CROHN'S DISEASE
The Role of the Intestinal Microflora

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Dr. Westergaard is professor of Internal Medicine in the Division of Digestive and Liver Diseases. Dr. Westergaard acknowledges that he has disclosed no financial interests or other relationships with commercial concerns related directly or indirectly to this program. He will not be discussing "off-label" uses in his presentation.

In 1932, Crohn, Ginzburg and Oppenheimer published a paper describing a series of 14 patients with chronic terminal ileal inflammation. (1) All patients underwent ileal resections because of obstruction or fistula formation. Histologic examination showed unspecific acute and chronic inflammation and, in some cases, giant cell and granuloma formation. Tuberculous enteritis, an important differential diagnosis, was ruled out by stains and cultures. The authors believed the condition represented a new disease entity – or a hitherto undescribed disease – and coined the term "regional ileitis." The disease is, of course, now known as Crohn's disease (CD) and affects not only the terminal ileum but most segments of the gastrointestinal tract. Current estimates of the prevalence of Crohn's disease in the U.S. suggest that about ½ million people are affected. An equal number of people suffer from ulcerative colitis (UC), the other disease in the inflammatory bowel disease (IBD) complex. The etiology of these two inflammatory bowel diseases has remained elusive. (2) Over the years, tremendous efforts have been expended in the pursuit of an infectious cause of Crohn's disease. The granulomatous inflammation so typical for Crohn's disease shares similar characteristics with intestinal mycobacterial infection; atypical mycobacterial infection (M. paratuberculosus) as a cause of CD is still a favored hypothesis of some IBD investigators. (3)

Over the past decade, there has been real progress in our understanding of inherited susceptibility, especially to Crohn's disease, from genetic studies in families with Crohn's disease and from studies of the inflammatory response in animal models of intestinal inflammation. (4-9)

Genetics of Crohn's disease

Dr. Kirsner, at the University of Chicago, one of the first clinical investigators of inflammatory bowel disease in the U.S., recognized early on that there was familial clustering of Crohn's disease and ulcerative colitis **(Fig. 1)** . (10) This observation was later extended to studies of identical twins with CD or UC where a high concordance rate (58%) of Crohn's disease was noted. (11) The concordance rate for UC was only 10%. The completion of the Human Genome project and the identification of multiple microsatellite markers spread throughout the genome have spurred the search for potential susceptibility genes for IBD. The common approach has been to use linkage analysis in nuclear families (i.e., several members with IBD) and controls to identify chromosomal loci that are linked to IBD. Hugot was the first to use this approach successfully to link a locus on chromosome 16 to Crohn's disease. (12) The linkage has since been corroborated by several independent studies in both the U.S. and Europe, but not in Japan. (13-15) The locus has been named IBD1. Since then, eight more loci have been identified (IBD2 to IBD9) on chromosomes 1, 3, 5, 6, 10, 12, 14, and 19, respectively **(Table 1)**. The identified loci contain 200-300 genes and only a few of the susceptibility genes within these loci have

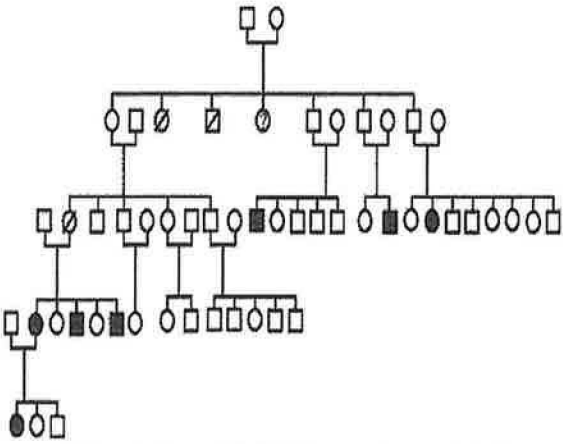

Fig. 1 Familial Clustering of CD

IBD SUSCEPTIBILITY LOCI

LOCUS	CHROMOSOME	GENE
IBDI	16Q12	NOD2 (CD)
IBD2	12P	? UC
IBD3	6P	HLA (IBD)
IBD4	14Q11	? (CD)
IBD5	5Q31	OCTN (CD)
IBD6	19P13	? (IBD)
IBD7	1P36	? (IBD)
IBD8	16P	? (IBD)
IBD9	3p	DLG5

Table 1

been identified. The first gene to be identified was in the IBD1 locus. Two groups independently identified the gene as NOD2/CARD 15 (see later). (16, 17) Homozygosity for mutations of the gene increases the relative risk of developing Crohn's disease 40-fold. Recently, the genes in the IBD5 and IBD9 susceptibility loci have been identified. The gene in the IBD5 locus encodes an organic cation transporter (OCTN1 & 2) which transports long-chain fatty acids and carnitine into mitochondria. (18) The gene in IBD9 called DLG5 encodes a protein involved in cell contacts. (19) DLG5 binds to β-catenin and is expressed in tight junctions in the intestine. It is currently not known how these proteins confer susceptibility for Crohn's disease. With the rapid advances in genomics and with the use of genome-wide SNP maps (SNP = single nucleotide polymorphism), the identification of the genes in the remaining IBD loci is expected within the next few years.

Animal models of IBD

A number of animal models of intestinal inflammation have been developed in the past decade (**Table 1**). They can be divided into four categories: 1) spontaneous development of inflammation; 2) induced by exogenous agents; 3) barrier dysfunction; and 4) disruption of cytokine or T-cell function. The inflammation in most of these models is driven by increased production of IL-12 which activates CD4 cells to produce the pro-inflammatory cytokines IFN-γ and TNF- α and cause mucosal inflammation. **(Table 2).**

- ANIMAL MODELS OF COLITIS

SPONTANEOUS	EXOGENOUS AGENT	BARRIER DYSFUNCTION	IMMUNE DYSFUNCTION
COTTON-TOP TAMARIN	DEXTRAN SODIUM SULPHATE(DSS)	mdr1a $^{-/-}$ MICE	Stat4 transgenic mice
SAMP1/Yit MICE	OXAZOLONE	N-CADHERIN $^{-/-}$ MICE	TNF ^{ARE}
	TRINITROBENZENE SULPHONIC ACID (TNBS)		TCRa $^{-/-}$
			TGFb $^{-/-}$
			IL-10 $^{-/-}$
			HLA-B27 transgenic rats

Table 2

For example, transgenic Stat 4 mice overproduce IL-12 which stimulates IFNγ production and mice mutant for TNF ^{ARE} have increased production of TNF-α. IL-12 production is controlled by IL-10 and TGF-β produced by regulatory T cells (T3) which suppress the inflammatory response. Thus, IL-10 and TGF- β knockout mice develop colitis due to unopposed IL-12 production. The exogenous agents trinitrobenzene sulphonic acid (TNBS) and

oxazolone cause colitis when administered rectally in specific mice strains **(Fig. 2)**.

