Chapter 6

The *ATG16L1–T300A* allele impairs clearance of pathosymbionts in the inflamed ileal mucosa of Crohn's disease patients

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Gut, in press

ABSTRACT

Background & Aims

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.

Methods

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 were 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 showed 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.

INTRODUCTION

The intestinal lumen is inhabited by a large number of microbes that aid in the digestion of dietary products[1,2]. Healthy individuals are in symbiosis with the intestinal microbiota. Their intestinal immune system defends against pathogens[3] and is tolerant towards resident commensal microbes. A disruption of the delicate 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)[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[6,7]. CD has a discontinuous inflammation that can occur in the entire gastrointestinal tract, but is most typically located in the ileocolic region[7,8]. Genome wide association studies have created a comprehensive map of genomic susceptibility with over 160 loci that predispose for IBD[9]. Many of which are involved in anti-bacterial defense systems, including the innate immune system and secretion of anti-bacterial peptides by the Paneth cells. Still very little is known regarding the interactions between individual susceptibility variants and the specific microbial composition[10,11]. Polymorphisms in the *NOD2* gene have been linked to alterations in innate host immunity. In addition, polymorphisms in *ATG16L1* and *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[12-15]. Moreover, Paneth cells show an abnormal morphology in patients homozygous for the *ATG16L1-T300A* risk allele, which may affect the secretion of anti-bacterial peptides, such as defensins[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[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[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[22]. Compared to healthy controls, IBD patients have fewer bacteria with anti-inflammatory properties and/or

more bacteria with pro-inflammatory properties[3,20,23]. *Faecalibacterium prausnitzii (F. prausnitzii)* has anti-inflammatory properties and low numbers are associated with increased risk of post-resection recurrence of ileal CD. In contrast, pro-inflammatory adherent-invasive *Escherichia coli* (AIEC) are more abundant in CD patients[3]. Increased numbers of *Bacteroides*, Fusobacteria and *Escherichia coli (E. coli)* are associated with earlier relapse of CD in patients after ileocolectomy[3,24]. We hypothesized that the *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-T300;* P) and from 11 CD patients homozygous for the *ATG16L1* risk allele (*ATG16L1-T300A;* R)[9]. Biopsies were genotyped for the *NOD2* and *IRGM* genes as well. For paired analysis, 6 patients carrying the *ATG16L1* protective allele (PI and PN) and 9 patients with carrying the *ATG16L1* risk allele provided biopsies from both inflamed and non-inflamed regions (respectively RI and RN). Biopsies were immediately snap-frozen in liquid nitrogen and stored at -80°C until further processing. Patient data is described in Supplementary table S1.

DNA extraction

Total DNA was extracted from the biopsy samples using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Additionally, bead beating was performed using a Precellys 24 (Bertin Technologies, Montigny le Bretonneux, France) and glass beads at 5.5 ms^{-1} in three rounds of 1 min each with 30 sec pauses at room temperature in between. 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'-B-AGAGTTTGATCMTGGCTCAG-3' and 5'-B-AGGGTTCGATTCTGGC TCAG-3', where B represents the B linker followed by the 16S rRNA 8F and 8F-Bif primers, respectively[27]. PCR amplifications (in a volume of 50 µl) were performed using 1x FastStart High Fidelity Reaction Buffer, 1.8 mM $MgCl_2$, 1 mM dNTP solution, 5 U FastStart High Fidelity Blend Polymerase (Roche, CT, USA), 0.2 µM reverse primer, 0.2 µM of the barcoded forward primer (unique for 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 sec, annealing at 51°C for 45 sec 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, MA, USA) according to the manufacturer's instructions and eluted in 25 µl 1x low TE (10 mM Tris-HCl, 0.1mM EDTA, pH 8.0). Amplicon concentrations were determined by Quant-iT PicoGreen dsDNA reagent kit (Invitrogen, NY, USA) using a Victor3 Multilabel Counter (Perkin Elmer, MA, USA), the quality was assessed on a Bioanalyzer 2100 (Agilent, CA, 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, CT, 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)[28] with default parameters, including removing sequence artifacts using Denoiser[29] and chimera removal with ChimeraSlayer; clustering via uclust[30] at 97% similarity; then classified taxonomically using the RDP classifier[31] retrained with Greengenes[32]. In addition, for identification purposes down to the species level, using ARB as described by de Goffau *et al.*[25].

