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Mechanisms of bacterial-epithelial interaction in Crohn's disease

Ida Schoultz

Linköping University
FACULTY OF HEALTH SCIENCES

Division of Surgery
Department of Clinical and Experimental Medicine
Faculty of Health Sciences, SE-581 83 Linköping
Sweden

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Yes, there were times, I'm sure you knew.
When I bit off more than I could chew,
But through it all, when there was doubt,
I ate it up and spit it out.
I faced it all and I stood tall
And did it my way.

Till pappa och mamma
för att ni ständigt uppmuntrar
mig att se livet ur olika perspektiv

ABSTRACT

Crohn's disease (CD) is believed to be initiated when an individual, who has a genetic predisposition either leading to a disturbance in the barrier function and/or the innate immune system is exposed to triggering environmental factors, the most important being intraluminal bacteria. Genetic and functional studies have confirmed the Pattern-recognition receptors (PRRs), Nod2, TLR4 and NALP3, as important mediators of the inflammatory process associated with disease progression. However, the mechanisms that link enteric bacteria and barrier function in a background of genetic predisposition to CD are just beginning to emerge. The general aim of this thesis was therefore to more thoroughly investigate the mechanisms of bacterial-epithelial interaction in CD.

Here we present evidence suggesting that the small bowel is able to induce transcytosis of antigens after short term exposure to *Yersinia pseudotuberculosis.* This suggests that small bowel enterocytes are able to attain follicle associated epithelial (FAE) abilities and contribute to the barrier dysfunction observed in CD. Furthermore we report a positive effect of anti-TNFα treatment (infliximab) on the translocation of adherent invasive *E.coli* (AIEC) across the colonic mucosa of patients suffering from severe CD.

We also confirm the importance of the Nod-like receptors (NLRs) in the pathogenesis of CD by showing that combined polymorphisms in the genes encoding NALP3 and CARD8 confer susceptibility to CD among Swedish men and in addition to previous published results add a gender aspect on the genotype-phenotype relationship in CD. Finally, we show that Nod2 is rapidly subjected to ubiquitination followed by proteasomal degradation, hence providing important clues about how NLR regulation might occur in the cell, suggesting that the ubiquitin-proteasome pathway is an important factor to consider in the development of the disease.

In conclusion we report novel insights into the bacterial-epithelial interactions occurring in CD and contribute important clues about the origin of this disease.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to by their Roman numerals as follows;

I. ***Yersinia pseudotuberculosis* induces transcytosis of nanoparticles across human intestinal villus epithelium via invasin-dependent macropinocytosis**
Eva GE Ragnarsson*, Ida Schoultz*, Elisabet Gullberg, Anders Carlsson, Farideh Tafazoli, Maria Lerm, Karl-Eric Magnusson, Johan D Söderholm and Per Artursson. *Lab. Invest. 2008*; 88:1215-26

II. **Infliximab reduces bacterial uptake in mucosal biopsies of Crohn's colitis via microtubule-dependent pathway**
Ida Schoultz, Anders Carlsson, Elisabet Gullberg, Sven Almer, Magnus Ström, Maria Lerm, Derek M McKay, Jonathan M Rhodes and Johan D Söderholm. *In manuscript 2009*

III. **Combined polymorphisms in genes encoding the inflammasome components NALP3 and CARD8 confer susceptibility to Crohn´s Disease in Swedish men**
Ida Schoultz*, Deepti Verma*, Jonas Halfvarsson, Leif Törkvist, Mats Fredrikson, Urban Sjöqvist, Mikael Lördal, Curt Tysk, Maria Lerm[6], Peter Söderkvist and Johan D Söderholm. Accepted for publication in *Am J Gastro 2009.*

IV. **Ubiquitination and degradation of the Crohn's Disease associated protein Nod2 involves the E2 enzyme UBE2G2**
Ida Schoultz, Thomas Kufer, Tieshan Jiang, Narveen Jandu, Peter Söderkvist, Maria Lerm, and Johan D Söderholm. *In manuscript 2009*

*These authors contributed equally to these papers

ABBREVIATIONS

AIEC	Adherent invasive *E.coli*
ASC	Apoptosis speck like protein
CIITA	Class II transactivator
CARD	Caspase recruitment domain
CD	Crohn's Disease
CEACAM	Carcinoembryonic antigenrelated cell adhesion molecule
^{51}Cr-EDTA	^{51}Chromium-EDTA
DC	Dendritic Cell
EBBP	Estrogen-responsive B box protein
FAE	Follicle associated epithelium
IBD	Inflammatory bowel disease
IFNγ	Interferon gamma
IL	Interleukin
Isc	Short circuit current
NFκB	Nuclear factor-kappa B
NLR	Nod-like receptor
MAMP	Microbe associated molecular patterns
MDP	Muramyldipeptide
MLCK	Myosin light chain kinase
PD	Potential difference
PGN	Peptidoglycan
PRR	Pattern recognition receptor
TACE	TNF-alpha converting enzyme
TER	Transepithelial resistance
TJ	Tight junction
TLR	Toll-like receptor
TNFα	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
SLP	Surfactant-like protein
SNP	Single nucleotide polymorphism
UC	Ulcerative colitis

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1. INTRODUCTION

1.1 Crohn's Disease

The first series of patients suffering from non-tuberculosis, granulomatous small bowel inflammation was described in 1913 by the Scottish surgeon Dalziel (Dalziel, T. K. 2009) However it was not until Crohn, Ginzburg and Oppenheimer published a thorough report of 14 patients suffering from chronic granulomatous inflammation in terminal ileum in 1932 that the disease was defined and named (Crohn, B. B. et al. 1932). They illustrated a condition causing abdominal pain, emaciation, diarrhea and fever. In this report, referred to as the original description of Crohn's Disease (CD), surgical resection of all of the inflamed segments of the intestine was suggested to cure the patients, a statement which was going to cause patients unnecessary suffering during many years to come. Today, it is known that CD together with ulcerative colitis (UC) constitutes the main condition of chronic inflammatory bowel disease (IBD), characterized by relapsing inflammation, often segmentally distributed throughout the intestine. The disease can develop in the entire gastrointestinal tract, from the mouth to the anus, however the ileocaecal region followed by the colon are the areas most commonly affected. Previously, a disease with high mortality, CD can today be controlled by medical and surgical treatment, resulting in only a slight increase in mortality compared to the rest of the population (Card, T. et al. 2003). Nevertheless, CD is a difficult disease to live with. It is usually diagnosed in young adulthood and necessitates lifelong medical treatment and repeated surgery, often resulting in periods of hospitalization.

1.1.1 Symptoms and General treatment

Patients suffering from CD experience different symptoms dependent on where the inflammation exists, however it is well known that the disease is accompanied with abdominal pain, diarrhea, weight loss, fever and vomiting. Often signs and symptoms are very diffuse in the beginning and it is therefore not unusual that the correct diagnosis is delayed by months or even years**.**

As there is no cure available today, treatment is directed to relieve symptoms or prevent complications. Usually treatment consists of a combination of anti-inflammatory corticosteroids, 5-aminosalicylates, and immunosuppressive drugs, azathioprine or 6-mercaptopurine. Patients that do not respond to this standard therapy are treated with antibodies directed towards the pro-inflammatory cytokine Tumor necrosis factor alpha (TNFα) (infliximab or adalimumab), which prevents the

binding of TNFα to its receptor, thus resulting in a dampened inflammatory response. It is a highly effective medication and today fewer patients with active inflammation undergo surgical resection (Rutgeerts, P. et al. 2006). Surgical resection is currently used more restrictively either to relieve symptoms that do not respond to medical therapy or to correct complications.

1.1.2 Epidemiology and Pathogenesis

CD is a disease of the western world with the highest incidence rates in Scandinavia, Great Britain and North America. The frequency of the disease has constantly increased during recent decades and in Scandinavia 8-10 cases per 100 000 citizens are diagnosed with CD each year (Lapidus, A. 2006). In Asia, which has the lowest incidence rate, the number of CD patients is rapidly increasing and in Japan a seven fold increase has been observed since the 1990s (Yang, S. K. et al. 2001). The disease affects people of all ages, with a peak incidence between 15-30 years and a slight female predominance.

CD is a complex disease and the exact cause remains unknown. However, evidence suggests that genetic, immunological and environmental factors all contribute to the pathogenesis of the disease (figure 1). Today it is believed that Crohn's Disease is initiated when an individual who is genetically predisposed (allowing for a disturbance in the barrier function and/or the innate immune system), is exposed to triggering environmental factors.

Intraluminal bacteria are considered to be the main environmental factor in CD that drives inflammation, a finding confirmed by several reports. Recently elevated numbers of adherent-invasive *Esherichia coli* (*E.coli*) (AIEC) were found in the mucosa of affected patients. CD is more common in ileum and colon, areas rich in bacterial content. That the driving force of the inflammation seen in CD is the non-pathogenic intraluminal bacteria is further emphasized by findings from several mouse models of IBD. These show, with one or two exceptions, that mice developing disease in a conventional environment do not do so in a germ-free environment. Additionally, in most cases the disease is ameliorated, when mice are treated with antibiotics (Rath, H. C. et al. 2001). Furthermore, CD has been linked to bacteria such as *Yersinia* spp and *Mycobacterium paratuberculosis,* where the latter has been thoroughly investigated. A connection between *Yersinia spp* and CD was shown when DNA from *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* was found in an increased frequency in intestinal samples from CD patients.

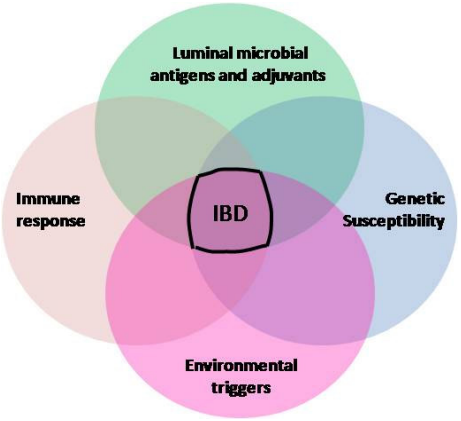


Figure 1. The various areas known to contribute to the inflammatory process associated with CD in a genetically susceptible host. Intraluminal bacteria are considered the most important environmental triggering factor and are necessary to initiate or reactivate disease expression.

In addition, several case reports have linked *Yersinia*-induced ileitis and lymphadenitis to the early phases of clinical CD. The involvement of *M. paratuberculosis* in the pathogenesis of CD has been thoroughly investigated, though the results so far are conflicting. Recently, DNA from *M. paratuberculosis* was shown to be increased in intestinal samples from patients suffering from CD as compared to controls (Feller, M. et al. 2007). The same group also observed elevated levels of antibodies towards *M paratuberculosis* antigens (Feller, M. et al. 2007). However, the involvement of *M paratuberculosis* is still a matter of debate and despite a lot of research in the area the results are controversial. Recently a two year study investigating the benefits of a combination therapy with antibiotics towards *M paratuberculosis*, and corticosteroids showed no difference in the number of patients relapsing after two or three years when given antibiotics or placebo respectively (Selby, W. et al. 2007).

