

Alterations of autophagic and innate immune responses by the Crohn's disease-associated *ATG16L1* mutation

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Abstract

Crohn's disease (CD) is driven by the loss of tolerance to intestinal microbiota and excessive production of pro-inflammatory cytokines. These pro-inflammatory cytokines are produced by macrophages and dendritic cells (DCs) upon sensing the intestinal microbiota by the pattern recognition receptors (PRRs). Impaired activation of PRR-mediated signaling pathways is associated with chronic gastrointestinal inflammation, as shown by the fact that loss-of-function mutations in the nucleotide-binding oligomerization domain 2 gene increase the risk of CD development. Autophagy is an intracellular degradation process, during which cytoplasmic nutrients and intracellular pathogens are digested. Given that impaired reaction to intestinal microbiota alters signaling pathways mediated by PRRs, it is likely that dysfunction of the autophagic machinery is involved in the development of CD. Indeed, the loss-of-function mutation T300A in the autophagy related 16 like 1 (*ATG16L1*) protein, a critical regulator of autophagy, increases susceptibility to CD. Recent studies have provided evidence that *ATG16L1* is involved not only in autophagy, but also in PRR-mediated signaling pathways. *ATG16L1* negatively regulates pro-inflammatory cytokine responses of macrophages and DCs after these cells sense the intestinal microbiota by PRRs. Here, we discuss the molecular mechanisms underlying the development of CD in the T300A *ATG16L1* mutation by focusing on PRR-mediated signaling pathways.

Key Words: *ATG16L1*; Crohn's disease; Autophagy; Innate immunity; Cytokine; Pattern recognition receptors

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Core Tip: The loss-of-function mutation T300A in autophagy related 16 like 1 (*ATG16L1*) increases the risk of Crohn's disease (CD). *ATG16L1* is a multifunctional protein involved in autophagy and innate immunity. The CD-associated *ATG16L1* mutation T300A leads to overgrowth of intestinal microbiota and enhanced pro-inflammatory cytokine responses, which disrupt intestinal immune homeostasis. In this minireview article, we have summarized the immunopathogenesis of CD in the presence of *ATG16L1* mutation.

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INTRODUCTION

Pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-12, and IL-23, underlie the immunopathogenesis of Crohn's disease (CD), as evidenced by the clinical efficacy of targeting these cytokines for the treatment of patients[1,2]. These colitogenic cytokines are produced by macrophages and dendritic cells (DCs) upon sensing the intestinal microbiota by the pattern recognition receptors (PRRs), which are classified into Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs)[3-6]. Thus, excessive pro-inflammatory cytokine responses caused by PRR activation play critical roles in the development of CD. This notion is fully supported by the identification of loss-of-function mutations in *NOD2* as one of the strongest risk factors for CD. *NOD2* is an intracellular PRR that senses muramyl dipeptide (MDP) derived from bacterial cell wall components and negatively regulates TLR-mediated pro-inflammatory cytokine responses[5,6].

Autophagy refers to the process during which cytoplasmic components and intracellular pathogens are delivered to the lysosome for degradation in the form of double-membrane-bound autophagosomes [7]. The autophagy related 16 like 1 (*ATG16L1*) protein plays an indispensable role in the initiation and completion of the autophagic process. In addition to its role in autophagy, *ATG16L1* has been shown to be involved in PRR-mediated innate immunity. *ATG16L1* negatively regulates pro-inflammatory and type I interferon (IFN-I) responses mediated by TLRs, NLRs, and RLRs[8]. More importantly, the loss-of-function mutation T300A in *ATG16L1* has been identified as a risk factor for CD in parallel with mutations in *NOD2*[6]. In this minireview article, we summarize the molecular mechanisms by which the T300A mutation in *ATG16L1* predisposes the host to CD development by focusing on the regulatory role of *ATG16L1* in PRR-mediated signaling pathways.

