro-inflammatory cytokines (including TNF-α and IL-1β) and promoting production of anti-inflammatory cytokines (including IL-10) [116].

Probiotics are also believed to function via the modulation of cell proliferation and apoptosis [118, 119]. The administration of 107 colony forming units (cfu)/ml of LGG and Clostridium butyricum in rats has been shown to increase epithelial cell proliferation rates in the small intestine, cecum and distal colon [118]. This increase in epithelial cell proliferation is believed to be due to the ability of probiotic strains to produce short chain fatty acids (SCFAs) via the fermentation of polysaccharides. The anti-apoptotic effects of probiotics have been demonstrated in an in vitro model, in which LGG was shown to prevent apoptosis in human and mouse colon cells [119]. This effect is believed to be due to the activation of the anti-apoptotic Akt/protein kinase B pathway and also by the inhibition of the pro-apoptotic p38/MAPK pathway [119]. Further studies demonstrated that the probiotic mixture VSL#3 was able to prevent the decrease in trans-epithelial resistance (TER) in T84 cell monolayers following incubation with pathogenic E. coli and Salmonella dublin strains [120]. Similarly,

Lactobacillus plantarum MF1298 was shown to prevent the decrease in TER caused by L. monocytogenes [121]. These studies indicate that probiotics may have the capacity to modulate barrier function. Therefore, probiotics have the potential to be beneficial in the treatment of IBD due to their capacity to prevent the colonisation of pathogenic bacteria and the decrease in TER, reduce inflammatory cytokine expression, enhance epithelial cell proliferation, inhibit apoptosis and provide metabolic energy for enterocytes [107].

There are opposing views as to the potential for probiotics to have the capacity to alter the gut microbiota in the normal and diseased gut. Tannock [108] suggested that it is unlikely that probiotics could significantly alter the intestinal microbiota as, even when administered in large numbers, the probiotic strain would only account for approximately 1% of the total bacterial count. However, numerous studies have indicated that probiotic administration can indeed alter the composition of the intestinal microbiota in animals [122], and humans [123, 124], although, in many cases, this alteration to the microbiota persists only for a short period following cessation of probiotic administration [125, 126]. Accordingly, continued, long-term probiotic administration may be required in certain settings [127].

Based on the results from studies in both animal models and clinical trials, there is evidence that a number of probiotic species assist in the reduction of inflammation and intestinal damage whilst others have no effect, depending on the disease setting [107]. For probiotics to become a legitimate therapeutic option for the treatment of IBD there needs to be more focus on (i) the determination of which probiotic strains have the greatest efficacy in a specific disease setting, (ii) whether these candidate probiotics are more effective alone, or in conjunction with other pro- or prebiotics, (iii) consideration of possible adverse side effects and knowledge of the effect of the probiotics on immune regulation in the intestinal mucosa, and (iv) the time that the species remains in the gastrointestinal tract, as this will determine the frequency and dose requirements [90]. It may also be the case that our genetic profile may predispose our responsiveness to probiotic treatment, as is the case with chemotherapy [128, 129]. Identification of these

"probiotic responsiveness genes" may lead to screening to determine i) whether a patient will be responsive to probiotic therapy, and ii) to which probiotics they would respond more efficiently [107].

1.11. Prebiotics

As the intestinal microbiota has been linked to the pathogenesis of IBD, probiotic treatment is an obvious avenue for therapeutic intervention. Another is via the administration of prebiotics. Prebiotics are described as, "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one, or a limited number of bacteria in the colon, thus improving host health" [130, 131]. The rationale behind prebiotic use is to elevate the endogenous numbers of beneficial bacterial strains including lactobacillus and bifidobacterium [132]. This increase will impart the beneficial effects seen by probiotic administration, including an increase in SCFA production, particularly butyrate, which can provide fuel for enterocytes, prevention of pathogenic adherence, production of anti-bacterial substances, and decreased luminal pH [133]. Common prebiotics include inulin and resistant maltodextrin, also oligosaccharides such as fructooligosaccharide (FOS), Germinated barley foodstuff (GBF) and galactooligosaccharide (GOS). The body of research involving the use of prebiotics to treat IBD is not currently as extensive as that underlying probiotic therapy.

One prebiotic that has demonstrated antagonistic effects in the intestine is FOS. FOS, administered as a dietary supplement (6% w/w of total diet), has been shown to stimulate lactobacilli and bifidobacteria, and increase SCFAs in the large bowel (a result that has been replicated in humans with ulcerative colitis) [107]. Therefore, FOS has been proposed to have the capacity to be beneficial in the IBD setting, however, it has also been demonstrated that, whilst FOS could decrease the colonisation of pathogenic bacteria, it actually increased translocation of bacteria, increased mucosal irritation and increased cecal and colonic myeloperoxidase (MPO) activity [134]. The proposed mechanism of injury by FOS is that elevated FOS levels in the cecum lead to rapid fermentation

by cecal bacteria, causing elevated levels of organic acids, which damage the mucosa of the cecum and colon [135]. Interestingly however, when FOS was administered by oral gavage in the trinitrobenzene sulfonic acid (TNBS) rat model of colitis, it was shown to decrease the severity of damage, indicated by increased lactic acid bacteria, lactate and butyrate production and decreased inflammation scores and MPO activity [136]. In contrast, a study investigating the effects of FOS in DSS colitis indicated that FOS-treated rats had no protection from inflammation [137]. These inconsistent findings may be due to differences in the model of colitis or possibly a result of alterations in the delivery of FOS, leading to different rates of fermentation, and in turn SCFA production.

A number of prebiotics have also been investigated in clinical trials, predominantly in UC patients. Germinated barley foodstuff (GBF) has been reported to reduce the severity of UC both clinically and endoscopically [138]. More recent studies have supported the use of GBF in UC and found that it can stimulate the growth of bifidobacterium and Eubacterium limosum [139]. GBF has also been reported to prolong the duration of remission in UC [140, 141]. GBF therefore has demonstrated beneficial effects in a number of smaller, open multi-centre trials, however a large, double-blind, placebo-controlled trial is still required.

1. 12. Synbiotics in IBD

In addition to probiotic and prebiotic administration, another viable option is to use both prebiotic and probiotic administration in conjunction, referred to as synbiotics [130, 142]. The rationale behind synbiotic treatment is that the desired probiotic and prebiotic (presumably with demonstrated efficacy on their own) would exert a beneficial effect greater than the additive effect if they were administered individually. It may however be the case that a prebiotic which is not efficacious when administered alone, stimulates the probiotic species, significantly elevating its beneficial effects. There are only a few studies demonstrating the positive effects of synbiotic therapy, however, it is becoming a more logical and viable treatment option [107].

Bomba et al. (2002) indicated that synbiotic combination of Lactobacillus paracasei and maltodextrin KMS X-70 led to a decrease in E. coli colonisation in the jejunum of piglets [143]. L. paracasei and FOS (Raftilose P95) combination led to an increase in lactobacillus and bifidobacteria and decreased clostridium and enterobacterium in weanling pigs [143]. Feeding the prebiotic mixture (6 g), Synergy 1 (a mixture of long-chain inulin and oligosaccharide), combined with B. longum in a double-blind, randomised controlled pilot study was able to improve sigmoidoscopy scores, decrease human β-defensin mRNA, TNF-α and IL-1α and reduce inflammation seen in rectal biopsies [144]. This study provides strong preliminary evidence that synbiotic therapy may be beneficial in the treatment of UC. GBF, which has demonstrated prebiotic benefits, has been shown to act by specifically increasing bifidobacteria and E. limosum [145], these may provide ideal candidates for synbiotic therapy. In addition, Kanamori et al. (2004) have demonstrated that synbiotic treatment with Bifidobacterium breve, L. casei Shirota and galactooligosaccharide for over 1 year led to an increase in fecal SCFA levels, increased fecal bifidobacteria and lactobacillus concentrations and improved the rate of body weight gain in patients with short bowel syndrome [146].

The potential benefits of synbiotic therapy are obvious, however, the great challenge, as is the case with probiotics and prebiotics alone, is to determine the best combination for each disease setting and each individual. The first attempts should be to combine probiotics and prebiotics which have demonstrated individual benefits to determine if there are additive effects, alternatively, a more structured approach would be to determine the specific properties that a prebiotic requires to be beneficial to the probiotic and select the prebiotic accordingly [107].

1.13. Sphingolipids, Gangliosides and IBD

Sphingolipids, in particular glucosylceramide, are abundant in the apical membrane in the absorptive epithelium in the gut, and are considered important

for the preservation of structural integrity during exposure to bile salts and enzymes [147]. The brush border sphingolipids may also support the insertion of transporters and receptors, necessary for the selective and effective transport of nutrients into the cells, although these aspects are poorly characterized. Sphingolipid composition changes when crypt cells differentiate to mature absorptive cells, reflecting the close connection between sphingolipid synthesis and mucosal regeneration and differentiation [148].

Gangliosides are negatively charged glycosphingolipids that consist of a hydrophobic ceramide and a hydrophilic oligosaccharide chain bearing one or more sialic acid residues in addition to a number of sugars, namely glucose, galactose, N-acetylglucosamine and N-acetylgalactosamine. Ceramide is an N-acylsphingosine in which the acyl residue is usually a saturated fatty acid with a chain length of more than 14 carbons [149].

1.13.1 Sphingolipid profile in the intestinal tract

Throughout the gastrointestinal tract sphingolipids are enriched in the apical membrane of the polarized epithelial cells. The sphingolipid profiles have been characterized by TLC, GLC and GLC–MS techniques with regard to sphingoid base, fatty acid and polar head group composition.

In the small intestine, about 40% of the lipids in the apical membrane of absorptive villous cells are sphingolipids, which is much more than in the basolateral membrane [147, 150]. Bouhours and Glickman analyzed sphingolipids of villous and crypt cells and found that glucosylceramide content increased with differentiation of absorptive villous cells, associated with alterations in fatty acid and sphingoid base composition [148]. Changes of the sphingolipid pattern also occur during fetal and neonatal development of the intestine to the mature absorptive organ [151]. Glycosphingolipid (Glyco-SL) levels of epithelial cells exceed those of the nonepithelial cells in small intestine and colon [152]. For example, epithelial cells of the colon contain three times as much glyco-SLs as the whole organ [153].

Gangliosides are negatively charged glyco-SLs containing sialic acid. Their presence in the mucosa was shown early [154]. Later several classes belonging to the GM, GD and GT types were found in the mucosa [155]. GM3 was previously found to be most abundant in the intestine and located primarily to the apical membrane. The levels of GM3 and the key enzyme in ganglioside synthesis, CMP-sialic acid: lactosyl ceramide sialyltransferase, are higher in villous than in crypt cells [156].

There are great similarities in the sphingolipid profiles throughout the gastrointestinal tract, but also some differences. In the rat small intestine, mono- and trihexosylceramide are major neutral glyco-SLs, with monohexosylceramide being high in the proximal third, and trihexosylceramide in the distal segment [157]. In monkeys concentration of gangliosides in small intestine is fourfold and that of neutral glyco-SLs twofold higher than in colon [157]. Although sphingosine is the major base in neutral glyco-SLs, phytosphingosine is abundant in ganglioside GM3 at both levels. Generally the high content of phytosphingosine is an important feature of small intestinal glyco-SLs [158].

In conclusion sphingolipid biosynthesis and cellular location are intimately linked to mucosal differentiation and maturation in the small intestine, and exhibit both specific and common features at different levels of the gastrointestinal tract.

1.13.2 Metabolism of glycosphingolipids in the intestinal tract

1. 13.2.1 Synthesis sphingolipids in the gut

Cells of the gastrointestinal tract have a rapid turnover rate, which makes the gastrointestinal mucosa one of the most rapidly proliferating tissues in the body, second only to the skin [159]. During the process, glyco-SLs and SM must be synthesized and degraded accordingly. Furthermore 6–10% of the polar lipids in chylomicrons secreted into chyle are SM [148]. The need of sphingolipids for mucosal renewal and lipoprotein secretion is difficult to estimate precisely, but may be of the order 1.5 g per day in humans [160]. Since there is no

evidence for any substantial uptake of plasma lipoprotein sphingolipids from blood to the mucosa, this amount must be supplied primarily by local de novo synthesis.

Synthesis of sphingolipids begins with the condensation of serine and palmitoyl-CoA, catalyzed by serine palmitoyltransferase, which is a ubiquitous enzyme and expressed also in the intestine [161]. The product, 3-ketosphinganine is reduced to sphinganine (dihydrosphingosine), which is subsequently acylated to dihydroceramide. Dihydroceramide is converted to ceramide by a desaturase that introduces a double bond at position 4 of the sphingoid base. Ceramide/dihydroceramide is synthesized by ceramide synthases that require ATP and formation of CoA derivatives of the fatty acids. These enzymes are located to the endoplasmic reticulum and use newly synthesized sphinganine or sphingoid bases from degraded sphingolipids [162].

1. 13. 2.2 Digestion and absorption of sphingolipids in the gut

Calculations based on available analytical data indicate that adult humans consuming an ordinary Western diet ingest about 0.3–0.4 g sphingolipids per day and the suckling baby ingesting milk consumes 50–150 mg SM per day as well as milk lactosylceramide and gangliosides [148].

In general, dietary intake of gangliosides is very low unless consuming whole-organ foods (i.e., brain), whole milk, buttermilk, or colostrum in high quantities [163]. Although sialic acid and gangliosides may be synthesized by the body, in newborn infants dietary sources are essential for correct development [164]. Infants receiving bovine-based milk formula may ingest as little as 20% of the ganglioside consumed by exclusively breast-fed infants [165]. One human pilot study in healthy population found that the ganglioside content consumed in healthy adults is less than 100 ug N-acetylneuraminic acid/4187 kJ daily [166]. The average consumption of ganglioside in patient with IBD is currently unknown, but assumed to not be higher than that of the general population.

Early studies raised the question whether dietary SM could directly contribute to the SM pools in atherogenic cholesterol- and triglyceride rich plasma lipoproteins and in chylomicrons [167, 168]. The answer was no. When radioactive SM, ceramide and glucosylceramide were fed to lymphatic duct cannulated and intact animals, little or no labeled SM, ceramide or glucosylceramide was absorbed intact into the chyle [169]. Although SM and glycosylceramides were found to be resistant to digestion by pancreatic enzymes, both substrates were sequentially hydrolyzed to ceramide, sphingosine and free fatty acids in vivo [170]. Free sphingosine and dihydrosphingosine were rapidly absorbed and metabolized in the mucosal cells to chylomicron palmitic acid [169]. A smaller portion of the sphingoid bases is reincorporated into mucosal ceramide and more complex sphingolipids [169, 171].

1.13. 2.3 Transport and degradation of gangliosides

According to Pagano's vesicle sorting theory [172], absorbed gangliosides have three fates: transport back to the plasma membrane immediately after being endocytosed; endocytosis to the Golgi apparatus for glycosylation to form more complex ganglioside species and transport by the endosome to the lysosome for degradation. Recently, glycolipid transport proteins have been identified in the cytosol that may play a role in non-vesicular transport of glycolipids within cells [173]. It is known that small amounts of gangliosides are able to move across the intestine to the blood as gangliosides have been found in serum lipoproteins, platelets and bound to albumin and leukocytes in the blood [174]. Animal feeding studies in our lab indicate localization of gangliosides in lipid rafts and transport out to plasma [175]. This route would suggest that dietary gangliosides changes ganglioside components made available to peripheral tissues by the gut.

Gangliosides degradation occurs on membrane surfaces and consists of sequential removal of individual sugar residues by hydrolytic exoglycohydrolases, assisted by activator proteins and negatively charged lipids [176, 177]. The end

product, ceramide, is eventually split into a long chain sphingosine base and a fatty acid by ceramidase. The degradation of gangliosides from plasma membrane reaches the lysosomal compartment mainly by an endocytic membrane flow through early and late endosomes [178]. More than ten different exohydrolases are known to be involved in glycosphingolipid degradation when vesicles reach the lysosome [178]. Enzyme deficiency results in accumulation as the corresponding lipid substrate and is sorted in the lysosomal compartment [178]. These undesirable disorders result in corresponding inherited lipid storage diseases [178].

1.13.3 Intestinal sphingolipids and IBD

Sphingolipid involvement in IBD can be related both to mucosal integrity, barrier and receptor functions and formation of sphingolipid messengers in epithelium and inflammatory cells. Bacterial and viral infections may trigger the onset or relapse of IBD. Interactions of bacteria and virus with sphingolipid receptors are therefore relevant both to the actions of intestinal pathogens and to IBD. At the site of inflammation, cytokines, eicosanoids and glycolipid and sphingolipid messengers are produced by the epithelium and by the immunocompetent cells [148].

1. 13.3.1 The receptor function and anti-infectious effects of sphingolipids

Since targeting of sphingolipids to the apical membrane is an important feature of the barrier formation, any abnormality in this process may be of interest in relation to the pathogenesis of IBD. These aspects of IBD are poorly investigated. One may, however, postulate that glucosylceramide and other neutral glycol-SLs have an important barrier role. It is therefore interesting to note, that multidrug resistance protein 1 (MDR1) participates both in transfer of glucosylceramide to the apical membrane of polarized cells [179] and in the translocation of glucosylceramide in the Golgi complex, making it available for the synthesis of other neutral glyco-SLs as lactosylceramide and

globotriaosylceramide [180]. The reason is that IBD has been linked to a downregulation and genetic polymorphism in IBD [181] and the MDR1a−/− mouse appears to be an appropriate model for spontaneous colitis [182]. Further exploration of the complex connections between glucosylceramide synthesis and MDR1 expression in relation to IBD is undoubtedly needed [183].

Some microorganisms and microbial toxins use glycosphingolipid to attach to the host cell as a way of mediating pathogenic effects. Viruses can bind to host cells via glycosphingolipid. Dietary sphingolipids may thus compete for the attachment sites and promote elimination of pathogenic organisms and toxins from the intestine and counteract bacterial translocation. In support of this hypothesis, milk fat consumption was associated with a reduced number of food-borne infections [184].

1. 13.3.2 Sphingolipid signaling in intestinal inflammation

Ceramide, although it functions as a proapoptotic molecule in many cell types, may also induce inflammatory response. Previous studies have shown that the proinflammatory response to TNFα and IL-1 involves activation of neutral SMase (sphingomyelinase), leading to the formation of ceramide [185]. NF-κB is a key molecule with proinflammatory properties and its activity was increased by ceramide in the small intestinal epithelial cells, due to reduction of IκB-a and IκB-b [186]. Similar activation of NF-κB was also identified in HT29 colon cells after SMase treatment and the cells appear more sensitive to acid SMase than N-SMase [187]. In addition, the formation of ceramide may be involved in the assembly of Toll-like receptor (TLR) in response to bacterial toxin such as lipopolysaccharide (LPS) [188]. Several microbial ligands such as LPS, p-fimbriae and the B-subunit of Shiga toxin were shown to increase the levels of ceramide and trigger a TLR4 dependent response in either leukaemia cells or epithelial cells [189, 190].