Disturbances in both the innate and adaptive immune system have been identified to contribute to the development of the disease. Previously the adaptive immune system has been the main focus of CD, where the CD4+ T helper cells (Th1/Th2) are the most important players, as they help eliminate both intracellular and extracellular microbes. Several reports have emphasized the importance of T-cells in the pathogenesis of CD and an inappropriate cytokine production by Th1 cells has been implicated in the disease course. However, it is now becoming evident that the immune responses involved are more complex than the traditionally dichotomous Th1/Th2 paradigm. The Th2 cytokines, interleukin (IL)-4 and IL-5, have been found in early stages in CD (Desreumaux, P. et al. 1997). This notion is further supported by findings from a mouse

model of CD, where establishment of chronic disease is mediated by IL-5 and IL-13, another prototypic Th2 cytokine (Bamias, G. et al. 2005). Recent evidence also suggests a critical role for the novel IL-23 dependent highly proinflammatory Th17 cell population in the pathogenesis of Crohn's Disease (Neurath, M. F. 2007), although further research is needed to completely understand their pathological function. In addition to the adaptive immune system, disturbances in the signalling pathways involved in innate immunity seems to be important for the progression of CD, in fact recent genetic and functional studies suggest that it may be the primary cause. The main focus has been on a set of germline pattern recognition receptors (PRRs), which mediate the initial recognition of microbes, through so called microbial associated molecular patterns (MAMPs). The PRRs comprise two major groups in the cell, the Nod like receptor (NLR) family, which is found intracellularly, and the Toll-like receptor (TLR) family, which mainly reside on cellular membranes. In 2001 the first connection between PRRs and CD was made as predisposing mutations were discovered and mapped to the nucleotide oligomerizing domain 2 (Nod2) locus, which encodes a cytosolic PRR that sense a component of bacterial peptidoglycan called muramyl dipeptide (MDP)(Girardin, S. E. et al. 2003; Hugot, J. P. et al. 2001; Inohara, N. et al. 2003; Ogura, Y. et al. 2001). Upon recognition of bacterial products Nod2 is activated and downstream signalling leads to the activation of NF-κB and hence production of the inflammatory driving cytokines IL-1β and TNFα. Since then, several susceptibility genes for CD have been discovered, including many genes encoding proteins involved in innate immunity. The important roles that the PRRs and the innate immune defence play have been further emphasized by the finding that a polymorphism in the gene encoding TLR4 was shown to confer susceptibility to the disease, resulting in increased sensitivity to gram-negative bacteria in patients carrying this specific alteration (Cario, E. et al. 2000; Franchimont, D. et al. 2004). Addintionally, a role for autophagy, a process involving the degradation of intracellular components via the lysosome, has been implicated in CD, specifically the autophagy dependent gene ATG16L1 was recently found to confer susceptibility to the disease (Hampe, J. et al. 2007a; Rioux, J. D. et al. 2007). Autophagy is suggested to be an innate defence mechanism against microorganisms and was recently shown to restrict the growth of cytosolic *Salmonella typhimurium* (Birmingham, C. L. et al. 2006a; Birmingham, C. L. et al. 2006b). Recent evidence suggests that the NLRs might provide a crucial link between recognition of bacteria in the cytosol and triggering of autophagy. In support of this, autophagy induced by *S. flexneri* infection was recently shown to be enhanced in the absence of Ipaf, another NLR known to mediate IL-1β secretion upon recognition of cytosolic flagellin (Suzuki, T. et al. 2007).

A dysfunctional barrier has also been associated with CD and patients suffering from the disease show an increase in paracellular permeability, which is a characteristic of intestinal inflammation (Bjarnason, I. et al. 1995). As previously mentioned, CD is a multifactorial disease and how the genetic, immunological and environmental factors described above contribute to the increased gut permeability is still under investigation. The inflammatory cytokine TNFα is known to play a central role in the barrier dysfunction, as illustrated by findings from cell culture experiments where TNFα has been shown to target epithelial tight junctions resulting in an increase in paracellular permeability. This is further supported by increased transcellular uptake of protein antigens in CD which has been associated with enhanced expression of TNFα(Soderholm, J. D. et al. 2004). In addition, a genetic contribution to the disturbed barrier function has been suggested as polymorphisms in the *Nod2* gene are associated with increased intestinal permeability (Meyer, U. et al. 2006). Furthermore, a disturbed secretion of the antimicrobial peptides, α-defensins and DMBT1, leads to a defect in the elimination of microorganisms and hence a higher bacterial content in the intestine, which may act as an inflammation-driving factor(Rosenstiel, P. et al. 2007; Wehkamp, J. et al. 2005).

1.2 The intestinal mucosal barrier

The intestinal mucosa is continuously exposed to a high content of bacteria and therefore needs to be specialised in controlling the invasion of foreign and dangerous agents. The high content of gastric acids and biliary juices in the stomach and duodenum, respectively, provides the first step in preventing invasion. Adhesion of microbes that survive this milieu is further prevented by the glycocalyx and the mucus layer covering the intestinal epithelium, which constitutes the physical barrier between the intestinal mucosa and luminal content. The integrity of this barrier is primarily maintained by enterocytes connected to each other via junctional complexes.

The lining of the small intestine (duodenum, jejunum and ileum) is characterized by numerous leaf-like projections called villi. The epithelium covering the mucosa consists of many different cell types with specialised functions. The enterocytes, are most abundant, and mediate the absorption of nutrients. On their luminal surface they posseses numerous microvilli, which serve to increase their absorptive ability. Scattered along the epithelium are also mucus-secreting cells, called goblet cells, and located at the base of the crypts are the Paneth cells, which prevent proliferation of microorganisms by the release of anti-bacterial factors, like defensins, TNFα, lysozyme and phospholipases. Enteroendocrine cells are also spread throughout the epithelium

releasing gastrointestinal hormones like secretin, neurotensin and somatostatin in response to changes in the microenvironment.

The epithelium that covers the small intestine constitutes either villus epithelium (VE) or follicle-associated epithelium (FAE). While the VE is specialised in digestion and absorption of nutrients and consists mainly of the cell types described above, the FAE also contains so called membranous or microfold (M) cells, specialised in antigen sampling and transport. The FAE covers the lymphoid follicles of Peyers patches in the intestine, an area characterised by a high content of lymphocytes. Once sampled by the M cells, antigens are captured by dendritic cells, which results in priming of T-cells and activation of the adaptive immune response. The exact distribution of the different types of epithelia in the intestine is not known, however FAE appear to be more abundant in the ileum as well as in the ileocaecal region.

In contrast to the small intestine the main function of the colon is absorption of electrolytes and water along with elimination of undigested food and waste. The mucosa is arranged in crypts where numerous straight tubular glands are present and do not form villi, which is a characteristic of the small intestine. It is covered by a single columnar epithelium containing the same cell types as described for the small intestine. However, Paneth cells and enteroendocrine cells are expressed to a lower extent, while absorptive and goblet cells are abundant. Goblet cells are more prevalent in the crypts than along the surface and their number increases distally towards the rectum. The absorptive colonic enterocytes express short irregular microvilli, with a glycocalyx absent of digestive enzymes. M-cells can be found in the epithelium covering the dome-like structure of the colonic lymphoid follicle (Cario, E et al 2000; Fujimura, Y. et al. 1992; Gebert, A. et al. 2004; Kucharzik, T. et al. 2000).

An important component of intestinal homeostasis is the proper function of the junctional complexes via which the enterocytes are connected. The paracellular space needs to be tightly regulated in order to avoid unnecessary invasion of foreign and dangerous antigens. However, several microbial pathogens have evolved countless strategies to interfere with junctional complexes in order to disrupt and cross the epithelial barrier. The junctional complex constitutes several groups of proteins (tight junction, adherence junction, desmosome and gap junction) that attach the enterocytes to each other at specific points. Tight junctions (TJs), located at the most apical part of the lateral membrane, are the major regulatory unit of the epithelial barrier (figure 2).

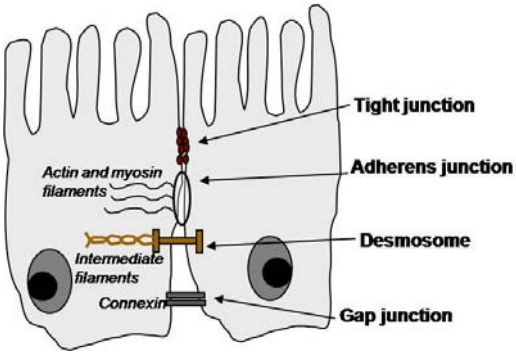
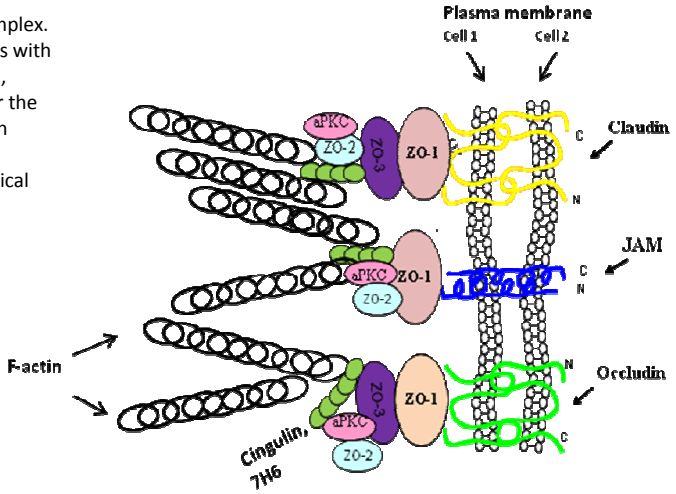


Figure 2. Schematic illustration of the junctional complex. Tight junctions are located at the most apical part and represent the major regulatory unit of the epithelial barrier

TJs consist of numerous protein families and form a network of linking strands, where the paracellular space is sealed by the transmembrane proteins occludin, claudin and junctional adhesion molecule-1 (JAM-1). Several scaffolding proteins, like zonula occludens (ZO)-1,-2,-3, cingulin and 7H6 antigen interact with both occludin and claudin and anchor the TJs to the F-actin filaments of the cytoskeleton (Citi, S. et al. 1988; Gumbiner, B. et al. 1991; Haskins, J. et al. 1998; Schneeberger, E. E. et al. 2004; Stevenson, B. R. et al. 1986; Zhong, Y. et al. 1993). JAM-1 is not a part of the TJ strands but is known to interact with occludin as well as scaffolding proteins like ZO-1, (figure 3) (D'Atri, F. et al. 2001; Liu, Y. et al. 2000). So far, 24 members of the claudin family have been identified, which are expressed differently in various tissues. They are also known to differ in distribution along the gastrointestinal tract, which might be account for the differences in paracellular permeability observed along the intestine. Numerous signalling proteins, like ZONAB, RhoA and Raf-1, are also known to interact with TJs and are proposed to be involved in the junctional assembly, barrier regulation and gene transcription reviewed by Schneeberger, E E et al 2004. The TJs primary function is to act as a regulated permeability barrier in the paracellular epithelial transport pathway as well as a fence in the plane of the membrane, preventing movement of membrane proteins, e.g ion channel diffusion from the apical to the basolateral region in the outer cell membrane.

Figure 3. The tight junction complex. Occludin and Claudin associates with scaffolding proteins ZO-1,2 & 3, cingulin and 7H6, which anchor the TJs to the F-actin. Tight junction assembly is mediated via phosphorylation involving atypical Protein Kinase C (aPKC).



1.2.1 Endocytosis

Large particles and molecules, like proteins and bacterial products that cannot pass through the cell membrane or the paracellular space can be taken up by the cell through invagination of the plasma membrane followed by vesicle formation, a process called endocytosis. This is an essential process that serves several purposes, making sure that the cell is supplied with necessary substances and mediating uptake of foreign antigens against which the body can initiate an effective immune response. Following endocytosis the engulfed substances are actively transported by transcytosis through the cytoplasm to their particular destination. These two processes are constantly manipulated by foreign microbes to establish an entry into the host. In order to keep an intact barrier function it is of great importance that these processes function correctly and that the cell can eliminate the foreign substance taken up. Classically three main types of endocytosis exist; macropinocytosis, caveolae mediated endocytosis, and clathrin-mediated endocytosis (figure 4).

Macropinocytosis is mediated through invagination of the cell membrane where bending of single surface lamellipodia gives rise to circular ruffles which ultimately are released in the cytoplasm as a vesicle (macropinosome) (Swanson, J. A. et al. 1995). It is a process by which considerable volumes of extracellular fluid can be internalised along along with dissolved molecules as well as larger particles such as viruses, bacteria and apoptotic cell fragments.

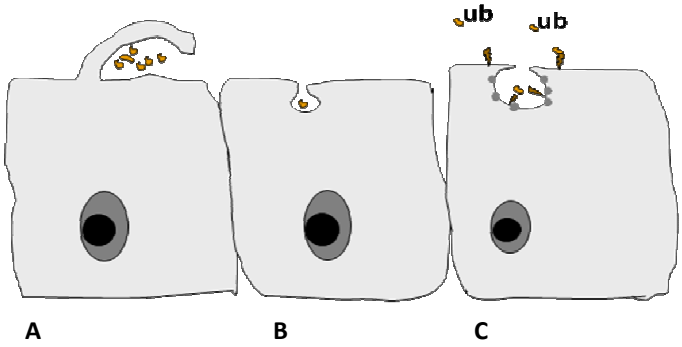


Figure 4. A) Macropinocytosis is mediated via invagination of the cell membrane and mediates uptake of dissolved molecules B) Caveolae-mediated endocytosis occurs at lipid rafts coated with caveolin. C) Clathrin-mediated endocytosis involves the formation of vesicles coated with clathrin and mediates uptake of receptors bound or not bound to their ligand as well as ubiquitinated cargo. Ub=ubiquitin.

