CHAPTER 5

The $ATG16L1$–$T300A$ allele impairs clearance of pathosymbionts in the inflamed ileal mucosa of Crohn’s disease patients.

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ABSTRACT

Objective: Crohn’s disease (CD) is caused by a complex interplay between genetic, microbial and environmental factors. ATG16L1 is an important genetic factor involved in innate immunity, including autophagy and phagocytosis of microbial components from the gut. We investigated the effect of inflammation on the composition of microbiota in the ileal mucosa of CD patients in relation to the ATG16L1 risk status.

Design: Biopsies (n=35) were obtained from inflamed and non-inflamed regions of the terminal ileum of 11 CD patients homozygous for the ATG16L1 risk allele (ATG16L1-T300A) and 9 CD patients homozygous for the ATG16L1 protective allele (ATG16L1-T300). Biopsy DNA was extracted and the bacterial composition was analyzed by pyrosequencing. Intracellular survival rates of adherent invasive Escherichia coli (AIEC) were analyzed by determining colony forming units (CFU) after exposure to monocytes isolated from healthy volunteers homozygous for the ATG16L1 risk or protective allele.

Results: Inflamed ileal tissue from patients homozygous for the ATG16L1 risk allele contained increased numbers of Fusobacteriaceae, whereas inflamed ileal tissue of patients homozygous for the ATG16L1 protective allele show decreased numbers of Bacteroidaceae and Enterobacteriaceae and increased Lachnospiraceae. The ATG16L1 allele did not affect the bacterial composition in the non-inflamed ileal tissue. Monocytes homozygous for the ATG16L1 risk allele showed impaired killing of AIEC under inflammatory conditions compared to those homozygous for the ATG16L1 protective allele.

Conclusion: CD patients homozygous for the ATG16L1 –T300A risk allele show impaired clearance of pathosymbionts in ileal inflammation indicating that ATG16L1 is essential for effective elimination of pathosymbionts upon inflammation.
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\section*{INTRODUCTION}

The intestinal lumen is inhabited by a large number of microbes that aid in the digestion of dietary products\textsuperscript{1,2}. Healthy individuals are in symbiosis with the intestinal microbiota. Their intestinal immune system defends against pathogens\textsuperscript{3}, and is tolerant towards resident commensal microbes. A disruption of the balance between the host organism and the intestinal microbiota triggers the activation of the intestinal immune system and initiates an inflammatory reaction, which is characteristic for intestinal disorders, such as inflammatory bowel diseases (IBD)\textsuperscript{4,5}.

IBD, mainly Ulcerative colitis (UC) and Crohn’s disease (CD) are inflammatory disorders that arise from a complex interplay between genetic susceptibility and environmental factors, where the mucosal immunity against commensal bacteria seems to play a crucial role\textsuperscript{6,7}. CD has a discontinuous inflammation that can occur in the entire gastrointestinal tract, but is most typically located in the ileocolic region\textsuperscript{7,8}.

Genome wide association studies have created a comprehensive map of genomic susceptibility with over 160 loci that predispose for IBD\textsuperscript{9}. Many of which are involved in anti-bacterial defence systems. However, very little is still known regarding the details of interactions between individual susceptibility variants and the specific microbial composition\textsuperscript{10,11}. Polymorphisms in the \textit{NOD2} gene have been linked to alterations in innate host immunity. In addition, polymorphisms in \textit{ATG16L1} and \textit{IRGM}, two components involved in macro-autophagy, disturb the elimination of specific bacteria after internalization through phagocytosis, linking disturbed autophagy to the pathogenesis of CD\textsuperscript{12-15}. Moreover, Paneth cells show an abnormal morphology in patients homozygous for the \textit{ATG16L1-T300A} risk allele, which may affect the secretion of anti-bacterial peptides, such as defensins\textsuperscript{16,17}. Collectively, this may alter the microbiota composition and promote survival of intracellular bacteria in the underlying tissues, leading to chronic intestinal inflammation. Accumulating evidence support a tight link between phagocytosis and the autophagy machinery\textsuperscript{18,19}.