INDUCTION OF AUTOPHAGY BY ATG16L1

ATG16L1 is an indispensable molecule for autophagic responses (Table 1). The autophagy process includes vesicle nucleation, vesicle elongation, vesicle completion, fusion with lysosome, degradation, and recycling[9]. Autophagy dysfunction is associated with neurodegenerative diseases, microbial infections, and aging[7]. Although autophagy has been identified as the primary cell response to the lack of nutrients, recent studies have highlighted the importance of autophagy in microbial infection and immune responses[9]. The autophagy process is negatively regulated by growth factors, which activate the mechanistic target of rapamycin (mTOR) and the phosphoinositide 3-kinase (PI3K)-AKT pathways[7,9]. On the contrary, nutrient starvation or rapamycin treatment promotes the autophagic process through the inhibition of mTOR. Thus, the PI3K-AKT-mTOR pathway negatively regulates autophagic process. On the molecular level, mTOR activation controls the initiation of autophagy by suppressing the activation of the primary initiation complex of autophagy, called Unc-51 Like autophagy activating kinase 1 (ULK1) complex, composed of ULK1/2, *ATG101*, *ATG13*, and *RB1CC1/FIP200*[9]. The formed ULK1 complex translocates to the site of the second complex, called the PI3K complex[9]. The latter PI3K complex recruits a number of *ATG* proteins to promote elongation and expansion of the autophagosome.

Two ubiquitin-like conjugation systems, the *ATG5-ATG12-ATG16L1* conjugation system and the microtubule-associated protein 1 Light chain 3 (LC3) conjugation system, play important roles in the elongation and expansion of the autophagosome[7,9]. The conjugation of the membrane lipid phosphatidylethanolamine with the soluble form of LC3 and formation of the *ATG5-ATG12-ATG16L1* complex is necessary for the maturation of autophagosomes[7,9,10]. Matured autophagosomes are fused with lysosomes for the degradation of cellular materials. Vesicles containing *ATG16L1* are necessary for

Table 1 Physiological functions of autophagy related 16 like 1

Function	Cell type	Ref.
Positive regulation		
Autophagy	ECs, DCs, macrophages	[9,10]
Regulatory T cell responses	DCs	[22]
Negative regulation		
IFN-I production by RLRs	MEFs	[11,12]
IFN-I production by TLR3 and TLR4	Macrophages	[13]
IL-1 β production by TLR4	Macrophages	[16,17,19]
IL-6 and IL-12 production by TLR2	DCs	[27]

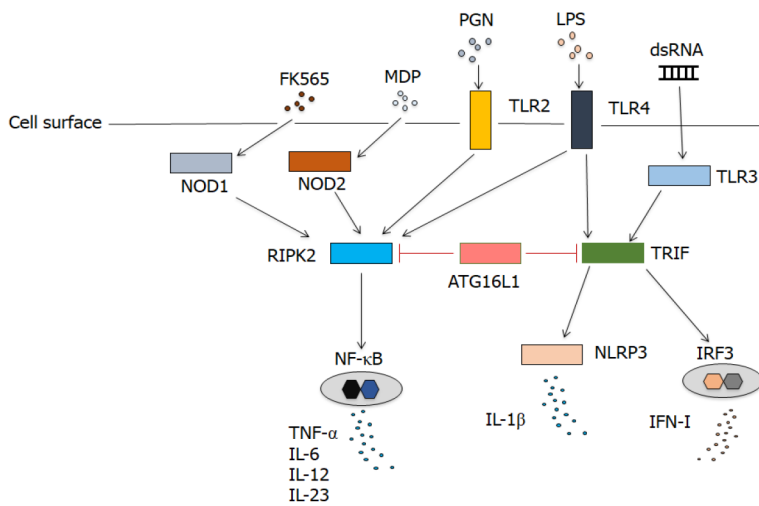
EC: Epithelial cell; DC: Dendritic cell; IFN-I: Type I interferon; RLR: Retinoic acid-inducible gene I-like receptors; MEF: Mouse embryonic fibroblast; TLR: Toll-like receptor; IL: Interleukin.

membrane trafficking and autophagosome formation[7,9,10]. Thus, ATG16L1 is an essential protein for the induction and completion of autophagic responses.