However, the role of ceramide often varies with the site and mechanism of formation and with cell type. In aortic endothelial cells, ceramide formed by neutral SMase in response to an oxidized phosphatidylcholine was reported to inhibit the LPS-induced IL-8 response [191]. Of particular interest is a

recent study showing that hydrolysis of SM at the apical membrane of intestinal Caco-2 cells by SMase attenuates the intoxication of the host cells by cholera toxin [192]. The study indicates a protective effect of intestinal SMase, particularly Alk–SMase, as it is an ecto-enzyme anchoring on the surface of microvilli membrane and also present in the intestinal content, with good access to SM at the apical membrane of mucosal cells. A reduction of Alk–SMase in human longstanding ulcerative colitis was previously reported [193].

While ceramide, sphingosine and S1P (sphingosine-1-phosphate) have divergent effects on intestinal inflammation [148], ganglioside, particularly galactosylceramide (GalCer) was reported to inhibit inflammation. Alpha–GalCer was found in vivo to inhibit Toxoplasma gondii-induced ileitis by overexpression of IFN-γ via a specific interaction with NKT cells [194]. Similar inhibition by alpha–GalCer on allergic airway inflammation was also reported [195]. The inhibitory effects of GalCer may be of species specificity, as one analogue of GalCer, CCL-34 but not GalCer, was found to stimulate NF-κB in a TLR4 dependent manner in Raw 264.7 cells [196]. Taking together, it suggests that gangliosides and the downstream metabolism products play important roles in regulating intestinal inflammation.

1.13.3.3 Interaction of sphingolipid signaling with eicosanoid and glycolipid signaling

The roles of eicosanoids in inflammation in IBD have been extensively investigated. An important progress in the recent years was the identification of the cross-communication between sphingolipid and eicosanoid signaling [148]. In eicosanoid metabolism, the key enzymes are PLA2 and COX2. PLA2 triggers the hydrolysis of phosphatidylcholine (PC) leading to increased release of both arachidonic acids and lysoPC. Arachidonic acid is the precursor of PGE2 and leukotrienes, and the lysoPC will further be converted to lysophosphatidic acid (LPA). PGE2, leukotriene, and LPA are potent factors promoting inflammatory responses. SM was reported previously to inhibit the PLA2 activity, indicating a protective effect of the lipids on pathogenesis of inflammation [197]. More

importantly, C1P (ceramide-1-phosphate) and S1P, the phosphorylated form of ceramide and sphingosine, were recently reported to activate PLA2 and induce expression of COX2, respectively [198]. C1P and S1P seem to act concertedly to stimulate the formation of PGE2 and promote the inflammation. Thus the kinases catalyzing the phosphorylation of ceramide and sphingosine may be novel targets for the development of antiinflammatory drugs.

PLA2 is also a key enzyme responsible for production of platelet activating factor (PAF), as it cleaves alkyl-acyl-glycerophosphocholines to form lyso-PAF, a precursor of PAF. In addition to the crosstalk between sphingolipid messengers and eicosanoids there may also be an interaction between sphingolipid- and glycerolipid messengers in the gut. PAF can be synthesized and released from the inflammatory mucosa and is implicated in IBD such as Cohn's disease, ulcerative colitis, and necrotizing enteritis in the newborn, which are associated with high extracellular levels of PAF. It is therefore of great interest that Alk–SMase was shown to hydrolyze and inactivate PAF [199]. PAF is considered to be hydrolyzed primarily by PAF-deacetylase. The effects of Alk–SMase on PAF represent an additional pathway which is well suited to act in the gut because of its location at the mucosal surface and the resistance of the enzyme to proteases. In addition, LPA (lysophosphatidic acid) derived from lyso-PC under the actions of lysophospholipase D is another lipid messenger that stimulates cell migration and inflammation. Alk–SMase can also hydrolyze lyso-PC with a phospholipase C activity and thus may compete with lysophospholipase D and reduce the formation of LPA [200].

1.13.4 Prevention of infection and immunity

Most of the functions in which gangliosides are implicated involve cell-cell recognition during normal differentiation. It is also well known that gangliosides can be "kidnapped" by pathogenic agents and toxins that use them as unintended receptors, and that this may be a mechanism by which gangliosides can prevent infection. Human-milk gangliosides have been involved in the

inhibition of Escherichia coli and Vibrio cholerae enterotoxins [201]. This inhibitory action was later attributed to the monosialoganglioside GM1, which has been identified as the unintended mammalian cell surface receptor for these enterotoxins [202]. More recently, the free milk oligosaccharide sialyllactose, which is the oligosaccharide present in GM1, has been identified as an inhibitor of cholera toxin adhesion to target tissues [203]. GM1 is found in human milk in very low concentrations.

Several studies support the notion that cell surface gangliosides function as "unintended" target receptors for bacterial adhesion in specific tissues; dietary gangliosides are putative decoys that interfere with pathogenic binding [204-206]. Such compounds could modify the intestinal microflora in the neonate and reduce the infectious capacity of pathogenic bacteria. Other findings also describe that sialylated compounds have growth-promoting effects on bifidobacteria and lactobacilli [207, 208].

One clinical study has tested the role of dietary gangliosides in infants [209]. In this study, the performance of a formula containing gangliosides from porcine brain (at concentration of 1.43 mg/100 kcal) was compared to that of a formula without gangliosides. The faecal E. coli counts in preterm infants fed the ganglioside-supplemented formula was lower than that observed in infants fed the standard formula for the first month of life. Furthermore, dietary gangliosides were also able to increase Bifidobacteria counts in faeces [209], which support a prebiotic role for these glycolipids. It is difficult to pinpoint the structural features of gangliosides responsible for specific biological effects; however, it is important to keep in mind that their carbohydrate portions either resemble or are identical to those of free oligosaccharides with proven prebiotic capabilities (Rueda, R. 2007).

Another notable aspect is the ability of gangliosides to modulate the development or behaviour of cells of the immune system. Some earlier studies suggested that gangliosides could be involved in the activation of T cells [210] and in the differentiation of different lymphocyte subpopulations [211, 212]. It was suggested that human milk gangliosides or gangliosides added to infant formulas might substantially contribute to the processes of proliferation,

activation and differentiation of immune cells, especially those from the intestine, in the neonate. More recently, animals fed with gangliosides showed an earlier development of cytokine-secreting cells, and a higher number of Th1 and Th2 cytokine-secreting lymphocytes in lamina propria and Peyer's patch lymphocytes after 4 weeks of feeding [213]. Another interesting finding was that dietary gangliosides increased the number of intestinal IgA-secreting cells [214] and the luminal content of secretory IgA in weanling mice [215]. According to these results, dietary gangliosides positively modulate the production and secretion of IgA at intestinal level, which constitutes a main mechanism of defense against microorganisms entering through the gastrointestinal tract. Ganglioside influences on in vitro intestinal lymphocyte proliferation were also reported [216]. Gangliosides elicited differential effects on intestinal lymphocyte proliferation depending on the presence and concentration of specific structures, suggesting that dietary gangliosides may influence the development of intestinal immunity by stimulating or inhibiting proliferative or inhibitory responses in intestinal lymphocytes during early infancy. However, it is not known if dietary gangliosides can influence immune function in adults and matured animals.

Recent publication describes the role of milk gangliosides, especially GD3 at early lactation (that is colostrum and transitional milk), in modulating immunity [217]. This publication indicates that because GD3 inhibits dendritic cell functionalities overall more than GM3, we may conclude that the immune modulating effect of the ganglioside fraction of breast milk might be more prominent in the commencement of lactation during which the milk contains the most GD3. Furthermore, cow’s milk contains much more GD3 and GM3 than breast milk, suggesting it maybe a good dietary supplement, but further studies are required to confirm if the GD3 and GM3 from cow’s milk has similar beneficial effects as human milk gangliosides.

A study in our lab has also suggested a role of gangliosides in the prevention of infection by parasites [218]. Results from this study showed that dietary gangliosides had a protective effect against Giardia muris infection in vivo and affected the survival of giardia lamblia trophozoites in vitro. Several recent

reports from our group describing the effects of dietary gangliosides on neonatal rats suggest yet another role for gangliosides. Dietary gangliosides are absorbed in the small intestine and transported to different sites, altering ganglioside levels in the intestinal mucosa, plasma and brain, and thus possibly having the potential to change enterocyte function [175]. Another report describes that dietary gangliosides enhance in vitro lipid uptake in weanling rats, probably by a modification in the physical properties of the brush border membrane [219], and dietary gangliosides have been noted to increase the content and composition of phospholipids containing PUFA in the weanling rat intestine [220]. These recent reports suggest that dietary gangliosides might have an effect on developing enterocyte function, but it is not known if they are also related to modulation of intestinal immunity and flora.

It also was demonstrated that dietary gangliosides inhibit acute inflammatory markers in intestinal mucosa and blood induced by systemic inflammation of Escherichia coli lipopolysaccharide, suggesting gangliosides have therapeutic potential in the treatment and management of acute local and systemic inflammatory diseases including IBD [221]. It is very interesting that most of inflammatory markers, such as prostaglandin E2, LTB4, IL-1β, and TNF-α were decreased in rats fed gangliosides. Similarly, gangliosides reduce LTB4, prostaglandin E2, IL-6 and IL-8 production in cultured infant bowel when exposed to LPS under hypoxic conditions [222]. Study in rats also indicated dietary ganglioside protects intestinal occludin tight junction protein in LPS-induced acute inflammation [223].

Clearly, further studies are required to clarify the mechanism and the potential application to other IBD models.

1. 14. Other pharmacological and Nutritional Therapy

The evolving goals of treatment in inflammatory bowel disease (IBD) are driven by advances that have increased expectations for more substantial outcomes of therapies beyond symptomatic improvement. The minimum acceptable therapeutic goals in IBD are the induction and maintenance of

corticosteroid-free remission. Preferably, therapeutic agents should lead to a reduction in hospitalizations and surgeries, and ideally to mucosal healing, which is anticipated to modify the natural history of these diseases [224]. Significant progress has been made in recent years in the management of IBD. The goals of therapy are to control the disease and induce a lasting remission; prevent relapses; and achieve normal nutrition, growth, and life-style. The treatment of IBD is pharmacological, nutritional, surgical and psychosocial.

1. 14.1 Other pharmacological therapy

The medical management of the chronic IBD, UC and CD, is constantly evolving. Over the past several years, biologic therapies have entered the market with great fanfare and cost, yet most patients with IBD will respond to conventional therapies if they are optimized [225]. Recent research has enlightened our approach to the dosing of aminosalicylates, long-term use of budesonide, and improving thiopurine response while minimizing adverse effects. Evidence is accumulating regarding old and new ways of using less frequently applied medications such as tacrolimus, cyclosporine, and methotrexate [226].

1. 14.1.1 5-Aminosalicylates

Aminosalicylates (5-ASA) continue to be the first-line agent for the induction and maintenance of remission in mild to moderately active ulcerative colitis (UC). 5-ASA dugs target the intestine to reduce inflammation. Advances in the use of 5-ASA from the middle part of the current decade were largely based around investigating the dose response at higher doses [224]. More recently, interest has focused on decreasing the frequency of dosing, largely to address one of the biggest challenges in 5-ASA therapy: poor adherence, which is reported to occur in up to 60% of patients on long-term treatment with 5-ASA [227] and is associated with an increased risk of disease flares [228].

1. 14.1.2 Budesonide

High-dose oral and intravenous corticosteroids effectively induce remission in both CD and UC, yet there are limited data showing a benefit of corticosteroids for maintenance of remission [229]. Given the prohibitive adverse effects of chronic corticosteroid use, it is hard to justify long-term dosing in most individuals [226]. Budesonide is a potent, topically active corticosteroid that undergoes substantial first-pass metabolism in the liver with minimal systemic effects [230, 231]. With controlled ileal release, the active agent is released primarily in the small intestine and right colon, making it an ideal agent to target CD in select patients. Budesonide was initially studied for inducing remission, and its role as a maintenance therapy in CD has been controversial [226].

1. 14.1.3 Immunomodulators

The thiopurine agents 6-mercaptopurine (6-MP) and azathioprine (AZA) have become mainstays of therapy in the treatment of corticosteroid-dependent or resistant UC and CD [226]. AZA/6-MP inhibits DNA synthesis due to its structural similarity to purines, thereby disrupting leukocyte proliferation [232]. They are also used to treat perianal CD, to prevent postoperative recurrence of CD, and to reduce immunogenicity to episodically administered biologic therapies.

AZA is rapidly converted into 6-MP; the enzyme thiopurine methyltransferase (TPMT) is responsible for converting 6-MP to the 6-methylmercaptopurine metabolite (6-MMP), which has been linked to hepatotoxicity [233]. Among the population, 89% of people have two wild-type (normal) TPMT gene alleles, 11% are heterozygous, and 0.3% are homozygous recessive [234]. Because the relationship between genotype and function does not always hold, phenotype testing for enzyme activity is preferred over genotyping [235].

1. 14.1.4 Antibiotics

It is proposed that the pathogenesis of IBD results, in part, from a dysregulated intestinal mucosal immune response to luminal bacterial antigens in genetically susceptible hosts [133]. Logically, it follows that alteration of the bacterial flora by antibiotics or probiotics may have a beneficial effect on intestinal inflammation. Antibiotics have been used with varying success in CD for colonic disease, perianal disease, and to prevent postoperative recurrence [224]. In UC, antibiotics have been consistently efficacious only for the management of pouchitis [236].

Although antibiotics are widely used as first-line medical therapy for perianal fistulizing CD, this practice has been based on results from small, uncontrolled studies. The most common reason for failure of antibiotic therapy is the development of adverse events. This occurrence has prompted recent trials of rifaximin, a nonabsorbable antibiotic with a high safety profile and activity against anaerobic bacteria and Escherichia coli [237]. Small preliminary studies showed potential for efficacy of rifaximin in the induction and maintenance treatment of pouchitis [224].

1. 14.1.5 Methotrexate

According to recent clinical data, methotrexate remained the second most commonly used immunosuppressive in IBD [238]. Methotrexate is effective for the induction and maintenance of remission in CD [226]. Three small studies have produced conflicting results in UC. Cummings et al. [239] performed a retrospective review of 42 UC patients treated with oral methotrexate at Oxford and Wycombe General Hospital in the United Kingdom. Indications for methotrexate were AZA intolerance (31/42) and lack of response (11/42). The mean dosage of methotrexate was 20 mg/wk for a median of 30 weeks (range: 7 – 395).

Overall, “ good ” efficacy (stopping steroids and tolerating methotrexate without adverse effects) was achieved in 54% of patients and “ partial” efficacy (steroid reduction but not discontinuation) in 18%. Of the patients for whom AZA was ineffective, only 3 of 11 achieved a good response, while 5 went for colectomy. Efficacy was good in 18 of 31 patients who were intolerant to AZA; 5 underwent colectomy. Adverse effects occurred in 23% of the patients, leading to cessation of therapy in 10%. Oral methotrexate (approximately 20 mg/wk) was well tolerated and effective in steroid-dependent or steroid-refractory patients. Efficacy was better in patients with AZA intolerance than in those in whom AZA was ineffective. As with any case series, this study had significant biases that could not be avoided or corrected, so it emphasizes the need for a randomized controlled trial of methotrexate in UC [226].

1.14.1.6 Cyclosporine

Intravenous cyclosporine fills an important role in the treatment of severe corticosteroid-refractory UC, typically as inpatient intravenous induction dosing, followed by outpatient oral dosing while patients are transitioned to long-acting purine analogues [226]. Although the variables in the studies done by a single centre have a statistically significant ability to predict cyclosporine response, further validation from different institutions is necessary before accepting the results of these single-center series [226]. Furthermore, even if these results are representative of a broad population of patients, the predictive value of the instruments may not be enough to support the decision to forgo a trial of cyclosporine therapy.

1. 14.2 Nutritional therapy

Although epidemiologic and basic research suggests that dietary factors play a role in the onset and course of IBD, currently recommendations cannot be

made for most patients, other than to eat a healthy, nutritionally adequate diet [240]. Diet manipulation has been evaluated in an effort to complement the benefits of drug therapy and replete nutritional deficits.

The beneficial effect of n-3 PUFAs in Crohn's disease patients with high C-reactive protein and erythrocyte sedimentation rate is supported by a randomized placebo-controlled trial of a supplement containing fish oil and antioxidants [241]. In patients receiving fish oil, greater proportions of docosahexaenoic acid and eicosapentanoic acid were incorporated into peripheral mononuclear cells as compared with arachidonic acid, and these patients exhibited lower production of both interferon-g, and prostaglandin-E2. Another study, looking at the role of oral vitamins [242], found that supplementation with vitamins C and E decreased oxidative stress, as estimated by breath pentane and plasma lipid peroxides, in patients with Crohn's disease as compared with normal individuals. Unfortunately, neither of these studies found that treatment reduced disease activity.

Short-chain fatty acids (SCFAs), of which butyrate is the most representative, are particularly worthy of note and are generated during the colonic fermentation of dietary fiber and other unabsorbable carbohydrates. A quantitative SCFA deficiency or their oxidation by colonocytes has been implicated in the physiopathology of UC [243, 244]. Factors that interfere in SCFA oxidation by the colon can weaken the epithelium. Patients with active UC have been found to have a decreased colonic oxidation of butyrate that normalizes when remission is achieved [245]. SCFAs may also be effective in treating antibiotic induced diarrhea due to their participation in regulating water and electrolyte absorption in the colon. SCFAs cannot be provided by mouth because they are not palatable, and they are absorbed by the upper gastrointestinal tract and rapidly metabolized by the liver.

The association between excessive consumption of carbohydrates and development of inflammatory bowel disease, especially Crohn's disease, probably indicates the differences in sugar consumption in Asia compared with Western Europe and North America rather than a true contributing factor [246]. New

feeding habits involve a high consumption of sugar and refined carbohydrates. Since the 1970s, various studies have indicated the high consumption levels of these products in patients with IBD, to the extent that they are now considered a risk factor for CD and UC [70]. Conversely, the consumption of citrous fruit, fruit juices and vegetables could lower the risk of the development of both diseases [70]. A particular study even showed an inverse relationship between the consumption of bran and the onset of CD [247]. To date, it has been impossible to determine whether the potentially protective effect is due to the action of the fiber or to other micronutrients contained in fruit and vegetables. The utility of low refined carbohydrate diets in the treatment of CD has been suggested by several authors, although extensive clinical trials have not confirmed the benefits of this measure [70].

Calorie-protein malnutrition causes humoral and cellular immunodeficiency. Its effects on the intestine lower the efficiency of the mucosal barrier, lead to alteration of the functionality of the mucosa-associated lymphoid tissue and to a greater risk of infection by bacterial translocation [70]. Hypoplasia of the intestinal villi perpetuates malabsorption and increases the risk of infections. Patients with IBD may have increased protein needs due to losses from inflammation of the intestinal tract, catabolism when an infection is present (i.e. abscess) and possibly for healing if patient requires surgery. Protein needs are assessed based on disease status and body weight. The recommended daily allowance (RDA) for protein is 0.8g/kg body weight. The majority of IBD patients free from renal disease require approximately 1.0–1.5g/kg body weight. However, some studies have suggested that the intake of proteins and calories might be higher in patients with IBD compared to controls [248, 249], although these data have not been uniformly observed and we do not know whether these factors are a cause or a consequence of the disease.