However, macropinosomes do not usually exceed the diameter of 1 µm. Immature dendritic cells (DC) are known to take up antigens via macropinocyosis (Swanson, J. A. et al. 1995). In other cells, such as macrophages, lymphocytes, fibroblasts and epithelial cells macropinocytosis is increased upon application of various stimuli (Amyere, M. et al. 2002; Amyere, M. et al. 2000; Hamasaki, M. et al. 2004; Lanzetti, L. et al. 2004; Meier, O. et al. 2004; Yang, Z. et al. 2005). In M-cells and entrocytes such increases have been observed in response to antigen stimulation (Conner, S. et al. 2003).

Caveolae-mediated endocytosis involves the formation of invaginations (caveolae, 50-80nm in diameter), which occur at cholesterol-enriched microdomains, known as lipid rafts, in the plasma membrane. In order for caveolae to form, the lipid rafts need to be coated with caveolin (Fra, A. M. et al. 1995; Shaul, P. W. et al. 1998). Internalization of the previously mentioned TJ protein occludin has been shown to occur via caveolae-mediated endocytosis (Shen, L. et al. 2005).

Clathrin mediated endocytosis involves the formation of clathrin coated vesicles, which begins with the recruitment and assembly of clathrin as well as adaptor and endocytotic accessory proteins at the plasma membrane (Heuser, J. 1980). The membrane curves into coated pits, which are sequentially severed from the plasma membrane as vesicles. Adaptor proteins are crucial for the assembly of clathrin-coated pits at the plasma membrane as well as for recognition of specific cytosolic motifs of the protein being internalized. Internalization of different plasma membrane proteins

such as receptors and their ligands has been shown to occur through clathrin-mediated endocytosis. Non-signalling receptors that mediate the uptake of nutrients, like low density lipoprotein receptors and transferrin receptors, are internalized either bound or not bound to their ligand via so called constitutive endocytosis (Goldstein, J. L. et al. 1982; Watts, C. 1985). This suggests that clathrin-mediated endocytosis functions in a more regulatory way to adjust the actual number of receptors present on the surface of the cell in response to environmental signals. Additionally, ubiquitin bound to the cargo protein has been shown to promote constitutive endocytosis when fused with certain reporter molecules (Barriere, H. et al. 2006; Goldstein, J L et al 1982; Haglund, K. et al. 2003; Nakatsu, F. et al. 2000; Watts, C 1985). On the contrary, ligand induced clathrin-dependent endocytosis mediates internalization of receptors upon binding of its particular ligand and involves the uptake of growth factors, like EGF, and their receptors (Hanover, J. A. et al. 1985). G-protein coupled receptors are also known to be internalized upon agonist binding (Rothman, J. E. et al. 1980). Several tight junction proteins have also been shown to be internalized via clathrin mediated endocytosis, like JAM-1(Ivanov, A. I. et al. 2004). Ivanov et al also report that occludin is internalized via this pathway and thus present conflicting data relating to how endocytosis of this particular protein is mediated and emphasizing the difficulty in elucidating the different endocytotic pathways.

1.3 Mucosal barrier dysfunction in Crohn's Disease

A compromised intestinal barrier has been proposed to play a crucial role in the development of IBD (Hollander, D. 1992; Meddings, J. B. 1997). As described above, several mechanisms work together to keep an intact barrier and under normal conditions only small amounts of protein antigens cross the intestinal epithelium. However, patients suffering from CD have an increased permeability of the small intestine to antigens as well as medium sized probes (Hollander, D. et al. 1986; Meddings, J B 1997; Soderholm, J. D. et al. 1999). This results in an increased exposure of antigens to immune cells ultimately leading to increased inflammation and gastrointestinal disease (Fiocchi, C. 1998; Hugot, J P et al 2001; Sanderson, J. D. 1993; Sartor, R. B. 2006). In addition this phenomenon has been observed in relatives of CD patients without evidence of disease, suggesting that increased intestinal permeability may be a primary etiological factor in CD (Hollander, D. 1993; Peeters, M. et al. 1997; Soderholm, J D et al 1999). Increased permeability is also observed in CD patients as well as their relatives when exposed to NSAIDS (May, G. R. et al. 1993; Soderholm, J D et al 1999; Zamora, S. A. et al. 1999). Moreover it has been reported that spouses to

patients suffering from CD have an increased permeability, data that further emphasize the importance of environmental factors in the development of disease. However, recent data contradict this finding by showing that first degree relatives living with the patient at the time of diagnosis did not differ in permeability as compared to relatives living in a separate household (Buhner, S. et al. 2006). At present it is not clear if the changes in barrier integrity observed in CD is an early event or rather a secondary phenomenon, a consequence to an already established inflammation.

1.3.1 Mechanisms of TNFα on the mucosal barrier

The pro-inflammatory cytokine TNFα, known to be upregulated in patients suffering from CD, has for a long time been considered a key driving mediator of the inflammatory process and is known to contribute to a dysfunctional barrier.

TNFα is synthesized by a wide range of different cells, including macrophages, T-cells, mast cells, granulocytes as well as non immune cells like fibroblasts and smooth muscle cells upon stimuli like bacterial agents or other inflammatory substances. TNFα exists either in a transmembrane bound form (tmTNFα) or is released as a soluble form (sTNFα) after cleavage by TNF-alpha converting enzyme (TACE). Both forms of the cytokine interact with a set of two distinct receptors TNF receptor tpe 1 (TNFR1) and TNF receptor type 2 (TNFR2), resulting in either activation of nuclear factor kappa B (NFκB) or apoptosis.

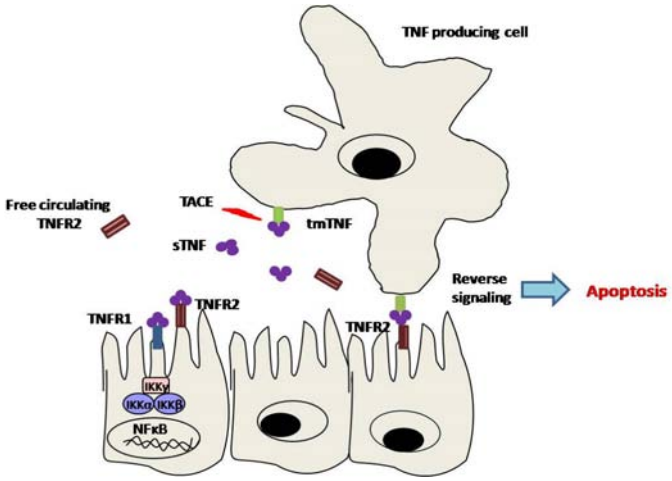


Figure 5. Once synthesized transmembrane TNFα (tmTNF) is expressed on the cell surface, soluble circulating TNFα (sTNF) is generated by the cleavage of tmTNF by TACE. sTNF bind both TNFR1 and 2 which results in the activation of NFκB, while an interaction between tmTNF and TNFR2 leads to reverse signalling and apotosis of the TNFproducing cell. TNFR2 exists as a transmembrane receptor but is also released as a circulating soluble receptor.

However, sTNFα have been found to have a higher affinity for TNFR1, while tmTNFα preferentially bind TNFR2. In addition, tmTNFα is known to act as a receptor as well and interaction with TNFR2 or a TNFα antagonist, so called reverse signalling, results in the activation of several signalling pathways leading to cytokine suppression or apoptosis (figure 5) as reviewed (Tracey, D. et al. 2008).

Extensive research has shown that TNFα affect the barrier in numerous ways. Experiments in the cell line HT29/B-6 have shown that TNFα decrease the trans-epithelial resistance (TER) as well as diminish the promoter activity of occludin (Mankertz, J. et al. 2000; Schmitz, H. et al. 1999). TNFα treatment also resulted in a decrease of junctional strands and a reduction in the depth of the TJs (Gitter, A. H. et al. 2000a; Schmitz, H et al 1999). Further, TNFα has been shown to affect TJs by inducing upregulation of the pore forming claudin 2, leading to enhanced paracellular permeability (Zeissig, S. et al. 2007). In the same cell line, TNFα was also shown to induce apoptosis, resulting in leaks in the epithelium and hence increased permeability (Gitter, A. H. et al. 2000b). Recently it was also shown that TNFα enhances the mRNA transcription of myosin-light chain kinase (MLCK) (Ma, T. Y. et al. 2005). This was also illustrated in the intestinal epithelial cell line Caco-2 where TNFα was found to synergize with interferon (IFN) γ to induce increased expression of MLCK (Wang, F. et al. 2005). Enhanced MLCK results in phosphorylation of myosin II regulatory light chain (MLC) and hence contractions of the perijunctional actomyosin ring. Under normal condition this mechanism adjusts the paracellular permeability in response to intraluminal stimuli like Na^+, glucose or bacteria (Turner, J. R. et al. 1997; Yuhan, R. et al. 1997). However, increased amounts of TNFα results in upregulation of MLCK, which disrupts the TJs and hence leads to increased paracellular permeability as shown by the finding that reorganisation of ZO-1, occludin and claudin 1 is accompanied by increased TER (Wang, F et al 2005). The synergistic effect observed in combination with IFNγ was recently suggested to be due to upregulation of TNFR2 by IFNγ. Wang et al show that IFNγ is necessary for TNFR2 upregulation and hence response to TNFα in Caco-2 cells (Wang, F. et al. 2006). To further support this finding TNFR2 has been shown to be upregulated on lamina propria T-cells in CD (Holtmann, M. H. et al. 2002). This observation could explain earlier findings that IFNγ enhances the effects of TNFα. Recently, it was shown that the elevated levels of TNFα that follow CD3 induced T-cell activation in mice drives MLC phosphorylation and hence contribute to the increased permeability observed in these mice (Clayburgh, D. R. et al. 2005). Furthermore, Clayburgh et al find no evidence for apoptosis during their three hour experiment, thus

suggesting that TNFα induced MLC phosphorylation is induced earlier in the cell while TNFα induced apoptosis might be a long-term effect.

In addition to the above described changes in paracellular permeability TNFα has also been shown to induce transcellular uptake of the protein antigen horseradish peroxidase (HRP) in T84 cells. Moreover increased endosomal uptake of HRP in histologically unaffected ileal mucosa of CD patients was correlated to increased mRNA expression of TNFα (Soderholm, J D et al 2004).

1.3.2 Mucosal barrier dysfunction in response to microbes

Translocation of enteric bacteria and bacterial products across the intestinal barrier has been proposed as a major factor in driving the inflammatory process associated with CD (Darfeuille-Michaud, A. et al. 2004; Martin, H. M. et al. 2004; Swidsinski, A. et al. 2002). Increased uptake of foreign antigens results in an enhanced inflammatory response and hence elevated levels of cytokines like TNFα and IFNγ.

Commensal bacteria have also been proposed to be of great importance for the exaggerated inflammation in CD. In support of this, intestinal epithelia under stress have been shown to perceive commensal bacteria as a threat (Nazli, A. et al. 2006). These data have been further confirmed by numerous mice model experiments, showing that mice developing disease in a conventional environment do not do so in a germ-free environment as reviewed by Rath, H C et al 2001.

Among pathogens associated with CD, *Escherichia coli* are the strain of the bacterial flora that has been most thoroughly investigated. Several studies report increased numbers of *E.coli* in the faeces and mucosa of CD patients (Darfeuille-Michaud, A. et al. 1998; Giaffer, M. H. et al. 1992; Liu, Y. et al. 1995; Swidsinski, A et al 2002). Adherent invasive *E.coli* (AIEC) represents a recently identified strain, which is characterized by the lack of several genes including *ipaC* plasmid*, afaD* and *tia*, encoding invasive determinants present in invasive *E.coli* known to be involved in acute gastrointestinal infections. Interestingly, AIEC were also found to be able to survive and replicate within macrophages without inducing cell death (Darfeuille-Michaud, A et al 1998). The bacterial strain was also observed to induce the secretion of high amounts of TNFα in macrophages (Glasser, A. L. et al 2001).

In 2004 two independent research groups reported increased numbers of AIEC in ileal and colonic mucosa of CD patients (Darfeuille-Michaud, A et al 2004; Martin, H M et al 2004). Adherence of the AIEC reference strain LF82 was recently shown to occur via

interaction of the virulence factor type 1 pili to the Carcinoembryonic antigen related cell adhesion molecule 6 (CEACAM6). CEACAM6 has been shown to be upregulated in ileal mucosa of CD patients and increased expression is induced by TNFα and IFNγ (Barnich, N. et al. 2007). The colonic AIEC identified have not been as thoroughly characterized as the ileal AIEC and whether adherence to the intestinal mucosa occurs via CEACAM6 is not known. However, all invasive strains found in the colonic CD mucosa were shown to encode type 1 pili as well as having the ability to induce IL-8 release from intestinal epithelial cell lines. AIEC, however, is not a specific pathogen only associated with CD as it has also been found in ileal and colonic control specimens though in low numbers (Darfeuille-Michaud, A et al 2004). AIEC, might therefore represent a normal bacterial flora that preferentially colonizes the mucosa of CD patients as this represents an environment where adherence and invasion is possible to a higher extent.