ATG16L1 AND INNATE IMMUNITY

ATG16L1 has been shown to attenuate proinflammatory cytokine responses in innate immunity (Table 1)[8]. RLRs, including RIG-I and melanoma differentiation-associated gene 5 (MDA5), are sensors for RNA viruses[3]. IFN-I, which is produced after viral RNA is sensed by RLRs, plays a protective role in host defense[3]. Mouse embryonic fibroblasts deficient in ATG5 displayed enhanced production of IFN-I after exposure to vesicular stomatitis virus due to enhanced activation of IFN regulatory factor 3 [11]. Enhanced production of IFN-I is associated with reduced viral load[11]. ATG16L1 is involved in the regulation of IFN-I mediated by RLRs. Two mitochondrial proteins, NLRX1 and its binding partner, Tu translation elongation factor, interact with ATG5, ATG12, and ATG16L1, and suppress RLR-induced IFN-I production and thereby promoting autophagy[12]. In addition, ATG16L1 has been shown to regulate IFN-I production by interacting with TLR3 and TLR4[13]. Samie *et al*[13] have provided evidence that macrophages deficient in ATG16L1 produced large amounts of IFN-I after stimulation with TLR3 and TLR4 Ligands (Figure 1). Mechanistically, the loss of ATG16L1 resulted in the accumulation of the toll-IL-1 receptor domain-containing adaptor inducing IFN- β protein (TRIF), leading to the excessive activation of TLR3- and TLR4-mediated signaling pathways. Interestingly, macrophages isolated from individuals bearing the CD-associated ATG16L1 T300A variant also exhibited enhanced IFN-I production upon stimulation with TLR3 and TLR4 Ligands[13]. Thus, ATG16L1 functions as a negative regulator of IFN-I production induced by TLR activation. Excessive activation of IFN-I signaling caused by ATG16L1 deficiency may protect against microbial infection. In fact, ATG16L1 hypomorphic mice displayed enhanced IFN-I signaling upon challenge with *Citrobacter rodentium*, which conferred protection from enteric pathogen infection[14]. This protection was mediated by mitochondrial antiviral signaling (MAVS) and stimulator of interferon genes (STING) proteins, because mice with hypomorphic ATG16L1 expression and deficient in MAVS or STING were not protected from the *C. rodentium* infection. Similarly, the clearance of *Salmonella typhimurium* from the intestine was augmented in mice with myeloid cell-specific ATG16L1 deficiency in an IFN-I-dependent manner[13]. IL-22 is a barrier protective cytokine that stimulates antimicrobial responses in the intestine[15]. IL-22 induces STING-dependent IFN-I signaling, which is augmented in the absence of ATG16L1[15]. Such enhanced IFN-I signaling promotes TNF- α production, leading to ileal inflammation, suggesting that ATG16L1 deficiency mediates pro-inflammatory TNF- α responses through cooperative interaction between IL-22 and IFN-I[15]. Taken together, these studies suggest that ATG16L1 dampens IFN-I production mediated by RLRs and TLRs. In turn, the lack of negative regulation of IFN-I signaling owing to the absence of ATG16L1 or the presence of ATG16L1 T300A variant mediates protection from microbial infection in the gastrointestinal tract in an IFN-I-dependent manner.

In addition to attenuating IFN-I production, ATG16L1 also suppresses IL-1 β production by macrophages[16,17]. Macrophages expressing ATG16L1 that lacks the coiled-coil domain produced large amounts of IL-1 β upon stimulation with lipopolysaccharide (LPS) (Figure 1)[17]. Pro-IL-1 β is processed into the mature form of IL-1 β by caspase-1[18]. Accumulation of TRIF is involved in enhanced IFN-I production in the absence of ATG16L1 or presence of the ATG16L1 T300A mutation[13]. Similarly, TRIF-dependent activation of caspase-1 leads to increased production of IL-1 β in macrophages lacking ATG16L1[17]. In a murine model of urinary tract infection, ATG16L1 deficiency promoted clearance of