Glutamine has been studied in the treatment of IBD due to its role as a fuel for rapidly replicating cells such as those lining the intestinal tract mucosa. In animal studies, glutamine has been found to improve gut mucosa and decrease

damage after certain drug treatments. There is insufficient evidence to date that glutamine treatment can improve gut health in patients with IBD [250].

Generally, it may be concluded that individual dietary compounds have only led to improvement in markers of inflammation, without a clear improvement in the clinical course of disease.

In summary, IBD is a chronic relapsing disorder associated with uncontrolled inflammation within the gastrointestinal tract, which reduces the quality of life in patients. The etiology appears multifactorial, including genetic, environmental, microbial and immune factors. Both animal and cells culture models of intestinal inflammation are indispensable for our understanding of the pathogenesis of IBD. Although these models do not represent the complexity of human disease and do not replace studies with patient material, they are valuable tools for studying many important disease aspects that are difficult to address in humans, such as the pathophysiological mechanisms in early phases of colitis. Apart from a total proctocolectomy for UC, there is no cure for IBD. Medications, however, aid in the induction and maintenance of remission, and target various points along the disordered immune pathway implicated in IBD. Many medical therapies inflammatory bowel disease, particularly immunosuppressants and modulators, are associated with important side-effects. There is a need for new treatments which are more effective, and less toxic and less expensive. Nutrition plays a role in IBD primarily in prevention and treatment of malnutrition and growth failure. Studies in both rats and cells culture indicate that gangliosides inhibits acute inflammatory makers in intestinal mucosa. Further studies are required to clarify the mechanism and the potential application for IBD.

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CHAPTER 2 RESEARCH PLAN

2.1 Introduction and Rationale

IBD is a chronic relapsing disorder associated with uncontrolled inflammation within the gastrointestinal tract. Defective epithelial barrier function, which can be measured as increased intestinal permeability, has been implicated in IBD. Paracellular permeability is controlled by a junctional complex of proteins and lipids which form different strands, commonly described as adherens-junction and tight junction (TJ). Pro-inflammatory signal cytokines can disrupt intestinal mucosa barrier function by changing the tight junction proteins.

Gangliosides are amphipathic glycosphingolipids found in human milk and cell membranes of various cells including the brush border and basolateral membrane of enterocytes. Animal and human cell studies suggest that ganglioside have anti-inflammatory effects during acute infection by modulating signal cytokine expression, such as decreasing TNFα, IL-1β, IL-6 , IL-1, LTB4, PGE2 and PAF. In order to promote ganglioside supplementation and prevent intestinal conditions such as IBD, it is crucial to understand the mechanism.

PLA_2 is a major enzyme produced in response to proinflammatory cytokines, such as LTB_4 and prostaglandin E_2, also involved in the biosynthesis of PAF. It is not clear the underlying anti-inflammatory effect of ganglioside resulting from decreased secretion of $sPLA_2$.

Caco-2 cells are human colonic adenocarcinoma cells that are able to express differentiation features characteristic of mature intestinal cells, such as enterocytes or mucus cells. These cells are valuable in vitro tools for studies related to intestinal cell function and structure.

The main focus of the present study is to determine if incubation with a mixture of gangliosides protects intestinal structure and improves barrier functions in vitro in a Caco-2 cell model of IBD. It is hypothesized that ganglioside treatment will decrease secretion of $sPLA_2$ and inhibit NF-kB

activation. Change in ganglioside and phospholipid content during infection was also examined. This Caco-2 cell model of IBD will be developed by incubation with dextran sulfate sodium (DSS), or LPS.

2.2 Objectives and Specific Aims

This research project was conducted to evaluate the LPS stimulated and DSS induced Caco-2 cells model of IBD and to investigate the effect of ganglioside supplementation. The focus was to determine if treatment with gangliosides can protect Caco-2 cells and improve barrier functions in vitro by altering the anti-inflammatory mechanisms.

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The specific aims of this research project are to:

- Develop an in vitro LPS treated enterocyte Caco-2 cells model of IBD to study the potential efficacy of treatment with gangliosides.
- Develop an in vitro DSS treated enterocyte Caco-2 cells model of IBD to study the potential efficacy of treatment with gangliosides.
- Determine whether incubation on the apical side with ganglioside in Caco-2 cells model of IBD lowers the pro-inflammatory cytokines HBD-2 and IL-23 levels.
- Determine whether incubation on the apical with ganglioside in Caco-2 cell model of IBD protects the disruption of epithelial tight junction complexes and improves epithelial integrity and barrier function.
- Determine whether ganglioside decrease the expression of $sPLA_2$ and further modulate the phospholipid profile of enterocyte cells membrane.
- Examine whether ganglioside supplementation modulates NF-kB activities.

- Examine how ganglioside incubation and DSS treatment alter plasma membrane ganglioside composition.

2.3 Hypotheses

Incubation with ganglioside will protect intestinal morphological structure and improve intestinal barrier function during infection by inhibiting pro-inflammatory cytokines, improving plasma membrane ganglioside content, decreasing secretion of $sPLA_2$ and/or inhibiting NF-kB activation. It is expected that ganglioside incubation will modify intestinal plasma membrane phospholipid, integrity and ganglioside content. It is specifically hypothesized that incubation of ganglioside in Caco-2 cells:

Hypothesis 1: Decreases the pro-inflammatory cytokines HBD-2 and IL-23 level in the media.

Hypothesis 2: Protects intestinal structure integrity and improves barrier function in a cell culture model of IBD

Hypothesis 3: Decreases the $sPLA_2$ secretion and modifies content of phospholipids.

Hypothesis 4: Inhibits NF-kB activation.

Hypothesis 5: Decreases TLR4 level.

Hypothesis 6: Increases ApoB 48 secretion.

Hypothesis 7: Increases plasma membrane ganglioside content.

In Chapter 3, LPS-induced IBD Caco-2 cells culture model is used to test hypotheses 1 to 4. DSS-induced IBD Caco-2 cells culture model is used to further determine hypotheses 1 to 4 in Chapter 4. Hypotheses 5 to 7 are addressed in Chapter 5.

CHAPTER 3

GANGLIOSIDE DECREASES HBD-2 AND IL-23 LEVEL IN A CACO-2 CELL MODEL OF IBD INDUCED BY LPS BY IMPROVING BARRIER FUNCTION AND INHIBITING NF-kB ACTIVATION

3.1 INTRODUCTION

Inflammatory bowel disease (IBD) is a term used to represent a chronic, relapsing disorder resulting from uncontrolled inflammation affecting the mucosa of small intestine and/or colon [1]. IBD includes Crohn disease (CD) and ulcerative colitis (UC). Although the etiology has not been clearly elucidated, CD and UC are thought to involve a complex interplay among genetic, environmental, microbial and immune factors [2]. Pathogenic bacteria deposit toxic and proinflammatory surface components, such as lipopolysaccharide (LPS), directly at the intestinal epithelial apical surface. The intestinal epithelium plays a crucial role in limiting direct contact of potential pathogenic bacteria with the mucosal epithelium, and produces several cytokines and chemokines in response to pathogenic bacteria [3].

Management of IBD is difficult and may consist of costly drug treatment including glucocorticosteroids, immunosuppressants, or biologics such as anti-tumor necrosis factor alpha (TNF-α). Some individuals with IBD do not respond to standard drug treatment, while others experience negative or toxic adverse effects [4]. Relapse is common, even after drug-induced remission has been achieved. There is a need for new treatment initiatives. Gangliosides exhibit anti-inflammatory functions in models of proinflammatory disease that have common features with IBD in both animal and human infant intestinal cells [5], [6].

Gangliosides are negatively charged glycosphingolipids that consist of a hydrophobic ceramide and a hydrophilic oligosaccharide chain bearing one or more sialic acid residents. Gangliosides are found ubiquitously in tissues and body fluids, including milk, and are abundantly expressed in the nervous system

[7]. Dietary gangliosides increase ganglioside content and decrease cholesterol and caveolin-1 content of lipid microdomains[8], disrupting membrane microdomain structure in the brush border membrane of the intestinal mucosa [6], [9], [10]. Cholesterol depletion of intestinal membranes inhibits cellular entry of pathogens and reduces generation of inflammatory signals. Feeding rats gangliosides results in reduced generation of inflammatory eicosanoids and cytokines by intestinal mucosa following acute exposure to bacterial endotoxin [8]. In vitro exposure of inflamed bowel to ganglioside reduces bowel necrosis and endothelin-1 production in response to LPS [6]. Ganglioside also suppresses production of nitric oxide, leukotriene B4, prostaglandin E2, hydrogen peroxide, interleukin-1β, interleukin-6 and interleukin-8 in infant bowel exposed to LPS and hypoxia [6].

In recent years, it has been shown that antimicrobial peptides are not only generated by the innate immune system, which is triggered immediately by microbial invasion, but also play a crucial role in acquired immunity [11]. Defensins are one member of the positively charged antimicrobial peptide family. Human β -defensin 2 (HBD-2) is one of the typical β -defensin family members that is widely distributed in the mucosal epithelium of the skin, respiratory, gastrointestinal, and urogenital tracts, and it can kill both Gram-positive and Gram-negative bacteria, as well as other pathogens [12]. Interleukin-23 (IL-23) is a heterodimeric cytokine composed of a p40 subunit, shared with IL-12, and a unique p19 subunit [13]. IL-23 has been proposed to play an integral role in the pathogenesis of IBD [14]. After binding to the appropriate receptor (IL-23R), IL-23 can stimulate the production of IL-17, TNF-α, and IL-6 from T-cells.

The family of nuclear transcription factor kappaB (NF-κB) proteins consists of five different members, which are namely p65 (RelA), c-Rel, RelB, p50 and p52. Nuclear factor-κB (NF-κB), a primary regulator of inflammatory responses, plays a critical role in a variety of physiological and pathologic processes [15]. Macrophages and epithelial cells isolated from inflamed gut specimens from IBD patients showed augmented levels of NF-κB p65 [16].

Alterations in activation of NF-kB may be one of the ways that ganglioside reduces inflammation in IBD, however this has not been previously explored.

Phospholipases A2 (sPLA2) are a widely distributed group of enzymes implicated in turnover of membrane phospholipids and lipid digestion. Activated sPLA2 catalyzes the release of fatty acids from membranes which is the first step in production of eicosanoids and other inflammatory mediators [17]. In addition to contributing to the inflammation pathway, sPLA2 is the main enzyme involved in the catabolism of micelle [18]. The relationship between amount of intestinal sPLA2 and inflammatory mediators in models of intestinal inflammation is not known.

Another likely pathogenic factor in IBD is intestinal hyperpermeability [19]. Impaired gut epithelial barrier function may lead to persistent immune reactions, thus augmenting gut inflammation [20]. A previous study in rats reported that dietary ganglioside inhibits degradation of gut occludin tight junction proteins during LPS-induced acute inflammation, thereby helping to maintain intestinal permeability [20].

In the present study, human adult intestinal epitheliums Caco-2 cells were used as a model for IBD. The cells were stimulated with LPS, a major structural component of Gram-negative bacteria to simulate the inflammation that occurs in IBD. It was hypothesized that ganglioside down regulates HBD-2 and IL-23 cytokine levels by improving intestinal barrier function and by decreasing NF-kB activation or synthesis of sPLA2.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Caco-2 Cell, Media and Reagents

The human colon carcinoma cell line CaCo-2 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained and subcultured at 37°C in an atmosphere of 5% CO2, 95% air. When cells were 80%–90% confluent, cells were subcultured at a density of 1.6 × 104 cells/cm2 on 25 cm2 flasks using 0.25% trypsin-2.65 mM EDTA. The complete

medium for cell maintenance consisted of HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid)/carbonate-buffered EMEM, which contains 10% (v/v) FBS, 1% (v/v) antibiotic/antimycotic, 26 mM sodium bicarbonate, 10 mM HEPES, and 1 mM pyruvic acid. The medium was changed every 2 days. All studies were performed on confluent cells of the CaCo-2 subclone between passages 20 and 40. MEM-based enriched medium requiring little or no serum ("Opti-MEM") was purchased from Invitrogen. Sodium-salts of taurocholic- and oleic- (OA) acids were from Sigma Aldrich. LPS (E. coli 0114:B4) was purchased from Sigma Aldrich. Limulus Amoebocyte Lysate assay was obtained from from Charles River. Gangliosides were extracted from Zeta lipid-2 milk powder (Fonterra, Cambridge, New Zealand). Total gangliosides were measured as ganglioside bound N-acetyl neuraminic acid with N-acetyl neuraminic acid used as a standard [9]. The ganglioside mixture used in experiments was similar to the composition of human colostrum, predominantly GD3 with smaller amounts of GM3, GM1 and GD1a. An aliquot of ganglioside stock dissolved in chloroform/methanol 2:1 (v/v) was dried under N2 gas and dissolved in bowel culture medium (HMEM-4) to give a final concentration of 100 μg/ml. Ganglioside dissolution and micelle formation was facilitated by a sonication prior to filter sterilization of the ganglioside solution through a 0.2 μm pore syringe filter.

3.2.2 Stimulation of Cells

Caco-2 cells were cultured in six-well plates at a density of 2.5×10^7 cells/ml on 0.4 μm pore inserts in 6 well trans-well plates (Corning Costar, NY). The apical (upper) and basolateral (lower) compartments contained 0.5 ml and 2 ml of medium, respectively. This culture medium was changed every 2 days. CaCo-2 cells were then grown to confluence and were differentiated for 21 days, by which time a highly differentiated monolayer is formed [21]. These cells have villi and microvilli, and function as small intestinal enterocytes. Cells were divided into 4 groups: control, ganglioside supplement group (10 μ g/ml), LPS treatment group (100 μ g/ml for 48 hrs) and ganglioside plus LPS combination

treatment group. Ganglioside supplement to the culture medium was started on Day 19. The cells were incubated for 24 hrs with LPS (0.1 mg/ml) from the apical side on day 21. Culture media was collected and analyzed for HBD-2, IL-23 after stimulation and/or supplement. Cells from the original culture medium were harvested for NF-kB and Western blot analysis.

3.2.3 Determination of IL-23 and HBD-2 Proteins

Human IL-23 and HBD-2 proteins were assayed from cell supernatants by an enzyme-linked immunosorbent assay (ELISA) based on titerzyme ELISA Kits, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN; and Phoenix Pharmaceuticals Inc., respectively). The optical density of the wells was determined using a microplate reader (Eppendorf BioPhotometer) set to 450 nm with a wavelength correction set to 540 nm.

3.2.4 TEER Measurement

Transepithelial electrical resistance (TEER) was used as an index of confluence, integrity and barrier function of cell monolayers [22]. TEER of Caco-2 monolayers grown on collagen-coated filter inserts was measured using an epithelial voltohmmeter (EVOM; World Precision Instruments, Hamden, CT) and a pair of chopstick electrodes. Results are expressed as ohm/cm2. The TEER value obtained in the absence of cells was used as background.

3.2.5 NF-kB Activity

NF-kB activity was examined using a TransAM NF-kB transcription factor family assay kit (Active Motif, Carlsbad, CA, U.S.) according to the manufacturer’s instructions. The kit contains 96-well plates to which oligonucleotides containing an NF-kB consensus binding site. The activated NF-kB contained in nuclear extracts was able to bind specifically to these oligonucleotides. NF-kB was detected using specific antibodies. Five micromoles of Bay 117082 (an inhibitor of NF-kB) were used as a positive control for NF-kB inhibition.

3.2.6 sPLA2 Assay

Human synovial SPLA2 secreted into the media was measured using a commercial ELISA kit (Cayman Chemical, Ann Arbor, Mich.). Media samples were collected, centrifuged and supernatants stored at -80°C. Samples were analyzed according to the manufacturer's instructions. Briefly, 100 µl of diluted sample was applied in duplicate to each well of a 96 well plate coated with a monoclonal antibody specific for sPLA2. Next, 100 µl of acetylcholinesterase: Fab' Conjugate (AchE:Fab), which binds selectively to a different epitope on the sPLA2 molecule, was added to the wells and incubated overnight at 4°C. All solutions from the wells were removed and the wells were rinsed six times with a wash buffer, followed by the addition of 200 µl of Ellmen's Reagent and allowed to incubate 3 hrs in the dark at room temperature. The absorbance was read at 420 nm using model 550 Microplate Reader (Bio-Rad, Hercules, Calif.) and the results compared with a standard curve of sPLA2.

3.2.7 Statistical Analysis

Data is presented as mean ± SD. Data was analyzed by a one-way ANOVA, Duncan's multiple range test. There is no interaction between LPS and gangliosides when analyzed by a two-way ANOVA. A P value of ≤ 0.05 was considered statistically significant.

3.3 RESULTS

3.3.1 Ganglioside Supplement Decreased Inflammatory HBD-2 Cytokine

LPS stimulation significantly increased amount of HBD-2 compared to control. Ganglioside supplement alone did not affect HBD-2 level compared to control treatment. Cells cultured with ganglioside had significantly less HBD-2 following LPS stimulation (Fig. 3-1).

3.3.2 Ganglioside Supplement Decreased Inflammatory IL-23 Cytokine in LPS Treated Cells

LPS stimulation significantly increased inflammatory IL-23 cytokine compared with the control. Ganglioside supplement alone did not affect IL-23 level. Cells cultured in ganglioside produced significantly less pro-inflammatory IL-23 following LPS stimulation (Fig. 3-2).

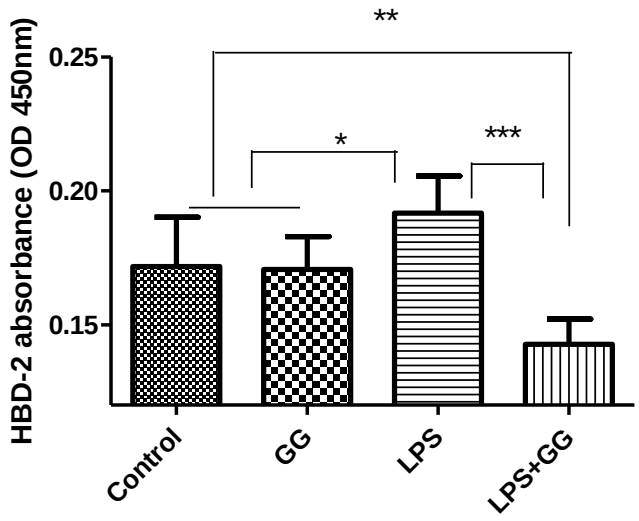

Fig. 3-1 HBD-2 level in cultured Caco-2 cells is decreased by ganglioside supplement after LPS stimulation. CaCo-2 cells were grown on transwells to monolayers for 21 days. Ganglioside supplement of cells started on day 19 from the apical chamber. LPS stimulation started on day 21. Cells were incubated with LPS at a dose of 0.1 mg/ml from the apical surface for 24 hrs. Apical medium was collected, HBD-2 was measured by ELISA. N=8 per group. The mean ± SD is shown. The asterisk denotes statistical significance at *P < 0.05, **P<0.01, *** P<0.005.