Additionally, to *E.coli* strains, several pathogenic bacteria have been implicated as contributing factors in the pathogenesis of disease. As previously mentioned, a connection between CD and *Yersinia spp* has been identified as DNA from *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* has been found in increased levels in intestinal samples from CD patients (Kallinowski, F. et al. 1998; Lamps, L. W. et al. 2003). To further support this case report, studies have linked *Yersinia* induced ileitis to the early phases of clinical CD (Homewood, R. et al. 2003; Zippi, M. et al. 2006). Furthermore, yersinia is known to cause a condition resembling CD with ileitis or ileocolitis as well as reactive arthritis.

Furthermore, invasion of *Yersinia pseudotuberculosis* is mediated via M-cells in the FAE, a route which is of particular interest in CD as the first observable sign of the disease is aphtoid ulcers in this region (Morson, B. C. 1972). Uptake of *Yersinia pseudotuberculosis* is mediated via two virulence factors, invasin and YadA, which together mediate adhesion, uptake and translocation of the bacteria across the intestinal epithelium (Eitel, J. et al. 2002; Isberg, R. R. et al. 1987). Ivasin exert its effect via interaction with β1-integrins, mainly expressed on M-cells. This indicates that *Yersinia spp* are more pathogenic to FAE than other parts of the intestine. As observed in mice infected with *Yersinia enterocolitica,* where vesicles form over the lymphoid follicle consequently leading to the degeneration of these sites (Autenrieth, I. B. et al. 1996). Despite the fact that the invasive route by which *Yersinia spp* crosses the epithelium has been thoroughly studied, little is known about what effects these bacterial strains have on the absorptive villus epithelium surrounding the FAE in the

intestine. The aim of paper I was therefore to investigate the effect of *Yersinia pseudotuberculosis* on the absorptive villus epithelium.

1.3.3 Mechanisms of infliximab in Crohn's Disease

During the last decade anti-TNFα treatment has gained widespread acceptance as standard therapy in the medical management of patients suffering from severe CD. The monoclonal antibody infliximab, with specificity for TNFα, was first shown to have beneficial effects on the healing of colonic ulcers in an open label study in 1995 and was later registered as Remicade and used for treatment of active, severe CD as well as fistulising disease (van Dullemen, H. M. et al. 1995).

Several studies have reported the beneficial effects of infliximab on immune cells. Binding of infliximab to tmTNFα on the surface of monocytes isolated from CD patients results in the induction of apoptosis. Lugering et al propose a mechanism where infliximab binds tmTNFα, which results in upregulation of the Bcl-2 family members, Bax and Bak and ensuing cytochrome C release from mitochondria. In the cytosol, cytochrome C binds apoptotic protease activating factor -1 leading to the activation of caspase-9 which initiates a cascade of caspases and hence apoptosis. This was further confirmed in a jurkat T cell line, where Bax was shown to be upregulated in the presence of infliximab (ten Hove, T. et al. 2002). Moreover, ten Hove et al observed an increase of apoptotic T cells in the gut mucosa of CD patients. This data was further confirmed by Di Sabatino, A. et al. 2004, who reported increased apoptosis of Lamina propria T cells ten weeks after infliximab treatment. Monocytes and T-cells mediate an important source of proinflammatory cytokines and hence elimination results in diminished inflammation.

Infliximab has also been shown to reduce the increased paracellular permeability associated with CD. In 2002 this was proved by Sunenaert et al as a diminished urinary secretion of ^{51}Cr-EDTA was observed four weeks after a single infusion of infliximab (Suenaert, P. et al. 2002). Additionally, Zeissig et al showed that enterocyte apoptosis is upregulated in CD patients but is restored after two weeks after infliximab treatment (Zeissig, S. et al. 2004). Further, they showed that TER is increased after infliximab treatment, however expression of occludin, claudin 1 and 4 did not change markedly after treatment. Thus, suggesting that restoration of the barrier is mainly due to downregulation of apoptosis in this study. Normalisation of inflammatory-driven epithelial apoptosis by anti TNFα treatment has previously been shown in SAMP1/YitFc mice, commonly used as a model of spontaneous ileitis (Marini, M. et al. 2003).

Furthermore, infliximab has been shown to downregulate cell adhesion molecules like CD40 on the microvessels in the mucosa of affected subjects as well as diminish circulation and expression of its ligand CD40L (Danese, S. et al. 2006). Thus, disrupting the production of innate and adaptive inflammatory mediators, which are important in the inflammatory process associated with the disease. CD40 is expressed on numerous cell types, while its ligand CD40L circulates in the body after being cleaved and shed from T helper cells. Upon binding, a complex signalling cascade is activated which ultimately converges on transcription factors like NF-κB and AP-1 reviewed by Danese, S. et al. 2004.

Even though the mechanisms by which infliximab cause relief of the symptoms associated with CD are beginning to emerge it is still unknown if this treatment affect the translocation of bacteria across the intestine. As intraluminal bacteria are a main contributor to CD we sought out to investigate the effect of infliximab, in paper II, on the invasion of colonic AIEC in patients suffering from CD.

1.4 Nod like receptors (NLRs) and Crohn's Disease

The NLR family resides, as previously mentioned intracellularly and meditates initial recognition of bacterial products to orchestrate an immediate inflammatory response against the invading organism. Nod2 was the first gene polymorphism discovered to be associated with CD and opened up a new perspective of the disease directing the focus to the innate immune system and the PRRs. The Nod2 protein, most commonly expressed in epithelial cells and monocytes, constitutes a cytosolic PRR sensing MDP, the minimal peptioglycan motif common to both gram negative and positive bacteria(Girardin, S E et al 2003; Inohara, N et al 2003). The Nod2 protein is composed of two amino-terminal caspase activating and recruiting domains (CARDs), a NACHT (named after the protein families NAIP, CIITA, HET-H and TP1) domain, and a carboxyl-terminal leucine-rich repeat (LRR) domain (Hugot, J P et al 2001; Ogura, Y et al 2001). Nod2 is activated upon recognition of MDP by the LRR domain and downstream signalling is triggered by a homophilic interaction of the CARD domains of Nod2 and the serine-threonine kinase RIP2 (Ogura, Y et al 2001; Tanabe, T. et al. 2004). This ultimately results in the activation of NF-κB through the formation of a complex with IKKγ (NEMO) (Abbott, D. W. et al. 2004) and subsequently a release of inflammatory cytokines like TNFα, IL-1β and IL-6, can be observed in the mucosa of affected subjects (Fiocchi, C 1998; Podolsky, D. K. 2002). Three genetic variants, L1007fs, G908R, R702W, all within the coding region of *Nod2/CARD15*, have been genetically associated with susceptibility to CD in European and American populations (Ahmad, T. et al. 2003;

Hampe, J. et al. 2007b; Hugot, J P et al 2001; Ogura, Y et al 2001). Among patients carrying the mutations the R702W variant is represented by 32%, the G908R by 18% and the L1007fs by 31% (Lesage, S. et al. 2002). The mechanism by which these mutations cause susceptibility to Crohn's disease is still poorly understood. *In vitro* studies demonstrate defective NFκB activation after stimulation of cells expressing the specific CD associated mutations with bacterial ligands (Bonen, D. K. et al. 2003; Chamaillard, M. et al. 2003; Inohara, N et al 2003; Ogura, Y et al 2001). This evidence has given rise to the question: How can a diminished sensing and response to bacteria due to mutated Nod2 be associated with increased production of NF-κB targets in patients suffering from CD? Within this conceptual framework, three main views have emerged on how these mutations are associated with CD.

The most recent model, showed that macrophages from knock-in mice expressing the truncated form of Nod2 containing the frameshift mutation L1007fs, upon stimulation with MDP, produced increased amounts of IL-1β (Maeda, S. et al. 2005). Further, they also found that the knock-in mice were more susceptible to dextran sodium sulfate (DSS) induced colitis and consequently providing evidence for the frameshift mutation associated with CD being a gain-of-function variant resulting in elevated levels of IL-1β. It is, however, important to remember that these are results from mouse models only and does not give any explanation to the fact that epithelial cells expressing the different CD associated Nod2 mutations have a defective NF-kB activation in response to stimulation with MDP. Moreover, the results do not match the findings that peripheral-blood mononuclear cells isolated from CD patients carrying the frameshift mutation show a defect in IL-1β production rather than an increase.

The second model postulates that the lost ability of intestinal epithelial cells to activate NF-kB, when expressing mutated forms of the Nod2 protein, might be a cause for a defective production of α-defensins, small antimicrobial peptides known to be able to enhance the innate inflammatory response towards foreign microbes. Recently, α-defensins were suggested to contribute to the intestinal host defence as α-defensin-5 has been shown to posses antimicrobial activity against several bacteria, like *E.coli* and *S. typhimurium* (Porter, E. M. et al. 1997). It has been shown that patients suffering from CD generally have a reduced expression of human α-defensin-5 and 6. This reduction is observed to be more pronounced in patients carrying the *Nod2* mutations (Wehkamp, J. et al. 2004). To further support this it has also been shown that Paneth cells in Nod2 deficient mice have a defective production of mRNA encoding α-defensins (Kobayashi, K. S. et al. 2005). A decreased level of α-defensins could also explain the fact that cultured intestinal epithelial cells have a reduced capacity to

restrict proliferation of *S. typhimurium* (Hisamatsu, T. et al. 2003). The data supporting this model is difficult to interpret as a whole. For example, studies examining the level of α-defensins in patients carrying the Nod2 mutations are based on the measurement of mRNA and hence do not reveal anything about the actual amount of peptide secreted (Wehkamp, J et al 2004).

The third view investigates the possibility that Nod2 might act as a negative regulator of IL-12 production, which is induced by TLR2 upon recognition of peptidoglycan (PGN). This model was first confirmed by experiments in antigen-presenting cells from mice lacking and expressing Nod2, respectively. Co-stimulation with PGN and increasing concentrations of MDP resulted in a dose-dependent inhibition of IL-12 production in mice expressing a functional Nod2 protein (Yang, Z. et al. 2007). The hypothesis is that this regulation is absent in patients carrying the CD associated Nod2 mutations, and PGN might elicit an excessive IL-12 response, which contributes to the inflammation seen in CD by creating a milieu that could support Th1 cell induced colitis. This was further supported by the finding that monocyte-derived DCs from patients with Nod2 mutations had an increased production of IL-12 in response to PGN. Further, preincubation of the same cell type from normal individuals with MDP resulted in a lower IL-12, IL-10 and IL-6 production when stimulated with TLR ligands as compared to cells not being preincubated (Watanabe, T. et al. 2004). However, this model does not explain the increased level of cytokines like TNFα, IL-1β, IL-10, observed in CD. In fact studies have shown that patients carrying the Nod2 mutations have reduced production of these particular cytokines in response to PGN and several TLR ligands.

Although contributing with important clues about the origin of the disease, none of the above described models provide a full explanation of the inflammation seen in CD and it is evident that other factors play an essential role in the development of the disease. The importance of the NLR family was recently strengthened by the finding that common variants in a regulatory region downstream of the *NALP3* locus contribute to CD, and was also associated with increased IL-1β secretion (Villani, A. C. et al. 2009). Mutations in the *NALP3* gene have previously been associated with rare autoinflammatory conditions characterized by excessive IL-1β production, e.g. CAPS (cryopyrin associated periodic syndromes), that consists of Familial Cold Autoinflammatory syndrome, Muckle-Wells syndrome and Chronic Infantile Neurological Cutaneous and Articular syndrome (Feldmann, J. et al. 2002; Hoffman, H. M. et al. 2001). NALP3 shares several structural similarities with Nod2 and is a member of the recently identified NALP3 inflammasome, a crucial molecular platform

regulating activation of caspase-1 and processing of IL-1β – two key mediators of innate immunity. To form the inflammasome NALP3 associates with apoptosis-associated speck-like protein (ASC) via a PYD-PYD (pyrin domain) interaction (figure 6)(Martinon, F. et al. 2002).

