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New insights into the mechanims of prebiotics and microbiota on intestinal defense

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A mi abuelo y a mi padre,
mis dos grandes modelos a seguir

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por permitirme conocer el mundo de la investigación
y darme la oportunidad de realizar este trabajo

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y Dpto. de Farmacología de la Facultad de Farmacia,
al Institute of Genetics and Molecular Medicine of Edinburgh

A mis compañeros y amigos del laboratorio, por enseñarme, alegrarme
y, sobre todo, por cuidarme en los momentos más díficiles

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A mi familia

A ti

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Abbreviations

AAD
Antibiotic-associated diarrhea

AIEC
Adherent-invasive *Escherichia coli*

AOM
Azoxymethane

AP
Alkaline phosphatase

APC
Antigen-presenting cell

ATG16L1
Autophagy related 16-like 1

BCA
Bicinchoninic acid

BSA
Bovine serum albumin

CARD
Caspase recruitment domain family

CD
Crohn's Disease

CDAI
Crohn's Disease Activity Index

CFGR
Core Facility for Germ-free Research

CFU
Colony forming units

ConA
Concanavalin A

COX
Cyclo-oxygenase

CRF
Corticotropin-releasing factor

CVD
Cardiovascular disease

DAI
Disease Activity Index

DBMB
Department of Biochemistry and Molecular Biology II

DMEM
Dulbecco's Modified Eagle's Medium

DP
Degree of polymerisation

DSS
Dextran sulfate sodium

EDTA
Ethylenediaminetetraacetic acid

ELISA
Enzyme-linked Immunosorbent Assay

ERK1/2
Extracellular signal-regulated protein kinase 1/2

FOS
Fructooligosaccharides

FOXP3
Forkhead box p3

GF
Germ-free

GI
Gastrointestinal

GLP
Glucagon-like peptide

GM-CSF
Granulocyte-macrophage colony-stimulating factor

GMOS
Goat milk oligosaccharides

GOS
Galactooligosaccharides

GPCR
G protein-coupled receptor

GRO-α
Growth-regulated oncogene alpha

GWAS
Genome-Wide Association Studies

HBSS
Hanks' Balanced Salt Solution

HIV-1
Human immunodeficiency virus-1

HMOS
Human milk oligosaccharides

hPBM
Human peripheral blood monocyte

H&E
Hematoxylin & eosin

IBD
Inflammatory Bowel Disease

IEC
Intestinal epithelial cell

IFN-γ
Interferon gamma

IgA
Immunoglobulin A

IκB
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor

IL
Interleukin

IL23R
Interleukin-23 receptor

IRGM
Immunity-related guanosine triphosphatase family M

JAK
Janus kinase

JNK
c-Jun NH_2-terminal kinase

KGF
Keratinocyte growth factor

KO
Knock-out

LC3
Microtubule-associated light chain 3

LDL
Low density lipoprotein

LPS
Lipopolysaccharide

MAP
Mycobacterium avium subspecies *paratuberculosis*

MAPK
Mitogen-activated protein kinase

MCFA
Medium-chain length fatty acids

MDP
Muramyl dipeptide

***MHC* / MHC**
Major histocompatibility complex

MLNC
Mesenteric lymph node cell

MPO
Myeloperoxidase

MSMC
Mice spleen mononuclear cell

MUC
Mucin, oligomeric mucus/gel-forming

Myd88
Myeloid differentiation factor 88

NDOS
Non-digestible oligosaccharides

NEC
Necrotizing enterocolitis

NFκB
Nuclear factor kappa-light-chain-enhancer of activated B cells

NK
Natural killer

NO
Nitric oxide

NOD
Nucleotide-binding oligomerization domain

NSAID
Non-steroidal anti-inflammatory drugs

OCP
Oral contraceptive pills

PAMP
Pathogen-associated molecular pattern

PBS
Phosphate buffered saline

PCR
Polymerase chain reaction

PE
Phycoerythrin

PGF
Pseudo germ-free

PI3K
Phosphatidylinositol-3-kinase

PPARγ
Peroxisome proliferator-activated receptor gamma

PRR
Pathogen-recognition receptor

PSC
Primary sclerosing cholangitis

PUFA
Polyunsaturated fatty acids

RegIII-γ
Regenerating islet-derived protein 3 gamma

qRT-PCR
Quantitative reverse-transcription polymerase chain reaction

RPMI
Roswell Park Memorial Institute medium

S100A8
S100 calcium binding protein A8

SBH
Sterile bacterial homogenate

SCFA
Short chain fatty acids

Scid
Severe combined immunodeficiency

SEM
Standard error of the mean

SPF
Specific pathogen free

SSI
STAT-induced STAT inhibitor

STAT
Signal transducer and activator of transcription

TFF3
Trefoil factor 3

TGF-β
Transforming growth factor beta

Th
Lymphocyte T helper

TLR
Toll-like receptor

TNBS
Trinitrobenzene sulfonic acid

TNF
Tumor necrosis factor

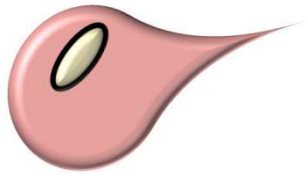
Treg
Regulatory lymphocyte T

UC
Ulcerative Colitis

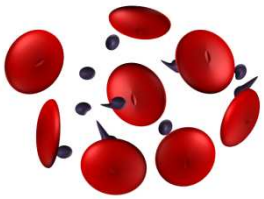
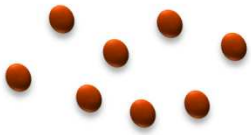
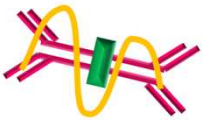
WT
Wild type

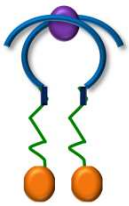
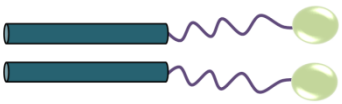
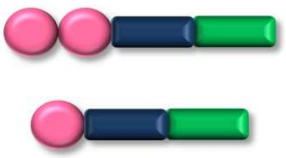
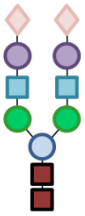
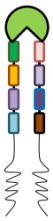
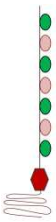
ZO-1
Zonula occludens 1

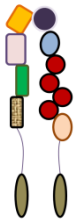
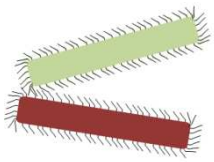
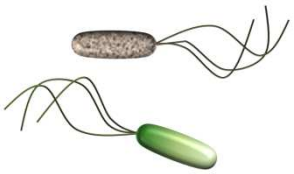
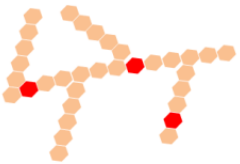
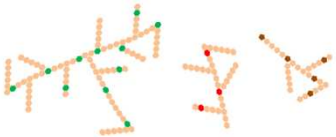
Drawing legend

Drawing	Name	Function
	Enterocytes	Intestinal epithelial cell. Microvilli on the apical surface increase surface area for the digestion and transport of molecules from the intestinal lumen. They also have a secretory role and act as antigen-presenting cell.
	Paneth cells	Innate immunity. Produce antimicrobial peptides, thus limiting bacterial presence at the crypt space.
	Goblet cells	Innate immunity. Secrete mucus, creating a microenvironment in close proximity to the epithelial surface that limits bacterial contact both physically and chemically.
	Hormone producing enteroendocrine cells	The enteroendocrine system consists of at least 15 different cell types, classified based on their main hormonal products. Essential regulators of digestion, gut motility, appetite and metabolism.
	Intestinal stem cells	Self-maintenance is the fundamental stem cell requirement. They are located within small intestinal and colonic crypts and they are able to form a regenerative crypt containing all cell lineages (enterocytes, goblet, Paneth and enteroendocrine cells).
	Microfold cells (M cells)	Differ from normal enterocytes in that they lack microvilli on their apical surface. Its main function is the selective endocytosis of antigens, transporting them to intraepithelial macrophages and lymphocytes, which then migrate to lymph nodes where an immune response can be initiated.

Drawing	Name	Function
	Endothelial cells	Thin layer of cells (endothelium) that lines the interior surface of blood vessels and lymphatic vessels. Its functions include fluid filtration, blood vessel tone, hemostasis, neutrophil recruitment and hormone trafficking.
	Monocytes	Innate immunity. Monocytes are the first cells attracted to the focus of inflammation through the bloodstream. There, they produce pro-inflammatory cytokines, sustaining and enhancing the inflammatory response. They differentiate into macrophages.
	Macrophages (resident)	Phagocytose cellular debris and pathogens. They act in both innate and adaptive immunity, stimulating lymphocytes and other immune cells to respond to pathogens.
	B lymphocytes (B cells)	Adaptive immunity. Antigen-presenting cells and antibody producers. Develop into memory B cells.
	T lymphocytes (T cells)	Adaptive immunity. Play a central role in cell-mediated immunity.
	Intraepithelial lymphocytes	Components of the gut-associated lymphoid tissue (GALT). They do not need priming. Upon encountering antigens, they immediately release cytokines and cause killing of infected target cells.

Drawing	Name	Function
	Dendritic cells	Innate immunity. Cytoplasmic extensions are interdigitated among the epithelial cells in order to sample antigens and present them to T cells in the *lamina propria* and the underlying lymphoid follicles. Dendritic cells can also travel to draining lymph nodes to interact with T cells.
	Natural killers (NK)	Innate immunity: cytotoxic lymphocytes with major role in the host-rejection of both tumours and virally infected cells. Adaptive immunity: they are able to readily adjust to the immediate environment and formulate antigen-specific immunological memory.
	Neutrophils	Innate immunity. During the acute phase of inflammation, particularly as a result of bacterial infection, they are one of the first-responders of inflammatory cells to migrate towards the site of inflammation.
	Platelets and erythrocytes	Erythrocytes (red blood cells) carry oxygen and collect carbon dioxide. Platelets (thrombocytes) are a natural source of growth factors and are involved in hemostasis, leading to the formation of blood clots.
	Antimicrobial peptides	These peptides are potent, broad spectrum antibiotics and may also have the ability to enhance immunity by functioning as immunomodulators.
	Secretory Immunoglobulin A (IgA)	Antibody that plays a critical role in mucosal immunity. IgA is the main immunoglobulin found in the gastrointestinal tract and provide protection against microbes. The secretory component protects from proteolytic enzymes.

Drawing	Name	Function
	Toll-like receptors (TLR)	Type I transmembrane proteins expressed by innate immune cells of the intestinal epithelum and the *lamina propria*. Consist of at least 11 members that recognize not only microbial components, but also damaged host cell components such as nucleic acids and other “internal” ligands.
	Toll-like receptor 4 (TLR4)	Plays a fundamental role in pathogen recognition and activation of innate immunity. This receptor has been implicated in signal transduction events induced by lipopolysaccharide found in most Gram-negative bacteria.
	NOD-like receptors (NLR)	Large family of cytoplasmic proteins comprising over 20 members. Among the NLR family members, Nod1 and Nod2 were the first identified and are sensors of bacterial components involved in the modulation of the intestinal inflammatory and apoptotic response.
	Glycoproteins	Proteins that contain oligosaccharide chains (glycans) covalently attached to polypeptide side-chains. Glycoproteins are often important integral membrane proteins, where they play a role in cell-cell interactions.
	Intercellular adhesion molecule 1 (ICAM1)	Cell surface glycoprotein which is typically expressed on endothelial cells and cells of the immune system. When activated, leukocytes bind to endothelial cells.
	Vascular cell adhesion molecule 1 (VCAM1)	Mediates the adhesion of lymphocytes, monocytes, eosinophils and basophils to vascular endothelium. It also functions in leukocyte-endothelial cell signal transduction.

Drawing	Name	Function
	Very late antigen 4 (VLA4)	Normally expressed on leukocyte plasma membranes. Activated leukocytes undergo the conformational change in the VLA4 integrin, necessary to confer high binding affinity for the endothelial adhesion molecules.
	Gut commensal flora	Complex of microorganism species that live in the digestive tract but do not normally produce diseases.
	Bacterial pathogens	They are always associated with human diseases (strict pathogens) or they can be opportunistic pathogens, taking advantage of preexisting conditions that enhance susceptibility of patient to cause a disease or a more serious disease.
	Lipopolysaccharide (LPS)	Found in the outer membrane of Gram-negative bacteria, recognized by TLR4, acts as an endotoxin and elicits strong immune responses.
	Fructooligossacharides (FOS)	Chains of fructose units linked together by β(2,1) linkages. Found in plant roots like onions, dahlia, wheat and chicory. They are widely used in infant formulas for their prebiotic effect and in patients with intestinal disorders.
	Non-digestible oligosaccharides (NDOS)	Resist digestion and are thus capable of reaching the large intestine in substantial amount. Selectively stimulate the growth and/or activity of one, or a limited number, of microbial species in the gut microbiota that confer health benefits to the host.

Introduction

I. *Inflammatory Bowel Disease. Generalities*

Inflammatory Bowel Disease (IBD) integrates a group of disorders characterized by recurrent, destructive inflammation of the gastrointestinal (GI) tract. They include the two most common forms, Crohn's Disease (CD) and ulcerative colitis (UC), and the more rare collagenous colitis, lymphocytic colitis and atypical microscopic colitis. CD and UC are characterized by a chronic intestinal inflammation, typically intermittent, resulting in diarrhea, abdominal pain, bloody faeces and, in children, growth arrest. Both disorders differ in terms of their distribution of the GI tract and in their macroscopic and histological features. It is not always possible to distinguish between CD and UC. The definitive differentiation often evolves during the course of the disease.

There are few diseases which have changed faces to such an extent as the IBD.[1] Although historic figures such as Alfred the Great and Bonnie Prince Charles have been proposed to have suffered from CD and UC, respectively by clinicians turned historian,[2, 3] it is obvious that the two disease entities were rare until the 20th century. It was not until Dr. Burrill B. Crohn, in 1932, introduced the term "regional ileitis" for the disease that was later named after him, when CD became a distinct clinical entity from UC.[4] Thereafter, there have been an increasing number of incidence studies published either for both UC and CD, or for one of these conditions, for different populations and different time periods. However, most of these studies have generally dealt with small populations and/or short time periods; in most instances less than 10 years. In spite of these shortcomings there are remarkable similarities in the temporal trends for UC and CD.[5]

IBD was primarily recognized in westernized countries following the rise of the industrial revolution. Its incidence dramatically increased during the 20th century. IBD is most prevalent in developed nations such as Canada, the United States and Western Europe. As developing countries, such as India and China, became industrialized, the incidence of IBD has risen in parallel. Additionally, as individuals move from areas of low to high prevalence of IBD, first-generation offspring acquire the same risk of developing IBD as the local population.[6]

Another consistent finding in the descriptive epidemiology of IBD up to recently is that

the incidence of UC is higher than for CD. The highest annual incidence of UC was 24.3 per 100,000 person-years in Europe, 6.3 per 100,000 person-years in Asia and the Middle East, and 19.2 per 100,000 person-years in North America; for CD was 12.7 per 100,000 person-years in Europe, 5.0 person-years in Asia and the Middle East, and 20.2 per 100,000 person-years in North America. The highest reported prevalence values for IBD were in Europe (UC, 505 per 100,000 persons; CD, 322 per 100,000 persons) and North America (UC, 249 per 100,000 persons; CD, 319 per 100,000 persons).[7]

IBD often occurs at the particularly vulnerable periods of childhood and adolescence, with potentially adverse effect on growth, quality of life and psychosocial functioning. Actually, the incidence of IBD is increasing in both periods.[8] There are many similarities in terms of the clinical features and therapeutic options, irrespective of the patient's age. Because of patient's age and the chronicity of the disease, the care of the patient should be performed in a pediatric gastroenterological center to optimize diagnostics and therapy.[9] Customizing treatment for the individual with IBD is critical, but it is especially important when that patient is a child or teenager. The same medications that are used to treat adults with IBD are also used for children. Thus, drug dosages for a child must be carefully tailored to suit their age, size and weight; in addition to existing symptoms, location of inflammation and previous response to treatment.[10]

Most patients with IBD experience concerns regarding fertility and pregnancy at some time of their reproductive life. Evidence compiled over many years indicates the importance of controlling disease activity at the time of conception and during pregnancy to optimize the outcome for mother and baby. Therefore, it is favorable that conception takes place when IBD is in remission. Fetal mortality-spontaneous abortion, stillbirth or neonatal death, is not higher for IBD patients, but there is an increased risk of preterm delivery and low birth weight in mothers with IBD, depending on the disease activity. The majority of women with IBD will have a normal outcome of pregnancy, however. In ¾ of the patients disease activity is not influenced by pregnancy. Acute flares normally appear during the first trimester or after delivery.[11]

What make IBD particularly challenging is its still unknown cause, its unpredictable

presentations and symptoms, the less than optimal treatments, and a continuous rise in its incidence and prevalence in many areas of the world. This variability and its related uncertainties have long been recognized, and the notion that IBD may actually represent a constellation of diseases or syndromes rather than the single entities of CD and UC has been taken into consideration at least since the 1970s. Over the years many theories have been proposed to explain IBD pathogenesis, ranging from infectious to psychosomatic, social, metabolic, vascular, genetic, allergic, autoimmune and immune-mediated.[12]

II. *Crohn's Disease*

CD shows segmental, non-continuous, subacute or chronic inflammation that can affect the entire digestive tract from the mouth to the anus, with a predilection for the distal ileum. Pathological and histological features include segmental transmural inflammation (affect all wall layers, although the deeper layers may be more severely affected) with microerosions, fissures, ulcerations, granulomas, infiltrations and dilated lymph vessels. Typical symptoms include cramping abdominal pain, diarrhea, rectal bleeding, fever and weight loss.

Clinical symptoms do not necessarily correspond with patient's endoscopic or histological findings or with individual laboratory parameters. For this reason, indices have been developed to assess disease activity and guide treatment. The most widely used is the Crohn's Disease Activity Index (CDAI),[13] albeit new scores are being developed, such as the Lemann score; this instrument should take into account damage location, severity, extent, progression, and reversibility, as measured by diagnostic imaging modalities and the history of surgical resection.[14]

CD is associated with various complications that often affect clinical management decisions. CD colonic complications include strictures, fistulas and abscess.[15]

III. *Ulcerative Colitis*

UC is a recurrent inflammatory disease of the colon and rectum characterized by ulcerations. Disease starts in the rectum and spreads to a varying extent in a proximal

(oral) direction. Pathological and histological features include diffuse circular inflammation affecting only the mucosal membrane with ulcerations, crypt abscesses, infiltrates and reduced numbers of goblet cells. Patients suffer from bloody diarrhea, cramping pain, anorexia and weight loss. The terminal ileum is rarely involved (backwash ileitis). UC variants are ulcerative proctitis (rectum), proctosigmoiditis (rectum and sigmoid colon), left sided colitis (rectum, sigmoid and descending colon), acute self-limited colitis (due to infection or drug, acute rather than chronic symptoms, usually heals completely), pancolitis (entire colon) and fulminant colitis (entire colon).[16, 17]

When a specific diagnosis is necessary it may be important and necessary to look at the colonic mucosa, both grossly and microscopically. Mucosal biopsy can be very helpful in distinguishing among different IBD forms, especially UC, because crypt distortion, which is a hallmark.[18] In UC, the inflammation is continuous and affects the entire circumference of the mucosal membrane. The endoscopic and histological findings therefore correlate more closely with disease activity. In the active stage there is a granulocyte inflammation with reduction of the goblet cells. During remission is not expected to observe mucosal lesions: however, often rarification of the crypt architecture and isolated growth of pseudopolyps are observed.[19] Different indices have been designed to assess disease activity and take into account clinical and laboratory parameters and/or endoscopic findings. An example is the Rachmilewitz Index.[20]

UC colonic complications include anorectal lesions and fistulas (uncommon), toxic megacolon, perforation, stricture and massive hemorrhage. The ultimate grade of UC is the fulminant colitis. This colitis can lead to what is known as toxic megacolon, i.e., the dilation of the colon, causing an intraluminal hemorrhage. It is a severe complication associated with considerable risk of requiring immediate surgical intervention. Most perforations are associated with the development of toxic megacolon; however, perforation may occur in UC in the absence of toxic dilation. Strictures are the most common local complication of UC. Goulston and McGovern suggested that the amount of narrowing is caused by contraction of the muscularis mucosa rather than irreversible fibrosis. However, there is always the concern that strictures in UC may represent an area of dysplasia and/or focus of adenocarcinoma.[21]

IV. *Diagnosis, symptoms and complications of IBD*

The natural history of CD and UC is highly variable, but most typically follows a course of relapses and remissions. Generally, symptoms of UC tend to be uniform; most patients complain of abrupt onset passage of blood, diarrhea and weight loss. Each acute relapse typically has similar clinical features. CD shows greater variability between patients in clinical features due to its greater anatomical distribution potentially involving any part of the GI tract, its transmural distribution and its propensity to give raise to complications such as strictures and fistulae. Also, as the disease evolves involving different parts of the GI tract, the clinical features in any one patient may also change through time.[22]

The presence of the following symptoms raises suspicion for an IBD: more than 2 bowel movements per day, water stool, blood or mucus in the stool, diarrhea persisting for more than 4 weeks, cramping abdominal pain, recurrent episode of such symptoms, increased urge to defecate, nocturnal defecation, feeling of incomplete bowel emptying and fever. Nonetheless, there are differential diagnoses for IBD: infections (*Yersinia sp.*, *Campylobacter jejuni*, *Clostridium difficile*, chlamydiae, amoebae, tuberculosis, opportunistic infections in AIDS -acquired immunodeficiency syndrome-), pseudomembranous colitis, eosinophilic gastroenteritis, ischemic colitis, microscopic colitis (collagenous and lymphocytic colitis), diversion colitis, graft-versus-host disease, radiation colitis, drug-induced colitis, endemic sprue, Behçet's disease (*immune-mediated small-vessel systemic vasculitis that often presents with mucous membrane ulceration and ocular problems*) and irritable bowel syndrome.[23]

Extraintestinal manifestations and complications of IBD include musculoskeletal, neurological, mucocutaneous, mucosal, ocular, hepatopancreatobiliary, pulmonary (recognized less frequently than other manifestations), renal and urological manifestations, thromboembolic events, anaemia, osteopenia and osteoporosis.[24-28] Extraintestinal symptoms of IBD occur in about 60% of patients. It is important to acquire knowledge on these extraintestinal manifestations of CD and UC to start the respective treatment early. Perhaps even more important, these extraintestinal symptoms can be the primary manifestation of both disorders. Therefore, they have to be recognized as extraintestinal manifestations to adequately treat the intestinal disease. Moreover,

patients who have one extraintestinal manifestation seem to be at increased risk of developing further extraintestinal symptoms.[24] The symptoms can occur as initial symptoms, especially joint pain and cutaneous manifestations, such as erythema nodosum or pyoderma gangrenosum.[29] Rheumatological complications have focused on peripheral arthritis and spondylitis, and less is known about soft tissue rheumatism, specifically the fibromyalgia syndrome. Nevertheless, Buskilda *et al.* proved that this syndrome is common in IBD, particularly CD.[30]

Up to 50% of patients with IBD are affected by hepatopancreatobiliary manifestations during the course of their disease course.[31] Occasionally, diagnosis of primary sclerosing cholangitis (PSC) in chronic liver disease leads to the diagnosis of UC.[32, 33] PSC is a chronic inflammatory cholangiopathy that results in fibrotic structuring of the intrahepatic and extrahepatic bile ducts. The majority (>80%) of PSC patients in the west have concomitant IBD at some point in their lifetime, whereas 2.5-7.5% of patients with IBD develop PSC. Although the pattern of intestinal inflammatory activity has been likened to that of UC (as opposed to CD), the clinical phenotype of IBD in PSC is unique, being characterized by rectal sparing, backwash ileitis and either pancolonic involvement or predominantly right-sided disease. PSC patients with IBD have reduced survival and an increased risk of cholangiocarcinoma when compared with PSC patients without IBD.[34] There is rare (<1%) incidence of pancreatitis, vasculitis, pericarditis, myocarditis, autoimmune hemolytic anemia and thrombotic diseases.[19]

In most patients, extraintestinal complications are caused by a lack or (rarely) an excess of exogenous and endogenous substances in the body as a result of the disturbed bowel function. Of special importance are vitamins (B_6, B_{12}, folate, D), trace elements (iron), proteins, bile acids, oxalic acid and water. Deficiencies can lead to the following symptoms: anemia, osteopenia, osteoporosis and osteomalacia, sensory disturbances (zinc and B_{12} deficiencies) and peripheral polyneuropathy. Changes in absorption can cause gallstones (impaired bile acid absorption) and kidney stones.[19]

IBD predisposes to the development of colorectal carcinomas. In UC the highest risk occurs in those patients with extensive colitis for more than 10 years. This risk is

accentuated if the disease began at an early age, and appears to be less if the inflammation is confined to the left side of the colon. Crohn's colitis, while initially thought to have a low risk is now recognized to have a risk comparable to that of UC.[35, 36] Numerous extraintestinal cancers such as hepatobiliary and pancreatic malignancies are also noted to be more prevalent in IBD patients particularly with co-existing PSC. Somewhat ironically, however, the medications used to control the inflammation in IBD may also be responsible for the development of other cancers.[37]

It is important to highlight that not all the complications are strictly physicals. IBD patients show a highly variable spectrum of psychosocial problems. The most prevalent problems reported addressed sleep disturbances, sexual impairments, coping, mood disorders, high stress, anxiety and depression.[38-42]

V. *Etiopathology of IBD*

Presently there is a general consensus among basic IBD investigators that both CD and UC are the result of the combined effects of four basic components: global changes in the environment, the input of multiple genetic variations, alterations in the intestinal microbiota and aberrations of innate and adaptive immune responses (**fig. 1**).

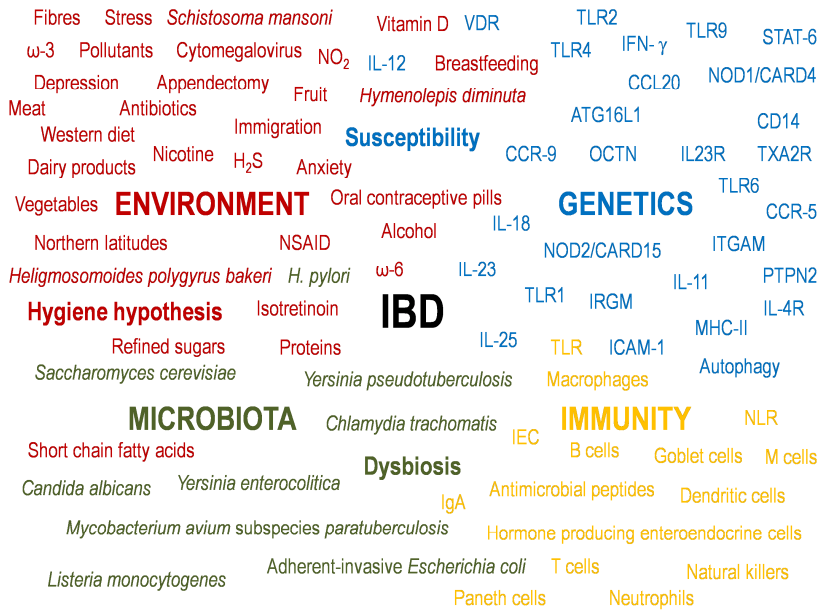


Figure 1. Etiopathology of IBD.

There is also agreement on the conclusion that none of these four components can by itself trigger or maintain intestinal inflammation.[12]

1. Environment.

i. Smoking. Smoking has long been known to affect IBD. A meta-analysis implicated smoking as a risk factor for CD and a protective factor for UC.[43] Compared to the general population, CD subjects are significantly more likely to be smokers and UC subjects to be ex-smokers, respectively.[44] In patients with CD, smoking worsens prognosis by increasing the frequency of disease flares and the need for surgery, in addition to increasing postoperative recurrence. Smoking cessation is a key therapeutic strategy in patients with CD. In contrast, there is convincing evidence that smoking cigarettes may improve the disease severity or have a "protective" effect in some patients with UC.[45]

Smoking may influence the development of IBD through nicotinic acetylcholine receptors, which are present in mucosal epithelial cells of the bowel and on T lymphocytes (T cells).[46] Clinical trials of nicotine replacement in UC have yielded modest yet inconsistent results; thus, nicotine alone may not be the sole component of smoking that influences IBD. Other proposed mechanisms are that chemicals in smoking modulate cellular immunity, alter cytokine levels, modify colonic mucus production, and predispose to the development of microvascular thrombi or altered blood flow.[6]

ii. Hygiene and microorganisms. The hygiene hypothesis states that a lack of early childhood exposure to infectious agents, symbiotic microorganisms (e.g., gut flora or probiotics) and parasites increases susceptibility to allergic diseases by suppressing natural development of the immune system. It is hypothesized that the T-helper lymphocyte (Th) type 1 (Th1) polarized response is not induced early in life, leaving the body more susceptible to developing Th2-induced disease.[47] The hygiene hypothesis posits that as western societies "cleaned up" they facilitated enhanced infant survival rates and the reduction of potentially lethal infectious diseases, but that a variety of immune-mediated diseases emerged subsequently/in parallel.[48]

- ***Helicobacter pylori.*** *H. pylori*, a pathogen involved in peptic ulcer disease, is a

bacterium that is associated with larger family size, multiple siblings and poor sanitary conditions. A meta-analysis of 23 studies reported that CD and UC are negatively associated with *H. pilory. H. pylori* increases the expression of T cell regulatory genes, such as forkhead box p3 (*FOXP3*), resulting in an anti-inflammatory response.[49]

- **Helminths.** Helminths are complex multicellular organisms adapted to live in immune competent hosts. Helminths have developed the ability to induce immune host regulatory cells that suppress inflammation; thus, colonization may be associated with a reduced prevalence of IBD.[50] Exposure to the roundworm *Heligmosomoides polygyrus bakeri*, fluke *Schistosoma mansoni*, or tapeworm *Hymenolepis diminuta* can protect animals from trinitrobenzene sulfonic acid (TNBS)-induced colitis. Helminths also protect mice lacking interleukin (IL)-10 from chronic colitis. Colonization with *H. polygyrus bakeri* inhibits development of colitis and will reverse established colitis in IL-10 deficient mice.[51] Animals with helminths show decreased pro-inflammatory Th1 and Th17 responses, increased anti-inflammatory regulatory T cells (Treg) and innate regulatory cell responses, and protection from intestinal inflammation. Helminth exposure may provide a novel therapeutic approach to treat IBD.[51]

- ***Mycobacterium avium* subspecies *paratuberculosis* (MAP)**. Originally identified in the mid-1980s, this *Mycobacterium* has been the center of much debate for almost three decades, with some reports supporting and others denying its possible etiological role in CD. MAP is detectable in the intestinal tissue and blood of a subset of CD patients, and anti-mycobacterial drugs have been found, in some cases, to improve disease.[6] The last serious study that attempted to provide a definitive answer to this possibility has been a large clinical trial in which patients with CD were given a combination of three anti-mycobacterial antibiotics or placebo, and followed up for the evaluation of remission and clinical activity. At the end of 2 years no evidence of sustained benefit was detected, strongly suggesting that the elimination of MAP in CD does not significantly affect the clinical course, a response that should be interpreted as denying an etiological role of MAP in this form of IBD. This study, however, has not completely settled the issue as some investigators and patient groups still believe in the causative role of MAP in CD.[12]

- **Adherent-invasive *Escherichia coli* (AIEC).** Boudeau *et al.* described the

isolation of *E. coli* strains from the ileal mucosa of CD patients with the capacity of adhere to, and invade intestinal epithelial cells (IEC) *in vitro*, and to survive and replicate intracellularly within macrophages without inducing host cell death.[52, 53] The same group later showed that AIEC are specifically associated with the ileal mucosa in CD, while they are uncommon in control or UC tissues. It is not entirely clear whether these AIEC are pathogens or commensals, but an argument against an etiological role in CD is that treatment with antibiotics that are effective against coliforms fails to cure CD patients.[12]

• **Other microorganisms.** Over the past decades, various pathogenic agents have been implicated in the development of IBD, including *Listeria monocytogenes*, *Candida albicans*, *Chlamydia trachomatis*, *Saccharomyces cerevisiae*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis,* cytomegalovirus and measles virus. To date, however, these data have not been confirmed and antibiotics have not been effective in larger studies.[23, 54, 55]

• **Dysbiosis.** Tamboli *et al.* introduced the term dysbiosis to suggest that the equilibrium between protective and harmful bacteria in healthy people is broken in IBD, thus resulting in chronic intestinal inflammation.[56] Is principally associated with CD.[57] Although the impressive list of documented microbial alterations in IBD patients was recently reviewed,[58] the original question remains if dysbiosis is just a secondary phenomenon in IBD or truly causal.

iii. Medications.

• **Non-steroidal anti-inflammatory drugs (NSAID).** A prospective cohort study of more than 76,000 women identified an increased absolute risk of both UC and CD in women who used NSAID at least 15 days per month.[59] NSAID can cause damage to the intestinal mucosa of the stomach, small bowel and colon. NSAID increase intestinal permeability by inhibiting cyclo-oxygenase (COX), which in turn reduces prostaglandin production. Reduced prostaglandin production has been implicated in IBD through the inhibition of tumor necrosis factor (TNF) and the induction of anti-inflammatory cytokines such as IL-10.[60]

• **Oral contraceptive pills (OCP).** A meta-analysis suggested that the use of OCP was positively associated with UC and CD.[61] A prospective cohort study found that

women who continued taking OCP were at a threefold increase risk of developing a relapse of CD; this effect was amplified among women who were prescribed OCP and smoked.[62] The mechanism of action by which OCP increase the risk of IBD is unknown, but the effect may be promoted by estrogen and moderated by progesterone.[63] Alternatively, estrogen may play a pathogenic role in IBD through a process of multifocal GI infarction due to its thrombogenic potential.[61]

- **Isotretinoin.** Is a retinoid (i.e., a vitamin A derivative) used to regulate epithelial cell growth and to treat severe acne and certain cancers. Retinoids are involved in the regulation of the intestinal mucosal immune response. The United States Food and Drug Administration's Medwatch program reported several cases of IBD following the prescription of isotretinoin as an acne medication.[64] However, results of case-control studies have been inconsistent.[6] There may be an association between certain acne treatments and IBD, but there may simply be an association between severe acne and IBD, two inflammatory entities occurring in the same age group.[65]

- **Antibiotics.** Colonization of the gut begins after birth through the introduction of bacteria by infant diet, hygiene level and medication exposure.[66] Antibiotic exposure in childhood is hypothesized to disrupt the development of the body's natural tolerance to enteric bacteria, which may lead to IBD. Several studies have demonstrated a positive association between antibiotic use and the development of IBD.[67]

iv. Appendectomy. Appendectomy is negatively associated with the development of UC, particularly among children experiencing appendicitis before 10 years of age.[6, 68] The main hypothesis that has been proposed to explain the probable protective effect of appendectomy in development and disease severity of UC is that the excision of the appendix may have an immune modulating effect that protects against the development of UC.[69] Some studies in animals have supported this. Cheluvappa *et al.*, utilizing the first murine appendicitis model, have shown that, although appendicitis alone or appendectomy alone were not protective, appendicitis followed by appendectomy provided significant protection against subsequent experimental colitis.[70] The mechanism by which appendectomy protects against UC is not clear; however, the appendix may have a physiological role in antigen sampling and regulating the immunological response

to intestinal flora. Furthermore, IBD is characterized by a shift in the balance toward a Th1/Th17 cell-mediated inflammatory response in CD and a Th2 response in UC (see **section VII: physiopathology of IBD**). A study by Andersson and colleagues suggests that appendicitis is mediated by Th1 cells, which may explain the inverse associations between appendicitis and UC.[71]

In contrast, the relationship between appendectomy and CD is less clear.[6, 68] A meta-analysis demonstrated a significant risk of CD following an appendectomy. However, a considerable proportion of the risk of developing CD was observed within the first year following an appendectomy, a time when incipient CD may lead to undue appendectomies. After 5 years, the risk of CD was no longer significant, suggesting that a biological association between appendectomy and the development of CD is less likely.[72]

v. Nutrition. A Western diet may be associated with an increased risk of IBD.[44] The role of processing and cooking should be taken into account. Dietary intake in Western populations has dramatically shifted to a high-fat, high carbohydrate diet over the past-half century, nearly in concordance with the development of the disease.[73] A high consumption of simple carbohydrates was linked with CD development. Further analyses specified that the key factor was the industrial way of preparing and refining sugars.[74] Intake of Western high fat-high carbohydrate diet promotes expansion of pathobionts in the GI tract, resulting in decreased abundance of commensals. Metabolism of the emerging pathobionts leads to increased host exposure to detrimental bacterial products (i.e., H_2S) and reduced exposure to beneficial products, such as short chain fatty acids (SCFA). This results in increased immunogenic antigen exposure, prompting inflammatory cytokine production of both antigen-presenting cells (APC) (i.e., IL-12p40) and T cells (i.e., interferon gamma (IFN-γ)) that increase intestinal inflammation.[75]

High-fibre intake was associated with a significant risk reduction of CD but not UC.[76] Fibres, via their fermentation end-products (SCFA), show clear anti-inflammatory properties, but are also capable of reducing intestinal permeability and bacterial translocation; e.g., soluble plant fibres, like green banana and broccoli, inhibit *E. coli* translocation across M cells (microfold cells), being increased in the presence of low

concentrations of an emulsifier commonly used in processed foods.[53] The fact that patients may avoid a high-fibre diet in case of diarrhea, or CD patients with underlying strictures may avoid fibre to minimize symptoms, might account in a large part for the conflicting results observed on fibre-rich food and IBD.[74]

Saturated and unsaturated fats may play a role in the inflammatory response through modulation of Toll-like receptors (TLR) in macrophages.[77] Various studies have associated an increased intake of ω-6 polyunsaturated fatty acids (PUFA) (i.e., beef, pork, corn, sunflower oils and polyunsaturated margarines) and a lower intake of ω-3 PUFA (fish) with increased incidence of both CD and UC.[78] Long-chain PUFA are precursors of eicosanoids for the synthesis of prostaglandins and leukotrienes important in inflammation, as well as components of immune cell membranes.[79] Several case-control studies revealed an increased consumption of ω-6 PUFA linoleic acid before diagnosis of UC.[80, 81] Linoleic acid can be metabolized to arachidonic acid. Pro-inflammatory in nature, eicosanoid metabolites of arachidonic acid are increased in the mucosa of UC patients.[80]

Dairy products have been suggested as a risk factor, since IBD is more common in “dairy-based” countries, than in “soy-based” ones.[82]

There are some general trends suggesting that high-refined sugar consumption, a high-caloric diet, regular intake of processed fat, diets low in fish (ω-3 PUFA), fruit and dietary fibre are associated with IBD development,[83] whereas high fruit, vegetables, and dietary fibre consumption appear to decrease the risk. A clearer association can be seen with protein intake since UC patients have a higher relapse risk when consuming lots of meat, protein and alcohol.[74] The mechanism by which fruits and vegetables confer protection may be related to their ability to modify enzymes involved in clearing reactive oxygen species.[84]

Breastfeeding, which protects infants against many other immune-mediated diseases, may also reduce the risk of developing IBD as it has been reported by several meta-analyses; however, different studies have produced conflicting evidence in which breastfeeding was found to be a significant risk factor for pediatric CD.[68] Breastfeeding

helps develop oral tolerance to microflora and food antigens, which may prevent IBD.[85] Lactoferrin, which is present in breast milk, but is absent in formula, may have anti-inflammatory properties, and may have antibacterial and antiviral effects.[86]

vi. Geographical risk factors. The prevalence of IBD has been considered in the context of a north-south gradient, with a higher prevalence reported in countries with northern latitudes.[87] One potential explanation is differences in exposure to ultraviolet light, resulting in relative vitamin D deficiencies in northern countries.[6] Activated vitamin D (i.e., calcitriol) may modulate the innate immune system by downregulating a Th1 pro-inflammatory response.[88]

Interestingly, several cases of immigrant families affected by IBD have been reported and seem to be of exceptional interest towards a better understanding of disease etiopathogenesis. The first case of CD in a family of immigrants with three offspring was described by Katsanos *et al.*[89] A family with three children, one 22 year-old male and two 18-year-old twin females emigrated from southern Albania to northwest Greece. The whole family lived in the same house and had no previous history of bowel or other chronic diseases. After 9 years, CD was diagnosed in the boy. One of the twins was diagnosed with ileal CD and six months afterwards, the second twin underwent emergency appendectomy due to acute appendicitis; four months later she was diagnosed with ileal CD. Genetically predisposed individuals seem to be vulnerable to lifestyle modification.

vii. Ambient air pollution. Air pollution directly affects pulmonary diseases and has also been associated with a variety of non-pulmonary diseases including myocardial infarction, appendicitis and rheumatoid arthritis. Air pollution-mediated inflammation has been implicated as the cause of several adverse health effects. Similar pro-inflammatory processes occur in IBD. Children and young adults living in areas with high SO_2 concentrations are at an increased risk of developing UC; moreover, the same population living in areas of high NO_2 concentrations is at an increased risk for CD.[90]

In a study conducted by Ananthakrishnan *et al.*, total air emissions of criteria pollutants

appear to be associated with hospitalizations for IBD in adults.[91]

The mechanism by which air pollution influences the development of IBD remains unclear. Exposure of the bowel to air pollutants occurs via mucociliary clearance of particulate matter from the lungs as well as ingestion via food and water sources. Gaseous pollutants may also induce systemic effects. Plausible mechanisms mediating the effects of air pollutants on the bowel could include direct effects on epithelial cells, systemic inflammation and immune activation, and modulation of the intestinal microbiota.[92]

viii. Stress. Our digestive tract has an autonomous functioning but also has a bidirectional relation with our brain known as brain-gut interactions. This communication is mediated by the autonomous nervous system and the circumventricular organs located outside the blood-brain barrier.[93] A dysfunction of these brain-gut interactions, favored by psychological stress, is most likely involved in the etiology and pathophysiology of digestive diseases such as IBD due to the chronic, relapsing and remitting nature of this disease.[68, 94, 95]

It is difficult to define stress. Activation of the stress response is highly dependent on the patient's perception of stress, depending of coping strategies, life experience and personal resources.[96] Thornton and Andersen suggested that personality traits can modulate the relationship between stress and the immunological reaction to it (e.g., neuroticism, perfectionism and alexithymia).[97]

There are different mechanisms by which the course of IBD can be influenced by stress: (1) Non-specific effects; are mediated by substance P, vasoactive intestinal protein, several neuropeptides, neurotransmitters and hormones. Stress stimulates the secretion of corticotropin-releasing factor (CRF). Endogenous CRF mediates the stress-induced inhibition of the upper GI tract motility and stimulation of colonic motility. (2) Immunological mechanisms; physiologic stress leads to a signaling cascade through the hypothalamus-pituitary-adrenal axis and the autonomous nervous system which culminates in immune responses and inflammation. Chronic or acute stress can alter

profiles of cytokines (e.g., IL-1β, IL6, IL10, IL4, and TNF-α) or of hormones such as cortisol. (3) Intestinal permeability; stress can also lead to increased intestinal permeability and stimulate secretion of ions, water, mucus and even immunoglobulin A (IgA), as a result of alterations in the cholinergic nervous system and mucosal mast cell function. This increased permeability in turn reduces mucosal barrier function and alters bacteria-host interaction. (4) Indirect effects; stress can also indirectly affect clinical course of IBD. These indirect effects are exerted through behaviors known to promote relapse and include poor medication adherence and smoking.[96, 98]

We have to keep in mind that stress is not a preferential IBD initiator, but it is thought to be one of the triggers of relapses and symptom exacerbation in patients with IBD.[41, 42, 98] IBD is associated with anxiety and depression, both after diagnosis and even for some years before diagnosis.[48] A peculiar study conducted by Shiga *et al.* examined the rate of relapse in IBD patients before and after the Great East Japan Earthquake (March 11th, 2011). Among the UC patients, disease was active in 167 patients and inactive in 379 patients before the earthquake. A total of 86 patients relapsed; the relapse rate was about twice that of the corresponding period in the previous year. The relapse rate did not differ in CD. Multivariate analyses revealed that UC, changes in dietary oral intake and anxiety about family finances were associated with the relapse.[99]