Several studies have demonstrated that IBD patients have an altered microbiota composition compared to healthy individuals, showing a reduced diversity and an increase in mucosa-adherent bacteria\textsuperscript{3,10,20,21}. Gut microbiota undergo remodeling during the active phase of CD and differ between remission and relapse phases of disease, though it is unknown what drives this process\textsuperscript{22}. Compared to healthy controls, IBD patients have fewer bacteria with anti-inflammatory properties and/or more bacteria with pro-inflammatory properties\textsuperscript{3,20,23,23}. \textit{Faecalibacterium prausnitzii} has anti-inflammatory properties and low numbers of it are associated with increased risk of post-resection recurrence of ileal CD. In contrast, pro-inflammatory adherent-invasive \textit{Escherichia coli} (AIEC) are more abundant in CD patients\textsuperscript{3}. Increased numbers of \textit{Bacteroides}, fusobacteria and \textit{E. coli} are associated with earlier relapse of CD in patients after ileocolectomy\textsuperscript{3,24}. We hypothesized that the \textit{ATG16L1}
genotype may directly affect bacterial handling by the ileal mucosa in CD patients, favoring a pro-inflammatory state.

In the present study, we studied the interrelationship between the ATG16L1 genotype and the composition of microbiota in the inflamed and non-inflamed ileal mucosa of CD patients. In addition, monocytes from healthy volunteers were used to study the effect of the ATG16L1 genotype on the processing and killing of AIEC.

MATERIAL AND METHODS

Tissue specimens

Ileal mucosal biopsies were obtained from CD patients at the University Medical Center Groningen, the Netherlands. All protocols for obtaining and studying human tissues were approved by the institution’s Medical Ethical Committee UMCG. All patients gave written informed consent.

Intestinal biopsies were obtained from macroscopically inflamed and non-inflamed ileal mucosa from 9 CD patients homozygous for the ATG16L1 protective allele (ATG16L1-300) (P) and from 11 CD patients homozygous for the ATG16L1 risk allele (ATG16L1-T300A) (R). Biopsies were genotyped regarding to the NOD2 and IRGM genes as well. For paired analysis 9 patients with risk allele provided biopsies from both inflamed and non-flamed regions (respectively RI and RN) and 6 did so from the patient with protective allele (PI and PN). Biopsies were immediately snap-frozen in liquid nitrogen and stored at -80°C until further processing. Patient data is described in Supplementary table 1.

DNA extraction

Total DNA was extracted from the biopsy samples using the QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer’s instruction, with an additional bead beating cell lysis step using a Precellys 24 (Bertin Technologies, France) and glass beads at 5.5 ms⁻¹ in three rounds of 1 minute at room temperature. DNA was eluted from the columns by 2 sequential washes with 250 µl of low salt buffer.²⁵,²⁶

Pyrosequencing

Amplicon libraries for pyrosequencing of the 16S rDNA V1-V3 regions were generated using a barcoded forward primer consisting of the 454 Titanium platform A linker sequence, a key (barcode) that was unique for each sample, and the 16S rRNA 534R primer sequence 5’-ATTACCGCGGCTGCTGG-3’, and a reverse primer consisting of a 9:1 mixture of two oligonucleotides, 5’-BAGAGTTTGATCMTGGCTCAG-3’ and 5’-BAGGGTTGATTCTGGCAG-3’, where B represents the B linker followed by the 16S rRNA 8F and 8F-Bif primers, respectively.²⁷ PCR amplifications were performed using 1x FastStart High Fidelity Reaction Buffer, 1.8 mM MgCl₂, 1mM dNTP solution, 5 U FastStart High Fidelity Blend Polymerase (Roche, USA), 0.2 µM reverse primer, 0.2 µM of the barcoded forward primer (unique for
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Each sample (and 1 µl of template DNA). PCR was performed using the following cycle conditions: an initial denaturation at 94°C for 3 min, 25 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 45 s and extension at 72°C for 5 min, and a final elongation step at 72°C for 10 min. Amplicons (20 µl) were purified using AMPure XP purification (Agencourt, USA) according to the manufacturer’s instructions and eluted in 25 µl 1x low TE. Amplicon concentrations were determined by Quant-iT PicoGreen dsDNA reagent kit (Invitrogen, USA) using a Victor3 Multilabel Counter (Perkin Elmer, USA), the quality was assessed on a Bioanalyzer 2100 (Agilent, USA). Amplicons were mixed in equimolar concentrations to ensure equal representation of each sample. A 454 sequencing run was performed on a GS FLX Titanium PicoTiterPlate with a GS FLX pyrosequencing system (Roche, USA).

