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Innate immune defence: NOD2 and autophagy in the pathogenesis of Crohn's disease

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Summary

Crohn's disease (CD) is a chronic inflammatory disorder of the gut with a poorly understood aetiology. Epidemiological studies suggest that the disease occurs in genetically susceptible individuals as a consequence of defects in mucosal barrier function and disregulated immune recognition of commensal gut flora. Of more than 30 genetic loci associated with CD, two genes with important polymorphisms, encoding the intracellular bacterial sensor NOD2/CARD15 and the autophagic regulator ATG16L1, have gained particular prominence as they suggest an important paradigm of CD pathogenesis. Both proteins exert crucial functions in innate immune defence through intracellular bacterial recognition and destruction of bacteria. This review focuses on the physiological functions of the protein products of both genes and discusses how innate immune defences are linked to autophagic processes through recruitment of ATG16L1 by the bacterial sensor NOD2 at sites of microbial infection.

Key words: *intracellular sensor; autophagy; Crohn's disease; NOD2/CARD15; ATG16L1*

Introduction

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal mucosa that presents clinically most often with (bloody) diarrhoea, abdominal cramping and weight loss. Mucosal inflammation can affect any segment of the gastrointestinal tract and frequently exhibits a discontinuous anatomic pattern along the gut axis, characterised by aphthous lesions, ulceration, and commonly transmural granulomatous inflammation. CD is one of the two major manifestations of inflammatory bowel disease (IBD) with an estimated prevalence of 50–200 patients per 100 000 persons in Western countries. The precise aetiology of CD is not well understood but is thought to be related to a combination of genetic and environmental factors that impact on normal host-microbe interactions. Such interactions are of particular importance in the gastrointestinal tract, as it is not only the major entry portal for environmental microbes into the body but it is also continuously exposed to a vast normal microbial flora in the intestinal lumen.

Several mechanisms are involved in maintaining a physical and functional barrier between luminal microbes and the interior of the body. Of particular importance is the mucosal immune system, which can be functionally divided into innate and adaptive immunity. Each system of

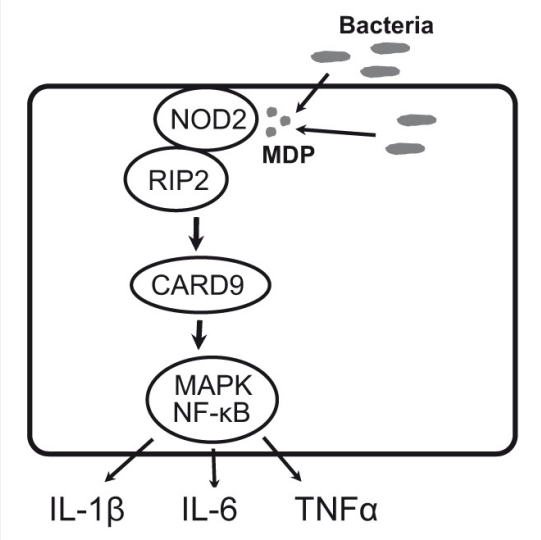

Figure 1

Innate immune activation through NOD2. Muramyl dipeptide (MDP) present in the cell wall of Gram positive and Gram negative bacteria activates the intracellular sensor NOD2 (CARD15), which interacts through CARD-CARD binding with RICK/RIP2 (CARD3) and with CARD9. Activation of several other adaptor proteins leads to activation of NF-κB and mitogen activated protein kinases (MAPK; JNK, p38, ERK) and secretion of proinflammatory cytokines.

immunity relies on different cell types, gene products, and distinct modes of action. Innate immunity is evolutionarily much older than adaptive immunity, and is found in practically all multi-cellular organisms, while adaptive immunity is limited to jawed vertebrates and their descendants (cartilaginous and bony fish, reptiles, birds, and mammals). Rapid host defence against invading bacteria involves primarily innate immune mechanisms. Recognition of conserved microbial molecules known as microbial-associated molecular patterns (MAMPs) is mediated by pattern recognition receptors (PRR), such as the membrane-anchored Toll-like receptors (TLR), which sense microbial components in the extracellular space or in certain intracellular membrane-bound compartments, and by the cytoplasmic NOD-like receptors (NLRs). On the effector side, antimicrobial peptides, which are secreted into the intestinal lumen by specialised epithelial cells, such as the Paneth cells, contribute to mucosal protection. Other innate immune mechanisms involve macrophages and antigen-presenting cells (APC), which take up microbes and their antigens, and activate and orchestrate adaptive immune responses via processing and presentation of antigens.