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, Leiden, The Netherlands). The purity of monocytes was evaluated by fluorescent staining with CD14-FITC antibody (Miltenyi Biotec). Cell cultures of primary monocytes were performed in RPMI-1640 medium in T75 flasks (Greiner bio-one, Alphen aan den Rijn, The Netherlands) 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% CO_2. Monocytes were plated at $5*10^5$ cells per ml on coverslips in 12-well plates (Greiner bio-one) with or without the presence of phorbol 12-myristate 13-acetate (PMA, 100 µM; Sigma-Aldrich, MO, USA), TNF-α and IL-1β to mimic inflammation.

Survival assay

The bacterial survival/killing was measured by the gentamicin protection assay as previously described[33]. Briefly, cells were infected at a multiplicity of infection (MOI) of 100 bacteria per monocyte. After 30 min of incubation at 37°C with 5% CO_2, infected monocytes were washed twice with sterile PBS and fresh culture medium containing 50 µg/ml of gentamycin was added to kill extracellular bacteria. After incubation of 1 hour, infected cells were washed twice with PBS and lysed by 1% Triton X-100 (Sigma) for an additional 5 min at room temperature. Total cellular lysate was plated onto Luria Bertani (LB) agar plates and incubated overnight at 37°C. 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, TNF-α and IL-1β.

RNA Isolation and Quantitative real-time PCR

Total RNA was extracted from tissue specimens with trizol (Sigma), reverse transcribed and analyzed for gene expression using real time PCR (ABI PRISM 7700 sequence detector; Applied Biosystems, NY, 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 S2. Quantitative PCR (Q-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

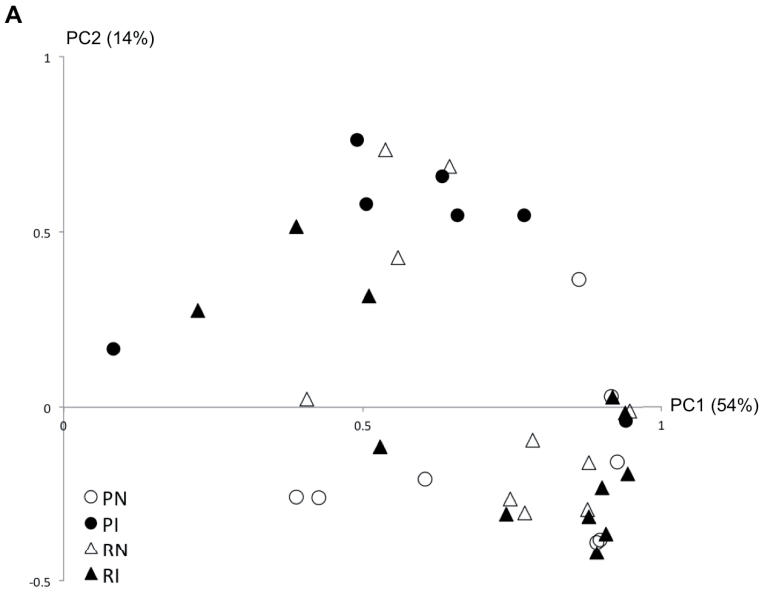
Principal component analysis (PCA) was performed to find clusters of similar groups of samples or species. All tests were performed with PASW Statistics 18 (SPSS, USA). As gut microbial species abundances are not normally distributed, non-parametric tests were used as described in the text. Differences in 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 no significant difference between the bacterial burden of the patients homozygous for *ATG16L1* protective allele (P) and risk allele (R) in inflamed and non-inflamed state (Supplementary figure S1). 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 S2).

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* protective allele (P) and risk (R). 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; Figure 1A). PC2 was positively correlated with *Lachnospiraceae* and negatively with *Bacteroidaceae* (both P<0.001; Figure 1B). Indeed, biopsies from the inflamed mucosa of CD patient homozygous for the *ATG16L1* protective allele showed reduced levels of *Bacteroidaceae* compared to biopsies from the non-inflamed mucosa of these patients (P=0.008; Figure 1B and C). Moreover, *Lachnospiraceae* were more common in the inflamed ileal mucosa of CD patients homozygous for the *ATG16L1* protective allele compared to the inflamed