Sequencing quality-control
Pyrosequencing produced a total of 632,726 reads of 16S rRNA with an average of 12,000 reads per sample ranging from 5,820 to 18,479 reads. Sequence analysis was performed using Quantitative Insights Into Microbial Ecology (QIIME) with default parameters, including removing sequence artifacts using Densi3 and chimera removal with Chimera Slayer; clustering via uclust at 97% similarity; then classified taxonomically using the RDP classifier retrained with Greengenes. In addition, for identification purposes down to the species level, using ARB as described by de Goffau et al.

Monocyte isolation from human peripheral blood
Heparinized blood was obtained from 8 healthy volunteers homozygous for either the ATG16L1 protective or risk allele (4 volunteers each). Human peripheral blood mononuclear cells were isolated using Lymphoprep gradients (Axis-Shield PoC As, Norway). Monocytes were further purified using CD14 monoclonal antibodies conjugated to micro-beads according to the manufacturer’s protocol (Miltenyi Biotec, Germany). The purity of monocytes was evaluated by fluorescent staining with CD14-FITC antibody (Miltenyi Biotec, Germany). Cell cultures of primary monocytes were performed in RPMI-1640 medium (PPA laboratories, Austria) supplemented with 10% (v/v) heat-inactivated fetal calf serum, penicillin (50 U/ml), streptomycin (50 µg/ml) and fungizone (5 µg/ml) at 37°C in a humidified atmosphere of 5% CO2. Monocytes were plated at 5*10^5 cells per ml on coverslips in 12-well plates with or without the presence of phorbol 12-myristate 13-acetate (PMA) (100 µM) (Sigma-Aldrich), TNFα and IL1β to mimic inflammation.

Survival assay
The bacterial survival/killing was measured by the gentamicin protection assay as previously described. The numbers of colony forming units (CFUs) were determined and represent
the AIEC that survived inside monocytes. To mimic inflammation, purified monocytes were activated overnight by PMA, TNFα and IL1β.

RNA Isolation and Quantitative real-time PCR
Total RNA was extracted from tissue specimens with trizol (Sigma-Aldrich, Netherlands), reverse transcribed and analyzed for gene expression using real time PCR (ABI PRISM 7700 sequence detector; Applied Biosystems, USA) as described before. 34. TaqMan® Gene Expression Assays for defensin 5 and 6 were obtained from Life Technologies (Bleiswijk, The Netherlands). CT values were normalized to the endogenous control (18S) and correlated inversely with initial mRNA levels. Primers and probes used are listed in Supplementary Table 2. Quantitative PCR analysis for total 16S rRNA (representative of bacterial load) enumeration was performed. 35. The ratio between 16S rRNA enumeration and 18S rRNA (representative of human tissue load) enumeration was calculated.

Statistical analysis
Principal component analysis (PCA) was performed to find clusters of similar groups of samples or species. All tests were performed with PASW Statistics 18 (SPSS, USA). As gut microbial species abundances are not normally distributed, non-parametric tests were used, as is described in the text. Differences in the CFUs and gene expressions were assessed by using the Mann-Whitney U test. All tests were two-tailed. P values of 0.05 or lower were considered significant.

RESULTS
There was not a significant difference between bacterial burden of the patients homozygous for ATG16L1 protective allele and risk allele in inflamed and non-inflamed state (Supplementary figure 1). The most abundant bacterial families in all the ileal mucosa samples were Bacteroidaceae (25%), Lachnospiraceae (18%), Enterobacteriaceae (9.4%), Ruminococcaceae (7.3%) and Fusobacteriaceae (6.4%) (Supplementary figure 2).

