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Inflammatory bowel disease: dysfunction of autophagy?

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Abstract: Recent genome-wide association studies identified single nucleotide polymorphisms within gene loci, encoding autophagy genes, e.g. the autophagy-related 16-like 1 (ATG16L1) and the immunity-related GTPase family M (IRGM), as an important risk factor for the onset of chronic inflammatory diseases such as Crohn's disease (CD) or rheumatoid arthritis. CD is characterized by a breakdown of the intestinal epithelial barrier function leading to an overwhelming and uncontrolled immune response to bacterial antigens. Autophagy, and therefore ATG16L1 and IRGM, are critically involved in the innate immune response to invading pathogens. Dysfunction of these molecules results in the increased survival of intracellular bacteria, defective antigen presentation and proinflammatory cytokine secretion. Interestingly, autophagy can also be regulated by other CD susceptibility genes, such as nucleotide oligomerization domain 2 or protein tyrosine phosphatase nonreceptor type 2, and the presence of the CD-associated variations within these genes results in comparable effects. ATG16L1 also plays a crucial role in maintaining Paneth cell function and morphology, while IRGM seems to be associated with mitochondrial function and apoptosis. Dysfunction of these molecules, i.e. of autophagy in vivo, is clearly associated with the increased bacterial infection and the onset of colitis. Interestingly, the phenotype of aberrant Paneth cells and dextran sodium sulphate-induced colitis in ATG16L1 hypomorphic mice closely resembles human CD. Taken together, the available data strongly suggest an important role for autophagy in maintaining intestinal homeostasis, and dysfunction of autophagy seems to be a major risk factor for the onset of chronic intestinal inflammation.

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Short title: IBD and autophagy dysfunction

Inflammatory Bowel Disease: Dysfunction of autophagy?

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Key words: Inflammatory bowel disease; Crohn's disease; autophagy; bacteria, ATG16L1

ABSTRACT

Recent genome wide association studies identified single nucleotide polymorphisms within gene loci, encoding autophagy genes, such as autophagy-related 16-like 1 (ATG16L1) and immunity-related GTPase family, M (IRGM) as important risk factor for the onset of chronic inflammatory diseases, such as Crohn's disease (CD) or rheumatoid arthritis. CD is characterized by a break-down of the intestinal epithelial barrier function leading to an overwhelming and uncontrolled immune response to bacterial antigens. Autophagy, and therefore ATG16L1 and IRGM, are critically involved in the innate immune response to invading pathogens. Dysfunction of those molecules results in increased survival of intracellular bacteria, defective antigen presentation and pro-inflammatory cytokine secretion. Interestingly, autophagy can also be regulated by other CD susceptibility genes, such as nucleotide oligomerization domain 2 (NOD2) or protein tyrosine phosphatase non-receptor type 2 (PTPN2) and the presence of the CD-associated variations within these genes results in comparable effects. In addition, ATG16L1 plays also a crucial role in maintaining Paneth cell function and morphology, while IRGM seems to be associated with mitochondrial function and apoptosis. Dysfunction of those molecules, meaning of autophagy *in vivo* is clearly associated with the increased bacterial infection and the onset of colitis. Interestingly, the phenotype of aberrant Paneth cells and dextran sodium sulphate (DSS)-induced colitis in ATG16L1 hypomorphic mice closely resembles human CD. Taken together, the available data strongly suggest an important role for autophagy in maintaining intestinal homeostasis and dysfunction of autophagy seems to be a major risk factor for the onset of chronic intestinal inflammation.