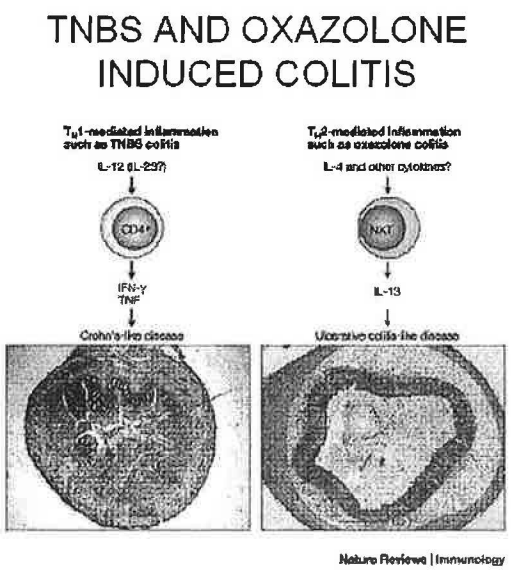

Figure 2

TNBS causes increased IL-12 production and results in a typical T_H1 response with recruitment of CD4 cells producing IFN-α and TNF and transmural inflammation, i.e., a Crohn's-like response. Oxazolone, on the other hand, causes only mucosal inflammation which is driven by NKT cells, producing IL-13. The inflammatory response seen in this model is typical for ulcerative colitis with only mucosal involvement. However, the response in this model is not a classic T_H2 response which is typically driven by IL-4. Finally, the importance of an intact epithelial barrier function is shown by the develop-ment of colitis in mice with disruption of barrier function (mdr1a$^{-/-}$ and N-adhesin$^{-/-}$). The important feature of these animal models is the fact that mucosal inflammation is prevented if these animals are raised in a germ-free environment. Thus, it is the presence of a normal intestinal flora that induces or maintains the inflammatory response. The search for a specific pathogen among the multitude of colonic bacteria has been unrevealing. Thus, antigens released from bacteria in the normal microflora which are tolerated in the normal host, cause mucosal inflammation in animals with a dysregulated immune system or decreased barrier function. The important lessons that have been learned from these models are that the inflammatory response is the result of either an excessive T-cell effector response or an ineffective or absent regulatory response.

The observations from the genetic studies of IBD and from the studies of intestinal inflammation in animal models have led to the current working hypothesis of IBD which states that Crohn's disease and ulcerative colitis result from an excessive immune response to the normal intestinal microflora in genetically susceptible persons.

Intestinal composition of microflora

The normal gastrointestinal flora is often referred to as the commensal flora (~ 'at table together') to indicate that it has no deleterious effects on the host. The gastro-intestinal tract from mouth to the rectum becomes rapidly colonized at birth by a variety of bacteria. (20) After weaning from a milk diet, the colonization at various places in the GI tract remains remarkably constant throughout life despite daily exposure to ingested bacteria. The number of bacteria in the human upper gastrointestinal tract (stomach, jejunum) remains low at $10^2 - 10^3$ bacteria/ml, presumably due to gastric acid

secretion and rapid transit **(Fig. 3)**. The bacteria in this location are mainly aerobic and gram-positive (lactobacilli and streptococci). The bacterial density increases in the distal ileum to 10^8/ml and the flora becomes more diverse with more anaerobic bacteria. The colon is the major site of bacterial colonization probably because of slow transit and low oxidation reduction potentials. The bacterial density in the colon has been estimated to be 10^{12} bacteria /g stool and 99% of the bacteria are obligate anaerobe. The composition of the normal flora has been difficult to characterize because many of the bacteria cannot be cultivated by current techniques. Older estimates suggested that there were at least 400-500 bacterial species in the colonic flora. A new approach to identify bacterial species is the utilization of sequence analysis of bacterial 16S ribosomal RNA (rRNA). (21-24) This new technique has been extremely useful in the identification of new bacterial species, in the generation of phylogenetic trees based on sequence similarity, and to establish that the bacterial domain contains 55 divisions (kingdoms) **(Fig. 4)**.

BACTERIAL DENSITY IN THE GI TRACT

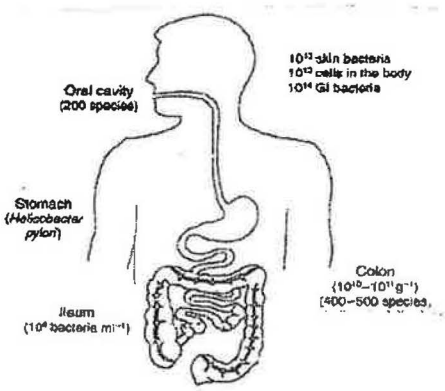

Figure 3

BACTERIAL DIVISIONS

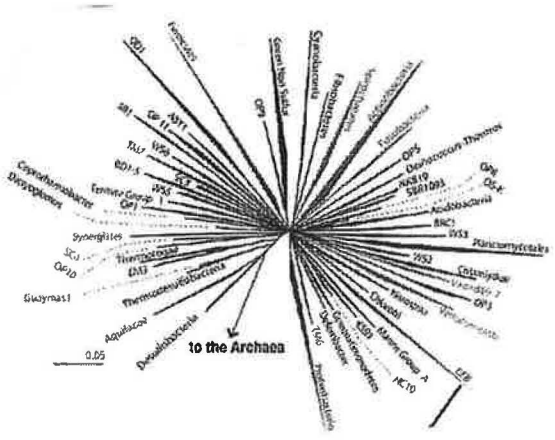

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Figure 4

Surprisingly, only eight of the known divisions are found in the human colonic flora and five of these are uncommon. The three major divisions are the CFB (cytophaga, flavobacterium, bacteroides), the firmicutes (Clostridia, Eubacteria) and proteobacteria. There are currently more than 200,000 rRNA sequences deposited in GenBank and 1822 of these sequences are derived from human intestinal flora. Gordon and coworkers recently provided an estimate of the

species' diversity by performing a relatedness analysis of these 1822 rRNA sequences. A cutoff of 98% sequence identity is commonly used to define species which yielded an estimate of about 800 species. (25)