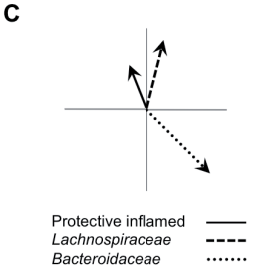


B

Correlation of bacterial groups with principal component 2
Spearman's rho test

Variable	%	P
Bacteroidaceae	25.0%	8×10^{-8} (-)
Lachnospiraceae	18.0%	2×10^{-6} (+)
Enterobacteriaceae	9.4%	0.224 (-)
Fusobacteriaceae	6.4%	0.127 (-)

(+) or (-) indicates positive or negative correlation



CHAPTER 6

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 *ATG16L1* risk allele are depicted by triangles, (RN, risk allele non-inflamed Δ; RI, risk allele inflamed ▲) 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 and describes the correlation of four bacterial families related to the PC2. (C) The arrows indicate that the protective allele and inflamed mucosa group is positively correlated with PC2 (P=0.001) and is associated with a high *Lachnospiraceae* abundance and lower numbers of *Bacteroidaceae.*

ileal mucosa of CD patients homozygous for the risk allele (P=0.042; Figure 1B and C). A paired analysis from available inflamed and non-inflamed patient biopsies, increased the resolution further in order to detect changes in the gut microbiota upon inflammation. For example, an increase in the number of *Fusobacteriaceae* is found upon inflammation in ileal biopsies of CD patients homozygous for the *ATG16L1* risk allele (P=0.046). This increase was not observed in an unpaired analysis, as patients who do not contain any *Fusobacteriaceae* in non-inflamed ileal mucosa will also not contain those bacteria 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 *ATG16L1* risk allele. Inflamed ileal mucosa from CD patients homozygous for the *ATG16L1* protective allele showed relatively low numbers of *Bacteroidaceae* (P=0.028) and more *Lachnospiraceae* (P=0.046) in comparison to the non-inflamed ileal biopsies. In addition, inflamed mucosa form CD patients homozygous for the protective *ATG16L1* allele had a lower score on PC3 (P=0.046), which is positively correlated with *Enterobacteriaceae* (P=0.016) and *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 *ATG16L1* risk allele contained more *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 *ATG16L1* protective (P) or risk allele (R) 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 *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 *ATG16L1* protective allele the localization is due to an (relative) increase in the *Lachnospiraceae* numbers and a decrease in the numbers of *Bacteroidaceae* (PC2) and a decrease of *Enterobacteriaceae* and/or *Fusobacteriaceae* (PC3; Figure 2A). In CD patients homozygous for the *ATG16L1* risk allele, this is due to either increased numbers of *Fusobacteriaceae*, a lack of changes or a combination of decreased numbers of