2. Genetic links.

In IBD, numerous genetic linkage studies have implicated loci in some chromosomal regions like holders of susceptibility genes for IBD. Genome-Wide Association Studies (GWAS) and subsequent Genome Scan Meta-Analysis of CD and UC as separate phenotypes implicated previously unsuspected mechanisms, such as autophagy, in pathogenesis and showed that some IBD loci are shared with other inflammatory diseases. The International Inflammatory Bowel Disease Genetics Consortium declare that nowadays the number of susceptibility loci/genes for IBD has been increased to a total of 163 that meet genome-wide significance thresholds, more than for any other human disease.[100, 101] 30 are classified as CD-specific, 23 are classified as UC-specific and 110/163 loci with both disease phenotypes; 50 of these have an indistinguishable effect size in UC and CD, while 60 show evidence of heterogeneous effects.[100, 102, 103]

Major IBD susceptibility pathways implicated through recent GWAS include the innate immune response (*NOD2*), the more specific acquired T cell response (*IL23R*) and autophagy (*ATG16L1*, *IRGM*) (**fig. 2**).[104] Differences in the genetic predisposition to UC and CD have also been recognized from earlier genetic studies. Mutations in the gene encoding the Nod2 protein were the first genetic associations reported specifically for CD. Mutations in *NOD2* result in defects in bacterial antigen processing.[105, 106] Other proteins that are important in the recognition and processing of bacterial components in the gut, such as the autophagy genes *ATG16L1* and *IRGM*, also have genetic variants associated with CD but not UC.[107] *IL23R* variants may exert a rather generic effect on chronic intestinal inflammation, although the effect size in UC does appear to be smaller than in CD.[108]

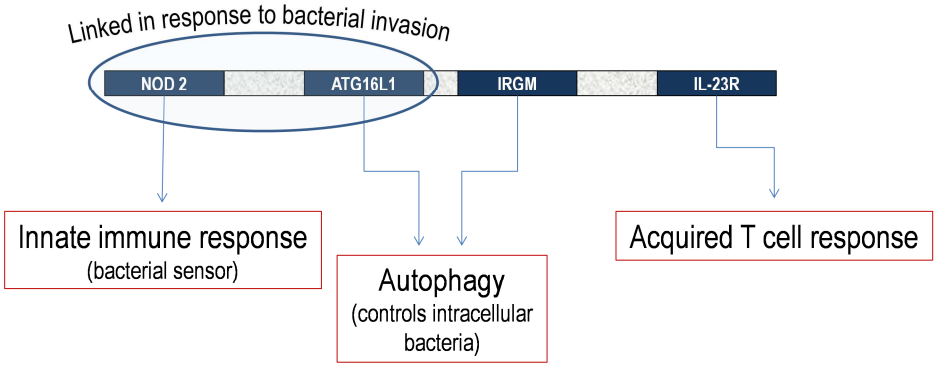


Figure 2. Major IBD susceptibility pathways.

i. Innate immune response: *NOD2* (nucleotide-binding oligomerization domain-containing 2). A mutation of *NOD2* in the IBD1 locus of the chromosome 16q21 (NCBI gene database)/16q12 (literature) is related with CD.[105, 106] *NOD2* encodes an intracellular receptor (Nod2), also named as caspase recruitment domain family (CARD), member 15 (CARD15), which plays an important role in the immune system (see **section VI: immunity in the intestinal mucosa**). Nod-deficient (Nod1- or Nod2-deficient) cells transduced with *NOD* constructs manifest autophagy upon exposure to Nod ligands; however, also occurs with lipopolysaccharide (LPS) stimulation in Nod2-deficient cells, indicating that Nod2 deficiency can be compensated by other innate responses.[109]

Biswas *et al.* have shown that Nod2 is an important regulator of crypt antimicrobial function.[110] The Nod2 ligand, muramyl dipeptide (MDP), was found to effectively induce the bactericidal activity of crypts. Nod2-deficient crypts stimulated with MDP or a non-specific inducer of secretion, carbamylcholine, could not induce effective bacterial-killing activity. In the intestines of Nod2-deficient mice or CD patients with *NOD2* mutations, Paneth cells are unable to control microbiota due to impaired bactericidal activity.[111] In addition, the genetic linkage of certain *NOD2*/*CARD15* mutations to barrier defects suggests that immune activation may be responsible for the early barrier defects observed in healthy relatives and in patients before disease reactivation.[112, 113] It is therefore likely that *NOD2* mutations in CD may increase disease susceptibility by altering interactions between ileal microbiota and mucosal immunity.[114]

ii. **Acquired T cell response: *IL23R***. Variations in the IL-23 receptor (*IL23R*) gen in the chromosome 1p31.3 have been unequivocally associated with CD and UC.[115] IL-23, along with IL-12, play his role determining whether the differentiation of T naïve cells goes to Th1 cells (IL-12) or Th17 cells (IL-23). Th17 cells are particularly interesting for his role in the organ-specific inflammation.[116] Functional analysis showed that the systemic inflammatory response was driven by IL-12 and not IL-23, whereas local intestinal inflammation required the presence of IL-23 and was independent of IL-12.[117] IL-23 is induced by pathogen-recognition receptor (PRR, see **section VI: immunity in the intestinal mucosa**) stimulation and is constitutively expressed in a small population of dendritic cells present in the *lamina propria* of the terminal ileum, although in patients with CD, CD14+ intestinal macrophages have also been reported to secrete large amounts of IL-23.[113] The IL-23/Th17 cell pathway defends against microbial infection by intestinal and other pathogens. However, IL-23 and the cytokines produced upon activation of Th17 cells contribute to tissue inflammation in general, and to IBD specifically, demonstrated in several studies of patients and mice.[118] These cytokines must therefore be carefully regulated during mucosal responses. Recently, Gallagher and colleagues have provided a potential functional explanation for *IL23R* mutation.[119] The rs11209026 mutation (G1142A/p.R31Q) resides in exon 9 which encodes a transmembrane region of IL-23R protein. The protective A allele causes alternative splicing, moves the stop codon and

results in removal of the transmembrane domain from the protein. The resulting soluble IL-23R protein (termed delta-9) binds IL-23, taking it out of circulation and decreasing Th17 cell differentiation.

iii. Autophagy: *IRGM* and *ATG16L1*. Since 2007, GWAS have found an unequivocally association between CD and this two autophagy-related genes. Results from several studies indicate that the defect of mammalian autophagy related 16-like 1 (*ATG16L1*) and immunity-related guanosine triphosphatase family M (*IRGM*) genes leads to increased susceptibility for CD. Autophagy is a highly conserved catabolic pathway in eukaryotic cells that plays a key role in maintaining cellular energy homeostasis.[120-122] Along with its metabolic function, autophagy is an important mechanism for innate immunity against invading bacteria, parasites and viruses.[123] This process also participates in the processing of antigens for antigen presentation in immune responses. Hence, it also has a role in immunological responses and host defense.[124]

IRGM gene is located in the chromosome 5q33.1 and codifies a 181 amino acids protein belonging to p47 kDa immunity-related guanosine triphosphatase (IRG) family. Sequence variants in the autophagy gene *IRGM* contribute to CD susceptibility.[125] Others have shown that mice deficient in one member of the family, Irgm1 (also known as LRG47) are highly susceptible to a wide variety of different intracellular bacterial and protozoan infections. Irgm1 plays a distinct role in the regulation of autophagy in macrophages and CD4+ T cells. During Th1 response, IFN-γ produced by activated natural killers (NK) and T cells induces Irgm1 expression to maximize host control of intracellular pathogens. While promoting IFN-γ-dependent autophagy in macrophages to enhance clearance of intracellular pathogens, Irgm1 inhibits autophagic death of activated CD4+ T cells to amplify Th1 effector populations.[126]

ATG16L1 gene is located in the chromosome 2q37.1 and codifies the Atg16L1 protein, an essential kinase for the initiation of the autophagy. Hampe and colleagues identified a susceptibility variant for CD in *ATG16L1*.[127] Atg16L1 forms an 800 kDa complex with Atg12 and Atg5. The Atg12-Atg5-Atg16L1 complex binds to membrane precursors causing its elongation to form a periferic autophagosoma, which encapsulates

cytoplasmic components. Then binds to a lysosome to form an autophagolysosome and proceed to the degradation of these components. Saitoh *et al.* have proposed that the coiled-coil domain of Atg16L1 also defines the site where LC3, microtubule-associated light chain 3, (homologue of yeast Atg8) is conjugated to phosphatidylethanolamine, an essential process for autophagy, by recruitment of an Atg3-LC3 intermediate to a source membrane of an autophagosome.[128] Following stimulation with LPS, a ligand for TLR4, Atg16L1-deficient macrophages produce high amounts of the inflammatory cytokines IL-1β and IL-18, indicating the importance of Atg16L1 in the suppression of intestinal inflammation.

3. Intestinal microbiota.

The GI tract is exposed to a wide variety of antigens: dietary components and, particularly, microorganism, such as bacteria, viruses and fungi. While the small bowel is normally not exposed to extensive colonization, in the colon, up to 60% of the stool mass consists of bacteria, with each gram of stool containing 10^{10} to 10^{12} bacteria;[129] about 1000 species, the vast majority belonging to two bacterial phylotypes, *Firmicutes spp.* and *Bacteroidetes spp*.[130]

In more recent years, it was postulated that the normal local flora, which lives in symbiosis with the GI tract, may play an important role in the development and perpetuation of IBD. This is supported by the observation that surgical exclusion of small bowel loops, resulting in loss of contact with bowel contents, can lead to disappearance of inflammatory changes in these bowel segments. Once the continuity of the bowel is re-established, recurrence of inflammatory changes may occur.[23] Mice raised in a germ-free (GF) environment or deficient in PRR pathways are frequently protected from colitis, but rapidly emerges when they are reconstituted with bacteria that are considered normal constituents of luminal flora, indicating that intestinal inflammation requires intestinal microbes.[118] In some instances, it has been possible to induce colitis in a susceptible murine strain with a single species of normal bacteria, for example, *Bacteroides vulgatus* in the IL-10 deficient mouse.[131]

Circumstantial evidence for this inference is provided by the observed therapeutic

benefits of antibiotic treatment in, at least, subsets of IBD patients, and recent findings suggesting that so-called “healthy bacteria” or probiotic combinations can ameliorate IBD.[58, 132] Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”.[133] The regular consumption of lactic acid bacteria in fermented dairy products, such as yogurt, was associated with enhanced health and longevity. Most probiotics belong to lactic acid bacteria, but new species and genera are being assessed for future use. Some other well-known probiotics are *Bifidobacterium spp.*, one strain of the Gram-negative bacterium *Escherichia coli* Nissle 1917 and the yeast *Saccharomyces boulardii*.[134]

Many studies have observed imbalances or dysbiosis in the GI microbiomes of IBD patients; in both CD and UC patients, there is decreased biodiversity and, in particular, a lower abundance of, and a reduced complexity in the *Bacteroidetes* and *Firmicutes* phyla.[135] In contrast to the general microbial dysbiosis theory, some researchers have suggested the involvement of specific taxa, for example the *Enterobacteriaceae* have been associated with the microbiota of patients with UC, and AIEC have been identified in the ileal mucosa of patients with CD. In addition, recent work analyzing intestinal biopsies and stool samples from patients with IBD and healthy subjects documented an association of the IBD disease status with alterations in the abundances of *Enterobacteriaceae, Ruminococcaceae* and *Leuconostocaceae*; while at genus level, *Clostridium* levels increased whereas butyrate producer *Roseburia* and succinate producer *Phascolarctobacterium* were significantly reduced in both UC and CD conditions.[136]

The GI microbiome influences dietary energy extraction, immune system development, vitamin production and drug metabolism, although most molecular and metabolic functions of the bacteria of the GI microbiome are yet uncharacterized. Morgan *et al.* identified 9 changes in bacterial clades associated with both CD and UC and 21 statistically significant differences in functional pathways and metabolic modules; microbial function was more consistently perturbed than composition. This allowed the identification of unique functional perturbations in the microbiomes of IBD patients: major shifts in oxidative stress pathways, as well as decreased carbohydrate metabolism and

amino acid biosynthesis in favor of nutrient transport and uptake. The microbiome of ileal CD was notable for increases in virulence and secretion pathways. This underscores the fact that phylogenetically diverse changes in the composition of the GI microbiome can be functionally coordinated and lead to major modifications in the metabolic potential of the microbiota.[135] It is now clear that the intestinal microbiota profoundly influences host metabolic and immune pathways and participates in human health and disease.[137]

4. Immune response.

The small intestine has an enormous surface area that is continuously exposed to dietary and microbial antigens. This antigens need to be tolerated by the innate and adaptive immune systems to maintain homeostasis, but pathogens must also be prevented from harming the host. Some of the mechanisms to achieve this balance include the existence of an efficient colonic columnic epithelial barrier, the expression of innate immune receptors, the production of antimicrobial peptides (e.g., production of α-defensins by the Paneth cells), secretion of IgA, autophagy of intracellular bacteria, constant sentinel-like surveillance by $CD16^{low}$ monocytes and efficient recruitment of granulocytes to areas in which barrier integrity has been compromised. All together mediate protection of the body against the microbiological onslaught on the mucosal surface. Unfortunately, in some cases the innate immune system's attempt to protect the host fails and chronic inflammation or other disorders occur.[138, 139] Each patient has his own individual disease-triggering antigen pattern.

VI. *Immunity in the intestinal mucosa*

The intestinal mucosa is a physical barrier that separates the lumen, which is in contact with the outside world, from the internal medium (**fig. 3**). As in the rest of the body, the intestinal mucosa is provided with innate and adaptive immune responses, but they have specific characteristics. One of them is that the immune response is mediated by both cells in the intestinal epithelium and the *lamina propria*, i.e., the layer located immediately underneath the epithelium. The first physical barrier that intestinal bacteria and food antigens encounter on the mucosal surface is represented by the mucous layer that covers the intestinal epithelium.[140] Mucous is organized in an inner firm layer and an outer loose layer that are produced by polymerization of gel-forming mucins, which are

secreted by goblet cells and expand in the lumen due to their capacity to bind water. The resulting mucin net is firm and dense in the inner layer, which is usually sterile, while the outer mucous layer appears to be more permeable and it is inhabited by commensal bacteria that find important nutrients in the mucin glycans.[141]

In contact with the inner mucous layer there is the intestinal epithelium that forms the second line of defense to bacterial invasion. The intestinal epithelium is a monolayer composed of four different cell types of epithelial lineage: enterocytes, the already mentioned goblet cells, hormone producing enteroendocrine cells and Paneth cells. Bacteria, viruses and fungi are efficiently restricted to the lumen by this barrier, and its efficiency is enhanced by the addition of functional and immune factors. Intestinal motility, especially in the colon, influences the luminal population mostly by maintaining an appropriate flux, i.e., by facilitating bacterial removal in faeces.[142]

Enterocytes and Paneth cells are the main players in the immune area. The former are majority and are specialized in transport functions. They are sealed by tight junctions. Enterocytes are important players but their role is far from being well defined. They may help shape the immune response in a number of ways, and they can also react directly with bacterial products because of their privileged position.[143] Paneth cells, located at the base of the crypts, produce antimicrobial peptides, thus limiting bacterial presence at the crypt space. These antimicrobial molecules are key mediators of host-microbe interactions, including homeostatic balance with colonizing microbiota and innate immune protection from enteric pathogens. Perhaps more intriguing, Paneth cells secrete factors that help sustain and modulate the epithelial stem and progenitor cells that cohabitate in the crypts and rejuvenate the small intestinal epithelium.[144]

Mucus secretory goblet cells create a microenvironment in close proximity to the epithelial surface that limits bacterial contact both physically and chemically, by acting as decoy ligands for bacterial receptors. The mucus layer is fundamental for the protection of the GI tract. Its anatomical distribution is consistent with the need to protect the epithelial monolayer and create a disconnection between the body and the luminal content. Indeed, the small intestine does not present a well defined mucus layer, as opposed to what

happens in the colon and in the stomach.[145] Muc2 (mucin 2, oligomeric mucus/gel-forming) is the major component of the mucus layer in the small and large intestine and mutations that involve *MUC2* are related with chronic intestinal inflammation as a result of uncontrolled IEC exposure to the commensal bacteria.[146, 147]

Hormone producing enteroendocrine cells comprise about 1% of all epithelial cells in the GI tract. The enteroendocrine system consists of at least 15 different cell types that can be classified based on their main hormonal products and on the ultrastructure of their secretory granules. A given enteroendocrine cell secretes one or more hormone or hormone-like substance, which is released directly into the *lamina propria* and diffuses into the capillaries. These hormones include gastrin, histamine, serotonin, cholecystokinin, somatostatin and glucagon-like peptides (GLP1 and 2). Although enteroendocrine cells are very scarce, they are essential regulators of digestion, gut motility, appetite and metabolism.[148]

In addition, the epithelium overlaying mucosal lymphoid follicles (called Peyer's patches in the small intestine) is composed of specialized M cells which display atrophied transport capacities and instead act as dedicated sampling instruments, passing luminal antigens into the follicle through transcytosis. Oddly enough, intestinal pathogens usually target these cells as point of entry to the mucosa, and this may be also the case for probiotic strains.[149-151] However, it is likely that minute passage of bacteria at these and other points occurs normally to facilitate some degree of host-microbiota contact.[152]

Underlying the intestinal epithelium, dendritic cells and macrophages in the *lamina propria* contribute decisively to the innate immune response. Dendritic cells cytoplasmic extensions are interdigitated among the epithelial cells in order to sample antigens and present them to T cells in the *lamina propria* and the underlying lymphoid follicles. Dendritic cells can also travel to draining lymph nodes to interact with T cells. Interspersed in the intestinal epithelium there are specific T cells (intraepithelial lymphocytes) that together with the Peyer patches/lymphoid follicles, and *lamina propria* T cells and B lymphocytes (B cells) (mainly IgA-producing B cells) form the intestinal adaptive immune system.

The intestinal mucosa maintains a state of so called “physiological inflammation”, i.e., a low level activation of immune cells with infiltration of the *lamina propria* but devoid of clinical symptoms.[153] This is a direct consequence of the presence of bacteria, since it is absent in GF animals.[154] Another key difference here is the epithelial turnover, which is normally quite high (the epithelium is entirely renewed every 5-7 days) and substantially reduced in GF conditions.[142] Each intestinal crypt contains 4-6 stem cells. They are able to form a regenerative crypt containing all cell lineages (enterocytes, goblet cells, Paneth cells and enteroendocrine cells).[155]

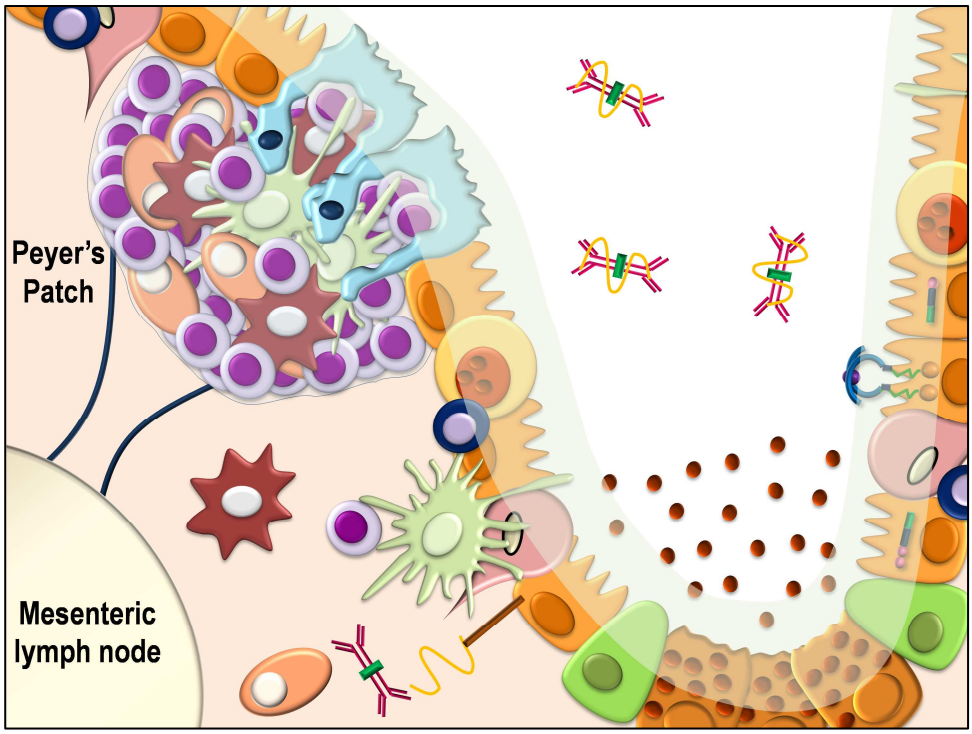


Figure 3. Immunity in the intestinal mucosa.

Innate immunity in the intestine and elsewhere relies on non-specific receptors, as opposed to the specific recognition of antigens used by the adaptive arm of the immune system. These receptors were initially called PRR and bind pathogen-associated molecular patterns (PAMP), i.e., not specific molecules, but types of molecules whose

structure differs substantially from eukaryotic ones.[116, 156] However these are not associated with pathogenicity, and the denomination microbial-associated molecular patterns (MAMP) was suggested instead. The picture has been complicated further by the realization that these receptors can in fact bind internal structures, which are produced especially in the context of tissue damage and inflammation, and therefore are referred to as damage-associated molecular patterns (DAMP). These terms are used exchangeably.

PRR comprise TLR, NOD-like receptors (NLR), the helicase family retinoic inducible gene I (*RIG-I*) and the differentiation associated gene or *MDA5*.[157] These receptors activate signaling cascades that finely tune the production of antimicrobial products and cytokines, depending on the signals delivered by the microbiota.[158] PRR signaling helps to regulate antigen specific adaptive immune response. The best studied PRR are TLR and NLR.

1. Toll-like receptors.

TLR are type I transmembrane proteins expressed by innate immune cells of the intestinal epithelium and the *lamina propria*, either at the cell surface or in endosomes. TLR consist of at least 11 members that recognize not only microbial components, including proteins, lipids and nucleic acids derived from bacteria, viruses and parasites, but also damaged host cell components such as nucleic acids and other "internal" ligands.[142] Cell and molecular localization of TLR together with their ligands are shown in **table 1**.

When examining TLR function in the intestine, one is confronted with the fact that innate immune cells, including enterocytes, express these receptors and are obviously exposed to an endless supply of ligands, yet no inflammatory response develops. Hence TLR mediated responses in the intestine are finely regulated. TLR are involved in intestinal homeostasis, including the regulation of the epithelial barrier, by modulating the production of IgA, the maintenance of intestinal integrity tight junctions and the expression of antimicrobial peptides such as RegIII-γ (regenerating islet-derived protein 3 gamma).[159] In addition, TLR signaling is also required to activate immune cells in the intestinal mucosa such as dendritic cells and macrophages.[139]

All TLR may signal through the adaptor protein MyD88 (myeloid differentiation factor 88). TLR3 and TLR4,[160] and possibly TLR2 and TLR5,[161, 162] can also signal through a MyD88-independent pathway that involves the adaptor molecule TRIF (Toll/interleukin-1 receptor domain-containing adapter-inducing interferon-β). Engagement of MyD88 activates a series of signaling molecules, including IRAK1/4 (IL-1 receptor-associated kinase 1/4) and TRAF6 (TNF receptor-associated factor 6), ultimately leading to the activation of the NFκB pathway (nuclear factor kappa-light-chain-enhancer of activated B cells), MAPK pathway (mitogen-activated protein kinase), JAK/STAT pathway (Janus kinase/signal transducer and activator of transcription) and PPARγ pathway (peroxisome proliferator-activated receptor gamma). Subsequent transcriptional activation of unique and common TLR target genes encoding pro- and anti-inflammatory cytokines and chemokines, as well as the induction of co-stimulatory molecules, control the activation of antigen-specific and non-specific adaptive immune responses by *lamina propria* cells (**fig. 4**). All of these various downstream effects are critically involved in the protection of the host homeostasis through control of milieu influences.[163]

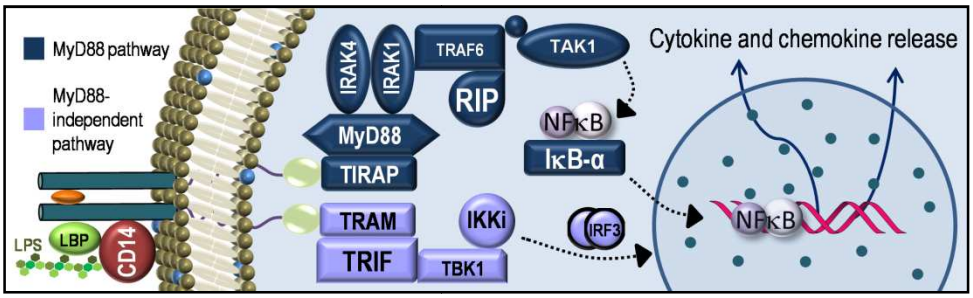


Figure 4. TLR4 signaling pathways. TLR4 may signal through MyD88-pathway or MyD88-independent pathway. Engagement of the adaptor molecule MyD88 activates a cascade ultimately leading to the activation of the transcription factor NFκB. MyD88-independent pathway involves the adaptor molecule TRIF leading to the activation of the transcription factor IRF3. Subsequently, the transcriptional activation of unique and common TLR target genes comes about.

The NFκB or Rel family consists of five different peptides that bind to each other forming dimers, being the prototype the one formed by p50/p65 (Rel A).[164-168] The inactive form is located in the cytosol bound to an inhibitory protein called IκB (α, β or ε).[166, 169-171] The activation of NFκB can occur within two pathways, the classical pathway

and the alternatively, although in both cases the result is the nuclear translocation of NFκB.[172]

MAPK family consists of a number of intracellular proteins with kinase activity.[173] The MAPK family comprises three different families: p42/44 extracellular signal-regulated protein kinase (ERK) MAPK, c-Jun NH_2-terminal kinase (JNK) and p38 MAPK.[174, 175] The p38 MAPK pathway is involved in the regulation of the response to pro-inflammatory mediators and cytokines.[176] Its inhibition has proven effective in treating several animal models of inflammation.[177] Moreover, in the intestinal mucosa of patients with IBD, JNK activation occurs primarily in areas with active disease and it is located in intestinal cells, macrophages and lymphocytes,[178, 179] indicating the involvement of JNK in the pathogenesis of IBD, although the mechanism has not been elucidated so far.

The JAK/STAT pathway is negatively regulated by several mechanisms including receptor dephosphorylation and activated STAT dephosphorylation by tyrosine phosphatases, and by specific endogenous inhibitors of JAK/STAT; proteins capable to limit the response mediated by cytokines, such as the SOCS (suppressors of cytokine signaling) or SSI (STAT-induced STAT inhibitor).[180, 181] Preliminary studies show an increase in SSI-3 levels in the mucosa of IBD patients, both in CD and UC.[182] SSI-3 has been implicated in the regulation of STAT-3, which is expressed in Treg and regulates inflammatory processes

PPARγ is expressed in high levels in the colon and plays an essential role in bacteria-induced inflammation. In fact, it has been demonstrated to regulate inflammation in animal models of experimental colitis and also in patients with UC, in which an altered colonic expression of this receptor is observed.[183] Thiazolidinediones, PPARγ ligands, show an anti-inflammatory effect in DSS- and TNBS-induced colitis.[184, 185]

The fine regulation of TLR responses is exemplified by TLR9. TLR9 is expressed on the cell surface of IEC, both on the apical and the basolateral membrane. *In vitro* studies in IEC cell lines have described that the basolateral stimulation of TLR9 mobilizes an inflammatory cascade, while the apical stimulation induces a signal that curtail

inflammatory responses to basolateral stimulation via different TLR, and therefore induces tolerance.[186] Other TLR may be restricted to the basolateral membrane or to intracellular locations, thus limiting responses to invading bacteria. However, it is not entirely clear how TLR responses are regulated in basal conditions.

Table 1. Expression patterns of Toll-like receptors (TLR) and their activators[142]

TLR	Ligands	Cell expression	Cellular localization
TRL1/2	Bacterial lipopeptides Protozoan parasite proteins (T cruzi Tc52, profillin)	Most cell types including DC and IEC	Plasma membrane
TRL 2	Bacterial lipoprotein/lipopeptides, peptidoglycan, lipoteichoic acid, porins, zymosan Viral structural proteins (Lipoarabinomannan) Helmint lipids Fungi cell wal components Endogenous HSP60, HSP70, HSP96, HMGB1β, hyaluronic acid	IEC, Paneth cells, peripheral mononuclear leukocytes, DC, monocytes and T cells	Plasma membrane
TRL 3	Viral single-stranded and double-stranded RNA, mRNAPoly(I:C), Poly(I:C_{12}U) Endogenous mRNA	IEC, DC, NK cells ant T cells	Endosomes
TRL 4	Bacterial lipopolysaccharide Viral envelope proteins Protozoan parasites Glycoinositolphospholipids (*Trypanosoma cruzi*) Fungi cell wall components Endogenous HSP22, HSP60, HSP70, HSP96, HMGB1β-defensin 2, extra domain A of fibronectin, hyaluronic acid, heparan sulfate, fibrinogen surfactant protein A, calprotectin	IEC, Paneth cells, macrophages, DC and T cells	Plasma membrane
TRL 5	Flagellin	IEC, Paneth cells, monocytes, DC, NK cells and T cells	Plasma membrane
TRL6/TLR2	Bacterial diacyl lipopeptides, lipoteichoic acid Phenol-soluble modulin, zymosan	IEC, high expression in B cells and DC, low in monocytes and NK	Plasma membrane
TRL 7	Viral single-stranded RNA Endogenous RNA	IEC, B cells, DC, monocytes and T cells	Endolysosome
TRL 8	Viral single-stranded RNA Endogenous RNA	IEC, monocytes, DC, NK cells and T cells	Endolysosome
TRL 9	Baterial, viruses and protozoan parasites, unmethylated CpG motifs Protozoan parasite Hemozoin (*Plasmodium*) Endogenous CpG DNA oligodeoxynucleotides	IEC, Paneth cells, DC, B cells, peripheral mononuclear leukocytes, macrophages, NK and microglial cells	Endolysosomes and plasma membrane
TRL 10	Unknown, may interact with TLR2 and TLR1	B cells, DC, monocytes and T cells	Intracellular
TRL 11	Cell surface uropathogenic bacteria, profillin-like molecule from *Toxoplasma gondii*		Plasma membrane

CpG: cytosine-guanine containing single-stranded oligodeoxynucleotides. DC: dendritic cell. HMGB1β: high mobility group box 1 beta. HSP: heat shock protein. IEC: intestinal epithelial cell. NK: natural killer.

The model of colitis induced by the administration of dextran sulfate sodium (DSS) has been shown to be very useful in studying host-microbial interactions. Although the pathogenesis of DSS colitis is not completely understood, it is widely accepted that the administration of DSS disrupts the intestinal barrier, possibly via interaction with surface lipids, and alters intestinal permeability, allowing intestinal microbiota to gain access to the intestinal mucosa.[187] It has been shown that the administration of ligands for TLR5, 2, 3 and 9 protects against DSS-induced colitis, while knock-out (KO) mice for TLR2, 4 and MyD88 are more susceptible to DSS colitis.[188]

Furthermore, TLR5 KO mice develop colitis spontaneously.[189] These results are the opposite of what would be expected based on the direct effects of TLR activation, and therefore suggest that TLR limit inflammation indirectly. On the other hand, studies that show that monoclonal antibody blockade of TLR4 suppresses DSS colitis,[190] and that constitutive activation of TLR4 in IEC in transgenic mice augments DSS-induced colitis, indicate the need to limit TLR responses in order to avoid excessive inflammatory responses.[188] However, interpretation of this evidence is complicated by the fact that DSS also stimulates monocytes.

2. NOD-like receptors.

NLR are a large family of cytoplasmic proteins comprising over 20 members. Among the NLR family members, Nod1 and Nod2 were the first identified and are sensors of bacterial components involved in the modulation of the intestinal inflammatory and apoptotic response.[191] Nod1 is expressed in IEC and recognizes Gram-negative peptidoglycan.[192] An elegant study showed that signaling through Nod1 constitutes the major pathway by which NFκB and NFκB genes are upregulated in cells infected with intracellular bacterial pathogens that do not activate TLR. This way, Nod1 in IEC provides the intestine with a backup mechanism to fight intracellular invasive Gram-negative enteric bacteria that can bypass TLR activation.[193]

Nod2 is expressed in IEC, with particularly high expression in Paneth cells in the small intestine, intestinal myofibroblasts, endothelial cells, granulocytes, and monocyte-derived cells, including macrophages, osteoblasts and dendritic cells.[194] Nod2 recognizes MDP, a

component of peptidoglycan of both Gram-positive and Gram-negative bacteria cell walls.[195] Nod2 is required for the secretion of antimicrobial peptides by Paneth cells. Paneth cells express a wide array of antimicrobial peptides, including α-defensins, lysozyme, phospholipase A2 (which has antimicrobial properties independent of its catalytic activity) and lectin RegIII-γ, that constitute an autonomous defense mechanism against harmful bacteria. RegIII-γ is produced also by intraepithelial lymphocytes and has been proposed to be essential for preventing bacterial contact with the epithelium.[196, 197]

NLR can assemble in response to several stimuli to form large multimolecular complexes that control the activation of the proteolytic enzyme caspase 1. Caspase-1 in turn cleaves the cytokine precursors pro-IL-1β and pro-IL-18; this is critical for the release of the biologically bioactive forms (IL-1β and IL-18) and triggers pro-inflammatory antimicrobial responses. These complexes are called inflammasomes. In general NLR inflammasomes contain the common adaptor ASC (apoptosis-associated speck-like protein containing a CARD). So far, four inflammasomes have been characterized in mouse models, named after the PRR regulating its activity: NLRP1, NLRP3, NLRC4 and AIM2 (a non-NLR-containing inflammasome).[198]

VII. *Physiopathology of IBD*

Immune cells of the innate system, such as dendritic cells and macrophages, but also IEC and myofibroblasts, can sense the intestinal microbiota and respond to diverse PAMP in a stereotypical manner.[140] Furthermore, luminal antigens are recognized by APC, namely macrophages, B cells and dendritic cells, and presented to naïve Th cells (Th0) through MHC-II (major histocompatibility complex, class II). At least four different types of CD4+ Th lymphocytes (Th1, Th2, Th17 and Treg) are involved in the immune response in IBD (**fig. 5**). These are the result of Th0 differentiation that ultimately depends on the cytokines produced in the inflammatory site. Differentiated Th cells in turn produce cytokines that regulate the immune response activating or directing B lymphocytes, macrophages, neutrophils and cytotoxic cells.[199]

Treg are CD4+ Foxp3+ regulatory T cells that play an essential role in intestinal homeostasis. Treg are characterized by the expression of Foxp3, which is considered to

confer their regulatory activity.[200] The expression of Foxp3 in peripheral Th0 is dependent on transforming growth factor (TGF)-β. Treg appear in the intestine after the oral administration of antigens and produce IL-10 and TGF-β1, that in general terms downregulate inflammation. Several pro-inflammatory cytokines inhibit Treg induction, including IL-6, IL-21, IL-23 and IL-27. Among them, IL-23 is the key for the inhibition of Treg during inflammation.[201] IBD patients appear to have lower numbers of Treg both in blood and colon, nevertheless it is important bear in mind that Treg isolated from these patients are functionally active *in vitro*.[201]

Th17 cells are a newly described subpopulation of Th cells, involved in the pathogenesis of IBD. This pro-inflammatory lymphocytes are characterized by the expression of the master transcription factor ROR-γt (retinoid-related orphan receptor-gamma t), as well as IL-17A, IL17F, IL-21, IL-22 and IL-26.[202] These cells differentiate under the influence of IL-1β, IL-6, IL-21 and IL-23, and TGF-β. The latter has been shown to be essential for their differentiation.[202] Th17 response is involved in the physiopathology of IBD, particularly in CD in which Th1 cell response is also concerned.[203-205]

Th1 cells express the STAT-4 and produce IL-2, IL-12, IL-18 and IFN-γ. While IFN-γ drives Th1 differentiation, IL-10, TGF-β and IL-4 inhibit it. IL-2, acting in an autocrine fashion, induces Th1 proliferation. The production of IFN-γ by Th1 cells activates macrophages, which in turn produce IL-12. Th2 cells express GATA-3 (GATA binding protein 3) and produce IL-4, IL-5, IL-6 and IL-13. Th2 cells proliferate in response to IL-4 and IFN-γ inhibits them. The activation of the Th2 response implicates B cells, increasing antibody neutralizing production.[206]

The pathology of CD is characterized by a high expression of IL-12/IL-23 and an associated predominance of CD4+ Th1/Th17 cells, leading to the secretion of pro-inflammatory cytokines such as IL-17, TNF-α and IFN-γ.[207, 208] UC has been considered as a Th2-mediated disease, with excessive production of IL-5 and IL-13.[209] However, there have also been different observations about mucosal Th1 and Th2 cytokines in IBD. Both UC and CD biopsies cultured *ex vivo* release high and comparable amounts of IFN-

γ.[210] Lower levels of IL-13 were found in the colonic mucosa of UC patients compared to CD patients and control subjects, and recent data on experimental colitis have suggested an anti-inflammatory effect of IL-13 in the gut.[140] Bernardo *et al.* have described the presence of a mixed cytokine profile with predominance of IL-6 and absence of IL-13 in supernatants of UC biopsies cultured *ex vivo*.[211] Collectively, these data should lead to reconsider the Th1/Th2 paradigm in CD and UC, and the notion that UC is a Th2-mediated disease remains controversial.

Unlike in healthy individuals, the equilibrium between pro- and anti-inflammatory cytokines is significantly deranged.[212-215] Deregulation of the immune reaction leads to excessive activation of pro-inflammatory cytokines. With the pathologically prolonged lifespan of disease-perpetuating T lymphocytes in patients with CD, the disease enters a chronic stage.

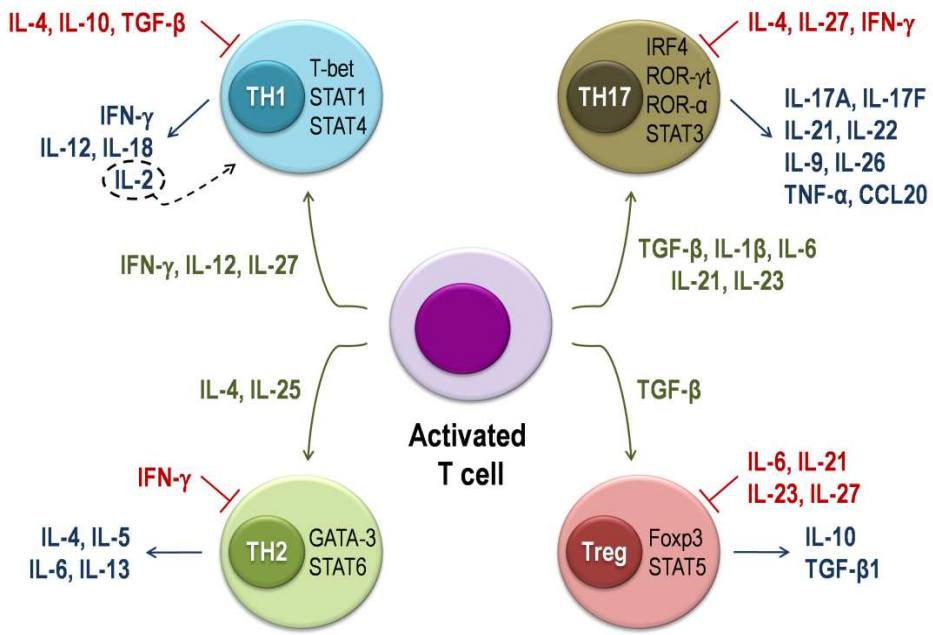


Figure 5. Th cell differentiation.

APC are pro-inflammatory *per se* (**fig. 6**). Activated IEC and resident macrophages secrete chemokines, providing a chemotactic gradient and favoring leukocyte extravasation.[206] Monocytes and neutrophils are the first cells attracted to the focus of inflammation. There, they produce mainly TNF, IL-1β, IL-6 and IL-12. Pro-inflammatory molecules are preferentially produced by the cells that have migrated to the inflammatory focus, rather than by resident macrophages. Resident intestinal macrophages have a limited capacity to respond to bacterial adjuvants owing to downregulation of their bacterial recognition receptors, such as TLR and CD14, the co-ligands for LPS.[216] Similarly, IEC normally have low levels of TLR, which allows epithelial cells to reside in the high bacterial concentration of the distal ileum and colon.[217]

Pro-inflammatory cytokines released by monocytes and neutrophils attract more neutrophils, T and B lymphocytes and regulate the inflammatory response. Newly recruited innate immune cells are particularly sensitive to activation by bacterial adjuvants like LPS, peptidoglycan and flagellin, sustaining and enhancing the inflammatory response. Anti-inflammatory cytokines are also produced as a result of the activation of macrophages and IEC, such as IL-1RA or IL-11, but they are unable to stop the massive inflammation in patients with IBD.[218]

Another key event in IBD progression is the expansion of the intestinal microvasculature. Angiogenesis (increased blood vessel density) in IBD sustains inflammation through alterations in the endothelial lining of these vessels. Angiogenesis increases the area of endothelium available for exchange, but also for extravasation of blood constituents into surrounding tissue to increase disease severity in IBD.[219] The leukocyte extravasation cascade involves multiple steps, including tethering/rolling, activation, adhesion, spreading and transmigration.[220] Inflammatory cytokines upregulate local endothelial expression of cell adhesion molecules such as VCAM1 (vascular cell adhesion molecule 1) and ICAM1 (intercellular adhesion molecule 1), that cause circulating leukocytes to adhere to the inflamed endothelium.[221] These adhesion molecules are necessary for circulating cells to be able to stick to the activated endothelium, which is the first step in the extravasation of mononuclear cells and polymorphonuclear cells into the inflammatory focus. Moreover, activated leukocytes

undergo the conformational change in the VLA4 integrin (very late antigen 4), necessary to confer high binding affinity for the endothelial adhesion molecules.[222] In addition, adhesion molecules mediate migration of the extravasated immune cells through the stroma to the source of maximal chemokine production, as well as through the epithelium to the lumen, where they produce crypt abscesses.[223] Finally, intestinal endothelial cells also express CD40, an important immune co-stimulatory molecule for T cells and platelets, which express CD40 ligand (CD40L), providing a mechanism for targeted recruitment of T cell subsets to the small intestine rather than the colon.[224]

Inflammatory mediators cause vasodilation and hyperemia in the mucosa, favoring fluid output, with the consequent edema formation.[225] These mediators damage the endothelial barrier function. The contraction of endothelial cells and the increased vascular permeability worsen the situation, allowing protein extravasation. During inflammation of the intestinal mucosa, neutrophils that migrate across the endothelium and through the extracellular matrix to the base of the epithelial layer must undergo an additional transepithelial migration step in order to reach the lumen. IEC form a tight barrier whose permeability is regulated by the apical junction complex, which consists of proteins from adjacent cells interacting to form the tight junctions and adherens junctions. Crossing this barrier is necessary for neutrophils to defend against extracellular pathogens in the lumen, and also plays an important role in inflammatory pathology.[226] Infiltration of neutrophils is associated with tissue damage at mucosal surfaces via mechanisms that include increased barrier permeability, epithelial apoptosis and the release of damaging effectors such as proteases, and reactive oxygen and nitrogen species.[227, 228]

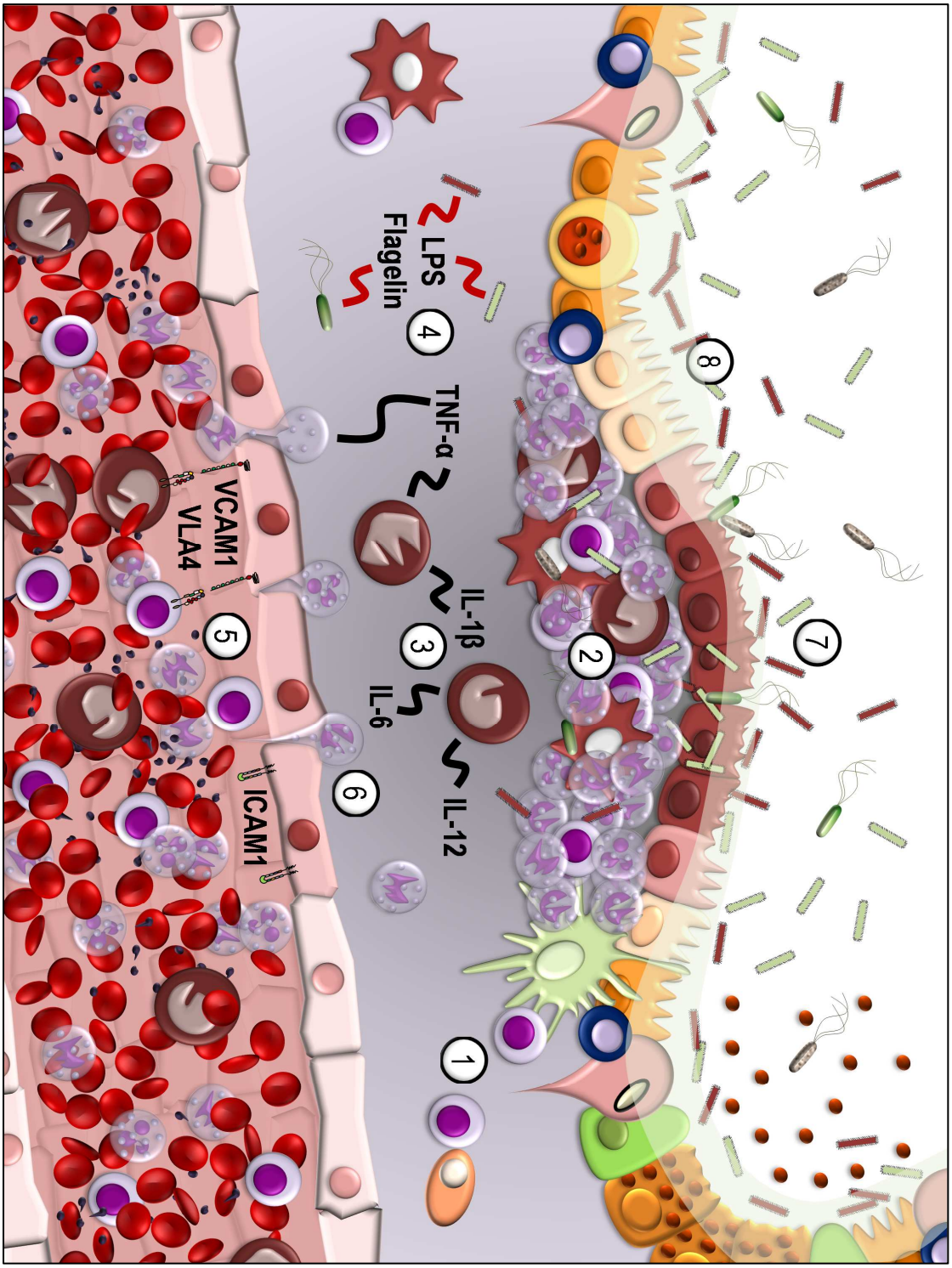
As IBD progresses, cytokine-mediated inflammation and epithelial apoptosis disturb the intestinal barrier, allowing the penetration of gut flora beyond the *lamina propria*, causing intense inflammatory responses,[229, 230] while also provoking endothelial microvascular permeability. Clinical symptoms associated with IBD, due to the altered epithelium, include diarrhea, weight loss, malnutrition, and changes in fluid and electrolyte homeostasis. IBD patients also suffer intestinal motility disturbances; in general terms the motility is diminished in this disease.[231] There is a reciprocal relationship between the

intestinal epithelium and the inflammatory process in the mucosa that may be affected either in both directions.