Differences in the gut microbiota modification upon inflammation
Non-parametric analyses and PCA of the abundance of bacterial groups shows that there are no significant differences in microbial composition in the non-inflamed ileal mucosa of CD patients homozygous for the ATG16L1 risk (R) or protective (P) allele. However, an unpaired analysis of the principal components and the individual microbial groups revealed significant differences in the composition of the gut microbiota upon inflammation in the 2 CD patient groups (Figure 1). In the inflamed ileal mucosa, an upward shift with regard to principal component 2, which accounts for 14% of the variation within the data, was observed in patients homozygous for the ATG16L1 protective allele (PC2, p=0.01). PC2 was
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positively correlated with \textit{Lachnospiraceae} and negatively with \textit{Bacteroidaceae} (both \(p<0.001\)). Indeed, biopsies from the inflamed mucosa of CD patient homozygous for the \textit{ATG16L1} protective allele showed reduced levels of \textit{Bacteroidaceae} compared to biopsies from the non-inflamed mucosa of these patients \((p=0.008)\). Moreover, \textit{Lachnospiraceae} were more common in the inflamed ileal mucosa of CD patients homozygous for the \textit{ATG16L1} protective allele compared to the inflamed ileal mucosa of CD patients homozygous for the risk allele \((p=0.042)\).

A paired analysis, on patients from which both inflamed and non-inflamed biopsies were available, increased the resolution further with respect to detecting changes in the gut microbiota upon inflammation. For example, an increase in the number of \textit{Fusobacteriaceae} is found upon inflammation in ileal biopsies of CD patients homozygous for the \textit{ATG16L1} risk allele \((p=0.046)\). This increase was not observed in an unpaired analysis as patients who do not have any \textit{Fusobacteriaceae} in non-inflamed ileal mucosa will also not have them in inflamed ileal mucosa. The analysis of paired inflamed and non-inflamed biopsies in the same patient confirmed the mucosal dysbiosis in CD patients homozygous for the \textit{ATG16L1} risk allele. Inflamed ileal mucosa from CD patients homozygous for the \textit{ATG16L1} protective allele showed relatively low numbers of \textit{Bacteroidaceae} \((p=0.028)\) and more \textit{Lachnospiraceae} \((p=0.046)\) in comparison to the non-inflamed ileal biopsies. In addition, inflamed mucosa from CD patients homozygous for the protective \textit{ATG16L1} allele had a lower score on PC3 \((p =0.028)\), which is positively correlated with \textit{Enterobacteriaceae} \((p=0.016)\) and \textit{Fusobacteriaceae} \((p<0.001)\), indicating that these latter species are underrepresented in the mucosa of these patients. In contrast, as described above, the inflamed ileal mucosa of CD patients homozygous for the \textit{ATG16L1} risk allele contained more \textit{Fusobacteriaceae} than in the non-inflamed parts of their ileum \((p=0.046)\).

A plot of the differences in microbial composition, which is a subtraction of non-inflamed from inflamed mucosa, in relation to the principal components 2 and 3 in CD patients homozygous for the \textit{ATG16L1} protective (P) or risk (R) allele is shown in Figure 2A. This plot demonstrates the effect of inflammation on the mucosal microbiota of the 2 patient groups. CD patients with the protective allele are clustered together in the upper left part of Figure 2A, indicating that upon inflammation the microbiota of the different patients of this group is modified in a similar pattern. In contrast, the differences in the paired samples of CD patients homozygous for the \textit{ATG16L1} risk allele are either close to zero in both dimensions (center), or located on the right or near to the bottom of the plot, opposite to the samples from patients with the protective allele. In CD patients homozygous for the \textit{ATG16L1} protective allele the localization on Figure 2A is due to a (relative) increase in the \textit{Lachnospiraceae} numbers and a decrease in the numbers of \textit{Bacteroidaceae} (PC2) and a decrease of \textit{Enterobacteriaceae} and/or \textit{Fusobacteriaceae} (PC3). In CD patients homozygous