Effect of microflora on host

The effect of the intestinal microflora on the host has mainly been studied in small animals by comparison of germ-free and conventionally raised animals fed similar diets. The germ-free environment is associated with a number of anatomical and physiological differences compared to animals with a normal microflora. The germ-free animals have thinner intestinal walls, smaller intestinal villi, decreased intestinal motility, decreased cardiac output and intestinal blood flow, and lack of bile acid transformation. (20) Conventionally raised mice have 60% more fat stored in adipocytes than germ-free mice although there is no weight difference between the two groups. Gordon's group at Washington University has shown in an elegant series of studies that conventionally raised mice have increased intestinal monosaccharide absorption, increased hepatic triglyceride synthesis and increased lipoprotein lipase activity compared to germ-free mice on the same high carbohydrate diet. (26)

Hooper et al used a novel technique to study the effect of a simplified microflora on gene expression in the ileum of germ-free mice colonized with a single bacterium, Bacteroides thetaiotaomicron, a common member of the intestinal microflora in both mice and man. (27) Isolated mRNA levels detected with microarrays representing 25,000 mouse genes were compared between groups of germ-free and colonized mice. Significant changes (>2-fold) were observed in 118 probe sets in the colonized mice with an increase in 95 and a decrease in 23. The cellular origin of some of these changes was further defined by isolating cells from villus tips, crypts, and lamina propria, and the fold changes in expression of mRNA were measured by quantitative PCR **(Table 3)**.

Colonization with B. thetaiotaomicron caused an increase in genes involved in nutrient digestion and absorption (SGLT-1, colipase, L-FABP), and in barrier function (sprr2a, DAF) and a decrease in glutathione S-

REGULATION OF ILEAL GENES BY B.THETAIOTAOMICRON

Gene	Fold Δ (relative to germ-free)
Na^+/glucose cotransporter (SGLT-1)	2.6 ± 0.9
Colipase	6.6 ± 1.9
Liver fatty acid–binding protein (L-FABP)	4.4 ± 1.4
Metallothionein I (MT-I)	−5.4 ± 0.7
Polymeric immunoglobulin receptor (pIgR)	2.6 ± 0.7
Decay-accelerating factor (DAF)	5.7 ± 1.5
Small proline-rich protein 2a (sprr2a)	205 ± 64
Glutathione S-transferase (GST)	−2.1 ± 0.1
Multidrug resistance protein (Mdr1a)	−3.8 ± 1.0
Lactase-phlorizin hydrolase	−4.1 ± 0.6
Adenosine deaminase (ADA)	2.6 ± 0.5
Angiogenin-3	9.1 ± 1.8

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transferase and multi-drug resistance protein (Mdr1a). There was no evidence of mucosal inflammation in the colonized mice although there was an increase in the number of IgA-secreting plasma cells compared to germ-free mice. Thus, the introduction of a single bacterium into the GI tract of germ-free mice has a profound effect on gene expression in the ileum.

Intestinal microflora and the immune system

Germ-free animals have a poorly developed intestinal immune system with few and poorly organized Peyer's patches, rare IgA-secreting plasma cells in the lamina propria and small mesenteric lymph nodes and spleens compared to animals with a normal microflora. These differences illustrate that the development of the intestinal immune system is governed by signals from the microflora. (28-31) In the past, the difference between commensal bacteria and pathogens was defined by the ability to disrupt and cross the mucosal barrier. Pathogens have virulence factors (adhesins, invasins) that facilitate tissue invasion, whereas commensals lack these factors and are thought to remain in the intestinal lumen or mucus layer.

The development of the immune system in conventionally raised animals and by extension in man illustrates that there is ongoing cross-talk between commensals in the intestinal lumen and the immune system. Commensal bacteria may gain access to immune cells in the lamina propria through several different routes. The main entryway is thought to be M-cells, specialized epithelial cells located over Peyer's patches and follicle-associated epithelium (FAE) in the intestinal mucosa. (32) M-cells lack microvilli and take up bacteria by pinocytosis and present them to dendritic cells and macrophages in the subepithelial dome. In addition, commensal bacteria may enter the lamina propria through breaches in the mucosal barrier when cells are shed through the normal turnover of epithelial cells. Finally, it has recently been shown that a subset of dendritic cells that expresses the practalkine receptor (CX_3CR1) have the capability to extend dendrites into the intestinal lumen. (33) The ligand for the receptor is mainly expressed on the basolateral surface of enterocytes in the terminal ileum. It was further found that both commensal and pathogenic bacteria administered orally were taken up by this population of dendritic cells. Thus, there is continuous sampling of antigens from the commensal flora by M-cells and dendritic cells which serve as a surveillance system for the innate immune response. Antigens taken up by dendritic cells in Peyer's patches or lamina propria are processed for presentation to B- and T-cells with subsequent activation of the adaptive immune system. Despite continuous exposure to luminal antigens, there are no obvious signs of mucosal inflammation in normal mice or man. One of the reasons for the lack of inflammation is that a proinflammatory response such as the IL-12-driven T_H1 response is suppressed by production of the regulatory cytokines TGFβ and IL-10, which is illustrated in two recent studies. The first study used macrophages isolated from human jejunum and showed that macrophages did not express innate immune system receptors and did not produce proinflammatory cytokines (IL-1, IL-6, IL-12, TNF) in response to bacteria or LPS as opposed to blood monocytes from which macrophages are derived **(Fig. 5)**. (34)

The macrophages, however, maintained avid phagocytic and bacteriocidal activity. The down regulation of cytokine production was shown to be due to TGFβ secretion from mast cells and intestinal epithelial cells. In the second study, it was shown that isolated dendritic cells from human colons were conditioned by intestinal epithelial cells to produce IL-6 and IL-10 but not IL-12, which promoted a non-inflammatory T_H2 response. (35) The factor secreted by epithelial cells was shown to be a newly-described chemokine, thymic stromal lymphopoietin (TSLP). Blockage of TSLP secretion reversed cytokine production in favor of IL-12 and a T_H1 inflammatory response. Hence, it is apparent that a complex control system has evolved to modify and regulate cytokine production and to prevent a proinflammatory response to commensal bacteria.