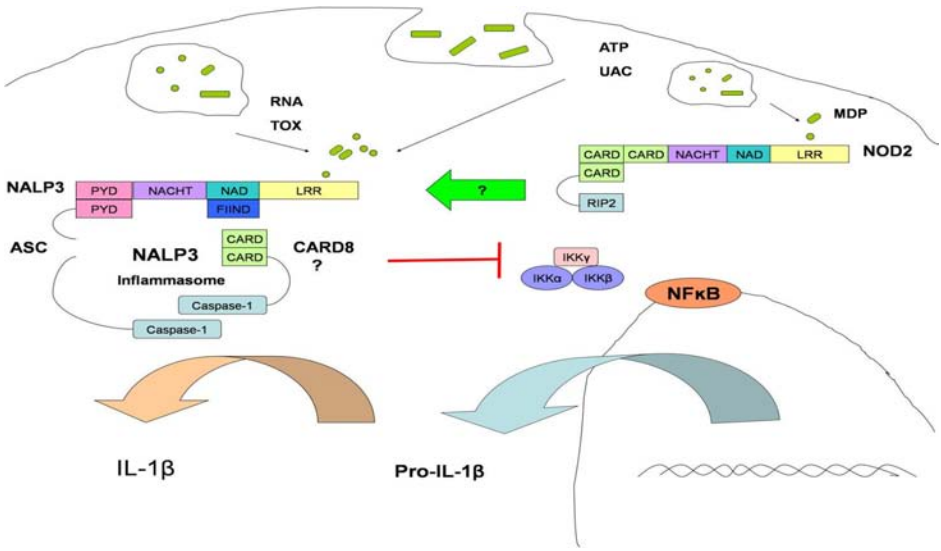


Figure 6. Structure and function of the inflammasome. NALP3, ASC and probably CARD8 together form the inflammasome. Activation of NALP3 leads to the assembly of the inflammasome and activation of caspase-1, which results in the cleavage of pro-IL-1β and secretion of mature IL-1β. CARD8 has also been found to act as a modifier of NFκB response in the context of pro-inflammatory signals. Crosstalk between NALP3 and Nod2 has also been identified and MDP-induced IL-1β processing requires of these proteins.

The tumor-upregulated CARD-containing antagonist of caspase 9 (TUCAN, more commonly CARD8) has also been identified as a binding partner of NALP3, however it is still a matter of debate as to whether this protein is a member of the inflammasome (Agostini, L. et al. 2004). Several stimuli, like bacterial toxins and RNA as well as ATP and uric acid crystals released from dying cells, have been reported to activate the NALP3 inflammasome (Kanneganti, T. D. et al. 2006; Mariathasan, S. et al. 2006). Upon assembly, the NALP3 inflammasome activates caspase-1, which ultimately leads to the cleavage of pro-IL1β and secretion of mature IL-1β, hence leading to an elevated inflammatory response. A connection between NALP3 and Nod2 has also been suggested as MDP induced IL-1β secretion was found to require both Nod2 and the NALP3 protein (Pan, Q et al 2007) (figure 6). To further emphasize the importance of

the inflammasome, a single nucleotide polymorphism (SNP), C10X, in the gene encoding CARD8, located on chromosome 19, has been significantly associated with the disease (Fisher, S. A. et al. 2007; McGovern, D. P. et al. 2006). The polymorphism results in a premature stop codon, which leads to the expression of a truncated protein. However, these results have been subject to controversy, and several independent investigators have shown on the contrary, that the polymorphism is not associated with CD (Buning, C. et al. 2008; Franke, A. et al. 2007).

The exact mechanism for how an altered expression of CARD8 contributes to the pathogenesis of CD remains to be further elucidated. However, recent data suggest that several isoforms exists of CARD8 and individuals who are homozygous for the premature stop codon, C10X, still express a functional immunoreactive protein (Bagnall, R. D. et al. 2008). This could explain the contradicting results that have been reported from independent studies. However, little is still known about how this affects the formation and function of the NALP3 inflammasome.

To support the potential importance of the inflammasome in the pathogenesis of chronic inflammatory diseases it was recently reported that combined genotypes of *NALP3* (Q705K) and *CARD8* (C10X) are associated with increased susceptibility to rheumatoid arthritis (RA) and a more severe disease course (Kastbom, A. et al. 2008). Since the mucosal inflammation in CD is characterized by increased IL-1β production, as is the inflammation observed in RA the aim of paper III was to study whether these combined polymorphisms also confer susceptibility to CD.

1.4.1 The ubiquitin-proteasome pathway and innate immunity

Protein degradation through the ubiquitin-proteasome pathway is the major system of non-lysosomal proteolysis of intracellular proteins. It plays an important part in a broad range of fundamental cellular processes such as regulation of cell cycle progression, apoptosis, cell trafficking and modulation of the immune system and inflammatory responses. The pathway involves a cascade of enzymatic reactions leading to the conjugation of ubiquitin, a protein that is highly evolutionarily conserved in eukaryotes, to the substrate. Ubiquitination of a protein is a three step process and requires the enzymes E1 (ubiquitin-activating enzyme), E2 or UBC (ubiquitin-conjugating enzyme) and E3 (ubiquitin-ligase) (figure 7). Before ubiquitin can bind and target proteins, the C-terminus of ubiquitin must be activated. This is mediated by E1, which in an ATP-dependent manner forms a thioester bond with ubiquitin and transfer it to one of several E2. The E2s then transfer it to one or multiple lysine residues of the substrate together with one of many E3 ubiquitin ligases (Hershko, A. et al. 1998).

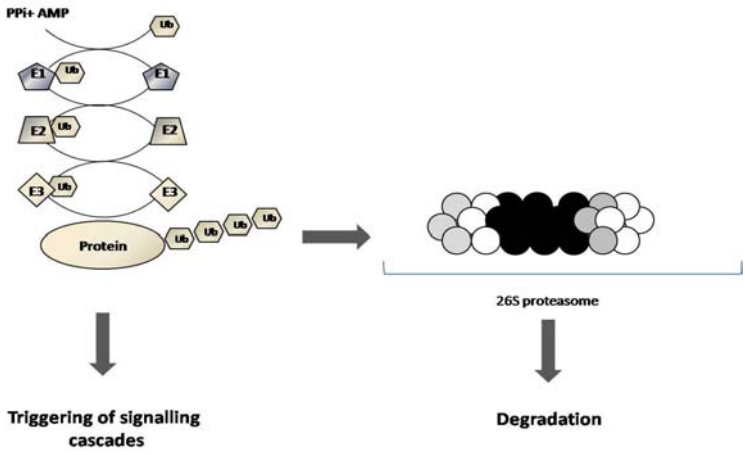


Figure 7. The ubiquitination process: Attachment of ubiquitin (Ub) to a protein is mediated via an enzymatic process involving three enzymes E1, E2, E3, which together mediate binding of ub to the substrate. Linkage of ubiquitin via lysine 48 (K48) generally mediates poly-ubiquitination, while mono-ubiquitination at K48 or K63 usually mediates the activation of several signalling cascades in the cell.

Once ubiquitin is attached to the substrate new molecules can be linked together via different lysine residues leading to different outcomes for the target protein. Ubiquitin chains linked via lysine residue number 48 (K48) are generally associated with proteasomal degradation, and are thought to direct the targeted protein for destruction by the 26S proteasome, while more regulatory functions of the cell are mediated via mono or poly-ubiquitination of Lysine residue number 63 (K63). Recently, it has been shown that chains linked together via K48 or K63 adopt different configurations, which could explain why linkage of different ubiquitin molecules results in different actions in the cell (Varadan, R. et al. 2004).

Several signalling mechanisms in innate immunity are known to be regulated via the ubiquitin-proteasome pathway and a number of E3 ligases, like the Tumor necrosis factor receptor-associated factors, have been shown to mediate the activation of downstream signalling cascades of the TLRs via K63 polyubiquitination (Deng, L. et al. 2000; Kayagaki, N. et al. 2007; Saha, S. K. et al. 2006; Wang, C. et al. 2001). Recently a more direct connection between the ubiquitin proteasome pathway and the PRRs was established as the E3 ligase, Triad3A, was identified to target selected TLRs for ubiquitination as well as degradation (Chuang, T. H. et al. 2004). Chuang et al found that Triad3A interacts with the E2 enzymes UBCH7 and possibly UBCH8 to enhance ubiquitination of TLR9 as well as mediating its degradation. Triad3A was also observed

to generate the degradation of TLR4. Moreover, depletion of Triad3A resulted in increased amounts of TLR9 and 4.

Further evidence link the NLR family to the ubiquitin proteasome system as the major histocompatibility complex (MHC) class II transactivator (CIITA) has been found to undergo ubiquitination (Greer, S. F. et al. 2003). Greer et al show that mono-ubiquitination in the N-terminal of CIITA stimulates its transcriptional potency, while subsequent addition of ubiquitin molecules in the C-terminus targets CIITA for degradation by the proteasome. Hence, suggesting that ubiquitination can act both as a positive and negative modulator.

To shed further light on the NLRs and how mutations in Nod2 and NALP3 contribute to the elevated cytokine production in CD we therefore sought out, in paper IV, to elucidate if Nod2 is regulated via this particular pathway.

2. AIMS OF THE THESIS

As discussed in the introduction several aspects of how immunological, environmental, and genetic factors interplay to establish the inflammatory process associated with CD still needs to be elucidated. The overall aim of the thesis was therefore to shed further light on essential mechanisms of bacterial-epithelial interaction occurring in CD.

The specific aims of the thesis were to:

I: Elucidate how enteric bacteria such as, *Yersinia pseudotuberculosis* affect transport processes of the absorptive villus epithelium in the intestine.

II: Investigate the effect of infliximab on the translocation of the colon specific AIEC strain HM427 across the colonic intestinal epithelium in patients suffering from severe Crohn's colitis.

III: Explore whether genetic variations in the inflammasome components, CARD8 and NALP3, confer susceptibility to CD.

IV: Elucidate if Nod2 is degraded via the ubiquitin-proteasome pathway.

3. METHODOLOGIES

The papers included in this thesis are based on experiments performed by a wide range of methodologies. The main techniques of each paper will be discussed below.

3.1 The Ussing Chamber

The Ussing chamber was first described by the two Danish physiologists Ussing and Zerhan in 1951 (Ussing, H. H. et al. 1951). Being a rather complicated methodology in the beginning, today's Ussing chambers are smaller, simplified and have revolutionised research within the field of intestinal permeability (Glasser, A L et al 2001). The technique makes it possible to study the transport of a wide range of substances across the intestinal mucosa and in combination measure electrophysiological parameters, like TER and short circuit current (Isc), and hence providing a thorough investigation of the permeability status of the intestinal specimen.

The technique is based on two half chambers, between which a surgical specimen or biopsy is mounted. The chambers are filled with a continuously oxygenated (95% O_2, 5 % CO_2) buffer, through a system that provides efficient mixing of the fluid and reduces the thickness of the unstirred water layer to physiological levels (Karlsson, J. et al. 1992). The chambers are kept at 37°C and monitoring of electrophysiological parameters is managed by two pairs of electrodes (figure 8).

A marker solution is added to the mucosal buffer, and at defined time intervals samples are redrawn from the serosal buffer as a measurement of passage across the intestinal mucosa. Several different types of markers can be used and mannitol and Cr^{51}-EDTA are examples of paracellular markers, where the latter is used in our laboratory together with horse radish peroxidase (HRP), as a measurement of transcellular passage.

The Ussing chamber technique was the main method in paper I, where the transport of nanoparticles was studied after stimulation of *Yersinia pseudotuberculosis* and paper II, where the effect of infliximab on the translocation of the AIEC strain, HM427, across the intestinal mucosa was elucidated (for experimental set up see individual papers).