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Figure 1: Principal component analysis of the microbial composition on the family level. A: Principal component (PC1) accounts for 54% of the variation in the data and PC2 represents 14% of the variation. Samples from patients homozygous for the \textit{ATG16L1} risk allele are depicted by triangles, (RI, risk allele inflamed ▲; RN, Risk allele non-inflamed Δ) and samples from patients homozygous for the protective allele are depicted with circles (PN, protective allele non-inflamed ○, PI, protective allele inflamed ●). B: The main correlation with PC2 with relevant bacterial groups is tabulated. This table describes the correlation of four bacterial families related to the PC2. C: The arrows in this figure indicate that the protective allele and inflamed mucosa group is positively correlated with PC2 (p=0.001) and contains relatively higher numbers of \textit{Lachnospiraceae} and lower numbers of \textit{Bacteroidaceae}. For the \textit{ATG16L1} risk allele, this is due to either increased numbers of \textit{Fusobacteriaceae}, a lack of changes or a combination of decreased numbers of \textit{Lachnospiraceae} and increased numbers of \textit{Bacteroidaceae} or \textit{Enterobacteriaceae}. The direction and strength of the correlations is shown by arrows in Figure 2B. Figure 2C shows the difference between the sum of the abundance of \textit{Bacteroidaceae}, \textit{Enterobacteriaceae} and \textit{Fusobacteriaceae} between paired (non-inflamed and inflamed) biopsies from both patient genotypes. It shows that inflammation leads to a significant decrease in these three bacterial groups (when considered as one group) in patients homozygous for the \textit{ATG16L1} protective allele. In contrast, these numbers remain approximately the same in patients with the \textit{ATG16L1} risk allele during inflammation. The modification of the gut microbiota, as expressed by this sum, is significantly different between the two patients groups (p= 0.01) and suggests that patients homozygous for the \textit{ATG16L1} risk allele respond differently or fail to respond properly to mucosal microbiota upon inflammation.

Since \textit{ATG16L1} mutations and \textit{IRGM} or \textit{NOD2} mutations are involved in bacterial clearance we also studied the effect of carrying the \textit{IRGM} rs13361189 and \textit{NOD2} rs2066844 (R702W)
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...variants. There were no patients homozygous for these *IRGM* and *NOD2* risk alleles and patients heterozygote for these risk alleles showed no differences in our principal component analysis.

Figure 2-A: Second principal component (PC2) that represents 14% of the variation in the data and the third principle component (PC3) representing 9% of the variation. Difference in subtraction of non-inflamed from inflamed state in 9 paired samples from patients homozygous for the *ATG16L1* risk allele are depicted by ▲ and from 6 samples from patients homozygous for the *ATG16L1* protective allele are depicted with ●. The figure shows that the difference in paired protective samples is positively correlated with PC2 (p=0.001). Figure 2-B: The direction and strength of the correlation with *Fusobacteriaceae*, *Enterobacteriaceae* and *Bacteroidaceae* is shown in arrows. Figure 2-C: shows the difference between percentage of *Bacteroidaceae* + *Enterobacteriaceae* + *Fusobacteriaceae* in inflamed and non-inflamed tissue (p=0.01). The figure shows the significant decrease in numbers of opportunistic bacteria in CD patients homozygous for the protective allele while the numbers stay approximately the same in CD patients homozygous for the *ATG16L1* risk allele.

**Survival assay for adherent invasive *E. coli***

The survival of the AIEC, established as colony forming units (CFU) after exposure to PMA-activated primary monocytes, was significantly higher using monocytes homozygous for the *ATG16L1* risk allele compared to monocytes homozygous for the *ATG16L1* protective allele, with an average of 173 vs. 50 CFU, respectively (p<0.05) (Figure 3-A). No significant difference in CFU was observed when monocytes were not activated by PMA, indicating that differences in *ATG16L1*-dependent killing of AIEC only become apparent under inflammatory conditions. IL1β stimulation of monocytes resulted in increased numbers of CFUs in both groups of monocytes (p<0.05), however, this increase for monocytes...
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homozygous for the ATG16L1 risk allele tended to be higher (average of 220 CFUs) than the one for monocytes homozygous for the ATG16L1 protective allele (average of 122 CFUs) (Figure 3-B). There were no significant differences between the survival rate of AIEC in the two types of monocytes with and without TNFα stimulation. However, as observed for IL-1β, CFU numbers using monocytes homozygous for the risk allele tended to be higher with an average of 96 vs 41 surviving AIEC in monocytes homozygous for the protective allele (Figure 3-C).