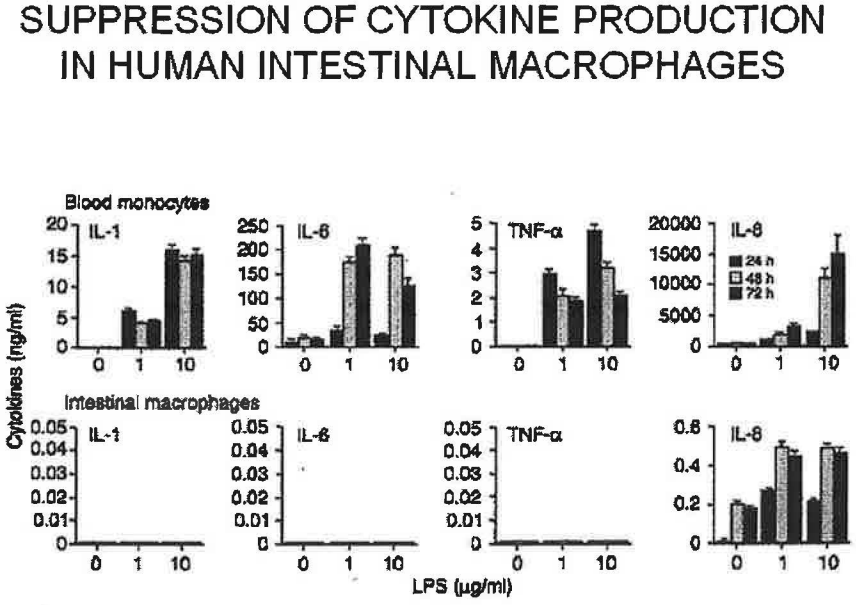


Figure 5

Intestinal defense against bacteria

a. ***Mucus production.*** There is constant mucus production and secretion from goblet cells in the small and large intestine which generate a layer of mucus on the mucosal surface. The mucus layer is thought to function as a protective shield of the epithelial cells against intraluminal bacteria and antigens, but it is difficult to assess the quantitative importance of mucus secretion in the defense against bacterial invasion. The mucus layer is absent over the M-cells which allows for unrestricted sampling of bacteria. Two other defense mechanisms have evolved to restrict bacterial entry into the mucosa: IgA secretion and secretion of bacteriocidal peptides.

b. ***IgA secretion.*** More than 80% of all plasma cells reside in the intestinal lamina propria where they secrete 3-5 grams IgA on a daily basis. (36) IgA is transported across the epithelial cells via the polymeric immunoglobulin receptor after association with the J-chain and the secretory component. IgA is transported into the intestinal lumen as dimers or polymers and attach to commensal bacteria which prevent entry into the host.

IgA B-cells originate in the germinal centers in Peyer's patches and isolated lymphoid follicles where B-cells, T-cells and dendritic cells reside. M-cells transport

commensal bacteria from the intestinal lumen to the germinal centers. The bacteria are taken up by macrophages where they are rapidly killed and by dendritic cells where they can survive up to 60 hours. Dendritic cells present commensal bacteria antigens to activated T-cells ($CD4_)$ which in turn stimulate IgM B-cells to class-switch to IgA production and develop antigenic specificity by somatic hypermutation (SHM) and, finally, to differentiate into IgA-secreting plasma cells and become part of the adaptive immune defense. The final step in B-cell maturation and differentiation takes place while the cells are in transit from germinal centers to mesenteric lymph nodes from which they return via the blood to the lamina propria. There is also a T-cell independent pathway for IgA production as the secretory IgA response measured in intestinal washings is almost identical in normal mice and T-cell-deficient mice. (37) This pathway is presumed to be confined to the lamina propria outside of Peyer's patches. Dendritic cells activated by lipopolysaccharide (LPS) have been shown to induce IgM B- cells to class-switch to IgA B-cells *in vitro,* independent of T-cells. The induction was stimulated by transforming growth factor (TGFβ) produced by lamina propria stromal cells. Secretory IgA in intestinal secretions has antibody specificity to antigens in bacterial cell walls of commensal bacteria. For example, studies in conventionally raised mice showed specific secretory IgA in intestinal washings against a common commensal in their flora (E. cloaca) but no detectable serum IgA or IgG against this bacterium. (38) Intestinal washings from germ-free mice had no detectable IgA binding to E. cloaca. Thus, the presence of commensal bacteria induces the generation of IgA secreting plasma cells in the lamina propria with antibody specificity to bacterial cell wall components which confines entry of commensal bacteria to the mucosal compartment and prevents a systemic immune response. (39)

It has been difficult to assess the relative importance of secretory IgA in the overall defense against the intestinal microflora. IgA deficiency is a common immune deficiency with a benign phenotype. Patients with IgA deficiency compensate by producing secretory IgM and do not suffer from increased susceptibility to intestinal infections. Recently, it was observed that mice with a deficiency of activation-induced cytidine deaminase (AID) develop massive expansion of lymphoid follicles in the small intestine and a 100-fold increase in anaerobe bacteria in the small intestine. (40) AID activity is required for class-switch recombination and somatic hypermutation. Consequently, these mice have massive accumulation of IgM plasma cells in the lymphoid follicles. The increase in anaerobe bacteria in the small intestine is presumably due to the inability to generate secretory IgM with high antibody specificity. Treatment with antibiotics eradicated the anaerobe flora and led to regression or disappearance of the lymphoid follicles. AID deficiency in man causes the hyper-IgM syndrome which is characterized by very low serum levels of IgA and IgG with normal or increased IgM, recurrent respiratory and gastrointestinal infections, and nodular lymphoid hyperplasia. (41) Thus, secretory IgA in intestinal secretions appears to protect against intestinal infections, and to control homeostasis of the commensal flora.

