Commensal microbes, immune reactivity and childhood inflammatory bowel disease

Cecilia Barkman

UNIVERSITY OF GOTHENBURG

Department of Infectious Diseases
Sahlgrenska Academy
University of Gothenburg
Sweden
2009

ISBN 978-91-628-7811-5
Department of Infectious Diseases
University of Gothenburg, Sweden

Printed by Intellecta Infolog AB
Västra Frölunda, Sweden 2009

Commensal microbes, immune reactivity and childhood inflammatory bowel disease.

Cecilia Barkman, Department of Infectious Diseases, University of Gothenburg
Guldhedsgatan 10A, 413 46 Göteborg, Sweden, cecilia.barkman@microbio.gu.se

Abstract

Inflammatory bowel disease (IBD) is characterized by chronic and relapsing intestinal inflammation of unknown etiology, but immune activation by the commensal microbiota probably plays a major role. The two major categories of IBD are ulcerative colitis and Crohn's disease. One fifth of the cases present in childhood and Sweden has a high and rising incidence of pediatric IBD. The aim of this thesis was to study the composition of the small and large intestinal microbiota and signs of activation on lymphocyte subsets in the blood circulation in children at the début of IBD, before initiation of treatment. Further, the requirements of commensal Gram-positive bacteria to initiate production of IL-12, a cytokine stimulating Th1 reactions in innate immune cells, was studied using blood obtained from healthy donors.

Blood, faecal and duodenal samples were obtained from children referred to a pediatric gastroenterology centre due to suspected IBD. Samples of the microbiota were cultivated quantitatively for aerobic and anaerobic bacteria. After establishment of diagnosis, the composition of the microbiota and lymphocyte subsets were compared between children with ulcerative colitis, Crohn's disease, symptomatic children found not to have IBD (diseased controls) and healthy controls. The microbiota mainly in children with ulcerative colitis was shown to be altered, with decreased counts of anaerobic Gram-positive bacteria such as bifidobacteria and clostridia. Whereas bifidobacterial counts normalized with treatment, clostridial populations remained low. Both children with ulcerative colitis and Crohn's disease had an increased fraction of Gram-negative bacteria in the stools, compared with controls. Blood cell subsets were analysed by flow cytometry for activation and memory markers. Children with ulcerative colitis displayed strong activation of circulating T cells, especially manifested as increased expression of β1-integins. Children with Crohn's disease had few memory B-cells, suggesting immunological immaturity.

Our studies further revealed that Gram-positive bacteria are major stimuli for IL-12 production in monocytes as long as they remain intact, but that fragments of Gram-positive bacteria inhibit IL-12 production in blood cells. This may be a physiological feedback circuit, since such fragments may signal that further activation of the phagocyte via the IL-12/IFN-γ loop is unnecessary. IL-12 production in response to intact Gram-positive bacteria required phagocytosis, activation of TLR2- and Nod2-receptors, demonstrated by chemical blocking, anti-human TLR antibodies, and binding of synthetic or natural ligands. Further, IL-12 production induced by intact Gram-positive bacteria required signalling via PIP3, NF-κB and JNK. These pathways differed from those inducing IL-12 production in response to LPS in interferon-γ primed monocytes. Thus, Gram-positive bacteria have unique and important immunomodulating properties that may influence IBD development.

Key words: inflammatory bowel disease, pediatric, ulcerative colitis, Crohn's disease, microbiota, T cells, B cells, cytokine, IL-12, monocytes, phagocytosis, intracellular signalling pathways

LIST OF PUBLICATIONS

The thesis is based on the following studies, which will be referred to in the text by their Roman numerals (I-III)

I. Barkman C., Saalman, R., Lindberg E., Wolving M., Ahrné S., Molin G., Adlerberth I., Wold A.E. **Intestinal microbiota at début of childhood inflammatory bowel disease.** In manuscript

II. Barkman C., Saalman, R., Rudin A., Wold A.E. **Activation, homing and memory markers on blood circulating B and T lymphocytes at début of childhood inflammatory bowel disease.** In manuscript.

III. Barkman C., Martner A., Hessle C., Wold A.E. **Soluble bacterial constituents down-regulate secretion of IL-12 in response to intact Gram-positive bacteria.** Microbes and Infection 10 (2008) 1484-93

To my family with all my love

ABBREVIATIONS

APC	Antigen presenting cell
CD	Crohn's disease
CARD	Caspase recruitment domain family member 15
CFU	Colony Forming Units
DAP	Diaminopimelic acid
IBD	Inflammatory bowel disease
IC	Indeterminate colitis
MDP	Muramyl dipeptide
Nod1	Nucleotide-binding oligomerization domain-1
Nod2	Nucleotide-binding oligomerization domain-2
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
PBMC	Peripheral blood mononuclear cells
PG	Peptidoglycan
SCFA	Short chain fatty acids
TLR	Toll like receptors
UC	Ulcerative colitis

TABLE OF CONTENTS

INTRODUCTION

Human beings and microbes have been evolving together for millions of years and this has led to a delicate interaction between the two. A life form can only persist as long as its integrity is maintained in relation to other particles and life forms and as long as it is able to interact with the environment. These factors are pivotal for healthy existence and a balance between the organism and its environment is normally kept. In inflammatory bowel disease, however, the balance is altered.

Childhood inflammatory bowel disease

Inflammatory bowel disease (IBD) is characterized by chronic and relapsing intestinal inflammation of unknown etiology. The children often experience symptoms of abdominal pain, diarrhoea, rectal bleeding, weight loss, lethargy and anorexia (3). The two major categories of IBD are ulcerative colitis (UC) and Crohn's disease (CD). In some cases, these two entities cannot be distinguished and the disease is termed "indeterminate colitis". IBD may also be accompanied by extraintestinal manifestations e.g. in the skin (erythema nodosum), eyes (episcleritis, iritis), liver (autoimmune hepatitis), bile ducts (sclerosing cholangitis) and joints (arthritis, ankylosing spondylitis). Chronic inflammation of the colon of long duration may lead to dysplasia and cancer development (9).

Although the onset of IBD may occur at any age, it peaks in adolescence or early adulthood (10). As many as 25 % of IBD patients receive their diagnosis during childhood or adolescence (11). Diagnosis of childhood IBD is based on clinical presentation, endoscopic histological and radiological features summarized in the Porto criteria (3).

Ulcerative colitis

The term ulcerative colitis was first used by Sir S. Wilks in London 1859 (12). The hallmark of UC is diarrhoea, which often contains blood and is accompanied by abdominal pain (Table 1). According to the definition of UC, the disease is restricted to the colonic mucosa and the distribution is continuous. 70-80 % of all children with UC have inflammation throughout the colon, pancolitis, upon disease presentation (11, 13). The histological picture includes crypt abscesses, i.e. presence of polymorphonuclear cells in the colonic crypts, and depletion of goblet cells (Table 2).

Crohn's disease

The disease is named after the American gastroenterologist B. Crohn, who described the condition in 1932 (14). Prominent symptoms of CD in children are abdominal pain, lethargy, anorexia, weight loss and growth retardation (Table 1). Rectal bleeding is less common in CD than in UC, while lethargy and anorexia is more frequent in CD (Table 1). CD is characterized by discontinuous inflammation that may affect any part of the gastro-intestinal tract, including the mouth. Colon involvement is more common in children than in adults and the entire colon may be affected, as in UC. As the disease progresses fistulae and strictures may develop. Histological presentation includes segmented and transmural inflammation with crypt abscesses. Characteristically, granulomas, i.e. aggregates of macrophages, giant cells and lymphocytes, may also be observed (Table 2).

Indeterminate colitis

The term indeterminate colitis is used when a distinct diagnosis of UC or CD cannot be established. Indeterminate colitis accounts for some 10-20 % of the patients in most IBD populations.

Table 1. Clinical findings in the two major categories of childhood inflammatory bowel disease.

Clinical symptoms	Ulcerative colitis	Crohn's disease
Diarrhoea	+++	++
Bloody stools	+++	+
Abdominal pain	+	+++
Lethargy	++	+++
Fever	+	++
Anorexia	++	+++
Weight loss	++	+++
Growth retardation	+	++
Delayed puberty	+	++
Oral ulcerations	-	+
Fistulae	-	+

+++ = typical findings, ++ = common, + = exists, -= absent

Data are adapted from Inflammatory Bowel Disease in Children and Adolescents: Recommendations for Diagnosis—The Porto Criteria (3) and from Swedish guidelines (Swedish Society for Pediatric Gastoenterology, Hepatology and Nutrition) (15)

Table 2. Endoscopic and histological findings in childhood inflammatory bowel disease

Ulcerative colitis	Crohn's disease
Endoscopic picture	
Continuous with variable proximal extension from rectum	Segmental distribution
Spontaneous bleeding	Skip lesions
Ulcers	Ulcers (aphthous, linear)
Loss of vascular pattern granularity	Cobblestoning
Friability	Strictures
Pseudopolyps	Fistulae
Erythema	Abnormalities in oral and/or perianal regions
Histological picture	
Mucosal involvement	Submucosal or transmural involvement
Continuous distribution	Patchy distribution
	Focal changes
Ulcers, crypt abscesses and distortion	Ulcers, crypt abscesses and distortion
Goblet cell depletion	Granulomas

Data are adapted from Inflammatory Bowel Disease in Children and Adolescents: Recommendations for Diagnosis—The Porto Criteria (3)

Epidemiology

Epidemiology is the study of the occurrence of a disease in a population. Many associate epidemiology with the study of acute outbreaks of infectious diseases but it is also an important tool for seeking the etiology of chronic idiopatic diseases such as IBD. Epidemiology can be defined as the study of disease incidence (in IBD literature = new cases per 100,000 persons per year) and prevalence (e.g. number of cases per 100,000 individuals at a particular time) in relation to demographic factors such as age, gender, race or ethnic group. Variations in incidence and prevalence in different populations, geographic regions and over time may yield clues to the causes of the disease.

The global incidence of IBD in adults

There are literally hundreds of articles describing the incidence and prevalence of IBD in adults. The topic has been reviewed by Loftus (16) and by Gismera and Aladrén (17). The highest incidences of IBD are reported from countries with a western life style, such as Scandinavia (18-23), northern Europe (24, 25), the United Kingdom (26-28) and North America (29-31) (Table 3). The incidences in southern Europe has been reported to be lower (24, 32-34) than in northern Europe, and incidence reported from Seoul in South Korea (35) and Lebanon (36) are even lower (Table 3).

Changes in incidence of IBD

The incidence of IBD is believed to be increasing in areas that have a relatively low incidence (16, 17, 37). For example, the incidence in South Korea doubled every four years between 1986 – 2005 (Table 3). In contrast, IBD is often said to have reached a plateau in high incidence areas (17) such as northern Europe, although a new study from Denmark reports a continuing increase in incidence (20).

Interestingly, in areas with a low incidence, such as South Korea, UC accounts for the increase of IBD (35), while in high incidence areas such as Denmark (20), Sweden (4) and Wisconsin USA (38) it is largely CD that accounts for the increased incidence.

Incidence of childhood IBD in Sweden

Sweden has one of the highest incidences of childhood IBD in the world and IBD is one of the most common chronic childhood diseases in Sweden and some other westernized countries. Particularly CD, has increased markedly in recent decades (1, 4) (Fig. 1). A predominance among male children has been reported in childhood CD not only in Sweden (4) but also in other countries with a high incidence of childhood IBD, such as Canada, the United Kingdom and Ireland (13, 29, 38).

Table 3. Examples of the incidence of the two major categories of IBD, ulcerative colitis and Crohn's disease in studies performed on well-defined populations.

Author	Location	Years	Incidence per 100 000	
			Ulcerative colitis	Crohn's disease
North America				
Lofus (31, 39)	Olmsted County, MN	1984-93	8.3	6.9
Bernstein (29)	Manitoba	1989-94	14.3	14.6
Bernstein (40)	Manitoba	1998-2000	11.8	13.4
Blanchard (30)	Manitoba	1987-96	15.5	15.6
Scandinavia				
Björnsson (41)	Island	1990-4	16.5	5.5
Moum (19)	Southeastern Norway	1990-3	13.6	5.8
Munkholm and Langholtz (21, 22)	Copenhagen Denmark	1980-7	9.2	4.1
Vind (20)	Copenhagen Denmark	2003-5	13.4	8.6
North Europe				
Shivananda (24)	8 cities of Northern Europe	1991-3	11.8	7.0
Russel (25)	The Netherlands	1991-4	10	6.9
Rodriguez (28)	United Kingdom	1995-7	11	8
Rubin (26)	Cardiff, United Kingdom	1995	13.9	8.3
Tsironi (27)	United Kingdom	1997-2001	8.2	7.3
South Europe				
Shivananda (24)	12 cities of Southern Europe	1991-3	8.7	3.9
Trallori (33)	Florence, Italy	1990-2	9.6	3.4
Asia				
Yang (35)	Seoul, South Korea	1986-90	0.34	0.05
		1991-5	0.87	0.22
		1996-2000	1.74	0.52
		2001-5	3.08	1.34
Abdul-Baki (36)	Lebanon	2000-4	4.1	1.4

Data are adapted from Loftus (16) Gismera *et al* (17) and Thia *et al (37)*

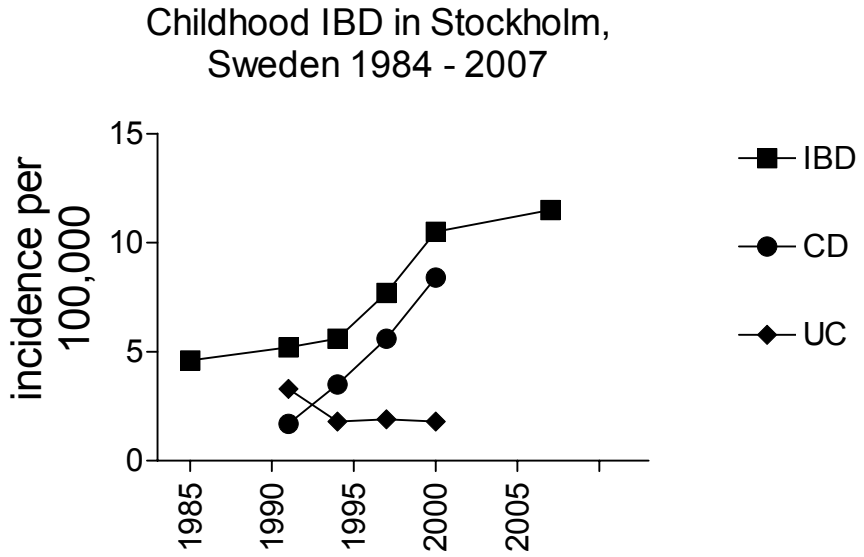


Figure 1. Childhood IBD in the Stockholm region, Sweden, based on data from Lindberg *et al* (1), Hildebrand *et al* (4) and Malmborg *et al.* (5)

Factors that may explain the increase and variations of the IBD incidence

The high incidence of IBD in northern Europe and the United States and the low incidence in developing countries have prompted the development of several theories. It is reasonable to assume that awareness of and knowledge about IBD is greater in areas of high incidence than in areas of low incidence.

Genetic factors may provide an explanation for the uneven global distribution of IBD. Several genes have been suggested to predispose an individual to the development of IBD (see section "Some theories behind the origin of IBD"). There is an increased risk of developing IBD if another family member, particularly a parent, has been diagnosed with the condition (42). Individuals of Jewish heritage are at greater risk of developing IBD than others (11).

Calculations based on several studies of non-Jewish populations in North America and Europe show that the risk of developing IBD, during a life time (assumed to be 70 years) is 5% with a first-degree relative with CD (proband) and 1.6% with a first-degree relative that have UC (proband) (43). In families of Jewish heritage the corresponding figures are 8% for CD and 5.2% for UC (43).

Results from twin studies have shown that if one monozygotic twin has CD the other has a 50% risk of also being affected while a dizygotic twin has only a 4% risk (44). In the case of UC, the corresponding figures are 19% in monozygotic twins, while no concordance was seen in dizygotic twins (44). These results indicate that genetic factors play a greater role in CD than they do in UC. However, the role of genetic factors would seem to be minor given that migrants who move from low to high incidence areas develop IBD at the same rate as individuals in the new country. This finding has been confirmed in migrants who move from South Asia to Europe (27, 45).

A number of life-style factors have been proposed to play a role in the development of IBD. Perinatal infections, including exposure to antibiotics, could influence later onset of IBD (46-48). However, it is difficult to interpret the findings of retrospective studies if the subjects have been asked to recall factors occurring many years earlier.

Active smoking appears to provide some protection from the development of UC, whereas it instead increases the risk of developing CD (20, 49). There is evidence to suggest that appendectomy, if performed early in life, may protect against UC (50-52).

Overall, the most likely factors for explaining the uneven geographical distribution of IBD would seem to be those of socioeconomic status; the growing incidence of IBD corresponds with westernization and industrialization and the lifestyle changes these imply.

Hygiene hypothesis

A promising hypothesis for explaining the high incidence of IBD in areas of high socioeconomic standard is the “hygiene hypothesis”. This hypothesis was formulated in 1989 by David Strachan to explain the increased incidence of allergy in North West Europe during the 20th century (53). He proposed that microbial stimulation early in life was required to properly develop and educate the immune system. Later, also the rising incidence of IBD has been associated with the hygiene hypothesis (54, 55) as IBD, in common with allergy, is more frequent in the first born child of families with several children (56). IBD has been shown to be more common in families with higher income and fewer family members (30, 57).

Especially CD is related to good sanitary conditions (58-60). In Scotland, the risk of developing CD was found to be greater among those who had access to hot tap water and a separate bathroom in early childhood (58). These results were later confirmed by Duggan *et al* (60). The fact that children living on a farm with regular contact with farm animals develop CD to a lesser extent supports the hygiene hypothesis (61). Further, children who live in a large family, or a family that owns a cat are less at risk of developing CD (59). However, a recent study from the United States shows that children of large families that keep pets are at greater risk of developing the disease (62).

Gut flora

Why is the gut flora interesting in IBD?

The surface of the human gastrointestinal tract is about the size of a tennis court and it is in constant contact with bacteria. The intestinal commensal flora comprises 10^{14} bacterial cells in total, while the human body only contains 10^{13} eukaryotic cells. Several findings have demonstrated the importance of the intestinal flora in IBD. Various animal models trying to resemble IBD requires bacterial presence for disease development, as animals bred under germ-free conditions remain healthy or develop less severe inflammation (63-65). Further, $CD4^+$ T cells, when activated by commensal bacterial antigens, may induce colitis when transferred to immune-deficient mice (66). In humans, treatment of CD with broad spectrum antibiotics or deviation of the fecal stream may ameliorate the disease (67-69). Further, treatment with probiotics has been reported to induce remission in patients suffering from UC (70-72).

Evidently there exists a mutual exchange between the intestinal bacteria and the human body. The commensal intestinal flora occupies an ecological niche, thereby preventing pathogens to colonize. Other bacteria such as lactobacilli are known to alter the intestinal pH (73). Gut bacteria can produce enzymes that can hydrolase amino acid conjugated bile acids into free bile acids which in turn influence bacterial function and gene expression enabling bacterial adhesion to intestinal epithelium (74). Intestinal anaerobic bacteria, metabolize dietary fibres to short chain fatty acids (SCFA) (75). SCFA such as acetate, propionate and butyrate are organic fatty acids with 2-6 carbon atoms. Especially, butyrate is the major source of energy for the colonic mucosa (76, 77). Butyrate have anti-inflammatogenic and anti-neoplastic properties (78-81). Roediger showed that colonic cells from patients with ulcerative colitis have decreased capacity to utilize butyrate as energy generating substrate (82).