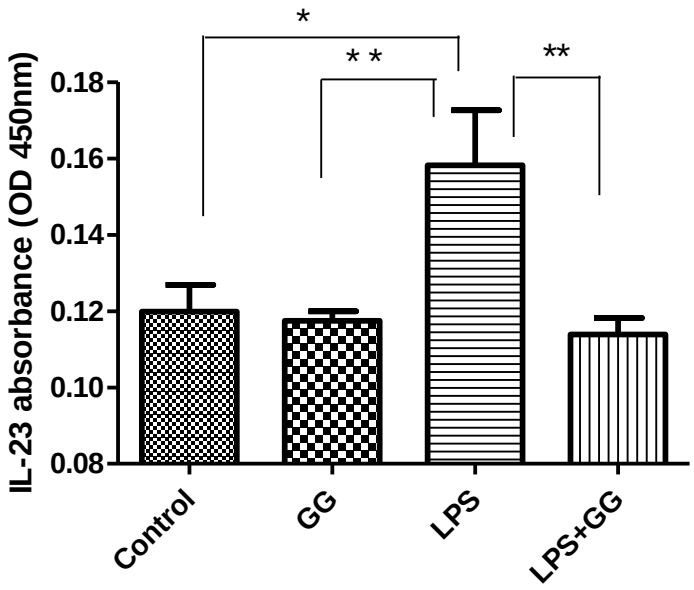

Fig. 3-2 IL-23 level in cultured Caco-2 cells is decreased by ganglioside supplement. IL-23 level was increased by LPS treatment, and this increase was reduced to level observed in the control cells. N=8 per group, values illustrated mean ± SD. The asterisk denotes statistical significance at *P < 0.05, **P<0.01.

3.3.3 Ganglioside Supplement Prevent Defective Barrier Function Caused by LPS at 8 hrs and 24 hrs

TEER was measured at 8 hrs and 24 hrs after LPS and ganglioside treatment. LPS treatment significantly decreased Caco-2 cells barrier function measured as reduced TEER (Fig.3-3). Ganglioside supplement alone did not affect TEER. Ganglioside supplement prevented Caco-2 cells defective barrier function caused by LPS at both 8 hrs and 24 hrs as measured by TEER.

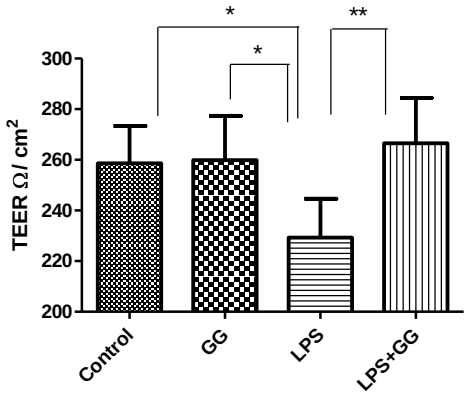

Fig. 3-3 A

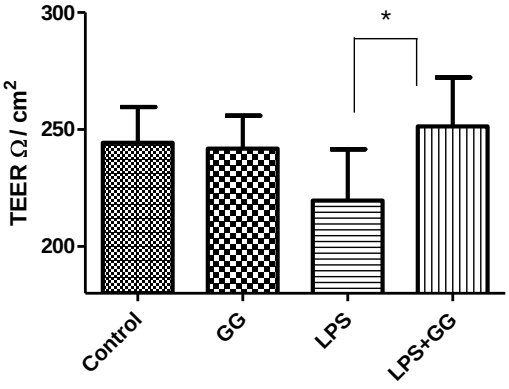

Fig. 3-3 B

Fig. 3-3 Ganglisode supplement increases TEER reduced by LPS treatment. TEER was measured at 8 hrs (Fig. 3-3 A) and 24 hrs (Fig. 3-3 B) after LPS and GG treatment in Caco-2 cell monolayers, grown on transwells for 21 days. N=8 per group, values illustrated the mean ± SD. The asterisk denotes statistical significance at *P < 0.05. **P<0.01.

3.3.4 Ganglioside Supplement Inhibited NF-kB Activation

LPS treatment significantly increased NF-kB P65 and P52 activation compared with the control (Fig.3-4 A and B). Ganglioside supplement did not affect NF-kB P52 or P50 activation (Fig. 3-4 B and C). Ganglioside supplement significantly inhibited NF-kB P65 activation by the LPS treatment (Fig.3-4 A). NF-kB P50 was also not affected by any of treatments (Fig. 3-4 C).

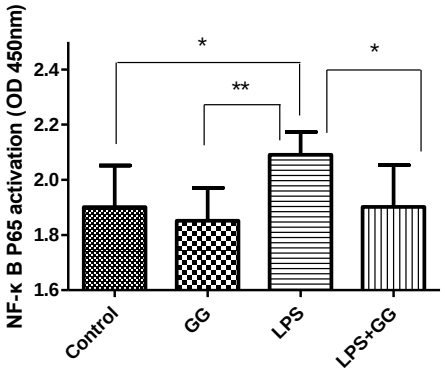

Fig. 3-4 A

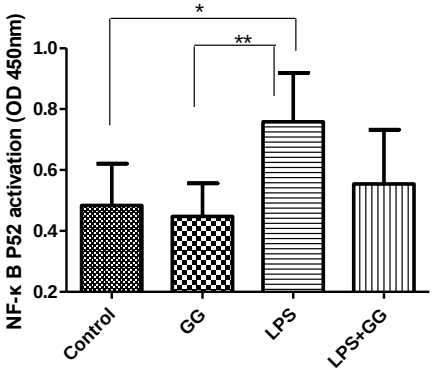

Fig. 3-4 B

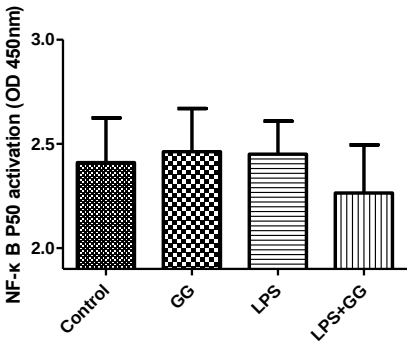

Fig. 3-4 C

Fig. 3-4 Ganglioside supplement inhibits NF-kB activation after LPS treatment. Caco-2 cells were harvested after each treatment. NF-kB activity was examined using a TransAM NF-kB transcription factor family assay kit (Fig. 4 A: NF-kB p65, Fig. 4 B NF-kB p52, Fig. 4 C: NF-kB p 50). N=6 per group, values illustrated the means ± SD. The asterisk denotes statistical significance at *P < 0.05. **P<0.01.

3.3.5 Ganglioside Supplement and LPS Treatment Increased sPLA2 Level in both Apical and Basolateral Medium

sPLA2 was measured in the apical and basolateral medium. Both LPS treatment and ganglioside treatment significantly increased amount of sPLA2 in the apical medium compared with the control treatment (Fig. 3-5A). Neither LPS treatment nor ganglioside treatment affected amount of sPLA2 in the basolateral medium (Fig. 3-5B). LPS treatment and GG supplement together significantly increased sPLA2 level in the basolateral medium (Fig. 3-5B).

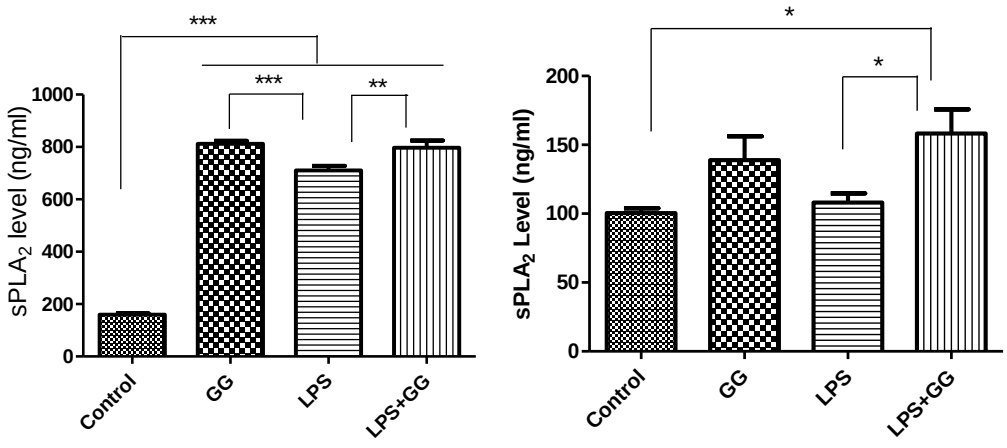

Fig. 3-5 A Fig. 3-5 B

Fig. 3-5 sPLA2 in cultured Caco-2 cells was increased by ganglioside and LPS treatment. Both apical and basolateral medium were collected after each treatment. Fig. 3-5 A illustrates the content of sPLA2 in apical medium, Fig. 3-5 B illustrates the content of sPLA2 in basolateral medium. N=8 per group, the mean ± SD is illustrated. The asterisk denotes statistical significance at *P < 0.05. **P<0.01.

3.4 DISCUSSION

Inflammatory bowel diseases have been postulated as being associated with both defects in the intestinal barrier and impaired immune function. This study reveals that barrier function and inflammation can be improved in a cell model of intestinal inflammation. The intestinal epithelial barrier regulates macromolecule trafficking between the lumen and the internal milieu and protects the host by preventing harmful solutes, microorganisms, toxins and luminal antigens from entering the body, including LPS [23]. Compromise or disruption of the intestinal barrier function causes deleterious effects and results in exposure of the host to luminal antigens and bacteria, leading to the immune response inflammation [24]. Impaired barrier functions have been described in a number of common gastrointestinal disorders, including IBD [25]. Elevated proinflammatory cytokine levels are present in patients with IBD [26]. Exposure of Caco-2 cells to LPS resulted in significantly increase of pro-inflammatory cytokines of HBD-2 and IL-23. Ganglioside in the media prevented the increase of both HBD-2 and

IL-23 after LPS treatment to levels less than that of the control treatment. HBD-2 is one of the most important host defense molecules noted for indelibility against various pro inflammatory signals such as tumor necrosis factor-α (TNF-α), LPS, bacteria and yeast [27]. HBD-2 is expressed in the colon during inflammation, particularly in ulcerative colitis (UC) [28]. Recent attention has focused on IL-23 as a key mediator contributing to inflammation in intestinal tissue. Genetic polymorphisms of the IL-23 receptor (IL-23R) are frequently observed in chronic IBD patients, signifying the importance of IL-23 and IL-23R signaling in IBD pathogenesis [29]. Accumulating data have demonstrated that IL-23 plays a crucial role in the pathogenesis of a number of immune-mediated inflammatory diseases by recruitment of several inflammatory cells and Th17 cells [30]. Expression of antimicrobial peptides is an important part of mounting an effective innate immune response with direct antimicrobial effects towards bacteria [31]. HBD-2 plays an important role in host defense and represents a link between innate and adaptive immune responses. In this investigation, exposure of Caco-2 cells to LPS resulted in significant increase of pro-inflammatory cytokines of HBD-2 and IL-23. Gangliosides supplement significantly decreased secretion of both HBD-2 and IL-23. The present findings suggest that gangliosides may prevent inflammation by inhibiting the secretion of HBD-2 and IL-23.

Intestinal epithelial cells are a key component to the physiologic barrier between the myriad of toxins, microbes, and antigens present in the gut lumen and the underlying mucosal immune system [32]. There is also increasing evidence that enterocytes are actively involved as full participants in generating an immune response at the mucosal level, and are not just the targets of inflammation [33]. Intestinal barrier serves as a first line of defense for the mucosal immune system. Dysfunction of this barrier represents a pivotal characteristic of both CD and UC that has been suggested to contribute to the pathogenesis of these disorders by initiating an inflammatory cascade triggered by increased passage of luminal antigens into the lamina propria [34]. The present results show that exposure of Caco-2 cells to LPS resulted in marked decrease of

barrier function measured via TEER. Ganglioside supplement prevented the LPS induced Caco-2 cell defective barrier function caused in this cell culture model.

The chronic mucosal inflammation in IBD is mediated by hyperactivation of effector immune cells, which produce high levels of pro-inflammatory cytokines like TNF-α, IL-6 and IFN-ϒ, resulting in tissue damage [35]. The nuclear transcription factor kappaB (NF-κB) has been identified as one of the key regulators in this immunological setting. Activation of NF-kB is markedly induced in IBD [35]. NF-kB regulates the expression of multiple immediate early genes involved in the immune, acute phase and inflammatory responses [36, 37]. NF-kB subunits are kept inactive in the cytoplasm by an endogenous inhibitor protein of the IkB family [38]. Under stimulation by IL-1, LPS, or bacteria, IkB is phosphorylated, selectively ubiquitinated and rapidly degraded. Once free of IkB, NF-kB translocates into the nucleus and induces target genes that have NF-kB binding sites [38]. NF-kB expression is activated by LPS in Caco-2 cells [39]. In IBD patients, the increased NF-kB expression in mucosal macrophages is accompanied by an increased capacity of these cells to produce and secrete TNF-α, IL-1 and IL-6 [16]. This finding reflects the central function of NF-kB in monocytes, which is the induction and control of pro-inflammatory cytokines. Previous studies have suggested gangliosides reduce pro inflammatory signaling in the intestine, including TNF-α, IL-1, IL-6 and IL-8 [5],[6]. However, the underlying mechanisms remain to be elucidated. In the present study, exposure Caco-2 cells to LPS resulted in increased activation of NF-kB P65. Ganglioside supplement prevented the increase in NF-kB P65 activation by LPS. Further studies are required to determine the mechanism underlying this effect.

One study reported that sPLA2 was increased in serum and colonic mucosa of patients with Crohn Disease and Ulcerative Colitis [40]. However, there have been no studies since that time therefore it remains unclear whether sPLA2 plays a role in IBD pathogenesis. In the IBD cell culture model of the present study, sPLA2 was found to be elevated by LPS and ganglioside treatment by an unknown pathway, suggesting the anti-inflammatory effect of gangliosides

is not related to sPLA2 pathway. Further research is needed to clarify the role of sPLA2 in IBD.

Early study suggested that highly purified preparations of mouse gangliosides have been demonstrated to bind to purified preparations of LPS [41] . In this study, the cells were incubated with ganglioside alone, LPS alone and the combination of the ganglioside and LPS, so, it can not be excluded any interaction between ganglioside and LPS in the results.

In summary, this study demonstrates that exposure of Caco-2 cells to LPS produced some of the inflammatory responses characteristic of IBD. Ganglioside supplementation prevented the secretion of inflammatory cytokines HBD-2 and IL-23 in response to LPS treatment. The anti-inflammatory effect of gangliosides may involve mechanisms to improve intestinal barrier function and inhibit NF-kB activation, but appears to be independent of sPLA2. Ganglioside is likely to have protective beneficial effects on IBD by down regulating proinflammatory signaling, either by inducing or by maintaining remission.

3.5 References

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CHAPTER 4

GANGLIOSIDES IMPROVE INTESTINAL INTEGRITY AND FUNCTION BY DECREASING sPLA2 AND DEPEND ON NF-kB ACTIVATION

4.1 INTRODUCTION

IBD including CD and UC are common causes of chronic disease in the developed world and represent an important public health issue [1]. IBD is a chronic and relapsing inflammatory disorder of the gut that demands long-lasting treatment targeting both flare-up periods and maintenance of remission. Both CD and UC have multifactorial etiologies which feature a complex interaction of host genetic susceptibility and environmental factors such as diet and gut microbiota.

Antimicrobial peptides are major immune system components, providing an important molecular basis for the body's resistance to interference by external factors. Human β-defensin-2 (HBD-2) is an important member of the defensin family of antibacterial peptides. The expression of human β-defensins 1 (HBD-1) and β -defensins 2 (HBD-2) in intestinal epithelial cells have been studied in vitro and in vivo [2]. The findings indicate that HBD-1 is expressed by epithelium of normal human colon and small intestine, with a similar pattern of expression in inflamed colon [2]. In contrast, little HBD-2 expression occurs in the epithelium of normal colon, whereas HBD-2 expression is more abundant in epithelium of inflamed colon [2]. HBD- 1 mRNA is expressed constitutively in colonic tissue irrespective of inflammation, while HBD-2 mRNA is expressed at very low levels in uninflamed colon but is induced in inflammation in patients with CD and UC [3].

The consequence of inflammatory bowel diseases (IBD) is cytokine-mediated severe local tissue damage. Recent attention has focused on IL-23 as a

key mediator that contributes to inflammation in intestinal tissue. Studies using IL-23 knockout mice or an IL-23 blockade system revealed that IL-23 plays a pivotal role in intestinal inflammation by stabilizing and activating the pathogenic T helper 17 (Th17) populations [4, 5].

The intestinal barrier regulates macromolecule trafficking between the lumen and the internal milieu and protects the host by preventing harmful solutes, microorganisms, toxins, and luminal antigens from entering the body [6]. Intestinal epithelial cells form a physical barrier to limit access of enteric microbes to the host and contribute to innate host defense by producing effector molecules against luminal microbes. Impaired mucosal barrier function and intestinal hyperpermeability is common in IBD patients [7]. One important component of the mucosal barrier is the apical and subapical intercellular junctions between epithelial cells, termed tight junctions and adherens junctions [8]. These structures seal the paracellular space and regulate permeability of the mucosal barrier. A large number of healthy first-degree relatives of patients with IBD have increased intestinal permeability, which suggests barrier dysfunction may be an early defect in IBD. Previous studies have demonstrated decreased expression of junction complex proteins in the intestinal mucosa of patients with IBD [9]. Impaired gut epithelial barrier function may lead to persistent immune reactions, thus augmenting gut inflammation.

NF-κB is a critical molecule regulating inflammatory and angiogenic processes. Sustained activation of NF-κB is detected in the intestinal lamina propria of patients with Crohn's disease [10]. Because sustained activation of NF-κB is thought to be pivotal in the pathophysiology of chronic colitis, inhibition of NF-κB activation has been proposed as an anti-inflammatory strategy in IBD [11].

To date, diverse chemical drugs, such as mesalazine, cyclosporine, the anti-tumor necrosis factor (TNF)-α drugs or antibodies and corticosteroids, have

been used for IBD therapy. These drugs have limitations owing to adverse effects, such as cramping, fever and aggravation of colitis. Moreover, continuous administration of anti-TNF drugs has been reported to increase vulnerability to opportunistic infections, such as mycobacterium tuberculosis [5]. There is need for new treatments for IBD, which are more effective, and less toxic and less expensive.

A ganglioside is a negatively charged glycosphingolipid that consists of a hydrophobic ceramide and a hydrophilic oligosaccharide chain containing a number of sugars, namely glucose, galactose, N-acetylglucosamine and N-acetylgalactosamine bearing one or more sialic acid residues. Gangliosides are present in both human and bovine milk. Animal studies show that inflamed intestinal mucosa has a lower ganglioside content than healthy intestinal mucosa [12]. Ganglioside supplementation increases ganglioside level and decreases proinflammatory cytokine production in intestinal mucosa [13], [12] and prevents hypoxia-induced bowel necrosis and cell injury in cultured infant bowel [14].