c. ***Secretion of antimicrobial peptides.*** Secretion of antimicrobial peptides by intestinal epithelial cells is part of the innate immune system and contributes to defense against luminal bacteria. Antimicrobial peptides are defined as short peptides (~ 30-40 amino acids) with bacteriocidal activity. One of the main families of antimicrobial peptides in animals and man is the defensin family. (42-46) The mammalian defensins

are classified as α- and β-defensins based on differences in disulfide bonds. α-defensins are stored in the granules of neutrophils (HNP1-4) and in the secretory granules of Paneth cells in the small intestine (HD5&6). β-defensins are secreted by epithelial cells in both the small and large intestine (HBD1&2). α- and β-defensins are synthesized as prepropeptides and after two cleavage steps, stored in granules as mature peptides. The secreted peptides are cationic and amphipathic which confers affinity for anionic phospholipids in bacterial membranes. Paneth cells are located at the base of intestinal crypts and are found only in the small intestine with a higher density in the distal small intestine. Paneth cells also secrete lysozyme and phospholipase A2, both of which have antimicrobial activity by their ability to degrade bacterial membranes. α-defensins have antimicrobial activity against E. coli, L. monocytogenes, S. typhimurium, S. aureus and Candida albicans. β-defensins are mainly active against gram-negative bacteria. The bacteriocidal activity is observed at low defensin concentrations (1-10 ug/ml). The mechanism of action of defensins is thought to be pore formation in bacterial membranes resulting in increased permeability and dissolution of the electrochemical potential. Recently, Hooper et al showed that Paneth cells in mice secrete yet another antimicrobial peptide, Ang4, which is a member of the angiogenin family known for its role in stimulating blood vessel formation. (47) Ang4 expression is restricted to the intestine and localizes to the secretory granules in Paneth cells. Ang4 has antimicrobial activity against gram-positive bacteria (E. faecalis, L. monocytogenes), whereas E. coli was resistant. Isolated crypts from mouse small intestine release α-defensins, Ang4, lysozyme and phospholipase A2 *in vitro* in response to incubation with bacteria or LPS. The synthesis of defensins is thought to be constitutive whereas the expression of Ang4 mRNA in mice increases during the weaning period as the normal microflora becomes established.

It has been difficult to demonstrate the importance of defensins in the intestinal immune defense. Temporary ablation of the Paneth cell population in a transgenic mouse model had no apparent effect on the mice or the commensal flora. In mice, the cleavage of the prepropeptides to mature defensins depends on the enzymatic activity of matrilysin. Knockout mice with disruption of the matrilysin gene are more susceptible to infection with Salmonella typhimurium and they had higher bacterial counts in the lower small intestine than wild-type mice. Conversely, mice transgenic for human defensin 5 (HD5) are more resistant to infection with S. typhimurium as they survived an oral dose that killed all the wild-type mice. (48) Intraperitoneal inoculation of S. typhimurium was equally lethal in both groups of mice indicating that intestinal secretion of HD5 provides protection against infection. The question whether defensins and angiogenin control the density or composition of the commensal flora in different compartments of the gastrointestinal tract still remains to be elucidated.

Pattern recognition receptors (PRRs)

The innate immune system senses the entry of commensal and pathogenic bacteria across the mucosa by a conserved primitive recognition system that detects so-called pathogen-associated molecular patterns (PAMPs) that are expressed in bacterial cell walls such as peptidoglycan , LPS and lipotheicoic acid. The molecular patterns are shared by commensal and pathogenic bacteria. The main pattern recognition receptors are the toll-like receptors (TLRs) and the NODs (nucleotide oligomerization domain).

a. ***Toll-like receptors (TLRs).*** The TLR family now includes 11 members that recognize different molecular motifs in bacteria and viruses **(Table 4)**. (49-51)

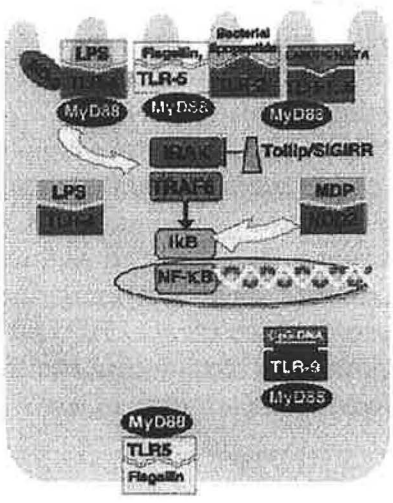
The ligands and origin of ligands for some of these receptors are shown in **Table 4**. Most of the TLRs are expressed in the cell membrane in macrophages, dendritic cells and also in intestinal epithelial cells. The intracellular domain of the TLRs is similar to the intracellular domain of the IL-1β. The extracellular domain contains multiple copies of leucine-rich repeats and is responsible for ligand recognition and binding.

TOLL-LIKE RECEPTORS

RECEPTOR	LIGAND	ORIGIN
TLR1	Triacyl lipopeptides	Bacteria
TLR2	Peptidoglycan Lipotheicoic acid	Gram pos bacteria
TLR3	Double stranded RNA	Viruses
TLR4	LPS	Gram neg bacteria
TLR5	Flagellin	Bacteria

Ligand binding elicits a complicated cascade of intracellular molecular interactions that involve several adaptor molecules **(Fig. 6).**

ACTIVATION OF TOLL-LIKE RECEPTORS



MyD88 is an essential adaptor molecule that recruits IRAK1 and 4 to the receptor complex and subsequent association with TRAF6 and TAK1 and, finally, phosphorylation of IκB and release of NF-kB, which translocates to the nucleus and induces transcription of proinflammatory cytokine and chemokine genes. The expression of TLR2 and TLR4 by intestinal epithelial cells is low despite constant exposure to commensal bacteria, but upregulated in chronic inflammation. TLRs signaling cascade can be blocked by intracellular molecules such as TolliP that associates with IRAK1 and prevents its phosphorylation and subsequent activation of NF-κB. Other negative regulators of TLRS signaling include IRAK-M, SOCS1, MyD88s and SIGiRR which interfere with various steps in the signaling cascade and presumably function to downregulate the inflammatory response. The role of TLR activation by commensal bacteria was recently investigated by inducing colitis with dextran sulfate (DSS) in four groups of mice: MyD88-deficient, TLR2-deficient, TLR4-deficient and wild-type. (52) The a priori hypothesis was that MyD88-deficient mice would be less susceptible to inflammation due to lack of TLR signaling **(Fig. 7)**.