Genetic, immunological and bacterial factors are believed to contribute essentially to the pathogenesis of IBD. According to current hypothesis, an epithelial barrier defect, coupled with a dysfunctional immune response of the innate as well as the acquired immune system to commensal flora, results in either excessive up- or impaired downregulation of inflammatory events, finally driving the development of chronic intestinal inflammation [1]. Recent data demonstrated that the intestinal flora is significantly altered in IBD, since mucosal microbial diversity is reduced in IBD, particularly in CD. Further, also species composition is disturbed, since the number of *Firmicutes* is reduced in the intestinal tract of IBD patients while concurrent increases in *Bacteroidetes*, and in CD only, *Enterobacteriaceae*, can be detected [2]. Genome wide association studies (GWAS) identified replicated variations in 99 gene loci being associated with IBD [3]. While 28 of those gene loci are associated with the onset of both, CD and UC, a large number is specifically attributed to the development of either CD or UC. In example, exclusively associated with CD are genes being members of the innate immune system, such as the intracellular bacterial-sensor nucleotide oligomerization domain 2 (NOD2) as well as the autophagy genes autophagy 16-like 1 (ATG16L1) and immunity-related GTPase family, M (IRGM) [3].

AUTOPHAGY

Besides its genetic association with Crohn's disease, dysfunction of autophagy has been implicated in numerous pathologies, such as many types of cancer or neurodegeneration [4]. On the one hand, the fundamental role for autophagy in maintaining cellular homeostasis consist in generating energy and degrading cytoplasmic compartments, damaged organelles and misfolded proteins. In autophagosomes, these structures are sequestered into double-membrane-enclosed vesicles and delivered to lysosomes for final degradation [4-6]. On the

other hand, autophagy is critically involved in regulating innate immune responses by providing first line defence against intracellular pathogens, such as *Listeria monocytogenes* (LM) or *Salmonella typhimurium* and recognizing intracellular viruses and by mediating antigen presentation via MHC class II molecules [7-9]. Consequently, dysfunctional autophagy is associated with defective bacterial handling, prolonged intracellular survival of pathogens and uncontrolled inflammation. Of particular interest, levels of autophagy proteins, such as ATG16L1, IRGM or LC3B-II are significantly decreased in the intestinal tissue of CD patients when compared to non-IBD control patients (Figure 1A) [10].

Autophagy occurs either as macroautophagy, microautophagy or chaperone-mediated autophagy. The activation of macroautophagy occurs during cellular stress, such as starvation or hypoxia, as well as in response to antigens or pathogens. A critical regulator of autophagosome formation is the molecular target of rapamycin (mTOR) [11]. Hereby, activated mTOR acts as an inhibitor of autophagy. Further, prolonged activation of autophagy finally results in the activation of mTOR as part of a negative feed-back mechanism [12]. Following inhibition of mTOR, the formation of autophagosomes, is mediated by two highly conserved protein conjugation systems. After activation of beclin-1, autophagosome assembly involves ATG12-ATG5 conjugation which is catalyzed by ATG7 and ATG10. The resulting ATG5-ATG12 conjugate is stabilized by a non-covalent complex with ATG16L1. This complex mediates, in addition to ATG7 and ATG3, the conversion of LC3B-I to LC3B-II by lipidation with phosphatidylethanolamine, what finally establishes the formation of functional autophagosomes. Those structures co-localize with lysosomes to finally degrade their content in the so-called autophagolysosomes [13].

AUTOPHAGY GENES AS RISK FACTORS FOR CD

To date, three genes being associated with autophagy in humans have been confirmed as CD susceptibility genes, namely ATG16L1, IRGM and Leucine-rich repeat kinase 2 (LRRK2) [3]. A fourth one, namely unc-51-like kinase 1 (ULK1) has only been associated in a study specifically looking on SNPs in autophagy-related genes. Though ULK1 is important for autophagosome formation by formation a complex with ATG1, the functional relevance of the CD-associated SNPs within the ULK1 gene locus is unknown[14]. Of note, none of those four genes has been associated with increased risk for developing UC.