In this review, we highlight new insights for two major CD susceptibility genes encoding the proteins' Nucleotide oligomerisation domain (NOD)2 / caspase activation recruitment domain (CARD)15, and autophagy-related protein (ATG) 16L1. Both gene products have important functions in innate immunity, as NOD2/CARD15 acts as an intracellular bacterial sensor and ATG16L1 is a key protein involved in autophagic processes. Although numerous other CD susceptibility genes have been described to date, these two proteins are among the best studied and provide a powerful paradigm of CD pathogenesis that can guide the detailed studies of the other genes. Other recent reviews provide more comprehensive discussion of the overall pathogenesis of CD [1].

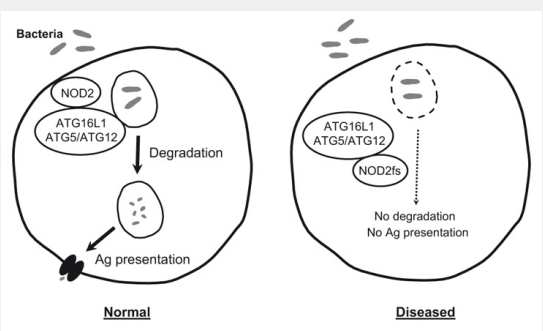

Figure 2

NOD2/CARD15 initiates the autophagic process. Normal) NOD2/CARD15 colocalises with the ATG16L1 at a bacterial entry site, leading to recruitment of the ATG5-ATG12 complex for autophagosome formation. After fusion of the autophagosome with the lysosome, bacteria are degraded and some constituents are used for antigen presentation via major histocompatibility complex (MHC) class II, leading to immune system activation and bacterial clearance. Diseased) Mutant NOD2/CARD15 fails to localise at the plasma membrane and retains ATG16L1 in the cytosol. Impaired autophagosome formation leads to ineffective bacterial degradation and diminished antigen presentation.

NOD2/CARD15: intracellular recognition of bacterial peptidoglycan

Epidemiological observations show a strong genetic contribution to CD susceptibility as siblings of affected individuals have up to a 20- to 40-fold increased risk of developing CD, and monozygotic twins have a 40% concordance rate for disease compared to 2–10% in heterozygotic twins [2]. Fine mapping of the first susceptibility locus (IBD1) in IBD-affected families demonstrated that *NOD2* (also termed *CARD15),* located in the pericentromeric region of chromosome 16, has the strongest association with CD susceptibility in North American and European populations [3, 4]. *NOD2* mutations in CD patients are notably absent in Asian populations, indicating that CD pathogenesis is not uniform across ethnic backgrounds [5]. *NOD2* encodes a tripartite protein consisting of two N-terminal caspase recruitment domains (CARDs), a centrally-located nucleotide binding oligomerisation domain (NBD/NACHT), and a C-terminal domain composed of leucine rich repeats (LRR) believed to be involved in ligand recognition. Of the more than 60 reported sequence variants of the NOD2 protein, three main variants, R702W, G908R, and 1007fs, have the strongest CD association [6–8]. Interestingly, all three main variants are located in the C-terminal end of the gene product within or close to the LRR region. In particular, the frameshift mutation, 1007fs, exhibits the most consistent association with CD across multiple studies and population groups [6–8]. In addition to CD, another granulomatous inflammatory disease, Blau syndrome, is associated with mutations in the centrally-located NBD domain of NOD2. The syndrome is a rare autosomal dominant disorder characterised by early-onset granulomatous arthritis, uveitis, and skin rash [9]. In contrast, NOD2 mutations are not found in patients with ulcerative colitis, the other major form of IBD [10].