However, MyD88-deficient mice showed severe morbidity and mortality as did TLR2- and TLR4-deficient mice, while all wild-type mice survived. Thus, signaling by TLRs not only activates the immune system but also induces protection of the epithelial barrier. It was further shown that MyD88-deficient mice failed to induce cytoprotective factors (heat shock proteins 25 and 72) and factors involved in epithelial repair (IL-6, TNF), whereas these factors increased significantly in wild-type mice after induction of colitis by DSS. Finally, wild-type mice treated for four weeks with broad-spectrum antibiotics to eliminate the commensal flora showed severe morbidity and mortality to DSS-induced colitis. The commensal-depleted mice did not express heat shock proteins 25 or 72. Thus, ligands from commensal bacteria continuously activate TLRs and upregulate cytoprotective factors in case of epithelial injury.

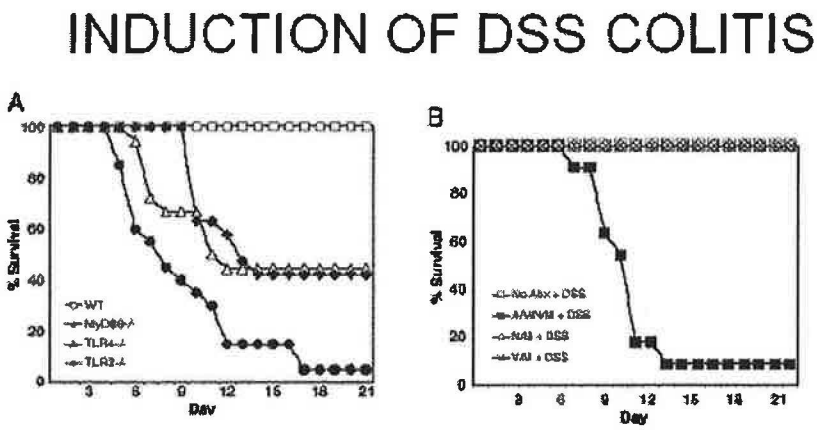
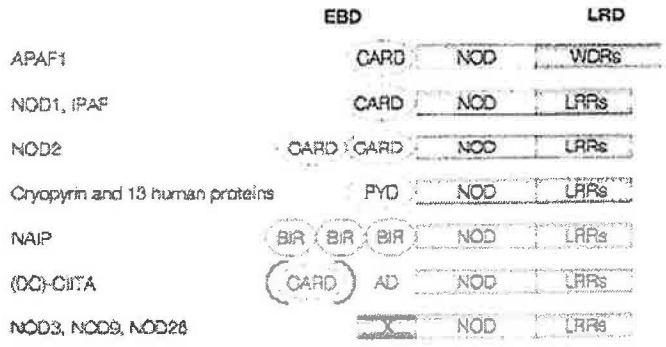


Figure 7

b. ***NOD proteins.*** The nucleotide binding oligomerization domain (NOD) proteins are members of a family of intracellular pattern recognition receptors (PRRs) which recognize distinct bacterial cell wall motifs and induce an inflammatory and apoptotic response. The NOD proteins serve as a detection system of intracellular infection and are part of the innate immune system. NOD homologs are found in plants, bacteria, fungi and animals and there are now more than 20 human homologs identified. (53)

The NOD proteins contain three functional domains: an effector binding domain (EBD), a central NOD domain, and a ligand recognition domain (LRD) **(Fig. 8).**



The NOD mediates oligomerization to dimers that is required for downstream activation of the effector domain molecules. The EBD of NOD1 and NOD2 contains one and two CARDs (Caspase Recruitment Domain) which belong to the death-domain fold family. The LRD of the NOD proteins contain leucine-rich repeats (LRRs) similar to the toll-like receptors. The ligands for

NOD1 and NOD2 are degradation products of bacterial cell walls. It was initially thought that peptidoglycan, a cell wall component of both gram-positive and gram-negative bacteria, was the ligand for NOD2. (54) It has now been convincingly shown by two independent groups that muramyl dipeptide (MDP), the minimal structural component of peptidoglycan, is the ligand for NOD2 signaling. (55, 56) MDP is composed of N-acetylmuramic acid, L-alanine and D-glutamic acid. Signaling by the NOD proteins after ligand recognition proceeds by NOD oligomerization which activates the CARD domain which, in turn, recruits another CARD-containing interacting molecule. NOD1 and NOD2 interact with RICK, a kinase, which then phosphorylates IKK (inhibitor of NF-kB) and NF-kB translocates to the nucleus and induces transcription of proinflammatory genes (TNF, IL-1β). Cells transfected with NOD2 and incubated with MDP showed a 15-fold activation of NF-κB. Replacement of L-alanine with D-alanine in MDP eliminated a NF-κB response indicating that ligand recognition is stereoselective. MDP is not recognized by NOD1 or TLRs. Thus, the NOD proteins can sense intracellular infections by gram-positive and gram-negative bacteria and mount an inflammatory response by NF-κB release or an apoptotic response by activation of caspases.

NOD2 mutations

In 1996, Hugot et al identified a susceptibility locus for Crohn's disease in the pericentromeric region of chromosome 16 by linkage study of nuclear families with Crohn's disease. (12) The locus was called IBD1 and the linkage was confirmed by several independent studies in other countries. (57-59) In 2001, two independent groups identified NOD2 as the gene within the IBD1 locus that confers susceptibility to Crohn's disease. (16, 17) The two groups used different approaches to identify the gene. Hugot's group used first microsatellite markers and then SNPs to narrow down the gene location while Ogura's group assumed that NOD2 might be the susceptibility gene as it had been mapped to microsatellite markers that overlapped the IBD locus. Both groups sequenced NOD2 and identified three mutations that were associated with Crohn's disease (a frameshift mutation 3020insC and two missense mutations). Both groups further showed that the mutated NOD2 failed to respond to MDP in an NF-kB expression system, i.e. loss of function mutations, and that the mutations were found in or near the LRR domain. It is estimated that NOD mutation confers about 18% of the genetic risk for Crohn's disease. Heterozygosity for the common NOD2 mutation increases the risk for disease 3 – 4-fold but homozygosity increases the risk 30 – 40-fold. The intestinal inflammation in CD is characterized by a high expression of NF-kB which is in contrast to experimental findings of lack of NF-kB activation in cells with NOD2 mutations. This discrepancy has been under intense scrutiny and, recently, two groups using slightly different models of NOD2 mutations reached opposite conclusions as to why NOD2 mutations are associated with CD. One group used mice where the NOD2 locus was replaced by the mutated allele 3020insC. (60) Mice homozygous for the 3020insC mutation developed normally and remained healthy. However, mice with the mutated allele were more susceptible to induced intestinal inflammation by dextran sulfate (DSS) than wild-type mice (more weight loss and higher mortality). Macrophages from these mice had higher NF-kB activation and significantly higher IL-1β secretion upon stimulation by MDP compared to macrophages from wild-type mice. The authors concluded that the 3020insC mutation in NOD2 is a gain of function mutation. The other group generated mice where NOD2