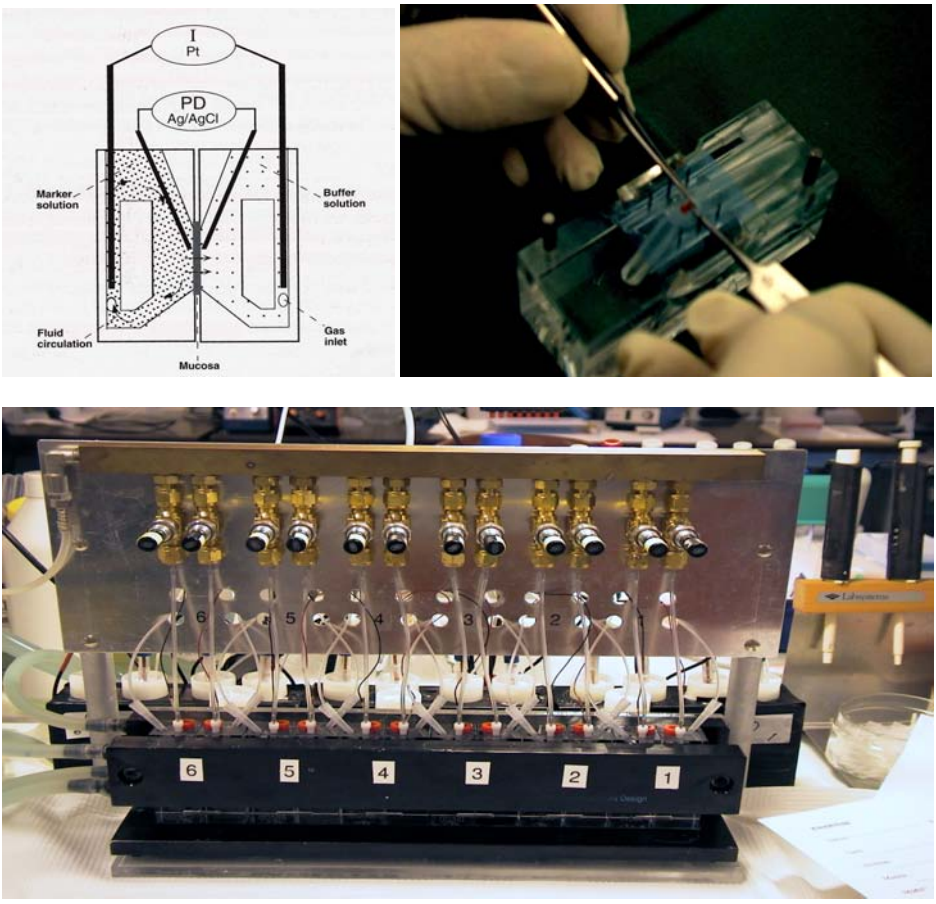


Figure 8. The Ussing Chamber system as used in the lab. Top left panel: Schematic illustration of the Ussing Chamber. Biopsies and surgical specimens are mounted in the chamber and a marker solution is added to the mucosal side of the tissue segment. One pair of electrodes monitors the potential difference during the experiment, while another set supply a current to the system. Top right panel: Mounting of an endoscopic biopsy in the Ussing Chamber. Lower panel: After mounting of biopsies, the chambers are filled with buffer and placed in the aerated 37°C Ussing chamber system.

3.1.1 Electrophysiology

The ability to maintain a transepithelial potential difference (PD) is a characteristic shared by all transporting epithelia and is dependent on the activity of all the electrogenic ion pumps generating a current across the cell membrane in combination with the epithelial barrier, mainly the TJs.

By looking at the epithelium as a parallel circuit consisting of paracellular and transcellular pathways the PD can be separated into short circuit current (Isc) and TER, based on Ohm's law (U= R • I), where Isc represents the current needed to nullify the PD and is dependent on the activity of the ion pumps and TER reflects the resistance of the paracellular route, hence mainly the TJs.

The electrophysiological parameters are monitored during the experiment by one pair of electrodes (connected via agar-salt bridges) which measure the spontaneous PD and one pair of platinum electrodes supplying current to the system. Since active ion transport requires energy production generally in the form of ATP, the basal PD or Isc can be used as a measurement of tissue viability. By passing the current (Isc) through the epithelium, the change in PD can determine the TER by Ohm's law PD= TER • Isc.

3.1.2 Considerations of the Ussing Chamber technology

The invention of the Ussing chamber has been ground-breaking for permeability studies not only in the field of gastroenterology. The Ussing chamber provides an excellent tool for thorough determination of changes in, as well as transport across, the intestinal epithelium upon stimulation by any substance of interest. The technique makes it possible to elucidate the transport across the entire mucosa and not only a single cultured cell monolayer. It also allows studies of different areas of the intestine of which suitable cell culture models are difficult to find. The system also enables thorough investigation of the transcellular and paracellular pathway by monitoring the electrophysiological parameters in combination with transport studies of different probes for the respective pathways. Thus, it gives a more detailed description of the condition of the mucosa during and after the experiment as compared to cell culture studies. The technique does suffer from several disadvantages. The most obvious being extraction of the mucosa from its normal environment resulting in deprivation of its circulation, lymph drainage and neuroendocrine regulation, which of course will affect the specimen and most likely also the permeability. In addition the measurement of the electrophysiological parameters is based on viewing the otherwise complicated epithelium as a parallel circuit where TER is considered a measurement of the paracellular permeability only. Given that the epithelium is not a static material, this simplification can result in a miscalculation of the TER as well as Isc. Aside from this, the method relies on careful handling of the tissue, in terms of transport to the laboratory as well as mounting it correctly in the chamber, to be able to generate reproducible results. To avoid misinterpretation of the experiments it is therefore important that the technique is performed in combination with other methods, like

confocal microscopy, to verify that the epithelium is still intact after performing the experiment. Cell culture experiments are also often used to confirm and verify results generated from Ussing chambers.

3.2 Cell culture experiments

Culturing of cells from diverging origins is pertinent in biomedical research. It provides an excellent milieu for studying signalling pathways, protein interactions as well as transcription of any gene of interest. In this thesis cell culture experiments were used for two purposes, transport studies across model epithelia (paper I) and for identifying particular protein interactions (paper IV).

In paper I cultured model epithelia of the human colonic adenocarcinoma cell line Caco-2 was used to address the question of what effect *Yersinia pseudotuberculosis* has on the absorptive villus epithelium surrounding the FAE. As FAE is mainly expressed in ileum, a site common for development of CD, we chose to use the Caco-2 cell line, which is able to differentiate into ileum like epithelium. Caco-2 cells were grown on permeable filters and after exposure to *Yersinia pseudotuberculosis* the transport of nanoparticles was measured across the monolayer (figure 9) (for detailed experimental set up see paper I).

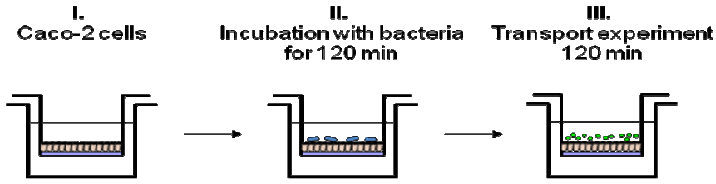


Figure 9. Experimental set up of transport experiment: Briefly, Caco-2 cells were grown on filters for 18-22 days, after differentiation to ileum like epithelium *Yersinia pseudotuberculosis* was added to the apical side of the epithelium and incubated for 120 minutes. Bacteria were then removed, epithelia washed and nanoparticles were added to epithelium. After another 120 minutes, samples were redrawn from the basolateral compartment and assessed for flow cytometry in order to determine the transport ability of the epithelium.

Caco-2 cells are widely used as a model system for the intestinal epithelium. Although being of colonic origin the cells spontaneously differentiate, when grown under standard conditions, into a polarized monolayer expressing common features of the small intestine. This is characterized by the formation of domes, apical brush borders and the ability to synthesize hydrolases, such as sucrose-isomaltase, lactase, and α_1-

antitryptase (Chantret, I. et al. 1988; Kenny, A. J. et al. 1982; Molmenti, E. P. et al. 1993). Similar findings have been observed by studying the different properties of the specialised membrane called the surfactant-like protein (SLP), synthesized by both enterocytes and colonocytes. Engle et al showed that the small intestine associated protein α_1-antitryptase increased rapidly after confluency and remained high, while the surfactant protein A (common in the SLP of colon) decreased even after a third day of confluency (Engle, M. J. et al. 1998). Thus, the features of the Caco-2 cells are dependent on the time of differentiation. Nevertheless, it is important to conclude that differentiated Caco-2 cells do not express several genes associated with mature enterocytes, like glucoamylase and angiotensin-1 converting enzyme and hence can only be viewed upon as a simple model of the human small bowel epithelium. In addition it should be emphasized that the cell line is derived from a colonic adenocarcinoma and carries mutations in genes altering intracellular signalling, which might affect the cell monolayers ability to transport antigens.

Several other cell lines do exist and in addition to Caco-2, the colon adenocarcinoma cell line HT-29 has been shown to undergo small bowel properties, as those characterized for Caco-2, when cultured in glucose free medium (Augeron, C. et al. 1984; Wice, B. M. et al. 1985; Zweibaum, A. et al. 1985; Zweibaum, A. et al. 1983). This cell line could therefore have been used as an alternative to Caco-2 in these experiments. However, they do not express an apical brush border, instead, irregular microvilli can be observed. Other cell lines also exist, like the Int 407 (also known as I407) cell line of human origin, isolated from jejunum and ileum from a two month old human embryo. This cell line has been shown not to differentiate to the same extent as Caco-2 (Ismail, M. 1999), which can be due to the fact that the cell line was established by co-culturing the embryonic cells with the human cervix carcinoma cell line, HeLa, known to be capable of overgrowing many other cells in mixed cultures (Gartler, S.M. 1968). Further, the Health protection agency, representing several clinically oriented culture collections including the European Collection of Cell Cultures, list int 407 as a cell line possible misidentified as it has been found to be genetically indistinguishable from HeLa cells and therefore suggests that this cell line should be considered a HeLa cell line.

In paper IV, cell culture experiments were performed to elucidate particular protein interactions through immunoprecipitation and affinity assays. Immunoprecipitation assays were performed in the colonic adenocarcinoma cell line SW480, which organizes into multilayers without any enterocytic differentiation (Chantret, I et al

1988), and was specifically chosen as it is known to expresses endogenous Nod2 (Hisamatsu, T. et al 2003).

Immunoprecipitation assays are commonly used to elucidate interactions between different proteins in the cell and were used in this paper to identify a binding of endogenous Nod2 to ubiquitin (see paper IV for experimental set up.) By using an antibody that targets the protein of interest, its interactions with other proteins can be determined by immunoblotting (figure 10a). For accurate and reliable results the technique is dependent on highly specific antibodies. This can be a problem when investigating proteins towards which there are no antibodies available or the antibody existing is of poor quality. To avoid this problem recombinant DNA technology is used to label proteins with a polypeptide protein tag, like FLAG, Myc, haemagglutinin (HA) or polyhistidine (His), which are expressed in the human embryonic kidney (HEK) 293T cell line. These tags make both proteins highly specific for antibodies or affinity beads generated towards the tag, which subsequently can be used to target the protein studied (figure 10b).

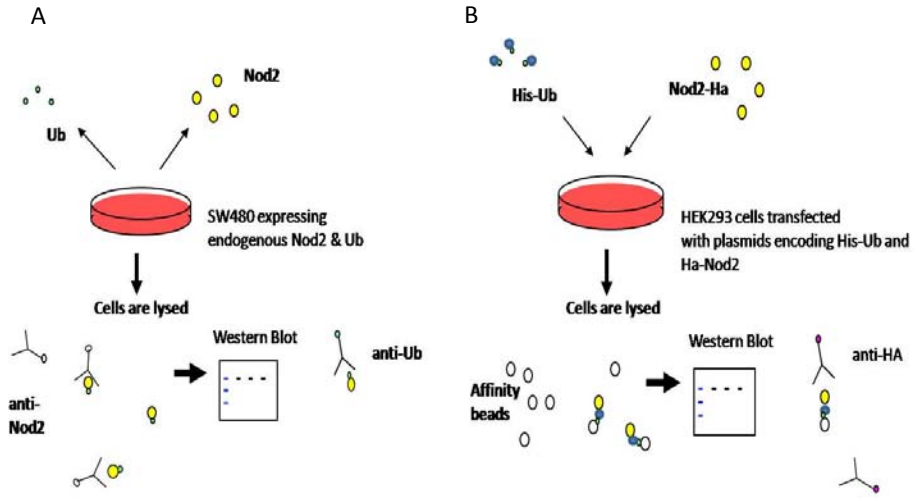


Figure 10. Schematic drawing illustrating the different principals for immunoprecipitation (A) and affinity assay (B). Immunoprecipitation assays rely on antibodies directed towards the protein of interest for the detection of protein-protein interactions, while affinity assays are more specific and involves tagged proteins and highly specific antibodies directed towards these tags.

Even by improving the technique, it is still possible that in affinity assays other proteins bridge the two proteins of interest together and thus give a false positive result. Other problems involve the correct incorporation of the polypeptide protein tag and correct expression of the tagged protein. In addition, antibodies produced towards the tag, sometimes only recognize the target protein when fused with the protein at a certain position, for example the N or C-terminal. It is therefore crucial to include appropriate controls and to view results from immunoprecipitation assays with caution, as this method relies on overexpression of proteins in cells constituting an environment clearly different from the normal surrounding of the protein.

3.3 Genotyping assays

To study the SNPs of interest in paper III we used three different genotyping assays, which all are based on the polymerase chain reaction (PCR).