In addition to intestinal inflammation, IBD presents a wide variety of symptoms affecting various distant organs of the GI tract (see **section IV: diagnosis, symptoms and complications of IBD**). Most of these symptoms are due to the effect of pro-inflammatory cytokines produced in excess during intestinal inflammation.

The efficient repair of GI mucosal injuries is essential in the preservation of the epithelial barrier to luminal antigens. Rapid re-sealing of the intestinal epithelial barrier is initially accomplished by migration of viable epithelial cells from the wound edge into the denuded area ("restitution") and only later by cell proliferation.[232] This migration has been modelled in various ways, most commonly in mechanically wounded monolayers of cell lines or cells in primary culture, and in wounded human or animal tissue. Evidence from these models indicates that migration is a highly complex process, which is likely to involve the tightly controlled spatial and temporal interaction of multiple factors, such as extracellular soluble factors (e.g., growth factors, trefoil peptides, cytokines) and matrix components (e.g., collagen, laminin, fibronectin); signaling molecules activated by the interaction of these factors with cell surface receptors (e.g., protein kinases, phospholipases, low-molecular-weight GTPases); factors which regulate adhesion to other cells (e.g., E-cadherin) and to matrix components (e.g., integrins, hyaluronic acid receptors); factors which regulate detachment from the extracellular matrix (e.g., urokinase-type plasminogen activator, matrix metalloproteinases); and molecules which regulate cytoskeletal function (e.g., Rac), that allow the formation of specialized cellular processes termed lamellipodia.[233]

Figure 6 (right). Physiopathology of IBD. (1) Luminal antigens recognized by APC. **(2)** Active IEC and resident macrophages secret chemokines, providing a chemotactic gradient and favoring leukocyte extravasation (grey background). **(3)** Monocytes and neutrophils release inflammatory cytokines. **(4)** Innate immune cells activated by bacterial adjuvants. **(5)** Inflammatory cytokines upregulate local endothelial expression of cell adhesion molecules. **(6)** Leukocyte extravasation. Vasodilation and hyperemia in the mucosa. Edema formation. **(7)** Interstitial leukocytes interact with various substances resulting in the formation of reactive oxygen and nitrogen species that cause damage to the epithelium and mucosa gap. **(8)** Restitution and cell proliferation.


LPS
Flagelin
4
TNF-α
IL-1β
3
IL-6
IL-12
2
7
8
1
6
VCAM1
VLA4
5
ICAM1

Selective inhibition of most of the pro-inflammatory cytokines attenuates the onset of experimental colitis.[234, 235] In addition to inducing the expression of inflammatory genes, NFκB simultaneously stimulates the expression of various protective molecules, such as TNF-induced protein 3 (formerly A20), CARD15, COX-2, β defensins, PPARγ and its own inhibitor, IκB-α, that inhibit inflammatory responses.[236]

NFκB is activated in the tissues of IBD patients and its inhibition can attenuate experimental colitis;[237] accordingly, the NFκB pathway was thought to have predominantly pro-inflammatory activities. Selective gene deletion studies have, however, shown that NFκB has both beneficial and detrimental effects on inflammation, with strikingly different functions in different cell types. Studies revealed colonic inflammation and pancolitis in mice after 3 weeks of age when the NFκB response in the intestinal epithelium was specifically ablated.[238] The authors of this study indicated that the inhibition of NFκB response favors the disruption of the intestinal barrier integrity, resulting in bacterial translocation and intestinal inflammation.[238]

VIII. *Animal models of IBD*

Much of the recent progress in the understanding of mucosal immunity has been achieved by the study of experimental animal models of intestinal inflammation.[239, 240] 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. At least 66 different kinds of animal models have been established to study IBD.[241] Selected mouse models are broadly categorized into 6 categories according to the defect in mucosal immunity that is believed to be most important for the onset of the disease: (1) impaired T cell regulation; (2) excessive effector cell function; (3) spontaneous IBD; (4) perturbations of the epithelium; (5) involving chemical or environmental stressors; and (6) as yet unclassified (**table 2**).

Table 2. Animal models of IBD[239-250]

Category	Model	Known defects / Type of response
Impaired T cell regulation	CD4+ CD45RB^{hi} T cell transfer model	Decreased number of Treg cells; high Th1 cytokines production; involvement of IL-10/TGF-β
	CD4+ CD62L+ T cell transfer model	Decreased number of Treg cells; predominance of Th1/Th17 cells
	IL-2-deficient mice	Decreased number of CD4$^+$ CD25$^+$ T cells; elevated immunoglobulin secretion
	IL-10-deficient mice	Increased epithelial permeability; IL-12/23-dependent Th1/Th17; lack TGF-β signaling
	CRF2-4-deficient mice	Unresponsive to IL-10
	Transgenic epsilon 26 (Tgε26) bone marrow transfer model	T cell and NK cell deficiency
	T cell receptor (TCR) α-chain-deficient mice	Loss of regulatory B cell function; increased numbers of Th2 cells. Production of IL-4
	TGF-β_1-deficient mice	Decreased numbers of Treg cells
Excessive effector cell function	STAT-4 transgenic mice	High production of Th1 cytokines; low levels of Th2 cytokines
	IL-7 transgenic mice	Sustained survival of colitogenic IL-7Rα-expressing memory CD4(+) T cells
	TNF-α "knockin" (TNF$^{\Delta ARE}$) mice *	Increased TNF-a production
	A20-deficient mice *	Increased response to LPS
	STAT3 deficiency in myeloid cells	Increased response to LPS; resistance to IL-10 regulation
	B7-related protein-1 (B7RP-1)-Fc transgenic	B cell hyperplasia; increased levels of IgE (Th2 pathway)
	CD40 ligand transgenic mice *	Increased numbers of activated T cells (IL-12/23-driven inflammation)
Spontaneus IBD	C3H/HeJBir mice	Impaired innate responses to TLR ligands; increased numbers of bacterially reactive T cells
	SAMP1/YitFc (Samp) mice	Epithelial cell defects; expanded B cell population; increased numbers of activated T cells
Perturbations of the epithelium	Multi-drug resistance gene 1α (mdr1a)-deficient mice	Altered epithelial barrier; enhanced IEC responses to bacteria, chemokine overexpression
	N-cadherin dominat negative mutant chimeric mice	Barrier function
	Gαi2-deficient mice	Defective epithelial barrier; defective regulatory B cells
	IKK-γ (NEMO)/IKKαβ deficiency in IEC	Barrier function/innate immunity
	Intestinal trefoil factor-deficient mice (with DSS)	Goblet cell dysfunction; impaired epithelial repair
	Keratin 8-deficient mice	Increase in TCRβ-positive/CD4-positive T cells, enhanced Th2 cytokine
	MUC1/2-deficient mice	Expansion of IL-17-producing lymphoid cells/IEC hyperproliferation and apoptosis defects
Involving chemical or environmental stressors	Dextran sulfate sodium (DSS)	Direct epithelial erosion with macrophage activation; Th1/Th2 cytokines production
	Trinitrobenzene sulfonic acid (TNBS)/ethanol enema	Ethanol breaks mucosal barrier/TNBS results in acute necrosis; macrophage/T cell activation
	Oxazolone/ethanol-induced colitis	Contact hypersensitivity reaction; Th2 cytokine production
As yet unclassified	HLA-B27/β2M transgenic rat *	Activation of Th1 cells
	Wiskott-Aldrich syndrome protein (WASP)-deficient mice	Treg cells
	Lymphopenic T cell receptor transgenic mice	Low numbers of circulating CD4+ T cells
	Fucosyltransferase transgenic mice	Disturbances in cell surface glycosylation (spontaneously develop colitis)

IBD: Inflammatory Bowel Disease. IEC: intestinal epithelial cell. LPS: lipopolysaccharide. NK: natural killer. TLR: Toll-like receptor.
* Multiorgan inflammation not limited to intestine.

Most of these models are based either on chemical induction, immune cell transfer or gene targeting, and only in some models disease occurs without any exogenous manipulation.[243]

Chemical models use different substances that disrupt the intestinal barrier to induce colonic inflammation.[251] Among these, the models of murine colitis induced by the administration of TNBS or DSS are the most widely used.[252, 253] Besides their similarities to multiple aspects of human IBD, the DSS and the TNBS murine models have several outstanding characteristics: the onset and duration of inflammation is immediate and controllable, there are no artificial genetic deletions or manipulations that are not found in human IBD,[254] and since there is a barrier disruption, they are better to study the implication of the defense against microorganisms.[255]

The most used KO model of IBD is the $IL10^{-/-}$ mice. These animals develop a spontaneous cecal inflammation and colitis at 2-4 month of ages that features many characteristics observed in human IBD. The inflammation in this model is Th1-driven, similar therefore to CD inflammation in humans. Transgenic models of IBD are well represented by the HLA-B27 model of rat colitis. In this model HLA-27 and human microglobulin 2 mu transgenic rats develop chronic colitis that, among other characteristics, develops some extraintestinal complications resembling those seen in IBD patients like spondyloarthropathy, with peripheral and axial joint, dermatologic complications and male genital inflammation.[256] Finally, in the most representative transference model CD4+ $CD45RB^{high}$ T cells from healthy wild type mice are transferred to a syngenic mice that lack B and T cells.[257] After 5-8 weeks pancolitis and intestinal inflammation is observed in recipient mice with features that are similar to those of human CD. This model presents the advantages that early symptoms of inflammation can be studied as well as the perpetuation of the disease.[257]

1. Dextran sulfate sodium-induced colitis.

First report on the use of DSS dates back in the year 1985, when Ohkusa *et al.* published their investigation on DSS-induced colitis in hamsters.[258] Thereafter, DSS colitis was induced also in mice by Okayasu *et al.* in 1990.[259] It consists of the

administration of DSS polymers dissolved in drinking water, at different concentrations (1-5%), to mice, rats, hamsters or guinea pigs. DSS is cheap and widely available. It is easy to store (at room temperature) and has a shelf life of up to 3 years. Preparation of DSS for induction of colitis is also quite simple: dissolved in tap water and administered *ad libitum*; hence, unlike most chemical induction models, no general anesthesia is required.[260] DSS is a sulfated polysaccharide with a highly variable molecular weight, ranging from 5 kDa to up to 1400 kDa. It was found that the molecular weight of DSS is a very important factor in the induction of acute and chronic colitis or colitis-induced dysplastic lesions (carcinogenicity). The severity of colitis and carcinogenic activity differs with the administration of DSS at different molecular weights (i.e., 5 kDa, 40 kDa and 500 kDa).[261]

By first interfering with intestinal barrier function, and next stimulating local inflammation, DSS is often used to induce a form of mouse colitis that mimics the clinical and histological features of IBD that have characteristics of UC. Although the exact mechanisms involved have not been completely elucidated, it is believed that DSS is directly toxic to gut epithelial cells of the basal crypts and therefore affects the integrity of the mucosal barrier, thus allowing luminal bacterial translocation and the subsequent infiltration of granulocytes and mononuclear immune cells.[187] Petersson *et al.* demonstrated a correlation between decreasing mucus barrier and increasing clinical symptoms during the onset of colitis.[262] The main manifestations of the colonic insult are animal body weight loss, diarrhea, rectal bleeding and shortening of the colon. The lesions observed during this phase have been associated with increased production of macrophage-derived pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α.[263] Laroui and colleagues recently suggested that DSS associates with medium-chain length fatty acids (MCFA), such as dodecanoate, in the colonic lumen prior to induction of colitis. MCFA are present at high concentrations in the colonic lumen and that the colonic epithelium absorbs and partially metabolizes MCFA. DSS complexed to MCFA form nanometer-sized vesicles ~200 nm in diameter that fuse with colonocyte membranes. The arrival of such vesicles in the cytoplasm affects major epithelial cell pathways and consequently reduces intestinal barrier functions that initiate intestinal inflammatory signaling cascades.[264]

As T and B cell deficient C.B-17 scid (severe combined immunodeficiency) or Rag1$^{-/-}$ mice also develop severe colitis, the adaptive immune system obviously does not play a major part (at least in the acute phase) in this model. Hence, the acute DSS colitis model is particularly useful to study the contribution of innate immune mechanisms of colitis.[252] In addition, the DSS model has been shown to be suitable to study epithelial repair mechanisms. Studies with TLR4$^{-/-}$ and MyD88$^{-/-}$ mice suggest that TLR signaling is required to limit bacterial translocation after DSS induced intestinal epithelial injury, suggesting that TLR signaling is important for the maintenance of the epithelial barrier.[159, 163]

The contribution of the intestinal bacteria to DSS colitis is somewhat controversial.[265] DSS treatment with clinically effective antibiotics in human IBD, like metronidazol or ciprofloxacin, results in amelioration in the intestinal inflammatory process induced by this polymer,[266] and treatment with bacterial products or probiotic organisms can ameliorate DSS colitis.[267, 268] On the other hand, the bacterial product LPS does not seem to have a role in the induction of DSS colitis, as the LPS-resistant mouse strains C3H/HeJ and C3H/HeJBir remain very susceptible to DSS-induced colitis. Further, other studies have shown that GF mice develop DSS colitis to the same or even more severe degree as conventional mice,[269-271] whilst other studies support the importance of commensal bacteria in the development of colitis in this experimental model.[272-274]

Chronic model of DSS colitis exists. In susceptible strains, the administration of DSS for several cycles (e.g., 7 days DSS, 14 days water) results in chronic colitis, and if combined with a single initial dose of the genotoxic colon carcinogen azoxymethane (AOM), in inflammation-associated colorectal cancer.[275] Patients with UC have an increased risk for the development of colon cancer.[37] As colonic inflammation is suggested to play a key role in IBD-related colorectal cancer, the AOM/DSS model is a very useful tool to study mechanisms linking inflammation to colon carcinogenesis.[276]

Both acute and chronic models are highly reproducible. In addition, it is one of the most commonly used models of IBD, and has been a good model to study various aspects of IBD such as therapy, pathogenesis (especially the role of permeability and

epithelial destruction in initiation of IBD), genetic predisposition and IBD loci, and bowel malignancy secondary to IBD.[260]

2. Trinitrobenzene sulfonic acid/ethanol enema-induced colitis.

The TNBS-induced colitis was initially described in 1989 by Morris *et al.* in rats,[277] although its use has also been extended to mice. Basically, it consists of the application of an enema of TNBS dissolved in a solution of ethanol/water, at different doses depending on the laboratory animal used: 10 to 30 mg in rats, or 0.5 to 4 mg in mice. The role of ethanol is to promote the disruption of the intestinal barrier, thus allowing the access of TNBS to the intestinal *lamina propria*, which exhibits a direct toxic effect and acts as a hapten, activating the host immune response of the intestine to colonic autologous or microbiota proteins.[278] The result is a severe and prolonged degenerative inflammation of large parts of the colon that shares several clinical and molecular characteristics with CD, including severe transmural inflammation with cell infiltration and ulcers, diarrhea, rectal prolapse, anorexia and weight loss.[279]

Furthermore, both the administration of TNBS-ethanol to mice and human CD produce Th1-driven inflammation, characterized at the initial stage by the infiltration of macrophages and neutrophils producing high levels of pro-inflammatory cytokines such as TNF-α, IL-1β or IL6, followed by T cell infiltration, mainly of the CD4+ phenotype, that produce IL-4 and IFN-γ.[252, 279] Although there are differences between human IBD and TNBS colitis in rodents, its simplicity and reproducibility clearly contribute to its extensive use in the evaluation of novel strategies in human IBD.

It is important to highlight that the induction of colitis in the TNBS model depends on the genetic background of the animal strain used and the individual microflora of the animal facilities. Since different TNBS lots show a striking variability in their capacity to induce colitis, initial studies are needed to find the optimal colitis-inducing TNBS/ethanol dose in a given microenvironment.[239]

3. CD4+ T cell adoptive cell transfer model of colitis.

In 1993 an additional mouse IBD model system was described by Powrie *et al.*, in

which intestinal inflammation developed spontaneously after reconstitution of SCID mice with CD4+ T cells.[280, 281] These naïve T cells react in a severe fashion on experiencing the gut antigens; become activated forming colitogenic T cells secreting cytokines, thus causing severe gut inflammation involving both small and large intestines, making this a model similar to CD.[257] It usually takes around 6–8 weeks, depending on the microbial populations present in the animal facility, to develop severe disease manifested by hunching, progressive weight loss and diarrhea. Histopathology reveals transmural inflammation, dense infiltrates involving neutrophils and crypt abscessation.[244]

As with virtually all mouse models of chronic colitis, T cells play an important role in disease pathogenesis. In addition, genetic background of the mouse is a major modifier of the disease with disease penetrance and severity. The advantages of using the IL-10$^{-/-}$ model is that it is a well-established Th1-mediated model of transmural colitis, which can be treated with various immunological agents (anti-TNF-α, anti-IFN-γ antibodies), antibiotics and probiotics. However, the onset and severity of disease are variable and in some cases disease requires many months to develop. Furthermore, continuous brother-sister mating (inbreeding) of these mice to maintain adequate numbers of animals results in a significant reduction in the penetrance and severity of disease over the course of a 2 years period. Because of these observations, coupled to the desire to more precisely "synchronize" the onset and severity of disease, the T cell transfer model of chronic colitis has become more widely used over the past years by a number of different investigators from around the world.[257]

It is important to note that the colitis-inducing potency of CD4+ T cells in the adoptive cell transfer models is determined by various parameters, including the activation state, CD45RB phenotype and IL-12 responsiveness.[282] One key finding using this model was that the reciprocal population of CD4+ CD45RB^{low} T cells from normal mice did not elicit colitis following adoptive transfer into immune deficient mice and,[280, 283] in fact, even suppressed the development of disease in immune deficient mice that also received pathogenic CD4+ CD45RB^{high} T cells. Further experiments demonstrated that this suppressive activity was present mainly within the CD4+ CD25+ T cell population, which, together with parallel studies by other investigators, indicated that CD25+ Treg cells play

a crucial role in the maintenance of self-tolerance and regulation of harmful inflammatory responses.[284]

Atreya *et al.* developed a modified version of this model by isolation of T cells using magnetic cell sorting. In this transfer model CD4+ $CD62L^{high}$ T cells from BALB/c mice were isolated and transferred into C.B-17 scid mice. This cellular population induces a chronic bowel inflammation with similar disease manifestations and kinetics as transferred CD4+ $CD45RB^{high}$ cells.[285]

IX. *Treatment of IBD*

Some medications used to treat CD and UC have been around for years.[10] Others are more recent breakthroughs. The most commonly prescribed medications fall into six basic categories:

1. Aminosalicylates.

Aspirin-like compounds that contain 5-aminosalicylic acid, such as sulfasalazine, balsalazide, mesalamine and olsalazine. These drugs, which can be given either orally or rectally, do not suppress the immune system but decrease inflammation at the wall of the intestine itself, and help heal both in the short- and long-term. They are effective in treating mild-to-moderate episodes of UC, both in the induction as in the maintenance of remission.[286, 287]

2. Corticosteroids.

These medications, which include prednisone, prednisolone and budesonide, affect the body's ability to begin and maintain an inflammatory process. Prednisone and prednisolone are used for moderate to severe CD and UC. Budesonide is used for mild to moderate ileal CD and right-sided colon CD. Corticosteroids can be administered orally, rectally or intravenously. Effective for short-term control of acute episodes (flares), they are not recommended for long-term or maintenance use because of their side effects.[288] If the use of steroids cannot be discontinued without suffering a relapse of symptoms, it may be helpful to add some other medications to help manage the disease. It is important not to suddenly stop taking this medication.

3. Immunomodulators.

These include azathioprine, 6-mercaptopurine, methotrexate and cyclosporine. This class of medications modifies the body's immune system so that it cannot cause ongoing inflammation. Usually given orally (methotrexate is injectable), immunomodulators are typically used in people for whom aminosalicylates and corticosteroids have not been effective, or have been only partially effective. They may be useful in reducing or eliminating reliance on corticosteroids. They also may be effective in maintaining remission in people who have not responded to other medications given for this purpose. Immunomodulators may take up to three months to begin working.

4. Biologic therapies.

These therapies are genetically engineered to target very specific molecules involved in the inflammatory process. The newest class of therapy to be used in IBD includes adalimumab, certolizumab pegol, infliximab and natalizumab. These medications are indicated for people with moderately to severely active disease who have not responded well to conventional therapy. They also are effective for reducing fistulas. Biologic therapies may be an effective strategy for reducing steroid use, as well as for maintaining remission. Recently, infliximab dependency has been described in children with perianal disease and no surgery prior to infliximab.[289] Three groups can be established within biologic therapies:

i. **Neutralizing pro-inflammatory cytokines.** IL-1β, TNF-α and IL-12/IL-18/IFN-γ axis.[290-292] They are molecules that bind to these pro-inflammatory cytokines, preventing binding to its receptor and, therefore, the execution of its biological activities.

ii. **Anti-inflammatory cytokines.** Recombinant human IL-10 and IL-11.[293, 294]

iii. **Interference with cell activation or cellular traffic.** Anti-CD4 antibody, with disappointing results due to its toxicity; anti-α_4 antibody;[295] and cell signaling inhibitors, like SB203580 (p38 MAPK inhibitor).

5. Antidepressants.

Studies with healthy volunteers have demonstrated that antidepressants can improve immunoregulatory activity and thus they may have a potential to positively impact the disease course in IBD.[296, 297]

6. Antibiotics.

Clinical and experimental studies suggest that the relative balance of aggressive and protective bacterial species is altered in these disorders. Antibiotics can selectively decrease tissue invasion and eliminate aggressive bacterial species or globally decrease luminal and mucosal bacterial concentrations, depending on their spectrum of activity.[298] Metronidazole, ciprofloxacin and other antibiotics may be used when infections occur.[299] They treat UC, CD and perianal CD. They are also used for post-surgical problems such as pouchitis (*inflammation of the ileal pouch; artificial rectum surgically created out of ileal gut tissue in patients who have undergone a colectomy*).

Alternatively, administration of beneficial bacterial species (probiotics), poorly absorbed dietary oligosaccharides (prebiotics), or combined probiotics and prebiotics (symbiotics) can restore a predominance of beneficial *Lactobacillus* and *Bifidobacterium* species.

Despite the wide range of drugs used in the treatment of IBD, therapeutic options available today are not entirely satisfactory. Firstly, there is no specific drug for the treatment of this disease, so that the drugs used are of inflammatory or immunosuppressive nature and, therefore, will suppress the immune response and inflammatory pathways globally. This can cause the individual to have a decline in defense capability, i.e., in their immune system. Furthermore, pharmacological treatments employed are characterized by a wide spectrum of reactions and, in some cases, as in the corticosteroids, resulting in dependence phenomena. Besides, there are situations where patients do not respond to a certain drug, or become refractory to it. Accordingly, the pharmacology of IBD is a field of intense research, and the search for new therapeutic options with a better toxicity profile is fully justified.[1]

X. *Prebiotics*

The term “prebiotic” was initially proposed by Gibson and Roberfroid,[300] and refers to dietary ingredients that promote “the selective stimulation of growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host”.[301] The prebiotic definition does not emphasize or target any specific bacterial group. However, it is generally assumed that a prebiotic should increase the number and/or activity of *Bifidobacteria* and *Lactobacilli*, as opposed to other more harmful genera like *Eubacterium*, *Faecalibacterium* or *Clostridium*, after treatment with prebiotics.[302]

Structurally, prebiotics are non-digestible carbohydrates, including monosaccharides (e.g., tagatose), oligosaccharides (short-chain carbohydrates) and polisaccharides (fibre). Prebiotics can be also industrially synthesized (e.g., enzymatically from sucrose) and can be incorporated into many foodstuffs, thus receiving high commercial interest. Moreover, due to their excellent safety profile and lack of serious side effects, the prebiotic therapeutic applications invite clinical trials on how to prevent various GI disorders. Of note, although the prebiotic market is mostly restricted to a handful of nutritional companies, new products are expected to be incorporated due to the increasing interests shown by pharmaceutical companies.[303] For these reasons, these products present a great added value from a sanitary and economical point of view, used in the food and drug industries as functional foods and nutraceuticals.[304-307] In addition, they are used as food additives and in the cosmetic industry.[308, 309]

1. Non-digestible oligosaccharides.

The terms non-digestible oligosaccharides (NDOS) and resistant oligosaccharides are synonymous with the resistant short-chain carbohydrates term and in practice all describe the same substances. This diverse group of substances are usually consumed in only small amounts from natural occurring sources, principally as fructans present in several plant roots and as the small α-galactosides (raffinose family) from legumes, with more complex galactooligosaccharides (GOS) found in breast milk.[310]

Nowadays, the prebiotics used in Europe and the United States are limited so far to

inulin and related variants such as fructooligosaccharides (FOS), and GOS, mainly as food ingredients.[311] There is also a rising interest in very novel carbohydrate products, as is the case of goat milk oligosaccharides (GMOS), for its similarity with the human milk oligosaccharides (HMOS).

i. Inulin and fructooligosaccharides. Both inulin and FOS are natural fructooligosaccharides found in plant roots like onions, dahlia, wheat and chicory. Generally consist of chains of fructose units linked together by β(2,1) linkages. Almost every molecule is terminated by a glucose unit. The total number of fructose or glucose units (= degree of polymerisation, DP) ranges mainly between 2 and 60. Inulin DP ranges between 20 and 60. FOS are produced by the partial enzymatic hydrolysis of inulin, consisting mainly of molecules with DP between 2 and 8.[306, 308] They are widely used in infant formulas for their prebiotic effect and in patients with intestinal disorders, such as diarrhea, IBD or necrotizing enterocolitis (NEC).[312-315]

ii. Galactooligosaccharides. The composition of the GOS fraction varies in chain length and type of linkage between the monomer units. They are mainly obtained by the action of the enzyme β-galactosidase on lactose, resulting in the production of 4'- or 6'-galactosyllactose, longer oligosaccharides, trans-galactosylated disaccharides and non-reducing oligosaccharides consisting of lactose molecules with one or more galactosyl residues linked by β(1,3), β(1,4) and β(1,6) bonds.[316] Since birth, the human body gets accustomed to regular intake of GOS. Breast milk provides a variety of GOS based on the lactose, next to the lactose itself.[317] In infants the usage of GOS has been shown to have a potential role in allergy prevention and reduction of infectious diseases.[318, 319] GOS supplementation has also been shown to reduce symptoms of stress-induced GI dysfunction.[320]

iii. Human milk oligosaccharides. Goat milk oligosaccharides. The presence and, particularly, the remarkable abundance of oligosaccharides in human milk as the third largest solid component, have led investigators to propose biological, physiological and protective functions to these molecules. The question, why would milk contain indigestible material, has challenged scientists studying milk for decades. Certainly, the

number and structural diversity of these molecules would allow more than one function.[321] More than a hundred different HMOS have been identified so far, but not every woman synthesizes the same set of oligosaccharides.[322] Recently, HMOS have been demonstrated to selectively nourish the growth of highly specific strains of *Bifidobacteria,* thus establishing the means to guide the development of a unique gut microbiota in infants-fed breast milk.[323] Certain oligosaccharides derived from the mammalian epithelial cells of the mother also share common epitopes on the infant's intestinal epithelia known to be receptors for pathogens. The presence of such structures in milk have been hypothesized to have evolved to provide a direct defensive strategy acting as decoys to prevent binding of pathogens to epithelial cells, thereby protecting infants from diseases.[321]

The original goal of supplementing infant formulas with oligosaccharide fractions was to mimic prebiotic effects of HMOS in non-breastfed infants. Inulin, FOS, GOS and even cow's milk oligosaccharides are much simpler in terms of its structure than those of human milk. Thus, the introduction of NDOS with similar impact to HMOS on the developing microbiota can be considered as a breakthrough in infant nutrition.[324] Is a paradox that infant formula, made from cow milk, lacks oligosaccharides similar to breast milk. For that reason, a group of the Chemical Engineering Department from the University of Granada, together with Puleva Biosearch Life, developed a method to purify GMOS. Goat milk is an optimal source of oligosaccharides for their similarity to human milk in concentration, complexity and variety.[325]

2. Prebiotic effects of non-digestible oligosaccharides.

The colonic microflora derive substrates for growth from the human diet as well as from endogenous sources such as mucins, the main glycoprotein constituents of the mucus which lines the walls of the GI tract. The vast majority of the bacteria in the colon are strict anaerobes and thus derive energy from fermentation. The two main fermentative substrates of dietary origin are non-digestible carbohydrates and proteins, peptides and amino acids which escape digestion in the small intestine.[301]

Fructans are classified according to differences in glycosidic linkages [β(2,1), β(2,6) or

both].[326] NDOS are not hydrolysed by enzymes secreted into the upper (small) GI tract because the glycosidic bonds present cannot be hydrolysed by the human enzymes. However, these glycosidic bonds can be hydrolysed by enzymes produced by bacteria present in the lower (large) GI tract; then, the hydrolysis products are often fermented by the bacterial population.

NDOS in the colon are fermented to SCFA, mainly, acetate, propionate and butyrate (**fig. 7A**), and a number of other metabolites such as the electron sink products lactate, pyruvate, ethanol, succinate, as well as the gases H_2, CO_2, CH_4 and H_2S (**fig. 7B**). As a whole, SCFA acidify the luminal pH which suppresses the growth of pathogen and leads to the acceleration of intestinal transit due to stimulation of colonic microbiota growth and, consequently, an increased gas production and water retention in faeces (**fig. 8**).[327-329] SCFA are rapidly absorbed by the colonic mucosa and contribute approximately 5-10% towards energy requirements of the host. Acetate is mainly metabolized in human muscle, kidney and heart. Brain propionate, which is cleared up by the liver, is a possible glucogenic substrate and it might contribute to inhibition of cholesterol synthesis. It might also play a role in the regulation of adipose tissue deposition. Moreover, butyrate is largely metabolized by the colonic epithelium where it serves as the major energy substrate as well as a regulator of cell growth and differentiation. In colon cancer cell lines, SCFA induce apoptosis. SCFA function as histone deacetylase inhibitors, which are pro-differentiation, pro-apoptosis, and can induce cycle growth arrest in cancer cells.[301]

SCFA also modulate inflammation and can affect several leukocyte functions. They suppress the production of pro-inflammatory mediators such as TNF-α, IL-6 and nitric oxide (NO). Butyrate can enhance the release of the anti-inflammatory cytokine IL-10. SCFA are involved with leukocyte chemotaxis, affecting migration to inflammatory sites. The anti-inflammatory effects of SCFA may be related to the activation of their cognate G protein-coupled receptors (GPCR) GPCR41 and GPCR43. Recent advances in the understanding of intestinal epithelial biology have included the identification of a likely SCFA uptake mechanism and of two SCFA GPCR that likely transduce the presence of luminal SCFA into neurohormona signals that can affect appetite, glycemic control, intestinal growth and gut motility.[330]

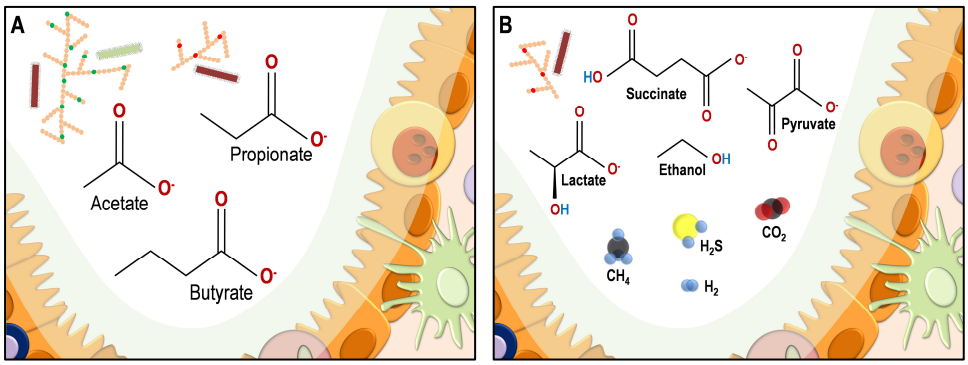


Figure 7. Fermentation products of prebiotics.
SCFA (short chain fatty acids) **(A)**, and electron sink products and gases **(B)**.

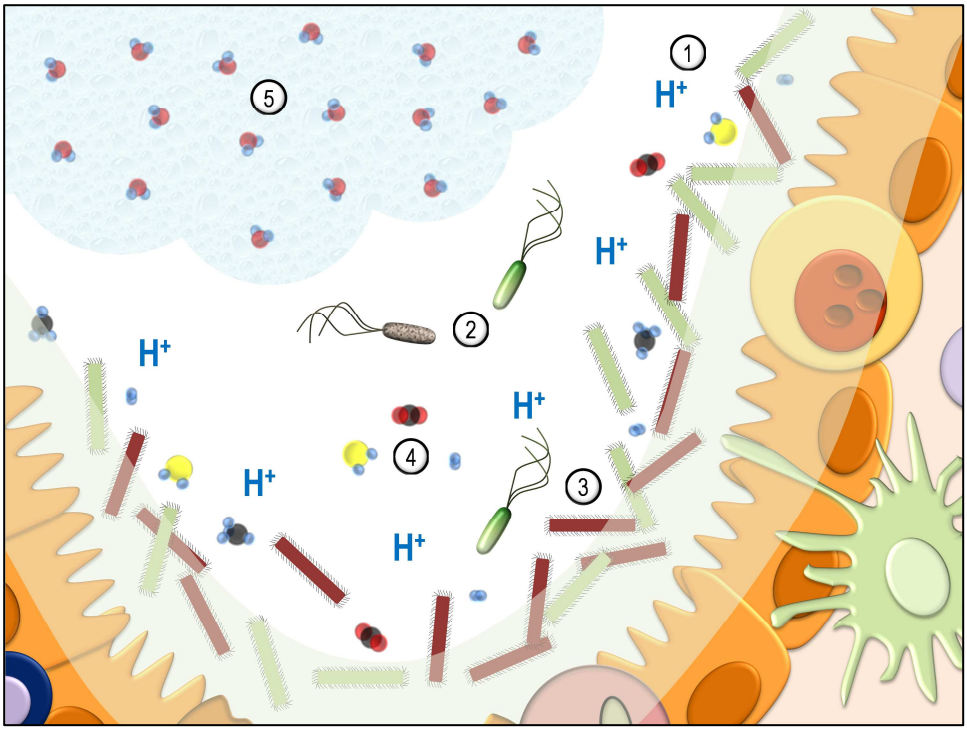


Figure 8. Prebiotic effects of non-digestible oligosaccharides. Acidify the luminal pH **(1)**, which suppresses the growth of pathogen **(2)** and leads to the acceleration of intestinal transit due to stimulation of colonic microbiota growth **(3)** and, consequently, an increased gas production **(4)** and water retention in faeces **(5)**.

On the other hand, proteins reaching and/or produced in the colon are fermented to branched chain fatty acids such as isobutyrate, isovalerate and a range of nitrogenous and sulphur-containing compounds. Unlike carbohydrate fermentation, products which are recognized as beneficial to health, some of the end products of amino acids metabolism may be toxic to the host, e.g., ammonia, amines and phenolic compounds. Consequently, excessive fermentation of proteins, especially in the distal colon, has been linked with disease states such as colon cancer and IBD, which generally start in this region of the large intestine before affecting more proximal areas. Thus, it is favorable to shift the gut fermentation towards saccharolytic fermentation over a prolonged period of time into the distal parts.[301]

3. Prebiotic-independent effects of non-digestible oligosaccharides.

In addition to the above, these NDOS may exert different actions independent of their prebiotic activity (**fig. 9**).

i. Inhibition of the adhesion of pathogenic bacteria to epithelial cells. HMOS can act as PRR and bind PAMP, acting as decoy receptors.[331, 332] Some HMOS resemble mucosal cell surface glycans, serve as soluble decoy receptors to prevent pathogen binding and reduce the risk of infections. The HMOS composition mirrors blood group characteristics, which depend on the expression of certain glycosyltransferases.[333] These enzymes are involved in the synthesis of glycoproteins and glycolipids of the surface of epithelial cells. In this manner, HMOS may act as analogues or homologues of PRR with which would interact specifically. Several studies have demonstrated the anti-adhesive antimicrobial effects of oligosaccharides. Xylooligosaccharides inhibit pathogen adhesion to enterocytes *in vitro*.[334] GOS have the highest anti-adhesion ability *in vitro* of all prebiotics tested according to Shoaf *et al.* They are able to inhibit the adhesion of enteropathogenic bacteria to Caco-2 cell cultures.[335]

HMOS have shown to competitively interact with HIV-1 (human immunodeficiency virus-1) for receptor binding sites *in vitro*.[336] Anti-adhesive antimicrobial effects may not be restricted to bacteria and viruses; they might also apply to certain protozoan parasites like *Entamoeba histolytica*, which causes amoebic dysentery or amoebic liver abscess.[337]

Furthermore, their effects may not only be relevant to enteric infections. Human milk often covers the mucosal surfaces in the infant's nasopharyngeal regions and occasionally reaches the upper respiratory tract during episodes of aspiration. Breast-fed infants are less likely to develop otitis media caused by *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* or *Haemophilus influenzae* and are also at lower risk to develop respiratory syncytial virus. Similarly, HMOS are absorbed and excreted with the urine, and they reduce uropathogenic *E. coli*-induced hemagglutination, suggesting that HMOS also reduce urinary tract infections.[333]

ii. Modulation of intestinal epithelial cell response. *In vitro* studies strongly suggest that HMOS can directly interact with the infant's IEC and reprogram the cell surface glycosylation.[323, 338] HMOS reduce cell growth and induce differentiation and apoptosis in cultured human IEC by altering growth-related cell cycle genes.[339] Fukasawa *et al.* identify marker genes that were upregulated in mice feeded with FOS. These genes were associated with the antigen presentation (*MHC-I* and *MHC-II*) and *IFN*, which are probably located in the upstream of IgA pathways, and those associated with phosphatidylinositol metabolism, which is an essential signal factor for the differentiation of various cells, including B cells.[340]

iii. Immune modulators. Regulation of the inflammatory response and modulation of cytokine production. HMOS may act as anti-inflammatory agents by inhibiting the complex formation between platelets and neutrophiles, which is increased during inflammatory processes.[341] Selectins bind to glycans that carry sialylated-Lewis blood group epitopes, which are very similar to HMOS. In fact, HMOS contain Lewis blood group antigens and are able to reduce selectin-mediated cell-cell interactions. Sialylated HMOS reduce leukocyte rolling and adhesion in an *in vitro* flow model with TNF-α-activated human endothelial cells.[342] Similarly, sialylated HMOS reduce platelet-neutrophil complexes formation and subsequent neutrophil activation in an *ex vivo* model with whole human blood.[341] Prebiotic oligosaccharides reduce pro-inflammatory cytokines in intestinal Caco-2 cells via activation of PPARγ and peptidoglycan recognition protein 3.[343] Interestingly, HMOS have been reported to be taken up by Caco-2 cells *in vitro*, suggesting that subepithelial cells may be modulated by these compounds *in vivo*. In fact,

they have been found to regulate cytokine production in human cord blood mononuclear cells *in vitro*, by directing the neonatal Th2 phenotype toward a more balanced Th1/Th2 profile.[344] The number of IFN-γ-producing CD3+ CD4+ and CD3+ CD8+ lymphocytes as well as IL-13-producing CD3+ CD8+ lymphocytes increases when cord blood T cells are exposed to sialylated HMOS.

Preliminary studies from our group have demonstrated that inulin, FOS and GMOS modulate IL-8 cytokine secretion in different intestinal epithelium cell lines (HT-29, Caco-2 and IEC-18) and IL-1β, IL-8 and TNF-α in a human monocyte/macrophage cell line (THP-1). Moreover, several NDOS have been useful in the treatment of induced-colitis in rodent models.[345-349]

iv. Brain development. Breast-fed preterm infants have superior developmental scores at 18 months of age and higher intelligence quotients at the age of 7. Brain development and cognition in part depend on sialic acid-containing gangliosides and poly-sialic acid-containing glycoproteins. Sialylated HMOS contribute to the majority of sialic acid in human milk.[333]

v. Regulation of the intestinal transport and permeability. NDOS increase net Ca^{2+} transport in Caco-2 cells via the paracellular route through tight junctions.[350] FOS enhance the apparent absorption rate of Ca^{2+}, Mg^{2+} and Fe^{2+}, and increase the hepatic Zn^{2+} and femur Mg^{2+} levels in mice.[351] Moreover, a 12-months study showed a significant increase in Ca^{2+} absorption that led to greater bone mineral density in adolescents ingesting 8 $g \cdot d^{-1}$ of short- and long-chain inulin fructans.[352] Oral and intravenous Ca^{2+} absorption have also been quantified in young adults following 8 weeks of supplementation with 8 g of inulin/FOS. Ca^{2+} absorption increased at least 3% in young adults with a mean calcium intake of 900 $mg \cdot d^{-1}$.[353]

In addition, the displacement of N_2 excretion to the colon and then faeces by oligosaccharide feeding is of great interest. Feeding rats a diet supplemented with inulin or FOS at a dose of 100 $g \cdot kg^{-1}$ for a few weeks decreases uraemia in both normal and nephrectomized rats.