Gram-positive and Gram-negative bacteria

Bacteria are divided into a number of phyla. Irrespective of genetic relatedness, bacteria can also be divided into Gram-positives and Gram-negatives based upon the staining method developed by the Danish pathologist Hans-Christian Gram (83). Bacteria are stained with crystal violet, fixed with iodide solution, destained with ethanol or acetone and then counter-stained with saffranin. In Gram-positive bacteria complexes of crystal violet are captured in the cytoplasm due to the thick and tightly meshed cell wall, while the stained complexes leak out of Gram-negative bacteria during ethanol/acetone treatment.

The cell wall of Gram-positive bacteria is composed of many layers of peptidoglycan, which is a polymer of N-acetyl-glucosamine, and N-acetylmuramic acid cross-linked by peptide bridges. Teichoic acid and lipoteichoic acid (LTA) are long negatively charged polymers, which are usually also present in Gram-positive bacteria (Fig 2).

The peptidoglycan layer of the Gram-negative bacteria is thinner and has fewer peptide bridges. It is covered by an outer membrane attached to the cell wall by lipoproteins, and is only found in Gram-negative bacteria. Proteins and lipopolysaccharide (LPS), the latter restricted to Gram-negative bacteria (Fig 2a), are attached to the outer membrane.

The peptide bridge differs between most Gram-positive and Gram-negative bacteria, as the third amino acid in the bridge is commonly a lysine in Gram-positive bacteria, but diaminopimelic acid (DAP) in most Gram-negatives (Fig. 2b) (84).

In addition, many Gram-negative bacteria have flagella, a tool for motility. These different characteristics of Gram-positive and Gram-negative bacteria are sensed by different receptors (see below) by the immune system in order to direct an appropriate immune response.

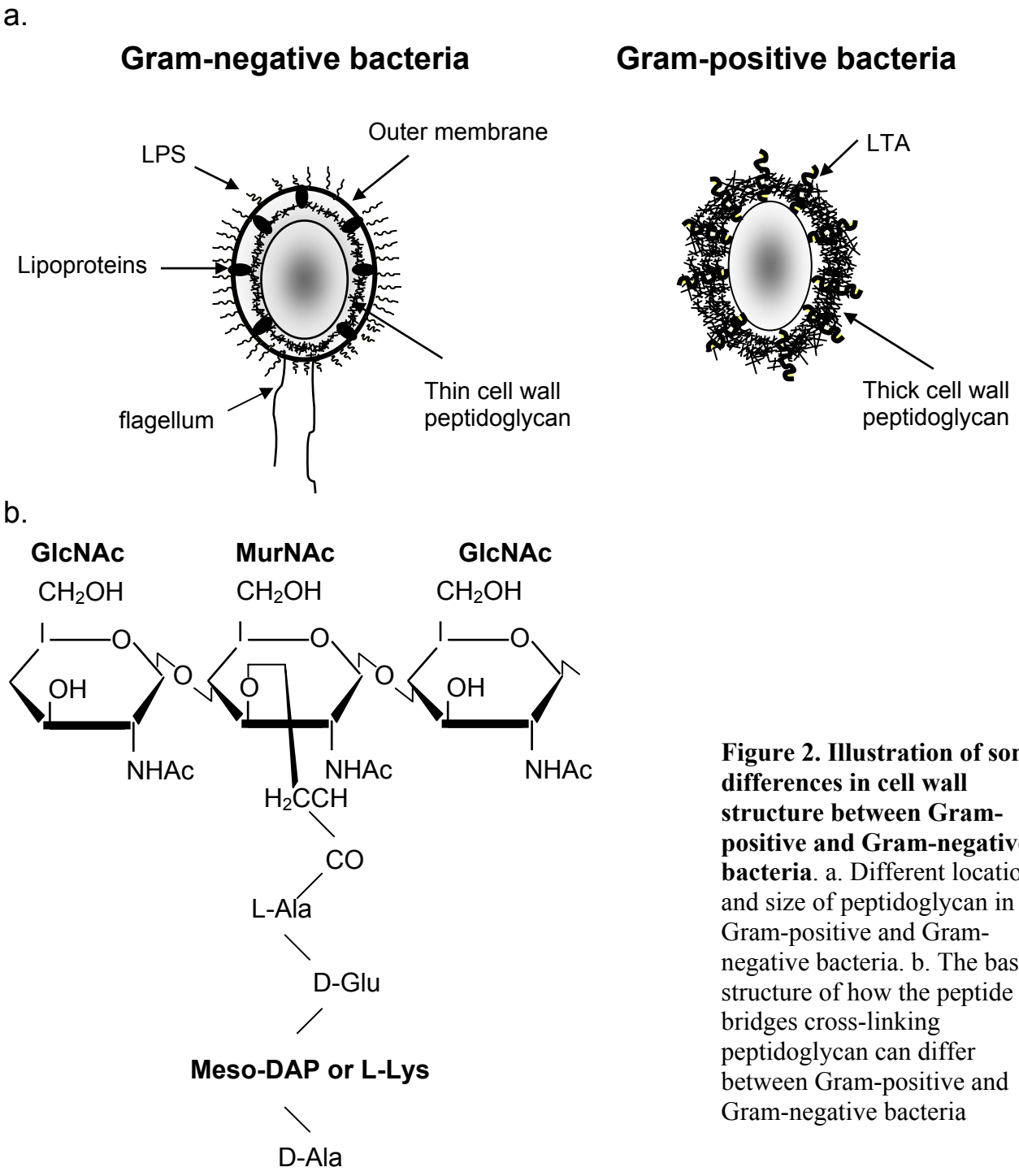


Figure 2. Illustration of some differences in cell wall structure between Gram-positive and Gram-negative bacteria. a. Different location and size of peptidoglycan in Gram-positive and Gram-negative bacteria. b. The basic structure of how the peptide bridges cross-linking peptidoglycan can differ between Gram-positive and Gram-negative bacteria

The composition of the normal gut microbiota

The intestinal microbiota is established in a gradual process in the first years of life (85). First, facultative bacteria establish, as the intestinal contents are rich in oxygen early in life. Anaerobes establish successively until a complex flora has been developed, composed of hundreds of species and characterized by anaerobic dominance (85). The more mature intestinal flora is composed of

species belonging to four bacterial phyla; *Firmicutes*, *Bacteriodetes*, *Proteobacteria* and *Actinobacteria* (85-87). Whereas some bacteria can perform both aerobic and anaerobic metabolism, so called facultative bacteria, others can only perform anaerobic metabolism, anaerobes.

The stomach and duodenum normally have a sparse flora due to the low pH and high motility, foremost lactobacilli, yeast and streptococci (88). In addition, one third of the human population is believed to be colonized with *Helicobacter pylori* in the stomach (89). The counts of bacteria in the stomach, duodenum and jejunum reach 10^2-10^3 per mL of contents. In the distal ileum species of lactobacilli, enterococci, members of *Enterobacteriaceae*, *Bacteroides* and clostrida can be found. The counts in the distal ileum are between 10^6-10^7 per mL of intestinal contents (88).

In the colon the motility is lower and the milieu anaerobic. The colon harbours more than 400 different species (88), however 99% of them belong to 30-40 different species (90). The total bacterial counts in the colon reach 10^{11} per g of faecal material. Approximately 99 – 99,9% of the bacteria in the colon are unable to utilize oxygen for their metabolism and are strictly anaerobic bacteria (88). The dominant phylum *Firmicutes* includes both strict anaerobic bacteria and facultative bacteria, including the obligate anaerobic clostridia, which are Gram-positive spore forming bacteria (Table 4). *Bacteroides* are Gram-negative anaerobes that are important members of the colonic microbiota. *Actinobacteria* contains *Bifidobacterium* spp, which are common in the colonic microbiota. Facultative or aerobic bacteria colonizing the colon are members of *Proteobacteria* (foremost the *Enterobacteriaceae* family) or *Firmicutes* (enterococci, streptococci, and staphylococci). About half of the bacteria in the colonic microbiota cannot be cultured, due to their strict anaerobic requirements (85). However, recently developed DNA based techniques such as cloning and sequencing or PCR using specific probes have been developed for analysis of the intestinal microbiota These studies confirm the dominance of *Firmicutes*, *Bacteriodetes*, *Proteobacteria* and *Actinobacteria* in the colonic microbiota (86, 91, 92). Still DNA based techniques can not replace culture techniques. Several interesting bacterial groups occur only in moderate numbers, foremost the facultative bacteria but since they contain the potentially pathogenic bacteria, bacteriologists have developed selective culture media to enable their detection and quantitation. Important groups of cultivable intestinal bacteria are exemplified below. The dominant bacterial groups in the intestinal microbiota are listed in Table 4.

Facultative bacteria

Facultative bacteria grow best in the precence of oxygen when they perform aerobic decomposition of carbohydrates to CO_2 and H_2O. They can also grow without oxygen, in which case they ferment carbohydrates to alcohols and or acids (93).

Enterobacteriaceae

This is a large family of Gram-negative rod-shaped bacteria, bacilli. They are either non-motile or motile utilizing flagella (94). All members can grow rapidly aerobically and anaerobically on non-selective (e.g. blood agar) or selective (e.g. Drigalski) media. Intestinal commensals in this family include *Escherichia, Klebsiella, Citrobacter, Enterobacter* and *Proteus species* which can cause opportunistic infections, foremost urinary tract infections, but also septicemia. This family also include pathogens like *Salmonella* and *Shigella* species.

Enterococci

Enterococci are Gram-positive spherical bacteria. The cocci grow on non-selective media such as blood agar (94) but can also be isolated on selective media, (enterococcosel agar) where they form black colonies due to hydrolysis of esculin. Enterococci are catalase-negative, a characteristic which distinguishes them from staphylococci (see below). *E. faecalis* and *E. faecium* are the most common enterococcal species found in the commensal flora.

Staphylococcus

Staphylococci are Gram-positive cocci, which grow in patterns resembling clusters of grapes. The name is derived from the Greek staphylé, meaning “a bunch of grapes”. Staphylococci are able to grow on media containing 10% sodium chloride (94) and have the enzyme catalase that converts hydrogen peroxide (H_2O_2) to water and oxygen. This characteristic is often used to distinguish staphylococci from other Gram-positive cocci growing under aerobic conditions. *S. aureus* are identified by production of coagulase, an enzyme that causes plasma to clot (94). The skin and mucosa are commonly colonized with staphylococci, foremost coagulase-negative staphylococci, but also *S. aureus. S. aureus*, is also able to cause a wide spectrum of diseases, such as septicaemia, pneumonia, osteitis, enterocolitis and skin infections. Many strains of *S. aureus* can produce a range of enterotoxins able to cause classical food poisoning and toxic shock syndrome, respectively, due to broad activation of T cells leading to massive cytokine production (95). In parallel with increased hygiene, Staphylococci have replaced *Enterobacteriaceae* as the first colonizers of the

infant's intestines (96, 97). In adults, they represent only a minor population (98).

Yeast

Yeast, mainly *Candida* species, are a common component of the human intestinal microbiota. Yeasts are fungi and have a cell wall composed of chitin (polymers of β1-4 bound N-acetyl glucosamine, GlcNAc), β- glucan (polymers of β1-3 or β1-6 bound glucose) and mannan (polymers of the sugar manose connected to protein via GlcNAc) (99).Yeast do not contain peptidoglycan, LTA or LPS. Different species of *Candida* are found in 80% of all healthy people (94).

Anaerobic bacteria

The majority of intestinal bacteria are anaerobes that cannot use oxygen-dependent metabolism. Several of these bacteria are extremely sensitive to oxygen and die rapidly in contact with air, others are more tolerant and can survive for some time.

Bacteroides

Bacteroides are Gram-negative anaerobic bacilli. The human colon is commonly colonized with *B. distasonis*, *B. fragilis B. ovatus* and *B.thetaiotaomicron*, which are species stimulated by growth on 20% bile. Colonisation with *Bacteroides* can cause infection in patients with disrupted mucosa (94).

Bifidobacteria

Bifidobacteria are Gram-positive bacilli with club-shaped ends. They are anaerobic but tolerate oxygen in the presence of CO_2. Species such as *B. adolescentis, B. bifidum, B. longum and B. infantis* are commonly found in the commensal flora of the large intestine (73). They digest carbohydrates yielding mainly acetate.

Lactobacilli

Lactobacilli are anaerobic Gram-positive bacilli whereas others can grow in moderate extent in the presence of oxygen. Species such as *L. acidophilus*, *L. fermentum*, *L. plantarum* and *L. gasseri* can be found in the human intestine (73) but in lower population counts than *Bacteroides* and bifidobacteria. Lactobacilli have a fermentative metabolism that result mainly in lactate production. This

lowers pH quite drastically and thereby making the milieu hostile to many other bacteria (73). This characteristic is exploited in the fermentation of food such as vegetables.

Clostrida

Clostridia constitutes a large heterogeneous group of anaerobes which usually stain Gram-positive at least in early stages of growth (73). Most species are obligate anaerobes, although tolerance to oxygen varies widely (73). In contrast to other Gram-positive bacteria, the peptidoglycan-linking peptide usually contains meso-DAP, as in Gram-negative bacteria (73). Prior to modern taxonomy clostridia were defined as Gram-positive anaerobes forming endospores, that may offer survival under poor conditions (94). Analyses of 16S ribosomal DNA have revealed that clostridia reside in 19 different clusters within the phylum *Firmicutes* together with other previously defined genera such as *Eubacterium and Ruminococcus* (100). Species in cluster XIVa and IV dominate the human adult intestinal flora (85, 86) (Table 4). Clostridia also include important pathogens that cause diseases by producing toxins. These include *C. botulinum,* causing botulinism, *C. perfingens*, causing gas gangrene and *C. difficile* that produce diarrhoea and severe colonic inflammation if allowed to proliferate freely (94), e.g. after treatment with broad spectrum antibiotics that kill competing intestinal bacteria.

The metabolism of many clostridia as well as *Eubacteria* and *Fusobacteria* belonging to *Firmicutes* class clostridia (100) results in the production of butyrate (93) which is the major energy source for colonocytes (76).

Table 4: Dominant groups of bacteria identified in the gut microbiota of healthy adults according to Adlerberth (unpublished)

Bacterial phyla and groups	Commonly detected species	% of microbiota
Anaerobic bacteria		
Firmicutes		
Clostridial cluster XIVa*	*Eubacterium rectale, E. eligens, E. halii, E. hadrum, E. contorum, Ruminococcus gnavus, R. obeum, R. torques, R. lactaris, Clostridium nexile, C. aminovalericum, C. clostridiiforme*	10-60
Clostridial cluster IV	*Faecalibacterium prausnitzii, Ruminococcus bromii, R. callidus, Clostridium orbisindens, C. sporosphaeroides, Eubacterium siraeum, Subdoligranulum variable*	9-49
Clostridial cluster XVIII	*Clostridium ramosum*	0-12
Clostridial cluster IX	*Megasphaera elsdenii, Veillonella parvula, V. atypica*	0-11
Clostridial cluster XI	*Clostridium bifermentans, C. difficile***	0-7
Clostridial cluster XVI	*Clostridium innocuum, Eubacterium cylindroides*	0-2
Clostridial cluster I	*C. perfringens, C. butyricum, C. disporicum,*	0-1
Lactobacilli	*Lactobacillus acidophilus* group (e.g. *L. gasseri*), *L. paracasei, L. rhamnosus*	0-1
Bacteroidetes		
Bacteroides	*Bacteroides vulgatus, B. thetaiotamicron, B. caccae, B. fragilis, B. uniformis, B. ovatus, B. distasonis, B. stercoris, B. eggerthii, B. merdae*	6-36
Actinobacteria		
Bifidobacterium	*Bifidobacterium angulatum, B. adolescentis, B. pseudocatenulatum, B. longum, B. infantis, B. bifidum*	0-13
Atopobium cluster	*Collinsella aerofaciens, Egghertella lenta*	0-3
Facultative or aerobic bacteria		
Proteobacteria		
Enterobacteriaceae	*Escherichia coli, Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp, *Proteus* spp.	0-3
Firmicutes		
Streptococci	*S. salivarius, S. caprinus, S. anginosus, S. mutans, S. mitis, S. sangis, S. Parasanguinis*	0-11
Enterococci	*E. faecalis, E. faecium*	0-1

Defence systems

The presence of large quantities of potentially pathogenic microbes makes the defence of the mucosa the major task of the immune system.

Barrier function

The first line of defence against microbial invaders is the physiological and chemical barriers. The acid production in the stomach kills most ingested bacteria. The intestine is constantly moving and its walls are covered by mucus, hindering bacteria to attach to the intestinal epithelium. The epithelial cells themselves constitute a barrier to antigens and microorganisms. In addition antimicrobial factors including lysozyme and defensins are produced by Paneth cells; specialized cells located in the base of crypts in the small intestine. Lysozyme is an enzyme that disrupts the bacterial cell wall by hydrolysis of the bonds between the sugar units in peptidoglycan. Defensins perforate the bacterial membrane by forming pores (101).

Innate immune system

The second line of defence consists of the innate immune system. This type of defence exists in all animals. Its task is to rapidly detect, defeat and destroy foreign objects that disturb the integrity of the organism. It consists of cells such as granulocytes, thrombocytes, natural killer cells, monocytes and in the tissue macrophages and dendritic cells, the latter are specialized antigen presenting cells (APC). In addition soluble proteins like the complement system recognize microbial structures and contribute to their elimination. Monocytes, macrophages and dendritic cells have also a unique role in directing the third line of defence; the specific or adaptive immune response (see below).

Phagocytosis.

Phagocytosis is the process of engulfing foreign cells, or particles (phagos = eat, cytosis = cell). Monocytes and macrophages can starts to phagocytose all type of particels, which may be bacteria, non-organic particles or necrotic human cells. Ligands are incorporated into phagosomes which undergo a maturation process in which defensins, lysozyme and other destructive substances are recruited from the endoplasmic reticulum. In addition, enzymes that produce nitric oxide (NO) and pumps delivering H^+ are delivered into the phagosome, whereby the pH decreases. This process is termed phagosome maturation. The now mature phagosome, called phagolysosome, kills and dissolves the microbe or foreign object (99, 102). Phagocytosis is enhanced by complement system binding to foreign objects.

Macrophages not succeeding to eliminate foreign objects e.g. silica particles or *M. tuberculosis* bacteria can induce granuloma formation. A granuloma consists of macrophages surrounded by lymphocytes, plasma cells, collagen and fibroblasts. However, the macrophages in the granuloma have changed their appearance and are termed epitheloid cells, because of the morphological similarities with epithelial cells. Macrophages also fuse to form large multinucleated cells within granulomas, due to the local cytokine milieu. Granuloma formation is sometimes regarded as a cellular attempt to encapsulate an undefeated microbe or particle. Granuloma formation is a hallmark of CD, but granulomas are not always found in this disease.

Monocytes and macrophages detect microbial antigens

The monocytes and macrophages of the innate immune system recognize conserved microbial molecular patterns like LPS, LTA, flagella and peptidoglycan, microbial carbohydrates and nucleic acids (Table 5).