Phospholipases A2 (sPLA2) are a widely distributed group of enzymes implicated in turnover of membrane phospholipids and lipid digestion. sPLA2 is also crucial for inflammatory pathways, as sPLA2 is the first step in production of eicosanoids and other inflammatory mediators [15]. Human studies have documented that activity of sPLA2 is elevated in the intestinal mucosa and serum of patients with both Crohn's disease and ulcerative colitis [16]. sPLA2 hydrolyzes plasma membrane phospholipid to biologically-active lyso-PLs and free fatty acids, which can function as inflammatory signaling molecules. Intestinal mucosal integrity is a physical and metabolic barrier against toxins and pathogens in the lumen. It is not known how ganglioside and dextran sodium sulfate (DSS) affect the plasma membrane PL composition and subsequent inflammatory pathways.

It was reported that administration of DSS in mice induces colonic mucosal inflammation as well as ulceration, leading to severe weight loss [17]. However, precise mechanisms responsible for DSS-induced colitis remain unclear. One study in vitro indicated that DSS was absorbed and entered Caco-2

cells and bound to the nucleus of the cells under fluorescence microscopy [18]. It also indicated the binding of DSS may be involved in the cell cycle arrest of Caco-2 cells, which may be involved during initiation or throughout the early stages of DSS-induced colitis [18]. In the present study, IBD cell culture model was developed by culturing Caco-2 cells with DSS. The present study describes the role of exogenous gangliosides in modulating inflammatory response and anti-inflammatory mechanisms of human intestinal Caco-2 cells cultured with DSS. The hypothesis that ganglioside down regulates HBD-2 and IL-23 cytokine level by improving intestinal barrier function and altering NF-kB activation or synthesis of sPLA2 was examined.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Cell, media, reagents

The human colon carcinoma cell line CaCo-2 was obtained from the American Type Culture Collection (HTB-37, Rockville, MD). Cells were maintained and subcultured at 37°C in an atmosphere of 5% CO_2, 95% air. When cells were 80%–90% confluent, cells were subcultured at a density of 1.6×10^4 cells/cm^2 on 25 cm^2 flasks using 0.25% trypsin-2.65 mM EDTA. The complete medium for cell maintenance consisted of HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid)/carbonate-buffered EMEM, which contains 10% (v/v) FBS, 1% (v/v) antibiotic/antimycotic, 26 mM sodium bicarbonate, 10 mM HEPES, and 1 mM pyruvic acid. The medium was changed every 2 days. All studies were performed on confluent cells of the CaCo-2 subclone between passages 20 and 40. Dextran sodium sulfate (DSS) was purchased Sigma Aldrich. Gangliosides were extracted from Zeta lipid-2 milk powder (Fonterra, Cambridge, New Zealand) [19].

Ganglioside was extracted from zeta lipid-2 milk powder (Fonterra, Cambridge, New Zealand), purified through Sep-Pak C18 reverse-phase cartridges (Waters Corporation, Milford, MA, USA) and analyzed as ganglioside bound N-acetyl neuraminic acid (also known as sialic acid) as described

previously [14]. The ganglioside mixture used in experiments was similar to the composition of human colostrum, predominantly GD3 with smaller amounts of GM3, GM1 and GD1a. Ganglioside dissolution and micelle formation was facilitated by a 30 s sonication prior to filter sterilization of the ganglioside solution through a 0.2 μm pore syringe filter.

4.2.2 Stimulation of cells

Caco-2 cells were cultured in six-well plates at a density of 2.5×10^7 cells/ml on 0.4 μm pore inserts in 6 well trans-well plates (Corning Costar, NY). The apical (upper) and basolateral (lower) compartments contained 0.5 ml and 2 ml of medium, respectively. This culture medium was changed every 2 days. CaCo-2 cells were then grown to confluence and were differentiated for 21 days, by which time a highly differentiated monolayer is formed [20]. Cells were divided into 3 groups: control group cells, DSS treated group and ganglioside supplementation plus DSS treatment group. Ganglioside supplementation started on Day 19 of culture from apical medium. The cells were incubated for 48 hrs with 2.5% DSS from the apical side on day 21. Culture media was collected and analyzed for HBD-2, IL-23 by ELISA after stimulation or supplementation. Cells from the original culture medium were harvested for NF-kB and Western blot analysis.

4.2.3 Determination of IL-23 and HBD-2 Proteins

Human IL-23 and HBD-2 proteins were assayed from supernatants of cultures stimulated with LPS (100 μ g/ml) for 48 h with or without the ganglioside supplement by use of an enzyme-linked immunosorbent assay (ELISA) based on titerzyme ELISA Kits, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN; Phoenix Pharmaceuticals Inc., respectively). The optical density of the wells was determined using a microplate reader (Eppendorf BioPhotometer) set to 450 nm with a wavelength correction set to 540 nm.

4.2.4 TEER measurement.

Transepithelial electrical resistance (TEER) was used as an index of confluence and integrity of cell monolayers. TEER of Caco-2 monolayers grown on collagen-coated filter inserts was measured using an epithelial voltohmmeter (EVOM; World Precision Instruments, Hamden, CT) and a pair of chopstick electrodes. Results are expressed as ohm/cm2. The TEER value obtained in the absence of cells was used as background. The TEER was measured before addition of DSS (time zero) and then at various time intervals and expressed as the ratio of the TEER at time t to the initial value (at time zero) for each series. The TEER of monolayers without added DSS or ganglioside represented the controls for each experiment.

4.2.5 Electron microscopy

Cell monolayer 3 samples of each treatment were collected and prefixed in 2.5% w/v of glutaraldehyde/cacodylate buffer (pH 7.2) for 1.5 hours at room temperature. CaCo-2 cells were then washed 3 times in the same buffer for 15 minutes and were postfixed in 1% v/v osmium tetroxide buffer for 1.5 hours at room temperature. Next, cells were rinsed in distilled water; dehydrated in a series of 50%, 70%, 90%, and absolute ethanol for 10 minutes each, then for 10 minutes in each of 2 additional absolute ethanol washes; and embedded in Spurr's resin mixture (Spurr's resin/ethanol 50:50) overnight. Following polymerization at 70 °C for 12 hours, ultrathin sections were cut, stained with uranyl acetate and lead citrate, and viewed with a Hitachi H7000 Transmission Electron Microscope (TEM) (Tokyo, Japan), or with a Hitachi Scanning Electron Microscope S2500 (Tokyo, Japan) using the method of Ramsoondar [21] to examine the integrity of monolayers.

4.2.6 Phospholipid Measurement

Phospholipid composition of cell lysates was measured after different treatments.The cell monolayers were harvested by trypsinization with trypsin-EDTA and the cell lysate was immediately frozen and stored at -70 °C until assayed. Total lipids were extracted using a chloroform/methanol method [22]. Lipid extracts were dried under nitrogen gas, dissolved in chloroform and the total phospholipid content determined as phospholipid phosphorus [23]. Extracted total phospholipids were separated by thin-layer chromatography (TLC) on silica gel 60 plates using chloroform/methanol/acetic acid/water (60:18:21:1.8). Individual phospholipids were resolved on silica gel H TLC plates, by using chloroform-methanol-2-propanol-0.2% KOH-triethylamine (45:13.5:37.5:9:27, by vol) and identified by comparison to authentic phospholipid standards (Sigma-Aldrich, St. Louis, MO). Plates were visualized by spraying with 0.1% anilinonaphthalene sulfonic acid under UV exposure. Lipid fractions were recovered, and lipid phosphate content was measured according to the method of Itoh et al. [24].

4.2.7 NF-kB Activity

NF-kB activity was examined using a TransAM NF-kB transcription factor family assay kit (Active Motif, Carlsbad, CA, U.S.) according to the manufacturer's instructions. The kit contains 96-well plates to which oligonucleotides containing an NF-kB consensus binding site. The activated NF-kB contained in nuclear extracts binds specifically to these oligonucleotides. NF-kB was detected using specific antibodies. An inhibitor of NF-kB (five micromoles of Bay 117082)) was used as a positive control for NF-kB inhibition.

4.2.8 sPLA2 Assay

Human synovial SPLA2 secreted into the media was measured by Enzyme Immunoassay (ELISA) kit (Cayman Chemical, Ann Arbor, Mich.). Media samples were collected, centrifuged and supernatants stored at -80°C. Samples were analyzed according to the manufacturer's instructions. Briefly, 100 µl of diluted sample was applied in duplicate to each well of a 96 well plate

coated with a monoclonal antibody specific for sPLA2. Next, 100 µl of acetylcholinesterase: Fab' Conjugate (AchE:Fab), which binds selectively to a different epitope on the sPLA2 molecule, was added to the wells and incubated overnight at 4°C. All solutions from the wells were removed and the wells were rinsed six times with a wash buffer, followed by addition of 200 µl of Ellmen's Reagent and allowed to incubate 3 h in the dark at room temperature. The absorbance was read at 420 nm using model 550 Microplate Reader (Bio-Rad, Hercules, Calif.) and the results compared with a standard curve of sPLA2.

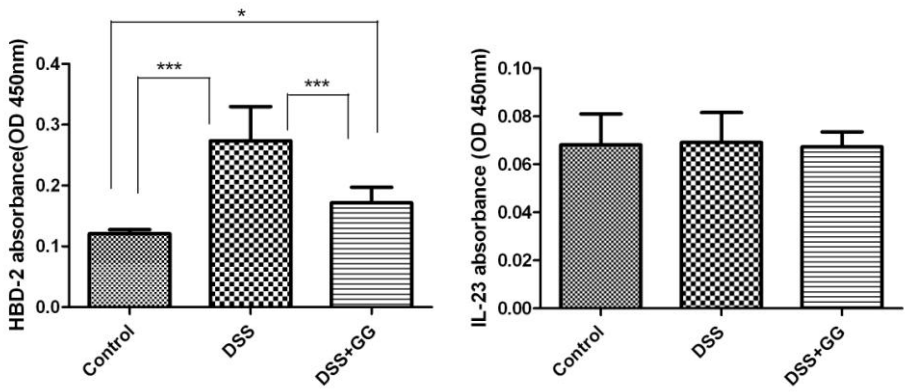
4.2.9 Statistical Analysis

Data was presented as mean ± SD. Data was analyzed by a one-way ANOVA, Duncan's multiple range test. A P value of ≤ 0.05 was considered statistically significant.

4.3 Results

4.3.1 Ganglioside Supplement Decreased Inflammatory HBD-2 Cytokine but not IL-23

DSS treatment caused a significant increase in HBD-2 in the apical medium compared to control cells. Cells cultured with gangliosides exhibit significantly less HBD-2 in the apical medium following DSS stimulation, although an increase was still apparent compared with control cells (**Fig. 4-1**). There were no differences in IL-23 concentration between DSS treatment and ganglioside supplement compared to control cells.



A B

Fig. 4-1 Effect of gangliosides on human-β defensins -2 (HBD-2) (A) and Interleukin – 23 (IL-23) (B) production after DSS treatment. Human intestinal CaCo-2 cells were exposed to DSS treatment with or without ganglioside (at 10 μg/ml). When ganglioside was not present, DSS treatment induced significant production HBD-2 compared to the control. Ganglioside incubation reduced HBD-3 release from Caco-2 cells following DSS treatment. Each data point represents the mean ± the standard error of the mean (n=8 for HBD-2 level; n=7 for IL-23 level). ∗P<0.05, ∗∗P<0.005, ∗∗∗P<0.0001.

4.3.2 Ganglioside protected the disruption of intestinal tight junction and adhesion junction proteins

Examination of enterocyte Caco-2 cells with electron microscopy (EM) revealed intact tight junctions, adherens junctions, and desmosomes in control cells (Fig. 4-2 A). In contrast, severely disrupted and open tight junctions and desmosomes were observed in the DSS treated cells (Fig. 4-2 B). In Fig. 4-2 C, ganglioside supplement attenuated the disruption of intestinal junction complexes, including tight junction, adhesion junctions caused by DSS treatment.

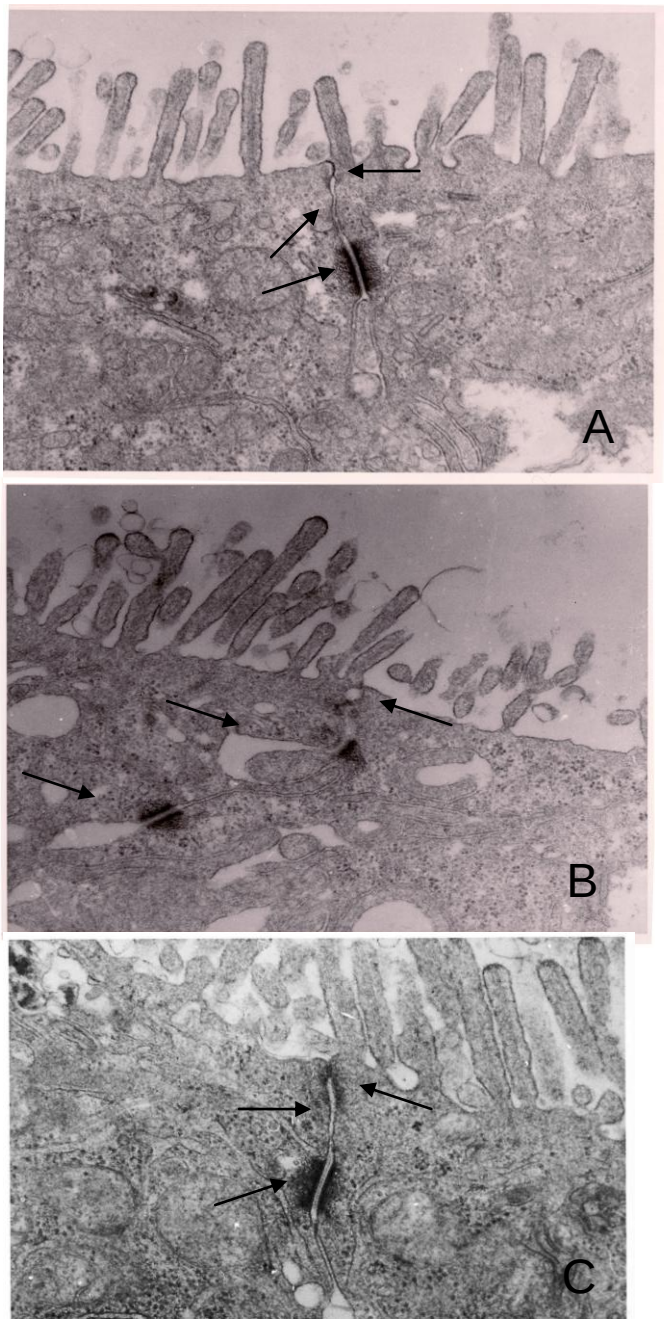


Fig. 4-2 Effect of DSS and ganglioside on the junction complexes in Caco-2 cells. Cells were exposed to DSS treatment with or without ganglioside incubation. Note the disruption of the tight junctions (TJ), adherens junction (AJ) and desmosomes (DS) in DSS treated cells (B), compared with control cells (A) or cells incubated with ganglioside (C). Arrows indicates examples of change of TJ, AJ and DS. Magnifications of A, B and C are same: ×2600.

4.3.3 Ganglioside improved barrier function of Caco-2 cells

TEER is an indication of epithelial barrier integrity, as decreased epithelial barrier function is accompanied by a reduction in TEER. DSS has been shown to increase mucosal permeability in mice [25]. To test the hypothesis that ganglioside enhances mucosal barrier integrity, TEER of the cells following DSS treatment with or without ganglioside supplement were compared. TEER was significantly reduced by DSS treatment compared with control cells, while, ganglioside supplement improved TEER approximately 10% compared with cells following DSS stimulation (Fig. 4-3), but not to levels of control.

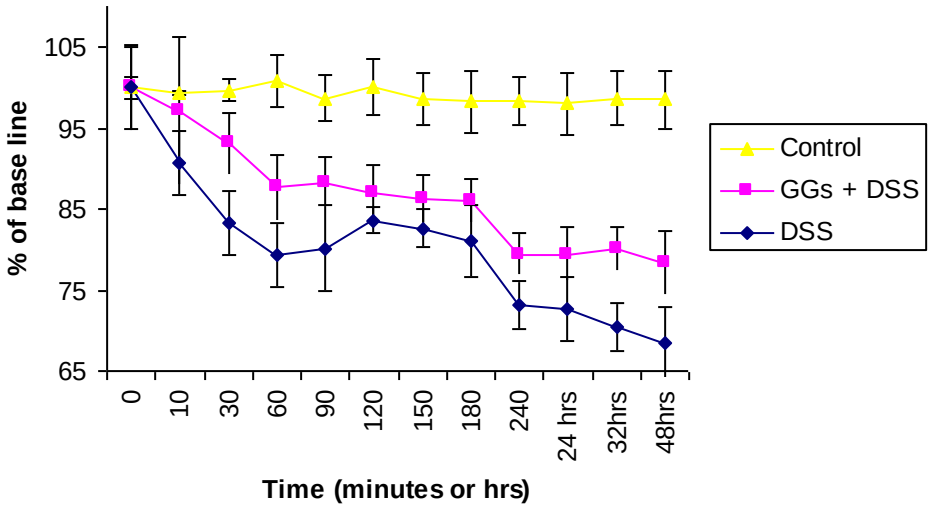
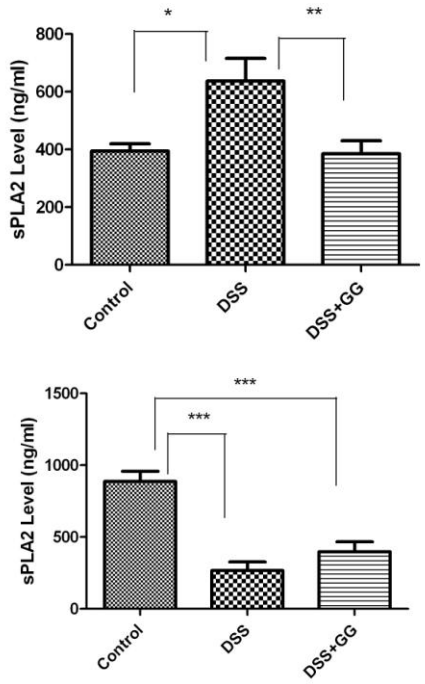


Fig. 4-3 Effect of ganglioside supplement and DSS treatment on Caco-2 barrier function. Caco-2 monolayers grown on filters in the presence (GGs+DSS) or absence (control) of were treated with 2.5% DSS. TEER was monitored from 0 to 48 hrs after addition of DSS to the media. The TEER is presented as % of the basal value in cells at the min not treated with DSS. Each data point represents the mean ± the standard error of the mean (n=6).