ATG16L1 represents a key molecule within the autophagy network being responsible for subcellular localisation of the autophagy machinery [15]. The SNP rs2241880 within the gene encoding ATG16L1 causes a switch from A to G allele at position 300. Presence of the disease-associated genotype GG results in substitution of threonine by alanine (T300A). The T300A variation is present in 58.1 % of CD patients (vs. 51.3 % of non-IBD control patients) and has been strongly associated with increased risk for developing CD, in particular ileal CD, but not UC [16-18]. It is well described that ATG16L1 protein is involved in the formation of functional autophagosomes, but recent evidence emerges that it plays also a crucial role for maintaining and regulating other cell functions, such as cytokine secretion and morphology and protein expression in Paneth cells. Of note, ATG16L1 knock-out mice die in the first day of their life since they are not able to deal with postnatal starvation [19].

A synonymous variation within the coding region of IRGM (rs10065172, c.313C>T) has been strongly associated with CD [17]. A further study revealed that this polymorphism exists in perfect linkage disequilibrium with a 20-kb deletion polymorphism upstream of the IRGM transcriptional start site. Interestingly, this deletion affects several transcription factor binding sites and hereby strongly impairs IRGM expression levels in a cell specific manner [20]. In addition, a number of polymorphisms within the promoter region or the 5'

untranslated region of IRGM are independently associated with CD [21]. Reduced expression of IRGM seems to be responsible for the decreased function of IRGM and a subsequent impairment in autophagy suggesting that a certain level of IRGM protein is necessary for proper protein function. Obviously, this level cannot be reached in cells carrying the CD-associated IRGM variants. The most important function of IRGM seems to consist in protecting the cells from invading bacteria. In contrast to ATG16L1-deficient mice, IRGM-deficient mice are vital, but less resistant to invading bacteria [22].

Recently, LRRK2 that plays a pivotal role for regulating autophagic activity has been confirmed as CD susceptibility [23]. Only one of the CD-associated SNPs is located within the coding region of LRRK2, namely rs376186 which results in decreased stability of the protein product leading to lower expression levels in Met2397 carriers [24]. LRRK2-deficient mice are more susceptible to DSS-induced colitis, since LRRK2 inhibits activation of NFAT1 which promotes the secretion of pro-inflammatory cytokines [25]. LRRK2 expression is elevated in intestinal tissue of CD patients and LRRK2-deficient cells are impaired in killing of intracellular bacteria [26] suggesting a possible role for LRRK2 in CD pathogenesis.

AUTOPHAGY AND BACTERIAL HANDLING

Recent studies demonstrate that presence of the CD-associated ATG16L1 variant results in increased numbers as well as prolonged survival and elevated replication of intracellular *Salmonella* *ssp.*, *E. coli* *ssp.* or *Shigella flexneri*. In particular, *adherent-invasive E.coli* (AIEC) are well associated with (ileal) CD and are known to colonize ileal CD lesions. ATG16L1 variant cells are not sufficient to limit the replication of AIEC, while their effectiveness against other *E. coli* strands is not impaired [27]. Further studies revealed that

enhanced intracellular survival and replication of AIEC in ATG16L1 (and IRGM or NOD2) mutant cells featuring dysfunctional autophagy also results in elevated secretion of the pro-inflammatory cytokines TNF and IL-6 finally promoting inflammatory conditions in the intestine [28]. Increased cytokine production in ATG16L1 mutant cells was also observed in a study using *Mycobacterium tuberculosis* (MTB). Here, mononuclear cells carrying the T300A variation featured increased IFN γ secretion when compared to ATG16L1 wild-type cells in response to MTB [29]. Of note, presence of the ATG16L1 variation obviously only affects the extent of autophagy following activation, while basal levels of autophagy remain unaffected when compared to ATG16L1 wild-type cells. However, T300A-variant carrying intestinal epithelial cells are clearly impaired in handling and capturing invading *Salmonella typhimurium* [30]. These observations strongly suggest a critical involvement of dysfunctional ATG16L1 and, consequently, autophagy in the pathogenesis of CD.