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Figure 1 Negative effect of autophagy related 16 like 1 on pattern recognition receptor signaling pathways. Autophagy related 16 like 1 (ATG16L1) negatively regulates pro-inflammatory and type I interferon (IFN-I) responses by toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). Production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-12, and IL-23 mediated by TLRs is suppressed by ATG16L1 through the inhibition of activation of receptor-interacting serine/threonine-protein kinase 2 and nuclear factor- κ B. Production of IL-1 β and IFN-I mediated by TLRs is suppressed by ATG16L1 through the inhibition of toll-IL-1 receptor domain-containing adaptor inducing IFN- β , NOD-, LRR-, and pyrin domain-containing protein 3, and interferon regulatory factor 3. MDP: Muramyl dipeptide; PGN: Peptidoglycan; TLR: Toll-like receptor; LPS: Lipopolysaccharide; NOD: Nucleotide-binding oligomerization domain; RIPK2: Receptor-interacting serine/threonine-protein kinase 2; ATG16L1: Autophagy related 16 like 1; IL: Interleukin; IFN: Interferon; TRIF: Toll-IL-1 receptor domain-containing adaptor inducing IFN- β protein; NF- κ B: Nuclear factor- κ B; NLRP3: NOD-, LRR-, and pyrin domain-containing protein 3; IRF3: Interferon regulatory factor 3; TNF: Tumor necrosis factor.

uropathogenic *Escherichia coli* through excessive production of IL-1 β [19]. Thus, ATG16L1 negatively regulates pro-inflammatory pathways mediated not only by IFN-I, but also by IL-1 β .

Regulatory T cells (Tregs) expressing forkhead box P3 (FOXP3) are a specialized T cell population that is indispensable for the establishment and maintenance of immunological self-tolerance[20]. Impaired activation of Tregs leads to the development of autoimmune disorders. *Bacteroides fragilis* (*B. fragilis*) has been considered to stimulate beneficial immunoregulatory functions through induction of Tregs[21]. Chu *et al*[22] provided evidence that ATG16L1 expressed in DCs was required for the induction of Tregs expressing FOXP3 upon exposure to outer membrane vesicles (OMVs) of *B. fragilis*. Oral administration of OMVs from *B. fragilis* protected wild-type mice from experimental colitis[22], and this effect was accompanied by increased proportions of Tregs expressing FOXP3 and IL-10. Such protective effect of oral administration of OMVs was not seen in mice with DC-specific ATG16L1 deficiency. Thus, ATG16L1 is involved in the maintenance of immune homeostasis through induction of Tregs expressing FOXP3.

Mutations in *NOD2* are the strongest risk factor for the development of CD[5,6]. MDP, a bacterial component derived from intestinal bacteria, is a prototypical *NOD2* ligand[23,24]. Activation of *NOD2* by MDP induces autophagy in macrophages, DCs, and fibroblasts, but not in cells harboring CD-associated *NOD2* mutations[25]. Physical interaction between *NOD2* and ATG16L1 is induced by the stimulation with MDP[25,26]. Thus, MDP activation of *NOD2* mediates bactericidal effects in an ATG16L1-dependent manner, and the presence of CD-associated *NOD2* mutations promotes overgrowth of intestinal bacteria, leading to excessive production of pro-inflammatory cytokines.

Receptor-interacting serine/threonine-protein kinase 2 (RIPK2) is a signaling molecule downstream of *NOD2* and TLRs[23,24]. It remains unclear whether ATG16L1 binds to RIPK2 after activation of *NOD2*. In this regard, we confirmed that ATG16L1 binds to the kinase domain of RIPK2 in overexpression studies[26,27]. In human DCs, ATG16L1 interacted with RIPK2 after the stimulation with MDP and this interaction suppressed NF- κ B-dependent proinflammatory responses mediated by TLRs[26, 27]. Transfection of intact *ATG16L1*, but not of *ATG16L1* with the T300A mutation, reduced TLR2-mediated NF- κ B activation in human embryonic kidney cells. In the physiological setting, NF- κ B activation, as assessed by the degradation of I κ B α and expression of phospho-I κ B α , was markedly suppressed in human DCs stimulated with TLR2 and *NOD2* ligands as compared to the effect of stimulation with a TLR2 ligand alone[26,27]. Furthermore, knockdown of *ATG16L1* by its specific siRNA increased IL-6 and IL-12p40 production by human DCs upon exposure to TLR2 and *NOD2* ligands as compared to the levels of those cytokines in cells transfected with control siRNA[26,27]. These studies strongly suggest that ATG16L1 functions as a negative regulator of TLR2-mediated pro-inflammatory cytokine responses (Figure 1).