4.3.4 Ganglioside treatment reduced the DSS-induced increase in sPLA2 in the basolateral medium

Secretion of sPLA2 is shown (Fig. 4-4). DSS led to a significant increase in total sPLA2 secretion compared with control cells in basolateral medium (Fig. 4-4 A). However, cells cultured with gangliosides secreted significantly less sPLA2 compared with DSS treatment (Fig. 4-4 A). Secretion of sPLA2 was significantly decreased by DSS treatment in apical medium compared with control cells (Fig. 4-4 B). There is no difference between DSS treatment with or without the ganglioside supplement in the apical medium (Fig. 4-4 B).

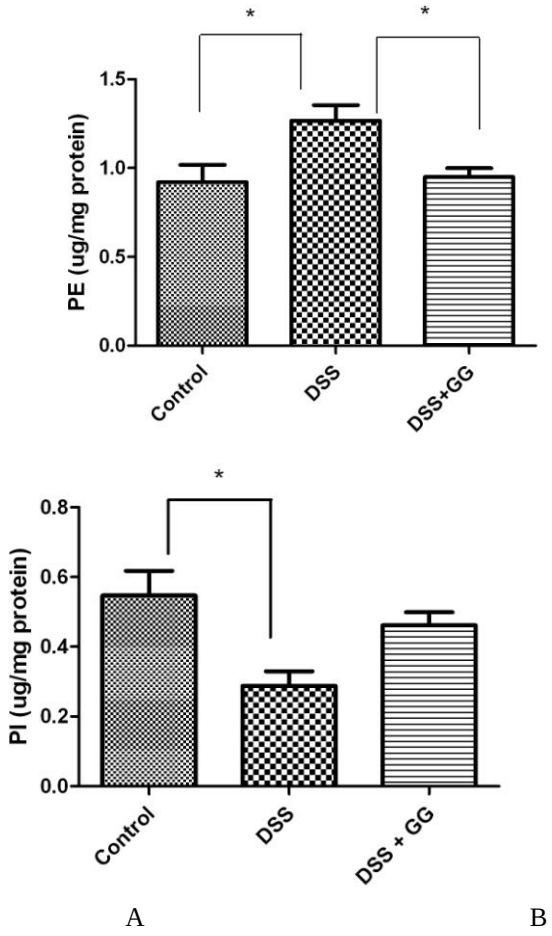


A B

Fig. 4-4 Effect of ganglioside incubation and DSS treatment on production of sPLA2 measured by ELISA. A: in basolateral medium. B: in the apical medium. Each data point represents the mean ± the SEM for different cells (n=10 for basolateral medium; n=12 for apical medium). ∗P<0.05, ∗∗P<0.005, ∗∗∗P<0.0001.

4.3.5 Ganglioside treatment attenuated change in cellular PE and PI content resulting from DSS treatment

There were no differences in total cellular PL content between the three treatments. DSS treatment led to significantly increase in PE and decrease in PI content compared with control cells (Fig. 5 A and B). Cells cultured with ganglioside exhibited similar cellular PE and PI content compared with control cells. There is no difference in PC, PS and SM content.



A B

Fig. 4-5 Phospholipid (PL) levels after DSS and ganglioside treatment were measured by thin-layer chromatography. A: PE change. B: PI change. PE: phosphatidylethanolamine, PI: phosphatidylinositol. Each data point represents the mean ± the standard error of the mean for different cells (n=7). ∗P<0.05, ∗∗P<0.005

4.3.6 DSS treatment increased NF-kB P65 activation.

NF-kB activation was significantly increased by DSS treatment compared with control cells (Fig.6). Cells cultured with ganglioside had a higher NF-kB activation after DSS treatment compared with cells not cultured with ganglioside (Fig. 6).

4.4 DISCUSSION

A cell model of IBD was developed to investigate roles of DSS in the pathogenesis of IBD and identify possible protective effects of treatment with exogenous gangliosides. In this in vitro model of IBD, DSS caused a significant increase in HBD-2 production. This is consistent with previous studies which have shown that although mRNA of HBD-1 is reduced in CD and UC patients, both HBD-2 and HBD-3 are induced with inflammation in CD and UC [26], [27]. Other studies have indicated that probiotic bacteria induces HBD-2 production in intestinal epithelial cells via NF-κB, leading to increased barrier function in the gut [28], [29]. Taken together these data suggest that β-defensins, in particular HBD-2, may have a key role in regulating innate immune responses to bacteria in the gut. In the present study, DSS-induced HBD-2 secretion was inhibited by ganglioside, indicating that ganglioside may attenuate the epithelial inflammatory response to DSS.

The DSS-colitis model shares many clinical and pathological features of human ulcerative colitis with regard to ulceration and loss of barrier function [17]. Using this model, the present study found that DSS caused mucosal injury as shown by disruption of intestinal tight junction and adhesion junction, leading to a decrease in epithelial integrity as measured by TEER. The present study demonstrated that ganglioside protects disruption of the tight junction complex caused by DSS treatment and also improved mucosal barrier function. This is consistent with animal model of IBD, where dietary ganglioside were shown to inhibit degradation of gut occludin tight junction protein during LPS-induced acute inflammation in rats [6].

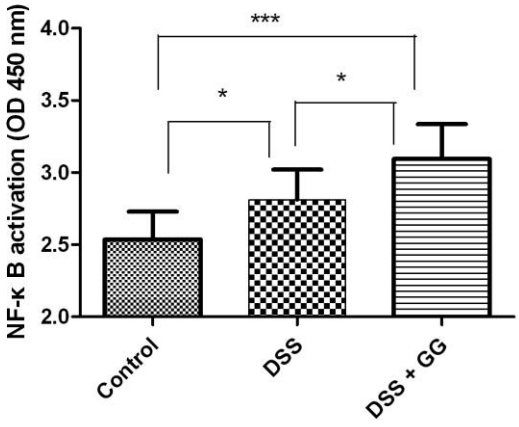


Fig. 4-6 Effect of ganglioside incubation and DSS treatment on activation of NF-kB P65 measured by ELISA. Each data point represents the mean ± the standard error of the mean for different cells (n=10). **P<0.005, ***P<0.0001.

Phospholipases have roles in diverse cellular responses, including phospholipid digestion and metabolism, host defense and signal transduction [30]. sPLA2 appears to be a key factor in the inflammatory process [30]. The present study demonstrated that DSS caused increase of sPLA2 in the basolaterial Caco-2 cell medium (Fig. 4-4 A). Provision of sPLA2 and PC leads to a reduction in TEER in Caco-2 cells [16]. In the present study, DSS also caused decrease in TEER (Fig. 4-3). More research is required to clarify whether TEER reduction is a primary or secondary event in the development of IBD. The present study also demonstrated that cells cultured with ganglioside expressed significantly lower sPLA2 level following DSS treatment in the basolaterial medium. sPLA2 enzymes catalyze hydrolysis of the sn-2 position of membrane glycerophospholipids, leading to production of free fatty acids and lysophospholipids. This reaction is of particular importance if the esterified fatty acid is arachidonic acid (AA), which is converted by downstream metabolic enzymes to various bioactive eicosanoids, including prostaglandins (PGs) and leukotrienes (LTs). A previous study in animals demonstrated that ganglioside down-regulates production of lipid inflammatory mediators of PGE2 and LTB4 in

rats [12]. This finding suggests ganglioside may down-regulate inflammatory mediators by decreasing sPLA2 level in this model.

Both PE and SM are known as inflammatory signaling factors involved in human IBD. The present study found that total cellular PL contents were unchanged. However, DSS led to significant increase in PE and decrease in PI (Fig. 4-5 A and B). This finding supports a study by Tazuke, et al. (2003) who also reported that intracellular phospholipids were changed by the inflammatory cytokine interleukin-6 in Caco-2 cells [31]. Collectively, this suggests phospholipids play an important role in the integrity of membrane in IBD. The present study also demonstrated that ganglioside incubation attenuated the change in cellular phospholipids (Fig. 4-5 A and B). The integrity of the intestinal mucus barrier is primarily maintained through the interactions of its principal glycoproteins, the mucins, with lipids [32]. Phospholipids, although accounting for a minor part of gastrointestinal mucus, are indispensable in the maintenance of an intact barrier function [33]. The finding suggests ganglioside may prevent inflammation and improve mucus barrier function by maintaining the integrity of the intracellular PL membrane.

Macrophages and epithelial cells isolated from inflamed gut specimens from IBD patients showed augmented levels of NF-κB p65 [34]. The present study demonstrated that treatment with DSS significantly increased NF-κB p65 activation. Ganglioside incubation further augmented the levels of NF-κB p65 following DSS treatment. In a previous experiment, ganglioside treatment without DSS had no effect on the activation of NF-κB p65 (For details, please see chapter 3). Obviously, NF-κB is clearly involved in some pro-inflammatory epithelial signalling cascades, but on the other hand, there exist recent data from different experimental models in knockout mice, which clearly demonstrate an anti-inflammatory overall function of NF-κB in colonic epithelial cells [35]. Another study also reported the induction of HBD-2 expression through Fusobacterium nucleatum in human gingival epithelial cells was independent of NF-κB activation [36]. Further research is needed to clarify the role of NF-κB p65 in IBD.

The present study describes an in-vitro enterocyte cell-culture model that enables investigation of the effects of exogenous ganglioside treatment on IBD. Ganglioside supplementation decreases the inflammatory HBD-2, which is an important anti-microbial peptide of the innate immune system, in response to DSS treatment. The anti-inflammatory effect of gangliosides may be involved in mechanisms protecting tight junction structures and intracellular phospholipid integrity, thereby maintaining intestinal barrier function and lowering the inflammatory response, respectively. The mechanism is dependent of NF-kB activation.

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CHAPTER 5

CACO-2 CELLS SUPPLEMENTED WITH GANGLIOSIDE INCREASES GANGLIOSIDE CONTENT AND apoB48 SECRETION

5.1 INTRODUCTION

Gangliosides are a family of acidic glycosphingolipids, one of the most structurally diverse and complex groups of amphipathic membrane lipids [1]. Gangliosides are found in plasma membranes of mammalian cells and are biologically important molecules involved in cell differentiation, proliferation, growth, adhesion, migration, signaling and apoptosis [2][3]. Ganglioside content and composition are species-specific and vary in different tissues, cells and biological fluids suggesting specific roles for ganglioside in distinct physiological processes.

Changes in ganglioside composition occur and play significant regulatory roles in normal physiological processes such as embryogenesis and lactation [4] and in pathological conditions such as tumor onset and progression [5]. Small amounts of dietary ganglioside enriched in GD3 increase membrane content of GD3 in intestinal mucosa of young rats [6]. GM3 localizes to the brush border membrane (BBM) and GD3 localizes to the basolateral membrane (BLM) [6]. Gangliosides interact with growth-factor receptors, the extracellular matrix, and neighbouring cells, whereas the backbone sphingosine and other long-chain or sphingoid bases, ceramides, and sphingosine 1-phosphate, activate or inhibit protein kinases or phosphatases, ion transporters, and other regulatory machinery [7]. Considering that the intestine undergoes important transformations in the structure, composition and absorption of nutrients during postnatal development, it is likely that ganglioside incubation induced modifications in ganglioside content occur that alter protein function in the plasma membranes of intestinal cells.

The etiology of IBD, including UC and CD, remains unclear. It is hypothesized that, in genetically susceptible individuals, inappropriate and

ongoing activation of a mucosal immune response against luminal antigens is a major cause of the inflammation [8]. The processes that monitor the luminal contents and modulate the mucosal response are probably central to functional integrity of the mucosa and health [8]. Thus, the mucosal immune system is poised to detect bacteria and antigens at the mucosal surface and to drive an appropriate response. In active IBD, the balance between protective and commensal luminal bacterial species is lost and, due to increased mucosal permeability and insufficient mucosal bacteria clearance, the commensal flora and pathogenic bacteria enter into the lamina propria, inducing destructive inflammatory responses [9]. This prompts an exaggerated immune response with activation of the innate and adaptive elements of the mucosal immune system [10]. The activation of the innate immune system depends on recognition of microbes by pattern recognition receptors such as Toll-like receptors (TLRs). The TLR family consists of 13 members, and each has a different type of ligand. TLR4 is responsible for recognition of lipopolysaccharide (LPS), a principal component of the bacterial outer membrane or cell wall [9]. Uncontrolled activation of TLR4 may lead to the loss of mucosal barrier integrity and aggravation of the inflammatory response within the gut epithelial mucosa [11].

Crohn's disease of the upper part (duodenum) and middle part (jejunum) of the small intestine may interfere with the absorption of proteins, sugars, iron, vitamins, and fats. This widespread malabsorption in Crohn's disease may lead to weight loss and malnutrition. Apo B exists in human plasma as two isoforms, apo B-48 and apo B-100. Apo B-100 is the major physiological ligand for the LDL receptor. It is a large monomeric protein, containing 4536 amino acid residues [12]. Apo B-48 is present in chylomicrons and chylomicron remnants and plays an essential role in the intestinal absorption and transport of dietary fats [13]. Developmental deficiencies in luminal lipid digestion by pancreatic lipases and solubilization by bile acid micelles that result in “physiological” fat malabsorption in the newborn and in IBD, as well as compensatory mechanisms such as gastric lipase digestion, have been well studied and are reviewed elsewhere [14][15]. Apo B-48 is exclusively synthesised in the small intestine and is requisite for

chylomicron assembly in enterocytes. ApoB-48 is a protein unique to chylomicrons secreted from the small intestine and serves as the main structural apolipoprotein of the buoyant, triglyceride (TG)-rich chylomicrons that deliver dietary lipids to tissues. After most of the lipids in the chylomicron have been lipolyzed, ApoB-48 is returned to the liver as part of the chylomicron remnant, where it is endocytosed and degraded. It is not clear whether ganglioside will improve lipid absorption.

In chapter 3 and 4, using LPS and DSS induced in vitro models of IBD ganglioside treatment exhibited potential anti-inflammatory effects by decreasing HBD-2, modulating integrity of phospholipids membranes, inhibiting production of sPLA2 and activation of NF-kB. The present study was designed to determine how these two models with or without ganglioside incubation altered ganglioside content and whether LPS and ganglioside modulates TLR4 and apoB-48 secretion.

5.2 MATERIALS AND METHODS

5.2.1 Cell culture

Human colon cancer CaCo-2 cells (passage 44-54) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Earle's Minimum Essential Medium (EMEM) containing 10% (v/v) fetal bovine serum (FBS), 1% (v/v) antibiotic/antimycotic, 26 mM sodium bicarbonate, 10 mM HEPES and 1 mM pyruvic acid. Cells were grown as adherent monolayers in 75 cm^2 T-flasks under standard incubator conditions (humidified atmosphere, 5% CO_2, 37°C) with medium replaced every 2-3 days. Monolayers were subcultured on reaching 80-90% confluence at a split ratio of 1:3 (1 T75 flask and 2 T150 flasks) using 0.25% trypsin/0.03% ethylenediaminetetraacetic acid (EDTA).

For DSS stimulation, Caco-2 cells were cultured in six-well plates at a density of 2.5×10^7 cells/ml on 0.4 μm pore inserts in 6 well trans-well plates (Corning Costar, NY). The apical (upper) and basolateral (lower) compartments contained 0.5 ml and 2 ml of medium, respectively. This culture medium was

changed every 2 days. Cells were divided into 4 groups: control group cells, ganglioside incubation cells, DSS treated cells and ganglioside supplementation plus DSS treatment cells.

Ganglioside was extracted from zeta lipid-2 milk powder (Fonterra, Cambridge, New Zealand). The ganglioside mixture used in experiments was similar to the composition of human colostrum, predominantly GD3 with smaller amounts of GM3, GM1 and GD1a. Ganglioside dissolution and micelle formation was facilitated by a 30 s sonication prior to filter sterilization of the ganglioside solution through a 0.2 μm pore syringe filter. Ganglioside supplementation of cells started on day 19 of culture and continued on day 21 at a concentration of 10 μg/ml of ganglioside. Cells were incubated for 48 hrs with 2.5% DSS from the apical side on day 21. Cells were harvested after different treatments for ganglioside analysis.

For LPS stimulation, cells were incubated for 24 hrs with LPS (0.1 mg/ml) from the apical side on day 21. Culture media was collected and analyzed for apoB48 content after stimulation or supplementation with ganglioside. Cells from the original culture medium were harvested, TLR4 and apoB48 was measured by Western blot.

5.2.2 Determination of cell protein

The amount of protein in cell supernatants was determined using the bicinchoninic acid (BCA) assay [16]. Cell supernatants were diluted 1 in 5 with ddH_2O. Bovine serum albumin standards and diluted cell supernatants (10 μl) were each mixed with 190 μl of a 50:1 mixture of BCA solution and 4% (w/v) $CuSO_4 \cdot 5H_2O$ for 30 min at 37°C. The absorbance at 562 nm was measured with a microplate reader (Molecular Devices Co., USA).

5.2.3 Ganglioside extraction and purification

Total lipid was extracted from cell supernatants using the Folch method [17]. Aliquots of cell supernatant 0.9 ml were mixed with 18 ml of chloroform/methanol (2:1, v/v) and incubated overnight on a shaker. Distilled water was added to give a final ratio of 5:1 chloroform methanol (2:1, v/v)/water. The upper aqueous phase containing gangliosides was collected. To increase the yield of gangliosides, the lower organic phase was washed twice with Folch upper phase solution (chloroform/methanol/water, 3:48:47 by vol.). The upper aqueous phases containing gangliosides were pooled together and purified by passage through Sep-Pak C_{18} cartridges (Waters Corporation, Milford, MA, USA) pre-washed with 10 ml methanol, 20 ml chloroform/methanol (2:1, v/v) and 10 ml of methanol as described by Williams and Mcluer [18]. The upper phase extract was loaded onto C_{18} cartridges. Cartridges were washed with 20 ml of distilled water to remove salts and water-soluble contaminants. Gangliosides were eluted with 5ml of methanol and 20 ml of chloroform/methanol (2:1, v/v), dried under nitrogen gas and redissolved in 500 µl of chloroform/methanol (2:1, v/v). Gangliosides were stored at -80°C until analysis.

5.2.4 Analysis of total ganglioside content

Total gangliosides were measured as ganglioside-bound N-acetyl neuraminic acid (GG-NANA) as described by Suzuki [19].

5.2.5 High Perforance Liquid Chromatography

Prior to MS analysis, the ganglioside extracts were subjected to reverse-phase chromatography with a Poroshell 120 EC-C18 column using an Agilent 1260 Infinity LC system (Santa Clara, CA). Two mobile phases were used; phase A was composed of a 50:50 water/isopropanol mixture containing 5 mM ammonium acetate and 0.05% acetic acid, phase B consisted of 100% methanol. A gradient elution consisting of an increase in mobile phase B from

70% to 95% over 6 minutes was used, with a total LC run time of 12 minutes at a flow rate of 0.5 µl/min. The retention time of the detected ganglioside species ranged from 3 to 9 minutes.