Table 5. Examples of monocytes and macrophage receptors detecting microbial structures

Receptor	Location	Ligand
C-lectin (e.g mannose receptors)	membrane	Carbohydrate structures found on microorganisms (e.g. N-acetyl glucosamine in peptidoglycan)
CD14	membrane (can also exist in soluble form)	LPS in complex with LPS binding protein.
Nod1	cytoplasma, endosomes	Meso-diaminopimelinic acid (DAP) in peptidoglycan from Gram-negative bacteria
Nod2	cytoplasma, endosomes	Muramyl dipeptide (MDP) in peptidoglycan from Gram-positive and Gram-negative bacteria
Scavenger receptors (class A-J)	membrane	Negatively charged macromolecules like LPS, teichoic acid and necrotic cells.
TLR1	membrane, possibly in endosomes?	Lipoproteins, in a hetrodimer association with TLR2
TLR2	membrane, possibly in endosomes	Lipoprotein LTA TLR2 functions forms heterodimers with TLR1 or TLR6
TLR4	membrane, possibly in endosomes?	LPS and LPS binding protein in association with CD14
TLR5	membrane	Flagellin, a protein composing flagella of Gram-negative bacteria
TLR6	membrane, endosomes	Lipoproteins, mycoplasma, TLR6 forms hetrodimers in association with TLR2
TLR7	ER, endosomes	Single stranded RNA from virus
TLR8	ER, endosomes	Single stranded RNA from virus
TLR9	ER, endosomes	CpG motifs in microbial DNA

TLR= Toll-like receptors, ER= Endoplasmic reticulum, Data are adapted from O' Neill (8) and Mölne & Wold (99)

Intracellular signalling

Recognition of microbial structures by monocytes or macrophages through their receptors prompts intracellular signalling. This is a cascade of signals that are transmitted through different adaptor molecules and that ultimately initiate the transcription of genes encoding for inflammatory mediators such as various surface molecules and cytokines. These contribute to alert and recruit further immune effector cells. The pathways of intracellular signalling are currently a

field of intense research. The signalling pathways for TLR4 are reasonably well defined, but the signalling pathways that go through TLR2 and Nod receptors are poorly known.

Signalling via Toll-like receptors

The TLRs are expressed as homo or hetero-dimers on the surface or in the endosomes of immune cells and intestinal epithelial cells (103). The ligand binding parts of the receptors have a leucin rich repeat (LRR) structure. The cytoplasmic part of the TLR receptor is called TIR (a shortening of Toll/IL-1 receptor homology domain). Ligand binding of TLR is believed to induce conformational changes that bring the dimers of the TIR domains together. The conformation of the TIR domain allows recruitment of an adaptor molecule. TLR2 utilizes MAL (also called TRAP) to recruit MyD88 (6) (Fig. 3). TLR4 can signal both through MAL/MyD88 or through the TRAM and TRIF pathways (6) (Fig. 3).

The MyD88 signalling pathway signals via the IRAK family and TRAF6. TRAF6 in turn activates the TAK-1 complex. The signal can take several pathways from TAK-1. One route, which involves several kinases, leads to activation of mitogen-activated protein kinases (MAP kinases) such as p38 or JNK (6) which, when activated, stimulate formation of transcription factors c-jun and AP-1. This then prompts the transcription of genes for cytokine production (99). Alternatively, a signal through TAK-1 may pass through the IKK-complex, which phosphorylates IkB. This leads to its degeneration and release of NFκB, which makes it possible for NFκB to enter the nucleus and start transcription of genes for cytokines.

In addition TLR4 signalling may, instead of passing MyD88, go through TRAM and TRIF, via Rip1, TAK-1, and the IKK-complex, which phosphorylates IκB and enables NFκB to start transcription. Alternatively, from TRIF the signal may go via IRF3 and lead to the transcription of genes for various proteins, such as cytokines (6, 7) (Fig. 3).

The intracellular Nod receptors have a leucin rich repeat LRR structure, which is involved in binding the ligand (8). Nod1 and Nod2 are believed to involve Rip2 before promoting formation of the IKK-complex, which is then followed by NFκB activation (Fig 3) (8).

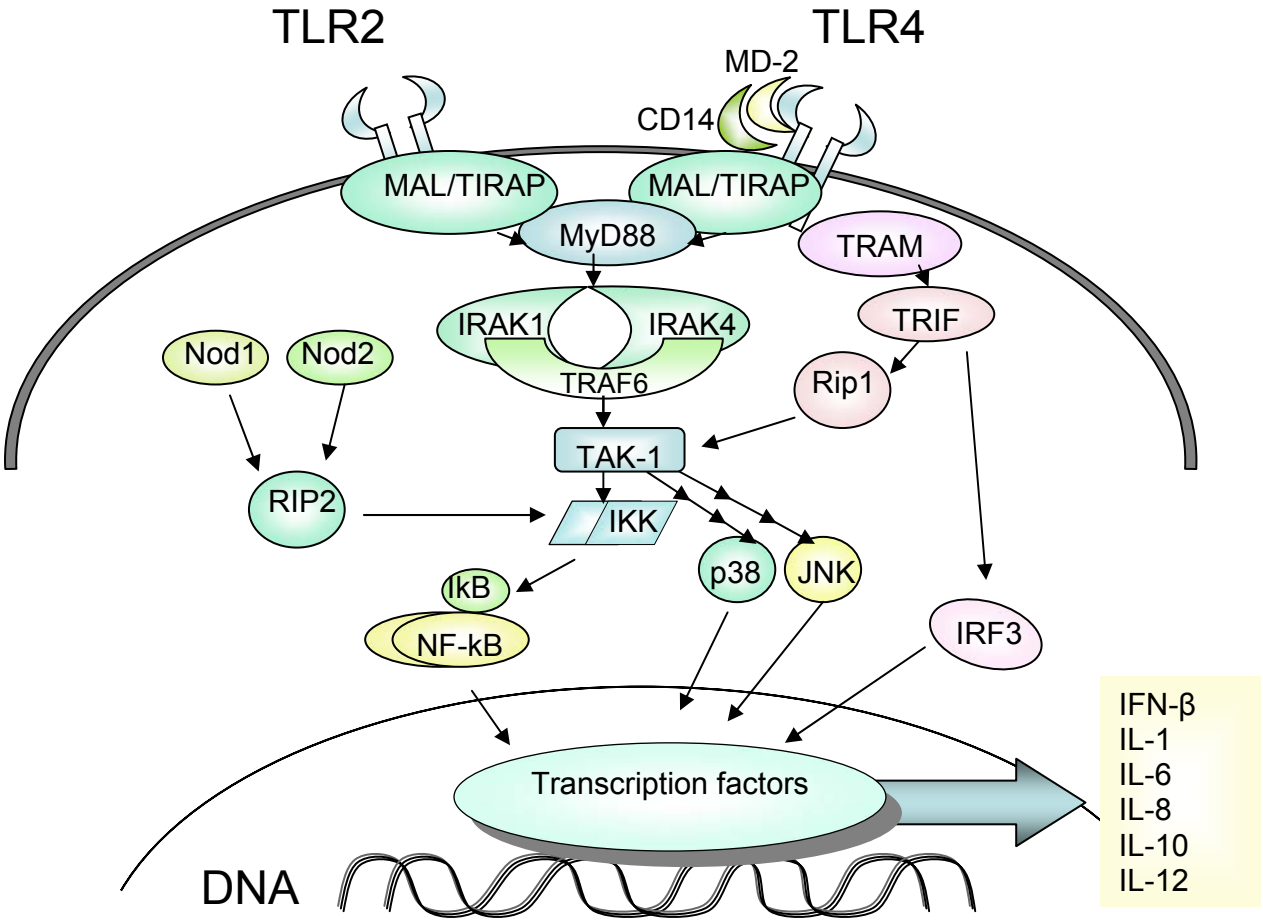


Figure 3. The intracellular signalling pathways involved in TLR2, TLR4, Nod1 and Nod2 signalling. Both TLR2 and TLR4 can signal through MyD88 leading to activation of the transcription factor NF-κB, or via several kinases and p38 or JNK activate other transcription factors (e.g. AP-1). Nod1 and Nod3 can also activate the same transcription factors but utilize Rip2. Data are adapted form Kaisho & Akira, Fizgerald & Chen and O'Neill (6-8).

Acquired immune system

The third line of defence constitutes of the acquired, previously termed "specific", immune system. This defence system exist in all vertebrate animals and it has an almost infinite ability to detect structures e.g. on virus and bacteria. However, this system is not as quick to respond as the innate immune system the first time it encounters a foreign structures. Nevertheless, the acquired system develops a memory enable fast response on subsequent encounters. The major lymphocytes of the acquired immune system are $CD4^+$ T cells, $CD8^+$ T cells, B cells and antibody producing plasma cells.

Lymphocytes are produced in the bone marrow. Here the B cells receive their specific antigen receptors, which consist of a membrane bound antibody. After binding a specific antigen and receiving help form T cells the B cells can proliferate and become plasma cells, which produce large quantities of antibodies specific for a particular structure, an antigen. An activated B cell can also become a memory cell, a cell primed to respond rapidly the next time an antigen is encountered.

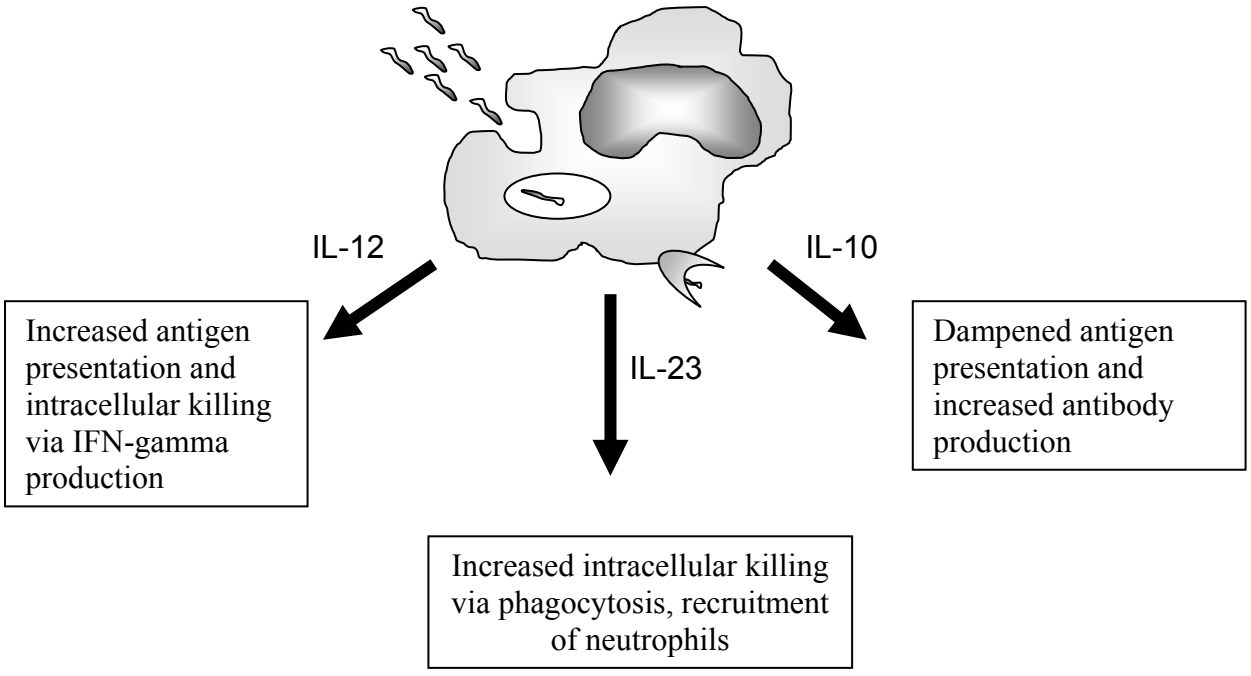
The T cells produced by stem cells in the bone marrow develop further in the thymus, where they receive their highly specific T cell receptor (TCR). Before leaving the thymus, the T cells undergo positive and negative selection, to ensure a functional receptor not reacting to structures of the own body. T cells that enter the blood/lymph system express either CD4 or CD8. These molecules determine on which type of antigen presenting molecule; MHC II or MHC I respectively, the T cell recognizes its antigenic peptide (see below). $CD4^+$ T cells may be T-helper cells or regulatory T cells. Regulatory T cells are able to down regulate other $CD4^+$ T cells to become activated, probably by close interaction (104).

It is believed that the condition during the primary encounter between a naive T cell and its specific antigen determines the maturation pathway taken by the T cell. $CD4^+$ helper cells are commonly divided into Th1 and Th2 cells. Th1 cells produce cytokines activating macrophages, such as IFN-γ. Th2 cells were originally defined as producing IL-4 and IL-5, which are important mediators inducing IgE antibody production and allergy, but sometimes other cytokines, including IL-10 and IL-6 are included in the Th2 cytokines. IL-4, IL-6 and IL-10 are B cell activating cytokines.

Antigen-presenting cells as links between innate and acquired immunity

Antigen-presenting cells, which are macrophages or dendritic cells, are present in all tissues, including the intestinal mucosa. They take up foreign objects and antigens. The APC then presents pieces of the antigen on its MHC molecule to T cells in the nearest lymph node. Peptides from virus infected, apoptotic or necrotic cells are presented by MHC I to $CD8^+$ T cells, whereas phagocytosed antigens are presented on MHC II to $CD4^+$ T cells. In order for the T cell to become activated, it requires additional co-stimulatory signals, including stimulatory cytokines and binding to co-stimulatory molecules on the APC surface. These signals prompt the T cells to divide and mature into effector cells, performing the mission they are designed for.

Antigen-presenting cells that sense danger signals with their receptors, via intracellular signalling, will produce cytokines that both direct the innate (line 2) and modulates the acquired immune defence (line 3) systems (Fig. 4). T cells that recognize an antigen in a milieu of IL-12 will mature into Th1 cells (105), which by production of IFN-γ (se below) will increase the intracellular killing by the macrophage and stimulate antigen presentation and proliferation of T cells (106). A milieu rich in IL-10 will counteract such development. T cells that mature in a milieu of IL-23 will instead develop into T cell producing IL-17, a cytokine that is as yet not well defined but nevertheless increases neutrophil recruitment and intracellular killing by macrophages (107).



Some cytokines and their roles in the communication between antigen-presenting cells and T cells affecting subsequent immune response are listed below.

Interleukin-12 (IL-12)

IL-12 is a heterodimeric protein formed by two subunits; 35-kDa light chain (known as p35) and a 40-kDa chain (known as p40). The bioactive form of IL-12 consists of these the two subunits and is called p70. The p40 unit of IL-12 is sometimes called IL-12, yet this is a subunit shared by another newly identified cytokine; IL-23 (108). IL-12 is a central cytokine able to bridge the gap between the innate and specific immune system, it is produced by monocytes, macrophages and dendritic cells (cells in tissue with an enhanced ability to present antigens to the specific immune system, but which are less able to perform phagocytos). IL-12 induces maturation of T cells into Th1 cells (109), which are $CD4^+$ T cells producing IFN-γ thereby promoting activation of macrophages and $CD8^+$ T cells. Further it stimulates proliferation and production of IFN-γ from T cells and NK-cells (110), resulting in a positive feedback circuit that enhance macrophage microbicidal functions.

Interferon -γ (IFN-γ)

IFN-γ is a monomeric cytokine consisting of 143 amino acids. It is produced by T cells and NK cells and is able to increase macrophage activation including cytokine production, antigen presentation and intracellular killing of phagocytosed microbes (106).

Interleukin-10 (IL-10)

IL-10 is a homodimer consisting of 160 amino acids. It is produced by monocytes, macrophages, dendritic cells and activated T cells. It is a potent cytokine able to suppress monocyte and macrophage functions and decrease antigen presentation. IL-10 decreases IL-12 and IFN-γ production, thereby counteracting Th1 responses. Further, IL-10 stimulates B cells leading to antibody production (111).

Interleukin-17 (IL-17)

IL-17 is produced by certain $CD4^+$ T cells known as Th17 cells. However, other cells such as $CD8^+$ T cells also produce IL-17. IL-17 activates macrophages to produce neutrophil recruiting cytokines (112).

Interleukin-23 (IL-23)

IL-23 is a heterodimeric protein formed by two subunits; the p40 unit shared with IL-12 and another subunit known as the p19 unit (108). IL-23 is believed to induce maturation and/or activation of T cells that produce IL-17 (112).

Interleukin-6 (IL-6)

IL-6 is a monomeric protein formed by 184 amino acids. It is produced by many cells but in particular by macrophages. This cytokine is an inducer of the acute-phase reaction in the liver. Through its action a range of proteins are induced including C-reactive protein (CRP), complement factors and fibrinogen. IL-6 induces fever and activates B cells leading to antibody production (99).

Signs of T and B cell activation and development into memory cells

T cells exiting from the thymus and B cells exiting from the bone marrow that have never encountered their specific antigen are termed naïve cells. Both T and B cells circulate between lymph nodes to find the antigen appropriate for their specific receptor. Certain proteins on the lymphocyte surface to enable this circulation. One example of such a protein is CD62L (see below). A lymphocyte that has found its appropriate antigen, become activated, which is shown by its expression of different types of activation markers. After termination of an immune response, most lymphocytes that have participated die. However, a fraction turns into long-lived memory cells that are characterized by other surface markers. By using flow cytometry (FACS) to study different expressions of surface molecules, a picture of the different maturation stages of various subsets of lymphocyte may be achieved. A selection of such surface markers is described below.

CD19

CD19 is a central molecule expressed on B cells, but is not usually present on plasma cells, the terminal maturation stage for B cells which produces massive

quantities of antibodies (113). This molecule forms a complex with two other molecules (CD21; receptor for complement and CD81; unknown ligand). This complex modulates the threshold for the B cell antigen receptor and mediates intracellular signalling (114).

CD62L

CD62L is expressed on both naïve and memory T and B cells. It mediates the initial tethering and rolling on endothelial surfaces in the specialized venules in the secondary lymphoid tissues. This, a prerequisite for extravasation from the blood circulation into peripheral lymph nodes and Peyer's patches (113).

CD38

CD38 is a transmembrane glycoprotein. The extracellular part has an enzyme function involved in Ca^{2+} mobilization (115). It is expressed on immature and activated B and T cells, but not on resting mature peripheral lymphocytes (113, 115).

HLA-DR

HLA-DR is an acronym for Human Leukocyte Antigen which denotes the human variety of major histocompability complex (MHC). HLA-DR is one of the varieties of human MHC class II. This molecule is expressed on APC and is used by the APC to present foreign peptides for $CD4^+$ T cells. Activated T cells also express HLA-DR (113).

CD25

CD25 is the α-chain of the IL-2 receptor; a cytokine able to induce proliferation, maturation and survival of T cells. This receptor is expressed on activated T cells, B cells and monocytes (113). A subset of $CD4^+$ T cells with high expressions of CD25 that also express intracellular CTLA-4 and FOXP3 (a transcription factor) are regarded as regulatory T cells (116).

CD27

CD27 is regarded as a marker for memory B cells. Binding of CD27 to its ligand CD70 yields crucial signals to the B cell enabling it to enter the pathway to become a plasma cell (117). However, CD27 can also be expressed on activated T cells and when binding to the ligand CD70, induces proliferation, cytokine production and generation of cytotoxic T cells (113).