NF- κ B activation mediated by TLRs and NOD2 is tightly regulated by Lys (K63)-linked polyubiquitination of RIPK2[23,24,27,28]. As for the molecular mechanisms accounting for the suppression of TLR2-mediated NF- κ B activation and pro-inflammatory cytokine production, ATG16L1 has been shown to inhibit polyubiquitination of RIPK2[26,28]. NOD2 activation by MDP also inhibited polyubiquitination of RIPK2 through the induction of interferon regulatory factor 4 (IRF4)[23,24]. Overexpression studies revealed that ATG16L1 and IRF4 act cooperatively to suppress K63-linked polyubiquitination of RIPK2 [27]. Given that physical interaction between RIPK2 and IRF4 or ATG16L1 is induced after NOD2 activation by MDP, it is likely that NOD2 downregulates TLR-mediated proinflammatory cytokine responses through binding of ATG16L1 and IRF4 to RIPK2. This idea is fully supported by the fact that RIPK2 expression level is markedly elevated in the colonic mucosa of patients with CD and ulcerative colitis (UC), and it correlates with the levels of pro-inflammatory cytokines, such as TNF- α and IL-6[29]. Furthermore, the percentages of lamina propria DCs expressing ATG16L1 and IRF4 in the colon inversely correlate with the expression levels of TNF- α and IL-6[27]. Collectively, these studies support the idea that ATG16L1 acts in concert with NOD2 to suppress excessive pro-inflammatory cytokine responses mediated by TLRs and thereby maintains intestinal homeostasis.

ATG16L1 AND CD

The polymorphism Thr300Ala (or T300A) in the coding region of the *ATG16L1* gene confers increased risk for the development of CD[6,10,16]. This polymorphism is a loss-of-function mutation, which affects the induction of autophagy against invading bacteria and is associated with gut bacterial overgrowth and pro-inflammatory cytokine responses[6,10,16]. Recent studies have successfully elucidated some of the molecular mechanisms accounting for the development of CD in the presence of the ATG16L1 T300A variant. Given that ATG16L1 is constitutively expressed in epithelial cells, especially Paneth cells and myeloid cells, these studies have highlighted the importance of ATG16L1-mediated signaling pathways in innate immune cells for the immunopathogenesis of CD[17,25,28,30,31].

Paneth cells are localized at the base of the crypts in the ileum, and they contribute to the maintenance of intestinal homeostasis through the secretion of antimicrobial peptides (AMPs) and inhibition of intestinal bacterial overgrowth[32]. Mice with hypomorphic expression of ATG16L1 and ATG16L1 T300A-knockin (KI) mice exhibit increased proportions of Paneth cells with abnormal phenotypes, as assessed by lysozyme localization and granule morphology[30-32]. Moreover, Paneth cells from patients with CD carrying ATG16L1 T300A have unusual granule morphology and accumulation of AMPs, with both having been observed also in mice deficient in ATG16L1 or expressing ATG16L1 T300A[32]. Furthermore, defective function of Paneth cells in the absence of ATG16L1 or the presence of the *ATG16L1* T300A mutation led to higher susceptibility to TNF- α -mediated necroptosis and accumulation of the endoplasmic reticulum stress sensor IRE1 α , indicating that necroptosis and endoplasmic reticulum stress are involved in the pathogenesis of CD[33]. Thus, the ileal mucosa of patients and mice bearing ATG16L1 T300A is characterized by the defective function of Paneth cells, which results in the overgrowth of intestinal bacteria. This notion is supported by the fact that CD patients bearing the *ATG16L1* T300A mutation display impaired clearance of pathogenic bacteria in the ileal mucosa[34]. It is well established that CD occurs as a result of the interplay between genetic susceptibility and environmental factors. Cigarette smoking is a risk factor for developing CD[35]. Interestingly, cigarette smoking has been suggested to amplify effects of the *ATG16L1* T300A mutation, triggering Paneth cell defects, thereby causing chronic intestinal inflammation[31].