Individuals with one of the three major disease-associated *NOD2* alleles have a 2- to 4-fold increased risk of developing CD, while homozygous or compound heterozygous carriers have a 20- to 40-fold increase in risk [3, 4, 6–8]. Despite their strong association with CD, mutated *NOD2* alleles are neither sufficient nor necessary for the development of CD, since they occur in healthy individuals, with estimates most commonly around 0.5–2% in the general population, and 60–70% of CD patients show no *NOD2* mutations [11]. These data show that mutant *NOD2* must be considered a predisposing risk factor for CD, which is likely to act in synergy with other factors, both genetic and environmental, in causing disease. Furthermore, the phenotypic penetrance of mutant NOD2 is low in absolute terms, but relatively high compared to other genes. In fact, NOD2 is only one of multiple (>30) genes associated with CD, many of which have even lower phenotypic penetrance. To date, all published genetic variants together can account for only 30% of the genetic risk of developing CD, indicating that either many more undescribed genes are involved and/or that complex gene constellations play a role.

NOD2 is constitutively expressed in myeloid cells, particularly macrophages, neutrophils, and dendritic cells, and in Paneth cells of the small intestine [12, 13]. It can be induced by TNFα and IFN-γ in intestinal epithelial cells [14]. NOD2 acts as a microbial sensor as it detects muramyl-

dipeptide (MDP), a peptidoglycan found in a wide range of both Gram-positive and Gram-negative bacteria. Consequently, the sensor contributes to innate detection of diverse bacterial pathogens, including *Listeria monocytogenes, Salmonella typhimurium and Staphylococcus aureus* [15–18]. Several of these pathogens (e.g. *L. monocytogenes*) invade host cells and enter the cytoplasm, where they shed cell wall components that are directly detected by NOD2. However, other pathogens (e.g. *S. aureus*) are minimally invasive, raising the question how MDP from such extracellularly-located bacteria can be sensed by the intracellular sensor NOD2. Several mechanisms have been proposed that can explain MDP access to cytoplasmic NOD2. For example, MDP can be delivered into the cytoplasm by phagolysosomes [19], specialised bacterial secretion systems [20], subversion of peptide transport pathways, such as the intestinal peptide transmembrane transporter hPEPT [21], or bacterial toxins that form membrane pores [16]. These data, combined with its cytoplasmic location, suggest that NOD2 is not primarily a sensor for the vast majority of extracellular bacteria in the intestine, but rather an "emergency sensor" for true bacterial threats that can gain access to the cytoplasm. Consistent with such a specialised role, NOD2 must bind to the inner side of the cell membrane for proper activation. This membrane binding is impaired in the 1007fs frameshift mutation of NOD2, underlining the importance of the subcellular localisation of NOD2 for its function [22, 23].

Upon activation NOD2 associates through its CARD domains with the RICK/Rip2 (CARD3) protein kinase and CARD9, leading to activation of NF-κB, as well as mitogen-activated protein kinases (MAPK), and subsequent induction of pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6 [24–27] (fig. 1). TNFα is likely to play an important role in CD pathogenesis, because anti-TNFα therapy is effective [28] and TNFα overproduction causes the development of CD-like intestinal inflammation in mice [29, 30]. Many of the anti-inflammatory drugs used to treat CD inhibit activation of NF-κB, a transcription factor involved in TNFα gene induction, which supports the hypothesis that aberrant NF-κB activation, caused by exposure to enteric bacteria or perhaps other stimuli, is critical to the development of CD [31, 32]. In support of this notion, CD patients carrying NOD2 variants show increased mucosal NF-κB activation related to elevated mucosal endotoxin [33] In this context, NOD2 mutations appear to have the overall effect of promoting NF-κB activation in the inflamed intestine.