Comparable findings were obtained for IRGM. In mice, sufficient IRGM1 expression plays a crucial role for protecting the animals from pathogenic bacteria or protozoans. It has been shown that functional IRGM1 plays a key role in regulating the maturation of pathogen-containing vacuoles as well as the adhesion and motility of activated macrophages. Consequently, IRGM1-deficiency results in increased susceptibility of the mice to pathogens, such as MTB, *Listeria monocytogenes*, *Salmonella typhimurium* or *Toxoplasma gondii* and finally in systemic infections [22,31,32]. Singh et al. could clearly demonstrate that IRGM is crucial for IFN γ -mediated autophagy and for the elimination of intracellular *Mycobacterium tuberculosis* in human macrophages [33]. Further studies demonstrated that siRNA-induced knock-down of IRGM in human cells resulting in a defect of the autophagy machinery favours the persistence of AIEC resulting in increased pro-inflammatory responses [27,28]. As mentioned above, a certain threshold level of IRGM expression seems to be necessary for proper molecule function. This observation has been confirmed by McCarroll et al, since the

efficiency of the autophagy machinery against invading *Salmonella typhimurium* in human cells can be reduced by siRNA-mediated knock-down of IRGM, but enhanced by overexpression of IRGM [20]. Further confirmation comes from a study showing that a SNP that increases IRGM expression contributes to protection from MTB [34]. The necessary threshold level can obviously not be reached in presence of the CD-associated polymorphisms and lower levels of IRGM expression have been detected in lymphocytes from CD patients [21]. Interestingly, a recent study demonstrated that the microRNA family, miR-196, which is overexpressed in the inflamed intestinal epithelium of CD patients, causes a downregulation of the protective IRGM variant, but does not affect levels of the disease-associated variant. The resulting decrease in IRGM expression levels contributes to impaired autophagy and enhanced intracellular replication of AIEC [35]. Additionally, IRGM exerts a high affinity to mitochondrial cardiolipin, translocates to the mitochondria and induces either mitochondrial fission or depolarization. While IRGM-induced mitochondrial fission is necessary for controlling intracellular mycobacteria, IRGM-induced mitochondrial depolarization is associated with autophagy-independent cell death suggesting a role for IRGM not only in controlling pathogen invasion but also in regulating damaging inflammation as can be observed in CD [36]. Of note, the IRGM CD risk variant is associated with increased seropositivity for anti-flagellin antibodies in CD patients [37].

AUTOPHAGY AND NOD2

Recent studies clearly demonstrated a close functional correlation between ATG16L1 and other IBD susceptibility genes, such as NOD2 in regulating autophagy. Similar to ATG16L1, NOD2 has been associated with a severe structuring and/or penetrating CD phenotype featuring ileal disease [18,38]. NOD2 serves as an intracellular receptor for the

bacterial wall component, muramyl-dipeptide (MDP) and initiates cellular antibacterial responses by activating the innate immune system [39]. Presence of the CD-associated NOD2-variants results in a dysregulated response of the intestinal epithelium to bacterial antigens leading to uncontrolled pro-inflammatory events *in vitro* and *in vivo* [40,41]. MDP seems to represent a powerful activator of autophagy via NOD2 [7,8,42]. In dendritic cells (DC), NOD2-mediated autophagy is crucial for handling of invading bacteria as well as for antigen presentation and the induction of antigen-specific CD4⁺ T-cell responses via major histocompatibility complex (MHC) class II molecules. Interestingly, DC from CD patients carrying either the CD-associated ATG16L1 or NOD2 variations are defective in autophagosome formation, bacterial trafficking and antigen presentation [8]. As a possible mechanism, *Travassos et al.* showed that NOD2 is crucial for the initiation of autophagy by recruiting ATG16L1 to the cell membrane at the site of bacterial entry. Cells featuring CD-associated NOD2 polymorphisms are unable to direct ATG16L1 to the plasma membrane and are deficient in handling of invading *Shigella flexneri* [7]. Presence of the CD-associated ATG16L1 variant resulted in increased secretion of the pro-inflammatory cytokines IL-1 β and IL-6 from peripheral blood mononuclear cells from CD patients in response to stimulation with NOD2 ligands [43]. These observations strongly suggest that NOD2 as well as autophagy play a key role for the innate immune system and present the functional mechanism how dysfunction of autophagy can essentially contribute to the onset of chronic intestinal inflammation. Of note, the combined presence of CD-associated variations 3020insC within the NOD2 gene and T300A within the ATG16L1 gene is associated with the development of anti-Saccharomyces cerevisiae antibodies (ASCA) in CD patients [37].