CD45RA/RO

The CD45 proteins are found on all cells of haematopoietic origin except erythrocytes (113). This molecule associates with the T cell receptor and is necessary for its signalling (118). The extracellular part of the CD45 molecule differs between the naïve T cells; expressing CD45RA, and memory T cells express CD45RO (119).

CD29

CD29 is the common β1-chain that can link with a variety of α-chains, to constitute the VLA integrin family (120). Integrins exists on most human cells including lymphocytes and epithelial cells, enableing adhering interactions between cells and between cells and extracellular matrix proteins e.g. laminin, fibronectin and collagen (120) (the α-chains can also associate with other β-chains) (Table 6).

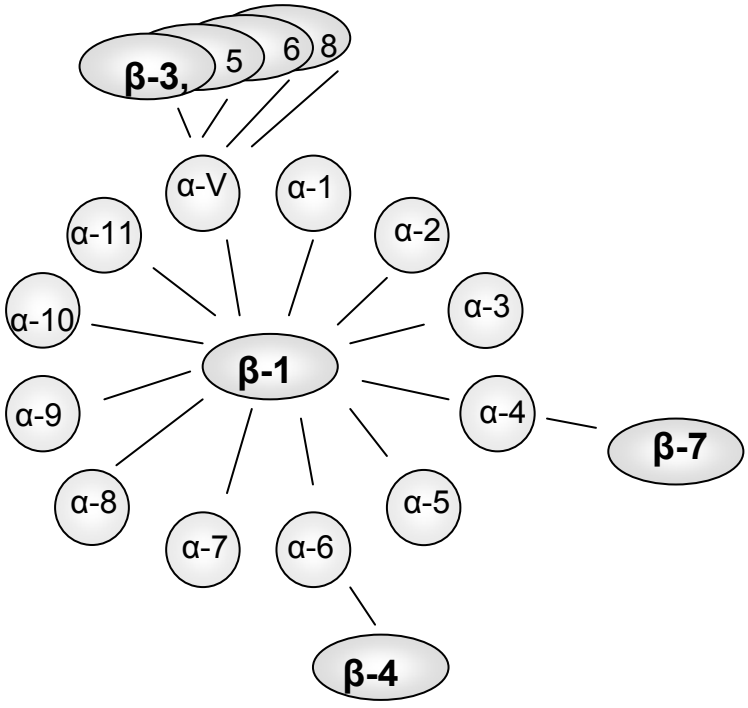


Figure 5. The β1-chain or CD29 can associate with several different α-chains, which also can associate with different β-chains.

Table 6. Examples of associations between β1-chain (CD29) and α-chains, and their interaction with parts of tissue.

	Other name on α- chain	Other names	Distribution	Ligand/function
β1/ α1	CD49a	VLA-1	Widespread	Adherence to collagen and laminin
β1/ α2	CD49b	VLA-2	Widespread	Adherence to collagen and laminin
β1/ α3	CD49c	VLA-3	Widespread	Adherence to laminin and fibronectin
β1/ α4	CD49d	VLA-4	Lymphocytes, monocytes	Involved in cell-cell adhesion, binding to VCAM-1 on endothelium (121), enables extravasation. Adherence to fibronectin
β1/ α5	CD49e	VLA-5	Widespread	Adherence to collagen and laminin
β1/ α6	CD49f	VLA-6	Widespread	Adherence to collagen and laminin

Data are adapted from Hemler (120).

Some theories behind the origin of IBD

IBD is a group of diseases which probably have multifactorial etiology. Major influences are thought to derive from the affected individual's genetic factors, intestinal flora and immune defence system (Fig. 6).

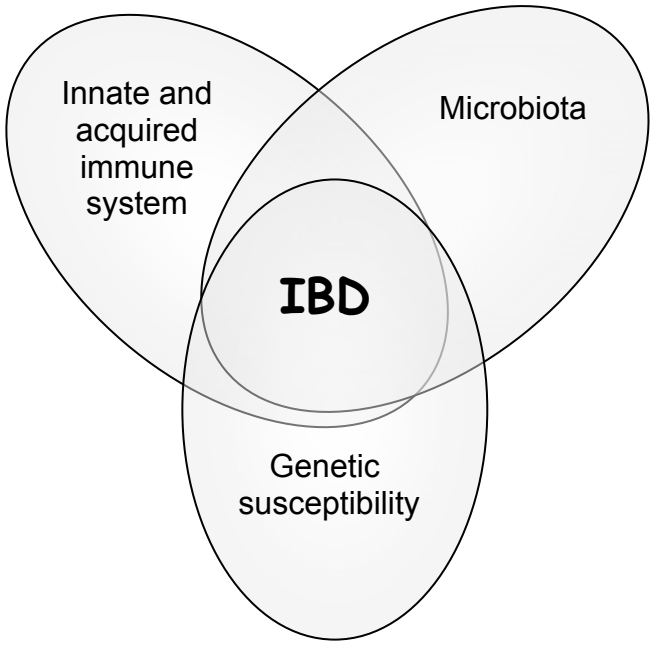


Figure 6. Suggested factors contributing to IBD

Genes associated with IBD

The first gene to be associated with development of CD was CARD15/NOD2 (Caspase recruitment domain family member 15/Nucleotide-binding oligomerization domain-2) (122, 123), coding for the Nod2 which is the receptor for the MDP unit of peptidoglycan (124). One of at least three types of mutations (R702W, G908R and 1007fsinsC) are present in 25-30% of CD patients of European ancestry. However, these mutations are not present to the same extent in Asian, American or African patients with CD (125). Mutations in CARD15 are common in children with ileal CD (11).

Mutations in CARD15 are believed to cause defective binding of the Nod2 receptor to MDP. But studies report conflicting consequences of having one of the three most common mutations in CARD15. Van Heel et al concluded after functional analysis that mutaions in the CARD15 caused "loss of function" in the Nod2 receptor (126). However, interactions between MDP and the Nod2 receptor have been reported to induce a down regulating effect on production of pro-inflammatory cytokines (127, 128). Nod2 receptors are present not only in monocytes and macrophages but also in Paneth cells. The latter cells are present in the crypts of the ileum and produce anti-microbial peptides such as α-defensins. Patients with CD and mutations in CARD15 have been shown to have decreased defensin production (101). This may result in increased luminal bacterial association with the intestinal epithelium, especially within the crypts.

The multidrug resistant gene (MDR1) encodes for an efflux transporter for drugs. This efflux pump is expressed at the apical surface of epithelial cells in the distal small bowel and colon (129). Some variants of MDR1 have been associated with UC (130, 131). Mice with depleted Mdr1 develop a severe, spontaneous intestinal inflammation, which can be prevented and treated by antibiotics (132).

Genomic-wide analysis and candidate gene studies have indicated an association between the human leucocyte antigen (HLA) region and susceptibility to IBD, especially UC (133). However, plausible evidence support that there exists more than one susceptibility locus within the large HLA region that could contribute to development of IBD (134).

According to Satsangi et al (134) these three susceptibility genes (CARD15/NOD2, HLA and MDR1) are those with the strongest evidence for

IBD development, but others also exist. Furthermore, new susceptibility genes are emerging, such as mutations in the locus coding for the receptor of IL-23 (135). IL-23 is a central cytokine in the differentiation of T cells into Th17 T cells, which produce the cytokine IL-17 (136).This is a cytokine able to induce neutrophil recruitment to an inflammatory focus (107). In some animal models, IL-23 has been shown to play a central role in mediating chronic and autoimmune inflammatory conditions (137).

Alterations of the intestinal flora associated with IBD

Environmental microbes may influence the development of IBD. Theoretically they may initiate, perpetuate and/or aggravate disease development.

Mycobacterium avium subspecies *paratuberculosis* has been proposed to cause CD. This suggestion is based partly on the fact that MAP causes spontaneous granulomatous enterocolitis with diarrhoea in e.g. cattle (138) and that MAP has been cultured from tissue from patients with CD (139). However, despite intense research to identify a pathogen, no evidence has been found that could provide an explanation for disease development.

Alterations of the commensal flora, such as raised counts of adherent/invasive *E. coli* (140, 141), or species of *Bacteroides* (142, 143) may contribute to the development of IBD. Gram-negative bacteria are in some aspects more inflammatogenic than Gram-positive bacteria. For example, Gram-negative bacteria induce production of more prostaglandinE2 (PGE2) than Gram-positive bacteria (144) Prostaglandins are forceful vasodilators contributing to mucosal oedema, recruitment of peripheral leukocytes, fever and pain (145).

However, microbes may also have protective properties, in addition to what was discussed earlier under hygiene hypothesis. Several studies performed on adults with IBD, have indicated a decrease of bifidobacteria or lactobacilli (146-148), which thereby would be candidates for protective microbes. Clinical trials with bacterial therapy have shown some positive results treating patients with IBD with probiotics (70, 71).

Recent findings in adult patients with IBD have demonstrated reduced colonization by *Faecalibacterium prausnitzii* (148), belonging to *Firmicutes* cluster IV. This is one of the dominant members of the intestinal microbiota (85) and a producer of butyrate.

Factors of the defence system

The mucus layer provides important defence against bacterial adhesion and interactions. Defective mucin production may therefore contribute to the development of IBD (149, 150).

Patients with IBD display increased numbers of lymphocytes, neutrophils, plasma cells, macrophages and dendritic cells in the lamina propria (2, 151, 152). Children with IBD demonstrate increased activation of macrophages (CD40 expression) in both ileum and colon the intestine (151). Patients with IBD and especially UC have an increased amount of IgG antibodies in the intestine (152), pointing at different immune responses in the two entities of IBD. Patients with IBD also have increased production of a range of cytokines in the intestinal mucosa (Table 7). Cytokines like TNF-α, IL-1β, IL-12 and IL-23 are preferentially produced by macrophages and monocytes that have migrated to the area of inflammation, rather then by resident macrophages. Resident macrophages have a limited capacity to respond to bacterial adjuvants due to down-regulated TLR receptors and CD14 (153). However, the two major categories of IBD, CD and UC, demonstrate different cytokine patterns in the mucosa, indicating different immune responses in these two diseases.

Patients with IBD have increased expression of TLR4 in the intestinal epithelial cells of both inflamed and non-inflamed tissue (103), while patients with CD have instead decreased expression of TLR3 (103). This could be a result from the inflammatory process or be a sign of defective receptor expression.

Table 7. Local cytokine expression in the intestine associated with the two major forms of IBD, data are adapted from Sartor (2)

Cytokine	Ulcerative colitis	Crohn's disease
	Innate response	
IL-1β	↑	↑
TNF	↑	↑↑
IL-6	↑	↑
IL-12	n	↑
IL-23	n	↑
	T cell response	
IFN-γ	n	↑
IL-5	↑	n
IL-13	↑	n
IL-17	n	↑

AIMS

The aims of the present study were:

To study the composition of the small and large intestinal microbiota at début of childhood inflammatory bowel disease.

To study the composition of lymphocyte subsets in the circulation at début of childhood inflammatory bowel disease.

To study which structures of Gram-positive bacteria are responsible for induction of IL-12 production and the intracellular pathways involved.

MATERIAL AND METHODS USED IN THE STUDIES

A brief summary of the material and methods used in paper I-III are presented here along with short descriptions. For more detailed information please see each individual paper.

Study I-II

Children included in studies I-II

Children included in study I–II had symptoms suggestive of IBD and were referred to the Gastroenterology Unit at the University Hospital in Gothenburg, Sweden, between 2001 and 2006 (Table 8). None of the patients had received any medical treatment for IBD, including anti-inflammatory drugs, antibiotics or probiotics three months before study samples were taken.

Table 8. Summary of participating children in study I-II

Study	Number of children at start	Excluded children	Ulcerative colitis	Crohn's disease	Diseased controls	Healthy controls
Study I	72	10	18	11	22	11
Study II	61	11	18	9	23	-

Samples from children referred to the gastroenterology unit were colleted before diagnosis, with the exception of faecal samples from IBD patients in remission (Fig 7).

Diagnosis of the patients was based on clinical presentation, endoscopic, histological features and radiological abnormalities i.e. the Porto criteria (3). Children in whom IBD diagnosis was excluded and who did not have any other intestinal inflammation were classified as diseased controls (DC). Due to low numbers children with other types of inflammatory condition e.g. allergic colitis, celiac disease, infectious colitis, unspecific colitis were excluded, as well as one single case of indeterminate colitis.

In study I, we included as controls eleven healthy volunteer children (HC) contributing with faecal samples.

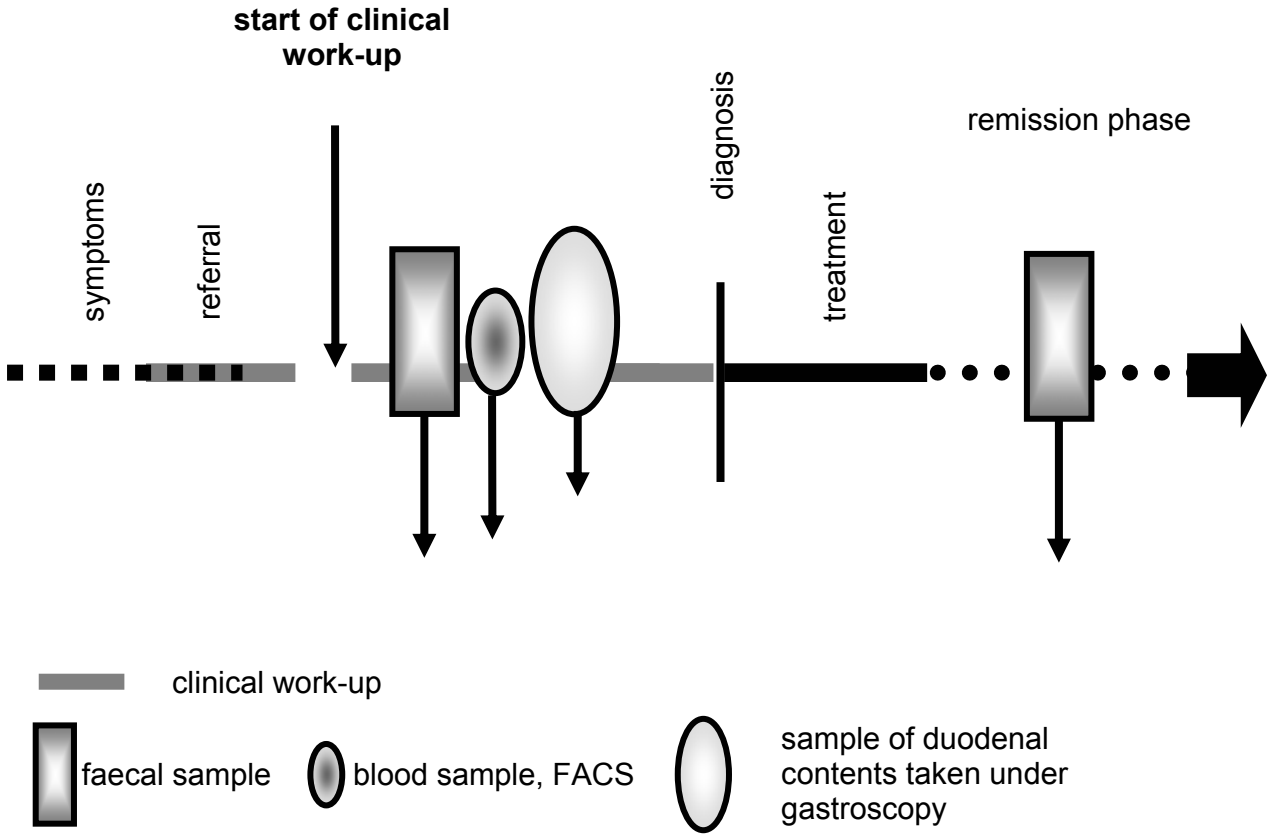


Figure 7. Children with symptoms suggestive for IBD were referred to the Gastroenterology Unit at the University Hospital. The faecal sample was taken before bowel cleansing for colonoscopy. Blood samples and duodenal contents were obtained in connection with gastroscopy. Some of the children who received IBD diagnosis were asked for a faecal sample when they were in remission phase. This was done to study if the changes seen in the microbiota at the début of the disease persisted in the remission phase. Samples of the microbiota were cultured within 24h.

Sampling in study I-II

Stool samples from children, referred to the pediatric gastroenterology unit, were obtained the day before endoscopy of the gastrointestinal tract prior to cleansing for colonoscopy. Blood and duodenal contents were sampled in connection with gastroscopy.

The stool and duodenal content samples were placed in sterile tubes, kept cold (about +8° C) and in anaerobic condition until cultivation within 24h from sampling. Anaerobic condition was ascertained upon arrival to the laboratory.

Venous blood samples were collected in heparinized tubes and kept at room temperature until analysed by flow cytometry within 24h from sampling.

Cultivation of microbiota (study I).

A calibrated spoon-full of faeces or duodenal content was serially diluted in ten-fold steps in sterile peptone water. The dilutions were plated on non-selective and selective media (Table 10). The detection limit for duodenal samples was 33 ($10^{1.52}$) colony forming units (CFU)/mL. For stool samples the detection limit was 330 ($10^{2.52}$) CFU/g faeces.

Aerobic cultures were performed overnight or for two days (staphylococcus and yeast plates). Anaerobic cultures were performed in anaerobic jars (MedPak Inc., Montvale, NJ), using pre-reduced agar plates. For the isolation of anaerobic spore formers (clostridia) a portion of undiluted duodenal contents or a 1:10 dilution of stool sample were mixed with an equal volume of 99% ethanol on a shaker at room temperature for 30 min. After this treatment, which kills vegetative cells, the sample was diluted, plated and cultured on Brucella blood agar in anaerobic conditions as described above (Table 10).

Enumeration and identification of the microbiota

Free-laying colonies appearing on selective or non-selective media were counted in the dilution giving 50 to 100 colonies and presented as CFU. Colonies were Gram-stained, examined in microscope and subcultured for purity. Pure isolates were frozen at -70°C until further analysed.

Table 9. Bacterial groups and culture media including conditions of incubation.

Bacteria	Culture conditions		Medium
	time (days)	atmosphere	
Total anaerobes	3	anaerobic	Brucella blood (154)
Sporeformers (*Clostridium* spp)	3	anaerobic	Brucella blood (alcohol treated sample)
Bacteroides spp	3	anaerobic	Bacteroides bile esculin
Bifidobacterium spp	3	anaerobic	Beerens agar
C. difficile	3	anaerobic	CCFA*
Total aerobes	1	aerobic	Colombia blood (155)
Enterobacteriaceae	1	aerobic	Drigalski
Staphylococcus spp	2	aerobic	Staphylococcus agar
Enterococcus spp	1	aerobic	Enterococcosel agar (156)
Yeasts	2	aerobic	Sabouraud agar

*Cycloserine Cefoxitin Fructose egg yolk agar

The total numbers of facultative bacteria as well as the numbers of Gram-positive and Gram-negative facultative bacteria were enumerated on Colombia blood agar. The total number of anaerobes and the number of Gram-positive and Gram-negative anaerobic isolates were quantified on Brucella blood agar. Only colonies that were unable to grow under aerobic condition were included in the counts of anaerobic bacteria. However, sparse growth under aerobic condition was accepted for Gram-positive rods resembling *Lactobacillus* or *Bifidobacterium* spp.

All bacterial groups were quantified on their respective selective media (Table 9).