To identify SNPs present in the genes encoding NALP3, CARD8 and Nod2, the conventional TaqMan® Genotyping assay was used. The assay involves the presence of two probes conjugated each with a fluorescent marker, with the capacity to bind specifically to the SNP of interest or to the wild type allele. Upon interaction, the marker is released from the probe and generates a fluorescent signal specific for the wild type allele or the allele carrying the SNP of interest. This method is rapid and very effective as a large number of samples can be analyzed at the same time. However, the method relies on pre-generated probes, and hence the manufacturer sets a limit to which genes can be analyzed.

As there was no probe available for the frameshift mutation, L1007fs, which results from the incorporation of an extra nucleotide, it was detected by Megabace™ SnuPe genotyping kit and separated from the wild type allele in a size dependent manner by using denaturating high performance liquid chromatography (dHLPC) (WAVE®Transgenomics).

The Megabace™ SnuPe genotyping kit mediates the incorporation of a fluorescent stop nucleotide at the position where the extra inserted nucleotide is present in the variant allele. Detection of the frameshift variant could then be detected by evaluating the fluorescence of the sample in the Megabace™ 100 DNA. Even though accurate identification of the frameshift mutation is achieved, the method is time-consuming and consequently we continued evaluating the samples by dHLPC. In this study the length of the generated PCR product was used to separate the wild type from the variant allele.

By performing a primer extension assay a specific stop nucleotide detecting the inserted nucleotide prevent further transcription of the DNA strain which leads to a difference in size between the variant and wild type allele. By keeping a high denaturating temperature the DNA remains single stranded and different lengths can be detected. Although, being a reliable and consistent technique samples that were considered positive for the variant allele were confirmed by sequencing.

4. RESULTS AND DISCUSSION

This thesis is based on four papers, each of which gives a different perspective on the bacterial-epithelial interaction associated with a dysfunctional intestine and CD. Detailed descriptions of the results are given in each respective paper. Briefly, Paper I suggests a role for the absorptive villus epithelium in the small bowel as an entry route for antigens via macropinocytosis after exposure to pathogenic bacteria. In paper II, we present evidence that infliximab not only restores the paracellular permeability of the colon mucosa of affected subjects, but also decreases the translocation of the adherent invasive *E.coli* (AIEC) HM427. Paper III and IV emphasize the role of the NLRs in the pathogenesis of CD, showing that common polymorphisms in *NALP3* and *CARD8* in combination are associated with CD among Swedish men and, in addition, demonstrate that the Nod2 protein is regulated via the ubiquitin-proteasome pathway.

4.1 Paper I: Small bowel- like enterocytes can be primed to sample antigens upon interaction with *Yersinia pesudotuberculosis*

This study shows that *Yersinia pseudotuberculosis* can induce uptake of nanoparticles via macropinocytosis in small bowel-like enterocytes as well as in ileal surgical specimens, findings that further support the existence of an antigen sampling mechanism independent of M-cells and Payer's patches.

Recently, it observed that M-cells can develop in mice lacking Payer's patches (Jang, M. H. et al. 2004). Apical expression of β1-integrin has been suggested to be a specific feature of M-cells as this particular integrin has only been identified on the basolateral surface of enterocytes in the villus epithelium (Schulte R et al 2000; Isberg RR et al 1990). In agreement with this, Jang et al observed that the M-cells residing in the villus epithelium were able to internalize *Salmonella, Yersinia* and *E.coli* expressing invasin as well as gut bacterial antigens.

M-cells developing in the villus epithelium might therefore represent an alternative pathway for the induction of antigen-specific immune responses.

Here we report that enterocytes residing in the absorptive villus epithelium can express M-cell like features after exposure to *Yersinia pesudotuberculosis,* as measured by the expression of β1-integrin as well as gained endocytotic ability. These findings support the notion that M-cells might develop from enterocytes under inflammatory conditions.

As previously mentioned, we also observed an increased ability of enterocytes to internalize nanoparticles via macropinocytosis after bacterial exposure. This conclusion was based on transport experiments performed in the presence of the following inhibitors affecting specific pathways involved in endocytosis; Chlorpromazine (clathrin-mediated endocytosis), Methyl-β-cyclodextrin (caveolae-mediated endocytosis), Nystatin (caveolae-mediated endocytosis and macropinocytosis) and 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), which previously has been used as a selective inhibitor of macropinocytosis. In the present study, all of them had an inhibitory effect on the nanoparticle transport. These findings underline the difficulty in determining specific endocytotic pathways. Macropinocytosis is particularly challenging to study, as there are no markers or drugs available that specifically interfere with this process. It has been shown that this pathway require proteins common to other endocytotic pathways and therefore all of the used inhibitors may have inhibitory effects on macropinocytosis (Jones, A. T. 2008). Thus, we cannot exclude the possibility that the observed internalization of nanoparticles involve other endocytotic pathways. However, based on previous knowledge according to the sizes of the endocytotic vesicles, macropinocytosis seems to be the most suitable process for nanoparticle uptake. Vesicles formed in caveolae-mediated endocytosis are reported to be only 5-10nm and would therefore not be able to transport the 150nm particles used in this study. Clathrin-mediated endocytosis requires the recruitment and assembly of clathrin at the plasma membrane, triggered by external stimuli preferentially by the subject undergoing endocytosis and is therefore unlikely to be involved in the internalization of the nanoparticles. Moreover, antigen sampling in immature DCs has been observed to be mediated via macropinocytosis. This process has also been shown to increase upon various stimuli, which further strengthens our suggestion that the nanoparticle uptake is mediated via macropinocytosis (Amyere, M et al 2002; Amyere, M et al 2000; Hamasaki, M et al 2004; Lanzetti, L et al 2004; Meier, O et al 2004; Yang, Z et al 2005). Nevertheless, further studies are needed to exclude the involvement of other pathways. For example, dynamin has been reported to be a competent inhibitor

of clathrin-mediated endocytosis, which could be used to further elucidate the nanoparticle uptake. By using specific antibodies towards clathrin and caveolin microscopy studies may reveal involvement of these proteins.

In conclusion, we have identified a possible mechanism for enterocytes of the absorptive villus epithelium to participate in antigen sampling upon interaction with *Yersinia pseudotuberculosis.* These results have also been confirmed in human ileal tissue, where EIPA inhibited transport of nanoparticles. Thus, it is also possible that antigen uptake might increase upon exposure to other enteric bacteria. In support of this notion, it has been reported that *Salmonella typhimurium* induces transcytosis of flagellin across polarized epithelial cells (Lyons, S. et al. 2004). Internalized flagellin then interacts with TLR5, which leads to the production of pro-inflammatory cytokines. In addition, several other bacterial species express invasin, which interacts with β1-integrin, and might affect the absorptive epithelium in the same way as *Yersinia pseudotuberculosis.* It is also possible that there are other receptors which upon interaction with specific bacteria induce antigen uptake via enterocytes. Recently, AIEC was shown to bind CEACAM6, however whether this interaction results in increased antigen sampling is not known. Thus, further investigation is needed to fully understand the implications of the results presented here. Of particular interest would be to elucidate if commensal bacteria could induce antigen uptake via macropinocytosis across the mucosa of CD patients.

4.2 Paper II: Infliximab decrease translocation of the invasive adherent *E.coli* (AIEC) strain HM427 across the colonic mucosa of patients suffering from severe Crohn's colitis.

During the last decade infliximab has become widely used as standard therapy of patients suffering from severe CD. Several reports have reported the beneficial effects after treatment, but none of them have investigated the effect of infliximab on the translocation of bacteria.

Intraluminal bacteria constitute the major inflammatory driving factor behind the inflammation associated with CD and it is therefore of great importance to elucidate the effects of infliximab on the translocation of bacteria across the intestinal mucosa. As the colon represents one of the most common locations for development of CD, we chose to investigate whether infliximab treatment alters the adherence and invasion ability of the adherent invasive *E.coli*, HM427, found specifically in the colonic mucosa of affected individuals.

AIEC has previously been suggested to represent a strain present in the normal gut flora that preferentially colonizes the mucosa of CD patients. Here we report that infliximab decreases the translocation of the AIEC strain HM427 across the intestinal mucosa of CD patients.

Further, we confirmed the previously observed positive effects of infliximab on the paracellular permeability. As noted in paper II we found a decreased transport of $Cr^{51}EDTA$ after infliximab treatment. A recovery of the barrier function in terms of paracellular permeability was also observed by measurements of TER, which was closer to control values after treatment. Studying the changes in TER during the first thirty minutes of bacterial exposure revealed a normalisation in the infliximab-treated group compared to controls. Similarly, the Isc values were observed to move closer to those of the controls after treatment.

In order to exclude paracellular passage of bacteria confocal microscopy studies need to be performed to evaluate the integrity of the tight junctions. Furthermore, this would reveal any redistribution of proteins constituting the TJs. Previous results have shown that anti-TNFα treatment does not alter the expression of the common TJ proteins occludin, claudin 1 and 4 (Zeissig, S et al 2004). However, it has not been elucidated if bacterial exposure after treatment rearranges the TJ proteins. It is therefore essential that the above mentioned microscopy studies are performed. Increased amounts of TNFα have previously been observed to disrupt TJs via the interaction of MLCK. The restoration of the paracellular permeability might therefore be due to less free TNFα molecules and thus a reduced disprution of TJs. Additionally, a decrease in the number of translocated bacteria will result in a diminished inflammatory response, including production of TNFα. Infliximab might therefore exert its positive effect on the paracellular permeability by not only limiting the number of free TNFα molecules, but also by diminishing the internalisation of intraluminal bacteria. It has previously been observed that IFNγ induced expression of TNFR2 is required for Caco-2 cells to respond to TNFα. Recently, it was reported that infliximab enhances the production of TNFR2, resulting in increased release of the receptor, which potentiates the effect of infliximab (Ebert, E. C. 2009). It would therefore be interesting to investigate how exposure of bacteria after treatment affects the expression pattern of TNFR2.

Our results show less translocation of HM427 across the colonic mucosa of CD patients after anti-TNFα treatment. However, the mechanism behind the decreased translocation still needs to be elucidated.

When characterised by Martin H et al, HM427 was observed to adhere to rather than invade the intestinal model epithelia, as shown by the finding that 80% of the original inoculum was adherent to the I407 monolayer after five hours, while only 2% were invasive.

In the present study, we have not investigated the mechanism via which adherence of HM427 occurs and whether this process is altered after anti-TNFα treatment. It is therefore possible that even though the invasion of the bacteria is decreased, the amount of bacteria adhering to the mucosa is unchanged. It would therefore be interesting to perform a gentamicin assay to elucidate the number of intramucosal bacteria. Recently it was shown that the ileal AIEC strain LF82 adhere to the intestine via an interaction between type 1 pili and CEACAM6, which is expressed in the intestinal mucosa. However it remains to be elucidated whether CEACAM6 is involved in the adherence and internalisation of HM427. HM427 does express type 1 pili and it is therefore highly possible that an interaction via CEACAM6 mediates adherence and internalisation of the bacteria. In addition, several reports have identified expression of CEACAM6 in the colon, which have been shown to be enhanced in the presence of IFNγ (Baranov, V. et al. 1994; Fahlgren, A. et al. 2003).

The mechanism by which invasion of HM427 is mediated also needs to be investigated. In the present study we provide data suggesting that HM427 is taken up via active transport as colchicine reduces the translocation of bacteria in non-treated CD patients. Further experiments, using additional endocytotic inhibitors, therefore need to be performed to identify the specific pathway via which HM427 is taken up.

In conclusion, it is tempting to speculate that the preference of HM427 to adhere rather than invade the epithelial monolayer might trigger a bacterial uptake of antigens via a similar mechanism as suggested for *Yersinia pesudotuberculosis* in paper I. Although, this study focuses on the colonic epithelium, it would be of interest to study HM427's ability to induce endocytosis of antigens and bacterial products.

4.3 Paper III: The inflammasome components NALP3 and CARD8 are associated with Crohn's disease in Swedish men

Here, we report that common polymorphisms in the genes encoding NALP3 and CARD8 are associated with an increased risk of developing CD among men, but only when carried in combination. These data strengthen the common knowledge that the NLRs constitute important factors in the development of the disease.

An interesting observation is that these polymorphisms only confer susceptibility among men, hence suggesting that different mechanisms might participate in disease development in men and women, respectively. However, in order to understand the impact of these observations on the course of the disease they have to be verified in larger cohorts. Villani, A C et al (2009), recently published a thorough report showing that common polymorphisms in the *NALP3* gene do not confer susceptibility to Crohn's disease. This is in agreement with our results as the Q705K polymorphism does not confer susceptibility on its own, but only in combination with the C10X polymorphism in the *CARD8* gene. It would therefore be of interest to elucidate whether the investigated variants of NALP3 as well as the Q705K also contribute to susceptibility in combination with the *CARD8* polymorphism in a larger cohort. This would be necessary in order to exclude the possibility that the results presented here are just established by chance. Nevertheless, our data further emphasize the complexity of the disease and provide additional perspectives that might have to be taken into consideration to fully understand the pathogenesis of CD.