Paradoxically, however, transient transfection experiments in epithelial cells have suggested that the NOD2 alleles associated with CD code for proteins that can no longer activate NF-κB in response to MDP, suggesting that the disease-associated alleles exhibit a loss-of-function phenotype relative to the wild-type protein [34, 35]. In this alternative model, NOD2 mutations can compromise host-microbial homeostasis by altering the defence capacity of the mucosal immune system or by weakening the barrier function of the intestinal epithelium. For example, diminished antimicrobial defence due to impaired NOD2-dependent secretion of antibacterial peptides by ileal Paneth cells may facilitate the entry of bacteria into the mucosa and thereby weaken the epithelial barrier [12, 13]. Interestingly, ileal CD, particularly in patients carrying the 1007fs mutation of *NOD2*, is characterised by a decrease in antimicrobial α-defensin production by Paneth cells [36, 37]. However, this explanation cannot readily account for disease locations other than the ileum, yet CD can affect any part of the gastrointestinal tract. Other explanations have focused on dysregulated inflammatory responses related to the adaptive mucosal immune system. For example, decreased production of the anti-inflammatory cytokine, interleukin-10, in CD patients carrying the $NOD2^{1007fs}$ mutation may be associated with increased susceptibility to inflammation, as suggested by the observation that IL-10 deficient mice develop chronic enterocolitis with histopathological features resembling human CD [38, 39]. Mutant NOD2 may also be impaired in its ability to inhibit pro-inflammatory signalling through the extracellular microbial sensor, TLR2, consistent with the idea that normal NOD2 suppresses mucosal inflammatory responses elicited by extracellular microbial stimulation [40, 41]. Despite these investigative efforts, the exact mechanism by which CD-associated mutations in NOD2 contribute to the pathogenesis of CD remains a matter of informed speculation at present, although new, potentially more comprehensive concepts continue to emerge. One of these envisions a link between NOD2 and the critical cellular process of autophagy.

Maintenance of intracellular homeostasis via autophagy

Autophagy, literally self-digestion, is an important, evolutionarily-conserved process of cellular degradation where cytoplasmic cargo is delivered to the lysosomal compartment for proteolytic degradation [42]. It includes micro- and macro-autophagy, in which large cellular structures are degraded through both selective and nonselective mechanisms, and chaperone-mediated autophagy, in which only soluble proteins are degraded in a selective manner [43]. Autophagy occurs ubiquitously in eukaryotic cells and is activated as an adaptive catabolic process in response to different forms of metabolic stress, including nutrient deprivation, growth factor depletion and hypoxia. Critical for the maintenance of intracellular homeostasis, autophagy acts as an intracellular cleaning and recycling system, which eliminates defective cytosolic proteins or damaged organelles and prevents abnormal accumulation of protein aggregates. It is essential for survival, differentiation and development [42].

Dysfunction of autophagy has been associated with a variety of human diseases such as cancer, neurodegenerative disorders, liver and cardiac disease, as well as aging [42, 44]. The autophagic machinery also plays an important role in innate host defence against viral, bacterial and parasitic infections by selectively delivering microorganisms to lysosomes in a process termed xenophagy (digestion of foreign constituents). Defective autophagy has been associated with increased susceptibility to infections, both in vitro and in vivo [45]. Several studies have linked autophagy to host defence against intracellular bacterial pathogens, e.g. *group A streptococcus* [46], *Staphylococcus aureus* [47], *Listeria monocytogenes* [48] and *Shigella*

flexneri [49]. The importance of autophagy is further underlined by the discovery that a number of pathogens have strategies to escape autophagic degradation [48, 49], thus pointing to autophagy as being at the centre of host-pathogen interactions.