AUTOPHAGY AND PTPN2

Recent studies also demonstrated a close functional correlation between the IBD susceptibility gene, protein tyrosine phosphatase non-receptor type 2 (PTPN2), ATG16L1, NOD2 and autophagy in general. PTPN2 has been well characterized as a key regulator of immune-related signalling pathways and functions [44]. PTPN2 expression is, at least partially, regulated by ATG16L1 and NOD2 [10,45]. On a functional level, PTPN2 seems also to be involved in NOD2-mediated autophagy, since presence of the CD-associated PTPN2 variations results in impaired autophagosome formation in human monocytes in response to MDP [45]. Dysfunction of PTPN2 is associated with decreased expression of autophagy genes, such as IRGM and ATG16L1. Interestingly, presence of the CD-associated variations within the PTPN2 gene in primary colonic lamina propria fibroblasts from CD patients resulted in defective autophagosome formation and increased intracellular numbers of invading *Listeria monocytogenes* (Figure 1B+C). Further, loss of PTPN2 in intestinal epithelial cells resulted in elevated IEC apoptosis, which was dependent on defective autophagy in these cells [10]. All of these events have been well associated with the onset of IBD confirming the role for dysfunctional autophagy in IBD pathogenesis.

ATG16L1 AND PANETH CELL FUNCTION

Besides its role in the handling of invading pathogens, ATG16L1 has been critically associated with the function of Paneth cells located within the crypts of Lieberkühn in the small intestine. Paneth cells represent a specialized epithelial cell type which is important for the secretion of antimicrobial factors into the intestinal lumen via production and secretion of characteristic cytoplasmic granules. In ATG16L1 hypomorphic (ATG16L1^{HM}) mice, Cadwell *et al.* found severe abnormalities in Paneth cell morphology and in their granule exocytosis pathways. In particular, ATG16L1^{HM} mice lack the antibacterial enzyme, lysozyme, in the

mucus of ileal sections compared to wild-type mice. Additionally, lysozyme was diffusely detectable in a number of Paneth cells in ATG16L1^{HM} mice accompanied by aberrant, disorganized granule as well as decreased numbers of granules. Further characteristic features of ATG16L1^{HM} Paneth cells were degenerated mitochondria, and the absence of apical microvilli. These abnormalities were in 100 % concordance with the ATG16L1^{HM} genotype suggesting an impaired exocytosis pathway in Paneth cells featuring defective autophagy. However, resistance to *Listeria monocytogenes* was not affected by the altered release of Paneth cell granules in these animals [46]. Further, Paneth cells from ATG16L1^{HM} mice revealed a gain of function with respect to the expression of molecules being involved in pro-inflammatory responses, such as peroxisome proliferator-activate receptor (PPAR) signalling as well as the adipocytokines, leptin and adiponectin [46]. Of special interest, Paneth cells derived from CD patients homozygous for the ATG16L1 CD risk allele feature similar abnormalities in Paneth cell morphology and granule secretion as ATG16L1^{HM} mice [46] strongly suggesting a key role for dysfunctional ATG16L1 and, subsequently, autophagy, in the pathogenesis of CD.