![Figure 3: Differences in colony forming units (CFUs) of AIEC obtained from monocytes isolated from healthy volunteers homozygous for the ATG16L1 protective (P) or the risk allele (R) with and without simulation of inflammation by PMA, IL1β and TNFα. Figure 3-A show that there are no significant differences in CFU in volunteers with the protective allele both in stimulated conditions with PMA and un-stimulated conditions. In contrast, significantly higher numbers of survived AIEC bacteria were observed in stimulated monocytes with PMA or IL1β from volunteers homozygous for the ATG16L1 risk allele compared to the un-stimulated situation (3-A and B) (p<0.05). Figure 3-C shows the same trend for TNFα however, the increase in CFU is not significant.

Differences in inflammation-related gene expression:
The NFκB mediated inducible nitric oxide synthase (iNOS) was equally increased in the inflamed ileum of both ATG16L1 genotypes (supplementary figure 3-A). Except for one patient homozygous for the ATG16L1 protective allele, the defensins 5 and 6 gene expression (paneth cell function) decreased upon inflammation in all other patients (p<0.05 for risk allele group) (Figure 4-A and 4-B).
The expression of various other cytokines; COX2, IL1β (related to pro-inflammatory type 1 macrophages) and IL10, MRC1 and TGF- β gene (related to tissue repair type 2 macrophages) were not significantly different in the inflamed ileum of both patients groups (supplementary figure 3).
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Figure 4: Differences in Defencin 5 and 6 gene expression between inflamed and non-inflamed paired samples of patients homozygous for \textit{ATG16L1} risk (R) and protective allele (P). Figure 4-A and 4-B show that there is no significant difference in the gene expressions between inflamed biopsies of both groups of patients (PI and RI).

**DISCUSSION**

In this study, we show that CD patients homozygous for the \textit{ATG16L1} risk allele are unable to adequately clear pathosymbionts, such as \textit{Enterobacteriaceae}, \textit{Bacteroidaceae} and \textit{Fusobacteriaceae}, during ileal inflammation in comparison to CD patients who are homozygous for the protective allele. Biopsies of inflamed terminal ileum from patients homozygous for the \textit{ATG16L1} protective allele contain markedly less pathosymbionts and have relatively more \textit{Lachnospiraceae} than their counterparts homozygous for the risk allele. In contrast, no differences were observed between both groups when comparing the microbial composition of biopsies obtained from non-inflamed parts of the ileum. Using an \textit{in vitro} inflammation model for isolated monocytes we furthermore demonstrate the \textit{ATG16L1-T300A} risk allele impairs xenophagy of invading opportunistic pathogens.

CD has been proposed to be the consequence of an immune response towards a variety of environmental inflammatory triggers in a genetic susceptible host \textsuperscript{37,38}. This can include the uncontrolled immune response against a variety of either pathogenic or non-pathogenic bacteria in the gut\textsuperscript{4,5}. Certain pathogenic bacterial species have been suspected as inflammatory triggers in CD. Specifically the higher abundance of AIEC in pathogenesis of ileal CD and the role of potential pathogens such as \textit{B. fragilis} and fusobacteria with regard to the recurrence of CD after ileocolonic resection have been described before\textsuperscript{24}. Additionally, it has been shown that the numbers of some non-pathogenic bacteria such as \textit{F. prausnitizii} with beneficial anti-inflammatory effects on the epithelium significantly decreases in CD patients\textsuperscript{3}. These findings suggest a difference in the immune response between patients homozygous for \textit{ATG16L1} risk and protective allele with regard to killing invasive opportunistic pathogens, but that this is only observed in inflamed tissue. The
higher abundance of *Lachnospiraceae* in biopsies from inflamed parts of CD patient’s ileum homozygous for the protective allele could be due to the immune system’s ability to distinguish non-pathogenic microbiota from pathosymbionts when harboring this *ATG16L1* gene variant.