Members of the *Enterobacteriaceae* family were speciated using the API20E biotyping system (API Systems, SA, La Balme les Grottes, Montalieu-Vercieu, France).

Enterococci were identified by there growth and colony appearance on enterococcosel agar, where they cause esculine hydrolysis, and typical Gram-stained apperence.

The identity of staphylococci was confirmed, in addition to Gram-staining and growth on selective medium, by positive catalase reaction. Coagulase-positive staphylococci were identified as *S. aureus*, while other staphylococci were defined as coagulase-negative (CoNS).

Bacteroides and *Clostridial* species were identified using Rapid ID32A biotyping system (API Systems SA, La Balme les Grottes, Montalieu-Vercieu, France). *Clostridial* typing was confirmed in the department of Food Technology, Engineering and Nutrition, University of Lund, Sweden by sequencing, after amplification of the 16S rRNA gene. Sequences were compared with those stored in GenBank by using Advanced BLAST similarity search option (157) available online at htt:/www.ncbi.nlm.nih.gov/. *Clostridium difficile* was isolated on CCFA, identified by colony morphology and typical smell, and speciated by the use of Rapid ID 32A.

Bifidobacteria were, in addition to Gram-staining, microscopic appearance and growth on Beerens agar, identified by PCR using specific primers pairs for the genus *Bifidobacterium* (158). Specific primers were used to further identify the species i.e. *B.longum*, *B. adolescentis, B. bifidum* and *B. breve.*

Lactobacilli were enumerated on Rogosa agar. To exclude bifidobacteria, which also grow on this medium, PCR with specific primers for bifidobacteria (see above) was used. Lactobacilli were identified using PCR with group - and species–specific primers described earlier (159).

Phenotypic analysis of lymphocyte populations (study II).

Venous blood samples, collected in heparinized tubes, were analysed by four-colour flow cytometry, to study the fraction and absolute numbers of different lymphocyte populations. A FacsCalibur equipped with CellQuest software

(Becton-Dickinson Erembodegem, Belgium) was used and 10,000 lymphocytes were recorded. The software FlowJo 7.2.5 (Tree Star Inc., Ashland, OR) was used to perform gating. The following markers were analysed on $CD4^+$ and $CD8^+$ T cells: HLA-DR, CD38, CD62L, CD29, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD45RA, CD45RO. In addition we used CD2 and CD3 to define T cells. On B cells defined as lymphocytes expressing CD19, we examined the expression of the memory marker for B cells CD27.

TruCOUNT assay was used to determine the absolute count of various subsets of lymphocytes found in the blood. Undiluted blood was stained with PerCP-conjugated anti CD45 antibodies in a TruCOUNT tube (Becton-Dickinson). In a dot plot of CD45 versus SSC, lymphocytes were defined on the characteristics of low SSC and high expression of CD45. A further dot plot was created to identify the beads using FL1 versus FL2 plot. The beads were defined as having high FL1 and FL2 properties. The absolute cell counts for lymphocytes were calculated by the use of the following formula: events of lymphocytes/events of beads multiplied with number of beads per TruCOUNT tube/blood volume. All reagents were from Becton-Dickinson.

Study III

Microbes, microbial products and non-microbial particles (Study III)

B. adolescentis (CCUG 18363, Culture Collection of University of Göteborg, Göteborg Sweden) and *B. dentium* (CCUG 17360) were cultured anaerobically on horse blood agar (Substrate Department, Clinical Bacteriology, Sahlgrenska University Hospital) for 3 days at 37°C. *E. coli* (serotype O6K13H1) was cultured on horse blood agar aerobically for 24h. *Candida albicans* (ATCC 64549) was cultivated in Saubouraud dextrose broth (Substrate Department, Sahlgrenska University Hospital) on a shaker at 37°C for 24 h. Bacteria were harvested in endotoxin-free Dulbecco's PBS (PAA laboratories, Linz, Austria), washed, suspended at 10^9 cells/ml (determined by optical density at 570 nm) and inactivated by UV-light for 17 min. Yeast cells were inactivated by 70°C for 30 min. Negative viable counts confirmed inactivation.

For disruption of bacterial cells, UV-inactivated bacteria were sonicated (amplitude 85 under cooling for 20 min, Vibra cell, Sonics & Materials Inc, Danbury, CT), or 6 x10^9 bacteria were pelleted by centrifugation, suspended in 500µl of lysozyme (15mg/ml, Sigma) and incubated for 2h at 37°C. Lysozyme-treated bacteria were re-suspended and used without further purification steps. For inactivation of surface protein, 6 x10^9 pelleted bacteria were treated with proteinase K (400µg/ml, Sigma) for 2h at 37°C, or suspended in 500 µl 3.7% paraformaldehyde solution, incubated for 15 min at room temperature, washed x

3, and re-suspended in the original volume of PBS. Gram-stained bacterial suspensions were examined in the microscope (Nikon eclipse E600 with camera and software, Nikon, Tokyo, Japan).

Cell wall fragments and phosphopeptidomannan from *C. albicans* were gifts from Dr. Nahid Kondori, Dept. of Clinical Bacterology, University of Gothenburg. Peptidoglycan from *S. aureus*, LPS from *E. coli* (055B5) and N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) were from Sigma (St Louis, MO). LTA from *S. aureus* (DSM20233) isolated by n-butanol extraction was kindly provided by C. Draing University of Konstanz, Germany. $Pam_3Cys\text{-}SK_4$ was purchased from Calbiochem (La Jolla, CA), 2.8 and 4.5 μm magnetic beads from Dynal Biotech (Oslo, Norway) and 0.81μm Latex beads from BD (Franklin Lakes, NJ).

Antibodies and inhibitors (study III)

Cytochalasin B (Sigma) was used to block cytoskeletal rearrangement, Wortmannin (KY12420) to inhibit PI3K, SB 203580 to inhibit p38, SP600125 to inhibit JNK, and IKK inhibitor VII to inhibit NF-κB release (Calbiochem, La Jolla, CA). Antibodies against TLR2, TLR4 and isotype control were from Serotec (Oxford, UK).

Preparation and stimulation of mononuclear cells (study III).

Peripheral blood mononuclear cells (PBMC) were prepared from blood-donor buffy coats (Blood Bank, Sahlgrenska University Hospital) by density gradient centrifugation (20 min, 820g) (Lymphoprep, Nyegaard, Norway) at room temperature. Cells were washed and suspended at $2x10^6$/ml in RPMI 1640 with 2mM glutamine (Gibco, Edinburgh, UK), 0.01% gentamycin (Sigma) and 5% inactivated AB-serum (Sigma), and seeded in 96-well plates (Nunc, Roskilde, Denmark). Endotoxin levels of medium and serum were ≤ 1 EU.

Bacteria were added to achieve a final concentration of $5x10^6$/ml, optimal to elicit IL-12 (160), whereafter the cultures were incubated at 37°C in 5% CO_2 atmosphere. Supernatants were harvested after 24h, previously found optimal (160). PBMC viability after 24h was determined by nucleic acid dye 7-amino-actinomycin D (7-ADD) staining (BD) and flow cytometry (FACS-Calibur equipped with FlowJo software, BD, Tree Star Inc., Ashland, OR).

For blocking experiments, PBMC were incubated with soluble bacterial components for 30 min at 37°C, or with antibodies for 1h at 4°C (final concentration: 10 μg/ml), before stimulation. In some experiments, cells were pre-incubated with LTA, peptidoglycan, fragmented or intact bacteria, washed and re-suspended in medium before re-stimulation with intact *B. adolescentis* ($5x10^6$/ml).

IL-12 (p70) and IL-10 ware quantified using ELISA-kits (Eli-pair, Diaclone, Besançon, France) with detection limits of 25 pg/ml and 40 pg/ml, respectively. IL-6 was determined by ELISA using purified anti-human IL-6 (MQ2-13A5) and biotinylated anti-human IL-6 (MQ2-39C3) (Pharmingen) as described previously (161). The limit of detection was 125 pg/ml.

Assessment of bacteria – monocyte interactions (study III)

To study phagocytosis of *B. adolescentis* and *B. dentium* by monocytes PBMC were incubated with bacteria for 30 min. Thereafter the cells were washed, centrifuged onto glass slides (Cytospin, Shandon Southern, Runcorn, UK) and stained (Diff-Quick, Dade Behring AG, Düdingen, Switzerland). Sixty monocytes per slide were examined for internalized bacteria in a blinded fashion and the average number of bacteria ingested per monocyte was calculated.

To study the interaction between monocytes and bacteria in relation to IL-12 production we used FACS. UV-killed bacteria were incubated with fluorescein isothiocyanate (FITC) (Sigma) in 0.1 M $NaHCO_3$ for 1h at room temperature and washed before stimulation of PBMC. After incubation at 37°C for 22 h, the last 5 h in the presence of GolgiPlug (BD Pharmingen, San Diego, CA), cells were detached and washed in FACS-buffer. Non-specific staining was blocked with human γ-globulin (R&D Systems). Cells were stained with PE-anti-CD14 (BD), fixed with paraformaldehyde, permeabilized with saponin and stained for intracellular IL-12 with APC-anti-IL-12 (p40/p70) (554576, BD Pharmingen).

RESULTS

Due to publication of the theses on the web, results are only briefly presented here not to interfere with later publication as original papers. The results are presented in more detail in the accompanying papers/manuscripts.

Intestinal microbiota of the small bowel in children at début of IBD (paper I)

We studied the composition of the small intestinal microbiota in children at the début of IBD. The contents of the small intestine were examined in children referred to paediatric gastroenterology clinic because of suspected IBD. No differences were observed differences between the groups of UC, CD, and DC regarding bacterial numbers (presented in paper I Fig. 1a-c) or presence of particular bacterial groups (presented in paper I, Table 4).

Intestinal microbiota of the large bowel in children at début of IBD (paper I)

When examining the composition of the intestinal flora by culturing stool samples, we included eleven healthy children for comparison (HC). Comparison of the intestinal flora revealed that primarily children with UC had altered composition of the intestinal flora. The ratio of anaerobes to facultatives in large intestinal microbiota were decreased in children with UC (presented in paper I Fig 2a), compared to all other groups, i.e. CD, DC and HC. The decreased ratio of anaerobic to facultative bacteria were not due to increased counts of facultative bacteria but decreased counts of anaerobic bacteria (presented in paper I) Children presenting with CD had similar ratio of anaerobes to facultative bacteria as DC and HC (presented in paper I).

Enumeration and speciation of anaerobic bacteria on selective media revealed that Gram-negative *Bacteroides*, was found in similar levels in all diagnostic groups while children presenting with UC had altered counts of bifidobacteria and clostridia (assessed on the ability to form spores) (presented in paper I, Fig. 3a-c). We also calculated the ratio of Gram-negative to Gram-positive bacteria on non-selective aerobic and anaerobic media (presented in paper I Fig 4). This result showed that both children with UC and CD had a higher ratio of Gram-negative bacteria to Gram-positive bacteria.

We considered whether the dysbalanced microbiota seen primarily in children with UC could be a secondary phenomenon due to decreased transit time in the intestine. We therefore compared the microbiota composition in patients with UC having 1-3 or > 3 stools/day. Interestingly, the decreased counts of anaerobes were more pronounced in children with UC who had moderate stool frequency than in those with high stool frequency (presented in paper I).

Further, we questioned if the altered microbiota seen in the active phase at début of IBD also persisted at remission phase. As presented in paper I the counts of bifidobacteria were normalised, but not the counts of spore forming clostridia. The ratios of Gram-negative to Gram-positive bacteria were mostly normalized in remission phase (presented in paper I).

Activation profile expressed on $CD4^+$ and $CD8^+$ T cells (paper II)

We wanted to study the composition of lymphocyte subsets in the circulation of children at début of IBD to reveal whether different lymphocyte activation patterns could be detected in different disease groups. The results are presented in paper II. Children with IBD diagnosis and DC had comparable numbers of lymphocytes including $CD4^+$ and $CD8^+$ T cells in the blood. In general, both children with UC and CD displayed increased activation of T cells, when studying the expression of HLA-DR, CD 38 and CD62L. The children with UC also showed a more pronounced pattern of activation in the $CD8^+$ T cells compared to CD and DC.

The expression of β-1 integrins (CD29) was clearly altered in children with UC both in the $CD4^+$ and $CD8^+$ T cells (presented in paper II). The children with UC had significantly different integrin expression both compared with CD and DC (presented in paper II).

Memory markers on circulating T and B cells (paper II)

A fraction of activated T cells and B cells remain in circulation as memory cells, enabling a rapid response to the same antigen at subsequent times. Memory T cells express CD45RO, whereas naive T cells express CD45RA. We examined the ratio between naive and memory T cells in children with UC, CD and DC. No different expression of the ratio between the groups was observed. However, fewer children with CD were examined for these markers, and the tendency seen in paper II need to be further examined.

Examination of the memory population in B cells revealed a distinct difference in expression between the examined children. Children with Crohn's disease had significantly altered expression of the memory marker CD27 on the B cells both compared with UC and DC (presented in paper II).

Soluble bacterial constituents down-regulate secretion of IL-12 in response to intact Gram-positive bacteria (paper III)

Earlier studies by Hessle *et al* have demonstrated the Gram-positive bacteria induce high production of IL-12 (p70) while Gram-negative bacteria induce more IL-10 in mononuclear cells from blood (160). We therefore wanted to further explore which structures of Gram-positive bacteria were involved in induction of IL-12 production. We also wanted to study which intracellular pathways in monocytes that were involved in producing IL-12 response to Gram-positive bacteria.

Intact Gram-positive bacteria induce more IL-12 than other microbial stimuli

A range of microbial and non-microbial stimuli were compared for their capacity to induce production of IL-12 (p70), IL-6 and IL-10 from human PBMC. As shown in Table 1 paper III, intact Gram-positive bacteria induced large amounts of IL-12 (1ng/ml) from freshly isolated PBMC while Gram-negative bacteria induced much less (25pg/ml) and LPS induced no detectable IL-12 production. Pre-treatment of the PBMC with IFN-γ permitted LPS to induce some IL-12 and the IL-12 response to Gram-negative bacteria was augmented. However, pre-treatment of the PBMC with IFN-γ also increased the IL-12 production induced by Gram-positive bacteria. Yet, no IL-12 production was detected from Gram-positive cell wall fragments LTA, MDP or peptidoglycan despite IFN-γ pre-treatment. In contrast to IL-12 production, IFN-γ pre-treatment decreased IL-10 production in PBMC. Yeast or compounds from the yeast cell wall could not induce IL-12.

IL-12 is produced by monocytes that have ingested bacteria

Previously Hessle *et al* had shown that monocytes, purified by adherence, are the major producers of IL-12 in response to intact Gram-positive bacteria among blood cells (160). We examined PBMC incubated with FITC-stained Gram-positive bifidobacteria and stained for intracellular IL-12 and surface CD14. The

results revealed that monocytes with intracellular IL-12 also had ingested bifidobacteria (presented in paper III).

Disruption of the Gram-positive bacterial particle abrogates its capacity to induce IL-12

We next studied how different treatments of intact Gram-positive bacteria affected their capacity to induce IL-12 production. UV-inactivated *B. adolescentis* were sonicated, formalin fixed, or treated with protease or lysozyme, before being added to PBMC. Treatments disrupting the bacteria, such as sonication and lysozyme treatment, resulted in minimal IL-12 production, while bacteria treated with proteinase or formalin still were efficient IL-12 inducers (presented in paper III). The decreased IL-12 production by fragmented Gram-positive bacteria was not due to an increased IL-10 production, a cytokine known to dampen IL-12 expression.

Fragments of Gram-positive bacteria inhibit IL-12 production

We found that fragmented Gram-positive bacteria or soluble constituents of Gram-positive bacteria such as peptidoglycan, LTA or MDP, dose-dependently inhibited the production of IL-12 induced by intact *B. adolescentis* (presented in paper III).

This inhibitory effect of soluble constituents from Gram-positive could be due to interaction of the fragments with the same receptors as intact bacteria without inducing IL-12. However, the results indicated that phagocytosis of Gram-positive bacteria were only marginally reduced if cells were pre-treated with constituents like peptidoglycan and LTA. We hypothesized that specific interaction between bacterial components and TLR2 (LTA) and/or Nod2 (MDP) were responsible for the inhibitory effect seen by soluble constituents. Results shown in paper III indicate that TLR2 but not TLR4 are of importance for IL-12 production from PBMC stimulated with Gram-positive bacteria.

Polymerization of the cytoskeleton is required to trigger IL-12 production in response to Gram-positive bacteria

As Gram-positive bacteria needed to be present in particular form to trigger IL-12 production, we hypothesized that phagocytosis was necessary. Blocking of phagocytosis with cytochalasin, dose-dependently reduced production of IL-12 in response to intact Gram-positive bacteria (presented in paper III).

Different intracellular signalling pathways involved in IL-12 production from Gram-positive bacteria and LPS after IFN-γ pre-treatment

We further wanted to investigate intracellular signalling pathways involved in IL-12 production form monocytes stimulated with Gram-positive bacteria. We compared the intracellular pathways with those involved in IL-12 production induced by LPS/IFN-γ stimulation. Intracellular signalling pathways were blocked by inhibitors and the effect on IL-12 production was studied and compared with IL-6 production. Inhibitors were used in concentrations leaving > 96% viable cells after 24h.

Inhibition of the transcription factor NF-κB by inhibition of IKK complex abolished IL-12 production in response to bifidobacteria and reduced the IL-12 response to IFN-γ/LPS. The IL-6 responses to both stimuli were reduced but not abolished (presented in paper III).

Wortmannin, an inhibitor of PI3K, has earlier been reported to increase IL-12 production in response to IFN-γ/LPS treatment (162-164). This finding was readily reproduced (paper III, figure 4). In striking contrast, the same inhibitor strongly *reduced* IL-12 production induced by intact bifidobacteria (paper III, figure 4). The levels of IL-6 and IL-10 were not altered by PI3K inhibition.

Further we found that blocking of the JNK pathway significantly reduced IL-12 production in response to intact bifidobacteria (paper III, Fig. 5), while low doses of the inhibitor rather tended to increase IL-12 production in response to IFN-γ/LPS (paper III, Fig. 5). IL-6 production in response to both bifidobacteria and LPS were unaffected by blocking of the JNK pathway but the Blocking the MAP-kinase p38 mainly reduced in response of IL-6 production, induced both with bifidobacteria and LPS (paper III, Fig. 5). However, high concentration of the p38 inhibitor only reduced IL-12 production in response to intact bifidobacteria (paper III, Fig. 5).

DISCUSSION

Despite intense research the etiology of IBD is still unknown. Epidemiological studies have shown that IBD is a disease with highest incidence in countries with high socioeconomic status. Further, that IBD incidence has started to increase in areas where it earlier has been a very low incidence and in which the society are becoming more westernized (37). This implicate that raise in incidence are associated with westernized type of living. Genetic factors are of importance for the disease development. However, genetic factors may not explain why individuals moving from low to high incidence countries develop IBD in the same extent as those in the new country. A tempting explanation to the raised incidence of IBD in countries of high socioeconomic standard is the "hygiene hypothesis" discussed earlier in the introduction.