The modulator of the autophagic process ATG16L1 and Crohn's disease

Multiple autophagy-related (ATG) proteins are required for the correct execution of the (macro-)autophagic process, which first involves formation of a double membrane-lined cytosolic vesicle, the autophagosome. Fusion of the autophagosome and lysosome is then followed by breakdown of the autophagosome in the lysosomal compartment and subsequent degradation of the constituents [43]. One of the key proteins involved in the execution of the autophagic process is the modulator, ATG16L1, a part of a large complex with ATG5-ATG12, which is responsible for the membrane localisation of the autophagic machinery and formation of the autophagosome [50]. In addition, several other proteins are required for the correct execution of the autophagic program. The ATG16L1 protein has a central coiled-coil domain, which mediates self-multimerisation, and seven tryptophan-aspartic acid dipeptide (WD) repeats in the carboxy-terminal domain whose function is unknown [51]. Cells deficient for ATG16L1 show ineffective recruitment of the ATG5-ATG12 complex, leading to failed formation and activation of the autophagosome [52].

Several genome-wide association studies have shown that a single nucleotide polymorphism, leading to a threonine to alanine substitution (T300A) in the carboxy terminal WD repeats of ATG16L1, is associated with increased susceptibility to CD in adult and paediatric patients [53–59]. Importantly, although the physiological function of this domain is largely unknown, studies in human intestinal epithelial cells (Caco-2) have revealed that the CD-associated ATG16L1*300A variant leads to impaired capture of internalised *Salmonella typhimurium* within autophagosomes [60]. These data suggest that this variant is associated with a loss-of-function phenotype characterised by an impaired ability to engage the autophagic machinery in response to intracellular bacteria.

Recently, new mouse models with genetically engineered mutations in the *Atg16l1* gene have revealed important functions of this autophagic modulator in innate immunity [52, 61]. In one study, mice with deletion of the entire coiled-coil domain of *Atg16l1* die in the first day of life [52]. Interestingly, macrophages from these mice respond to the TLR4 ligand, LPS, with enhanced caspase-1 dependent IL-1β and IL-18 production indicative of inflammasome activation [52]. Increased IL-1β production also occurred after stimulation with ligands of other TLRs, such as TLR3, TLR7 and TLR9, but not for TLR2 and TLR5. Furthermore, chimeric mice deficient for ATG16L1 in haematopoietic cells show significantly elevated IL-1β and IL-18 cytokine levels in a model of dextran sulfate sodium (DSS)-induced colitis. In parallel, the chimeric mice exhibited high mortality, which could be reversed by injection of antibodies against IL-1β and IL-18. These studies point toward a key role of ATG16L1 in regulating inflammasome activation, although the specific mechanisms remain unclear.

Another ATG16L1 study has focused on Paneth cells, the specialised epithelial "defence" cells found mainly in the distal ileum. A major function of these cells is to secrete peptides with antimicrobial activity into the crypt lumen, which provides an important innate immune defence against bacterial invasion. Using a gene trap vector inserted into the intronic region of the *Atg16l1* gene, mice with suppressed ATG16L1 expression were generated. ATG16L1-suppressed mice exhibited normal overall morphology of ileum and colon, but the Paneth cells of these mice have aberrant, disorganised granules and decreased granule numbers, indicating that ATG16L1 plays a role in regulating the exocytosis pathway of Paneth cell granules. Interestingly, Paneth cells from ATG16L1-suppressed mice-raised in an enhanced barrier facility showed no morphological differences compared to wild-type littermates and suggested that exogenous factors are needed to cause Paneth cell abnormalities [62]. Indeed, infection of ATG16L1-suppressed mice with murine norovirus generated abnormalities in granule packaging and unique patterns of gene expression in Paneth cells [61]. In response to DSS challenge the virus-infected mutant mice presented with increased muscularis propria thickness, increased number of lymphoid aggregates and mucosal atrophy in the ileum resembling features seen in CD patients. These pathologies were prevented by treatment with broad spectrum antibiotics [61]. The data suggest that impaired protein function in the transgenic ATG16L1 mouse model may partly mimic the observations in CD patients homozygous for the ATG16L1 CD risk allele [61]. They further indicate that a combination of several (environmental) factors may alter the interaction of mucosa with commensal and pathogenic microbes, which is crucial for the development of an inflammatory response in genetically susceptible hosts. However, to truly understand the pathophysiological implications of dysfunctional proteins associated with CD, mouse models with specific mutations that code for a gene product equivalent to the human ATG16L1 T300A are needed.