To further emphasize the results presented here, functional studies are essential. Of special interest is to elucidate whether the Q705K polymorphism is a gain of function mutation, resulting in an overactive NALP3 inflammasome. This would provide essential information and further strengthen the hypothesis that different mechanisms might be of importance for disease development in men and women. In support of this, estrogen has been shown to exert anti-inflammatory effects. Recently, estrogen was shown to decrease the expression of the Macrophage migration inhibitory factor in female rat colon leading to a reduction of IL-1β production (Houdeau, E. et al. 2007). Furthermore, estradiol has been shown to down-regulate the IL-1 receptor type I in uterine epithelial cells and thereby limiting the cells' ability to respond to IL-1β (Schaefer, T. M. et al. 2005). It is therefore possible that estrogen plays a role in modulating the proinflammatory response in women, hence suggesting that other mechanism and signalling cascades might be of greater importance for development of disease among women.

Recently, the estrogen-responsive B box protein (EBBP) was shown to be associated with an enhanced secretion of IL-1β as well as interacting with caspase-1, NALP1 and pro-IL-1β (Munding, C. et al. 2006). EBBP is expressed in various tissues, including colon and its mRNA expression is upregulated by estrogen (Liu, H. L. et al. 1998). Thus, EBBP is a fascinating protein to study as it might also interact with NALP3. Of great interest would be to elucidate whether women suffering from Crohn's disease have an

increased expression of EBBP compared to men, which could lead to an overactive inflammasome.

Lately, the involvement of CARD8 as a component of the inflammsome complex has been debated. In 2004, Agostini et al showed that endogenous expressed NALP3 co-immunoprecipitates with CARD8 in THP-1 cells. However, they fail to identify an interaction in transfection experiments using tagged NALP3. In addition, CARD8 is no longer included as a member of the inflammasome in recent reviews by the same group (Petrilli, V. et al 2007). The primary function of CARD8 might therefore be as an inhibitor of NFκB as well as apoptosis. It would therefore be interesting to investigate whether combined polymorphisms of C10X *CARD8* and *EBBP* in combination confer susceptibility to CD among women.

In conclusion, our data support recent findings suggesting that different cytokine expression patterns exist in men and women. It would therefore be essential to investigate the IL-1β levels among male and female patients. A different expression pattern could also explain the fact that certain patients do not respond to anti-TNFα treatment. Furthermore, our results raise the question of whether these patients would rather respond to IL-1 targeted therapy. Moreover, the NALP3 inflammasome might represent a molecular platform important for the development of disease in populations where the Nod2 variants are uncommon, as the data presented here are in the presence of a wild type *Nod2* gene.

4.4 Paper IV: The Crohn's disease associated Nod2 protein is regulated via the ubiquitin-proteasome pathway

The identification of the ubiquitin-proteasome pathway as a regulatory mechanism of the Nod2 protein might represent an important finding that may shed further light on how the common CD variants of this gene cause increased NFκB activation.

The importance of ubiquitination has previously been emphasized as the NLR family member CIITA is known to undergo ubiquitination and subsequently degradation by the proteasome. Furhtermore, TLR4 and 9 have been shown to be regulated via ubiquitination through involvement of the E3 ligase Triad3A.

Here we report that Nod2 is ubiquitinated and degraded via the proteasome. This action was further found to be mediated through the involvement of the E2 enzyme UBE2G2. In addition, Nod2 ubiquitination seems to increase upon stimulation of MDP. Even though our results show that the L1007fs mutation is ubiquitinated to the same

extent as the wild type protein, the protein turnover for the variant has not been elucidated. In addition, it is important to investigate whether Nod2 undergo mono- or poly-ubiquitination via attachment of lysine residue 48 or 63. As previously mentioned, ubiquitination via K48 is generally associated with proteasomal degradation, while linkage via K63 has more regulatory functions in the cell.

In support of this, Greer et al propose a model where mono-ubiquitination enhances the transcriptional potency of the protein and poly-ubiquitination leads to proteasomal degradation (Greer, S F et al 2003). However, they do not identify whether this occurs via residue K48 or K63. Even though UBE2G2 has been shown to promote poly-ubiquitination via K48 linkage (Li, W. et al. 2007), it would be interesting to identify whether Nod2 undergo the same regulation as CIITA. Mono-ubiquitination at K48 or K63 might be needed as a second signal for Nod2 activation, promoting Nod2's ability to sense MDP or possibly the ability to mediate autophagic clearance of endocytosed antigens.

Recently, autophagy induced by *S. flexneri* was shown to be enhanced in the absence of Ipaf (Suzuki, T et al 2007), thus, suggesting a possible link between the NLRs and autophagy. As Nod2 functions as an intracellular receptor for bacterially-derived MDP, it would be of great interest to study whether Nod2 promotes autophagy. If so mono-ubiquitination of Nod2 might act as a trigger of this particular process.

To elucidate whether the identified ubiquitination of Nod2 is implicated in the pathogenesis of CD it would be of great importance to more thoroughly study the ubiquitination of the identified Nod2 variants. It is possible that wild type Nod2 as well as the L1007fs variant is able to undergo poly-ubiquitination, however they might differ in their ability to undergo mono-ubiquitination. Additionally, it would be of great interest to investigate the ubiquitination of the other genetic variants of Nod2 associated with CD.

A possible explanation to equal ubiquitination observed, could be that both wild type Nod2 and the L1007 variant can undergo poly-ubiquitination, however they might differ in their ability to undergo mono-ubiquitination. In addition the degradational rate of the Nod2 variants would be of interest to study.

In conclusion we have identified the pathway via which Nod2 is regulated. It is therefore tempting to speculate that other NLRs might be regulated via the same pathway. Consequently, we also present a new pathway that might be of importance in the development of CD and consequently polymorphisms in genes encoding proteins regulating Nod2 may contribute to the susceptibility to Crohn's disease.

5. CONCLUSIONS

- Exposure of invasin expressing *Yersinia pseudotuberculosis* results in increased trancytosis of nanoparticles across the Caco-2 monolayer and ileal mucosa. This process is possibly mediated via macropinocytosis.

- Upon interaction with invasion-expressing *Yersinia pseudotuberculosis* β1-integrin expression is increased and redistributed to the apical side of Caco-2 monolayers and ileal tissue.

- Infliximab decreases the elevated paracellular permeability associated with active disease in patients suffering from severe Crohn's colitis, as measured by ^{51}Cr-EDTA flux.

- Treatment with infliximab reduces the translocation of the adherent invasive *E.coli* across the mucosa of Crohn's colitis patients.

- The Q705K polymorphism of *NALP3* and C10X polymorphism of *CARD8* do not confer susceptibility to Crohn's disease on their own, but in combination they increase the risk for men to develop Crohn's disease in a study population comprising 498 Swedish patients.

- The Nod2 protein is ubiquitinated and degraded via the proteasome, a process possibly mediated via the E2 enzyme UBE2G2.

6. SUMMERIZING DISCUSSION

The results presented in this thesis open up new perspectives to be taken into consideration to completely understand the complexity of CD.

In this thesis, evidence is presented that suggests that enterocytes of the absorptive villus epithelium upon bacterial infection can be triggered to antigen sample via macropinocytosis. This additional pathway of antigen sampling might be of importance in patients suffering from CD, where a dysfunctional barrier is present. Indeed, commensal bacteria have been shown to be a threat for epithelia under stress. This would result in an increased amount of bacterial antigens in the cytosol and hence more stimuli for intracellular receptors like the NLRs. In individuals expressing a wild type Nod2 protein this would lead to the activation of NF-κB and thus an inflammatory response to clear the bacterial infection. Moreover, Nod2 might act as a promoter of autophagy of macropinocytosed antigens. In order for this to occur a secondary signal may be necessary, in the form of mono-ubiquitination. In the epithelium of CD patients carrying mutations in the gene encoding Nod2, this may result in the loss of mono-ubiquitination and hence autophagic clearance of antigens could not occur. A second possible mechanism is that mono-ubiquitination is needed in order for Nod2 to be activated by MDP. Patients expressing a defect in their Nod2 protein might therefore be able to activate the protein but not mediate poly-ubiquitination. This would result in a constitutively active Nod2 protein able to activate NF-κB but not able to undergo degradation by the proteasome. However, Nod2 mutations alone cannot explain the pathogenesis of CD, as the most common mutation (L1007fs) is present in <30% of affected individuals. In patients suffering from CD, who express a wild type form of Nod2 dysfunction of other NLRs might be of importance. In support of this we demonstrate that polymorphisms in the genes encoding the NLR family member NALP3 and CARD8 contribute to CD among men. It is therefore possible that increased IL-1β levels in patients carrying the wild type gene of Nod2 might be explained by the presence of an overactive NALP3 inflammasome caused by the expression of mutated forms of NALP3 in combination with a CARD8 not being able to act as a suppressor of NF-κB activation. These results also emphasize that a gender difference in the development of CD might exist, as the combined genotypes of *NALP3* and *CARD8* only confer susceptibility in men.

To further emphasize that enhanced bacterial uptake is an important driving factor behind the inflammation of CD, we observe a reduced translocation of adherent invasive *E.coli* across the intestinal mucosa of patients suffering from Crohn's colitis after anti-TNFα treatment.

In conclusion, mechanisms of bacterial-epithelial interactions are crucial for the development of CD. The present thesis opens up new perspectives of the pathogenesis of CD which may shed further light on the complexity of the disease. However, further research is necessary to fully understand how CD develops. Of special interest is to elucidate the mechanisms by which the NLRs contribute to disease outbreak and an enhanced inflammatory response in terms of cytokine production.

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8. SVENSK SAMMANFATTNING

Crohns sjukdom är en kronisk inflammatorisk tarmsjukdom av okänd orsak. Flera faktorer har dock identifierats som bidrar till sjukdomens förlopp. En genetisk predisposition har visat sig vara av stor betydelse och tros orsaka en störd tarmbarriär och/eller inflammatoriskt svar mot invaderande bakterier samt antigen. Hos en predisponerad individ gör tarmbakterier att en inflammation drivs på och dessa två faktorer i kombination tros orsaka sjukdomen.

För övervakning av tarmlumen sker vanligtvis ett kontrollerat upptag av bakterier och antigen via M-celler i det så kallade follikel associerade epitelet i tunntarmen. Det omgivande villus epitelet har ofta antagits att ha en mindre roll i upptag av antigen. I det första arbetet presenterar vi dock resultat som tyder på att enterocyter lokaliserade till villusepitelet kan ta upp nanopartiklar via macropinocytosis efter att de utsatts för *Yersinia pseudotuberculosis.* Denna observation tyder på att andra vägar för antigenupptag existerar i tarmen, vilka kan spela en betydande roll vid sjukdomar som Crohns där bakterier har en central roll. Med anledning av detta är det därför av särskilt intresse att vi i arbete II observerar en normalisering av den ökade passagen av den adherenta invasiva *E.coli* stammen HM427 över tjocktarmen hos Crohnpatienter, efter anti-TNFα behandling.

Det finns också genetiska bevis för att sjukdomsutvecklingen till stor del beror på en störd interaktion mellan bakterier och slemhinna. Till exempel är det känt att varianter i genen som kodar för Nod2 ökar risken för insjuknande i Crohns sjukdom. Nod2 är en intracellulär receptor som bidrar till det inflammatoriska svaret i och med igenkänning av bakteriepeptider. Flera Nod-lika receptorer har visat sig vara associerade med Crohns sjukdom och denna grupp av proteiner utgör därför en viktig faktor att studera. I arbete III visar vi att förändringar i de gener som kodar för beståndsdelar i den så kallade NALP3 inflammasomen, en plattform för igenkänning av nedbrytningsprodukter från tarmen, bidrar till Crohns sjukdom hos män. Detta ger dessutom en inblick i att orsakerna till utvecklingen av Crohns kan skilja mellan män och kvinnor. Avslutningsvis har vi identifierat ubiquitin-proteasomevägen som den mekanism via vilken Nod2 regleras i cellen. Dessa båda arbeten bidrar tillsammans till en ökad förståelse för hur varianter i gener associerade med Crohns sjukdom kan påverka signalvägarna i cellerna och därmed bidra till den kroniska inflammationen.

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