Intestinal flora and IBD

In parallel with increased hygiene, the composition and turn-over of the intestinal microbiota changes (97, 165, 166). The intestinal microbiota may play a crucial role in the initiation and/or maintenance of IBD and changes in the microbiota may explain the association between hygienic life-style and high risk of IBD. There are limited data of the microbiota of patients with childhood IBD. Most of the studies where the composition of the flora is studied are performed on adults, often with long-standing disease and who have received treatments that may influence to composition of the flora. Disease-specific alterations in the microbiota might best be studied in new-onset IBD patients before instituted treatment. Children with IBD may be an especially interesting group to study because the disease less likely has been long-standing as childhood UC commonly presents as pancolitis and children with CD frequently have involvement in large intestine. Thus, patients are likely to seek immediate contact with medical care. Further, children are less likely to self-medicate or adjust their diet in response to gastrointestinal symptoms.

Our results in paper I indicated that children with UC at début of the disease had decreased counts of anaerobic bacteria mainly due to decreased counts of bifidobacteria and clostridia, two common groups of Gram-positive anaerobes in the intestinal microbiota. A decrease of this type of bacteria may, thus, initiate, aggravate or perpetuate IBD. The mechanism by which these bacterial groups might exert beneficial effect can only be speculated upon. Probiotics including both bifidobacteria and lactobacilli may exert beneficial effects by inducing increased mucin production, thereby improving the barrier functions (167, 168) in the intestine. Furthermore, bifidobacteria and lactobacilli have been

associated with immunoregulatory functions by their ability to down-regulate TNF-α and IL-1 (71, 169). Treatment of patients with mild to moderate UC, not responding to conventional therapy, with a mixture of Gram-positive bacteria, bifidobacteria, lactobacilli and a strain of *Streptococcus salvarius* (a mixture called VLS#3), resulted in a remission/response rate of 77% (70). However, administration of VLS#3 accomplished only transient bacterial colonization and after a month the faecal flora returned to the pre-treated state, a phenomena earlier described as colonisation resistance (170). However, our findings that soluble constituents from fragmented Gram-positive bacteria down-regulate responses to intact bacteria may be of importance for healthy immune balance in the mucosa.

The decreased counts of anaerobes could also be a consequence of the disease, as diarrhoea leads to aeration of the gut contents and less favourable conditions for the anaerobes. We did not, however, find any association between stool frequency and alteration of the microbiota. However, the fact that bifidobacterial counts and total counts of anaerobes returned to normal upon remission could indicate that the altered flora was due to the inflammatory process.

However, colonization by clostridia seemed to be reduced both in the active and remission phase of UC. The clostridia that were found in diseased and healthy control children mainly belonged to cluster I. Thus, the clostridia making up the greatest fraction of adult microbiota, cluster XIVa and IV had not yet established, alternatively were not able to be cultured. To our knowledge, this is the first demonstration of a potential beneficial effect of clostridia belonging to cluster I. Many *Fusobacterium*, *Eubacterium* and *Clostridium* spp may produce butyrate (butyric acid) as a result from fermentation of carbohydrates. Butyrate is the preferred SCFA as energy substrate for colonocytes (76, 82). SCFA produced by strictly anaerobic bacteria have an ability to maintain the electrolyte and water balance (79), which may help to control diarrhoea. The short chain fatty acid butyrate is also the main source of energy for colonocytes (76) and is anti-inflammatogenic and anti-neoplastic (77, 79-81).

If the butyrate producers are severely altered or absent from the microbiota, substrate deficiency will make the epithelium leaky. Leaky epithelium will consequently lead to diarrhoea and increased food and bacterial antigen translocation over the gut wall, resulting in inflammation, enhanced epithelial destruction and further leaky epithelium.

Colonic epithelial cells isolated from patients with UC have been shown to be less capable of utilizing butyrate as fuel compared to cells from non-IBD patients (82). Roediger therefore suggested that UC is a colon starvation disease due to energy deficiency (82). Our results could be interpreted along the same lines, although we would not propose that the disease are due to unwillingness of epithelial cells from patients with UC to metabolize butyrate, but it may instead be an adaptation to a relative absence of butyrate in the colon of these patients due to a reduction of the particular anaerobic bacteria that produce butyrate, namely clostridia. Our results are in accordance with recent findings of a lack of *Faecalibacterium prausnitzii* in adult patients with IBD (148). This is one of the dominant members of the clostridial cluster IV in the intestinal microbiota.

Intestinal bacteria may also be causative agents inducing or aggravating the inflammation characterizing IBD. Interestingly, we found that both UC and CD patients had a significantly greater proportion of Gram-negative bacteria in their flora then diseased controls and healthy controls. Gram-negative bacteria are in some aspects more inflammatogenic then Gram-positive bacteria. They induce more PGE2 (144) and more IL-6 and IL-8 than do Gram-positive bacteria (171). Perhaps more importantly, they are active even when degraded, as LPS in soluble form have practically equal stimulating properties as intact Gram-negative bacteria (172). Whereas Gram-positive bacterial fragments induce much less cytokine production than intact Gram-positive bacteria when used to stimulate monocytes.

Lymphocyte profiles in children at début of IBD

In paper II we investigated circulating lymphocytes and their expression of some selected markers of activation, homing and memory at début of childhood IBD. None of the children included in the study had consumed medical treatment including antibiotics, probiotics and anti-inflammatory drugs that may modify the lymphocyte populations. We therefore believe that our results accurately reflect the situation at début of childhood IBD.

Children with UC demonstrated a more pronounced activation of T cells than children with CD and DC. This may be a result from the larger area involved in UC frequently presented with pancolitis. However, a majority of the children with CD had also extensive colon involvement, which is quite common in childhood CD. The pronounced expression of β1-integrins on T cells in début of UC compared to both CD and DC reveals pronounced T cell activation (173). The VLA integrins are expressed by T cells that have been activated by antigen

in the lymph node, maturated to effector T cells and are ready to home to inflamed tissue. This may also reflect a more pronounced inflammatory activation in UC. Another explanation could be the different organ involvement, in UC and CD. Interestingly, increased expression of β1-integrins on $CD4^+$ T cells has been observed earlier in autoimmune diseased such as systemic sclerosis, Behcet's disease and Vogt-Koyanagi-Harada disease (174, 175). UC is more often associated with autoimmune disorders than is CD, accordingly, the autoantibody, p-ANCA (perinuclear anti-neutrophil cytoplasmic antibodies) (176) is common in patients with UC, but not in CD. Our results suggest further studies of β1-integrins in patients with UC.

As discussed earlier under section "hygiene hypothesis", microbial exposure in early infancy may be of importance by hindering initiation, of disease development later in life. Epidemiological studies have shown that IBD is more common in highly developed industrialized nations than in poor countries. One hypothesis is that good sanitary conditions lead to decreased stimulation of the immune system in early childhood, resulting in poor maturation of normal tolerance mechanisms (53). The intestinal flora evokes low-grade inflammation (177) that may provoke maturation of the immune system. Especially CD has been linked to hygiene hypothesis and good sanitary conditions (55, 61). In paper II we show that children at the début of CD have decreased fraction of memory B cells compared to both DC and children with UC. Much of the research done on the mechanism in CD focuses on the microbiota and interactions with T cells. However, some experimental mouse models propose a protective role of B cells in chronic colitis (178, 179). Thus, the altered proportion of memory B cells (presented in paper II) could be a predisposing condition for CD. In agreement with the "hygiene hypothesis" our results suggest that, immune inexperience may predispose to CD, perhaps by insufficient development of normal tolerance mechanisms that could result in altered development of memory B cells.

Gram-positive bacteria need to be intact to induce high IL–12 production in monocytes, soluble constituents from Gram-positive bacteria inhibit such production.

The activation of monocytes/macrophages is fundamental in anti-microbial defence. IL-12 secreted by monocytes/macrophages in response to microbial products induces secretion of IFN-γ from T and NK cells, which, in turn, enhances the bactericidal capacity of the phagocytes (105). The IL-12/IFN-γ loop is required both to combat intracellular pathogens (180) and other extracellular bacteria such as streptococci, staphylococci and pneumococci (181-183). In paper III, we show that intact Gram-positive bacteria are the best

inducer of IL-12 (p70) in monocytes and that fragmented Gram-positive bacteria and components of the Gram-positive cell wall inhibit IL-12 production. We compared the IL-12 inducing capacity in PBMC between several microbes and microbial components. Clearly, only intact Gram-positive bacteria induced IL-12 production in the range of ng/mL. In addition, pre-treatment with, the T cells cytokine, IFN-γ further increased the IL-12 production after stimulation with intact Gram-positive bacteria and also resulted in detectable levels of IL-12 after stimulation with LPS. IFN-γ enhances the bactericidal capacity of monocytes/macrophages (106) and also enhances further IL-12 production, indicating how T cells can assist monocytes in combating enemies.

Gram-positives and Gram-negatives have different capacity to resist chemical (lysozyme) and disrupting treatment (sonication), disruption of different Gram-positive bacteria requires more time/effort (own unpublished observations). Assistance from acquired immune system seems therefore appropriate. That fragmented Gram-positive bacteria inhibited IL-12 production seems logical, as fragments of the cell wall signals that the enemy has been defeated and further phagocytos assistance is not needed. In accordance, resistance of lactobacilli to lysis within macrophages has been correlated with their capacity to induce IL-12 (184). IL-6 was induced both of intact Gram-positive bacteria and constituents from the cell wall. This cytokine instead promotes specific immunity and healing processes (185).

The IL-12 production in monocytes was dependent on interaction with TLR2 and Nod2 receptors, as blocking of these receptors separately inhibited the IL-12 production. Also phagocytosis of intact Gram-positive bacteria was mandatory for IL-12 production from monocytes as blocking of phagocytos with cytochalasin inhibited IL-12 production. Non-microbial particles (plastic or latex beads) induced no bioactive IL-12 (p70), not even when presented together with cell wall fragments of Gram-positive bacteria. Thus, particles exposing Gram-positive bacterial structures seemed to be an optimal trigger of IL-12 production in monocytes. However, proteins on the intact Gram-positive bacteria were ruled out as important for IL-12 production as formalin and protease treatment left their IL-12 inducing capacity intact.

IL-12 production in response to Gram-positive bacteria and IFN-γ/LPS not only induce different range of IL-12 but also utilize different intracellular signalling pathways

To study the intracellular signalling pathways involved in IL-12 production in monocytes we compared the results with LPS stimulation after pre-incubated

with IFN-γ. IFN-γ/LPS is a often used stimuli to study IL-12 production (163, 186). The enzyme PI3K is crucial in phagocytosis and phagosome maturation (187-189). We blocked, PI3K with Wortmannin, this strongly and dose-dependently inhibited IL-12 production in response to intact Gram-positive bacteria. In sharp contrast, blocking of PI3K strongly increased IL-12 production induced by IFN-γ/LPS, which was in accordance with previously published data (162-164), and has been attributed to the fact that PI3K down-regulates TLR signalling (190).

As phagocytosed Gram-positive bacteria and LPS are different in structure it is not surprising that they engaged different intracellular pathways. Although, both Gram-positive bacteria and IFN-γ/LPS stimuli seemed to involve transcription factor NF-κB, Gram-positive bacteria seemed to involve the JNK pathway. On the contrary, inhabitation of the JNK pathway instead tended to increase IL-12 production in response to IFNγ/LPS, in accordance with previous reports (164).

We propose that the principal physiological function of IL-12 production in monocytes/macrophages is to trigger IFN-γ production in NK and T cells that activates the monocytes/macrophages. Such activation increases the capacity to digest the sturdy and thick cell wall containing up to 50 layers of peptidoglycan. The presence of fragments of Gram-positive bacteria does on the contrary turn of this signal, as increased macrophage activation is not needed.

ACKNOWLEDGEMENTS (in Swedish)

Det är väldigt många som på olika sätt har bidragit till att jag har kunnat genomföra detta arbete, tusen tack till er ALLA. Några av er vill jag speciellt tacka:

Min huvudhandledare Agnes Wold, en fascinerande kvinna med en enorm kapacitet, humor och åsikter om det mesta. Tusen tack för att du givit mig möjlighet att genomföra detta arbete och för ditt ihärdiga manusläsande.

Min bi-handledare Robert Saalman; utan dig och alla barnen hade det inte blivit någon avhandling. Jag vill speciellt tacka dig för din förmåga att uppmuntra mig och ingjuta mod i mig under slutfasen av detta arbete.

Min bi-handledare Christina Hessle, Tusen tack för allt du lärt mig om labbandets konst och för alla gånger jag har fått ringa och konsultera dig om diverse saker. Men trots det har jag ändå saknat dig sedan du slutade, både ditt kunnande och din vänskap.

Sedan vill jag rikta ett enormt stort tack till kvinnorna som gör vår arbetsplats till en av de trevligaste arbetsplatserna man kan tänka sig……..Ni är helt fantastiska, jag kan inte nog prisa er, utan er ja då…blev det absolut ingen avhandling. Nu vet alla att det är Eva, Ingela och Jolanta som jag talar om.

Ett stor tack vill jag även rikta till Ia, jag är mycket tacksam för ditt kunnande och din värme utan dig hade det inte heller blivit en avhandling.

Tack även till Anna M och för vårt samarbete på IL-12 artikeln, dina friska tag och idéer var ovärderliga. Tack även till Sofia, som tålmodigt har lyssna på mig, du är en klippa! Tack även till Anna Ö, för att du förgyller jobbet och speciellt tack för musiken. Tack även till alla andra goa medarbetare och doktorander i vår grupp Erika, Forough, Anna L, Fei, Susann S, Susanne H, Bodil, Elisabeth och Åsa. Tack även till alla härliga medarbetare på avdelningen: Eva J, Ingela, Ivar, Elin, Marianne, Kerstin, Lisa, Johanna och Christine. Tack Inger för våra diskussioner, speciellt de som inte handlar om forskning.

Ett stort tack vill jag även rikta till vänner som inte längre syns så ofta på avdelningen, Nahid Ka. Nahid Ko. Liljana, Louise, Natascha, Carola, Maja, Anna-Lena, Anna-Clara, Henrik, Britta. Givetvis vill jag tacka Lena som förgyllt många dagar och kvällar i labbet.

Tack Anna R för vårat samarbete, dina kunskaper och idéer har varit ovärderliga.

Ett stort tack till tålmodige och goa Gaby, som varje gång vänligt talar om hur saker och ting skall göras.

Tack även till medarbetare på Klinisk immunologi för er support och hjälp, speciellt tack till Ulla, Marianne, Marie, Ingela, Inger, Susanne, Florence och Mirjana.

Tack, till alla medarbetare på Rematologen för er support och vänskap, speciellt tack till, alltid lika glada Anna-Carin, Eva S men även Helen som en gång har tillhört Reumatologen.

Ett stort tack vill jag rikta till alla på Drottning Silvias Barn och Ungdoms Sjukhus, avdelning 334 som hjälpt till att genomföra detta arbete, speciellt tack till; Anette, Britt, Kerstin och Britt-Marie.

Tack även till alla barn, vänner och till hela ”La familja” (släkten), som alla på olika sätt har backat upp mig, speciellt tack till mina syskon med familjer och tack även till Siv. Tack pappa för att du tror på mig. Mamma….utan ditt stöd och ovärderliga insatser skulle detta inte ha fungerat.

Thomas och Tom; er support och kärlek som hjälpt mig att genomföra detta arbete betyder alldeles för mycket för att kunna formuleras på ett papper.

REFERENCES

1. Lindberg, E., B. Lindquist, L. Holmquist, and H. Hildebrand. 2000. Inflammatory bowel disease in children and adolescents in Sweden, 1984-1995. *J Pediatr Gastroenterol Nutr* 30:259-264.

2. Sartor, R. B. 2006. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nature clinical practice* 3:390-407.

3. 2005. Inflammatory bowel disease in children and adolescents: recommendations for diagnosis--the Porto criteria. *J Pediatr Gastroenterol Nutr* 41:1-7.

4. Hildebrand, H., Y. Finkel, L. Grahnquist, J. Lindholm, A. Ekbom, and J. Askling. 2003. Changing pattern of paediatric inflammatory bowel disease in northern Stockholm 1990-2001. *Gut* 52:1432-1434.

5. Malmborg P, Montgomery S, Grahnquist L, and Hildebrand. H. 2008. Pattern of pediatric inflammatory bowel disease in Northern Stockholm 2002-2007. In *World Congress of Pediatric Gastroenterology, Hepatology and Nutrition. 2008*, Iguassu, Brazil;.

6. Kaisho, T., and S. Akira. 2006. Toll-like receptor function and signaling. *J Allergy Clin Immunol* 117:979-987; quiz 988.

7. Fitzgerald, K. A., and Z. J. Chen. 2006. Sorting out Toll signals. *Cell* 125:834-836.

8. O'Neill, L. A. 2006. Targeting signal transduction as a strategy to treat inflammatory diseases. *Nat Rev Drug Discov* 5:549-563.

9. Eaden, J. A., K. R. Abrams, and J. F. Mayberry. 2001. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 48:526-535.

10. Biank, V., U. Broeckel, and S. Kugathasan. 2007. Pediatric inflammatory bowel disease: clinical and molecular genetics. *Inflamm Bowel Dis* 13:1430-1438.

11. Griffiths, A. M. 2004. Specificities of inflammatory bowel disease in childhood. *Best practice & research* 18:509-523.

12. Wilks, S. 1859. Morbid appearances in the intestines of Miss Bankes. *Medical Times & Gazette*:264-265.

13. Sawczenko, A., and B. K. Sandhu. 2003. Presenting features of inflammatory bowel disease in Great Britain and Ireland. *Archives of disease in childhood* 88:995-1000.

14. Crohn, B. B., L. Ginzburg, and G. D. Oppenheimer. 1932. Regional ileitis. *JAMA*:1323-1329.

15. Society, S. P. 2008-2009. Vårdprogram IBD. Swedish Paediatric Society, Gastro/Nutrition section.

16. Loftus, E. V., Jr. 2004. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. *Gastroenterology* 126:1504-1517.

17. Gismera, C. S., and B. S. Aladren. 2008. Inflammatory bowel diseases: a disease (s) of modern times? Is incidence still increasing? *World J Gastroenterol* 14:5491-5498.

18. Björnsson, S., J. H. Johannsson, and E. Oddsson. 1998. Inflammatory bowel disease in Iceland, 1980-89. A retrospective nationwide epidemiologic study. *Scand J Gastroenterol* 33:71-77.

19. Moum, B., M. H. Vatn, A. Ekbom, E. Aadland, O. Fausa, I. Lygren, J. Sauar, T. Schulz, and N. Stray. 1996. Incidence of ulcerative colitis and indeterminate colitis in four counties of southeastern Norway, 1990-93. A prospective population-based study. The Inflammatory Bowel South-Eastern Norway (IBSEN) Study Group of Gastroenterologists. *Scand J Gastroenterol* 31:362-366.

20. Vind, I., L. Riis, T. Jess, E. Knudsen, N. Pedersen, M. Elkjaer, I. Bak Andersen, V. Wewer, P. Norregaard, F. Moesgaard, F. Bendtsen, and P. Munkholm. 2006. Increasing incidences of inflammatory bowel disease and decreasing surgery rates in Copenhagen City and County, 2003-2005: a population-based study from the Danish Crohn colitis database. *Am J Gastroenterol* 101:1274-1282.

21. Munkholm, P., E. Langholz, O. H. Nielsen, S. Kreiner, and V. Binder. 1992. Incidence and prevalence of Crohn's disease in the county of Copenhagen, 1962-87: a sixfold increase in incidence. *Scand J Gastroenterol* 27:609-614.