Intracellular sensor NOD2 and autophagic modulator ATG16L1: Implications for immune defense

TLRs are important receptors for microbial components in the extracellular space and regulate autophagy induction, act in the recruitment of autophagy regulators to phagosomal membranes, and are involved in the initiation of innate immune responses against potentially harmful microbes [63, 64]. Similarly, recent data suggest that the intracellular bacterial sensors, NOD1 and NOD2, are also important for the autophagic response to invasive bacteria [65, 66]. Their stimulation by peptidoglycans can induce autophagy and antibacterial defence. Both sensors colocalise with the autophagy protein ATG16L1 on the cytosolic side of the plasma membrane at the bacterial entry site and initiate the autophagocytic process (fig. 2). This pro-cess is independent of the downstream signaling adaptor molecule, RIP2, and the transcription factor, NF-κB [66]. Both of these signaling pathways were previously found to be key mediators of NOD2 functions. Interestingly, the CD-asso-

ciated frameshift mutation of NOD2 (L1007insC) encodes a shortened protein that does not localise to the plasma membrane [22, 23]. Cells homozygous for this mutation failed to recruit ATG16L1 to the plasma membrane with the consequence of impaired clearance of invading bacteria by autophagosomes (fig. 2), although co-immunoprecipitation assays showed that the shortened NOD2 retains the normal ability to bind ATG16L1 [66]. These results indicate that mutant NOD2 can suppress the function of ATG16L1 by retaining it in the cytosol and thereby preventing it from localising to the plasma membrane. Besides the defect in autophagy induction and bacterial killing, mutant NOD2 leads to inefficient antigen presentation with the consequence of an inadequate generation of CD4+ T cell immune response, as shown with dendritic cells from CD patients homozygous for $NOD2^{1007fs}$ [65]. Impaired induction of autophagy, when stimulated with MDP or peptidoglycan from Gram-positive bacteria, was also shown in cells from donors homozygous for the CD-associated variant ATG16l1* 300A [66].

These data suggest that colocalisation of NOD1 or NOD2 with ATG16L1 at the cell membrane is a crucial step in the initiation of the autophagic process probably independent of the activation NF-κB. Such NF-κB-independent autophagy has also been observed in *Drosophila melanogaster* and suggests an evolutionarily-conserved host defense mechanism [67]. Defects in this pathway, particularly in individuals that are bearing CD risk alleles for either *NOD2* or *ATG16L1*, may lead to failed bacterial killing due to impaired lysosomal degradation, inefficient immune-mediated bacterial clearance and consequently to mucosal inflammation. This pathophysiological hypothesis may also provide a mechanism by which mutant NOD2 may increase susceptibility to CD.

Conclusion

Intracellular bacterial sensors and bacterially-induced autophagy (xenophagy) are evolutionarily- conserved elements of the innate immune response against invading bacteria. Exciting recent progress has been made in understanding a mechanistic link between the proteins encoded by two genes strongly associated with susceptibility to CD, the cytoplasmic bacterial sensor, NOD2, and the autophagic regulator, ATG16L1. Despite this progress, important questions remain: How exactly is the autophagic process regulated by microbial stimulation? What are the structural explanations for, and larger signaling implications of, the failure of mutant NOD2, particularly the form encoded by $NOD2^{1007f}$, to colocalise with ATG16L1 at the plasma membrane? Can this failure be circumvented pharmacologically and would such an intervention attenuate intestinal inflammation? Do other CD-associated NOD2 mutants exhibit a similar phenotype toward ATG16L1? Could selective stimulation of bacterially-induced autophagy be used for CD treatment? Answers to these questions will not only provide important insights into the biological mechanisms of innate immune defense against pathogenic bacteria, but may also suggest novel targets for immunomodulatory treatment of CD.

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