AUTOPHAGY AND COLITIS

Interestingly, all of the mentioned abnormalities in Paneth cell morphology and function were absent when the ATG16L1^{HM} mice were raised in an enhanced barrier facility, but could be completely introduced again following infection of the ATG16L1^{HM} mice with murine norovirus (MNV) strain CR6 for 7 days strongly suggesting a critical role for a virus-plus-susceptibility gene interaction [47]. Infection with MNV also caused a unique gene expression pattern in ATG16L1^{HM} Paneth cells favouring the expression of genes being associated with intracellular protein traffic, targeting and localization as well as with amino

acid metabolism [47]. ATG16L1^{HM} mice only displayed a severe intestinal injury response to dextran sodium sulphate (DSS) treatment when they were infected with MNV CR6 at least 7 days before DSS administration, but not in the absence of the virus or when the virus infection occurred at the same time as the begin of DSS treatment. Of note, the virus pre-infected ATG16L1^{HM} mice featured multiple hallmarks being characteristic for human CD, such as increased inflammation in the muscularis, increased numbers of lymphoid aggregates, subserosal fibrosis, hypertrophy of the muscularis propria, mucosal atrophy, ileal involvement and ulcerations [47]. The extent of the inflammatory reaction was critically dependent on the presence of TNF, IFN γ and commensal bacteria, since treatment with anti-TNF antibodies, anti-IFN γ antibodies and antibiotics resulted in a marked decrease in the inflammatory response [47]. These observations strongly suggest that virus-plus-susceptibility gene interactions in addition to environmental factors and commensal bacteria are critically involved in the pathogenesis of chronic intestinal inflammation. Further, there is also evidence for functional connection between IRGM and virus infection, since IRGM is targeted by a large number of RNA viruses and the formation of autophagosomes during infection with Measles virus, Hepatitis C virus and human immunodeficiency virus-1 is critically dependent on IRGM [48]. Further evidence for a pathogenetic role for ATG16L1 in the development of colitis comes from a study using mice lacking the conserved coiled-coil domain of ATG16L1. Such functional deficiency of ATG16L1 results in increased activation of the inflammasome in response to lipopolysaccharide (LPS) causing increased secretion of IL-1 β and IL-18. Also, these mice are more susceptible to DSS-induced colitis [19].

SUMMARY

A number of studies clearly provide evidence for the involvement of genetic variations within autophagy genes in the pathogenesis of CD. Dysfunction of autophagy, caused by genetic

variations within CD susceptibility genes has been well shown to result in defective handling of intracellular bacteria. In addition, while ATG16L1 is critical for Paneth cell function and regulating the secretion of pro-inflammatory cytokines, IRGM has been associated with mitochondrial depolarization or fission and apoptosis. Dysfunction of ATG16L1 and IRGM *in vivo* has been clearly associated with increased susceptibility to bacterial infection and the onset of colitis (Figure 2). All of these effects can be observed during human CD. These observations demonstrate a crucial role for autophagy in maintaining cellular homeostasis and strongly suggest that dysfunction of autophagy contributes essentially to the onset of chronic intestinal inflammation in human.

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Figure 1: Autophagy is altered in Crohn's disease patients. (A) Protein levels of ATG16L1 and IRGM are decreased in colonic biopsies derived from CD patients when compared to non-IBD control patients. (B) Loss of PTPN2 results in decreased formation of LC3B positive autophagosomes, but increased levels of intracellular *Listeria monocytogenes*. (C) In primary colonic lamina propria fibroblasts from CD patients, presence of the CD-associated PTPN2 variation impairs autophagosome formation and favours the onset of intracellular *Listeria monocytogenes*.

Figure 2: Current hypothesis of IBD pathogenesis. An epithelial barrier defect favours the penetration of commensal and pathogenic bacteria, food antigens and toxins into the gut mucosa. Genetic variations, in example, in autophagy-related genes contribute to the barrier defect and cause a dysregulated immune response to those molecules. The aberrant immune response results in a dysbalance between pro-inflammatory and anti-inflammatory cytokines finally establishing the onset of chronic intestinal inflammation.