22. Langholz, E., P. Munkholm, O. H. Nielsen, S. Kreiner, and V. Binder. 1991. Incidence and prevalence of ulcerative colitis in Copenhagen county from 1962 to 1987. *Scand J Gastroenterol* 26:1247-1256.

23. Jacobsen, B. A., J. Fallingborg, H. H. Rasmussen, K. R. Nielsen, A. M. Drewes, E. Puho, G. L. Nielsen, and H. T. Sorensen. 2006. Increase in incidence and prevalence of inflammatory bowel disease in northern Denmark: a population-based study, 1978-2002. *Eur J Gastroenterol Hepatol* 18:601-606.

24. Shivananda, S., J. Lennard-Jones, R. Logan, N. Fear, A. Price, L. Carpenter, and M. van Blankenstein. 1996. Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). *Gut* 39:690-697.

25. Russel, M. G., E. Dorant, A. Volovics, R. J. Brummer, P. Pop, J. W. Muris, L. P. Bos, C. B. Limonard, and R. W. Stockbrugger. 1998. High incidence of inflammatory bowel disease in The Netherlands: results of a prospective study. The South Limburg IBD Study Group. *Dis Colon Rectum* 41:33-40.

26. Rubin, G. P., A. P. Hungin, P. J. Kelly, and J. Ling. 2000. Inflammatory bowel disease: epidemiology and management in an English general practice population. *Alimentary pharmacology & therapeutics* 14:1553-1559.

27. Tsironi, E., R. M. Feakins, C. S. Probert, D. S. Rampton, and D. Phil. 2004. Incidence of inflammatory bowel disease is rising and abdominal tuberculosis is falling in Bangladeshis in East London, United Kingdom. *Am J Gastroenterol* 99:1749-1755.

28. Garcia Rodriguez, L. A., A. Gonzalez-Perez, S. Johansson, and M. A. Wallander. 2005. Risk factors for inflammatory bowel disease in the general population. *Alimentary pharmacology & therapeutics* 22:309-315.

29. Bernstein, C. N., J. F. Blanchard, P. Rawsthorne, and A. Wajda. 1999. Epidemiology of Crohn's disease and ulcerative colitis in a central Canadian province: a population-based study. *American journal of epidemiology* 149:916-924.

30. Blanchard, J. F., C. N. Bernstein, A. Wajda, and P. Rawsthorne. 2001. Small-area variations and sociodemographic correlates for the incidence of Crohn's disease and ulcerative colitis. *American journal of epidemiology* 154:328-335.

31. Loftus, E. V., Jr., M. D. Silverstein, W. J. Sandborn, W. J. Tremaine, W. S. Harmsen, and A. R. Zinsmeister. 2000. Ulcerative colitis in Olmsted County, Minnesota, 1940-1993: incidence, prevalence, and survival. *Gut* 46:336-343.

32. Tsianos, E. V., K. H. Katsanos, D. Christodoulou, I. Dimoliatis, A. Kogevinas, and R. F. Logan. 2003. Continuing low incidence of Crohn's disease in Northwest Greece. *Dig Liver Dis* 35:99-103.

33. Trallori, G., D. Palli, C. Saieva, G. Bardazzi, A. G. Bonanomi, G. d'Albasio, M. Galli, G. Vannozzi, M. Milla, O. Tarantino, F. Renai, A. Messori, A. Amorosi, F. Pacini, and A. Morettini. 1996. A population-based study of inflammatory bowel disease in Florence over 15 years (1978-92). *Scand J Gastroenterol* 31:892-899.

34. Tragnone, A., G. Corrao, F. Miglio, R. Caprilli, and G. A. Lanfranchi. 1996. Incidence of inflammatory bowel disease in Italy: a nationwide population-based study. Gruppo Italiano per lo Studio del Colon e del Retto (GISC). *International journal of epidemiology* 25:1044-1052.

35. Yang, S. K., S. Yun, J. H. Kim, J. Y. Park, H. Y. Kim, Y. H. Kim, D. K. Chang, J. S. Kim, I. S. Song, J. B. Park, E. R. Park, K. J. Kim, G. Moon, and S. H. Yang. 2008. Epidemiology of inflammatory bowel disease in the Songpa-Kangdong district, Seoul, Korea, 1986-2005: a KASID study. *Inflamm Bowel Dis* 14:542-549.

36. Abdul-Baki, H., I. ElHajj, L. M. El-Zahabi, C. Azar, E. Aoun, H. Zantout, W. Nasreddine, B. Ayyach, F. H. Mourad, A. Soweid, K. A. Barada, and A. I. Sharara. 2007. Clinical epidemiology of inflammatory bowel disease in Lebanon. *Inflamm Bowel Dis* 13:475-480.

37. Thia, K. T., E. V. Loftus, Jr., W. J. Sandborn, and S. K. Yang. 2008. An update on the epidemiology of inflammatory bowel disease in Asia. *Am J Gastroenterol* 103:3167-3182.

38. Kugathasan, S., R. H. Judd, R. G. Hoffmann, J. Heikenen, G. Telega, F. Khan, S. Weisdorf-Schindele, W. San Pablo, Jr., J. Perrault, R. Park, M. Yaffe, C. Brown, M. T. Rivera-Bennett, I. Halabi, A. Martinez, E. Blank, S. L. Werlin, C. D. Rudolph, and D. G. Binion. 2003. Epidemiologic and clinical characteristics of children with newly diagnosed inflammatory bowel disease in Wisconsin: a statewide population-based study. *J Pediatr* 143:525-531.

39. Loftus, E. V., Jr., M. D. Silverstein, W. J. Sandborn, W. J. Tremaine, W. S. Harmsen, and A. R. Zinsmeister. 1998. Crohn's disease in Olmsted County, Minnesota, 1940-1993: incidence, prevalence, and survival. *Gastroenterology* 114:1161-1168.

40. Bernstein, C. N., A. Wajda, L. W. Svenson, A. MacKenzie, M. Koehoorn, M. Jackson, R. Fedorak, D. Israel, and J. F. Blanchard. 2006. The epidemiology of inflammatory bowel disease in Canada: a population-based study. *Am J Gastroenterol* 101:1559-1568.

41. Björnsson, S., and J. H. Johannsson. 2000. Inflammatory bowel disease in Iceland, 1990-1994: a prospective, nationwide, epidemiological study. *Eur J Gastroenterol Hepatol* 12:31-38.

42. Russell, R. K., and J. Satsangi. 2004. IBD: a family affair. *Best practice & research* 18:525-539.

43. Halme, L., P. Paavola-Sakki, U. Turunen, M. Lappalainen, M. Farkkila, and K. Kontula. 2006. Family and twin studies in inflammatory bowel disease. *World J Gastroenterol* 12:3668-3672.

44. Halfvarson, J., L. Bodin, C. Tysk, E. Lindberg, and G. Jarnerot. 2003. Inflammatory bowel disease in a Swedish twin cohort: a long-term follow-up of concordance and clinical characteristics. *Gastroenterology* 124:1767-1773.

45. Yang, S. K., E. V. Loftus, Jr., and W. J. Sandborn. 2001. Epidemiology of inflammatory bowel disease in Asia. *Inflamm Bowel Dis* 7:260-270.

46. Hildebrand, H., P. Malmborg, J. Askling, A. Ekbom, and S. M. Montgomery. 2008. Early-life exposures associated with antibiotic use and risk of subsequent Crohn's disease. *Scand J Gastroenterol* 43:961-966.

47. Wurzelmann, J. I., C. M. Lyles, and R. S. Sandler. 1994. Childhood infections and the risk of inflammatory bowel disease. *Digestive diseases and sciences* 39:555-560.

48. Card, T., R. F. Logan, L. C. Rodrigues, and J. G. Wheeler. 2004. Antibiotic use and the development of Crohn's disease. *Gut* 53:246-250.

49. Odes, H. S., A. Fich, S. Reif, A. Halak, A. Lavy, D. Keter, R. Eliakim, J. Paz, E. Broide, Y. Niv, Y. Ron, Y. Villa, N. Arber, and T. Gilat. 2001. Effects of current cigarette smoking on clinical course of Crohn's disease and ulcerative colitis. *Digestive diseases and sciences* 46:1717-1721.

50. Koutroubakis, I. E., I. G. Vlachonikolis, and E. A. Kouroumalis. 2002. Role of appendicitis and appendectomy in the pathogenesis of ulcerative colitis: a critical review. *Inflamm Bowel Dis* 8:277-286.

51. Andersson, R. E., G. Olaison, C. Tysk, and A. Ekbom. 2003. Appendectomy is followed by increased risk of Crohn's disease. *Gastroenterology* 124:40-46.

52. Andersson, R. E., G. Olaison, C. Tysk, and A. Ekbom. 2001. Appendectomy and protection against ulcerative colitis. *N Engl J Med* 344:808-814.

53. Strachan, D. P. 1989. Hay fever, hygiene, and household size. *BMJ (Clinical research ed* 299:1259-1260.

54. Bach, J. F. 2002. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 347:911-920.

55. Feillet, H., and J. F. Bach. 2004. Increased incidence of inflammatory bowel disease: the price of the decline of infectious burden? *Curr Opin Gastroenterol* 20:560-564.

56. Hampe, J., K. Heymann, M. Krawczak, and S. Schreiber. 2003. Association of inflammatory bowel disease with indicators for childhood antigen and infection exposure. *Int J Colorectal Dis* 18:413-417.

57. Klement, E., J. Lysy, M. Hoshen, M. Avitan, E. Goldin, and E. Israeli. 2008. Childhood hygiene is associated with the risk for inflammatory bowel disease: a population-based study. *Am J Gastroenterol* 103:1775-1782.

58. Gent, A. E., M. D. Hellier, R. H. Grace, E. T. Swarbrick, and D. Coggon. 1994. Inflammatory bowel disease and domestic hygiene in infancy. *Lancet* 343:766-767.

59. Bernstein, C. N., P. Rawsthorne, M. Cheang, and J. F. Blanchard. 2006. A population-based case control study of potential risk factors for IBD. *Am J Gastroenterol* 101:993-1002.

60. Duggan, A. E., I. Usmani, K. R. Neal, and R. F. Logan. 1998. Appendicectomy, childhood hygiene, Helicobacter pylori status, and risk of inflammatory bowel disease: a case control study. *Gut* 43:494-498.

61. Radon, K., D. Windstetter, A. L. Poluda, B. Mueller, E. von Mutius, and S. Koletzko. 2007. Contact with farm animals in early life and juvenile inflammatory bowel disease: a case-control study. *Pediatrics* 120:354-361.

62. Amre, D. K., P. Lambrette, L. Law, A. Krupoves, V. Chotard, F. Costea, G. Grimard, D. Israel, D. Mack, and E. G. Seidman. 2006. Investigating the hygiene hypothesis as a risk factor in pediatric onset Crohn's disease: a case-control study. *Am J Gastroenterol* 101:1005-1011.

63. Aranda, R., B. C. Sydora, P. L. McAllister, S. W. Binder, H. Y. Yang, S. R. Targan, and M. Kronenberg. 1997. Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of CD4+, CD45RBhigh T cells to SCID recipients. *J Immunol* 158:3464-3473.

64. Dianda, L., A. M. Hanby, N. A. Wright, A. Sebesteny, A. C. Hayday, and M. J. Owen. 1997. T cell receptor-alpha beta-deficient mice fail to develop colitis in the absence of a microbial environment. *Am J Pathol* 150:91-97.

65. Taurog, J. D., J. A. Richardson, J. T. Croft, W. A. Simmons, M. Zhou, J. L. Fernandez-Sueiro, E. Balish, and R. E. Hammer. 1994. The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. *J Exp Med* 180:2359-2364.

66. Cong, Y., S. L. Brandwein, R. P. McCabe, A. Lazenby, E. H. Birkenmeier, J. P. Sundberg, and C. O. Elson. 1998. CD4+ T cells reactive to enteric bacterial antigens in spontaneously colitic C3H/HeJBir mice: increased T helper cell type 1 response and ability to transfer disease. *J Exp Med* 187:855-864.

67. Rutgeerts, P., M. Hiele, K. Geboes, M. Peeters, F. Penninckx, R. Aerts, and R. Kerremans. 1995. Controlled trial of metronidazole treatment for prevention of Crohn's recurrence after ileal resection. *Gastroenterology* 108:1617-1621.

68. D'Haens, G. R., K. Geboes, M. Peeters, F. Baert, F. Penninckx, and P. Rutgeerts. 1998. Early lesions of recurrent Crohn's disease caused by infusion of intestinal contents in excluded ileum. *Gastroenterology* 114:262-267.

69. Harper, P. H., S. C. Truelove, E. C. Lee, M. G. Kettlewell, and D. P. Jewell. 1983. Split ileostomy and ileocolostomy for Crohn's disease of the colon and ulcerative colitis: a 20 year survey. *Gut* 24:106-113.

70. Bibiloni, R., R. N. Fedorak, G. W. Tannock, K. L. Madsen, P. Gionchetti, M. Campieri, C. De Simone, and R. B. Sartor. 2005. VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. *Am J Gastroenterol* 100:1539-1546.

71. Furrie, E., S. Macfarlane, A. Kennedy, J. H. Cummings, S. V. Walsh, A. O'Neil D, and G. T. Macfarlane. 2005. Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. *Gut* 54:242-249.

72. Sartor, R. B. 2004. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology* 126:1620-1633.

73. Sneath, A. H. P., S. N. Mair, M. E. Sharpe, and G. J. Holt, eds. 1986. *Bergey's manual of Systematic Bacteriology*. Williams &Wilkins, Baltimore, MD.

74. Pumbwe, L., C. A. Skilbeck, V. Nakano, M. J. Avila-Campos, R. M. Piazza, and H. M. Wexler. 2007. Bile salts enhance bacterial co-aggregation, bacterial-intestinal epithelial cell adhesion, biofilm formation and antimicrobial resistance of Bacteroides fragilis. *Microb Pathog* 43:78-87.

75. Wong, J. M., R. de Souza, C. W. Kendall, A. Emam, and D. J. Jenkins. 2006. Colonic health: fermentation and short chain fatty acids. *Journal of clinical gastroenterology* 40:235-243.

76. Roediger, W. E. 1980. Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. *Gut* 21:793-798.

77. Velazquez, O. C., H. M. Lederer, and J. L. Rombeau. 1997. Butyrate and the colonocyte. Production, absorption, metabolism, and therapeutic implications. *Adv Exp Med Biol* 427:123-134.

78. Kruh, J. 1982. Effects of sodium butyrate, a new pharmacological agent, on cells in culture. *Molecular and cellular biochemistry* 42:65-82.

79. Cummings, J. H. 1983. Fermentation in the human large intestine: evidence and implications for health. *Lancet* 1:1206-1209.

80. Siavoshian, S., J. P. Segain, M. Kornprobst, C. Bonnet, C. Cherbut, J. P. Galmiche, and H. M. Blottiere. 2000. Butyrate and trichostatin A effects on the proliferation/differentiation of human intestinal epithelial cells: induction of cyclin D3 and p21 expression. *Gut* 46:507-514.

81. Domon-Dell, C., Q. Wang, S. Kim, M. Kedinger, B. M. Evers, and J. N. Freund. 2002. Stimulation of the intestinal Cdx2 homeobox gene by butyrate in colon cancer cells. *Gut* 50:525-529.

82. Roediger, W. E. 1980. The colonic epithelium in ulcerative colitis: an energy-deficiency disease? *Lancet* 2:712-715.

83. Popescu, A., and R. J. Doyle. 1996. The Gram stain after more than a century. *Biotech Histochem* 71:145-151.

84. Girardin, S. E., L. H. Travassos, M. Herve, D. Blanot, I. G. Boneca, D. J. Philpott, P. J. Sansonetti, and D. Mengin-Lecreulx. 2003. Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. *J Biol Chem* 278:41702-41708.

85. Adlerberth, I., and A. E. Wold. 2009. Establishment of the gut microbiota in Western infants. *Acta Paediatr* 98:229-238.

86. Frank, D. N., A. L. St Amand, R. A. Feldman, E. C. Boedeker, N. Harpaz, and N. R. Pace. 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A* 104:13780-13785.

87. Sartor, R. B. 2008. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 134:577-594.

88. Finegold, S. M. 1986. Normal human intestinal flora. *Ann Ist Super Sanita* 22:731-737.

89. Blaser, M. J. 1993. Helicobacter pylori: microbiology of a 'slow' bacterial infection. *Trends Microbiol* 1:255-260.

90. Tannock, G. V. 1995. *Normal Microbiota. An introduction to microbes inhabiting the human body.* Chapman and Hall, London.

91. Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K. E. Nelson, and D. A. Relman. 2005. Diversity of the human intestinal microbial flora. *Science* 308:1635-1638.

92. Wang, M., S. Ahrne, B. Jeppsson, and G. Molin. 2005. Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. *FEMS microbiology ecology* 54:219-231.

93. Lim, D. 1998. *Microbiology*. WCB/McGraw-Hill.

94. Murray, R. P., S. K. Rosenthal, S. G. Kobayashi, and A. M. Pfaller. 1998. *Medical Microbiology*. Mosby, St. Louis, Missouri.

95. Dinges, M. M., P. M. Orwin, and P. M. Schlievert. 2000. Exotoxins of Staphylococcus aureus. *Clinical microbiology reviews* 13:16-34, table of contents.

96. Lindberg, E., F. Nowrouzian, I. Adlerberth, and A. E. Wold. 2000. Long-time persistence of superantigen-producing Staphylococcus aureus strains in the intestinal microflora of healthy infants. *Pediatric research* 48:741-747.

97. Adlerberth, I., E. Lindberg, N. Åberg, B. Hesselmar, R. Saalman, I. L. Strannegård, and A. E. Wold. 2006. Reduced enterobacterial and increased staphylococcal colonization of the infantile bowel: an effect of hygienic lifestyle? *Pediatric research* 59:96-101.

98. Lindberg, E., I. Adlerberth, B. Hesselmar, R. Saalman, I. L. Strannegård, N. Åberg, and A. E. Wold. 2004. High rate of transfer of Staphylococcus aureus from parental skin to infant gut flora. *J Clin Microbiol* 42:530-534.

99. Mölne, J., and A. E. Wold. 2007. *Inflammation*. Liber AB, Stockholm.

100. Collins, M. D., P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. Farrow. 1994. The phylogeny of the genus Clostridium: proposal of five new genera and eleven new species combinations. *International journal of systematic bacteriology* 44:812-826.

101. Wehkamp, J., J. Harder, M. Weichenthal, M. Schwab, E. Schaffeler, M. Schlee, K. R. Herrlinger, A. Stallmach, F. Noack, P. Fritz, J. M. Schroder, C. L. Bevins, K. Fellermann, and E. F. Stange. 2004. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. *Gut* 53:1658-1664.

102. Aderem, A., and D. M. Underhill. 1999. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* 17:593-623.

103. Cario, E., and D. K. Podolsky. 2000. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun* 68:7010-7017.

104. Baecher-Allan, C., J. A. Brown, G. J. Freeman, and D. A. Hafler. 2001. CD4+CD25high regulatory cells in human peripheral blood. *J Immunol* 167:1245-1253.

105. Romagnani, S. 1999. Th1/Th2 cells. *Inflamm Bowel Dis* 5:285-294.

106. Boehm, U., T. Klamp, M. Groot, and J. C. Howard. 1997. Cellular responses to interferon-gamma. *Annu Rev Immunol* 15:749-795.