was ablated ($NOD2^{-/-}$).(61) These mice also remained healthy without evidence of intestinal inflammation. Furthermore, they did not have increased susceptibility to DSS-induced colitis. Isolated macrophages from NOD2 mice failed to respond to MDP, whereas macrophages from wild-type mice showed activation of NF-kB and secretion of IL-6 and TNF. There was no difference in survival when wild-type and NOD2 mice were challenged with Listeria monocytogenes intravenously or intraperitoneally. However, intragastric challenge with L. monocytogenes caused more morbidity and higher recovery of bacteria from spleen and liver in NOD2 than in wild-type mice. Expression of NOD2 has been shown to be high in Paneth cells in the intestinal crypts which was confirmed in this study. It was further shown that the expression of defensins was significantly lower in NOD2 mice compared to wild-type, and decreased upon infection with L. monocytogenes. Thus, lack of NOD2 function in Paneth cells leads to decreased defensin expression and presumably decreased secretion and renders these mice more susceptible to bacterial infections. The absence of NOD2, therefore, represents a "loss of function" mutation in this study. It is of interest that patients with CD and NOD2 mutations primarily have ileal disease where Paneth cells have the highest density. (62) Furthermore, it has recently been shown that expression of α-defensins is reduced in ileal tissue in patients with NOD2-associated CD. (63) NOD2 mutations are found in up to 50% of central Europeans with CD, but are absent in Japanese or African-American patients. The frequency of NOD2 mutations in healthy individuals has been estimated to be 1%. Thus, the NOD2 mutations are the first identified genetic risk factors of CD and functional studies are beginning to unravel a defect in the innate immune system. There is another mutation in NOD2 which is not associated with CD. This mutation causes the Blau syndrome (arthritis, uveitis and skin rash). (53) The mutation is in the dimerization domain of the molecule and causes NF-κB activation. NOD2 mutations are, of course, just one of the risk factors of CD and identification of genes within the other susceptibility loci (IBD2-IBD9) is much anticipated. Knowledge of the function of these genes may ultimately lead to development of new treatments.

New Treatments of CD

The mucosal inflammation in CD is thought to proceed as illustrated in **Fig. 9**.

POINTS OF INTERVENTION IN THE INFLAMMATORY CASCADE

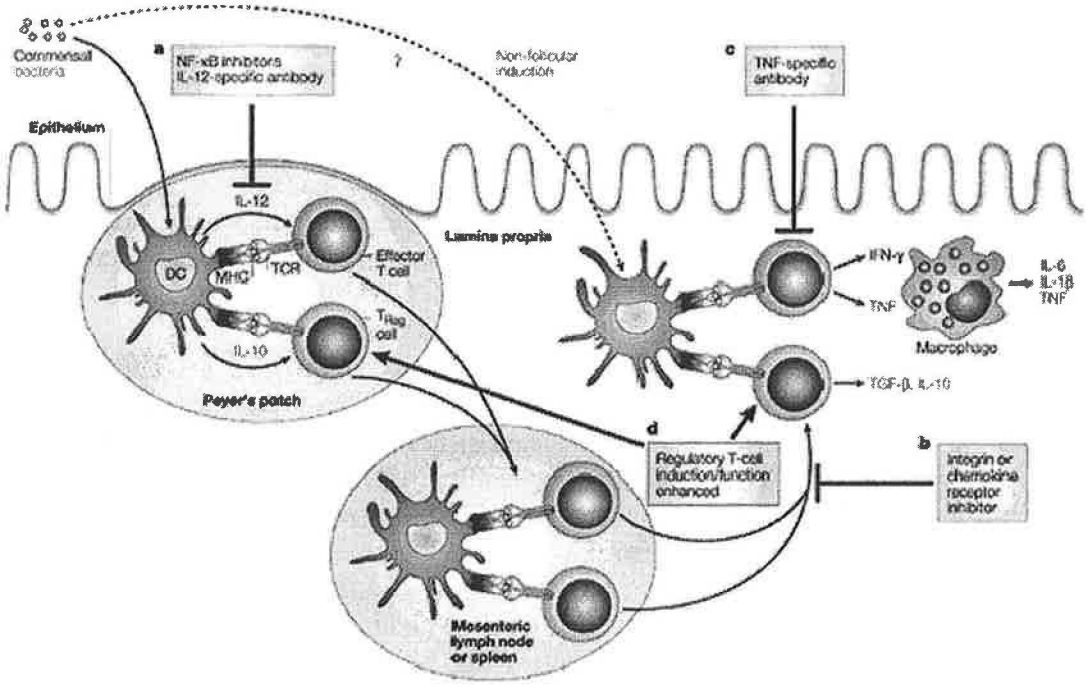


Figure 9

Commensal bacteria or degradation products are taken up by dendritic cells in the Peyer's patches in the lamina propria and recognition by TLRs or NODs activates the innate immune system by release of NF-κB and production of proinflammatory cytokines with a T_H1 profile (IL-12). (64) Dendritic cells also present bacterial antigens via the MHC complex and costimulatory molecules for engagement with T-cell receptors (TCR) on naïve CD4 cells, to initiate a response of the adaptive immune system. The cells are transported to the mesenteric lymph nodes, become activated, express $\alpha4\beta7$ integrin, and return via the blood to the lamina propria through interaction with a specific integrin receptor (MADCAM1). Activated CD4 T_H1 cells produce IFNγ and TNF, which stimulate macrophage cytokine production (IL-1β, IL-6, TNF). According to this scheme, there are several points in the inflammatory cascade where therapeutic interventions may be attempted.