5. 2.6 Mass Spectrometry

All MS measurements were obtained using an Agilent 6430 Triple-Quad LC/MS system (Santa Clara, CA) operating in negative ion mode. Deprotonated gas-phase ions of the various ganglioside species were obtained using electrospray ionization, with the electrospray needle held at -4500 V. The MS was operated in multiple reaction monitoring mode (MRM). Briefly, a library of theoretical precursor ions was generated for over 600 gangliosides of specific ceramide and carbohydrate composition. The first quadruple mass filter was set to scan for these specific precursor ions, allowing each to sequentially pass into the hexapole collision cell where ions were fragmented using collision induced dissociation (CID). Gangliosides readily lose a sialic acid moiety when subjected to CID [20], thus the second quadrupole mass filter was set to only allow this characteristic fragment ion (m/z 290) to reach the detector. The CID and ion source voltage for each ganglioside class (ie GM, GD, and GT) was optimized using the Agilent Optimizer software. Data acquisition and analysis was carried out using the Agilent Mass Hunter software package.

5.2.7 Ganglioside library screening

For each class of ganglioside (ie GM, GD, and GT), a theoretical m/z library was generated in which the ceramide composition was varied in its extent of saturation and total carbon chain length. Ganglioside masses were calculated assuming a spingosine of d18:1 and dihyrosphingosine of d18:0. The fatty acid (FA) side chain was then varied from 10 to 26, and also included the odd carbon FA species. The library also included mono- and polyunsaturated FA. In summary, for each ganglioside of a specific carbohydrate compositon, there was 52 variations on the ceramide composition included in the library search. Thus in

total, the library consisted of 624 gangliosides from 12 classes (GM1, GM2, GM3, GM4, GD1, GD2, GD3, GD4, GT1, GT2, GT3, GT4).

5.2.8 Western blot analysis

Protein extracts from harvested Caco-2 cells were prepared in TKM buffer (50 mM Tris, pH 7.5, 150 mM KCl and 5mM MgCl2) using a Dounce homogenizer. The total protein content was determined using a BCA assay. For Western blotting aliquots containing 20 μg of total protein from each sample were separated on sodium dodecyl sulphate-polyacrylamide gels SDS-PA (10%) and then transferred to polyvinylfluoride (PVDF) membranes (Immobilen-P, Millipore Corp.) in accordance with standard procedures. Incubation with the primary antibodies to TLR4, apoB48 and ß-actin was conducted overnight at 4˚C. Immunodetection was performed using the alkaline phosphatase (AP)-conjugated anti-mouse and anti-rabbit IgG (Sigma). AP was detected using a mix containing 0.45% nitroblue tetrazolium (NBT, Serva) and 0.35% bromo-4-chloro-3-indolyl phosphate toluidinium salt (X-Phosphat, Serva) in AP buffer (100 mM NaCl, 50 mM MgCl2 and 100 mM Tris, pH 9.2). Primary antibodies were used in a 1:100 dilution. Secondary antibodies were used at concentrations suggested by the suppliers.

5.2.9 Statistical Analysis

All values are displayed as the mean ± the standard error of the mean for a sample size. Data was analyzed by a one-way ANOVA, Duncan's multiple range tests. A *P* value of less than 0.05 was considered statistically significant.

5.3 RESULTS

5.3.1 Total ganglioside change after DSS treatment or ganglioside incubation

Total ganglioside content in Caco-2 cells was significantly increased after ganglioside supplementation from apical medium (Fig. 5-1), compared with the control cells. DSS treatment alone did not significantly affect ganglioside content.

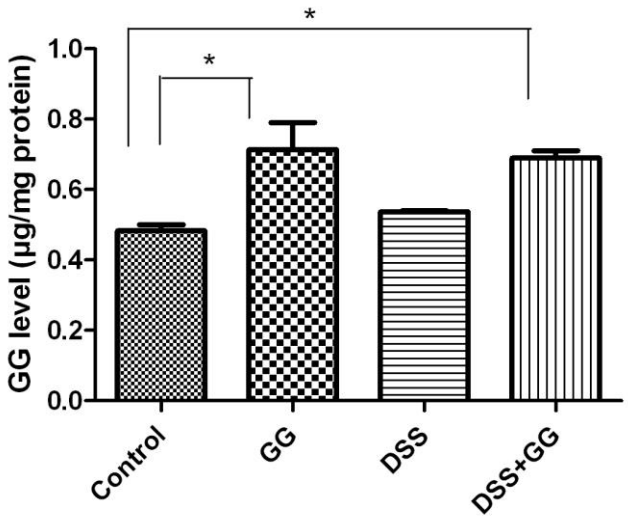


Fig. 5-1 Total content of ganglioside in Caco-2 cells after DSS and ganglioside treatments. Values are means ± the standard error of the mean for a sample size of n=3 (3 different cell passages). ∗ $P < 0.05$.

5.3.2 Quantitation of individual gangliosides in Caco-2 cells after ganglioside incubation or DSS treatment

GD3 increases for incubating cell with ganglioside, less so for ganglioside and DSS treatment cells (Fig. 5-2). GT3 and GT1 increase for GG treated cell groups. DSS results in a large decrease in GD1, an affect that can be inhibited by GG slightly.

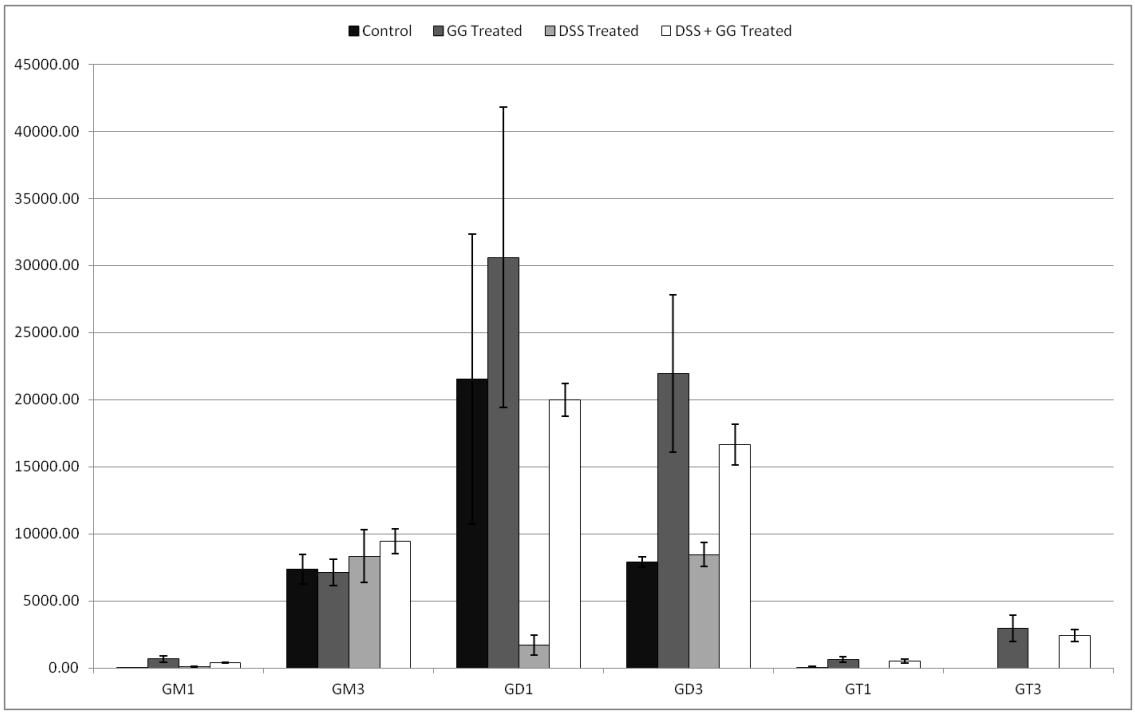


Fig. 5-2 Ganglioside composition of Caco-2 Cells after different treatment. Values are expressed as the mean ± the standard error of the mean for 4 different cell passages.

5.3.3 TLR4 was not affected by ganglioside or LPS.

There was no significant change in the TLR4 protein level with any of the treatments (Fig. 5-3).

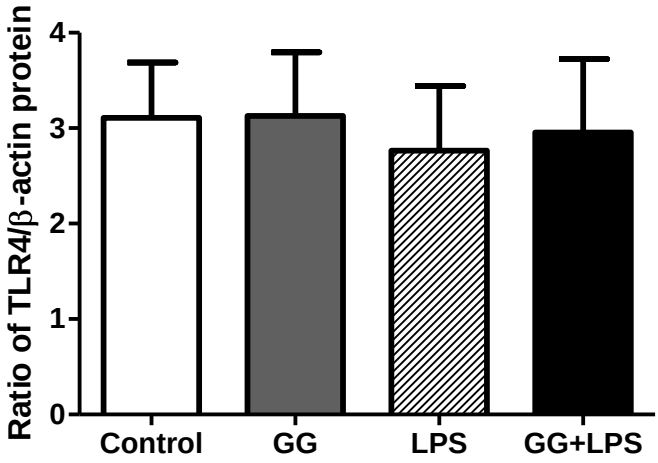
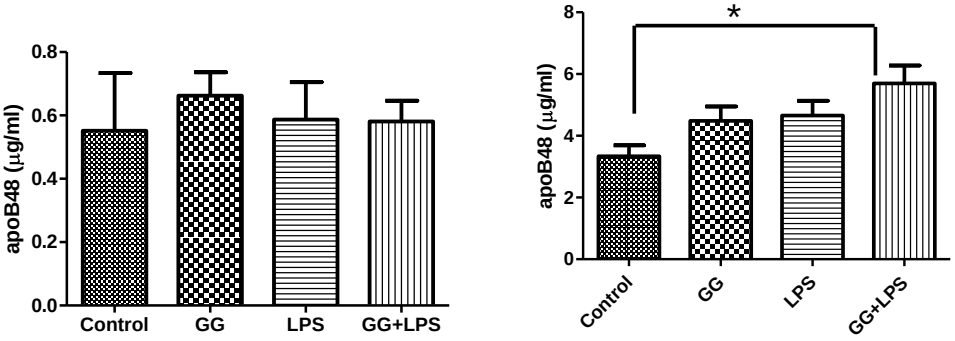


Fig.5-3 TLR4 protein level in Caco-2 cells after LPS or ganglioside treatments. Values are means ± the standard error of the mean for a sample size of n=12. No significant difference was observed.

5.3.4 apoB48 change in medium and cell lysates after LPS stimulation and ganglioside incubation

Incubation with ganglioside or LPS stimulation did not affect apoB48 level in the cell media (Fig.5-3 A). In Caco-2 cells lysates, apoB48 protein was significantly increased after ganglioside incubation in combination with LPS stimulation (Fig.5-4 B).



A B

Fig. 5-4 apoB48 in the media (panel A) and in the cell lysates (panel B) after incubation with LPS or ganglioside. Values are means ± the standard error of the mean for a sample size of n=10. ∗ P< 0.05.

5.4 DISCUSSION

Changes in ganglioside content and composition have been reported in human milk during lactation and intestinal development [21], [22]. The present study shows that proving a mixture of bovine gangliosides to Caco-2 cells in culture increased total ganglioside content, including content of GD3, GT3 and GT1. DSS treatment has no effect on total ganglioside content, however, DSS treatment resulted in a large decrease in GD1. Enhanced ganglioside content following incubation of cells with ganglioside is supported by the observation that feeding gangliosides significantly increase total ganglioside GD3 content in the intestinal mucosa in rats [6]. Taken together, it suggests that gangliosides content are affected by disease status, intestinal development and dietary supplement.

Dysregulated innate immune responses to commensal bacteria contribute to the development of inflammatory bowel disease (IBD). TLR4 is overexpressed in the intestinal mucosa of IBD patients and may contribute to uncontrolled inflammation [23]. TLR4 is responsible for recognizing endotoxin (LPS) from Gram-negative bacteria and is thus important in the activation of the innate immune system [24]. The present study found that LPS did not increase the cell content of TLR4, nor did ganglioside treatment effect this. Another study in human adipose tissue and adipocyte primary cultures also indicated that TLR4 was not activated by fatty acids [25]. Human data on TLR4 polymorphisms are not consistent, either. For example, there is no association in Scottish CD patients and in the Guangxi Zhuang IBD patients in China [26]. More research is required to clarify the role of TLR4 in the pathogenesis and treatment of IBD.

After uptake and re-esterification of dietary fatty acids, triglyceride synthesis and chylomicron assembly take place through a series of events culminating in a physical interaction between microsomal triglyceride transfer protein (MTTP) as a neutral lipid donor and a large hydrophobic transport protein, apolipoprotein B (apoB) [27]. Chylomicrons are the physiological carriers of absorbed dietary lipids and apoB-48 plays a crucial role in chylomicron assimilation and rapid transport as an exclusive protein of the chylomicron

particles [27], thereby playing an essential role in intestinal absorption of dietary fats [13]. In the present study, neither LPS or ganglioside alone had an effect on apoB48 production by Caco-2 cells. ApoB48 concentration (indicator of secretion) was also not affected by LPS stimulation without ganglioside. However, incubation of cells with ganglioside significantly increased the cellular concentration of apoB48 after LPS stimulation. Prevalence of nutritional deficiencies and malnutrition has been well documented in IBD, especially in Crohns disease [15]. Factors that contribute to malnutrition and nutrient deficiencies in patients with IBD, include reduced nutrient intake, malabsorption and inflammation. The intestinal brush border membrane contains approximately 20% glycosphingolipids in the brush border lipid [28]. The dominant ganglioside is GM3, which in the rat is 7 times more concentrated in neonatal than in adult intestine [29]. Another animal study also indicated dietary ganglioside enhances in vitro glucose uptake in weanling rats [30]. The tissue- and age-specific composition may contribute to the intestinal adaptation to diet that alters nutrient absorption.. Taken together, these findings indicate that gangliosides may improve malnutrition in IBD by increased apoB48 expression and lipid absorption.

In conclusion, the present study indicates that incubation of Caco-2 cells with ganglioside increases the content and composition of cellular ganglioside and increases apoB48 content in cells after LPS stimulation, thus indicating ganglioside may play a role in ensuring the absorption of dietary fats in cells exposed to enterotoxin.

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CHAPTER 6

THESIS CONCLUSIONS AND FUTURE DIRECTIONS

6.1 THESIS CONCLUSIONS

The hypotheses tested in this thesis were addressed as follows:

Hypothesis 1: Ganglioside supplementation will lower levels of pro-inflammatory mediators of HBD-2 and IL-23.

Two IBD culture models were investigated in Caco-2 cells. In the LPS induced model, LPS treatment resulted in significantly higher production of both pro-inflammatory mediators of HBD-2 and IL-23. Cells incubated with ganglioside had significantly less production of HBD-2 and IL-23 after LPS stimulation (Chapter 3). Ganglioside incubation alone has no effect on levels of HBD-2 and IL-23 (Chapter 3). In the DSS induced Caco-2 cell model, a similar trend was observed for HBD-2 in that DSS treatment resulted in significantly higher HBD-2 level. Cells cultured with ganglioside exhibited significantly less HBD-2 (Chapter 4). Neither DSS treatment nor ganglioside incubation significantly altered the level of IL-23. This study suggests that LPS and DSS induce two different models of IBD. This observation is also supported by the complex etiopathogenesis known for IBD. Due to the discrepancies in IL-23 levels, further research is needed to evaluate other cells and animal models for studying the role of IL-23 in the physiology and the pathophysiology of IBD. Meanwhile, the increase of IL-23 in IBD has been well documented and is very convincing, it may suggest LPS induced IBD model is better than DSS induced IBD model in Caco-2 cells in term of IL-23 secretion.

Hypothesis 2: Ganglioside supplementation will protect intestinal structural integrity and improves barrier function in a cell culture model of IBD.

LPS stimulation significantly decreased intestinal barrier function measured by TEER (Chapter 3). DSS treatment led to severely disrupted and opened tight junctions and desmosomes (Chapter 4). DSS also caused significant loss of intestinal barrier function measured by TEER (Chapter 4). Incubation of cells with ganglioside attenuated the disruption of intestinal junction complexes, including tight junction, adhesion junctions (Chapter 4). Incubation of cells with ganglioside results in an improved intestinal barrier function after both LPS and DSS compared to these chemicals alone. This study suggests that providing gangliosides may exhibit a protective therapeutic effect on intestinal integrity and barrier function.

Hypothesis 3: Ganglioside supplementation will decrease $sPLA_2$ secretion and modifies cellular content of phospholipids in Caco-2 cells.

Incubation of cells with gangliosides or LPS increased the secretion of $sPLA_2$ in the apical medium. Combination of LPS stimulation and ganglioside incubation also increased $sPLA_2$ in the basolateral medium compared to control or LPS alone (Chapter 3). DSS treatment significantly increased $sPLA_2$ level in the basolaterial medium (Chapter 4). However, cells incubated with ganglioside after the DSS treatment had significantly lower level of sPLA2 in the basolaterial medium (Chapter 4). DSS treatment led to a significant increase in PE and decrease in PI compared with the control cells (Chapter 4). Ganglioside incubation protects the cellular change in content of PE in Caco-2 cells after DSS treatment (Chapter 4).

This study suggests that LPS and DSS induced significant increase in sPLA2 level in the basolaterial medium. Ganglioside incubation inhibited production of $sPLA_2$ level in the DSS induced IBD model, but not in LPS induced IBD model. Ganglioside also protects the cellular phospholipid integrity. Due to discrepancies in $sPLA_2$ levels between LPS and DSS treatment, further research is needed to evaluate other cells and animal models for studying the roles of $sPLA_2$ and the pathophysiology of IBD.

Hypothesis 4: Ganglioside supplementation inhibits NF-kB activation.

Stimulation of Caco-2 cells with LPS significantly increased activation of NF-kB p65 and p52 (Chapter 3). Ganglioside alone exhibited no effect on NF-kB activation (Chapter 3). However, cells cultured with ganglioside have significantly lower level of NF-kB p65 after LPS stimulation (Chapter 3). DSS treatment significantly increased activation of NF-kB p65. Cells cultured with ganglioside exhibited higher activation of NF-kB p65 after DSS treatment (Chapter 4). This study indicates that incubation of Caco-2 cells with ganglioside inhibits NF-kB activation in a LPS induced Caco-2 cell model of IBD, but not in the DSS induced cell culture model of IBD. Due to differences in the activation of NF-kB with LPS and DSS treatments, further research is needed to evaluate beneficial effect of ganglioside on other cells and animal models for studying the roles of NF-kB and the pathophysiology of IBD.

Hypothesis 5: Ganglioside supplementation decreases TLR4 level.

Both LPS stimulation and ganglioside have no apparent effect on TLR4 level (Chapter 5). This study indicates that the mechanism of ganglioside anti-inflammatory activity is independent of TLR4 in the LPS-induced Caco-2 cell culture mode of IBD.

Hypothesis 6: Ganglioside supplementation increases ApoB 48 secretion.

Incubation of Caco-2 cells with ganglioside alone has no effect on apoB48 secretion (Chapter 5). ApoB48 protein was significantly higher after ganglioside incubation following LPS stimulation Caco-2 cells (Chapter 5). This suggests that ganglioside treatment may positively impact on the absorption of dietary fats during states of intestinal inflammation.