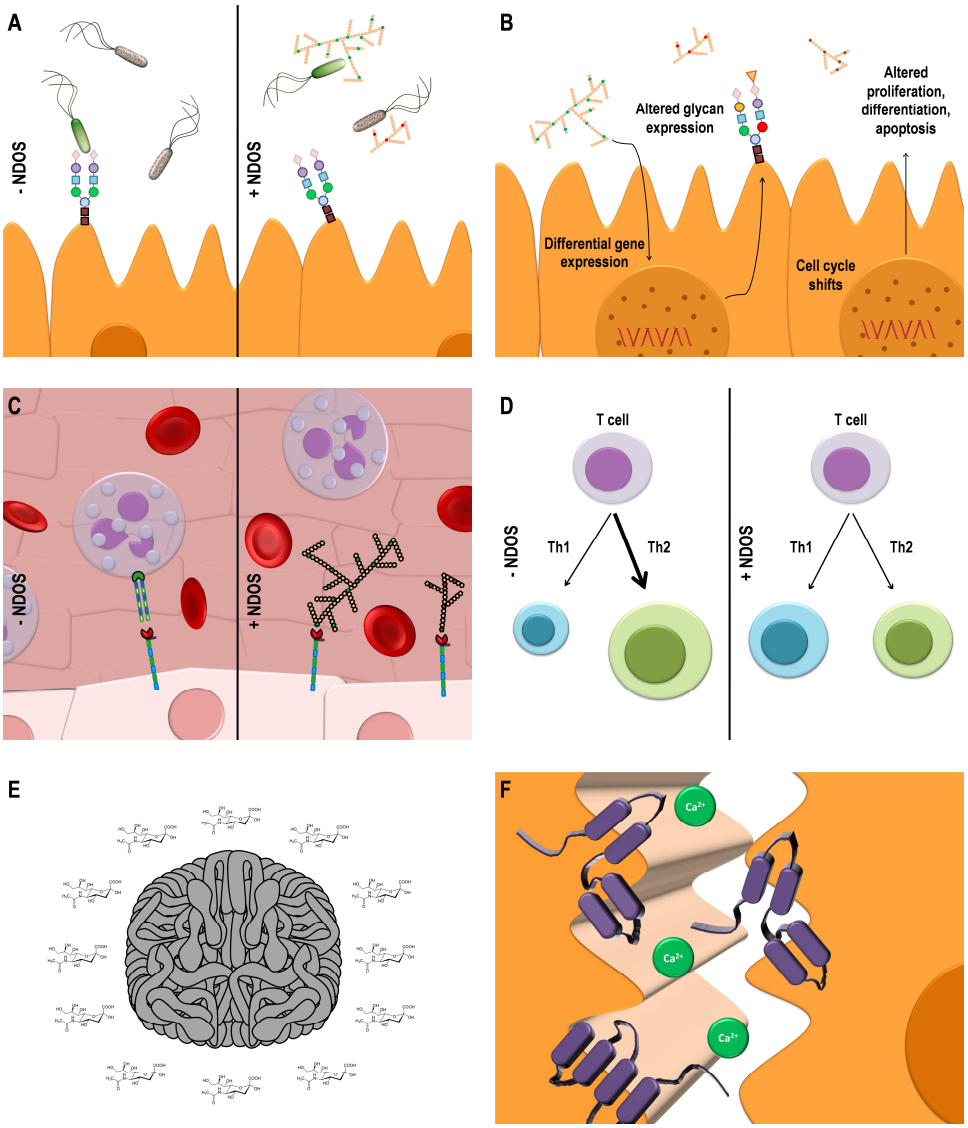


Figure 9. Prebiotic-independent effects of non-digestible oligosaccharides. (A) Antiadhesive antimicrobials that serve as soluble glycan receptor decoys and prevent pathogen attachment. **(B)** Directly affect intestinal epithelial cells and modulate their gene expression, which leads to changes in cell surface glycans and other cell responses. **(C)** Reduce selectin-mediated cell–cell interactions in the immune system and decrease leukocyte rolling on activated endothelial cells, potentially leading to reduced mucosal leukocyte infiltration and activation. **(D)** Modulate lymphocyte cytokine production, potentially leading to a more balanced Th1/Th2 response. **(E)** Provide sialic acid as a potentially essential nutrient for brain development and cognition. **(F)** Increase net Ca^{2+} transport via the paracellular route through tight junctions.

It has been proposed that their osmotic effect in the small intestine allows the transfer of urea into the distal ileum and the large intestine, where a highly ureolytic microflora proliferates. When fermentable oligosaccharides intake is high, the amount of NH_4 required to sustain maximal bacterial growth may become insufficient, and blood urea is then required as a ready source of N_2 for protein synthesis by caecal bacteria.[354]

4. Prebiotics on diseases.

Several intestinal diseases, in particular those of mucosal inflammation, such as UC, pouchitis, diversion colitis, short bowel syndrome and obesity, are thought to result from dysbiosis.[330] The production of SCFA, in addition to their selective promotion of host-friendly bacteria in contraposition to other more harmful genera, have led to the consideration of prebiotics in the treatment or prevention of conditions like constipation, diarrhea, IBD, NEC, septic shock, diabetes and allergies to dietary protein.[312-314, 327, 328, 355-357] The possible benefit is enhanced by the low toxicity generally ascribed to these compounds. However, it should be emphasized that in some cases prebiotics/probiotics may be deleterious, as in the case of patients with acute pancreatitis treated with probiotics.[358]

i. Diarrhea. *Passage of 3 or more loose or liquid stools per day, or more frequently than is normal for the individual.* The Working Group for Probiotics and Prebiotics of the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) conducted a multicenter trial to determine the efficacy of administering a combination of prebiotics, specifically inulin and FOS, for the prevention of diarrhea and antibiotic-associated diarrhea (AAD). A total of 105 children (aged 6 months-11 years) with common infections were enrolled. They received antibiotic orally treatment plus inulin and FOS in age-dependent doses with a maximum dose of 5 $g \cdot d^{-1}$ (n=51) or a placebo (maltodextrin) (n=54) for the duration of the antibiotic treatment. The administration of the two prebiotics was not effective for preventing diarrhea and AAD. The overall frequency of diarrhea was low, and the study was underpowered.[359]

However, when small amounts (2 $g \cdot d^{-1}$) of FOS or a placebo (maltodextrin) were administered over 3 weeks to 35 healthy infants (aged 7-19 months), greater numbers of

Bifidobacteria and lower numbers of *Clostridia* were found in the stool. Fewer children were afflicted with diarrhea, and fewer diarrheal episodes were observed, in the FOS compared with the placebo group. Additionally, FOS supplementation was accompanied by less flatulence, vomiting and fever events.[360] In a study of 244 healthy participants traveling to high- or medium-risk destinations for traveler's diarrhea, 10 $g \cdot d^{-1}$ of inulin ingested 2 weeks prior to travel and 2 weeks during travel reduced the prevalence and resulted in less severe attacks of diarrhea.[361]

Despite established positive effects of NDOS on the intestinal microflora, and some promising results of animal experiments, there is not enough evidence to medically recommend prebiotics for the prevention or treatment of diarrhea.[312]

ii. Infections. *Invasion of a host organism's bodily tissues by disease-causing organisms, their multiplication, and the reaction of host tissues to these organisms and the toxins they produce.* As we have seen above, one of the most promising non-prebiotic effects for the use of NDOS in the prevention of diseases is the inhibition of the adhesion of pathogenic bacteria to human epithelial cells. The anti-adhesive antimicrobial effects of HMOS may contribute to the lower incidence of intestinal, upper respiratory and urinary tract infections in breast-fed compared with formula-fed infants.[333, 362] With the intention to mimic breast milk oligosaccharides it is important to test the usefulness of different NDOS in formulas. Bruzzese *et al.* conducted a study where 342 healthy infants were enrolled and randomized to a formula additioned with a mixture of GOS and FOS or to a control formula. The incidence of intestinal and respiratory tract infections was monitored for 12 months. The incidence of gastroenteritis was lower in the supplemented group than in the controls. The number of children with more than 3 episodes tended to be lower in prebiotic group. The number of children with multiple antibiotic courses/year was lower in children receiving prebiotics. A transient increase in body weight was observed in children on prebiotics compared to controls during the first 6 months of follow-up.[319]

Breast-feeding is the predominant postnatal transmission route for HIV-1 infection in children. However, a majority of breast-fed infants do not become HIV-infected despite continuous exposure to the virus through their mothers' milk over many months. As

mentioned previously, HMOS competitively interact with HIV-1 for receptor binding sites *in vitro*.[336]

iii. Necrotizing enterocolitis. *Devastating disease that affects mostly the intestine of premature infants. The wall of the intestine is invaded by bacteria, which cause local infection and inflammation that can ultimately destroy the wall, leading to perforation of the intestine and spillage of stool into the infant's abdomen, which can result in an overwhelming infection and death.* In preterm infants with immature GI tracts, development of NEC may be associated with a variety of factors, such as colonization with pathogenic bacteria, secondary ischemia, genetic polymorphisms conferring NEC susceptibility, anemia with red blood cell transfusion and sensitization to cow's milk proteins.[363]

Several studies have demonstrated increased *Bifidobacteria* and decreased pathogenic bacteria in the stool of preterm infants fed prebiotic-containing formula with GOS and/or FOS in comparison with control infants. Similar effects of increased *Bifidobacteria* colonization were seen with the use of symbiotics.[364]

Butel *et al.* developed an experimental model of NEC using gnotobiotic quails associated with faecal flora specimen belonging to premature infants. They have shown that onset of intestinal lesions requires a combination of low endogenous lactase activity, lactose in diet, intestinal stasis and colonization by lactose-fermenting bacteria such as *Clostridia* or faecal flora specimens from premature infants suffering from NEC. The protective role of *Bifidobacteria* was demonstrated in this model through a decrease in clostridial populations and in butyric acid. FOS dietary supplementation was shown to enhance this effect with an increase in the bifidobacterial level and consequently a greater decrease in *Clostridia*.[314]

Riskin and colleagues reported that premature infants fed with low dose of lactulose had more *Lactobacilli*-positive stool cultures and less intolerance to enteral feeding. They also tended to have fewer episodes of late-onset sepsis, lower incidence of NEC, and their nutritional laboratory indices were better, especially calcium and total protein. This

pilot study supports the safety of supplementing preterm infants' feeds with low doses of lactulose. It also demonstrated trends that may suggest positive prebiotic effects.[365]

Nevertheless, Srinivasjois *et al.* systematically reviewed randomized controlled trials evaluating the safety and efficacy of prebiotic oligosaccharide supplementation in preterm infants and concluded that supplementation with prebiotic oligosaccharides was safe but did not result in decreased incidence of NEC.[366]

iv. Irritable bowel syndrome. *Chronic abdominal pain, discomfort, bloating and alteration of bowel habits.* The major studies have used FOS and GOS mainly, in varying doses (3.5-20 $g \cdot d^{-1}$) and for varying durations (4-12 weeks). Two trials using FOS, (6 and 20 $g \cdot d^{-1}$ respectively) found no significant impact on symptoms at the study endpoints, although in the latter high dose study, prebiotics actually worsened symptoms at the study mid-point. Two studies have demonstrated symptom improvement, with FOS (5 $g \cdot d^{-1}$) lowering composite symptom score and trans-GOS (3.5 $g \cdot d^{-1}$) lowering flatulence and bloating and improving global symptom relief. However, in the latter study patients randomized to a higher dose of trans-GOS (7 $g \cdot d^{-1}$) reported higher composite symptom scores. These data would suggest that both the type and dose of prebiotic is important in determining any clinical benefit in this syndrome, with some evidence that higher doses may have a negative impact on symptoms.[367]

v. Colorectal cancer. *Uncontrolled cell growth in the colon or rectum, or in the appendix.* Dietary carbohydrates recognized in prevention of colon cancer include β-glucans, dietary fibres, fructans and resistant starch.[317] In the 1970s, many reports suggested that increased colorectal cancer prevalence was a result of low-fibre diets. These assumptions were predominantly based on differences in colorectal cancer rates among nations and regions with high- and low-fibre intakes; this type of data clearly lacks causal evidence.[368] Many studies suggest that prebiotics counteract colon carcinogenesis by the production of SCFA. Inulin-type fructans are fermented extensively by large bowel microflora to lactic acid and SCFA contributing to the protective effects and apoptosis induction (see **section X**, **subsection 2: prebiotic effects of non-digestible oligosaccharides**). Furthermore, compared with other anaerobes in the GI tract,

Lactobacilli and *Bifidobacteria* have enzymes with lower activities, such as β-glucosidase, β-glucuronidase, urease, azoreductase and nitrate reductase, which are involved in the formation of mutagens and carcinogens.[369] Inulin-type fructans are known to induce apoptosis of colonic cells with mutations in their DNA. Protective effects of NDOS rise with increasing structural complexity (DP and branching). This could be attributed to the lower fermentation rate of inulin compared to FOS, being able to reach the distal parts of the colon. Similarly, elimination of carcinogenic colonic cells by inulin is more effective than FOS, again pointing to the importance of structural differences.[317]

Data from tumour models further demonstrated that a reduced number of colonic tumours in inulin/FOS-supplemented animals coincided with enhanced NK cell cytotoxicity.[370]

As prebiotics are also known to work synergistically with probiotics to elicit beneficial effects on commensal populations and overall gut health, symbiotics have been explored as potential therapeutic agents in colorectal cancer. Studies using *Bifidobacteria* and *Lactobacillus* strains in conjunction with prebiotics, such as inulin and FOS, have been found to off-set carcinogenesis. Direct immune modulation has also been found with *B. lactis* and *L. rhamnosus*, which were shown to decrease IL-2 and inducible NO synthase, the enzyme responsible for NO production. As colorectal cancer can arise from untreated colitis, these studies suggest potential use of probiotics/prebiotics/symbiotics as an anti-inflammatory therapeutic, utilized not only in pre-established colorectal cancer cases, but also as a preventative measure in patients exhibiting symptomatic signs of colitis or early-stage colorectal cancer.[371]

vi. Cardiovascular disease. *Any disease that affects the cardiovascular system, principally cardiac disease, vascular diseases of the brain and kidney, and peripheral arterial disease.* The adequate intake level of 14 g of fibre per 1000 kcals of energy consumed is based on protection against cardiovascular disease (CVD); so the data for this relationship are strong. Fibre intake consistently lowers the risk of CVD and coronary heart disease primarily through a reduction in low density lipoprotein (LDL) levels. The results of randomized clinical trials are inconsistent, but suggest that fibre may play a

beneficial role in reducing C-reactive protein levels, apolipoprotein levels and blood pressure, all of which are biomarkers for heart disease. Water-soluble fibres (specifically, β-glucan, psyllium, pectin and guar gum) were most effective for lowering serum LDL cholesterol concentrations, without affecting high density lipoprotein (HDL) concentrations. Other soluble fibres, glucans and pectins, have recognized ability to lower blood lipids and the regulations in individual countries determine labeling and claims.

vii. Obesity *(excess body fat)***, type II diabetes** *(high blood glucose in the context of insulin resistance and relative insulin deficiency)* **and metabolic syndrome** *(cluster of the most dangerous heart attack risk factors, including central obesity, hypertension, dyslipidemia and insulin resistance)***.** The beneficial effects of prebiotics have been mainly explained by their ability to regulate lipid metabolism (decrease serum and liver lipids -steatosis-), body weight and fat mass development, glycemia (lowered postprandial glycemia), low-grade inflammation and peptide hormones controlling hunger and satiety (anorexigenic peptides) in normal and in obese rats, mice and hamsters; and to exert antidiabetic (improvements in glucose tolerance and partially restored insulin secretion), antihypertensive and anti-inflammatory effects.[372, 373] Prebiotics exhibit anti-obesity potential owing to their fermentation in distal gut and the impact on gut microbiota composition, which have different physiological prospects. The underlying mechanisms driving the response are not clear. However, there are some links associated with production of SCFA, decrease in bacterial derived LPS and alteration in gut hormones production. The major effect of inulin supplementation appears to be its influence on production of GI hormones like GLP-1, peptide YY, ghrelin and other related peptide hormones, both in rodents and in humans. These hormones modulate several physiologic functions such as insulin secretion (incretin effect), GI motility and appetite regulation by modulating secretion of neuropeptides in major hypothalamic appetite centers. These factors may all contribute to the anti-obesity potential of prebiotics.[311]

viii. Type I hypersensitivity *(anaphylactic response rapidly initiated to allergenic exposure, consisting in an excessive activation of mast cells and basophils by IgE and resulting in an inflammatory response)***: food allergy** *(adverse immune response to a food protein)* **and atopic dermatitis** *(type of eczema, an inflammatory, relapsing, non-*

contagious and pruritic -itchy- skin disorder). Both animal studies and human clinical trials show that dietary intervention with oligosaccharides in early life could lead to the prevention of atopic dermatitis, food allergy, and/or allergic asthma.[374] 134 healthy term infants with a parental history of atopy were fed either a prebiotic-supplemented (8 $g \cdot l^{-1}$ GOS/FOS) or placebo-supplemented (8 $g \cdot l^{-1}$ maltodextrin) hypoallergenic formula during the first 6 months of life. Cumulative incidences for atopic dermatitis, recurrent wheezing and allergic urticaria were higher in the placebo group than in the intervention group.[318] Eiwegger *et al.* confirmed that HMOS impact allergen-specific T cell cytokine polarization *in vitro* in peanut allergic individuals.[344]

Furthermore, some experimental evidence suggests an important role for prebiotics, highlighting possible functional dependency of probiotics on prebiotic supplementation. Discrepancies have been seen in experiments testing the effectiveness of symbiotic mixtures. *Bifidobacterium breve* and oligosaccharide treatment used in a cohort of 29 patients with asthma, found a decrease in IL-5, IL-4 and IL-13, yet the same symbiotic mix tested in atopic dermatitis infants found no significant difference in IL-5 levels. Furthermore, in response to allergen-specific stimuli, decreases in IL-12 production were found in conjunction with unchanging levels of Treg.[371]

ix. Coeliac disease. *Delayed type hypersensitivity reaction which culminates in an autoimmune-like disorder. Is associated with the gut mucosa triggered by an inappropriate immune response to the dietary antigen gluten (α-gliadin).* The accepted treatment for coeliac disease involves avoidance of dietary gluten. The use of inulin in gluten-free bread is being introduced as a method for producing wheat replacement foods with an improved consistency in order to help individuals adhere to the strict dietary regime.[375]

5. Prebiotics on IBD.

Many animal studies have demonstrated the effectiveness of prebiotics in preventing and treating models of IBD, although the results often differ depending upon the compound used. The prebiotics have been mainly tested in the TNBS and DSS chemically-induced models of colitis, but data from HLA-B27 transgenic rats or IL-10 gene-deficient mice have also been reported. Treatment with oral inulin to rats exposed to

DSS resulted in the amelioration of damaged mucosa and a decreased severity of crypt destruction, an effect associated with a significant reduction in tissue myeloperoxidase (MPO) activity and in the mucosal release of inflammatory mediators.[376] Moreover, FOS supplementation has been shown to attenuate TNBS-induced colitis in rats, promoting the growth of beneficial lactic acid bacteria and increasing colonic butyrate levels.[377] The anti-inflammatory effect of GOS and active hexose-correlated compound (AHCC), a commercial product yielding a 74% content in oligosaccharides, were probed in the TNBS model of colitis in rats.[345, 346] However, another study has reported that no beneficial effect was observed in the DSS-induced colitis model in FOS-fed rats.[378] A similar inefficacy of GOS in TNBS-colitis rats have been reported,[379] although the lack of efficacy has not been consistent with other studies. In consequence, further studies are necessary to elucidate the mechanism involved in the beneficial effect of these compounds in intestinal function and their implication in human intestinal inflammation.

Several prebiotic mixtures and symbiotics have been tested in animal models in order to improve their activity. Thus, the combination of FOS and inulin showed anti-inflammatory activity in spontaneous colitis in HLA-B27 rats.[380] A TNBS model of colitis in rats was used in our laboratory. AHCC (100 or 500 $mg \cdot kg^{-1}$) and *Bifidobacterium longum* BB536 ($5 \cdot 10^6$ colony forming units $(CFU) \cdot rat^{-1} \cdot day^{-1}$) were administered together or separately for 7 days prior to colitis induction and then for another 7 days and compared with non colitic and TNBS rats. The results showed that both treatments had intestinal anti-inflammatory activity separately, which was enhanced when used in combination, as shown by changes in body weight gain, colonic weight to length ratio, MPO activity and inducible nitric oxide synthase (iNOS) expression.[348]

One of the common characteristics reported in most of these studies performed with prebiotics is the fact that the beneficial effects could be associated with the increased production of SCFA in the intestinal lumen. Of note, it has been suggested that intracellular butyrate oxidation is impaired in patients with UC, similar to that shown in the murine model of DSS-induced colitis. Thus, the energetic deficit in the colonocytes observed during IBD could be counteracted by prebiotic treatment. Since the direct intracecal infusion of lactic acid bacteria together with SCFA was able to reproduce the

intestinal anti-inflammatory effects of FOS in the TNBS-induced colitis, it has been suggested that fermentation of the prebiotic by lactic acid bacteria was the principal mechanism mediating their anti-inflammatory effect.[303]

These beneficial effects were associated to a reduction in the production of pro-inflammatory cytokines, including IL-6, IL-8, IL-1β and TNF-α, and even to an enhancement in the expression of regulatory type cytokines like TGF-β. However, the direct mechanism of action produced by prebiotics in the immune system has not been totally elucidated.[303]

The experimental models of colitis have also provided valuable information about the mechanisms of action implicated in the anti-inflammatory effects of prebiotics, supporting their potential role in the treatment of human IBD. Prebiotics have been shown to stimulate faecal and mucosal *Bifidobacteria* and *F. prausnitzii* in healthy people, while acetate and propionate increase immunoregulatory IL-10 production. Consequently, prebiotics have been investigated as a potential therapeutic target for CD.[381] In human subjects, a number of studies have investigated the combined use of probiotic and prebiotic combinations,[382, 383] but few have investigated the effect of prebiotics alone.

In 2003, Hussey *et. al* did an open-label human trial involving 10 children with CD. In a 6 weeks prospective pilot study, children with active disease were given, as their sole source of nutrition, a whey protein, FOS and inulin-containing formula (Peptamen® with Prebio), via nasogatric feeding. The children gained weight significantly and had significantly reduced CDAI, with nine out of ten children having scores indicating little or no disease activity, together with markedly reduced erythrocyte sedimentation rates, a non-specific biochemical marker of inflammation.[384]

Lindsay *et al.* conducted and open-label study using ten patients with active CD, all of whom were given 15 g of FOS for 3 weeks. The Harvey-Bradshaw index, a simplified version of the CDAI, was reduced markedly, and the patients had increased faecal bifidobacterial numbers. There was also an increase in the percentage of IL-10-positive dendritic cells, and the percentage of dendritic cells expressing TLR2 and TLR4.

Bifidobacteria has been linked to TLR4.[385]

Since these promising, but preliminary findings, two large randomised controlled trials have been published.[367] Neither demonstrated an impact of FOS/inulin at doses of 15 $g \cdot d^{-1}$ or 20 $g \cdot d^{-1}$ on CD activity; indeed both studies showed greater withdrawal in the prebiotic groups. Furthermore, neither of them resulted in higher *Bifidobacteria* or *F. prausnitzii* in the prebiotic groups compared with placebo.

This perhaps suggests that the prebiotic effect may be most pronounced at lower levels of inflammation, highlighting a need for studies investigating the role of prebiotics in disease maintenance.

Many patients do not respond well to standard therapies, which often have undesirable side-effects, therefore, an inexpensive and effective treatment based on the use of prebiotics or symbiotics could make a significant contribution to relieving the clinical and financial burdens of these diseases. The use of well-designed and tested products to treat CD and UC offers several potential advantages in that they are inexpensive, easy to administer, demonstrably safe, and have no side-effects. The few studies that have been reported on the therapeutic use of prebiotics and symbiotics in IBD to date have shown some promise for the future of this area of research, but more randomized controlled trials with larger patient cohorts need to be undertaken. There is no doubt that adequately powered clinical trials are required to take into account subtypes within the groups, both in terms of disease spectrum and individual microflora differences, because these factors may significantly influence overall results.[386]

Objectives

On the basis of the existing challenges in the field of IBD and its relationship with luminal modulatory influences, we set out to undertake the following objectives:

I. *Determine non-prebiotic effects of non-digestible oligosaccharides*

Different types of NDOS are used in the food and drug industries as functional foods and nutraceuticals due to their prebiotic effects.[304, 305] Given that prebiotics have a very low toxicity and have established benefits for the host, these products present a great added value from a sanitary and economic point of view.

Their direct prebiotic properties, such as the production of SCFA and the selective promotion of host-friendly bacteria,[327, 328] have led to the consideration of NDOS in the treatment or prevention of conditions like constipation, diarrhea, IBD, NEC, septic shock, diabetes and allergies to dietary protein.[312-314, 327, 328, 355-357]

In addition to the above, these NDOS may exert different actions independent of their prebiotic activity. For instance, they inhibit the adhesion of pathogenic bacteria to human epithelial cells *in vitro*, acting as a decoy receptor.[331, 332] They directly affect IEC and modulate their gene expression.[339] Prebiotics modulate cytokine production in IEC and in human cord blood mononuclear cells *in vitro*,[343, 344] potentially leading to a more balanced Th1/Th2 response. Moreover, NDOS provide sialic acid as a potentially essential nutrient for brain development and cognition,[333] and regulate the intestinal transport and permeability.[350, 353]

Our first objective in this Thesis is to assess the direct, bacteria-independent, non-prebiotic activity of traditionally used prebiotics such as inulin, FOS, GOS and GMOS on monocytes and T cells.[306, 308, 316, 323]

II. *Validate the possible use of prebiotics in the treatment of IBD*

IBD is regularly managed pharmacologically with drugs that downregulate the immune system such as corticoids, infliximab, aminosalicylates or azathioprine. All of these agents have a plethora of serious adverse effects which limit their application and they are not effective in all patients. Hence the search for new treatments with a low profile of adverse effects is much warranted.[1]

Many animal studies have demonstrated the effectiveness of prebiotics in preventing and treating models of IBD, although these results often differ depending upon the compound used. Clinical studies range from small evidence of benefit to no effects,[381, 387] although in this scenario the experimental conditions, which may be critical for the success of the therapy, are less flexible and therefore it is possible to have an effective treatment fail, for instance for an inadequate dose. Animal studies have employed chemically-induced models of colitis, namely TNBS and DSS, as well as other gene-targeted rodent models of spontaneous colitis, as HLA-B27 transgenic rats.[315, 380] While profusely used for preclinical testing, the TNBS and DSS models present several disadvantages because they are not strictly chronic (i.e., they heal with time) and they are not lymphocyte-driven as in the human disease. In consequence, further studies are necessary to elucidate the mechanism involved in the beneficial effect of these compounds in intestinal function and their implication in human intestinal inflammation. Some authors have advocated the use of the T cell transfer model of colitis to achieve a better prediction of human bioactivity.[388]

In order to fully validate the possible use of prebiotics such as FOS in IBD, it is important to demonstrate their bioactivity in such a model. Hence, our second objective is to verify the anti-inflamatory effect of FOS in the CD4+ CD62L+ T cell transfer model of colitis, and to establish the ideal conditions for clinical testing.

III. ***Verify the importance of the microbiota in the onset of IBD***

It is well known that we humans travel with a heavy luggage made up of ~10^{14} prokaryotic organisms, mostly bacteria, but also viruses and fungi. The GI tract is the home of the largest bacterial population, which is maximal in the cecum, followed by the colon and then ileum, jejunum and finally duodenum.[389] The realization of this fact immediately prompts the question, what are these germs for? The answer to this question is not as easy as it might seem. The study of laboratory animals in GF conditions, available now for ~50 years, soon revealed that mice and rats survive fairly well without bacteria. Reproduction and overall appearance and physiology are essentially normal. In fact, it was shown early on that GF mice survive much longer than the conventionally reared mice,[390] and this seemed to be the case also for rats.[391] This effect may be

dependent on age, so that absence of bacteria at an early age extends life while it may shorten it at later stages.

The gut maintains a complex relationship with the intestinal microbiota, probably more obliged than strictly symbiotic. Despite the enormous bacterial load carried by the GI tract and the sheer variety of species present, an exquisite balance is maintained at almost all times. The combination of an efficient, self repairing barrier, abundant mucus secretion, continuous luminal flow of contents, and a vigorous yet finely regulated immune system is capable of keeping a massive foreign population contained within the limits of the mucosa. This delicate equilibrium represents a well balanced opposition of considerable forces. However, this equilibrium can be altered substantially, resulting typically in inflammatory responses, as in IBD. Thus, intestinal inflammation may be the consequence both of an enhanced immune response or of a defect in barrier function.[142]

There is substantial evidence pointing at a deregulated immune response toward the normal microbiota as a pivotal factor in IBD. One of the main arguments that support the importance of the microbiota in this context is the fact that intestinal inflammation is very difficult to induce in experimental animals in GF conditions. A notable exception however is DSS colitis, which has been reported to be elicited in GF conditions with similar or enhanced severity than that in regular mice.[270, 271] Hence luminal bacteria may not play a key role in DSS colitis.

Thus, the third objective is to clarify this issue, i.e., verify the importance of the microbiota in the onset of IBD, comparing the colitis-inducing effect of DSS in mice reared in regular and GF conditions, as well as in an antibiotic-induced microbiota depletion model (“pseudo germ-free” -PGF- conditions).

Material & Methods

I. *Ethics statement*

All human samples were obtained upon informed consent given by the subjects and the protocol was approved by the Human Research Ethics Committee of the University of Granada (Granada, Spain). All animal procedures in this study were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and were approved by the Animal Experimentation Ethics Committee of the University of Granada (registry number 710).

II. *Experimental subjects*

Blood samples were collected from healthy volunteers, namely 10 subjects (6 females, 4 males, aged between 24-27 years). Conventional hemogram tubes were used for blood extraction (ethylenediaminetetraacetic acid (EDTA) - k_2 BD Vacutainer®). Blood extraction was carried out in the Hospital Universitario Virgen de las Nieves, Granada, Spain.

III. *Experimental animals*

All experimental animals were housed in air conditioned animal quarters with a 12 h light-dark cycle and were given free access to autoclaved tap water and food (Harlan-Teklad 2014, Harlan Ibérica, Barcelona, Spain) (**table 3**). All animals were sacrificed by cervical dislocation under isoflurane anesthesia.

1. Wistar rats.

28 female Wistar rats, obtained from Janvier (Janvier, Le Genest Saint Isle, France) were used. Rats were maintained at the Department of Biochemistry and Molecular Biology II (DBMB) (School of Pharmacy, University of Granada, Granada, Spain).

2. C57BL/6J wild type and TLR4$^{-/-}$ mice.

10 male B6.B10ScN-Tlr4$^{lps\text{-}del}$/JthJ (TLR4 KO or TLR4$^{-/-}$) mice, and their corresponding background-mates, 27 C57BL/6J wild type (C57BL/6J WT) mice, were used. Mice were purchased from Jackson (Jackson Laboratory, CA, USA) and were maintained in DBMB.

3. C57BL/6J wild type and Rag1$^{-/-}$ mice.

7 female C57BL/6J wild type and 22 Rag1$^{-/-}$ mice (C57BL/6J background) were obtained from Jackson (Jackson Laboratory, CA, USA) and were maintained at the

University of Granada Animal Facility (UGAF) (Biomedical Research Center, University of Granada, Granada, Spain).

4. Conventional and germ-free NMRI mice.

43 conventional (20 females and 23 males) and 40 GF NMRI mice (half females and half males) were used. All of them were obtained from Karolinska Institutet Core Facility for Germ-free Research (CFGR) (Comparative Medicine, Karolinska Institutet, Stockholm, Sweden). Conventional NMRI mice were raised and maintained in DBMB. GF NMRI mice were raised and maintained in Karolinska Institutet CFGR. The experiments were conducted at the respective institutions.

Table 3. Experimental animals

Animal species	Strain	Category	Weight (g ± SEM)	Sex	Used animals	Supplier/ maintenance	Purpose
Rat	Wistar	Haloxenic	217.3 ± 5.1	F	28	Janvier/ DBMB	Non-prebiotic (1)
Mouse	C57BL/6J	Haloxenic	29.5 ± 0.9	M	9	Jackson/ DBMB	Non-prebiotic (1)
		Heteroxenic (SPF)	27.3 ± 0.5	M	18		DSS (2)
		Heteroxenic (SPF)	19.2 ± 0.7	F	7	Jackson/ UGAF	Transference (donor) (3)
Mouse	B6.B10ScN-*Tlr4*$^{lps\text{-}del}$/JthJ	Heteroxenic (SPF)	30.2 ± 1.1	M	10	Jackson/ DBMB	Non-prebiotic (1)
Mouse	B6.129S7-*Rag1*^{tm1Mom}/J	Heteroxenic (SPF)	21.5 ± 0.2	F	22	Jackson UGAF	Transference (receptor) (4)
Mouse	NMRI	Haloxenic	29.6 ± 0.8	M	23	CFGR/ DBMB	DSS (2)
			20.1 ± 0.5	F	20		DSS (2)
Mouse	NMRI	Axenic (GF)	32.6 ± 0.6	M	20	CFGR/ CFGR	DSS (2)
			25.7 ± 0.4	F	20		DSS (2)

CFGR: Core Facility for Germ-free Research. DBMB: Department of Biochemistry and Molecular Biology II. DSS: dextran sulfate sodium. F: female. GF: germ-free. M: male. SPF: specific pathogen free. UGAF: University of Granada Animal Facility.

Mutation	Background Strain	Donor strain
Tlr4$^{lps\text{-}del}$ (5)	C57BL/6J	C57BL/10ScN
Rag1^{tm1Mom} (6)	C57BL/6J	129S7 via AB1 ES cell line (+$^{Hprt\text{-}bm2}$)

(1) Determining non-prebiotic effects (**obj. 1**)
(2) DSS-induced colitis (**obj. 3**)
(3) T cell transfer colitis (cell donor) (**obj. 2**)
(4) T cell transfer colitis (cell receptor) (**obj. 2**)

(5) Defective response to LPS stimulation
(6) Produce no mature T cells or B cells

IV. *Animal housing*

1. Haloxenic conditions.

Animals were housed in makrolon cages.

2. Heteroxenic conditions (specific pathogen free).

i. Department of Biochemistry and Molecular Biology II. Animals were housed per groups in makrolon cages equipped with 45 µm filter sheets (Tecniplast, 1290D420R, Buguggiate, VA, Italy). Males and females were kept separately.

ii. University of Granada Animal Facility. Animals were housed per groups in Individual Ventilated Cages (IVC) with an air insufflation and exhalation system with dual filter (pre-filter and HEPA filter). Males and females were kept separately.

3. Axenic conditions (germ-free).

Newborn litters of GF NMRI mice were placed and raised in special plastic isolators until they reached 16 to 18 weeks of age. Animals were maintained on autoclaved R36 Lactamin Chow (Lactamin). Males and females were kept separately. The GF status was checked weekly as routinely quality control by culturing fecal samples, both aerobically and anaerobically, at 20ºC and 37ºC for up to 4 weeks.

V. *Buffers and culture media*

- Antibiotic cocktail for depletion of colonic microbiota: ampicillin 1 $g \cdot l^{-1}$ (Applichem, Darmstadt, Germany), neomycin 1 $g \cdot l^{-1}$, metronidazole 0.25 $g \cdot l^{-1}$ and vancomycin 0.5 $g \cdot l^{-1}$.
- Antibiotic enriched PBS solution (antibiotic-PBS solution) with 500 $U \cdot ml^{-1}$ penicillin, 0.5 $mg \cdot ml^{-1}$ streptomycin and 12.5 $mg \cdot ml^{-1}$ amphotericin B.
- Cold saline buffer: 0.9% NaCl in distilled water.
- DMEM: Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%), 2 mM L-glutamine, 100 $U \cdot ml^{-1}$ penicillin, 0.1 $mg \cdot ml^{-1}$ streptomycin and 2.5 $mg \cdot ml^{-1}$ amphotericin B.
- Hanks' balanced salt solution (HBSS).
- Hypotonic lysis buffer: 0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM $Na_2EDTA \cdot 2H_2O$, pH

7.3.

- Glycine buffer: 50 mM glycine buffer with 0.5 mM $MgCl_2$, pH 10.5.
- MACS buffer: 0.5% bovine serum albumin (BSA) and 2 mM EDTA in PBS, pH 7.2.
- MPO buffer: 50 mM Tris base buffer with 0.5% Hexadecyl Trimethyl Ammonium Bromide (HTAB), pH 6.0.
- RPMI-1640 medium containing fetal bovine serum (FBS 10%), 2 mM L-glutamine, 100 $U \cdot ml^{-1}$ penicillin, 0.1 $mg \cdot ml^{-1}$ streptomycin, 2.5 $mg \cdot ml^{-1}$ amphotericin B and 0.05 mM mercaptoethanol.

VI. *Reagents*

Except where indicated, all reagents and primers were obtained from Sigma-Aldrich® (Madrid, Spain). Dynabeads® mRNA Purification Kit was obtained from Invitrogen (Madrid, Spain). Reverse transcription was achieved with the iScript™ cDNA Synthesis Kit and iQ™ Sybr® Green Supermix was used for amplification (Biorad, Alcobendas, Madrid, Spain). Human and rat ELISA kits were obtained from BD OptEIA™ (Madrid, Spain), except for human TNF-α ELISA kit, obtained from Invitrogen (Madrid, Spain). Mouse ELISA kits were obtained from eBioscience (San Diego, CA, USA). All the primary antibodies used in the magnetic cell separation were purchased from BD Pharmingen™ (Madrid, Spain); MACS Column, anti-Biotin, anti-PE and CD62L MicroBeads, and CD4+ CD62L+ T Cell Isolation Kit II from MACS Miltenyi Biotec (Cologne, Germany). DSS (cat Nº 160110) was obtained from ICN Biomedicals (Costa Mesa, CA, USA); average molecular weight: 36-50 kDa. Reinforced Clostridial Agar, MRS. Agar, AnaeroGen™ and CO2Gen™ pouches, and plastic anaerobic jars were purchased from Oxoid (Hampshire, England); Wilkins-Chalgren Agar from BD Pharmingen™ (Madrid, Spain); Blood Agar from Panreac (Barcelona, Spain). 70 µm nylon filters (Ref. 352350) were obtained from BD Falcon™ (Madrid, Spain); Vitro-Clud® Mounting Medium from Deltalab (Barcelona, Spain).

VII. *Non-digestible oligosaccharides*

NDOS used in this study were FOS, inulin, GOS and GMOS. FOS and inulin were kindly provided by BENEO Orafti® (Tienen, Belgium). Orafti® GR (inulin) is a food ingredient consisting mainly of chicory root inulin, a mixture of oligo- and polysaccharides

which are composed of fructose units linked together by β(2-1) linkages. Almost every molecule is terminated by a glucose unit. The total number of fructose or glucose units (DP) of chicory inulin ranges mainly between 2 and 60. Orafti® P95 oligofructose (FOS) is produced by the partial enzymatic hydrolysis of chicory-derived inulin, consisting mainly of molecules with DP between 2 and 8.

GOS were a kind gift from Vivinal FrieslandCampina Domo (Needseweg, Holland). Vivinal® GOS Syrup (GOS) (product number 502675) contains 57% oligosaccharides on dry matter. The stock of GOS at 5 $g \cdot l^{-1}$ was prepared taking into account the content of oligosaccharides on dry matter.

GMOS were obtained by Dr. Guadix, working in collaboration with us, as previously described.[325] Briefly, pasteurized skimmed goat milk was used as source and a two-stage tangential ultrafiltration nanofiltration process was selected. Multitubular ceramic Ceram Inside membranes (TAMI Industries) made of ZrO_2-TiO_2, with three channels (25 cm long) of 3.6 mm hydraulic diameter, membrane area of 0.0094 m^2, and molecular weight cutoffs of 50 and 1 kDa, respectively, were used. There were two separate, consecutive and continuous, diafiltration steps. The cumulated permeate from the first stage was collected and used as initial retentate in the second step. The retentate from the second step, containing the oligosaccharide fraction, was lyophilized. Quantification of oligosaccharides was performed by high-pH anion-exchange chromatography with pulsed amperometric detection with use of a Carbo Pac PA-1 column (250 x 4.6 mm i.d.) connected to a Dionex system equipped with a pulsed electrochemical detection and a Foxi Jr.® Fraction Collector (Isco Inc., Lincoln, NE, USA). Solutions of oligosaccharide standards were used to identify and quantify oligosaccharide peaks obtained in the chromatograms. A product containing > 80% of the original oligosaccharide content, only 5% ($w \cdot w^{-1}$) of lactose and virtually salt free, was obtained and used to carry out all the experiments.

VIII. *Sterile bacterial homogenate*

Faeces from conventional mice were collected and frozen at -80ºC. The day after, they were homogenized in a 1:4 proportion in distilled water, sonicated 15 min at 4ºC and spun (6164 g/10 min/4ºC). The supernatant was collected and spun again to remove debris

(4473 g/10 min/4ºC). The new supernatant was sonicated 10 min at 4ºC and filtered using a 70 µm nylon filter firstly and a 0.20 µm filter (Sarstedt, Ref. 83.1826.001) secondly. The filtering were frozen at -80ºC and lyophilized afterwards. The diet was made by Lantmännen Lantbruk (Kimstad, Sweden). 1 g SBH per 6 kg of chow was incorporated and pelletized.

IX. *Culture isolation, explants and in vitro experimental design*

Except were indicated, cells were plated at a density of $1 \cdot 10^6 \cdot ml^{-1}$ and a final volume of 500 µl in 24-well plates in a humidified 5% CO_2 atmosphere at 37ºC.

1. Mice spleen mononuclear cell (MSMC) isolation. Splenocytes.

Mice were sacrificed by cervical dislocation and the spleen was extracted aseptically. Cell suspensions were obtained by disrupting the tissues between dissecting forceps in DMEM medium. After centrifuging, cells were cleared of erythrocytes by suspension on hypotonic lysis buffer for 30 min on ice. Cells were washed once with fresh medium and were filtered using a 70 µm nylon filter to obtain a mononuclear suspension. Cells were plated at a density of $2 \cdot 10^6 \cdot ml^{-1}$ in DMEM medium and were stimulated with LPS (*E. coli* 055:B5) at a final concentration of 1 $\mu g \cdot ml^{-1}$. Cell culture medium was collected after 48 h.

2. Mesenteric lymph node cell (MLNC) isolation.

Mesenteric lymph nodes were extracted from the mice in the study using sterile technique and dissected mechanically. Cells were washed once with RPMI fresh medium and were filtered using a 70 µm nylon filter to obtain a mononuclear suspension. Cells were cultured in RPMI medium and were stimulated with concanavalin A (ConA) at a final concentration of 5 $\mu g \cdot ml^{-1}$. Cell culture medium was collected after 48 h.

3. Rat splenic monocyte and T lymphocyte isolation.

Female Wistar rats were sacrified by cervical dislocation and the spleen was extracted aseptically. Mononuclear suspensions were obtained as explained before (splenocytes). Using an antibody cocktail for either monocytes or lymphocytes, cells were separated magnetically with negative staining using Miltenyi technology (**fig. 10**). The primary antibodies used were CD11b-biotin (1:200), CD161a-biotin (1:200) and CD45RA-PE

(1:200) for lymphocytes; and CD161a-biotin (1:200), CD45RA-PE (1:200) and CD3-biotin (1:150) for monocytes. The cells were first incubated for 30 min with the primary antibodies and then with the magnetic anti-biotin and anti-PE MicroBeads. After the incubation, the cells were passed through the magnet and non-labeled cells were collected. Isolated monocyte and T lymphocyte populations were used separately. Separation and purification protocols were set up and validated by flow cytometry, using FACSCalibur™ (BD Biosciences®, California, USA). Monocytes were cultured in DMEM medium and were stimulated with LPS 1 µg·ml^{-1}. Lymphocytes were cultured in RPMI medium and were stimulated with ConA 5 µg·ml^{-1}. Cell culture medium was collected after 24 h for monocytes and 48 h for lymphocyte.

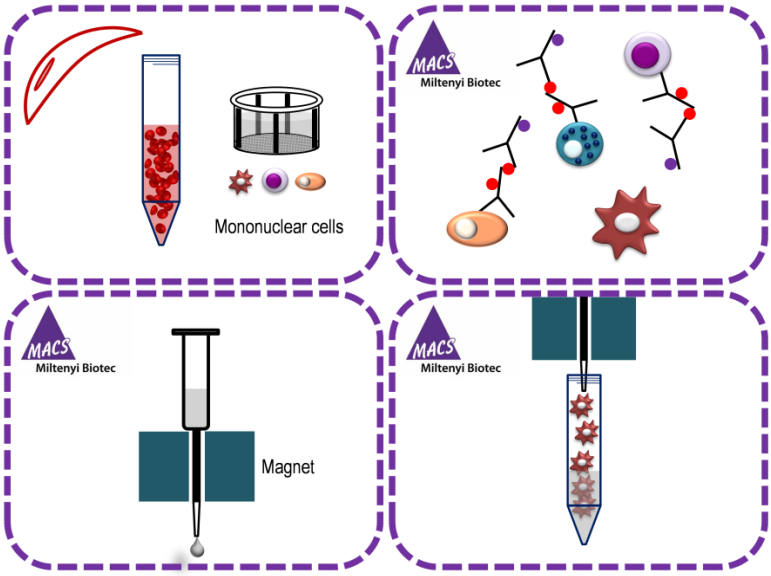


Figure 10. Rat splenic monocyte and T lymphocyte isolation with Miltenyi negative staining.