107. Mills, K. H. 2008. Induction, function and regulation of IL-17-producing T cells. *Eur J Immunol* 38:2636-2649.

108. Oppmann, B., R. Lesley, B. Blom, J. C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh, F. Zonin, E. Vaisberg, T. Churakova, M. Liu, D. Gorman, J. Wagner, S. Zurawski, Y. Liu, J. S. Abrams, K. W. Moore, D. Rennick, R. de Waal-Malefyt, C. Hannum, J. F. Bazan, and R. A. Kastelein. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13:715-725.

109. Manetti, R., P. Parronchi, M. G. Giudizi, M. P. Piccinni, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J Exp Med* 177:1199-1204.

110. Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nature reviews* 3:133-146.

111. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19:683-765.

112. McKenzie, B. S., R. A. Kastelein, and D. J. Cua. 2006. Understanding the IL-23-IL-17 immune pathway. *Trends Immunol* 27:17-23.

113. Barclay, A. N., H. M. Brown, S. K. A. Law, M. G. Tomlinson, and v. d. M. P.A. 1997. *The Leucocyte antigen*. Academic Press Limited, London, UK.

114. Doody, G. M., P. W. Dempsey, and D. T. Fearon. 1996. Activation of B lymphocytes: integrating signals from CD19, CD22 and Fc gamma RIIb1. *Curr Opin Immunol* 8:378-382.

115. Jackson, S. M., P. C. Wilson, J. A. James, and J. D. Capra. 2008. Human B cell subsets. *Advances in immunology* 98:151-224.

116. Wing, K., P. Larsson, K. Sandström, S. B. Lundin, E. Suri-Payer, and A. Rudin. 2005. CD4+ CD25+ FOXP3+ regulatory T cells from human thymus and cord blood suppress antigen-specific T cell responses. *Immunology* 115:516-525.

117. Agematsu, K. 2000. Memory B cells and CD27. *Histology and histopathology* 15:573-576.

118. Hermiston, M. L., Z. Xu, and A. Weiss. 2003. CD45: a critical regulator of signaling thresholds in immune cells. *Annu Rev Immunol* 21:107-137.

119. Trowbridge, I. S., and M. L. Thomas. 1994. CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. *Annu Rev Immunol* 12:85-116.

120. Hemler, M. E. 1990. VLA proteins in the integrin family: structures, functions, and their role on leukocytes. *Annu Rev Immunol* 8:365-400.

121. Elices, M. J., L. Osborn, Y. Takada, C. Crouse, S. Luhowskyj, M. E. Hemler, and R. R. Lobb. 1990. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell* 60:577-584.

122. Hugot, J. P., P. Laurent-Puig, C. Gower-Rousseau, J. M. Olson, J. C. Lee, L. Beaugerie, I. Naom, J. L. Dupas, A. Van Gossum, M. Orholm, C. Bonaiti-Pellie, J. Weissenbach, C. G. Mathew, J. E. Lennard-Jones, A. Cortot, J. F. Colombel, and G. Thomas. 1996. Mapping of a susceptibility locus for Crohn's disease on chromosome 16 [see comments]. *Nature* 379:821-823.

123. Ogura, Y., D. K. Bonen, N. Inohara, D. L. Nicolae, F. F. Chen, R. Ramos, H. Britton, T. Moran, R. Karaliuskas, R. H. Duerr, J. P. Achkar, S. R. Brant, T. M. Bayless, B. S. Kirschner, S. B. Hanauer, G. Nunez, and J. H. Cho. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411:603-606.

124. Girardin, S. E., I. G. Boneca, J. Viala, M. Chamaillard, A. Labigne, G. Thomas, D. J. Philpott, and P. J. Sansonetti. 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* 278:8869-8872.

125. Newman, B., X. Gu, R. Wintle, D. Cescon, M. Yazdanpanah, X. Liu, V. Peltekova, M. Van Oene, C. I. Amos, and K. A. Siminovitch. 2005. A risk haplotype in the Solute Carrier Family 22A4/22A5 gene cluster influences phenotypic expression of Crohn's disease. *Gastroenterology* 128:260-269.

126. van Heel, D. A., S. Ghosh, M. Butler, K. A. Hunt, A. M. Lundberg, T. Ahmad, D. P. McGovern, C. Onnie, K. Negoro, S. Goldthorpe, B. M. Foxwell, C. G. Mathew, A. Forbes, D. P. Jewell, and R. J. Playford. 2005. Muramyl dipeptide and toll-like receptor sensitivity in NOD2-associated Crohn's disease. *Lancet* 365:1794-1796.

127. Netea, M. G., G. Ferwerda, D. J. de Jong, T. Jansen, L. Jacobs, M. Kramer, T. H. Naber, J. P. Drenth, S. E. Girardin, B. J. Kullberg, G. J. Adema, and J. W. Van der Meer. 2005. Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release. *J Immunol* 174:6518-6523.

128. Watanabe, T., A. Kitani, P. J. Murray, and W. Strober. 2004. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat Immunol* 5:800-808.

129. Annese, V., M. R. Valvano, O. Palmieri, A. Latiano, F. Bossa, and A. Andriulli. 2006. Multidrug resistance 1 gene in inflammatory bowel disease: a meta-analysis. *World J Gastroenterol* 12:3636-3644.

130. Ho, G. T., E. R. Nimmo, A. Tenesa, J. Fennell, H. Drummond, C. Mowat, I. D. Arnott, and J. Satsangi. 2005. Allelic variations of the multidrug resistance gene determine susceptibility and disease behavior in ulcerative colitis. *Gastroenterology* 128:288-296.

131. Onnie, C. M., S. A. Fisher, R. Pattni, J. Sanderson, A. Forbes, C. M. Lewis, and C. G. Mathew. 2006. Associations of allelic variants of the multidrug resistance gene (ABCB1 or MDR1) and inflammatory bowel disease and their effects on disease behavior: a case-control and meta-analysis study. *Inflamm Bowel Dis* 12:263-271.

132. Panwala, C. M., J. C. Jones, and J. L. Viney. 1998. A novel model of inflammatory bowel disease: mice deficient for the multiple drug resistance gene, mdr1a, spontaneously develop colitis. *J Immunol* 161:5733-5744.

133. Satsangi, J., K. I. Welsh, M. Bunce, C. Julier, J. M. Farrant, J. I. Bell, and D. P. Jewell. 1996. Contribution of genes of the major histocompatibility complex to susceptibility and disease phenotype in inflammatory bowel disease. *Lancet* 347:1212-1217.

134. Van Limbergen, J., R. K. Russell, E. R. Nimmo, and J. Satsangi. 2007. The genetics of inflammatory bowel disease. *Am J Gastroenterol* 102:2820-2831.

135. Duerr, R. H., K. D. Taylor, S. R. Brant, J. D. Rioux, M. S. Silverberg, M. J. Daly, A. H. Steinhart, C. Abraham, M. Regueiro, A. Griffiths, T. Dassopoulos, A. Bitton, H. Yang, S. Targan, L. W. Datta, E. O. Kistner, L. P. Schumm, A. T. Lee, P. K. Gregersen, M. M. Barmada, J. I. Rotter, D. L. Nicolae, and J. H. Cho. 2006. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 314:1461-1463.

136. Steinman, L. 2007. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nature medicine* 13:139-145.

137. Hue, S., P. Ahern, S. Buonocore, M. C. Kullberg, D. J. Cua, B. S. McKenzie, F. Powrie, and K. J. Maloy. 2006. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J Exp Med* 203:2473-2483.

138. Sartor, R. B. 2005. Does Mycobacterium avium subspecies paratuberculosis cause Crohn's disease? *Gut* 54:896-898.

139. Thayer, W. R., Jr., J. A. Coutu, R. J. Chiodini, H. J. Van Kruiningen, and R. S. Merkal. 1984. Possible role of mycobacteria in inflammatory bowel disease. II. Mycobacterial antibodies in Crohn's disease. *Digestive diseases and sciences* 29:1080-1085.

140. Dickinson, R. J., S. A. Varian, A. T. Axon, and E. M. Cooke. 1980. Increased incidence of faecal coliforms with in vitro adhesive and invasive properties in patients with ulcerative colitis. *Gut* 21:787-792.

141. Rolhion, N., and A. Darfeuille-Michaud. 2007. Adherent-invasive Escherichia coli in inflammatory bowel disease. *Inflamm Bowel Dis* 13:1277-1283.

142. Swidsinski, A., J. Weber, V. Loening-Baucke, L. P. Hale, and H. Lochs. 2005. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *J Clin Microbiol* 43:3380-3389.

143. Conte, M. P., S. Schippa, I. Zamboni, M. Penta, F. Chiarini, L. Seganti, J. Osborn, P. Falconieri, O. Borrelli, and S. Cucchiara. 2006. Gut-associated bacterial microbiota in paediatric patients with inflammatory bowel disease. *Gut* 55:1760-1767.

144. Hessle, C. C., B. Andersson, and A. E. Wold. 2003. Gram-negative, but not Gram-positive, bacteria elicit strong PGE2 production in human monocytes. *Inflammation* 27:329-332.

145. Davies, P., P. J. Bailey, M. M. Goldenberg, and A. W. Ford-Hutchinson. 1984. The role of arachidonic acid oxygenation products in pain and inflammation. *Annu Rev Immunol* 2:335-357.

146. Mylonaki, M., N. B. Rayment, D. S. Rampton, B. N. Hudspith, and J. Brostoff. 2005. Molecular characterization of rectal mucosa-associated bacterial flora in inflammatory bowel disease. *Inflamm Bowel Dis* 11:481-487.

147. Fabia, R., A. Ar'Rajab, M. L. Johansson, R. Andersson, R. Willen, B. Jeppsson, G. Molin, and S. Bengmark. 1993. Impairment of bacterial flora in human ulcerative colitis and experimental colitis in the rat. *Digestion* 54:248-255.

148. Sokol, H., P. Seksik, J. P. Furet, O. Firmesse, I. Nion-Larmurier, L. Beaugerie, J. Cosnes, G. Corthier, P. Marteau, and J. Dore. 2009. Low counts of Faecalibacterium prausnitzii in colitis microbiota. *Inflamm Bowel Dis*.

149. Johansson, M. E., M. Phillipson, J. Petersson, A. Velcich, L. Holm, and G. C. Hansson. 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A* 105:15064-15069.

150. Einerhand, A. W., I. B. Renes, M. K. Makkink, M. van der Sluis, H. A. Buller, and J. Dekker. 2002. Role of mucins in inflammatory bowel disease: important lessons from experimental models. *Eur J Gastroenterol Hepatol* 14:757-765.

151. Perminow, G., D. H. Reikvam, L. G. Lyckander, P. Brandtzaeg, M. H. Vatn, and H. S. Carlsen. 2009. Increased number and activation of colonic macrophages in pediatric patients with untreated Crohn's disease. *Inflamm Bowel Dis*.

152. Brandtzaeg, P. 2001. Inflammatory bowel disease: clinics and pathology. Do inflammatory bowel disease and periodontal disease have similar immunopathogeneses? *Acta odontologica Scandinavica* 59:235-243.

153. Smythies, L. E., M. Sellers, R. H. Clements, M. Mosteller-Barnum, G. Meng, W. H. Benjamin, J. M. Orenstein, and P. D. Smith. 2005. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest* 115:66-75.

154. Slots, J. 1986. Bacterial specificity in adult periodontitis. A summary of recent work. *J Clin Periodontol* 13:912-917.

155. Ellner, P. D., C. J. Stoessel, E. Drakeford, and F. Vasi. 1966. A new culture medium for medical bacteriology. *Am J Clin Pathol* 45:502-504.

156. Isenberg, H. D., D. Goldberg, and J. Sampson. 1970. Laboratory studies with a selective Enterococcus medium. *Appl Microbiol* 20:433-436.

157. Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *Journal of molecular biology* 215:403-410.

158. Roy, D., and S. Sirois. 2000. Molecular differentiation of Bifidobacterium species with amplified ribosomal DNA restriction analysis and alignment of short regions of the ldh gene. *FEMS Microbiol Lett* 191:17-24.

159. Ahrné, S., E. Lönnermark, A. E. Wold, N. Åberg, B. Hesselmar, R. Saalman, I. L. Strannegård, G. Molin, and I. Adlerberth. 2005. Lactobacilli in the intestinal microbiota of Swedish infants. *Microbes Infect* 7:1256-1262.

160. Hessle, C., B. Andersson, and A. E. Wold. 2000. Gram-positive bacteria are potent inducers of monocytic interleukin-12 (IL-12) while gram-negative bacteria preferentially stimulate IL-10 production. *Infect Immun* 68:3581-3586.

161. Karlsson, H., C. Hessle, and A. Rudin. 2002. Innate immune responses of human neonatal cells to bacteria from the normal gastrointestinal flora. *Infect Immun* 70:6688-6696.

162. Martin, M., R. E. Schifferle, N. Cuesta, S. N. Vogel, J. Katz, and S. M. Michalek. 2003. Role of the phosphatidylinositol 3 kinase-Akt pathway in the regulation of IL-10 and IL-12 by Porphyromonas gingivalis lipopolysaccharide. *J Immunol* 171:717-725.

163. Fukao, T., M. Tanabe, Y. Terauchi, T. Ota, S. Matsuda, T. Asano, T. Kadowaki, T. Takeuchi, and S. Koyasu. 2002. PI3K-mediated negative feedback regulation of IL-12 production in DCs. *Nat Immunol* 3:875-881.

164. la Sala, A., M. Gadina, and B. L. Kelsall. 2005. G(i)-protein-dependent inhibition of IL-12 production is mediated by activation of the phosphatidylinositol 3-kinase-protein 3 kinase B/Akt pathway and JNK. *J Immunol* 175:2994-2999.

165. Adlerberth, I., B. Carlsson, P. de Man, F. Jalil, S. R. Khan, P. Larsson, L. Mellander, C. Svanborg, A. E. Wold, and L. A. Hanson. 1991. Intestinal colonization with Enterobacteriaceae in Pakistani and Swedish hospital-delivered infants. *Acta Paediatr Scand* 80:602-610.

166. Adlerberth, I., F. Jalil, B. Carlsson, L. Mellander, L. Å. Hanson, P. Larsson, K. Khalil, and A. E. Wold. 1998. High turnover rate of Escherichia coli strains in the intestinal flora of infants in Pakistan. *Epidemiol Infect* 121:587-598.

167. Caballero-Franco, C., K. Keller, C. De Simone, and K. Chadee. 2007. The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. *American journal of physiology* 292:G315-322.

168. Mack, D. R., S. Michail, S. Wei, L. McDougall, and M. A. Hollingsworth. 1999. Probiotics inhibit enteropathogenic E. coli adherence in vitro by inducing intestinal mucin gene expression. *The American journal of physiology* 276:G941-950.

169. Borruel, N., M. Carol, F. Casellas, M. Antolin, F. de Lara, E. Espin, J. Naval, F. Guarner, and J. R. Malagelada. 2002. Increased mucosal tumour necrosis factor alpha production in Crohn's disease can be downregulated ex vivo by probiotic bacteria. *Gut* 51:659-664.

170. Van Der Waaij, D. 1979. The colonisation resistance of the digestive tract in man and animals. *Zentralblatt fur Bacteriologie* (suppl. 7):155-161.

171. Hessle, C. C., B. Andersson, and A. E. Wold. 2005. Gram-positive and Gram-negative bacteria elicit different patterns of pro-inflammatory cytokines in human monocytes. *Cytokine* 30:311-318.

172. Hessle, C., L. Å. Hanson, and A. E. Wold. 2000. Interleukin-10 produced by the innate immune system masks in vitro evidence of acquired T-cell immunity to E. coli. *Scand J Immunol* 52:13-20.

173. Hynes, R. O. 2002. Integrins: bidirectional, allosteric signaling machines. *Cell* 110:673-687.

174. Kahan, A., F. Picard, C. J. Menkes, and B. Amor. 1991. Abnormalities of T lymphocyte subsets in systemic sclerosis demonstrated with anti-CD45RA and anti-CD29 monoclonal antibodies. *Ann Rheum Dis* 50:354-358.

175. Ohta, K., K. Norose, X. C. Wang, S. Ito, and N. Yoshimura. 1997. Abnormal naive and memory T lymphocyte subsets in the peripheral blood of patients with uveitis. *Curr Eye Res* 16:650-655.

176. Saxon, A., F. Shanahan, C. Landers, T. Ganz, and S. Targan. 1990. A distinct subset of antineutrophil cytoplasmic antibodies is associated with inflammatory bowel disease. *J Allergy Clin Immunol* 86:202-210.

177. Wold, A. E., and I. Adlerberth. 2000. Pathological consequences of commensalism. In *Persistent bacterial infections*. J. P. Nataro, M. J. Blazer, and S. Cunningham-Rundles, eds. ASM; oxford: Backwell Science, Washington, D.C. 115-144.

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

179. Mizoguchi, E., A. Mizoguchi, F. I. Preffer, and A. K. Bhan. 2000. Regulatory role of mature B cells in a murine model of inflammatory bowel disease. *Int Immunol* 12:597-605.

180. Romani, L., P. Puccetti, and F. Bistoni. 1997. Interleukin-12 in Infectious Diseases. *Clin Microbiology Reviews* Oct:611-636.

181. Cusumano, V., G. Mancuso, F. Genovese, D. Delfino, C. Beninati, E. Losi, and G. Teti. 1996. Role of gamma interferon in a neonatal mouse model of group B streptococcal disease. *Infect Immun* 64:2941-2944.

182. Rubins, J. B., and C. Pomeroy. 1997. Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia. *Infect Immun* 65:2975-2977.

183. Zhao, Y. X., and A. Tarkowski. 1995. Impact of interferon-gamma receptor deficiency on experimental Staphylococcus aureus septicemia and arthritis. *J Immunol* 155:5736-5742.

184. Shida, K., J. Kiyoshima-Shibata, M. Nagaoka, K. Watanabe, and M. Nanno. 2006. Induction of interleukin-12 by lactobacillus strains having a rigid cell wall resistant to intracellular digestion. *J Dairy Sci* 89:3306-3317.

185. Jones, S. A. 2005. Directing transition from innate to acquired immunity: defining a role for IL-6. *J Immunol* 175:3463-3468.

186. Snijders, A., C. M. Hilkens, T. C. van der Pouw Kraan, M. Engel, L. A. Aarden, and M. L. Kapsenberg. 1996. Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. *J Immunol* 156:1207-1212.

187. Vieira, O. V., R. J. Botelho, L. Rameh, S. M. Brachmann, T. Matsuo, H. W. Davidson, A. Schreiber, J. M. Backer, L. C. Cantley, and S. Grinstein. 2001. Distinct roles of class I and class III phosphatidylinositol 3-kinases in phagosome formation and maturation. *J Cell Biol* 155:19-25.

188. Simonsen, A., R. Lippe, S. Christoforidis, J. M. Gaullier, A. Brech, J. Callaghan, B. H. Toh, C. Murphy, M. Zerial, and H. Stenmark. 1998. EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. *Nature* 394:494-498.

189. Fratti, R. A., J. M. Backer, J. Gruenberg, S. Corvera, and V. Deretic. 2001. Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. *J Cell Biol* 154:631-644.

190. Fukao, T., and S. Koyasu. 2003. PI3K and negative regulation of TLR signaling. *Trends Immunol* 24:358-363.