Hypothesis 7: Ganglioside supplementation increases plasma membrane ganglioside content.

Total ganglioside content, including GD3, GT3 and GT1, was significantly increased after ganglioside incubation, (Chapter 5). DSS treatment decreased the content of GD1. This decrease can be prevented by ganglioside incubation (Chapter 5).

6.2 Thesis Summary

This thesis demonstrates in Caco-2 cells that: (1) LPS stimulation and DSS treatment result in some, albeit different, pathologies observed in IBD; (2) Ganglioside apparently exerts protective effects on IBD by down-regulating pro-inflammatory signals, especial HBD-2 levels; (3) Ganglioside at a dose of 10 μg/ml is apparently safe without side effects on Caco-2 cells; (4) Incubation with a mixture of gangliosides protect intestinal integrity of structure and improves barrier function; (5) Treatment with gangliosides protects cellular phospholipid and ganglioside integrity; (6) Ganglioside treatment resulted in lower $sPLA_2$ secretion and inhibits NF-kB activation in DSS and LPS models, respectively.

6.3 Significance of Thesis Research Findings

Human CaCo-2 cells may provide valuable insight into the physiology and pathophysiology of IBD. This is the first research to compare the effect of LPS and DSS on Caco-2 cells (Table 6-1 and Fig. 6.1). This study demonstrated that both LPS stimulation and DSS treatment are useful models for the study of aspects typical of IBD. The two IBD models used share some similarities and have some differences. For example, both LPS and DSS increased HBD-2 level, increased activation of NF-kB P65 and increased secretion of sPLA2 in the basolateral medium. Both LPS and DSS decreased intestinal barrier function measured by TEER. This finding is significant as it suggests that HBD-2, NF-kB p65, sPLA2 and TEER are important to characterize the pathogenesis and treatment of IBD.

In the LPS induced Caco-2 model of IBD, the research demonstrated that ganglioside decreases pro-inflammatory HBD-2 and IL-23 level. Our research suggests that this is due to improving barrier function and inhibiting NF-kB activation, independent of sPLA2 level. For the first time, the present study indicated that ganglioside may modulate innate immune responses and adaptive immunity in the intestinal epithelial cells by inhibiting HBD-2 level. HBD-2 plays an important role in host defense and represents a link between innate and adaptive immune responses. After binding to the appropriate receptor (IL-23R), IL-23 can stimulate production of IL-17, TNF-α, and IL-6 from T-cells. Therefore, IL-23 was proposed to play an integral role in the pathogenesis of IBD. The importance of this finding suggests that ganglioside in the intestine may exert protective effects on IBD by increasing HBD-2 and IL-23.

Ganglioside treatment also inhibited NF-kB activation after LPS stimulation, which may explain the mechanism for how ganglioside benefits IBD treatment. Expression and activation of NF-κB is strongly induced in the inflamed gut of IBD patients. This finding suggests that ganglioside exerts protective

effects on IBD by inhibiting NF-kB activation. Blocking of NF-κB pathway has become an attractive target for therapeutic interventions in IBD.

Ganglioside also increased secretion of apoB 48 after LPS stimulation. The implication of this important finding is that ganglioside may improve lipid absorption status in IBD. Fat intake is lower in UC and CD patients [1]. Medium chain triglycerides are often suggested for use in patients with fat malabsorption. Malnutrition is common in IBD and mechanisms involved include decreased food intake, malabsorption, increased nutrient loss, increased energy requirements, and drug–nutrient interactions. Nutrition plays a role in IBD primarily in prevention and treatment of malnutrition and growth failure [2]. Furthermore, in Crohns disease (CD), improved nutrition can induce remission, maintain remission, and prevent relapse [2].

In the DSS induced Caco-2 cell model of IBD, this research demonstrated that ganglioside incubation decreases secretion of sPLA2 in the basolateral medium after DSS treatment. The implication of this finding is that ganglioside may decrease pro-inflammatory eicosanoids of LTB_4 and PGE2 by inhibiting secretion of sPLA2. sPLA2 releases arachidonic acid from mucosal phospholipids, and the arachidonic acid cascade then leads to production of many inflammatory mediators.

The present study also indicated that ganglioside incubation was demonstrated to protect the integrity of cellular phospholipids. A direct implication of this finding is that ganglioside may improve plasma membrane barrier function in patient with IBD. The bilayer form of phospholipid is selectively permeable, allowing only certain molecules across the membrane to enter or exit the cell. Therefore, the integrity of cellular phopholipid is very important to maintain barrier functions.

Finally, the present study indicated that incubation of cells with a mixture of gangliosides increases GD3, GT3 and GT1 content. Ganglioside incubation attenuated PE change caused by DSS. A direct implication of this finding is that individual ganglioside may play different roles in IBD and gangliosides protect membrane integrity.

Table 6-1 Summary of major results of LPS model and DSS model.

	LPS	DSS	LPS+GGs	DSS+GGs
HBD-2	↑	↑	↓	↓
IL-23	↑	-------	↓	-------
Intestinal Integraty and Barrier Function	↓	↓	↑	↑
$sPLA_2$ (apical)	↑	↑	↑	↓
$sPLA_2$ (basolateral)	-------	↓	↑	↓
NF-kB P65	↑	↑	↓	↑
TLR4	-------	NA	-------	NA
ApoB 48	-------	NA	↑	NA

6.4 Future Directions

Research in the area of ganglioside and IBD is growing with numerous future opportunities to enhance knowledge in this exciting field. Based on an extensive review of the IBD and ganglioside literature there are three major areas that require more research attention: Pathogenesis of IBD and mechanism of action of ganglioside on IBD, role of individual gangliosides on IBD and broader clinical application. In the final section of this thesis, I will address these issues by providing examples of future follow-up studies that could be done to improve knowledge in these three areas.

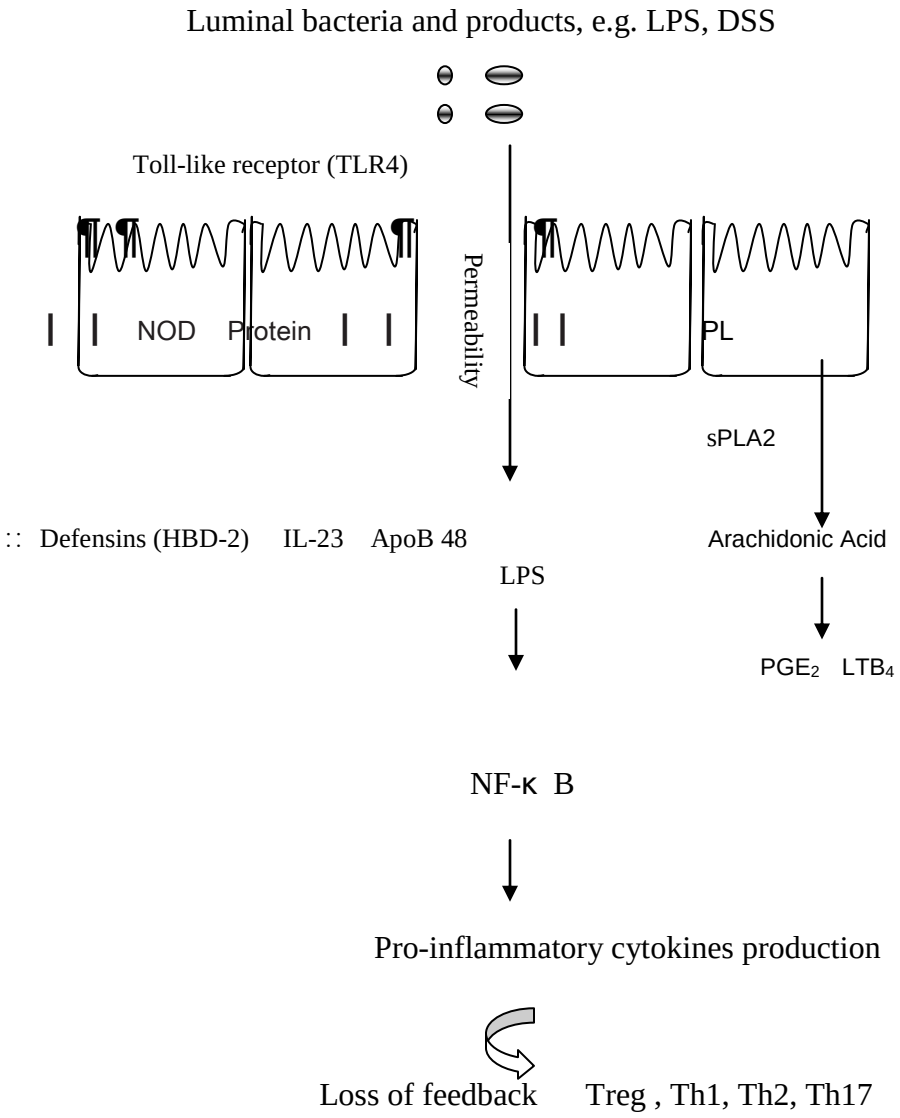


Fig. 6.1 Pathogenesis of IBD and potential mechanism by which gangliosides protect IBD from inflammation. Althought the pathogenesis of IBD is not clearly elucidated, it is thought involved in the complex interplay among bateria, intestinal defective barrier function, genetic factors and immune system. (1) Bacteria and its products, such as LPS is recognaized by Toll-like receptor and NOD, which will lead to the activation of NF-κ B and increase the production of pro-inflammatory cytokines. (2) Uncontrolled inflammation is caused by the loss of feedback of mimmune system. (3) Genetic defects play important role on IBD, including permeability of the epithelial barrier, innate immune and adaptive immunity. Gangliosides may modulate intestinal permeability, inhibit the

acitivation of NF-κ B, decrease expression of sPLA2 and balance the production of cytokines from immunce cells. IBD=inflammatory bowel disease; NOD=nucleotide oligomerization domain; LPS= lipopolysaccharide;; NF-kB=nuclear factor-κ B.

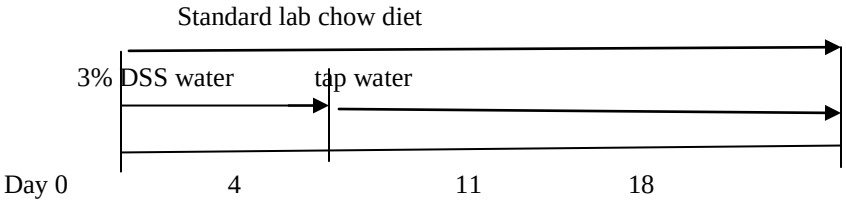
6.4.1 Pathogenesis of IBD and mechanism of action of ganglioside in IBD

It is well established that ganglioside exhibits biological effects through decreasing pro-inflammatory signals levels of prostaglandin E2, LTB4, IL-1β, and TNF-α in rats induced by LPS [3] and in an infant model of necrotizing enterocolitis induced by LPS and hypoxia [4]. This thesis expanded the previous knowledge by showing that gangliosides have anti-inflammatory effects in both LPS stimulated and DSS treated Caco-2 cells (Chapter 3 and Chapter 4). However, there are still some discrepancies about the mechanistic detail for $sPLA_2$ and NF-kB between LPS induced and DSS treated IBD model (Chapter 3 and Chapter 4). It is necessary to clarify the role of $sPLA_2$ and NF-kB in the pathogenesis of IBD and to investigate beneficial effects of ganglioside in other animals and cells culture models.

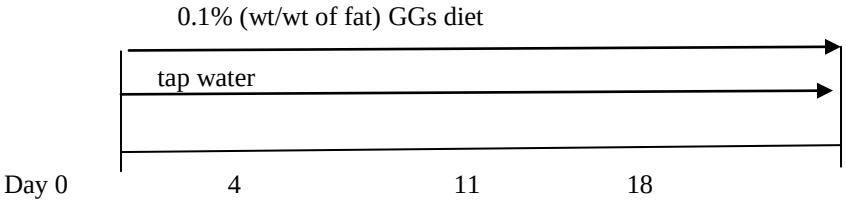
The pathogenesis of IBD is not completely understood, although considerable progress has been made due to the development of novel cell culture and animal models. The question arises as to which model of IBD is the most useful? An appropriate response is that the value of each model depends on the particular application. Many of the models induced by exogenous toxins, acetic acid, trinitrobenzene sulfonic acid in ethanol, formalin, and 3-5% dextran sulfate sodium (DDS) have been used to evaluate new anti-inflammatory drugs [5]. These models usually demonstrate acute, self-limiting colitis. Chemical-induced models of gut inflammation are the most commonly used and best described models of IBD. DSS colitis is a widely used chemically induced model of intestinal inflammation. It is necessary to examine the effect of ganglioside on a DSS induced animal model. The following animal study design can be used to investigate the role of gangliosides on prevention and treatment of IBD. The

findings of Caco-2 cell culture models can be further confirmed with the DSS animal model (please see Fig. 6.2), especially the mechanism of NF-kB activation, sPLA2 secretion, TLR4 expression, intestinal barrier function as well as HBD-2 and IL-23 expression.

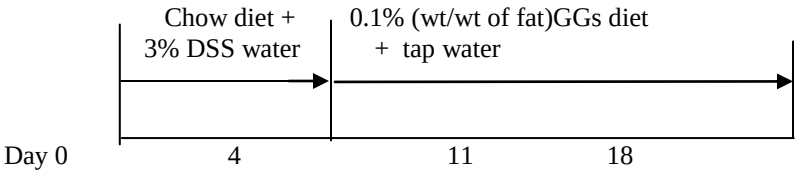
Control rats group (n=8)



GGs rats group (n=8)



GGs post-treated rats (n=8)



GGs pre-treated rats (n=8)

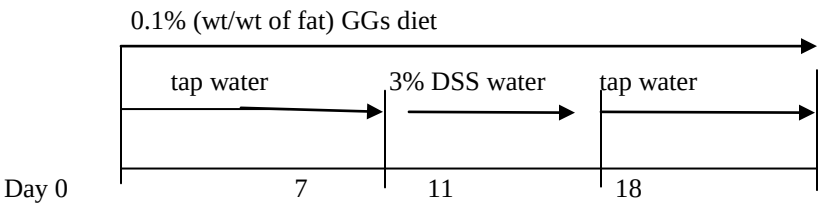


Fig. 6.2 DSS animal model study design. Animals will be divided into 4 groups (n=8 for each group).

6.4.2 Role of individual ganglioside on IBD

Gangliosides are sialic acid-containing glycosphingolipids concentrated predominantly in lipid rafts. Present in small amounts in the diet, dietary gangliosides are incorporated into tissues and exert potent effects on cellular functions [6]. Depending on location, concentration and form, individual gangliosides play important roles in recognition, attachment and translocation of cells, microbes and macromolecules across membranes and regulate cell signaling and protein function [6]. The present study used a dairy based mixture of Gangliosides, it also indicated that GD1 was decreased by DSS treatment and ganglioside incubation increased total ganglioside and GD3, GT3 and GT1 content (Chapter 5). Taken together, the results suggest that individual gangliosides may have different roles in IBD. It is necessary to investigate and compare the role of individual gangliosides on the prevention and treatment of IBD.

6.4.3 Broader Clinical Applications

The result of this thesis suggests clinical potential for ganglioside supplementation in the prevention and treatment of IBD. Along with in vivo ganglioside research findings from rats [7], [3] and cells in culture from infants [8], the present study has led to more research interest on the safety and efficacy of dietary gangliosides in IBD. Thus, this section will focus on other broad clinical applications.

6.4.3.1 Prevention of necrotizing enterocolitis

One of the most serious disorders and the single most serious gastrointestinal disorder occurring in neonates is necrotising enterocolitis (NEC) [9]. Similar with CD, NEC can arise in any area of the GI tract; however, the most common sites are the terminal ileum, caecum, and ascending colon [10]. Important elements in modulating the damage that results from NEC are the inflammatory cytokines interleukin 1, 3, 6, tumour necrosis factor (TNF), and platelet activating factor (PAF). Infection and enteral feeding are risk factors associated with NEC, whereas feeding human milk is protective [8]. Gangliosides are rich in colostrum and in membrane microdomains, which promote enterocyte growth and differentiation, and modulate Th1/Th2 responses [11]. One NEC cell culture model has indicated that ganglioside protect bowel in an infant model of NEC by suppressing proinflammatory signals [8]. One animal study also indicated that dietary GD3 protects newborn rats from NEC, in part, by augmenting mucosal regulatory immune responses [12]. Further mechanistic investigations on are required to confirm potential application of ganglioside in preventing NEC.

6.4.3.2 Prevention and treatment of diabetes

It has been increasingly accepted that chronic subacute inflammation plays an important role in the development of insulin resistance and type 2 Diabetes in animals and humans. Suppression of systemic inflammation in type 2 Diabetes improves glycemic control, which also points to a new potential therapeutic target for the treatment of type 2 Diabetes. Animal studies also show that gangliosides enhance glucose uptake [4] and have a cholesterol lowering effect in weanling rats [13]. The present study suggests gangliosides may have anti-inflammatory activity by deceasing NF-kB activation and down regulating sPLA2 secretion in Caco-2 IBD models. Studies in OB/OB mice and Cohen diabetic rat indicated that glucocerebroside ameliorates the metabolic syndrome by improving glucose tolerance and modulating cytokines levels [14].

Gangliosides may have beneficial effects on diabetes, as glucocerebroside is a metabolic intermediate in the metabolic pathway for synthesis of complex glycosphingolipids.

6.4.4 Limitation of Caco-2 cells in vitro study

Caco-2 cells are valuable in vitro tools for studies related to intestinal cell function and structure. However, the Caco-2 monolayers still inherit properties that make further refinement of this model desirable. For one, the tightness of the monolayer resembles more colonic than small intestinal tissue, resulting in poor permeabilities for hydrophilic compounds traversing the epithelium via the aqueous paracellular pathway [15]. On the other hand, the Caco-2 model is composed of solely absorptive cells, whereas the intestinal epithelium is a conglomerate of absorptive enterocytes and other cells such as goblet cells, endocrine cells, and M cells, with the mucus secreting goblet cells representing the second most frequent cell type [16]. A third limitation of the pure Caco-2 cell system is the frequently discussed potential overexpression of P-glycoprotein which may lead to higher secretion rates and consequently lower permeabilities in the absorptive direction [17]. Finally, it is well known that permeabilities of compounds that are transported via carrier-mediated absorption are lower in the Caco-2 cell system as compared to the human small intestine, probably also reflecting the colonic origin of this cell line. All the present results in vitro need to be confirmed in vivo.

In summary, this thesis used LPS and DSS treatment, represent two different IBD model to examine a breadth of mechanisms including IL-23 and HBD-2 proteins, barrier function with TEER, NF-κB activity, phospholipase A2, phospholipid composition and TLR-4 and apo48 protein expression in Caco-2

cells. Given the prevalence of IBD and the detrimental side-effects that patients experience with current pharmacological treatments, this work examined a milk-derived compound in advancing the field, exploring a potential less toxic and less expensive therapeutic role of gangliosides in treating IBD.

6.5 References

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