4. Macrophage-conditioned medium cultures.

Once rat splenic monocytes were isolated, they were cultured with the different treatments. After 24-hours incubation, supernatant was removed from the wells, spun (9.300 g/10 min/4ºC) and added to the just isolated rat splenic lymphocytes in a 1:1 proportion (250 µl DMEM-conditioned medium + 250 µl RPMI medium).

5. Human peripheral blood monocyte (hPBM) isolation.

9 ml per subject of peripheral blood was centrifuged (2.100 g/10 min/4ºC), ensuring that the blood was not hemolysed. Mononuclear cells were isolated by density centrifugation using Percoll (GE Healthcare, Bucks, UK). Cells were rinsed once with HBSS and plated. After 1 h in a cell culture incubator with 5% CO2/95% air at 37ºC, cells were rinsed 3 times with HBSS to remove non-adherent cells. Cells were stimulated with LPS at a final concentration of 1 $\mu g \cdot ml^{-1}$. Cell culture medium was collected after 24 h.

6. Mice colonic tissue fragments (explants).

TLR4 KO and C57BL/6J WT mice were sacrificed by cervical dislocation. The colon was removed and longitudinally opened so as to exhaustively eliminate fecal remains with an antibiotic-PBS solution. Next, colon surface was divided into several equal portions (approximately 0.5 $cm^2 \cdot fragment^{-1}$) and stored until culture onset in fresh antibiotic-PBS solution at 4ºC. After an hour of incubation with the different treatments, LPS was added at a final concentration of 10 $\mu g \cdot ml^{-1}$. When the culture period was over, 24 hours, culture supernatant was collected, as well as mice tissue fragments, and stored at -20ºC.

i. Protein extraction from explants. Explants were homogenized in cold saline buffer. The protein content was measured by the bicinchoninic acid (BCA) assay,[392] using BSA as standard.

7. Cell viability assay.

Cell viability was quantified with the Trypan blue exclusion assay. Cell suspensions were diluted 1:10 for MLNC, monocytes and lymphocytes, and 1:20 for splenocytes in 0.4% Trypan blue in PBS, incubated 2 min while shaking, and viable (unstained) and total cells were counted.

8. *In vitro* experimental design.

hPBM, rat splenic T lymphocytes and WT/TLR4 KO mouse splenocytes were cultured with the different NDOS at a concentration of 5 $g \cdot l^{-1}$. Rat splenic monocytes were cultured with the different NDOS at different concentrations ranging from 0.005 $g \cdot l^{-1}$ to 5 $g \cdot l^{-1}$. Additionally, the effect of macrophage-conditioned medium over lymphocytes was

studied. Mice explants were also cultured with the same NDOS. In some cases, specific cell signaling inhibitors were added to cells 30 min prior to treatment with the NDOS, namely Bay 11-7082 (inhibitor of IκB-α phosphorylation, 10 µM), wortmannin (inhibitor of PI3K, 1 µM, phosphatidylinositol-3-kinase), SB203580 (p38 MAPK inhibitor, 10 µM), PD98059 (ERK1/2 MAPK inhibitor, 10 µM) or SP600125 (JNK inhibitor, 10 µM).

X. *Colitic models and in vivo experimental design*

1. DSS-induced colitis. Conventional and germ-free NMRI mice.

Colitis was induced by adding 1-4% DSS to drinking water for 7 days. Some GF mice also received SBH in the diet. The status of the animals was monitored by general examination and specifically by means of the DAI (Disease Activity Index), a combined score for weight loss, diarrhea and hematochezia, which are 3 main signs of pathology in this model. Food intake, water intake and body weight were measured every day.

Mice were randomly assigned as follows. Conventional mice were divided into control (n=11), 1% DSS (n=11), 2% DSS (n=11) and 4% DSS (n=10) (**fig. 11**). GF mice were divided into control (n=8), 2% DSS (n=8), 4% DSS (n=8), 2% DSS + SBH (n=8) and 4% DSS + SBH (n=8) (**fig. 12**).

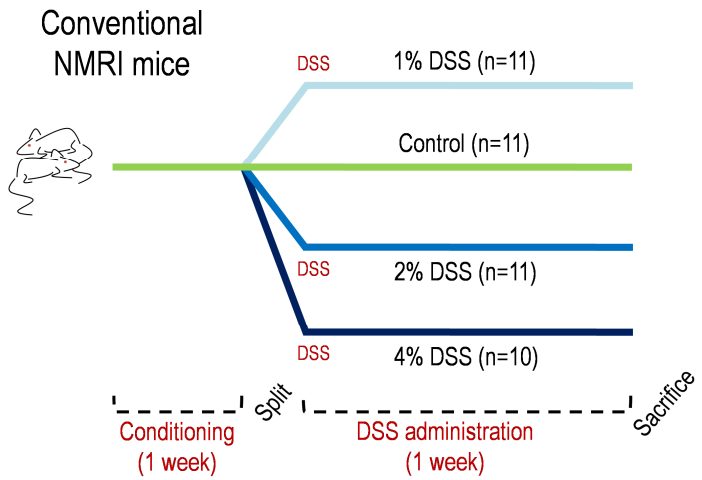


Figure 11. Dextran sulfate sodium (DSS)-induced colitis experimental design (I).
Conventional NMRI mice.

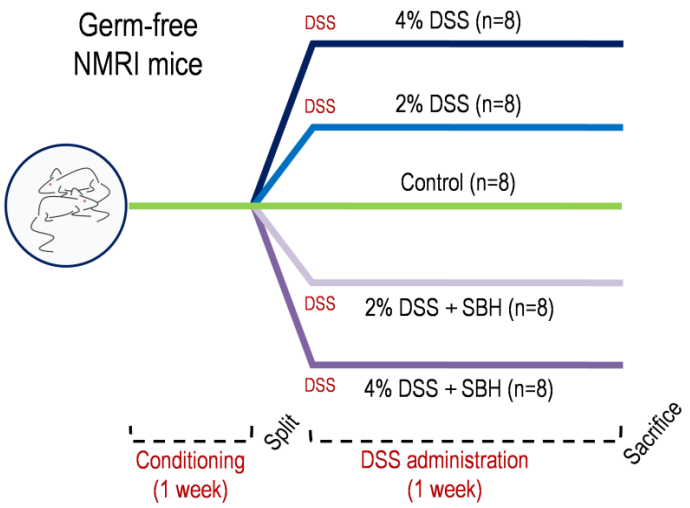


Figure 12. Dextran sulfate sodium (DSS)-induced colitis experimental design (II).
Germ-free NMRI mice.

The control groups did not receive DSS. The same batch of DSS was used both in conventional and GF mice to avoid differences in the activity. The remainder mice drank DSS supplemented water in the indicated proportion ($w \cdot v^{-1}$). Animals were sacrificed after 7 days by cervical dislocation under isoflurane anesthesia.

2. DSS-induced colitis. Conventional and pseudo germ-free C57BL/6J mice.

Acquired depletion of colonic microbiota was achieved in C57BL/6J mice (PGF mice) by the administration of an antibiotic cocktail in the drinking water. To test significant bacterial reduction, DNA from faeces was extracted with QIAamp® DNA Stool Mini kit (QIAGEN®, Hilden, Germany), quantified and analysed for total 16S by qPCR with a Stratagene (La Jolla, CA, USA) MX3005P real time PCR device, using a non-variant amplicon (16S sense: TCC TAC GGG AGG CAG CAG T; antisense: GGA CTA CCA GGG TAT CTA ATC CTG TT). Antibiotic treatment was applied for 4 weeks before DSS was started and was maintained until the end of the experiment. The same protocol used for conventional and GF NMRI mice was applied for conventional and PGF C57BL/6J mice, except that only one DSS dose was used, namely 3%. Mice were distributed into control (n=4), 3% DSS (n=4), pseudo germ-free control (PGF control, n=3) and pseudo germ-free 3% DSS (PGF 3% DSS, n=7) (**fig. 13**). The control groups did not receive DSS. The same batch of DSS was also used for these animals.

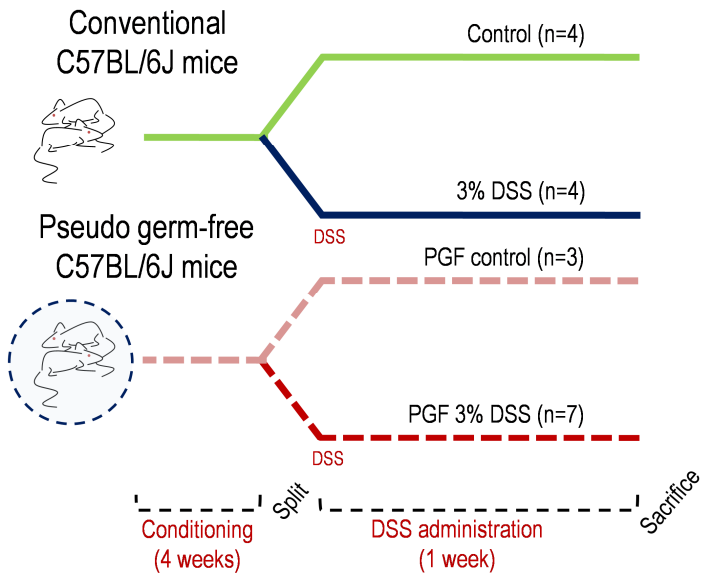


Figure 13. Dextran sulfate sodium (DSS)-induced colitis experimental design (III).
Conventional and pseudo germ-free C57BL/6J mice.

3. CD4+ CD62L+ T cell transfer colitis.

Female C57BL/6J mice were sacrificed by cervical dislocation and the spleen was extracted aseptically. Cell suspensions were obtained by disrupting the tissues between dissecting forceps in DMEM. After centrifuging, cells were cleared of erythrocytes by suspension on hypotonic lysis buffer for 30 min on ice. Cells were filtered using a 70 µm nylon filter to obtain a mononuclear suspension. Mononuclear cells were washed and resuspended in MACS buffer. CD4+ CD62L+ T cell isolation from spleen cells was performed using CD4+ CD62L+ T Cell Isolation Kit II. First, non-CD4+ T cells were indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies and anti-Biotin MicroBeads. The labeled cells were subsequently depleted by separation over a MACS Column. In the second step, CD4+ CD62L+ T cells were directly labeled with CD62L (L-selectin) MicroBeads and isolated by positive selection from the pre-enriched CD4+ T cell fraction. The CD4+ CD62L+ T cells were eluted in 100 µl of sterile PBS and administered intraperitoneally into C57BL/6J $Rag1^{-/-}$ mice (10^6 CD4+ CD62L+ T cells·$mouse^{-1}$). Wild type control mice were administered PBS sterile.

The status of the animals was monitored by general examination and specifically controlling body weight evolution, beginning the experiment after a 10% of body weight loss, about 8 weeks after the transfer. Colitic mice were randomly assigned to 2 different groups. The FOS CD4+ CD62L+ group (FOS, n=8) received by gavage 75 mg·day^{-1} of FOS, while the transfer colitic group (colitic, n=8) was administered vehicle (saline). A healthy control group (non colitic, NC, n=6) was also included in the experiment (**fig. 14**). Treatment was maintained until animals were sacrificed after 13 days by cervical dislocation under isoflurane anaesthesia. Food and water intake were measured every day.

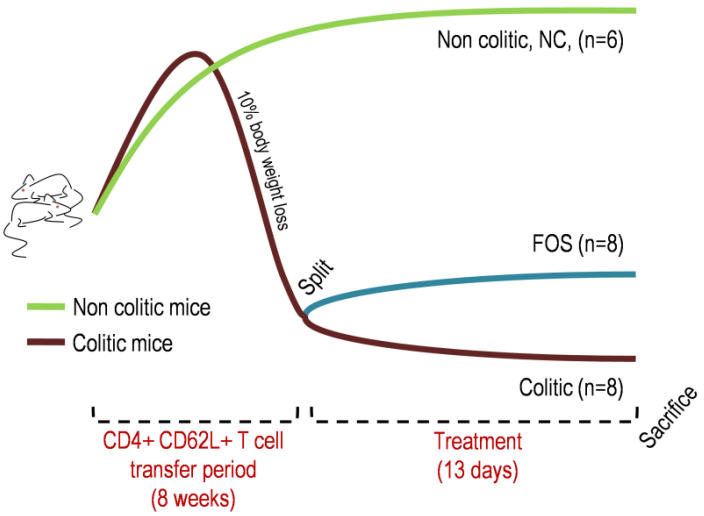


Figure 14. CD4+ CD62L+ T cell transfer colitis experimental design.

XI. *Assessment of colonic status*

1. Sampling.

After the animals were sacrificed, different organs were obtained: colon, duodenum, jejunum, ileum, liver, spleen and mesenteric lymph nodes, plus a blood sample. Animal faeces were also obtained. The entire colon was removed, gently flushed with saline and blotted on filter paper, placed on an ice-cold plate, cleaned of fat and mesentery and longitudinally opened so as to exhaustively eliminate fecal remains. Each specimen was weighed and its length measured under a constant load (2 g). A small segment was dissected from the intestine and used for RNA isolation. The colon was subsequently divided longitudinally in several pieces for biochemical determinations. The fragments

were immediately frozen in liquid nitrogen and kept at -80ºC until used.

2. Assessment of colonic damage.

i. DSS-induced colitis. The large intestine was longitudinally opened and scored for visible damage by a blinded observer on a 0 to 13 scale according to the following criteria: adhesions (0-3), hyperemia (0-3), fibrosis (rigidity, 0-3), deformation (0-2) and thickening (0-2).

ii. CD4+ CD62L+ T cell transfer colitis. The large intestine was longitudinally opened and scored for visible damage by a blinded observer on a 0 to 7 scale. The score was assigned as follows: adhesions (0-2), hyperemia (0-2), fibrosis (0-2) and thickening (0-1).

3. Histology.

Colon tissues were fixed in 4% paraformaldehyde ($w \cdot v^{-1}$). Five micrometer sections were obtained from paraffin-embedded colonic tissue and were placed on 3-amino propyl triethoxy silane coated slides. After being deparaffinized, sections were rehydrated in serial dilutions of ethanol and water. Sections were then stained with hematoxylin & eosin (H&E) and mounted. H&E staining was conducted to study morphology and check for integrity of the colonic tissue. Images were captured and digitalised using a Leica DMI3000B microscope equipped with a Leica DFC420 C Camera.

4. Inflammatory markers: myeloperoxidase and alkaline phosphatase activities.

Colonic tissue homogenization was carried out with the Protocol for Intestinal Tissue Homogenization in the Bullet Blender® (Next Advance, Inc., NY, USA). MPO activity was measured spectrophotometrically as the peroxidase enzymatic activity extracted from colonic tissue after homogenization (1:20 $w \cdot v^{-1}$) in MPO buffer. The homogenate was sonicated and subjected to 3 freeze-thaw cycles before measurement. The aim of this procedure is to allow efficient leakage of MPO from azurophilic granules in neutrophils. The enzymatic reaction was performed in MPO buffer with 0.0005% hydrogen peroxide and 0.168 $mg \cdot ml^{-1}$ o-dianisidine as substrate. Alkaline phosphatase (AP) activity was measured spectrophotometrically, using 5.5 mM disodium nitrophenyl phosphate as

substrate in glycine buffer. The samples were homogenized normally in saline. The sensitivity to the AP inhibitor levamisole was also tested. The protein content was measured by the BCA assay. AP and MPO enzymatic activities are expressed as mU·mg protein^{-1}.[253, 393, 394]

5. Colonic gene expression analysis. RNA isolation and purification. Quantitative Reverse-transcription Polymerase Chain Reaction (qRT-PCR).

Total RNA was isolated by the Trizol method (Invitrogen, Barcelona, Spain) and checked for integrity by electrophoresis in 1% agarose gel (w·v^{-1}). Orally administered DSS interferes with qPCR amplification of cDNA derived from multiple tissues.[395] Thus, Dynabeads® mRNA Purification Kit was used for the purification of DSS colonic mRNA from total RNA, following the protocol given by the manufacturer. In brief, the isolation protocol relies on base pairing between the poly A residues at the 3' end of most mRNA and the oligo $(dT)_{25}$ residues covalently coupled to the surface of the Dynabeads. Quantification was determined by the 260/280 nm absorbance ratio. 1 µg mRNA was subjected to reverse transcription, iQ™ Sybr® Green Supermix was used for amplification and specific DNA sequences were amplified with a Stratagene (La Jolla, CA, USA) MX3005P real time PCR device. The primers used are shown in **table 4**. Results are expressed as 2^{-ddCt} using GAPDH as reference gene

XII. *General techniques*

1. Cytokine determination by Enzyme-linked Immunosorbent Assay (ELISA).

When the culture period was over, culture supernatant was collected, spun (9.300 g/10 min/4ºC), aliquoted and frozen at -80ºC until assayed for cytokine content by commercial ELISA, following the protocols recommended by the manufacturer. The human cytokines determined were IL-1β, IL-8, IL-10 and TNF-α; rat cytokines were IL-1β, IL-2, IL-10, GRO-α (growth-regulated oncogene α), IFN-γ and TNF-α; and mouse cytokines determined were IL-6, IL-10, IL-17, IFN-γ and TNF-α. Plates (Nunc™ Inmuno plate, Roskilde, Denmark) were read at 450 nm using a plate reader (Tecan, model Sunrise-basic, Austria). Results are expressed as cytokine concentration (pg·ml^{-1}).

Table 4. Mouse primers (sequence 5'-3')

	Forward	Reverse
GAPDH	CAT TGA CCT CAA CTA CAT GG	GTG AGC TTC CCG TTC AGC
IL-1β	AAG GGC TGC TTC CAA ACC TTT GAC	TGC CTG AAG CTC TTG TTG ATG TGC
IL-10	CAG GAC TTT AAG GGT TAC TTG	ATT TTC ACA GGG GAG AAA TC
IL-17A	ACG TTT CTC AGC AAA CTT AC	CCC CTT TAC ACC TTC TTT TC
IL-22RA-1	CTG TTA TCT GGG CTA CAA ATA C	GTA CGT GTT CTT GGA TGA AG
IL-27RA	AAA CCT CAG CAC ATT GTT AC	TAC TAA CTC CTC TCT GAA TCC
IFN-γ	GCT CTG AGA CAA TGA ACG CTA CAC	TTC TTC CAC ATC TAT GCC ACT TGA G
TNF-α	CGT GGA ACT GGC AGA AGA GG	CAG GAA TGA GAA GAG GCT GAG AC
ZO-1	GGG GCC TAC ACT GAT CAA GA	TGG AGA TGA GGC TTC TGC TT
OCCLUDIN	ACG GAC CCT GAC CAC TAT GA	TCA GCA GCA GCC ATG TAC TC
TFF3	CCT GGT TGC TGG GTC CTC TG	GCC ACG GTT GTT ACA CTG CTC
MUC3	AAA GAT TAC CTC CCA TCT CC	TAA AAC TAA GCA TGC CCT TG
KGF	AAA GAA CGG CAG TAA ATA CG	CCA GCA TCC TCA AAA GTT AC
S100A8	GCC CTC TAC AAG AAT GAC TTC AAG	ATC ACC ATC GCA AGG AAC TCC
REGIII-γ	CAG AGG TGG ATG GGA GTG GAG	CAC AGT GAT TGC CTG AGG AAG AG
FOXP3	AAT AGT TCC TTC CCA GAG TTC	GGT AGA TTT CAT TGA GTG TCC

2. Bacteriological analysis of faeces.

A sample of faeces was collected the day mice were killed and stored at -80ºC until analysis. A total of 0.1 g of fecal material was weighed and homogenized in 0.9 ml of PBS. Serial dilutions (10^{-1}-10^{-7}) were made and 30 µl aliquots of each dilution were plated on the different culture media: Blood Agar (total aerobes), Wilkins-Chalgren Agar (total anaerobes), MRS (Man, Rogosa and Sharpe) Agar (lactobacteria), and Reinforced Clostridial Agar (clostridia). The plates were subsequently incubated aerobically or anaerobically at 37ºC for different time periods: 24 h for total aerobes and anaerobes; 48 h for lactobacteria and 72 h for clostridia. For the growth of clostridia, samples were boiled for 10 min at 80°C, allowing the formation of spores. For the generation of anaerobic conditions or CO_2 enriched environment, plates were incubated in the appropriate jars using Oxoid AnaeroGen™ or CO2Gen™ pouches. All the colonies appearing on the appropriate dilution were counted. The results are expressed as the logarithm values of

the number of CFU per gram of weight of cecal content.

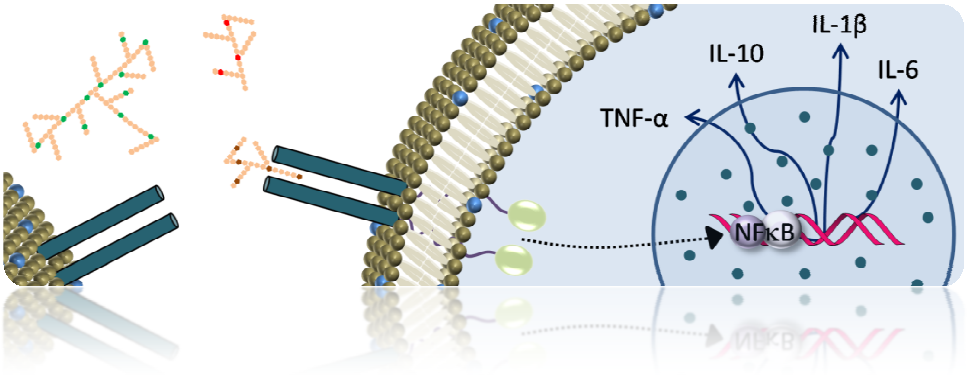
XIII. *Data and statistical analysis*

In all the experiments, samples were run at least in triplicate and results are expressed as mean ± standard error of the mean (SEM). In DSS-induced colitis, the obtained values are the result of the average values between males and females. The result is normalized to the protein content both in explant cytokine production as in MPO and AP activity. Graphs were made with the OriginPro 8 program (OriginLab Corporation, Northhampton, MA, USA). Differences among means were tested for statistical significance by One-way analysis of variance (ANOVA) and *a posteriori* Fisher LSD tests on preselected pairs. All analyses were carried out with the SigmaStat 3.5 program (Jandel Corporation, San Rafael, CA, USA). Differences were considered significant at $p<0.05$.

Results

Chapter I

Non-digestible oligosaccharides: determining non-prebiotic effects



I. *Effect of prebiotics on wild-type mouse splenocytes*

As a first approach, we examined the effect of NDOS on cytokine secretion by primary mouse splenocytes, which showed an increase in TNF-α secretion, in the order GMOS>inulin>FOS>GOS (**fig. 15A**). Under LPS stimulation TNF-α secretion was comparable in all groups; only inulin produced a slight enhancement, and GOS a small inhibition (**fig. 15B**). IL-6 was upregulated by GMOS, inulin and GOS, but not FOS (**fig. 15C**).

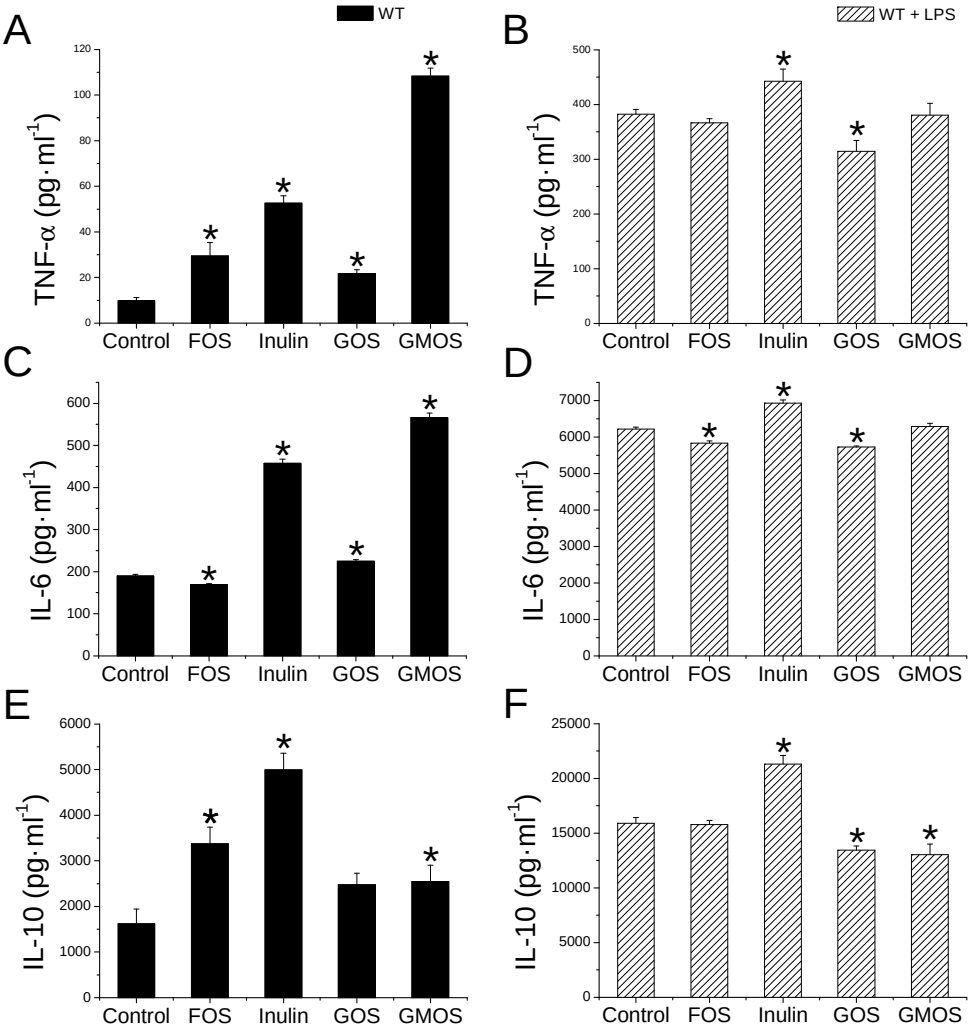


Figure 15. Effect of prebiotics on cytokine secretion by splenocytes from WT mice. Basal- (A, C, E) or LPS- 1 μg·ml^{-1} evoked secretion (B, D, F) is displayed. Cytokines measured are TNF-α (A, B), IL-6 (C, D) and IL-10 (E, F). Concentrations are expressed as means ± SEM (pg·ml^{-1}). *p<0.05 *vs.* control, n = 6 (IL-6) or 12 (TNF-α, IL-10). FOS: fructooligosaccharides. GMOS: goat milk oligosaccharides. GOS: galactooligosaccharides. WT mice: C57BL/6J wild type mice.

When splenocytes were stimulated with LPS there was very little additional effect of NDOS on IL-6 secretion (< 11.5%, **fig. 15D**). IL-10 was upregulated under basal conditions by inulin and FOS, and also by GMOS, albeit slightly (**fig. 15E**). LPS evoked a robust secretory response, which was further increased 34% by inulin and decreased by GOS and GMOS (15.6% and 18.1%, respectively, **fig. 15F**).

IFN-γ basal levels were below the detection level. FOS, GOS and GMOS caused a 25-59% inhibition of ConA-evoked secretion, while inulin had no effect (**fig. 16A**). IL-17 was also measurable only under ConA stimulation and was modestly inhibited by FOS and GOS (**fig. 16B**).

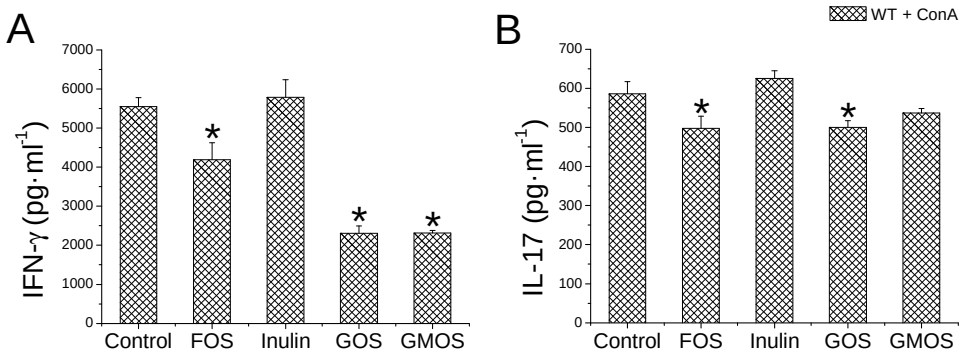


Figure 16. Effect of prebiotics on ConA-evoked cytokine secretion by splenocytes from WT mice. Cytokines measured are IFN-γ (A) and IL-17 (B). Concentrations are expressed as means ± SEM (pg·ml^{-1}). *p≤0.017 *vs.* control, n = 6. ConA: concanavalin A. FOS: fructooligosaccharides. GMOS: goat milk oligosaccharides. GOS: galactooligosaccharides. WT mice: C57BL/6J wild type mice.

II. *Effect of prebiotics in splenocytes from TLR4$^{-/-}$ mice*

We have previously established that NDOS bind and activate TLR4 receptors in IEC.[396] Therefore, we tested the possible involvement of this receptor in the effect of NDOS on splenocytes using TLR4$^{-/-}$ mice of the same genetic background (**figs. 17** and **18**). As expected, the results showed a much reduced cytokine secretion in these cells. In particular, the TNF-α response in TLR4$^{-/-}$ cells was severely curtailed in all cases (compare **figs. 17** and **15**) although LPS still produced a significant response (i.e., **fig. 17A** vs. **fig. 17B**). Inulin retained its relative effect in both basal and LPS stimulated conditions, while the other NDOS were practically devoid of effect. Similar effects were obtained for IL-6 secretion, except for a substantial increase of basal IL-6 levels brought

about by GMOS (**fig. 17C**). The effect of inulin on IL-10 secretion was also preserved in TLR4$^{-/-}$ cells, so that basal and LPS-induced release remained significantly elevated compared to the controls (**figs. 17E**, **F**). However, there was no effect with FOS. The inhibition of LPS-evoked secretion by GMOS was higher in these cells than in WT cells.

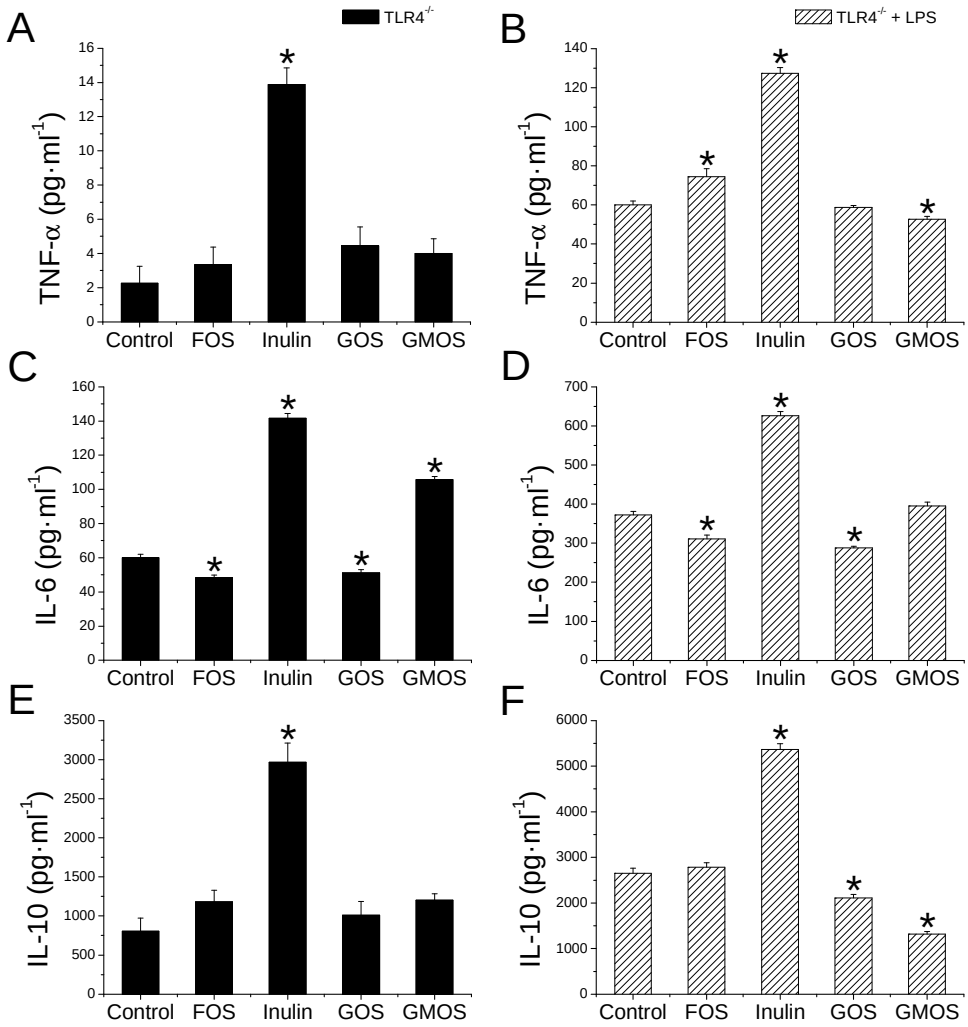


Figure 17. Effect of prebiotics on cytokine secretion by splenocytes from TLR4$^{-/-}$ mice. Basal- (A, C, E) or LPS- 1 µg·ml^{-1} evoked secretion (B, D, F) is displayed. Cytokines measured are TNF-α (A, B), IL-6 (C, D) and IL-10 (E, F). Concentrations are expressed as means ± SEM (pg·ml^{-1}). *p≤0.006 *vs.* control, n = 6 or 12 (basal TNF-α, basal IL-10). FOS: fructooligosaccharides. GMOS: goat milk oligosaccharides. GOS: galactooligosaccharides. TLR4$^{-/-}$ mice: B6.B10ScN-*Tlr4*$^{lps\text{-}del}$/JthJ.

In contrast with these effects, NDOS inhibition of IFN-γ levels was preserved in TLR4$^{-/-}$ cells (**fig. 18A**). In the case of IL-17 there was no further inhibition in TLR4$^{-/-}$ cells, but an

enhancement was carried out by inulin and GOS (51.8% and 26.8%, respectively, **fig. 18B**).

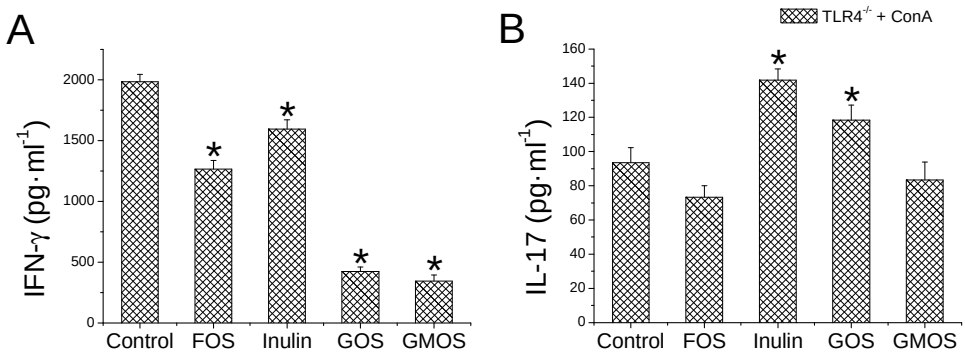


Figure 18. Effect of prebiotics on ConA-evoked cytokine secretion by splenocytes from TLR4$^{-/-}$ mice. Cytokines measured are IFN-γ (A) and IL-17 (B). Concentrations are expressed as means ± SEM (pg·ml^{-1}). *p<0.05 *vs.* control, n = 6. ConA: concanavalin A. FOS: fructooligosaccharides. GMOS: goat milk oligosaccharides. GOS: galactooligosaccharides. TLR4$^{-/-}$ mice: B6.B10ScN-*Tlr4*$^{lps\text{-}del}$/JthJ.

III. *Effect of prebiotics on* mice colonic tissue fragments (explants)

There was a profile-response comparable to that of mouse splenocytes, although non-significant due to a greater heterogeneity between samples (data not shown). However, some differences were observed. IL-6 and IL-10 release from TLR4 KO mouse explants was not reduced much as expected. On the other hand, IFN-γ and IL-17 were measurable under basal conditions, but with a very small release. In general, despite using a stimulus (LPS) 10 times greater than splenocytes, there was not a greater response. The only significant finding was an increased release of IL-10 given by the explants from WT mice when they were cultured with inulin (**fig. 19**).

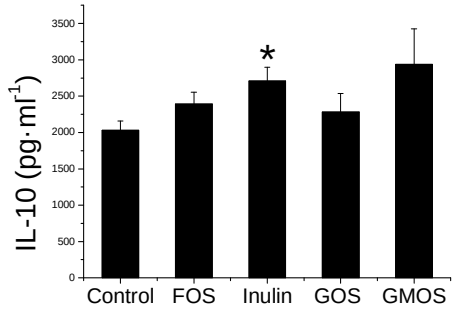


Figure 19. Effect of prebiotics on WT mice colonic tissue fragments. Cytokine measured is IL-10. Concentrations are expressed as means ± SEM (pg·ml^{-1}). *p<0.05 *vs.* control, n = 6. FOS: fructooligosaccharides. GMOS: goat milk oligosaccharides. GOS: galactooligosaccharides. WT mice: C57BL/6J wild type mice.

IV. *Effect of prebiotics on rat monocytes*

Because the results obtained suggested a predominant effect on monocytes / macrophages, we next focused on studying the effects of NDOS on monocytes. We used rat primary cells to enhance the yield of monocytes, which is very low in mice unless activated macrophages are used. We focused on FOS and inulin, which showed the most pronounced effects in splenocytes. As expected, LPS addition upregulated all cytokines, especially TNF-α and GRO-α (**fig. 20**, black bars). In basal conditions, FOS evoked the release of several cytokines by primary rat monocytes, including TNF-α, GRO-α and IL-10, but not IL-1β. The effect, compared to that of LPS, was highest for TNF-α and GRO-α. Only the concentration of 5 $g \cdot l^{-1}$ of FOS elicited secretion of GRO-α, while the effect was still observed at 0.5 $g \cdot l^{-1}$ in the case of TNF-α and IL-10, indicating a higher potency. Inulin had comparable effects.

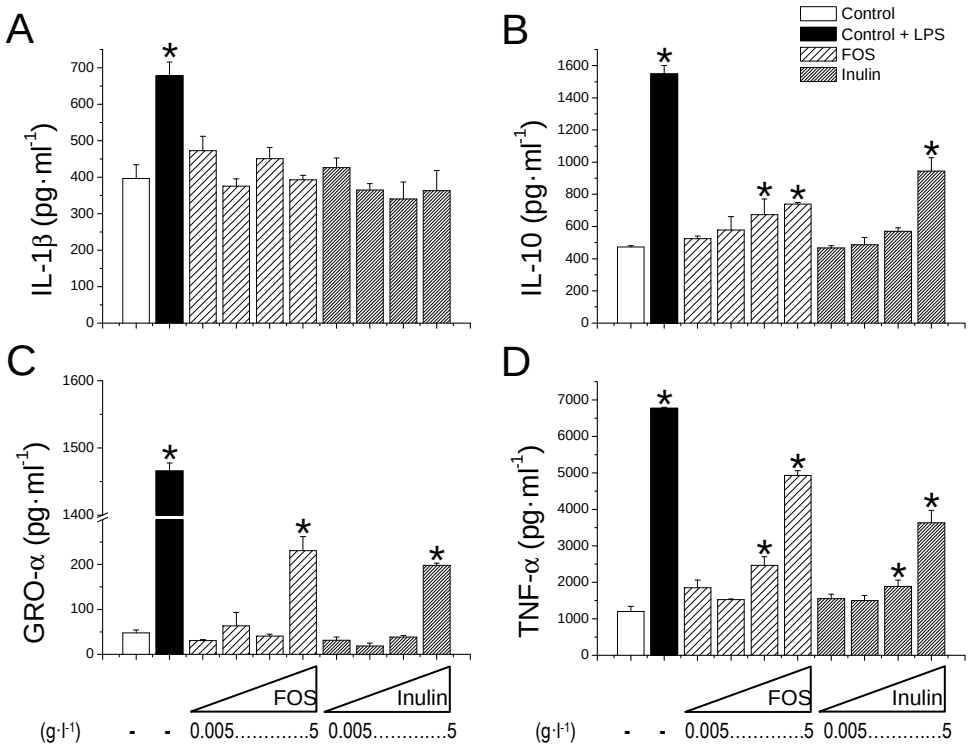


Figure 20. Effect of prebiotics on cytokine secretion by rat primary monocytes. Cytokines measured are IL-1β (A), IL-10 (B), GRO-α (C) and TNF-α (D). From left to right within treatments (FOS and inulin): 0.005 $g \cdot l^{-1}$, 0.05 $g \cdot l^{-1}$, 0.5 $g \cdot l^{-1}$, 5 $g \cdot l^{-1}$. Concentrations are expressed as means ± SEM ($pg \cdot ml^{-1}$). *$p \leq 0.028$ *vs.* control, n = 3. FOS: fructooligosaccharides. LPS: lipopolysaccharide.

Next, we assessed the signaling pathways involved in NDOS effects using pharmacological probes. The NFκB inhibitor Bay 11-7082 fully prevented FOS- (**fig. 21**) and inulin- (**fig. 22**) evoked cytokine secretion. Control release was also inhibited, especially in the case of IL-1β and IL-10, indicating an ongoing activation of the NFκB pathway even in the absence of external stimuli in these cells. There was also a marked (58.5-99.3%) inhibitory effect of SB203580 (p38 MAPK inhibitor) and of wortmannin (57.1-100%) (PI3K inhibitor) on all cytokines. A significant but relatively minor inhibition was obtained with the ERK1/2 MAPK blocker PD98059 (which actually increased FOS- and inulin-evoked IL-10 secretion). Finally, SP600125 (JNK inhibitor) partially inhibited the TNF-α and IL-1β response only.

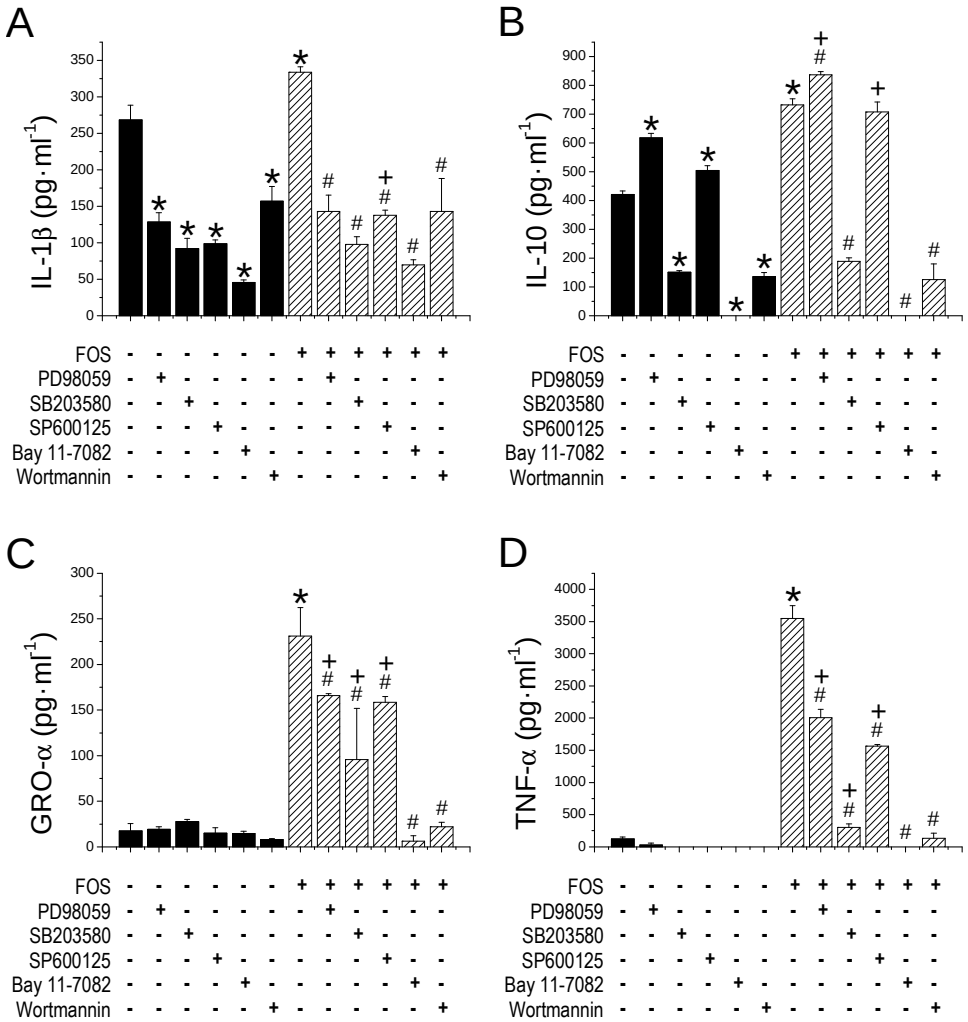


Figure 21 (left). Effect of specific inhibitors of cell signaling on FOS-evoked cytokine secretion by rat primary monocytes. The inhibitors used are PD98059 (ERK1/2 MAPK inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), Bay 11-7082 (inhibitor of IκB-α phosphorylation) and Wortmannin (PI3K inhibitor). Cytokines measured are IL-1β (A), IL-10 (B), GRO-α (C) and TNF-α (D). Concentrations are expressed as means ± SEM (pg·ml^{-1}). Black bars: control ± inhibitors; striped bars: FOS ± inhibitors. *p<0.05 *vs.* control (first black bar); ^{+}p<0.05 *vs.* each control + inhibitor; $^{\#}p$<0.05 *vs.* FOS (first striped bar), n = 3. FOS: fructooligosaccharides.