A higher abundance of the three pathosymbiont groups during inflammation namely *Enterobacteriaceae* (mostly *E. coli*), *Bacteroidaceae* (mostly *B. fragilis* group) and *Fusobacteriaceae*, in inflamed tissue of the terminal ileum of patients with the risk allele, in comparison with those with the protective allele, indicative of the impairment of the immune system of these patients to clear such bacterial groups. This could be a result of an impaired autophagy/xenophagy process. It remains elusive whether the invasion of the intestinal epithelial layer of patients homozygous for the risk allele by *Enterobacteriaceae*, *Bacteroidaceae* and *Fusobacteriaceae* is a cause or consequence of inflammation.

Our findings concerning the inability of the immune system of patients homozygous for the *ATG16L1* risk allele to properly handle invading bacteria is supported by the results of the killing/survival assay. The higher numbers of bacterial CFU show the inability of monocytes with the *ATG16L1* risk allele to effective process and remove AIEC cells upon inflammation. Interestingly, such a difference is not observed when monocytes were not exposed to PMA. PMA activates protein kinase C (PKC) and triggers reactive oxygen species (ROS) production, thereby causing oxidative stress and increased production of inflammatory cytokines, providing an *in vitro* model for inflammation\(^39\). In addition, we show that exposure to either IL-1β or TNF-α enhanced the survival of AIEC in monocytes homozygous for the risk allele compared to monocytes homozygous for the protective allele, though these differences were not significant. This may imply that the enhanced survival of AIEC in *ATG16L1-T300A* homozygous monocytes, as observed after PMA stimulation, is a result of a synergistic effect of various cytokines. This finding is in line with findings of Murthy *et al.* which show the inability of knock-in mice harboring the *ATG16L1* risk variant in effective clearance of the ileal pathogen *Yersinia enterocolitica* \(^40\). The increased survival of AIEC in stimulated *ATG16L1-T300A* homozygous monocytes is in line with the increased numbers of potential pathogens like *E. coli*, *B. fragilis* and fusobacteria in inflamed mucosa of CD patients homozygous for the *ATG16L1* risk allele. Furthermore, the fact that the *ATG16L1* allele did not affect AIEC survival in non-stimulated monocytes is in agreement with the observation that no differences in the microbiota composition were found in non-inflamed tissue of the 2 patients groups. We did not find significant differences in the expression of genes related to pro-inflammatory signaling type 1 macrophages and tissue repair type 2 macrophages in the inflamed biopsies between two genotypes of patients nor in biopsies form the non-inflamed ileum of those patients which could indicate that there is no differences in macrophages differentiation upon inflammation. However, the mechanism behind this
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inability and whether there is a difference in macrophages function upon activation needs to be further investigated.

Our hypotheses that the \textit{ATG16L1} risk allele impairs the autophagy process of pathogenic bacteria is in line with a previous study that showed that the \textit{ATG16L1} risk allele increases susceptibility to \textit{H. pylori} infection\textsuperscript{41}. Moreover, \textit{in vitro} studies revealed that the \textit{ATG16L1} risk allele incapacitates the autophagy process against \textit{Salmonella} in human epithelial cells\textsuperscript{42} and that siRNA knockdown of \textit{ATG16L1} impairs the autophagy process of AIEC in HeLa cells\textsuperscript{43}. In another study the \textit{ATG16L1-T300A} risk allele did not change the autophagy process against \textit{S. typhimurium} in mouse embryonic fibroblasts\textsuperscript{44}, which is also in agreement with our findings since differences only occur under inflammatory conditions.