Pro-inflammatory cytokine responses play an important role in the development of CD[1]. The *ATG16L1* T300A mutation has been shown to enhance pro-inflammatory cytokine responses in the intestine. Mice lacking ATG16L1 in hematopoietic cells were susceptible to dextran sodium sulfate (DSS)-induced colitis[17]. Aggravated DSS-induced colitis in mice lacking ATG16L1 was alleviated by blocking IL-1 β -mediated signaling pathways[17]. Furthermore, macrophages lacking ATG16L1 produced more IL-1 β upon stimulation with LPS[17]. As for the molecular mechanisms accounting for enhanced production of IL-1 β in the absence of ATG16L1, Saitoh *et al*[17] showed that ATG16L1 deficiency resulted in increased production of this cytokine through the TRIF-dependent activation of caspase-1. Thus, ATG16L1 deficiency predisposed mice to DSS-induced colitis by activating IL-1 β -mediated signaling pathways. In line with these data obtained in mice lacking ATG16L1 in hematopoietic cells, ATG16L1 T300A-KI mice displayed enhanced production of IL-1 β upon exposure to LPS[16]. These studies, which utilized ATG16L1-deficient and ATG16L1 T300A-KI mice, support the idea that intact ATG16L1-mediated signaling pathways limit pro-inflammatory cytokine responses triggered by activation of TLRs. In this regard, we and others have reported that ATG16L1 negatively regulates pro-inflammatory cytokine responses mediated by RIPK2, a downstream signaling molecule of TLRs and NLRs[27,28]. Binding of ATG16L1 to the kinase domain of RIPK2 inhibits polyubiquitination of RIPK2, followed by suppression of NF- κ B activation[27,28]. These studies strongly suggest that ATG16L1 activation maintains intestinal homeostasis and attenuates reactions against microbiota by inhibiting TLR-mediated pro-inflammatory cytokine responses in macrophages and DCs. Strong support for this idea also comes from the observations that colonic pro-inflammatory cytokine

expression inversely correlates with the percentage of CD11c⁺ DCs expressing ATG16L1 in patients with CD and that induction of remission is accompanied by accumulation of CD11c⁺ DCs expressing ATG16L1 in the gastrointestinal tract of patients with CD[27].

ATG16L1 negatively regulates IFN-I responses mediated by RLRs and TLRs[11-14]. Isolated macrophages from patients with CD bearing the *ATG16L1* T300A mutation produced more IFN-I upon stimulation with TLR3 and TLR4 ligands than macrophages from patients with intact ATG16L1[13,36]. Excessive production of IFN-I is involved in the immunopathogenesis of CD and UC. Expression levels of the IFN-stimulated genes was shown to be higher in the inflamed colonic mucosa of patients with CD or UC than in healthy controls[13]. Moreover, expression levels of IFN-stimulated genes rapidly declined in response to infliximab treatment. Although the presence of the *ATG16L1* T300A variant is associated with colitogenic IFN-I responses, the enhanced production of IFN-I may improve survival of patients with colorectal cancer[36].

Similar to the molecular mechanisms of chronic inflammation in the presence of CD-associated mutations in *NOD2*, the *ATG16L1* T300A mutation promotes the development of CD by causing impaired production of AMPs in Paneth cells and excessive secretion of TLR-mediated pro-inflammatory cytokines by macrophages and DCs. MDP activation of *NOD2* induces robust production of AMPs from Paneth cells, thereby preventing bacterial overgrowth in the intestine[5]. Paneth cells deficient in *NOD2* or bearing CD-associated *NOD2* mutations fail to produce AMPs[5]. With regard to the pro-inflammatory cytokine responses, activation of intact *NOD2* by MDP negatively regulates the production of TLR-mediated pro-inflammatory cytokines through the induction of IRF4[23,24]. In the absence of intact *NOD2* or the presence of CD-associated *NOD2* mutations, pro-inflammatory cytokine responses by DCs are markedly enhanced upon exposure to TLR ligands derived from the intestinal microbiota[5]. Thus, impaired function of Paneth cells and excessive pro-inflammatory cytokine responses by TLRs underlie the immunopathogenesis of CD in the presence of *ATG16L1* and *NOD2* mutations.

CONCLUSION

The autophagic protein ATG16L1 plays an indispensable role in the maintenance of intestinal homeostasis. The *ATG16L1* T300A mutation confers an increased risk for the development of CD as it is associated with increased bacterial burden and excessive pro-inflammatory cytokine responses in the gastrointestinal tract. Elucidation of the molecular mechanisms by which the *ATG16L1* T300A variant leads to the development of CD has provided new insights into the immunopathogenesis of CD induced by impaired induction of autophagy.

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FOOTNOTES

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