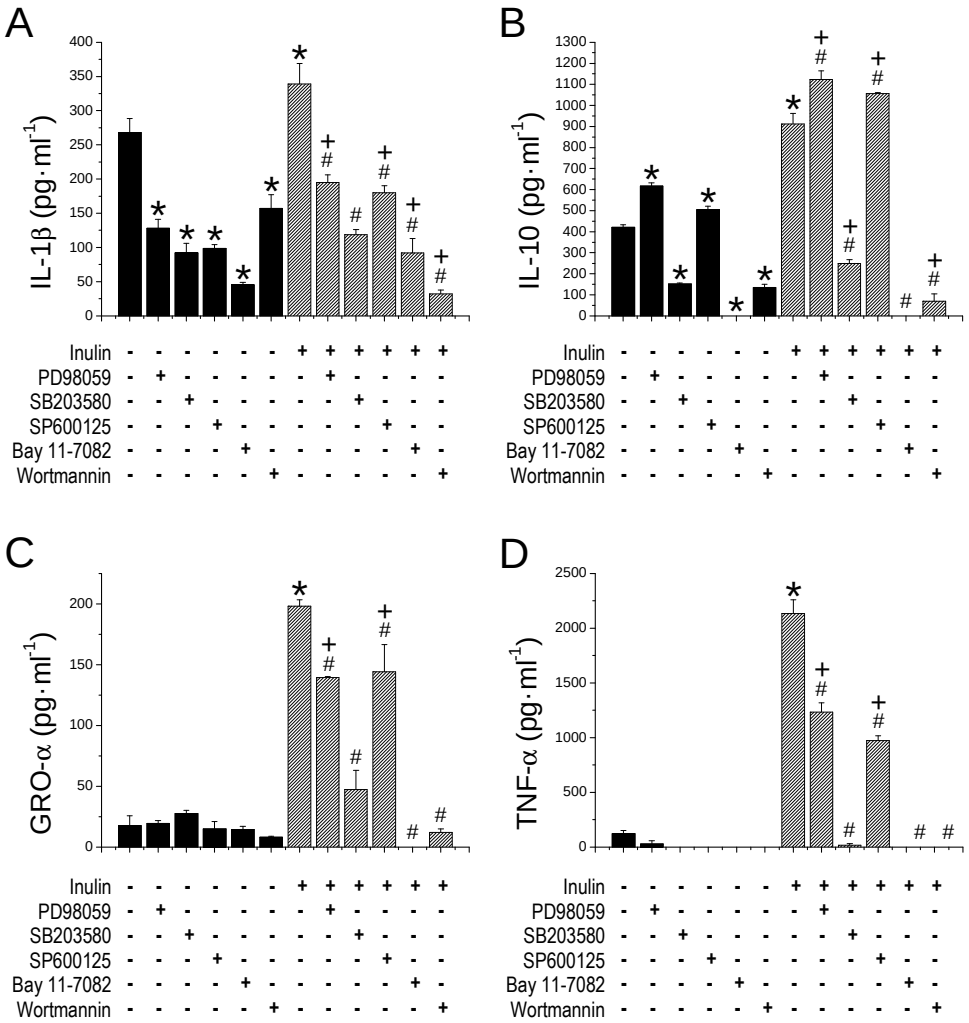


Figure 22. Effect of specific inhibitors of cell signaling on inulin-evoked cytokine secretion by rat primary monocytes. The inhibitors used are PD98059 (ERK1/2 MAPK inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), Bay 11-7082 (inhibitor of IκB-α phosphorylation) and Wortmannin (PI3K inhibitor). Cytokines measured are IL-1β (A), IL-10 (B), GRO-α (C) and TNF-α (D). Concentrations are expressed as means ± SEM (pg·ml^{-1}). Black bars: control ± inhibitors; striped bars: inulin ± inhibitors. *p<0.05 *vs.* control (first black bar); ^{+}p<0.05 *vs.* each control + inhibitor; $^{\#}p$<0.05 *vs.* inulin (first striped bar), n = 3.

V. *Effect of prebiotics on rat T lymphocytes*

T cells were isolated from the spleen of normal rats and treated with 5 g·l^{-1} of the different NDOS *in vitro*. There was no effect in basal conditions except for a 91% increase in IL-2 in inulin-treated cells (**fig. 23A**). Under ConA stimulation, there was a marked upshot in cytokine levels, as expected. No change was detected with NDOS treatment (**figs. 23B**, **C**).

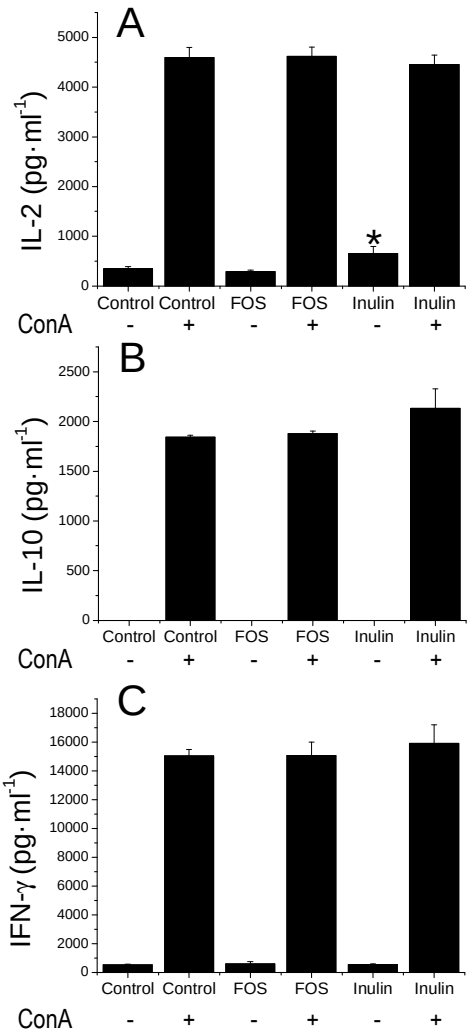


Figure 23. Effect of prebiotics on basal and ConA-stimulated cytokine secretion by rat primary T lymphocytes. Cytokines measured are IL-2 (A), IL-10 (B) and IFN-γ (C). Concentrations are expressed as means ± SEM (pg·ml^{-1}). *****p<0.05 *vs.* control, n = 3. ConA: concanavalin A; FOS: fructooligosaccharides.

VI. *Effect of prebiotics on macrophage-conditioned medium cultures*

FOS and inulin had negligible effects on rat T cells, rather than the inhibition suggested by the splenocyte data. We hypothesized that the effect may be indirect, i.e., mediated by monocytes, presumably via enhanced IL-10 secretion. In order to check this hypothesis we carried out an experiment in which monocytes were isolated as described in Material & Methods and treated with FOS or inulin at 5 g·l^{-1}. The conditioned medium was added then to T cells in an attempt to modulate cytokine expression by monocyte-derived factors. However, T cells were maximally stimulated by the medium in these

conditions, so no further regulation seemed possible (data not shown).

VII. *Effect of prebiotics on human peripheral blood monocytes*

At the concentration of 5 g·l^{-1} both FOS and inulin elicited TNF-α secretion (**fig. 24D**, $p<0.05$). IL-1β levels were also higher in the presence of NDOS but without reaching statistical significance (**fig. 24A**). IL-10 was significantly induced by both inulin and FOS (**fig. 24B**). There was no effect on IL-8 (**fig. 24C**). LPS addition to the control had only a weak increasing effect on IL-8, and failed to upregulate IL-1β, TNF-α or IL-10. When cells were co-treated with LPS and NDOS, cytokine secretion was generally higher than with NDOS alone, and the profile was comparable to that in basal conditions.

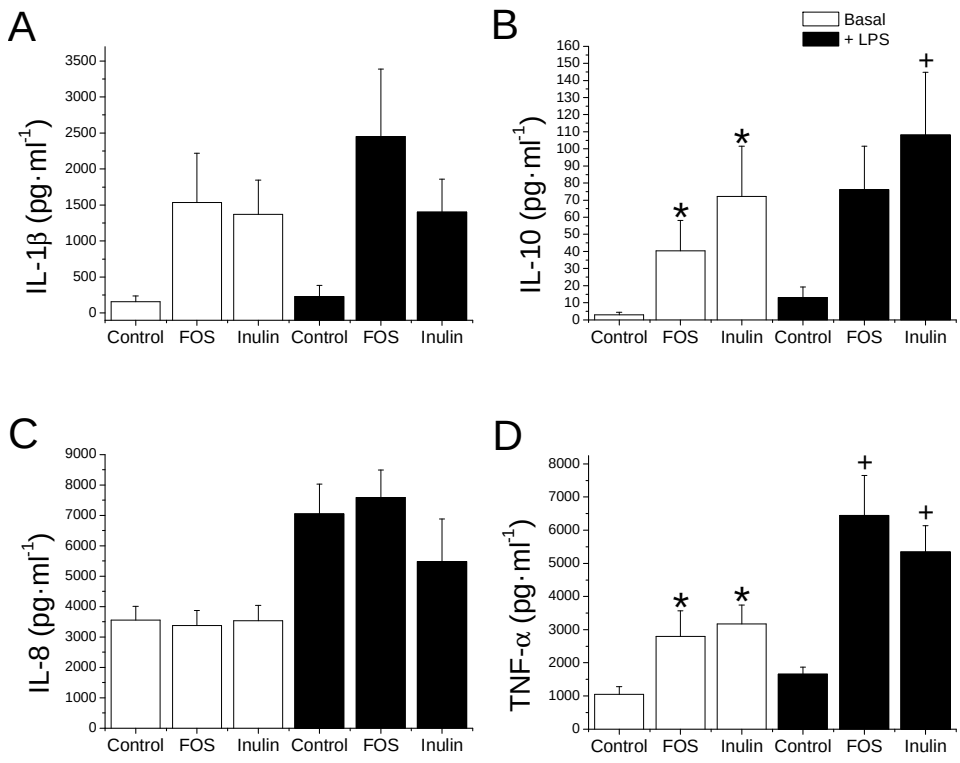
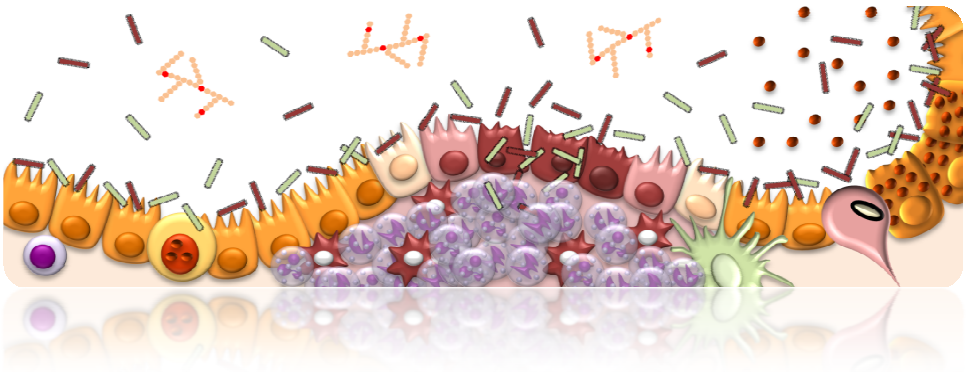


Figure 24. Effect of prebiotics on basal and LPS-stimulated cytokine secretion by hPBM. Cytokines measured are IL-1β (A), IL-10 (B), IL-8 (C) and TNF-α (D). Concentrations are expressed as means ± SEM (pg·ml^{-1}). *$p<0.05$ *vs.* control; $^{+}p<0.05$ *vs.* LPS, n = 10. FOS: fructooligosaccharides. hPBM: human peripheral blood monocytes. LPS: lipopolysaccharide.

Chapter II

Effects of fructooligosaccharides in the CD4+ CD62L+ T cell transfer model of colitis



I. *Colitis evolution and animal status*

Rag1$^{-/-}$ mice were monitored for 8 weeks after transfer for body weight evolution and overall status. The transfer of CD4+ CD62L+ T cells into Rag1$^{-/-}$ mice caused significant body weight loss, beginning between week 4 and 6 after transfer. The animals selected for this study showed a 10.42% average of body weight loss around week 8 and were then randomized for treatment with FOS (75 mg·day^{-1}) or vehicle (day 0). Mice administered FOS maintained a relatively stable body weight, which was increased by 3.42 ± 1.94% after 13 days (p<0.05 *vs.* colitic group), while the colitic group lost 6.06 ± 2.96% body weight since day 0 (p<0.05 *vs.* non colitic). As expected, non colitic mice gained weight steadily throughout the experimental period (6.5 ± 0.95% since day 0) (**fig. 25**, **table 5**). Food intake was comparable in the three groups, while colitic mice drank slightly more water than non colitic mice (4.05 ± 0.19 *vs.* 2.90 ± 0.20 ml·d^{-1}, p<0.05). This was effectively counteracted by FOS treatment (3.24 ± 0.08 ml·d^{-1}, p<0.05) (**table 5**). Lymphocyte transfer colitis was also characterized by a trend to splenomegalia, both in the control and FOS-treated mice (**table 6**).

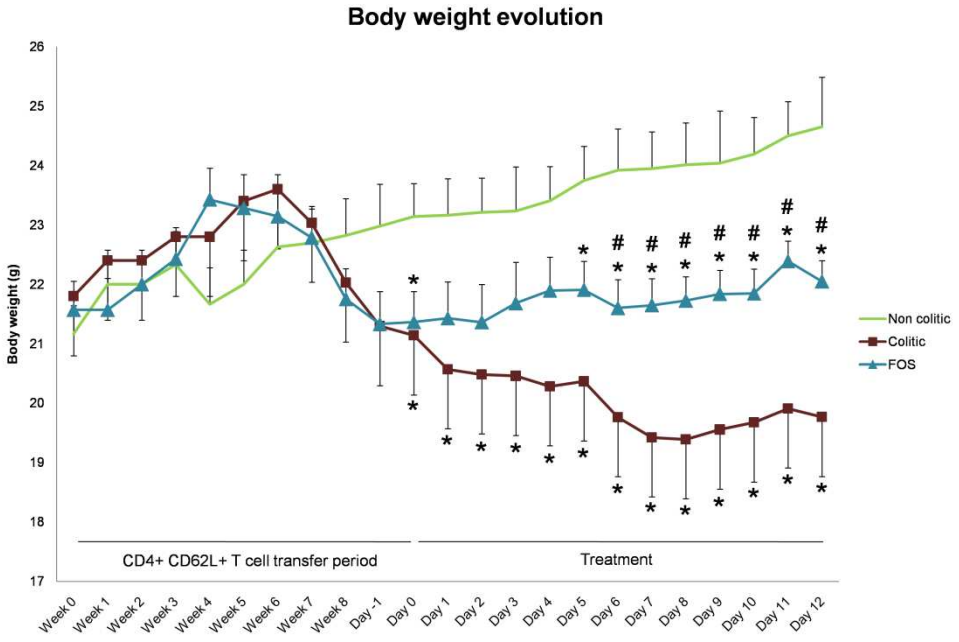


Figure 25. Body weight evolution. Body weight evolution during CD4+ CD62L+ T cell transfer period (8 weeks) and treatment (13 days). Body weight is expressed as means (g) ± SEM. Single green line: non colitic; brown line with squares: colitic CD4+ CD62L+ (colitic); blue line with triangles: fructooligosaccharides CD4+ CD62L+ (FOS). *p<0.05 *vs.* non colitic; #p<0.05 *vs.* colitic.

Table 5. Body weight (BW) evolution *(1)*, food *(2)* and water intake *(3)*

	BW week 0	BW week 4-6	BW day 0	BW day 12	Food Intake	Water Intake
Non colitic	91.62 ± 1.34	95.14 ± 0.63	100 ± 3.05	106.50 ± 0.95	2.97 ± 0.13	2.90 ± 0.20
Colitic	103.67 ± 3.40*	111.72 ± 1.04*	100 ± 3.81	93.94 ± 2.96*	3.04 ± 0.17	4.05 ± 0.19*
FOS	101.04 ± 1.20*	109.72 ± 1.28*	100 ± 2.41	103.42 ± 1.94#	2.83 ± 0.07	3.24 ± 0.08#

(1) % BW ± SEM. 100% has been normalized to the day 0. *(2)* g·mouse^{-1}·day^{-1} ± SEM. *(3)* ml·mouse^{-1}·day^{-1} ± SEM.
**p*<0.05 *vs.* non colitic; #*p*<0.05 *vs.* colitic. FOS: fructooligosaccharides.

Table 6. Morphological indicators of inflammation

	Colonic damage score	Colon weight:length ratio	Spleen weight *(1)*
	Arbitrary units	(mg·cm^{-1})·1000	(g spleen·BW mouse^{-1})·1000
Non colitic	0 ± 0	21.60 ± 1.36	1.34 ± 0.06
Colitic	2.00 ± 0.84*	39.39 ± 7.87*	3.82 ± 1.48
FOS	1.37 ± 0.44*	43.41 ± 4.13*	4.84 ± 2.30

(1) Referred to the animal relative weight.
Values are means ± SEM, **p*<0.05 *vs.* non colitic. BW: body weight. FOS: fructooligosaccharides.

II. *Colonic inflammatory status*

Mice from the colitic group exhibited a hyperemic mucosa with bowel wall thickening and increased adhesions and rigidity but no necrosis, resulting in a significantly augmented damage score (**table 6**). Treatment with FOS resulted in amelioration of these visible signs of colitis, although the change was not significant. Enhanced neutrophil recruitment to the mucosa was evidenced by a 2-fold increase in colonic MPO activity, a widely used inflammatory marker, comparing the colitic with the non colitic group (**fig. 26A**). This was fully prevented by FOS treatment (p<0.05). Colonic AP activity, a marker of intestinal inflammation and epithelial stress,[253, 397, 398] was also augmented 3-fold in the colitic control group, associated with a dramatic increase in the sensitivity to the specific inhibitor levamisole (**figs. 26B**, **C**). FOS treatment resulted in a 34% reduction in AP activity and a parallel effect on sensitivity to levamisole (p<0.05).

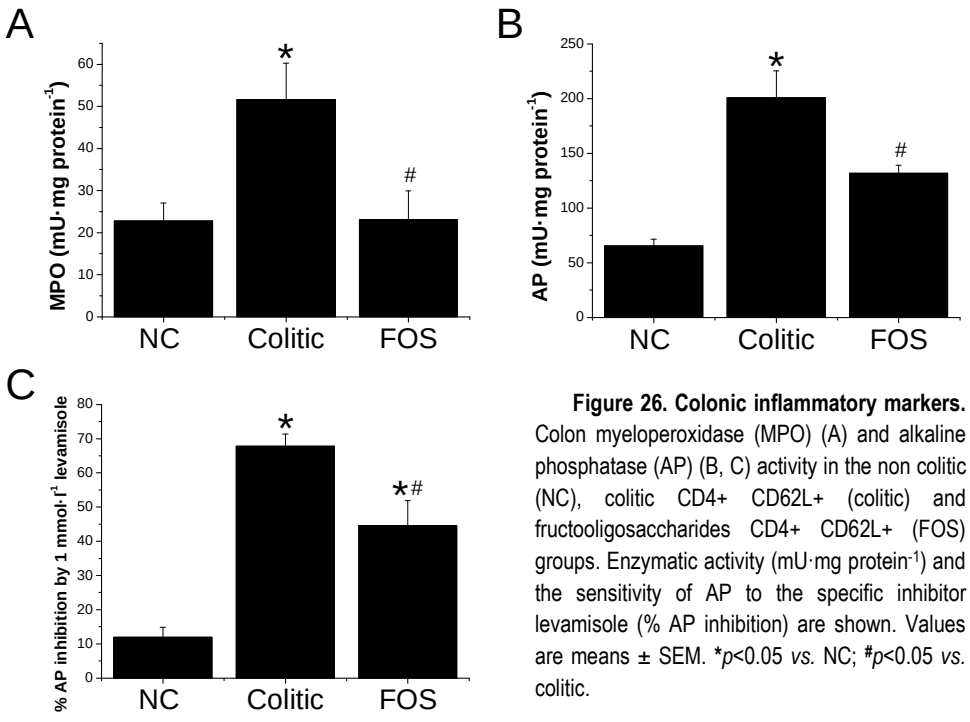


Figure 26. Colonic inflammatory markers. Colon myeloperoxidase (MPO) (A) and alkaline phosphatase (AP) (B, C) activity in the non colitic (NC), colitic CD4+ CD62L+ (colitic) and fructooligosaccharides CD4+ CD62L+ (FOS) groups. Enzymatic activity (mU·mg protein^{-1}) and the sensitivity of AP to the specific inhibitor levamisole (% AP inhibition) are shown. Values are means ± SEM. *p<0.05 *vs.* NC; $^{\#}p$<0.05 *vs.* colitic.

III. *Colonic expression of inflammatory markers assessed by qRT-PCR*

CD4+ CD62L+ transferred animals showed an increased colonic expression of the inflammatory markers S100A8 and TNF-α, while IL-1β was not significantly affected (**fig. 27**). Treatment with FOS fully normalized the expression of S100A8, one of the subunits that form calprotectin in mice (p<0.05 vs. colitic) (**fig. 27B**). While mRNA levels of IL-1β in the inflamed intestine were apparently lower in FOS-treated mice, there was no significant differences overall (**fig. 27A**). FOS had no effect whatsoever on TNF-α (**fig. 27C**).

IV. *Cytokine secretion by MLNC ex vivo*

This model of colitis is characterized by progressive expansion of the transferred T lymphocyte population, with a predominance of Th1/Th17 cells and a paucity of Treg cells, and, accordingly, basal and ConA-stimulated MLNC of the colitic group exhibited a heightened secretion of IFN-γ and IL-17 compared to non colitic mice (**fig. 28**). The basal level of IL-6, TNF-α and IL-10 was also higher in the colitic group than in the non colitic animals, but this did not reach statistical significance (**fig. 29**). ConA elicited nonetheless

a robust response in the colitic controls (**fig. 29**). FOS treatment had no effect on spontaneous (basal) cytokine secretion by MLNC, but it decreased IFN-γ, IL-17 and TNF-α ConA-evoked secretion by ~50% (p<0.05, **figs. 28** and **29**). There was no effect however on IL-6 or IL-10.

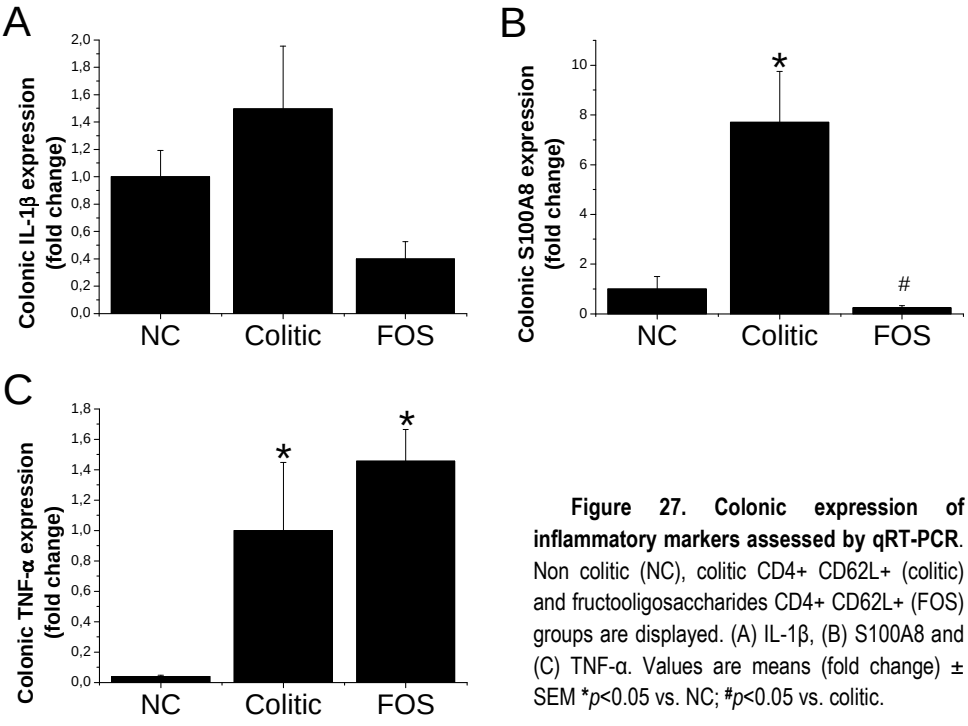


Figure 27. Colonic expression of inflammatory markers assessed by qRT-PCR. Non colitic (NC), colitic CD4+ CD62L+ (colitic) and fructooligosaccharides CD4+ CD62L+ (FOS) groups are displayed. (A) IL-1β, (B) S100A8 and (C) TNF-α. Values are means (fold change) ± SEM *p<0.05 vs. NC; #p<0.05 vs. colitic.

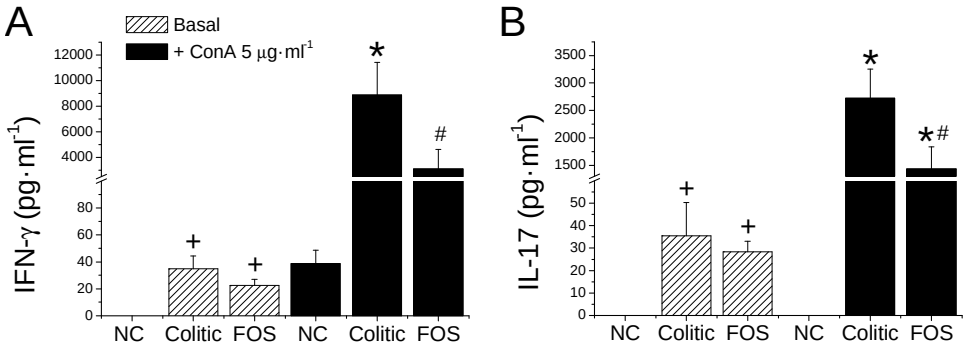


Figure 28. Cytokine secretion by MLNC (I). Non colitic (NC), colitic CD4+ CD62L+ (colitic) and fructooligosaccharides CD4+ CD62L+ (FOS) groups are displayed. Cytokines measured are IFN-γ (A) and IL-17 (B). Striped bars: basal secretion; white bars: ConA 5 μg·ml^{-1} secretion. Values are means (pg·ml^{-1}) ± SEM. +p<0.05 *vs.* basal NC; *p<0.05 vs. ConA NC; #p<0.05 *vs.* ConA colitic. ConA: concanavalin A. MLNC: mesenteric lymph node cells.

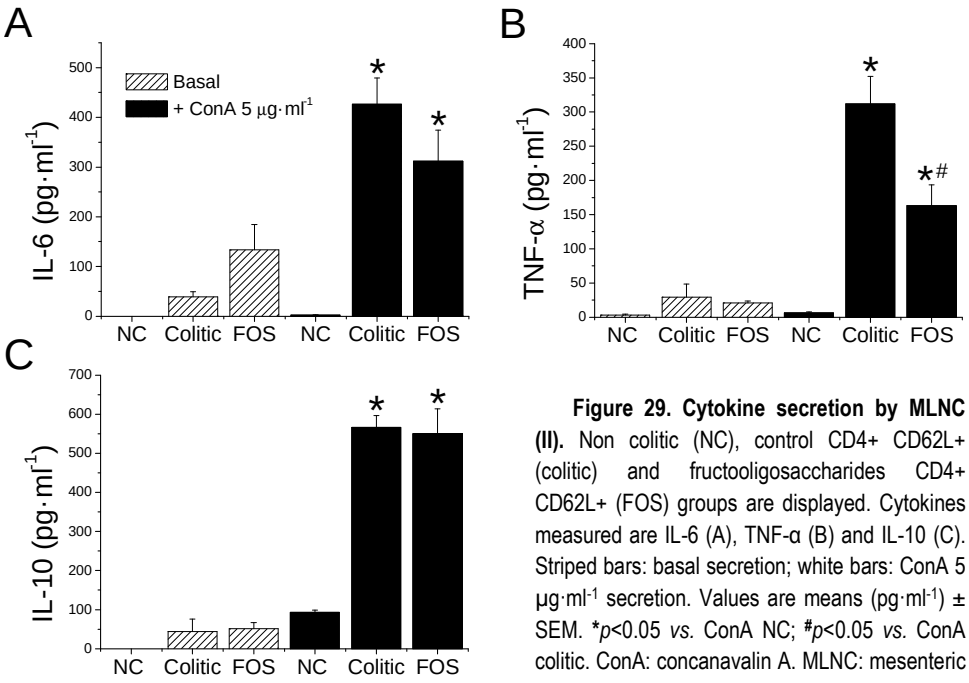


Figure 29. Cytokine secretion by MLNC (II). Non colitic (NC), control CD4+ CD62L+ (colitic) and fructooligosaccharides CD4+ CD62L+ (FOS) groups are displayed. Cytokines measured are IL-6 (A), TNF-α (B) and IL-10 (C). Striped bars: basal secretion; white bars: ConA 5 μg·ml^{-1} secretion. Values are means (pg·ml^{-1}) ± SEM. **p*<0.05 *vs.* ConA NC; #*p*<0.05 *vs.* ConA colitic. ConA: concanavalin A. MLNC: mesenteric lymph nodes cells.

V. *Bacteriological analysis of faeces*

In order to confirm that FOS exert prebiotic effects in this model of intestinal inflammation, a series of bacterial cultures were performed to determine their effect on faecal microbiota (**table 7**). The transfer of CD4+ CD62L+ T cells into Rag1$^{-/-}$ mice resulted in alterations in the microflora profile, increasing both aerobes and anaerobes, the latter significantly (p<0.05). FOS treatment resulted in an increased level of acid lactic bacteria (p<0.05 *vs.* non colitic). After 72 hours, no growth was observed in clostridia in any of the groups.

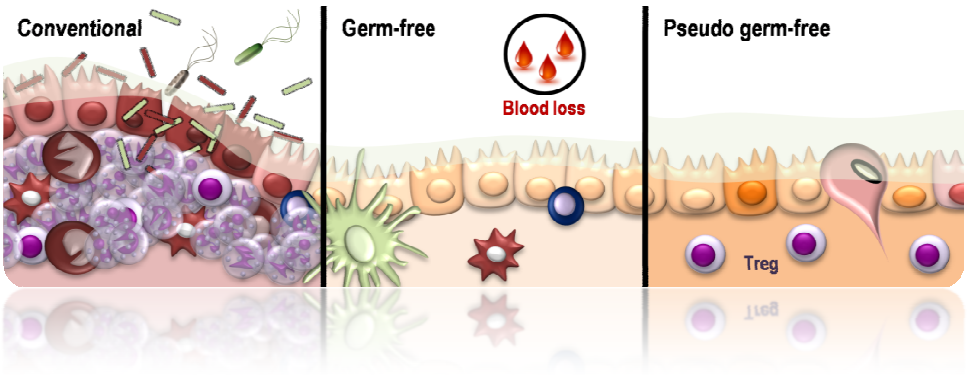
Table 7. Effects of FOS treatment on bacteria fecal levels

	Aerobes	Anaerobes	Lactic acid bacteria
	Log CFU		
Non colitic	10.77 ± 0.26	10.30 ± 0.29	10.91 ± 0.36
Colitic	11.86 ± 0.26	12.03 ± 0.37*	11.35 ± 0.24
FOS	11.79 ± 0.25	11.68 ± 0.19*	12.25 ± 0.16*

Values are means ± SEM, **p*<0.05 *vs.* non colitic. CFU: colony forming units. FOS: fructooligosaccharides.

Chapter III

DSS-induced colitis in conventional, germ-free and pseudo germ-free conditions



I. *Colitis evolution and animal status in conventional and GF NMRI mice*

Conventional and GF NMRI mice were monitored throughout the experiment for body weight evolution and overall status. The supplementation of DSS in drinking water caused significant body weight loss (**fig. 30**), diarrhea and hematochezia, beginning around day 4. As expected, non colitic mice did not suffer body weight loss during all the experiment, which was slightly increased after 7 days. Due to technical limitations no data on food or water intake are available from GF mice. Three GF mice died in the 4% DSS group, compared with one in the conventional mice group. In addition, one mouse died prematurely in the 2% DSS + SBH and another in the 4% DSS + SBH group.

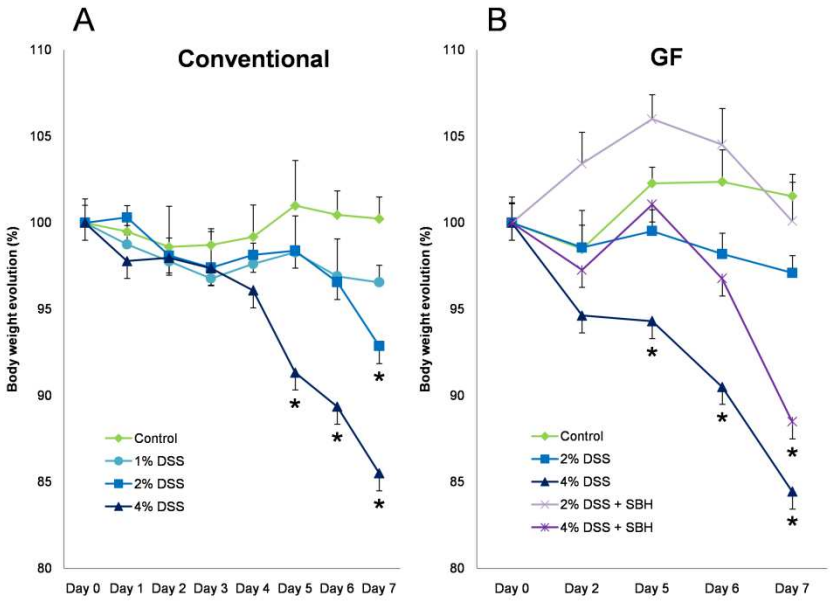


Figure 30. Body weight evolution in conventional and GF NMRI mice. Body weight evolution (%) during DSS-induced colitis in conventional NMRI mice (A) and GF NMRI mice (B). Body weight is expressed as percentage (%) ± SEM. Green line with diamonds: controls; light blue line with circles: 1% DSS; cerulean line with squares: 2% DSS; dark blue line with triangles: 4% DSS; light purple line with blades: 2% DSS + SBH; dark purple line with asterisks: 4% DSS + SBH. DSS: dextran sulfate sodium. GF: germ-free. SBH: sterile bacterial homogenate. *$p<0.05$ *vs.* control.

II. *Macroscopic parameters and colonic damage score in conventional and GF NMRI mice*

As expected, conventional NMRI mice treated with 1-4% DSS showed an inflammatory reaction characterized by loss of the mucosal vascular pattern, hyperemia

and occasional hemorrhage, edema and fibrotic features, resulting in a dose-dependent increase in the total colonic damage score compared with the non colitic group (**table 8**). Despite the apparent bowel wall thickening, the colonic weight to length ratio was not significantly increased (**table 9**). Interestingly, this parameter was actually lowered in the ileum of colitic animals (**table 9**). Mice with DSS colitis showed also hepatomegaly, significant with the 2% dose only (p<0.05), but not splenomegaly (**table 9**).

Table 8. Colonic damage score in conventional and GF NMRI mice [(1)]

	Adhesions	Hyperemia	Fibrosis	Deformation	Thickening	Total score [(2)]
Conventional						
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	**0.0 ± 0.0**
1% DSS	1.0 ± 0.2*	0.3 ± 0.1	0.5 ± 0.3	0.2 ± 0.1	0.1 ± 0.1	**2.1 ± 0.8***
2% DSS	0.7 ± 0.2*	0.9 ± 0.2*	1.2 ± 0.2*	0.2 ± 0.1	0.7 ± 0.2*	**3.7 ± 0.7***
4% DSS	0.9 ± 0.2*	1.8 ± 0.3*	1.5 ± 0.1*	0.5 ± 0.1*	0.8 ± 0.1*	**5.5 ± 0.5***
Germ-free						
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	**0.0 ± 0.0**
2% DSS	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.0*	0.0 ± 0.0	0.0 ± 0.0	**1.0 ± 0.0***
4% DSS	0.6 ± 0.2*	0.0 ± 0.0	2.0 ± 0.0*	0.0 ± 0.0	0.0 ± 0.0	**2.6 ± 0.2***
2% DSS + SBH	0.0 ± 0.0	1.0 ± 0.0*	1.5 ± 0.0*	0.0 ± 0.0	0.0 ± 0.0	**2.5 ± 0.0***
4% DSS + SBH	1.3 ± 0.2*	0.0 ± 0.0	1.5 ± 0.0*	0.0 ± 0.0	0.0 ± 0.0	**2.8 ± 0.2***

[(1)] Arbitrary units. Values are means ± SEM, *p<0.05 *vs.* control. [(2)] Sum of all the values (adhesion, hyperemia, fibrosis, deformation and thickening).
DSS: dextran sulfate sodium. GF: germ-free. SBH: sterile bacterial homogenate.

GF NMRI mice appeared significantly sicker than conventional mice, especially with the 4% dose, as they showed reduced spontaneous movement, increased huddling behaviour, eye and ear decoloration. After sacrifice, it was interesting to note that intestinal hyperemia was completely absent (**table 8**), the blood sample appeared pink rather than red, and the liver had a brownish color (4% DSS). Taken together, these findings are suggestive of substantial blood loss in DSS-treated GF mice. In addition to the lack of hyperemia, the colon of these animals was fragile and fibrotic but not

apparently thickened, resulting in a dose-dependent increase in the damage score that was, somewhat paradoxically, less pronounced than in conventional mice. This is due to a mismatch between animal pathology and regular scoring criteria. It is also worth mentioning that the colon was very difficult to clean of fecal material, which had a dark-blue colour and a petroleum-like consistency. Ileal weight to length ratio was reduced (p<0.05 for the 4% dose, **table 9**), but in contrast GF mice showed splenomegaly (4% dose) but no hepatomegaly. It is interesting to note that the GF mice that received a bacterial homogenate did show both spleen and liver enlargement at the 2% dose of DSS (**table 9**, 2% DSS + SBH), with hyperemia and an increased colonic damage score (**table 8**, 2% DSS + SBH). These effects were again lowered at the 4% DSS dose. The cecum of mice receiving 4% DSS + SBH was enlarged and filled with a petroleum-like stool.

Table 9. Morphological indicators of inflammation in conventional and GF NMRI mice

	Colon weight:length ratio	Ileum weight:length ratio	Spleen weight (1)	Liver weight (1)
	(mg·cm^{-1})·1000		(g organ·body weight mouse^{-1})·1000	
		Conventional		
Control	39.08 ± 3.84	27.93 ± 2.80	3.98 ± 0.94	37.41 ± 1.76
1% DSS	39.60 ± 6.85	22.96 ± 1.24	3.55 ± 0.57	41.55 ± 2.57
2% DSS	47.79 ± 3.12	20.62 ± 1.62*	3.61 ± 0.17	49.36 ± 4.16*
4% DSS	41.31 ± 2.48	19.11 ± 1.02*	3.20 ± 0.24	43.65 ± 1.86
		Germ-free		
Control	21.03 ± 0.93	21.36 ± 0.79	2.86 ± 0.09	36.35 ± 0.94
2% DSS	22.43 ± 1.48	21.14 ± 0.81	3.12 ± 0.31	36.73 ± 0.88
4% DSS	20.54 ± 1.01	15.10 ± 2.29*	5.01 ± 0.83*	35.21 ± 1.88
2% DSS + SBH	21.29 ± 1.51	21.60 ± 1.41	4.89 ± 1.00*	40.29 ± 2.27*
4% DSS + SBH	19.72 ± 0.65	16.01 ± 1.34*	4.94 ± 0.64*	34.05 ± 0.86

(1) Referred to the animal relative weight. Values are means ± SEM, *p<0.05 *vs.* control.
DSS: dextran sulfate sodium. GF: germ-free. SBH: sterile bacterial homogenate.

III. *Colonic inflammatory status in conventional and GF NMRI mice*

In conventional NMRI mice treated with DSS there was a dose-dependent increase in colonic MPO activity, compared with the non colitic group (**fig. 31A**). Colonic AP activity was also significantly greater in the colitic groups than in the control group (**fig. 31C**). In addition, the sensitivity of this enzymatic activity to levamisole *in vitro* was increased in colitic tissue compared with the control, consistent with neutrophil infiltration and the isoform shift previously described in enterocytes (**fig. 31E**).[253, 397, 398] In the three measurements, the increase was significant with 2 and 4% DSS (p<0.05). Conversely, GF NMRI mice exhibited no change in MPO activity (**fig. 31B**) and a substantially reduced increase in AP activity (**fig. 31D**), with no differences in the sensitivity to levamisole (**fig. 31F**). In fact, both MPO activity and AP sensitivity to levamisole were substantially lower (3-5 fold) in GF control mice than in conventional mice (compare panels A-B and E-F in **fig. 31**). The addition of SBH to DSS-treated GF mice had little effect on these parameters, although AP inhibition by levamisole *in vitro* was lowered in the 2% DSS + SBH group (p<0.05, **fig. 31F**).

IV. *Colonic expression of inflammatory markers assessed by qRT-PCR in conventional and GF NMRI mice*

Colonic mRNA levels of ZO-1, OCCLUDIN, IL-1β, IL-10, IL-17, IFN-γ, TNF-α, REGIII-γ and S100A8 were also studied. Conventional NMRI mice that drank 1%, 2% and 4% DSS-supplemented water showed a significant reduction (p<0.05) in ZO-1 and OCCLUDIN levels (p<0.05, **fig. 32A**). REGIII-γ level was increased by 2% and 4% DSS (p<0.05, **fig. 32B**), while S100A8 was dramatically induced by 4% DSS (p<0.05, **fig. 32C**). The magnitude of this increase actually masked the 18-fold surge in S100A8 detected in the 2% DSS group, which was substantial, but not statistically significant. Moreover, the 4% DSS group significantly increased the expression of IL-1β, IL-10 and IFN-γ, the latter also increased by 1% DSS group.

Conversely, there were fewer changes in colonic GF mRNA levels, so that only TNF-α was upregulated with 4% DSS with or without SBH (p<0.05, **fig. 32D**), and IFN-γ was downregulated with 4% DSS + SBH (p<0.05, **fig. 32F**).