Paneth cells produce different antimicrobial peptides, particularly the $\alpha$-defensins 5 and 6. The expression of HD-5 and HD-6 in the paired (uninflamed vs. inflamed) ileal biopsies from both genotypes were analyzed. Both HD-5 and -6 expression were strongly suppressed upon inflammation, but no significant differences were observed in HD-5 and -6 levels in non-inflamed ileum of the 2 genotypes nor between the inflamed ileum of those two patients groups. These findings could indicate that the observed differences in mucosal bacterial composition are most probably not due to \textit{ATG16L1}-related Paneth cell dysfunction. Genotyping of CD patients for genes that are involved in bacterial recognition and autophagy could reduce post-operative recurrence of CD after ileocecal resection. Antibiotica prophylaxis of recurrence has already been shown, but the use of more selective antibiotics to control the pathosymbionts could be even more beneficial, especially in patients homozygous for the \textit{ATG16L1} risk allele\textsuperscript{45}. Based on recent reports one could also posit that small molecule inducers of selective autophagy could contribute to enhanced bacterial autophagy in patients homozygous for the \textit{ATG16L1} risk allele. However additional studies are required to determine if the small molecules / FDA approved compounds possess both autophagy-dependent and autophagy-independent activity on immune pathways\textsuperscript{46}.

\textit{ATG16L1} is a crucial factor in the autophagy pathway that is associated with the innate immune system\textsuperscript{47}. Mutation in this specific loci seems to affect the regulation of the immune response against the intestinal microbiota but only under conditions of inflammation. The \textit{ATG16L1} risk allele in CD patients is associated with a higher abundance of pathosymbionts, such as \textit{Enterobacteriaceae}, \textit{Bacteroidaceae} and \textit{Fusobacteriaceae} in the intestinal epithelial layer during inflammation. In contrast, patients with the protective allele have higher numbers of commensal bacteria such as \textit{Lachnospiraceae} in their mucosal microbiota. These groups of bacteria may play a beneficial role in maintaining an anti-inflammatory balance since many of them are directly or indirectly involved in the production of butyrate, which stimulates the barrier function of the gut\textsuperscript{48}.  

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In conclusion this study shows that CD patients homozygous for the ATG16L1 –T300 risk allele display impaired clearance of pathosymbionts in ileal inflammation and that the killing of AIEC is impaired in activated monocytes homozygous for this risk allele, both indicating that ATG16L1 is essential for effective elimination of pathosymbionts upon inflammation.

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All authors have no conflict of interest to declare.

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## Supplementary Table 1: List of CD patients in the study with available data, including genotype, type of biopsy, age at the visit date, medicine used, and smoking condition is described.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>ATG16L1 genotype</th>
<th>NOD2 genotype</th>
<th>IRGM genotype (rs13361189)</th>
<th>Non-inflamed biopsy</th>
<th>Inflamed biopsy</th>
<th>Age at visit</th>
<th>Medicine used</th>
<th>Smoking state</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATG16L1 T300</td>
<td>Wild-type</td>
<td>Wild-type</td>
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The interaction between *ATG16L1* and gut microbiota

**Supplementary Table 2:** Primers and probes used in this study

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**Supplementary figure 1:** The 16S/18S enumeration ratios in the biopsies. The graph shows that there are no significant differences between biopsies of patients homozygous for *ATG16L1* risk and protective allele in inflamed (PI and RI) and non-inflamed (PN and RN) state. The median of 16S/18S ratio for PN group is 0.138 for PI, 0.053 for RN, 0.035 and for RI group 0.033.
Supplementary Figure 2: The percentages of abundance of the major bacterial groups in inflamed and non-inflamed ileal biopsies from patients homozygous for ATG16L1 risk or protective allele. Most abundant bacterial families are Bacteroidaceae (25%), Lachnospiraceae (18%), Enterobacteriaceae (9.4%), Ruminococcaceae (7.3%) and Fusobacteriaceae (6.4%).
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**Supplementary Figure 3**: The differences between iNOS, COX2 and IL1β (upper row of the figure) and IL10, TGFβ and MRC1 (lower row of the figure) gene expressions between paired inflamed and non-inflamed biopsies of patients homozygous for *ATG16L1* protective allele (PN and PI) and patients homozygous for risk allele (RN and RI). Bar shows the significance between groups (p<0.05).