Infliximab, an anti-TNF antibody, was introduced as a treatment for therapy-resistant CD in 1999 and has proven to be a valuable addition to the limited number of treatment modalities for CD. Infliximab binds TNF and, more importantly, induces apoptosis of T_H1 effector cells. However, about 40% of CD patients fail to respond to infliximab which underscores a need for new treatments.

Integrin antibodies

Antibodies to α4β7 integrin prevent lymphocytes to home back to lamina propria and have been shown to improve colitis in an animal model (cotton-top tamarin). The effect of natalizumab, an α4 antibody, has recently been examined in a study of 248 patients with moderate to severe CD in a multi-center study in Europe. (65) Patients were divided into four groups to receive two infusions of natalizumab or placebo at 0 and 4 weeks, and followed to week 12. Natalizumab was given at either 3 or 6 mg per kg body weight. One group received 3 mg/kg of natalizumab at week 0 and placebo at week 4. The response rate was measured with the Crohn's Disease Activity Index (CDAI). Clinical remission was defined as a score of less than 150 and a clinical response as a decline in CDAI score by at least 70 points. At the end of the study, at week 12, the rate of remission in the groups given natalizumab was not significantly different from the placebo group. The rate of clinical response in the two groups that received two infusions of natalizumab (3 mg and 6 mg/kg, respectively), however, was statistically significant at both 4 and 12 weeks when compared to placebo **(Table 5)**.

NATALIZUMAB STUDY

	PLACEBO	1 INFUSION 3 MG/KG	2 INFUSIONS 3 MG/KG	2 INFUSIONS 6 MG/KG
n	63	68	66	51
Remission week 4	14%	31%*	29%*	29%*
Remission week 12	27%	28%	42%*	39%
Response week4	29%	47%*	62%*	53%*
Response week 12	43%	50%	61%*	65%*

* $p<0.05$

Table 5

The clinical response is in the same range as that seen in treatment with infliximab. The number of patients who had adverse effects were similar in the four groups. Natalizumab has also been shown to have a significant effect in the treatment of patients with multiple sclerosis. However, natalizumab was withdrawn from the market in February 2005 after three patients (2 MS, 1 CD) developed progressive multifocal leukoencephalopathy (PML).

Anti-interleukin-12 antibody

IL-12 has a pivotal role in the induction of the T_H1 response in CD inflammation. (66)

Inhibition of IL-12 by an anti-IL-12 antibody in animal models of colitis reduces or resolves the inflammation. A fully humanized anti-IL-12 antibody has now been developed and the first results of a safety and dose-finding study in patients with active CD were recently published. (67) The primary endpoint of the study was safety and the secondary endpoints were rates of remission (CDAI < 150), and clinical response (decrease in CDAI by at least 100 points). The study only enrolled 79 patients who were divided into two cohorts to receive seven weekly subcutaneous injections of 1 mg or 3 mg/kg of the anti-IL-12 antibody or placebo. In cohort 1, there was a 4-week interval between the first and second injection while cohort 2 received the injections for seven consecutive weeks. The patients were followed for 18 weeks. The main adverse effect of the anti-IL-12 antibody was a local reaction at the injection site. There were nine cases of serious adverse effects which could not be attributed to the antibody. The clinical response and remission rates in cohort 1 were not statistically different between treatment groups and placebo. In cohort 2, the clinical response rate was significantly higher in the group receiving 3 mg/kg compared to 1 mg/kg and placebo, and was maintained throughout the study **(Fig. 10)**.

EFFECT OF ANTI-INTERLEUKIN 12 ANTIBODY IN ACTIVE CD

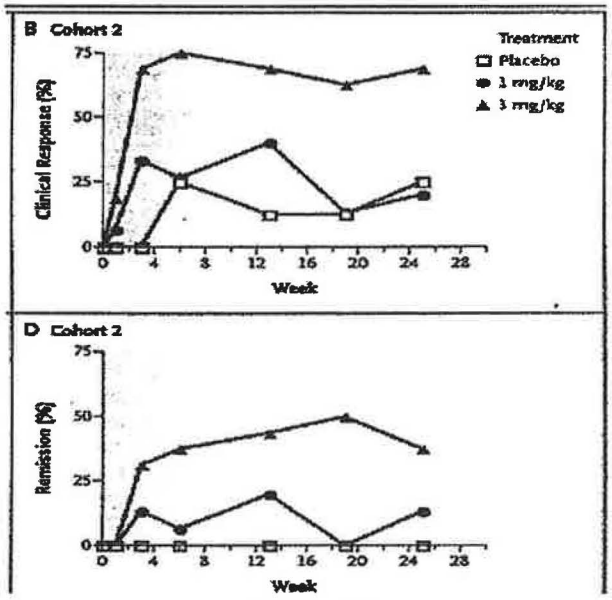


Figure 10

The clinical remission rates were not significantly different among the three groups in cohort 2. Thus, short-term administration of anti-IL-12 antibodies appears to be safe and, at 3 mg/kg, induces a substantial clinical response.

Treatments on the Horizon

Our knowledge of the complex interplay of cytokines and chemokines in intestinal inflammation is rapidly expanding and has led to development of new treatments which are currently in phase I or II clinical trials in CD. Some of the

compounds that are being evaluated are semapimod, a mitogen-activated protein kinase inhibitor which blocks production of TNF, IL-1 and IL-6, recombinant human IL-11 and an antagonist to chemokine receptor 9. These compounds interfere with proinflammatory cytokine signaling. Other agents that are being investigated for possible therapeutic effect in CD are omega-3 fatty acids and glucagon-like peptide 2.

Ultimately, only controlled clinical trials will prove whether any of these new agents will be of clinical benefit in the treatment of Crohn's disease. There was much hope that IL-10, which is effective in the treatment of several animal models of colitis, would also prove to be effective in active CD, but a large clinical trial failed to show a significant benefit. (68) Finally, a word of caution is warranted as the experience with natalizumab has shown that the long-term effect of interference with cytokine signaling is unknown.

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