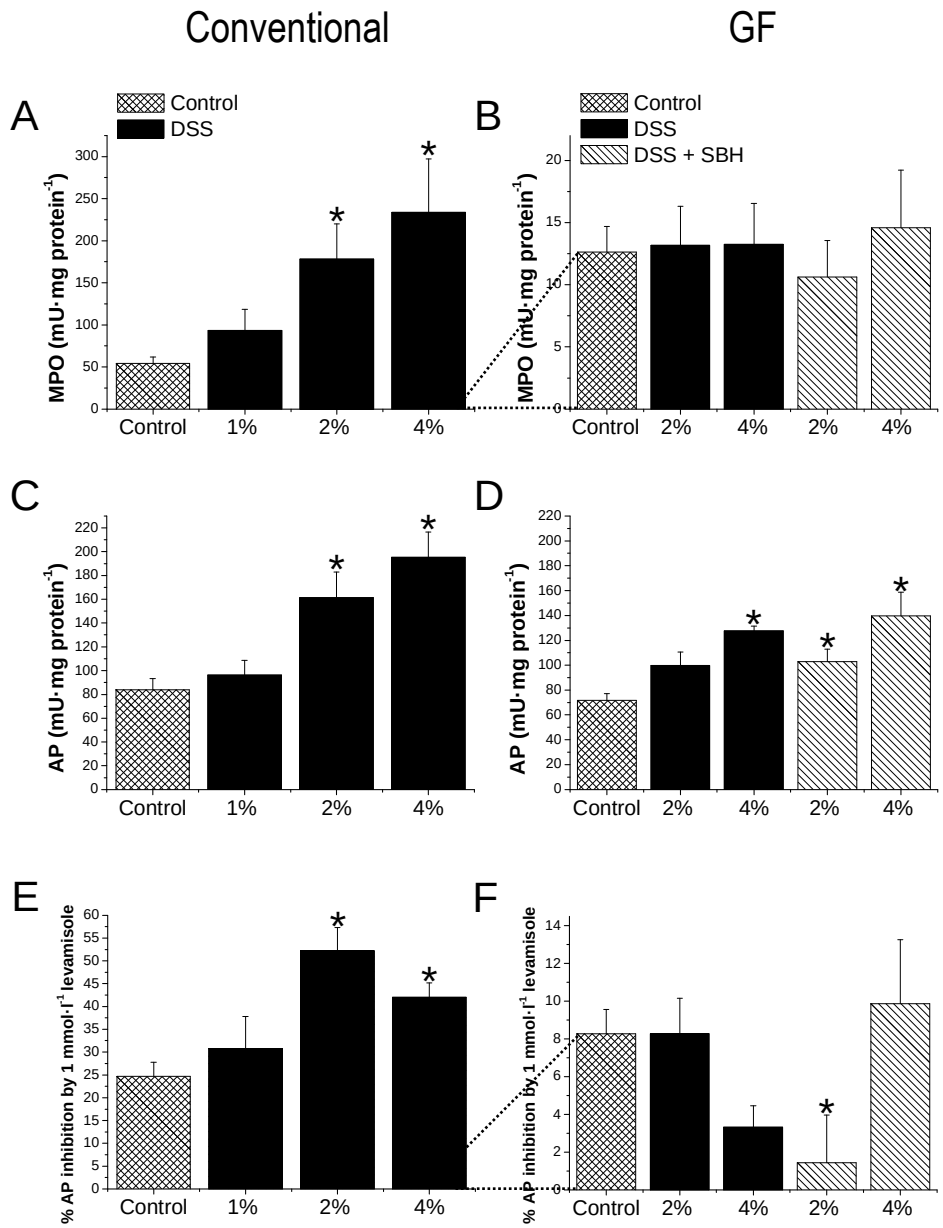


Figure 31. Colonic inflammatory markers in conventional and GF NMRI mice. Conventional NMRI mice (left) and GF NMRI mice (right). Colonic myeloperoxidase (MPO) (A) and alkaline phosphatase (AP) (B, C) activity. Grid columns: controls; black columns: DSS groups; columns with diagonal lines: DSS + SBH groups. Dotted lines show the difference of scale between conventional and GF panels. Enzymatic activity (mU·mg protein^{-1}) and the sensitivity of AP to the specific inhibitor levamisole (% AP inhibition) are shown. Values are means ± SEM. *p<0.05 *vs.* control. DSS: dextran sulfate sodium. GF: germ-free. SBH: sterile bacterial homogenate.

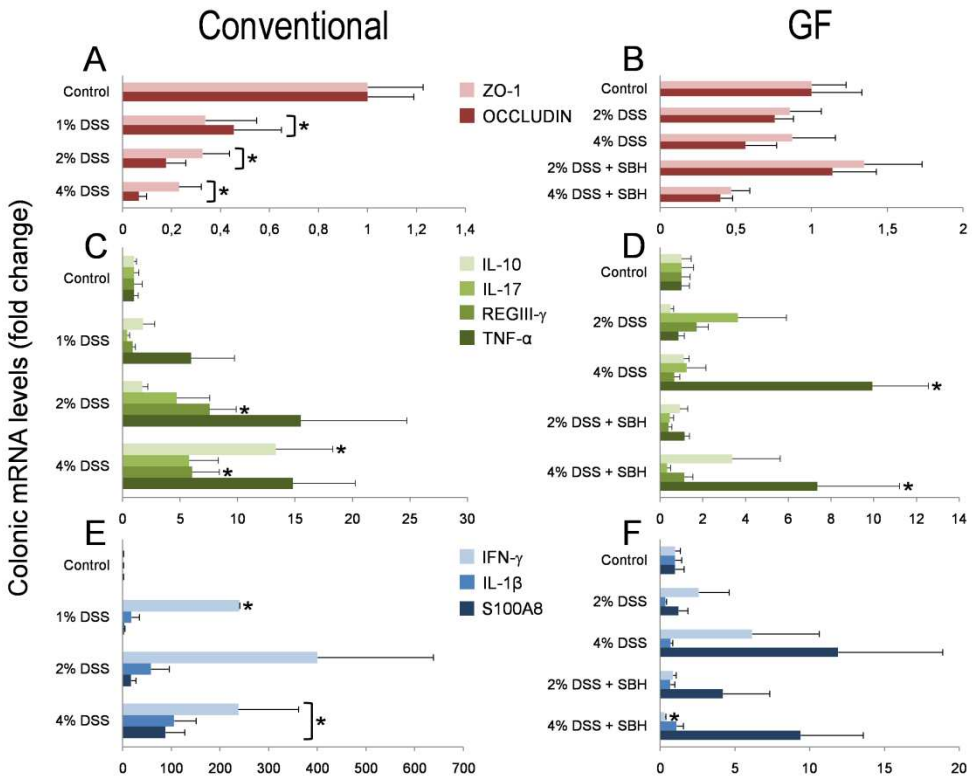


Figure 32. Colonic expression of inflammatory markers assessed by qRT-PCR in conventional and GF NMRI mice. Conventional NMRI mice (left) and GF NMRI mice (right). Colonic mRNA levels of ZO-1, OCCLUDIN, IL-10, IL-17, REGIII-γ, TNF-α, IFN-γ, IL-1β and S100A8 are displayed. Values are means (fold change) ± SEM. *p<0.05 *vs.* control. DSS: dextran sulfate sodium. GF: germ-free. SBH: sterile bacterial homogenate.

V. *Cytokine secretion by MLNC in conventional and GF NMRI mice*

In conventional NMRI mice, only IL-6 and IL-17 release was detected at baseline. Mice treated with 4% DSS showed no IL-17 basal output, but there was no other effect of DSS colitis at this level (**fig. 33A**). Under ConA stimulation there was a marked upshot in IL-6, IL-17, TNF-α and IFN-γ production, which was similar among the groups except for IFN-γ, increased in the three DSS groups (p<0.05 for the 2% dose only, **fig. 33C**). This cytokine also exhibited the highest level of production by MLNC.

As in conventional mice, MLNC from GF NMRI animals only showed basal release of IL-6 and IL-17. The latter was significantly diminished in both DSS groups (**fig. 33B**). Even with ConA stimulation, cytokine production was very weakly induced (compare **figs.**

33C and **33D**), and IFN-γ remained undetectable. The only effect of DSS colitis was a reduction of IL-17 output.

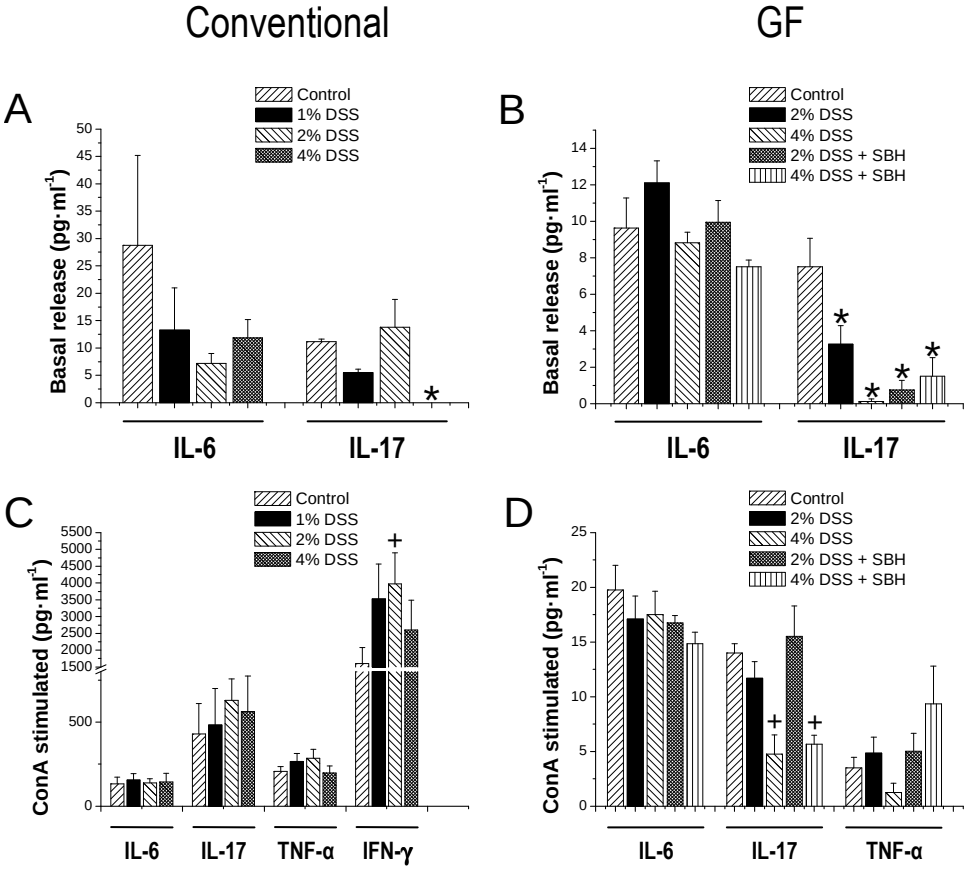


Figure 33. Cytokine secretion by MLNC in conventional and GF NMRI mice. Conventional NMRI mice (left) and GF NMRI mice (right). MLNC were cultured *ex vivo* in the presence (C, D) or absence (A, B) (basal release) of ConA 5 µg·ml^{-1} and the supernatant was collected at 48 h and analysed for cytokine secretion by ELISA. Cytokines measured are IL-6, IL-17, TNF-α and IFN-γ. Concentrations are expressed as means ± SEM (pg·ml^{-1}). **p*<0.05 *vs.* control; +*p*<0.05 *vs.* control ConA. DSS: dextran sulfate sodium. GF: germ-free. SBH: sterile bacterial homogenate.

VI. *Cytokine secretion by splenocytes in conventional and GF NMRI mice*

Splenocytes from conventional NMRI mice exhibited basal IL-6 and TNF-α secretion, which tended to be higher in the 1 and 2% DSS groups ($p<0.05$ for IL-6 in the 2% group only, **fig. 34A**). The same trend was apparent in cells treated with ConA, which showed a higher release of IL-6, IL-17, TNF-α and IFN-γ (see **fig. 34C** for significances). However, TNF-α was not significantly changed in any group.

In GF basal cultures, there were no differences in spontaneous (basal) cytokine release between the control and DSS groups (**fig. 34B**). ConA had almost no stimulatory effect on IL-6, IL-17 and TNF-α (compare **figs. 34C** and **34D**), while IFN-γ was not induced at all and remained undetectable. The 4% DSS-treated group showed a small but significant reduction in IL-6 secretion but no other effect. In contrast with MLNC, splenocytes obtained from the 4% DSS + SBH group showed a heightened basal IL-6 and TNF-α release (**fig. 34B**) and a higher IL-17 and TNF-α ConA-evoked production (**fig. 34D**). This was not observed in the 2% DSS + SBH group, which showed generally reduced cytokine output by splenocytes.

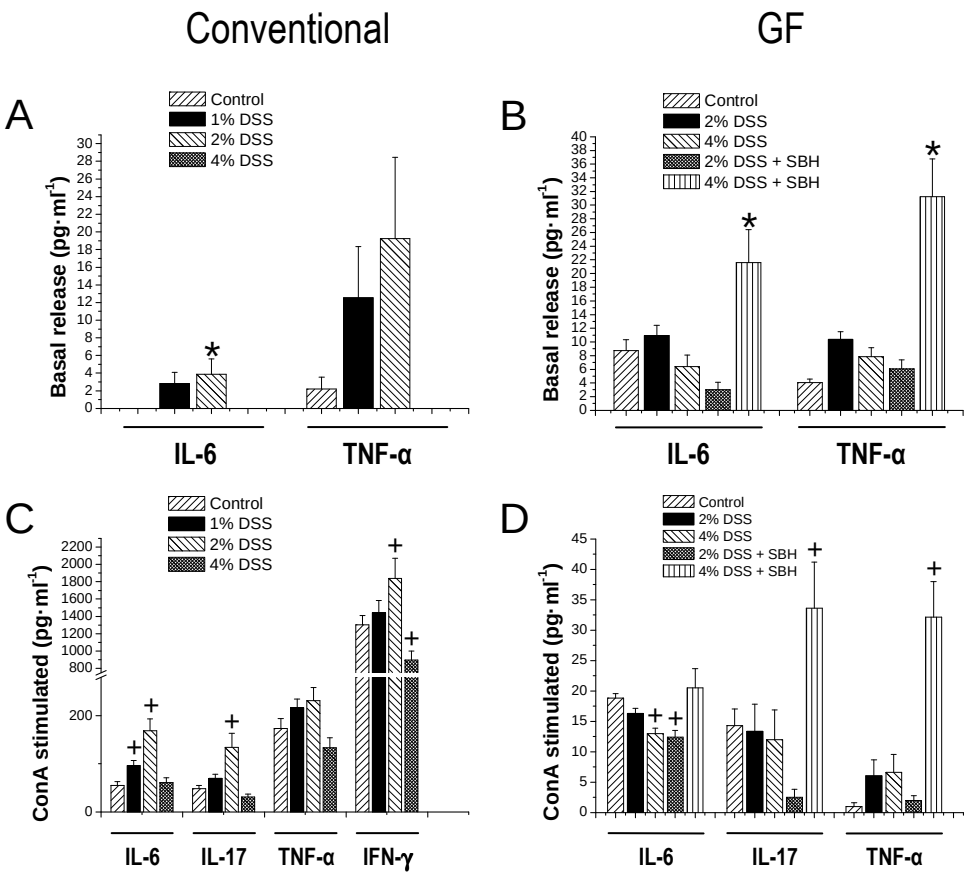


Figure 34. Cytokine secretion by splenocytes in conventional and GF NMRI mice. Conventional NMRI mice (left) and GF NMRI mice (right). Splenocytes were cultured *ex vivo* in the presence (C, D) or absence (A, B) (basal release) of ConA 5 μg·ml^{-1} and the supernatant was collected at 24 h and analysed for cytokine secretion by ELISA. Cytokines measured are IL-6, IL-17, TNF-α and IFN-γ. Concentrations are expressed as means ± SEM (pg·ml^{-1}). *****p<0.05 *vs.* control; **+**p<0.05 *vs.* control ConA. DSS: dextran sulfate sodium. GF: germ-free. SBH: sterile bacterial homogenate.

VII. *Acquired depletion of colonic microbiota recapitulates the altered response to DSS*

We next set out to verify whether a similar differential response could be obtained in conventional animals undergoing a substantial depletion of the colonic microbiota, using an antibiotic cocktail as described in Material & Methods. For simplicity we refer to this model as "pseudo-germ free" (PGF) mice. This experiment was carried out with C57BL/6J mice. Animals were treated with the antibiotic cocktail for 4 weeks, a time point when 16S bacterial DNA could no longer be amplified from faeces, indicating a massive bacterial depletion. At this point animals were randomized to receive DSS or normal drinking water, while the antibiotic cocktail was continued. PGF mice show changes in water absorption and colonic transporter expression compared to conventional mice. Those changes were also observed in GF NMRI mice. Besides, PGF mice also developed the characteristic caecum enlargement observed in GF mice.

As expected, non colitic mice did not suffer body weight loss during the experiment, and remained stable for the 7 days of the experiment (**fig. 35A**). Interestingly, while conventional 3% DSS mice experienced a substantial body weight loss, this was not observed at all in PGF 3% DSS animals. DAI was also much lower in 3% DSS-treated PGF mice (**fig. 35B**).

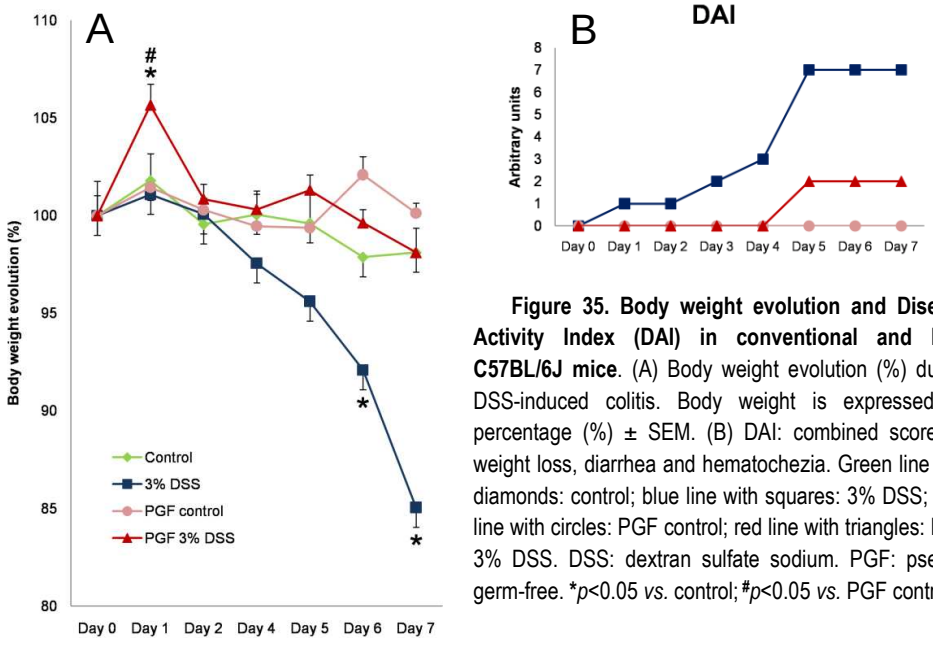


Figure 35. Body weight evolution and Disease Activity Index (DAI) in conventional and PGF C57BL/6J mice. (A) Body weight evolution (%) during DSS-induced colitis. Body weight is expressed as percentage (%) ± SEM. (B) DAI: combined score for weight loss, diarrhea and hematochezia. Green line with diamonds: control; blue line with squares: 3% DSS; pink line with circles: PGF control; red line with triangles: PGF 3% DSS. DSS: dextran sulfate sodium. PGF: pseudo germ-free. **p*<0.05 *vs.* control; #*p*<0.05 *vs.* PGF control.

There were no significant differences in food and water intake among the groups (data not shown). Conventional mice treated with 3% DSS showed a significant increase in colonic damage score compared with PGF 3% DSS mice (3.1 ± 0.1 *vs.* 0.9 ± 0.3, $p<0.05$), mainly due to enhanced hyperemia and fibrosis (data not shown). The colonic weight to length ratio was not significantly modified and hepatomegaly was not observed in any case (**table 10**).

Table 10. Morphological indicators of inflammation in conventional and GF C57BL/6J mice

	Colonic damage score	Colon weight:length ratio	Liver weight [(1)]
	Arbitrary units	(mg·cm^{-1})·1000	(g liver·BW mouse^{-1})·1000
Control	0.0 ± 0.0	78.3 ± 9.4	44.0 ± 2.9
3% DSS	3.1 ± 0.1*	87.6 ± 5.8	49.2 ± 2.4
PGF control	0.5 ± 0.3	52.7 ± 10.9	35.4 ± 2.3*
PGF 3% DSS	0.9 ± 0.3*	67.7 ± 7.2	34.3 ± 1.4*

[(1)] Referred to the animal relative weight. Values are means ± SEM, *$p<0.05$ *vs.* control.
BW: body weight. DSS: dextran sulfate sodium. PGF: pseudo germ-free.

Colonic MPO activity was substantially higher in the conventional 3% DSS group compared with control mice, while DSS treatment failed to affect this parameter in PGF mice (**fig. 36A**). In contrast, colonic AP activity was augmented by DSS only in PGF mice ($p<0.05$, **fig. 36B**). Paradoxically, this increase was associated with a lower sensitivity to levamisole *in vitro* ($p<0.05$, **fig. 36C**).

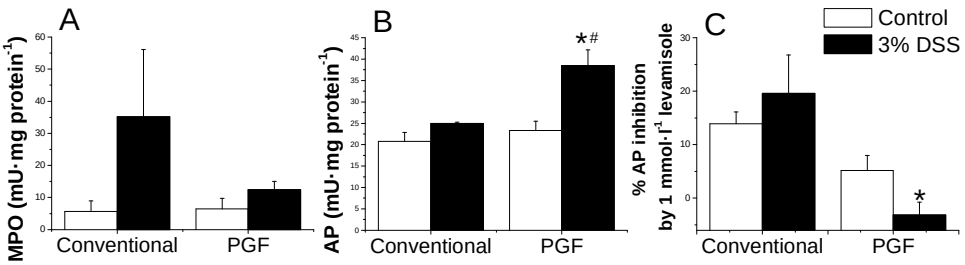


Figure 36. Colonic inflammatory markers in conventional and PGF C57BL/6J mice. Colonic myeloperoxidase (MPO) (A) and alkaline phosphatase (AP) (B, C) activity. White columns: control; black colums: 3% DSS. Enzymatic activity (mU·mg protein^{-1}) and the sensitivity of AP to the specific inhibitor levamisole (% AP inhibition) are shown. Values are means ± SEM. *$p<0.05$ *vs.* conventional control; #$p<0.05$ *vs.* PGF control. DSS: dextran sulfate sodium. PGF: pseudo germ-free.

TNF-α and REGIII-γ colonic expression was upregulated in 3% DSS mice (non-significant), but very weakly or not at all in PGF 3% DSS mice (**fig. 37B**). Controversially, S100A8 was induced not only by DSS administration (3% DSS, $p<0.05$, and PGF 3% DSS), but also by the antibiotic cocktail itself (PGF control, non-significant) (**fig. 37C**).

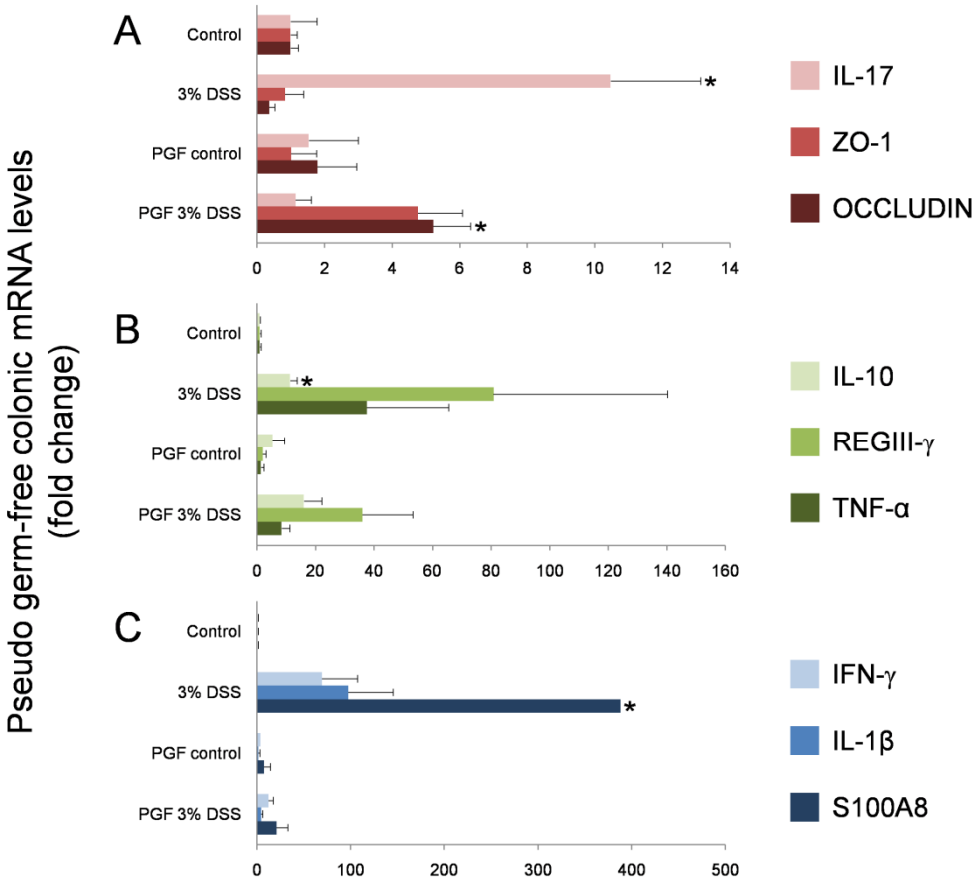


Figure 37. Colonic expression of inflammatory markers assessed by qRT-PCR in conventional and PGF C57BL/6J mice. Colonic mRNA levels of IL-17, ZO-1, OCCLUDIN, IL-10, REGIII-γ, TNF-α, IFN-γ, IL-1β and S100A8 are displayed. Values are means (fold change) ± SEM. *$p<0.05$ *vs.* control. DSS: dextran sulfate sodium. PGF: pseudo germ-free.

Histologically, the colonic wall of conventional 3% DSS mice showed a significant mucosal and submucosal infiltration, crypt elongation and epithelial erosions as major signs, while PGF 3% DSS mice samples showed a generally well preserved mucosal architecture with reduced infiltration and no major erosions (**fig. 38**).

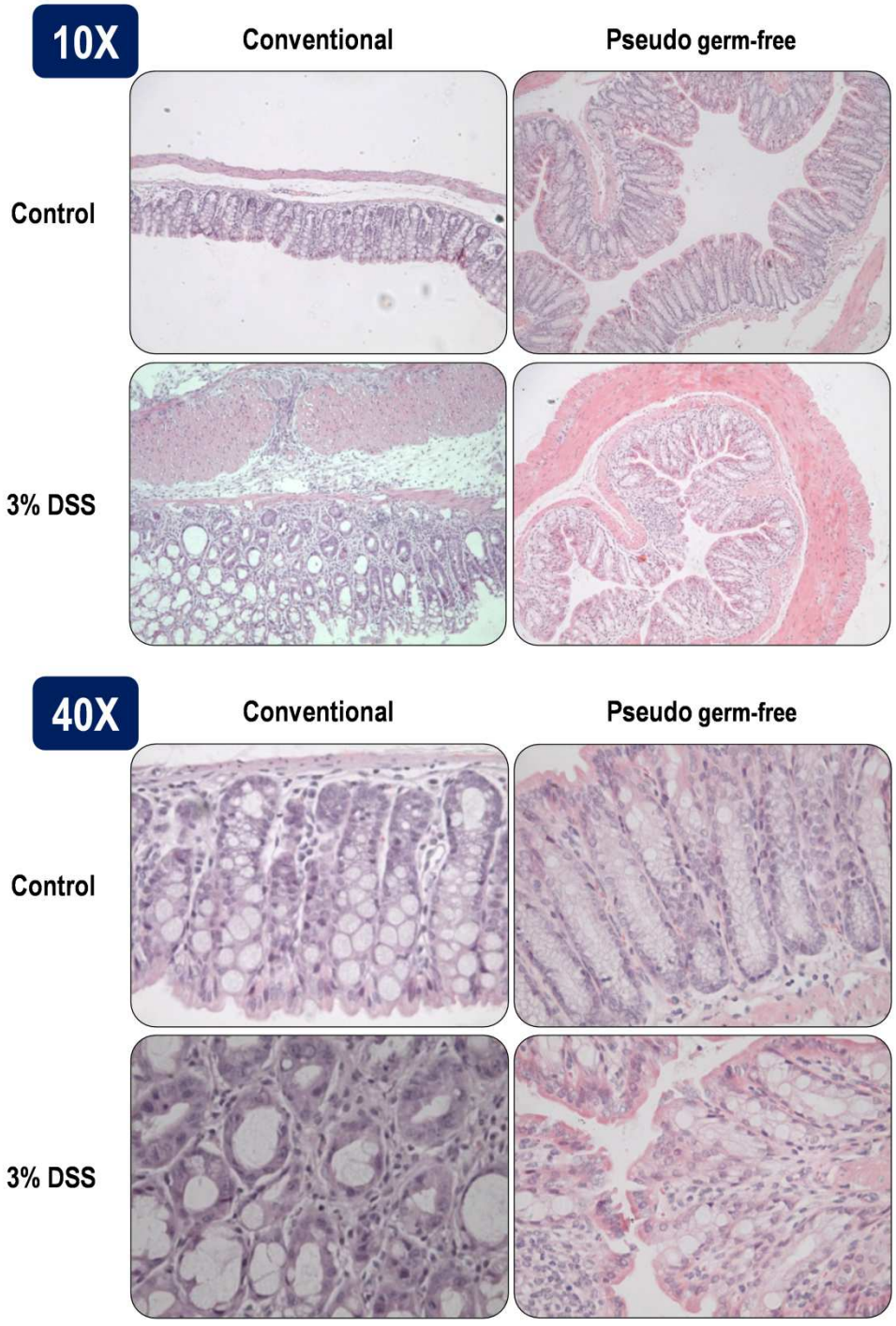


Fig. 38. Histological analysis. Colon of conventional and PGF C57BL/6J mice after they were sacrificed on day 7. Hematoxylin & eosin staining, 10X and 40X. DSS: dextran sulfate sodium. PGF: pseudo germ-free.

VIII. *Effect of conventional, GF and PGF conditions on determinants of mucosal barrier function*

In order to explore the possible mechanism underlying the discrepant results presented so far, we measured by RT-qPCR a number of parameters related to mucosal barrier function, based on the hypothesis that this plays a central role in the overall response to DSS. As shown in **fig. 37A**, the epithelial junctional genes OCCLUDIN and ZO-1 were downregulated in conventional 3% DSS C57BL/6J mice, although non-significant (as well as DSS-treated conventional NMRI mice, **fig. 32A**, $p<0.05$). Conversely, they were upregulated 5-fold in PGF 3% DSS C57BL/6J mice (DSS GF NMRI mice genes were unchanged).

Trefoil factor 3 (TFF3) and the mucin MUC3 were differentially affected by DSS depending on the "germ" status of mice. Thus, TFF3 was downregulated in conventional NMRI mice ($p<0.05$ in 2 and 4% DSS, **fig. 39A**) and was unchanged in GF conditions (**fig. 39C**), but it was upregulated almost 10-fold in PGF 3% DSS mice (**fig. 39E**).

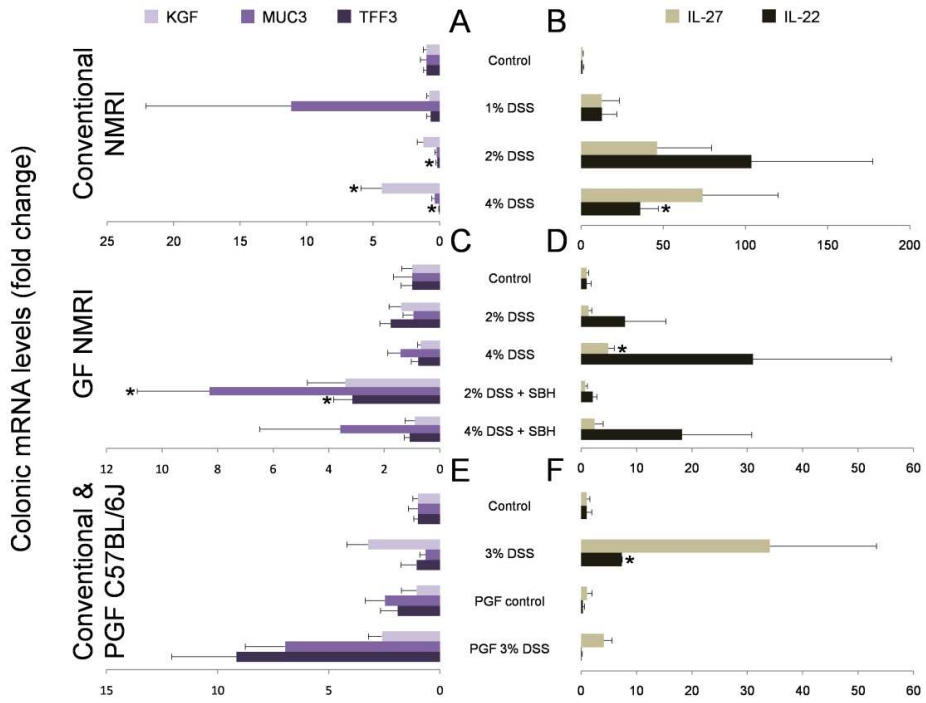


Figure 39. Mucosal barrier function analysis assessed by qRT-PCR (I). Colonic mRNA levels of KGF, MUC3, TFF3, IL-27 and IL-22 are displayed. Values are means (fold change) ± SEM. *$p<0.05$ *vs.* control. DSS: dextran sulfate sodium. GF: germ-free. PGF: pseudo germ-free.

MUC3 exhibited a similar profile although it did not reach statistical significance. The adittion of SBH to the 2% dose in GF NMRI mice upregulated both MUC3 and TFF3 genes (p<0.05, **fig. 39C**).

Keratinocyte growth factor (KGF), IL-22 and IL-27, with known epithelial proliferative actions, were generally increased in both conventional mice (NMRI and C57BL/6J) in response to DSS, but not, or much less, in GF or PGF conditions (**fig. 39**, check the scale). Because the changes in OCCLUDIN, ZO-1, MUC3 and TFF3 correlate with IL-10 expression in PGF mice, and this cytokine is known to promote epithelial barrier function, we hypothesized that previous normal exposure of PGF mice to bacteria and the resulting induction of Treg cells in the colonic mucosa may exert protective effects in these mice that are absent in GF mice. Thus, we measured the expression of the Treg marker FOXP3 (**fig. 40**). Indeed, DSS treatment resulted in robust increase of FOXP3 (~10-fold) in either conventional or PGF mice (**figs. 40A**, **C**), but not in GF mice. Interestingly, a trend toward augmented FOXP3 was noted in GF mice exposed to SBH (**fig. 40B**), suggesting that the luminal presence of bacterial components may ultimately compensate for the lack of Treg cells in adequate conditions.

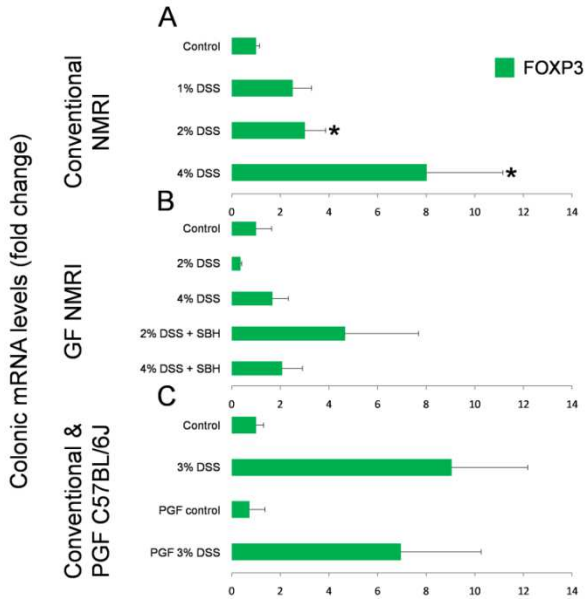


Figure 40. Mucosal barrier function analysis assessed by qRT-PCR (II). Colonic mRNA levels of FOXP3 are displayed. Values are means (fold change) ± SEM. *p<0.05 *vs.* control. DSS: dextran sulfate sodium. GF: germ-free. PGF: pseudo germ-free.

Discussion

The present PhD Thesis touches on several aspects of the microbiota/luminal contents-mucosal biology relationships. These relationships have great importance for the homeostasis of the gut, especially in connection with IBD. It is widely acknowledged that intestinal microbiota shapes the immune response, while the latter modulates the intestinal microbiota. Because IBD has been tightly related to dysbiosis and to an aberrant response to the microbiota in susceptible patients, modulation of the luminal bacterial content is considered a relevant and potentially powerfull target for therapeutic intervention. In this regard, prebiotics and probiotics offer promise of beneficial effects lacking undesirable side effects.

We have tackled 3 important and related aspects of this current theme:

First, we have characterized the direct, non-prebiotic effects of oligosaccharides on macrophages and lymphocytes. These oligosaccharides are known for their prebiotic effects, i.e., microbiota modulating, but before reaching the colon they are in contact with the intestinal mucosa and may reach the submucosa resulting in immune modulation.

Second, since many of the beneficial effects of functional foods described in animal models fail to be reproduced in humans, we planned the use of a truly chronic lymphocyte-driven model of colitis in order to validate the intestinal anti-inflammatory effect of FOS, one of the most clinically used prebiotics.

Third, several studies indicate that colitis may be induced using DSS in the absence of microbiota, and this is in contradiction with the attributed role of luminal bacteria on the induction of colitis and IBD in humans. Therefore, this issue was assessed using conventional, GF and PGF animals.

I. *Non-prebiotic effects*

The aim of this Thesis was to assess the posibility of prebiotics acting as immunomudulators on monocytes and T cells. We have previously established that these compounds exert significant actions on IEC.[396] Namely, an increased production of GRO-α, MCP-1 (monocyte chemotactic protein-1) and MIP-2 (macrophage inflammatory protein-2) is obtained in IEC18 cells, as well as IL-8 in Caco-2 and HT29 cells, via a

mechanism that appears to involve TLR4 ligation and the subsequent activation of the classical NFκB pathway. Since monocytes and, to a lesser extent, T lymphocytes, also express TLR4, it was logical to expect a response consistent with TLR4 activation. The data obtained in mouse splenocytes and in rat monocytes and T lymphocytes indicate that these cells respond to NDOS in a similar fashion to IEC, i.e., with enhanced cytokine secretion. As expected, the response to LPS is greatly diminished in TLR4$^{-/-}$ splenocytes compared to the controls, and this is the case also for the effect of NDOS on TNF-α, IL-6, IL-17 and IFN-γ, either with or without LPS. This suggests that TLR4 ligation is involved in all these effects. However, since we have no evidence of direct binding to the receptor, it is possible also that these compounds modulate the activity of TLR4 indirectly. At any rate, both LPS and NDOS elicited a small but significant response in TLR4$^{-/-}$ splenocytes, reflecting probably the ability to engage other receptors. Interestingly, IL-10 levels were less reduced by TLR4 KO. Also, the inhibition of IFN-γ release by T cells appears to be fairly independent of TLR4.

Because TNF-α is produced mainly by monocytes, while IFN-γ and IL-17 are produced by T cells, and IL-10 and IL-6 are released by both cell types, the results obtained point broadly at an activation of monocytes and an inhibition of T cells by NDOS. Therefore, we tested their effect on primary murine monocytes isolated from rat spleen. As explained, the content of monocytes in the mouse spleen is rather low and therefore makes it necessary to sacrifice a relatively high number of animals for *in vitro* experiments. Since our aim was to examine cell type effects regardless of species, we chose to use rat cells for the following experiments. We have focused on two of the NDOS, namely FOS and inulin, which have an identical chemical structure and differ only in the molecular size, and exhibited robust effects in splenocytes.

The results obtained in rat monocytes confirm that FOS and inulin evoke cytokine secretion, including IL-10, GRO-α and TNF-α. Pharmacological analysis of this response indicated that the NFκB pathway is chiefly involved, although p38 MAPK and PI3K also play a role, based on the effect of pharmacological inhibitors. This is consistent with the ligation of TLR4 as mechanism of action of NDOS.

In contrast, FOS and inulin had negligible effects on rat T cells, rather than the inhibition suggested by the splenocyte data. We hypothesized that the effect may be indirect, i.e., mediated by monocytes, presumably via enhanced IL-10 secretion. In order to check this hypothesis, we carried out an experiment in which monocytes were isolated as described in Material & Methods and treated with FOS or inulin at 5 $g \cdot l^{-1}$. The conditioned medium was added then to T cells in an attempt to modulate cytokine expression by monocyte-derived factors. However, T cells were maximally stimulated by the medium in these conditions, so no further regulation seemed possible (data not shown). Therefore we cannot establish the mechanism of IFN-γ and IL-17 downregulation by FOS/GOS/GMOS, although the hypothesis of indirect regulation seems plausible. Of course we cannot rule out possible differences related to species (mouse vs. rat).

Monocytes were also isolated from the blood of healthy human volunteers in order to confirm our results obtained in animal cells. Indeed, both FOS and inulin evoked cytokine secretion, namely IL-1β, TNF-α and IL-10. Only TNF-α and IL-10 were significantly induced, in agreement with the higher sensitivity observed in rat monocytes. However, there was no effect on IL-8. This cell population is little responsive to LPS, consistent with their non-differentiated/activated phenotype, and thus only IL-8 tended to increase after LPS addition. This is consistent with IL-8 being more sensitive to LPS than other cytokines in these cells.[399] The reason for this discrepancy is unknown. However, our data suggest strongly that NDOS stimulate monocytes via TLR ligation and activation of the NFκB pathway or, alternatively, by some other type of modulation of the TLR4 receptor. Of note, it is now accepted that TLR4 can be activated by a number of compounds other than LPS, including heat shock proteins, hyaluronan, heparan sulfate and other extracellular matrix components, the nuclear protein HMGB1β (high mobility group box 1 beta) and calprotectin (see **table 1**).[400, 401]

Prebiotics are dietary compounds well known for their ability to influence bacterial proliferation selectively in the gut, hence their “prebiotic” denomination. They have been ascribed other beneficial activities, such as interference with bacterial attachment to the epithelium by competing with glycan receptors or enhanced formation of SCFA in the

large bowel. So far just a few studies have focused on the possible direct actions of prebiotics on the intestinal mucosa.[402-404] In principle, TLR4 activation produces pro-inflammatory signals and, given that the intestinal mucosa is constantly exposed to bacteria and bacterial components, this might be expected to lead to inflammation. The subepithelial localization of monocytes in the mucosa implies that they would be engaged by LPS (or prebiotics) only through breaches in the epithelial layer, for instance in case of inflammation. However, it has been recently found that LPS is in fact absorbed normally in the gut, reaching the bloodstream and possibly influencing the host for instance in terms of metabolic equilibrium.[405] Indeed, prebiotics have been shown to transfer through epithelial monolayers, suggesting that the cells located in the subepithelial milieu may be modulated by these compounds.[344] Furthermore, lymphocyte modulation can be achieved at lower concentrations than the ones used in the present study for some prebiotic compounds, implying that direct immunomodulatory actions may be more important for some prebiotics than for others.[344] Anyway, the concentration used in our study is comparable to those in similar studies by other groups and are in line with oligosaccharide concentration in human milk.[339, 402, 406, 407] Therefore, while it is quite likely that mucosal monocytes are exposed to ingested prebiotics and thereby modulated by them, it is uncertain to what extent. At any rate, neither the intestinal microbiota, carrying high amounts of LPS and other bacterial products, nor prebiotics are inflammatory *in vivo*.

In fact, orally administered prebiotics are beneficial in a number of conditions, including metabolic syndrome, diarrhea and experimental intestinal inflammation, among others.[345, 346, 408] Our data suggest that prebiotics can be useful in colitis in part by enhancing mucosal barrier function. This putative mechanism of action is based on the hypothesis that failure of the mucosal barrier to contain the bacterial flora results in enhanced translocation and a more robust inflammatory response. There are various examples of paradoxical effects supporting this concept. Thus, granulocyte-macrophage colony-stimulating factor (GM-CSF) administration has been shown to be protective in experimental colitis acting on innate immunity mechanisms.[409] Accordingly, GM-CSF KO mice is more susceptible to inducible colitis.[410] Similarly, lack of expression of GRO-α/CXCL1, considered the main chemokine responsible for neutrophil recruitment in the

colon, is associated with augmented colitis.[411] In line with these findings, neutrophil depletion itself aggravates colitis.[412] As discussed above, to what extent orally administered prebiotics can effectively modulate mucosal monocytes is unknown. However, at the dose of 500 $mg \cdot kg^{-1}$ used by our group in rat colitis,[345, 346] it is reasonable to expect concentrations much higher than 5 $g \cdot l^{-1}$ in the lumen, suggesting that this activity is indeed feasible. Indeed, higher doses of prebiotics have been applied in experimental animals, further supporting this possibility.

Interestingly, human breastfeeding milk incorporates both complex oligosaccharides and monocytes, and maternal formulas often feature inulin or FOS in an attempt to replicate these oligosaccharides. It is tempting to speculate that milk and/or mucosal monocytes are activated in this fashion in the neonate to enhance immune protection for the first few weeks after birth. Milk also features κ-casein, which generates glycomacropeptide in the stomach, a peptide that also activates monocytes.[199, 399]

II. ***FOS and T cell transfer colitis***

Nutraceuticals are food related products that produce health benefits to the consumer beyond their basic nutritional value. IBD is an obvious possible target of nutraceuticals because of the important adverse effects associated to the drug therapy employed in this condition. Although it seems unrealistic to manage IBD patients on the basis of nutraceuticals or functional foods alone, these products may be useful coadjuvants due to their extremely low toxicity, as an add-on to regular nutrition. Not surprisingly, the food industry has been particularly active in the search and marketing of new products of this type. However, there is an obvious risk of overselling the claimed virtues of a given nutraceutical, and current regulations in Europe (EC 432/2012) require demonstration of specific qualities in terms of physiological benefit or prevention of disease. The goal of this Thesis was, therefore, to validate the intestinal anti-inflammatory activity of FOS in a lymphocyte-driven model of IBD.[257, 388] The advantage of this IBD model lies on its truly chronic nature and the lymphocyte-driven pathology, qualities that bring it closer to the clinical characteristics of human IBD. Colitis develops slowly and once established it may remain relatively stable for weeks or deteriorate slowly until animal death (spontaneous or by euthanasia due to ethical reasons). We chose to apply a postreatment protocol

because it is otherwise difficult to ensure disease consistency among mice, since colitis does not develop simultaneously in all animals, in contrast with the situation in chemically-induced models. In this experiment we established a 10% body weight loss as threshold to consider animals colitic, and they additionally exhibited other signs of disease such as reduced movement, huddling or diarrhea. At this time point animals showed also rectal prolapse, a sign of intestinal inflammation. Other mice transferred in parallel to the ones in this experiment and sacrificed at an earlier time point also showed increased colon weight:length ratio, colonic damage score and splenomegalia, augmented ConA-stimulated MLNC cytokine secretion, and so forth.

Consistent with chronic colitis, the untreated transferred mice continued to lose weight after the treatment period started, a trend that was promptly counteracted by FOS treatment. Since there were no significant differences in food intake, these effects are probably related to inflammation-evoked cachexia and FOS protection. Although not tested in the transfer model, experimental colitis has been shown to be associated to augmented systemic levels of IL-1β and leptin, resulting in anorexia and weight loss.[413, 414] Even though there was no anorexia in the colitic group in the present study (probably because of the mild degree of colitis), acute stress-associated cachexia is a logical explanation for weight loss. It is possible also that FOS supplementation provides a significant caloric input (in the form of SCFA), thus contributing to protection against body wasting. It is however uncertain to what extent this factor is significant given that FOS supplementation represented only 2.5% of dietary intake.

This initial benefit was confirmed by a significantly lower colonic MPO and AP activity, decreased S100A8 expression, and ConA elicited IFN-γ, IL-17 and TNF-α secretion by MLNC *ex vivo*. In addition, FOS-treated mice had a lower damage score (due to a favorable impact on adhesions and fibrosis), which was non-significant. It should be noted in this regard that lymphocyte transfer colitis is relatively mild compared with chemically-induced models. There is hardly any precedent of the use of this model for the testing of new treatments other than immunological manoeuvres,[415] so that we adapted for this purpose a score criterium used in TNBS and DSS in rats and mice. At any rate, FOS

treatment clearly failed to diminish colonic thickening. In this model this is accounted for by crypt enlargement, a typical sign of colitis, suggesting that this feature is unaffected by the prebiotic.

Mechanistically, it is interesting to consider the effect of FOS on colonic AP. There are two isoforms of this enzyme in mice, namely the intestinal form and tissue non-specific AP. In turn, the latter has three varieties, i.e., liver, kidney and bone AP, which are named after the organs where they are predominant, but in fact are expressed by multiple tissues. In particular, leukocytes express tissue non-specific AP of the bone or kidney isotype.[416] While intestinal AP is dominant in the small intestine, the colon expresses the tissue non-specific type, which probably represents a mix of the liver and bone/kidney AP.[253, 397] The three isoforms within the tissue non-specific gene have identical aminoacid sequence and differ only by the pattern of glycosylation, which in turn defines their sensitivity to levamisole, so that liver AP is resistant, and bone and kidney AP are sensitive. We have demonstrated that IEC undergo a change of isoform under severe stress from the liver to the bone/kidney isoform.[398] Thus, AP activity is increased in colitis as a consequence of both leukocyte infiltration and a shift of isoform in enterocytes. The effect of FOS on both enzyme activity and sensitivity to levamisole suggests therefore that it involves a beneficial impact on the epithelium.

FOS are thought to dampen intestinal inflammation by modulation of the enteric microbiota. Therefore we looked at changes in the microbiota to confirm that FOS behave as prebiotic in our experimental conditions. Indeed, we found a significant increase in lactic acid bacteria in FOS-treated mice. It should be noted however that the changes observed in the colitic group are relatively modest.[345] Although not explored so far as we can tell, it is possible that the contribution of dysbiosis is reduced in this model. On the other hand, we have established that FOS have direct immunomodulatory actions in IEC and monocytes in the absence of bacteria (unpublished results and [396]). These actions are consistent with stimulation of innate immune defense. Although apparently incongruent, such an effect may be associated with a reduced inflammatory response by a prompt and efficient control of mucosa invading microorganisms and antigens. For instance, Nenci *et al.* observed that conditional suppression of intestinal epithelial

expression of IKK-γ (IκB kinase gamma, also known as NEMO) or IKK-α/β, resulting in reduced activation of the NFκB pathway, produced a severe inflammatory response.[238] Thus, weaking the mucosal barrier function tends to aggravate colitis, and reinforcing it may have opposite effects. It is interesting to note in this regard that FOS reduce the invasion of IEC *in vitro* by *E. coli* LF82 enteroinvasive bacteria, due at least in part to an effect on enterocytes (unpublished results). Therefore FOS may act by both microbiota dependent and independent effects.

The dose used in this study, 75 $mg \cdot d^{-1}$, corresponds to 0.5 $g \cdot d^{-1}$ in the rat or 29 $g \cdot d^{-1}$ in humans on a body surface basis. In rats, FOS have been shown to exert colonic anti-inflammatory effects at doses ranging between 1 and 2 $g \cdot d^{-1}$, i.e., up to 4-fold higher than the dose used in this study,[315, 349, 377, 417] while at least some of the studies which failed to find therapeutic benefit employed lower doses.[378] In particular, a recent study showing beneficial effects of FOS in the HLA-B27 transgenic model of rat colitis used a dose of 8 $g \cdot kg^{-1}$, equivalent to roughly 3 times the dose used by us after correction for body surface.[315] This may be relevant especially when considering the evidence available in clinical studies. Recently, Benjamin *et al.* found no improvement in CD patients with FOS despite evidence of mucosal immunomodulation.[387] However, the dose used was 15 $g \cdot d^{-1}$, i.e., about half of the equivalent dose in our study, and a sixth of that applied in HLA-B27 rats.[315] Therefore, it is entirely possible that clinical studies may miss efficacy because of insufficient dosage, although of course we cannot rule out that human IBD responds differently to FOS than animal models.

III. *Bacteria and colitis*

IBD is generally considered a condition characterized by an abnormal, exacerbated inflammatory reaction directed against the enteric microbiota in genetically predisposed patients. An intense investigative effort has identified a number of genetic polymorphisms and environmental factors linked to IBD risk and severity in a complex manner. The importance of the microbiota in IBD has been revealed to a large extent by evidence obtained in animal models. As a rule, intestinal inflammation develops weakly or not at all in GF conditions in multiple types of models, including genetic (IL-10 KO, IL-2 KO),

lymphocyte transfer and chemically-induced models (TNBS).[298, 418-423] There is also clinical evidence pointing to a significant involvement of the microbiota in "human" IBD, particularly regarding CD.[424] In turn, the presence of a normal microbiota is critical for the development of Treg cells that act as counterbalances to mucosal pro-inflammatory stimuli.[419]

Therefore, enteric luminal bacteria have a profound impact on intestinal physiology and, specifically, on mucosal immune responses. TNBS and DSS colitis are the two most used IBD models for pathophysiological studies and, particularly, for the testing of pharmacological and nutritional treatments. While TNBS is recognized as a microbiota dependent model,[423] there have been conflicting reports regarding DSS. Thus, DSS colitis has been claimed to develop normally in the absence of bacteria,[269] or even to be a more efficient colitogenic stimulus in these conditions.[270] Nonetheless, other authors have reported a weak inflammatory response to DSS colitis in GF conditions,[425] and DSS colitis is amenable to antibiotic treatment.[426] The reasons for this discrepancy are unknown. Assessing whether DSS colitis is indeed independent of the intestinal microbiota is of interest because this would constitute an exception in the variety of animal models of IBD studied, as noted above. In addition, we had a more specific interest in this issue, because a microbiota independent IBD model would allow us to test the colonic anti-inflammatory effects of certain compounds without the interference of luminal microorganisms.

DSS is a high molecular weight polymer made out of α(1,6) D-glucose units with variable sulfate esters (up to 3 per sugar bond). It has been used widely since the 80s to induce experimental colitis, but the structurally related carrageenan was among the first colitogenic stimuli identified, some 15-20 years earlier.[260] The mechanism of colitis induction has not been fully elucidated but it appears to be related to direct epithelial disruption. Colitis ensues as a consequence of greatly increased permeability to luminal contents. The response occurs in a few days and is considered an acute type of colitis, but it can adopt a more chronic form by alternating DSS and water-only periods, or administering DSS continuously at low doses such as 1%. Inasmuch as inflammation is a result of enhanced interaction with luminal contents, it would be expected to be

downregulated in the absence of bacteria.

Interestingly, Kitajima *et al.* showed that GF mice (IQI/Jic strain) died early after treatment with 5% DSS was started, with an enhanced decrease in hematocrit and weight loss.[270] Postmortem analysis showed signs of luminal blood loss but little sign of inflammation. In contrast, using a chronic protocol with a 14 day administration of 1% DSS, GF animals again exhibited weight loss and signs of hemorrage compared with conventional animals, but inflammation was noted in the colon, and it was higher than that observed with conventional mice, which was of low grade. The authors concluded that early death in the acute DSS protocol was due to massive blood loss, preventing the development of inflammation, while the chronic protocol allowed colitis to ensue in GF animals, showing that these animals are more susceptible than regular (i.e., conventional) animals. In a previous study, NMRI mice were also shown to exhibit a high mortality rate after treatment with 5% DSS. This was not due to strain specific sensitivity because conventional NMRI mice responded normally.[269] However, the inflammatory status of the colon was not assessed.

Our results in the main experiment (GF *vs.* conventional mice), obtained with the same mouse strain, NMRI, are in agreement with this last study, in that conventional mice responded normally to DSS, with loss of body weight, increased colonic MPO and AP activity, augmented colonic expression of IL-10, IFN-γ, IL-1β, S100A8, REGIII-γ, plus higher secretion of IFN-γ by MLNC and of IL-6, IL-17 and IFN-γ by splenocytes *ex vivo*. In contrast, the colonic weight to length ratio was not significantly increased in these mice despite a markedly higher damage score, a somewhat atypical feature. The reason for this is unknown, but may be related to a strain specific reduced tendency to tissue edema and/or fibrosis.

The results obtained in GF mice were dramatically different. Although body weight loss was comparable to that in conventional mice, the colon showed few signs of inflammatory changes. Thus, there was no increase in colonic MPO activity or on the expression of IL-10, IL-17, IL-1β or REGIII-γ, while TNF-α was significantly augmented, despite the fact that the magnitude of the increase was lower than that in conventional mice. Although

colonic AP activity was significantly increased, it was less than in conventional animals, and its sensitivity to the specific inhibitor levamisole was not increased at all. There was also no increase in cytokine secretion by either MLNC or splenocytes, and some were actually significantly diminished, while IFN-γ was undetectable in all cases. Moreover, the basal (control) levels of a number of these parameters was markedly reduced compared with those in conventional mice, including colonic MPO activity and AP sensitivity to levamisole, colonic gene expression levels and ConA-evoked cytokine secretion by MLNC and splenocytes. Of note, colonic thickness was also much lower in GF animals, approximately 50%. These features are consistent with the well characterized atrophy of the intestinal mucosal immune system in GF animals. Finally, the damage score was also increased to a lower extent than that in conventional animals, and there was no hyperemia, thickening or deformation detected. Therefore, our data indicate unambiguously that DSS-induced colonic inflammation and immune responses are greatly diminished in GF mice in these conditions. DSS-treated GF mice did look sicker than regular mice in our experiment, and there were signs of blood loss, consistent with previous observations,[270] suggesting that while colitis was attenuated in GF conditions, blood loss was enhanced. Colonic inflammation, either experimental or clinical, is frequently associated with rectal bleeding or subclinical hematochezia, and DSS colitis in particular is a model characterized by a marked visible blood loss in faeces. Such bleeding is consistent with a mechanism of epithelial disruption, especially if subepithelial cells are further affected by DSS, resulting in capillary lesion and blood loss to the lumen. Because DSS is continuously administered to recipient animals, this process goes on indefinitely, and so does hemorrage.

On the other hand, DSS exerts anticoagulant effects, due to its similarity to heparin.[270, 427] However, it is unclear why GF and conventional mice (or even PGF mice, see below) should respond differently to this challenge. One possibility is that anticoagulant effects are potentiated by lack of DSS degradation by bacteria, which has been claimed to occur normally in conventional mice.[269, 428] However, this hypothesis has been disputed,[429] and does not explain the effect on PGF mice. Thus, this mechanism is unlikely to be involved in the observed differences.

Our data suggest that epithelial disruption is heightened in GF mice. Unfortunately, because of technical reasons histological analysis could not be carried out in this experiment. To further characterize the interaction between DSS and the microbiota, we started an additional experiment in which conventionally reared mice were depleted of the microbiota (PGF conditions) by treatment with an antibiotic cocktail. This approach allowed us to test whether acquired absence of luminal microorganisms had similar effects to those of inborn GF conditions. Indeed, the results obtained pertaining intestinal inflammation were largely comparable to those in the main experiment (the strain was different due to the lack of NMRI mice, and C57BL/6J mice were used, using 3% DSS as colitogenic dose). Thus, colitis was normally induced in conventional animals, while intestinal inflammation was practically nil in PGF mice treated with DSS, which showed an absence of colonic MPO activity and no change in gene expression levels such as IFN-γ, IL-1β and S100A8. At the histological level conventional DSS-treated mice showed substantial infiltration, submucosal edema, crypt distortion and epithelial erosions, while PGF mice receiving DSS exhibited a largely unaffected mucosal architecture, with weak infiltration and mild submucosal edema. This is in agreement with the findings of Kitajima *et al.*, who reported a normal mucosal histology in DSS-treated GF mice despite a marked blood loss and early death.[270]

A major difference between GF and PGF mice was that the latter not only had greatly diminished colonic inflammation, but also had no major signs of disease, i.e., they had no weight loss and looked healthy, in sharp contrast with the response of GF mice. Inasmuch as the latter are assumed to suffer enhanced blood loss or translocation of bacteria or bacterial products, PGF must be capable of maintaining mucosal barrier function more efficiently than GF mice, and actually more even than conventional mice. In order to explore this possibility we measured a set of markers of barrier function, like TFF3, the mucin MUC3, cytokines with epithelial regulatory functions (IL-22, IL-27 and KGF), and the cytoskeletal components ZO-1 and OCCLUDIN. Both ZO-1 and, especially, OCCLUDIN were downregulated in conventional mice, while GF mice showed little changes, and upregulation was observed in PGF conditions. A similar trend in conventional mice was observed with TFF3, while no changes were observed for GF

mice, and almost a 10-fold upregulation was observed in PGF, although non-significant. KGF was comparably induced in both conditions. MUC3 was not affected except in PGF conditions, where a substantial (albeit non-significant) increase was noted. IL-22 and IL-27, on the other hand, showed a behaviour comparable to those of most other inflammatory markers.

Another interesting observation is that colonic AP activity was paradoxically augmented in PGF animals in response to DSS. AP activity is increased in the inflamed intestine, associated with an augmented sensitivity to the specific inhibitor levamisole, owing to a change in isoform in epithelial cells and a higher contribution of neutrophil derived enzyme.[253, 398] This change was observed in the conventional animals, although without reaching statistical significance, probably due to the low intensity of DSS colitis in this experiment. Despite the indisputable dampening of DSS colitis in the PGF animals, AP activity was significantly augmented, while a reduction in the sensitivity to levamisole *in vitro* was obtained. It is thus tempting to speculate that modulation of epithelial AP is a part of the enhanced barrier function in these mice. Absence of an increased inhibition by levamisole suggests that enterocytes express a higher level of the liver isoform (expressed predominantly in basal conditions) or the intestinal isoform. Of note, the latter has well documented mucosal protective effects.[397, 430-432]

Whatever the mechanism, our data indicate that (1) in the absence or limited presence of luminal bacteria there is little inflammatory response to DSS; (2) GF mice appear to have enhanced blood loss despite greatly reduced colitis intensity; and (3) the epithelial response to DSS in mice with acquired rather than congenital deficiency of microbiota is consistent with enhanced barrier function, while colonic inflammation developing in conventional mice shows the opposite behaviour, and GF mice show essentially no change. The first finding is expected, as noted above. The second may be related to a reduction of epithelial proliferative luminal signals through activation of TLR receptors.[158] Interestingly, addition of SBH to DSS-treated GF mice resulted in lower weight loss during the experiment, despite a trend toward a higher colonic damage score and heightened splenomegaly. Although most inflammatory parameters were not affected, SBH enhanced cytokine secretion by splenocytes *ex vivo* using the 4% but not the 2% DSS dose. In

addition, SBH induced a specific increase in MUC3 and TFF3. Therefore, it appears that providing luminal bacterial components has limited but significant effects on DSS colitis. Reduced body weight loss points to reduced blood loss, possibly via enhanced epithelial compliance, but this is speculative because no specific parameters were measured. An indirect sign in this regard may be the ileal thinning. A decrease in the ileal weight to length ratio is observed with 2-4% DSS in conventional mice and with 4%, but not 2% DSS, in GF mice. DSS evoked blood loss and the subsequent fluid depletion may help to explain this finding. As noted, GF mice treated with 2% DSS have no weight loss, especially with SBH supplementation. The dose of SBH employed was 0.016%, which is certainly lower than that corresponding to the normal luminal load, so that it is likely that these effects are increased with higher SBH doses.

The third finding may be related to the well documented thinning of the mucus layer in GF animals.[262] Although not determined in our study, if mucus thickness is not substantially affected by short term removal of luminal bacteria, a greater epithelial resistance to DSS is expected compared with GF mice. In turn, there is evidence indicating that a contact with the intestinal microbiota through a breach increases Treg and increases the resistance of mice to a later TNBS challenge.[433] In fact, Treg induction is inhibited in GF animals due to the lack of luminal input.[434, 435] This is consistent with the observation that IL-10 is similarly increased in response to DSS in conventional and PGF mice, but not in GF mice. This is especially interesting considering the lack of inflammation in PGF animals, suggesting IL-10 production by resident Treg cells. In fact, direct measurement of FOXP3, a marker of Treg cells, confirms that this is indeed the case. IL-10 has well documented enhancing effects on mucosal barrier function.[436] Presumably, although IL-10 was also increased, the parallel upshot in inflammatory cytokines (IL-6, IL-17 and IFN-γ) in conventional mice, with known inhibitory effects on the expression of tight junction proteins (ZO-1 and OCCLUDIN),[437, 438] prevents the protective effects observed in PGF mice. This fact being further sustained by the inhibition in the expression of ZO-1 and OCCLUDIN observed in our conventional animals.

Conclusions

As a result of the studies performed in the present PhD Thesis we have reached the following conclusions:

I. Monocytes are activated by FOS and inulin, and possibly also by GMOS and GOS, via TLR4 stimulation and engagement of the NFκB and other pathways, such as p38 MAPK and PI3K, resulting in enhanced cytokine secretion. This is a direct action of these compounds that may be involved in their effects *in vivo*.

II. FOS are effective in lymphocyte transfer colitis at the dose of 75 $mg \cdot d^{-1}$ when given as a postreatment. This is a significant advancement in the preclinical evidence supporting the use of this prebiotic in IBD. The anti-inflammatory effect observed using this dose of FOS suggests that doses used in clinical assays might be insufficient.

III. DSS-induced colitis is greatly attenuated in the absence of luminal microorganisms, either inborn or acquired. Nevertheless, the overall status of the animals reared in GF conditions was greatly impaired, probably because of massive blood loss and a failure of the epithelial barrier function.

IV. There is a reinforcement of the epithelial barrier function in response to DSS, which depends critically on previous exposure to the microbiota. A previous exposure increases Treg cells, with the consequent IL-10 production and amelioration of epithelial and mucosal compliance. This reinforcement is overwhelmed by the normal inflammatory response in conventional animals.

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Resumen

La Enfermedad Inflamatoria Intestinal (EII) integra un grupo de trastornos caracterizados por una inflamación crónica, idiopática, destructiva y recurrente del tracto gastrointestinal, en la que se alternan periodos de remisión. Incluye las dos formas más comunes, la Enfermedad de Crohn (EC) y la colitis ulcerosa (CU), así como la colitis colagenosa, la colitis linfocítica y la colitis microscópica atípica. En la actualidad existe un consenso general entre investigadores básicos de la EII de que, tanto la EC como la UC, son el resultado de los efectos combinados de cuatro componentes básicos: cambios globales en el medio ambiente, múltiples variaciones genéticas, alteraciones en la microbiota intestinal y aberraciones de la respuesta inmune innata y adaptativa.[1]

La presente Tesis Doctoral toca varios aspectos de las relaciones existentes entre la microbiota y la biología de la mucosa. Estas relaciones tienen gran importancia para la homeostasis de la mucosa, especialmente en relación con la EII. Es ampliamente reconocido que la microbiota intestinal modula la respuesta inmune, así como la respuesta inmune moldea la microbiota intestinal.[2] Debido a que la EII ha sido fuertemente asociada a la disbiosis y a una respuesta aberrante a la microbiota, en pacientes susceptibles, la modulación del contenido bacteriano luminal se considera un objetivo relevante y potencialmente potente para la intervención terapéutica. En este sentido, los prebióticos y probióticos ofrecen prometedores efectos beneficiosos carentes de efectos secundarios indeseables.

Hemos abordado 3 aspectos importantes y relacionados con el tema actual:

I. ***Determinar efectos no prebióticos de oligosacáridos no digestibles***

En primer lugar, hemos caracterizado los efectos directos, no prebióticos, de diversos oligosacáridos no digestibles (NDOS) en macrófagos y linfocitos. Estos oligosacáridos son conocidos por sus efectos prebióticos, es decir, por la modulación de la microbiota intestinal, pero antes de alcanzar el colon están en contacto con la mucosa intestinal y podrían alcanzar la submucosa, resultando en modulación inmune.

Los prebióticos son ingredientes no digestibles que afectan beneficiosamente al organismo mediante la estimulación selectiva del crecimiento y actividad de

bifidobacterias y, en algunos casos, de lactobacilos, mejorando la salud.[3]

El mecanismo clásico de acción de los prebióticos incluye su digestión por la microbiota colónica. La mayoría de las bacterias colónicas son anaerobias estrictas y, por tanto, obtienen energía mediante fermentación. En la fermentación de prebióticos se producen ácidos grasos de cadena corta (AGCC), principalmente acetato, propionato y butirato. Los AGCC acidifican el pH del lumen intestinal inhibiendo el crecimiento de patógenos e incrementando la movilidad intestinal. Los AGCC producidos son absorbidos rápidamente a través de la mucosa colónica siendo utilizados como fuente de energía. Por otra parte, la utilización de prebióticos por la microbiota produce un aumento de la misma y, en consecuencia, un incremento de la masa fecal que estimula el tránsito intestinal.[4, 5]

Además de las acciones anteriores, estos NDOS pueden ejercer diferentes acciones independientes de su actividad prebiótica. Por ejemplo, inhiben la adhesión de bacterias patógenas a células epiteliales humanas *in vitro*, actuando como receptores señuelo.[6, 7] Actúan directamente sobre células epiteliales intestinales (IEC) y modulan su expresión génica.[8] Modulan la producción de citoquinas en IEC y en células mononucleares de sangre de cordón umbilical humano *in vitro*,[9, 10] equilibrando la respuesta Th1/Th2. Por otra parte, los NDOS proporcionan ácido siálico como un nutriente esencial para el desarrollo del cerebro y la cognición,[11] y regulan el transporte intestinal y la permeabilidad.[12, 13]

Hemos establecido previamente que estos compuestos ejercen acciones importantes en IEC.[14] En la linea celular IEC18 se obtuvo un aumento en la producción de GRO-α, MCP-1 (proteína quimiotáctica de monocitos-1) y MIP-2 (proteína inflamatoria de macrófagos-2), así como un aumento de IL-8 en las lineas celulares Caco-2 y HT29, a través de un mecanismo que parece implicar la unión al receptor Toll-like 4 (TLR4) y la posterior activación de la vía clásica de NFκB. Dado que los monocitos y, en menor medida, los linfocitos T, también expresan TLR4, era lógico esperar una respuesta coherente con la activación de TLR4. Los prebióticos utilizados han sido la inulina, los fructooligosacáridos (FOS), los galactooligosacáridos (GOS) y los oligosacáridos de leche

de cabra (OSLC) a una concentración de 5 $g \cdot l^{-1}$. Los datos obtenidos en esplenocitos de ratón, y en monocitos y linfocitos T de rata, indican que estas células responden a los NDOS de manera similar a las IEC, es decir, con una mayor secreción de citoquinas. Como era de esperar, la respuesta a lipopolisacárido bacteriano (LPS) disminuyó en gran medida en esplenocitos de ratones knock-out para TLR4 ($TLR4^{-/-}$ o TLR4 KO) en comparación con los controles, y éste es el caso también para el efecto de los NDOS sobre la producción de TNF-α , IL-6, IL-17 e IFN-γ, ya sea con o sin LPS. Esto sugiere que la activación de TLR4 está involucrada en todos estos efectos. Sin embargo, ya que no tenemos evidencia de que se produzca una unión directa al receptor, es posible también que estos compuestos modulen la actividad de TLR4 indirectamente. En cualquier caso, tanto los NDOS como el LPS provocan una respuesta pequeña, pero significativa, en esplenocitos de ratones TLR4 KO, lo que refleja probablemente la capacidad de activar a otros receptores. Curiosamente, los niveles de IL-10 se redujeron en menor medida en los ratones TLR4 KO. Además, la inhibición en la liberación de IFN-γ por células T parece ser bastante independiente de TLR4.

Debido a que TNF-α es producido principalmente por monocitos, mientras que IFN-γ e IL-17 son producidos por células T, e IL-10 e IL-6 son liberados por ambos tipos de células, los resultados obtenidos en términos generales indican que se produce una activación de monocitos y una inhibición de linfocitos por los NDOS. Por ello probamos el efecto de FOS e inulina sobre monocitos primarios aislados de bazo de rata. Los resultados obtenidos confirman que FOS e inulina promueven la secreción de citoquinas, incluyendo IL-10, GRO-α y TNF-α. Utilizando inhibidores de diversas vías de transducción de señal comprobamos que la vía de NFκB está principalmente involucrada, aunque p38 MAPK (proteínas quinasas activadas por mitógenos) y PI3K (fosfatidilinositol-3-quinasa) también desempeñan un papel. Esto es consistente con la activación de TLR4 como mecanismo de acción de NDOS.

Por el contrario, FOS e inulina tuvieron efectos insignificantes en linfocitos de rata, en lugar de la inhibición sugerida por los datos de esplenocitos. La hipótesis es que el efecto puede ser indirecto, es decir, mediada por monocitos, presumiblemente a través de un

aumento en la secreción de IL-10. Para comprobar esta hipótesis, se realizó un experimento en el que se aislaron monocitos y se trataron con FOS o inulina. El medio condicionado se añadió a continuación a las células T en un intento de modular la expresión de citoquinas por factores derivados de los monocitos. Sin embargo, no se produjo ningún cambio con respecto a los controles.

Los monocitos también fueron aislados de sangre de voluntarios humanos sanos con objeto de confirmar los resultados obtenidos en las células animales. En efecto, tanto FOS como inulina evocaron la secreción de citoquinas (IL-1β, TNF-α e IL-10).

En principio, la activación de TLR4 produce señales pro-inflamatorias y, dado que la mucosa intestinal está constantemente expuesta a las bacterias y componentes bacterianos, cabría esperar una respuesta inflamatoria. La localización subepitelial de los monocitos en la mucosa implica que serían activados por LPS (o prebióticos) sólo a través de aberturas en la capa epitelial, por ejemplo, en caso de inflamación. Sin embargo, recientemente se ha descubierto que el LPS es absorbido normalmente en el intestino, alcanzando el torrente sanguíneo y, posiblemente, influyendo en el hospedador, por ejemplo, en términos de equilibrio metabólico.[15] En efecto, se ha demostrado que los prebióticos transfieren a través de monocapas epiteliales, lo que sugiere que las células situadas en el medio subepitelial pueden ser modulada por estos compuestos.[10] Por lo tanto, mientras que es muy probable que los monocitos de la mucosa estén expuestos a los prebióticos ingeridos, y de ese modo modulados por ellos, no se sabe en qué medida. En cualquier caso, ni la microbiota intestinal, transportando altas cantidades de LPS y otros productos bacterianos, ni los prebióticos, son inflamatorios *in vivo*.

II. ***Validar el posible uso de los probióticos en el tratamiento de la EII***

En segundo lugar, ya que muchos de los efectos beneficiosos de los alimentos funcionales, descritos en modelos animales, no llegan a ser reproducidos en los seres humanos, se propuso el uso de un modelo de colitis verdaderamente crónico para estudiar el posible efecto antiinflamatorio intestinal de FOS, uno de los prebióticos más utilzados en la clínica.

A pesar del amplio abanico de fármacos empleados en el tratamiento de la EII, las opciones terapéuticas disponibles en la actualidad no son totalmente satisfactorias. En primer lugar, no existe ningún fármaco específico para el tratamiento de esta enfermedad, sino que se utilizan fármacos con carácter antiinflamatorio o inmunosupresor y que, por tanto, van a suprimir la respuesta inmune y las vías inflamatorias de manera global. Esto puede hacer que el individuo presente una merma en la capacidad de defensa, es decir, en su sistema inmune. Por otro lado, los tratamientos farmacológicos empleados se caracterizan por un amplio espectro de reacciones adversas y, en algunos casos, como en el de los glucocorticoides, dan lugar a fenómenos de dependencia. Además, existen situaciones en las que los pacientes no responden a un determinado fármaco, o incluso se hacen refractarios al mismo. Por todo ello, la farmacología de la EII es un campo de intensa investigación, y la búsqueda de nuevas opciones terapéuticas con un mejor perfil de toxicidad está totalmente justificada.[16]

Muchos estudios en animales han demostrado la eficacia de los prebióticos en la prevención y el tratamiento de modelos de EII, aunque estos resultados a menudo varían dependiendo del compuesto usado. Los cambios en la microbiota intestinal son la base para la actividad antiinflamatoria colónica de los prebióticos, incluyendo FOS.[17-21] Esto se ha puesto de manifiesto por la mayoría de los estudios en animales,[17, 22-24] aunque no por todos.[25, 26] La suplementación de FOS ha demostrado atenuar la colitis inducida por ácido trinitrobenceno sulfónico (TNBS) en ratas, promover el crecimiento de bacterias beneficiosas ácido lácticas y aumentar los niveles de butirato en colon.[17]

Aunque estos modelos animales no representan la complejidad de la enfermedad humana, son herramientas valiosas para el estudio de muchos aspectos importantes de la enfermedad que son difíciles de tratar en los seres humanos, tales como los mecanismos fisiopatológicos en fases tempranas de la colitis y el efecto de las estrategias terapéuticas emergentes. El aspecto clínico de la EII humana es heterogéneo, un hecho que también se refleja en el cada vez mayor número de ratones transgénicos o cepas específicas de ratón que desarrollan EII. La mayoría de estos modelos se basan en la inducción química, la transferencia de células inmunitarias o la manipulación

genética y, sólo en algunos modelos, la enfermedad se produce sin ningún tipo de manipulación exógena.[27]

Aunque se han utilizado profusamente para ensayos preclínicos, los modelos de colitis inducida por TNBS y por sulfato de dextrano (DSS) presentan varias desventajas debido a que no son estrictamente crónicos (es decir, se curan con el tiempo) y no están mediados por linfocitos como en la enfermedad humana. En consecuencia, son necesarios más estudios para dilucidar el mecanismo implicado en el efecto beneficioso de los prebióticos en la función intestinal y su implicación en la inflamación intestinal humana. Algunos autores han defendido el uso del modelo de colitis por transferencia de células T para lograr una mejor predicción de la bioactividad humana.[28]

Con el fin de validar plenamente el posible uso de los prebióticos, tales como FOS, en la EII, es importante demostrar su bioactividad en tal modelo. Por ello pretendemos verificar el efecto antiinflamatorio de FOS en el modelo de colitis por transferencia linfocitaria de células T CD4+ CD62L,[28, 29] y establecer las condiciones ideales para las pruebas clínicas.

Para ello se aislaron células T CD4+ CD62L+ de bazo de ratones C57BL/6J y se inyectaron por vía intraperitoneal en ratones receptores C57BL/6J Rag1$^{-/-}$. Tras 8 semanas desde el inicio de la transferencia linfocitaria los ratones desarrollaron colitis. Los ratones colíticos fueron asignados aleatoriamente a 2 grupos diferentes: un grupo tratado con FOS (75 mg·día^{-1}) y un grupo control al que se le administró vehículo (solución salina). Además se incluyó un tercer grupo de ratones control no colíticos. El tratamiento se mantuvo hasta que los animales fueron sacrificados después de 13 días por dislocación cervical bajo anestesia con isoflurano.

De acuerdo con la colitis crónica, los ratones control colíticos continuaron perdiendo peso después del inicio del período de 13 días de tratamiento, una tendencia que fue contrarrestada rápidamente por el tratamiento con FOS. Puesto que no hubo diferencias significativas en la ingesta de alimentos, estos efectos están probablemente relacionados con la caquexia derivada de la inflamación en los ratones control colíticos, mostrando

FOS un efecto protector.

Este beneficio inicial se confirmó por una actividad colónica mieloperoxidasa (MPO) y fosfatasa alcalina (AP) significativamente inferior, por una disminución de los niveles de expresión génica de S100A8, y por una disminución en la liberación de citoquinas pro-inflamatorias por células mesentéricas de nódulos linfáticos (MLNC) cultivadas *ex vivo* (IFN-γ, IL-17 y TNF-α). Además, los ratones tratados con FOS tuvieron una puntuación de daño macroscópico menor (debido a un impacto favorable en las adherencias y fibrosis), aunque no fue significativo.

Se cree que FOS amortigua la inflamación intestinal por modulación de la microbiota entérica. Por ello estudiamos los cambios en la microbiota fecal para confirmar que FOS se comporta como prebiótico en nuestras condiciones experimentales. Encontramos un aumento significativo en las bacterias ácido lácticas en los ratones tratados con FOS. Es posible que la contribución de la disbiosis se reduzca en este modelo. Por otra parte, hemos establecido que FOS tiene acciones inmunomoduladoras directas en IEC y en monocitos en ausencia de bacterias (resultados no publicados y [14]).

III. *Verificar la importancia de la microbiota para desarrollar EII*

En tercer lugar, varios estudios indican que la colitis puede ser inducida utilizando DSS en ausencia de microbiota, siendo contradictorio con el papel atribuido a las bacterias luminales en la inducción de la colitis y la EII en los seres humanos. Por lo tanto, este aspecto se evaluó induciendo colitis por DSS en ratones convencionales, en ratones libres de gérmenes (germ-free, GF), así como en un modelo de reducción drástica de microbiota inducido por antibióticos (condiciones "pseudo GF" -PGF-).

El intestino mantiene una compleja relación con la microbiota intestinal, probablemente más obligada que estrictamente simbiótica. A pesar de la enorme carga bacteriana del tracto gastrointestinal, y la gran variedad de especies presentes, un exquisito equilibrio se mantiene casi siempre. La combinación de una barrera eficaz auto-regenerativa, la secreción de abundante moco, el continuo flujo luminal de contenido y un sistema inmune finamente regulado, permite mantener la masiva población microbiana

contenida dentro de los límites de la mucosa. Sin embargo, este equilibrio puede ser alterado sustancialmente, lo que resulta normalmente en respuestas inflamatorias, como en la EII. Por lo tanto, la inflamación intestinal puede ser la consecuencia tanto de una respuesta inmune aumentada como de un defecto en la función de barrera.[2]

En cualquier caso, existen evidencias sustanciales que señalan hacia una respuesta inmune desregulada para con la microbiota normal, como un factor fundamental en la EII. Uno de los principales argumentos que apoyan la importancia de la microbiota en este contexto es el hecho de que la inflamación intestinal es muy difícil de inducir en los animales experimentales en condiciones GF.[30-36] Una notable excepción es la colitis inducida por DSS. Kitajima y col. asumieron que en ratones GF la colitis es inducida con la misma o mayor gravedad que en ratones normales.[37, 38] Por lo tanto las bacterias luminales no pueden jugar un papel clave en la colitis inducida por DSS.

Nuestros resultados en el experimento principal (GF *vs.* ratones convencionales) muestran que los ratones convencionales responden como cabría esperar al DSS, es decir, con pérdida de peso corporal, incremento de actividad MPO y AP, aumentando los niveles de expresión génica en colon (IL-10, IFN-γ, IL-1β, S100A8, REGIII-γ), además de mostrando una mayor liberación de citoquinas por MLNC (IFN-γ) y por esplenocitos (IL-6, IL-17 e IFN-γ) cultivados *ex vivo*.

Los resultados obtenidos en ratones GF fueron dramáticamente diferentes. Aunque la pérdida de peso corporal fue comparable a la de los ratones convencionales, el colon mostró pocos signos inflamatorios. Por lo tanto, no hubo un incremento en la actividad MPO o en la expresión colónica de IL-10 , IL-17 , IL-1β o REGIII-γ, mientras que TNF-α aumentó significativamente, a pesar del hecho de que la magnitud del aumento fue menor comparado con los ratones convencionales. Tampoco hubo aumento en la secreción de citoquinas e IFN-γ fue indetectable en todos los casos. El ratio peso:longitud del colon también fue mucho menor en los animales GF, aproximadamente del 50%. Estas características son consistentes con la atrofia bien caracterizada del sistema inmune de la mucosa intestinal en animales GF. Por último, la puntuación del daño macroscópico también aumentó en menor medida que en los animales convencionales,

no mostrando signos de hiperemia, engrosamiento o deformación colónica. Por lo tanto, nuestros datos indican inequívocamente que la inflamación colónica inducida por DSS y la respuesta inmune están disminuidas en gran medida en ratones GF.

Sin embargo, los ratones GF tratados con DSS se veían más enfermos que los ratones normales en nuestro experimento, y hubo señales de pérdida de sangre masiva, en consonancia con observaciones anteriores,[37] lo que sugiere que, si bien la colitis se atenuó en condiciones GF, la pérdida de sangre fue mayor.

Nuestros datos sugieren que la interrupción epitelial se ve aumentada en ratones GF. Para caracterizar mejor la interacción entre DSS y microbiota utilizamos el modelo PGF. Este enfoque nos ha permitido poner a prueba si la ausencia adquirida de microorganismos luminales, mediante el empleo de un coctel de antibióticos, tendría efectos similares a los observados en ratones GF innatos. En efecto, los resultados obtenidos relativos a la inflamación intestinal fueron en gran medida comparables a los del experimento principal. A nivel histológico, los ratones control tratados con DSS mostraron infiltración, edema submucoso, distorsión de las criptas y erosiones epiteliales, mientras que los ratones PGF tratados con DSS mostraron una arquitectura de la mucosa prácticamente intacta, con infiltración débil y edema submucoso leve. Esto está en consonancia con los hallazgos de Kitajima y col., que describieron una histología normal de la mucosa en los ratones GF tratados con DSS, a pesar de la marcada pérdida de sangre y muerte temprana.[37]

Una diferencia importante entre ratones GF y ratones PGF es que estos últimos no sólo habían disminuido considerablemente la inflamación del colon, sino que también carecían de signos de la enfermedad, es decir, no presentaron pérdida de peso y parecían saludables, en contraste con la respuesta de los ratones GF. Los ratones PGF deben ser capaces de mantener la función de barrera de la mucosa de manera más eficiente que los ratones GF y, de hecho, incluso más que los ratones convencionales. Con el fin de explorar esta posibilidad se midió un conjunto de marcadores de la función de barrera, como TFF3, MUC3, citoquinas con funciones reguladoras epiteliales (IL-22, IL-27 y KGF -factor de crecimiento de queratinocitos-) y componentes del citoesqueleto

(ZO-1 y OCLUDINA).

Cualquiera que sea el mecanismo, nuestros datos indican que (1) en ausencia o presencia limitada de bacterias luminales hay poca respuesta inflamatoria a DSS; (2) los ratones GF parecen sufrir una mayor pérdida de sangre a pesar de la reducida intensidad de la colitis; y (3) la respuesta epitelial a DSS en ratones con flora adquirida (PGF), en lugar de deficiencia congénita (GF), es consistente con la función de barrera mejorada.

El primer hallazgo se ha comentado anteriormente. El segundo puede estar relacionado con una reducción de las señales proliferativas epiteliales luminales a través de la activación de los receptores TLR.[39] El tercer hallazgo puede estar relacionado con la disminución en el espesor de la capa de moco en animales GF.[40] Si el espesor de la mucosidad no se ve afectado sustancialmente por la eliminación a corto plazo de las bacterias luminales con el coctel de antibióticos, se esperará una resistencia epitelial mayor al DSS en comparación con los ratones GF. A su vez, hay evidencias que indican que un contacto con la microbiota intestinal a través de una brecha aumenta las células T reguladoras (Treg) y aumenta la posterior resistencia de los ratones a la colitis inducida por TNBS.[41] De hecho, la inducción de Treg es inhibida en animales GF al no estar en contacto con antígenos luminales.[42, 43] Esto es consistente con la observación de que IL-10 se incrementa de manera similar en respuesta a DSS en ratones convencionales y PGF, pero no en ratones GF. Es especialmente interesante teniendo en cuenta la falta de inflamación en animales PGF, lo que sugiere la producción de IL-10 por células residentes Treg. Se ha documentado que IL-10 mejora la función de barrera de la mucosa.[44] Es de suponer que, a pesar de que IL- 10 también se incrementó en animales convencionales, la producción paralela de citoquinas pro-inflamatorias, con conocidos efectos inhibitorios sobre la expresión de las proteínas de unión estrecha (ZO-1 y OCLUDINA),[45, 46] evita los efectos protectores observados en ratones PGF. Este hecho se sustenta aún más por la inhibición observada en la expresión de ZO-1 y OCLUDINA en nuestros animales convencionales.

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Conclusiones

Como resultado de los estudios realizados en la presente Tesis Doctoral se ha llegado a las siguientes conclusiones:

I. Los monocitos son activados por FOS y por inulina, y posiblemente también por GOS y OSLC, a través de su unión a TLR4 y la posterior activación de la via de NFκB y otras vías, como la p38 MAPK y PI3K, dando lugar a una mayor secreción de citoquinas. Se trata de una acción directa de estos compuestos, la cual puede estar involucrada en sus efectos *in vivo*.

II. FOS es eficaz en la colitis inducida por transferencia linfocitaria a la dosis de 75 $mg \cdot d^{-1}$ al ser administrado como postratamiento. Es un avance significativo en la evidencia preclínica que apoya el uso de este prebiótico en la EII. El efecto antiinflamatorio observado utilizando esta dosis de FOS sugiere que las dosis usadas en ensayos clínicos podrían ser insuficientes.

III. La colitis inducida por DSS es atenuada en gran medida debido a la ausencia de microorganismos luminales, ya sea de manera innata o adquirida. Sin embargo, el estado general de los animales criados en condiciones GF se vio deteriorado en gran medida, probablemente debido a la pérdida masiva de sangre y a un fallo de la función de barrera epitelial.

IV. Se produce un refuerzo de la función de barrera epitelial en respuesta a DSS, el cual depende fundamentalmente de la exposición previa a la microbiota. Una exposición previa aumenta el número de células Treg, con la consiguiente producción de IL-10 y una disminución en el compromiso de la mucosa epitelial. Este refuerzo es sobrepasado por la respuesta inflamatoria normal en animales convencionales.