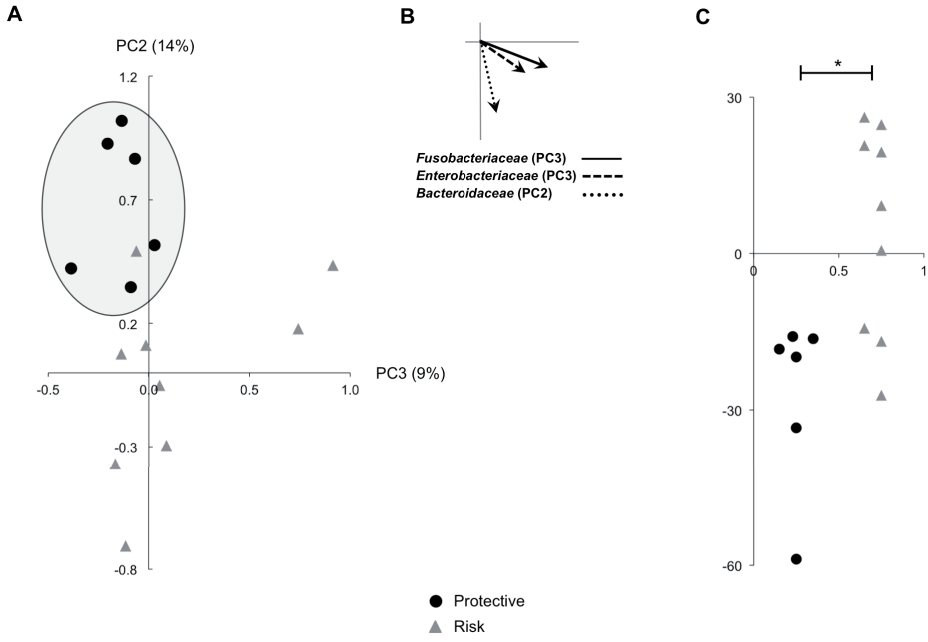


Figure 2. Differences in microbial composition in relation to principal components 2 and 3 in CD patients carrying the *ATG16L1* protective or risk allele. (A) Second principal component (PC2) that represents 14% of the variation in the data and the third principle component (PC3) representing 9% of the variation and shows that the difference in paired protective samples is positively correlated with PC2 (P=0.001). 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 paired samples from patients homozygous for the *ATG16L1* protective allele are depicted with ●. (B) The direction and strength of the correlation with *Fusobacteriaceae*, *Enterobacteriaceae* and *Bacteroidaceae* is shown in arrows. (C) 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 *ATG16L1* protective allele while the numbers stay approximately the same in CD patients homozygous for the *ATG16L1* risk allele.

Lachnospiraceae and increased numbers of *Bacteroidaceae* or *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 *Bacteroidaceae*, *Enterobacteriaceae* and *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 *ATG16L1* protective allele. In contrast, these numbers remain approximately the same in patients with the *ATG16L1* risk allele during inflammation. The modifications of the gut microbiota, as expressed by this sum, is significantly different between the two patients groups (P=0.01) and

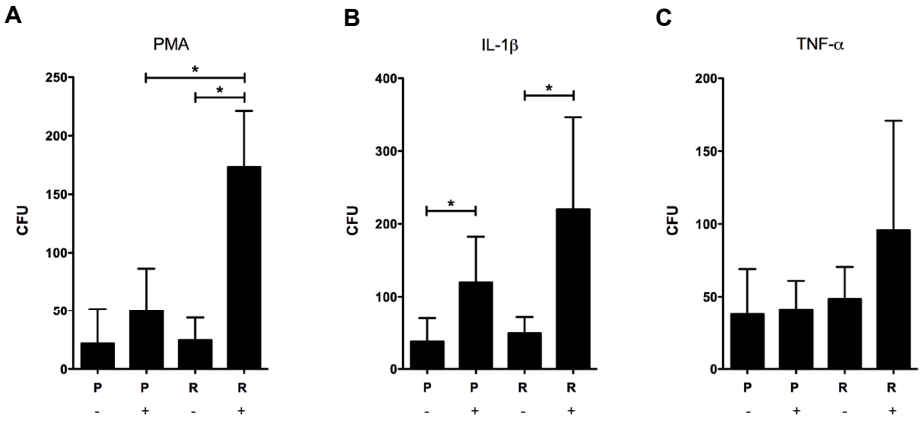


Figure 3. Differences in colony forming units (CFUs) of AIEC obtained from monocytes isolated from healthy volunteers homozygous for the *ATG16L1* protective (P) or the risk allele (R) with and without simulation of inflammation by PMA, IL-1β and TNF-α. (A) No significant differences in CFUs in healthy volunteers with the protective allele both in stimulated conditions with PMA and unstimulated conditions. (A/B) In contrast, significantly higher numbers of survived AIEC bacteria were observed in stimulated monocytes with PMA or IL-1β from volunteers homozygous for the *ATG16L1* risk allele compared to the unstimulated situation (*P<0.005). (C) The same trend for TNF-α is observed, however, the increase in CFU is not significant.

suggests that patients homozygous for the *ATG16L1* risk allele respond differently or fail to respond properly to mucosal microbiota upon inflammation.

Since ATG16L1, IRGM and NOD2 are involved in bacterial recognition and clearance, we also studied the effect of carrying the *IRGM* (rs13361189) and *NOD2* (rs2066844; R702W and rs2066845; G908R) 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.

SURVIVAL ASSAY FOR ADHERENT INVASIVE *E. COLI*

The survival of AIEC, established as colony forming units (CFUs) 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 3A). No significant difference in CFUs was observed when monocytes were not activated by PMA, indicating that differences in *ATG16L1*-dependent killing of AIEC only become apparent under inflammatory conditions. IL-1β stimulation of monocytes resulted in an increased numbers of CFUs in both groups of monocytes (P<0.05), however this increase for monocytes homozygous for *ATG16L1* risk allele

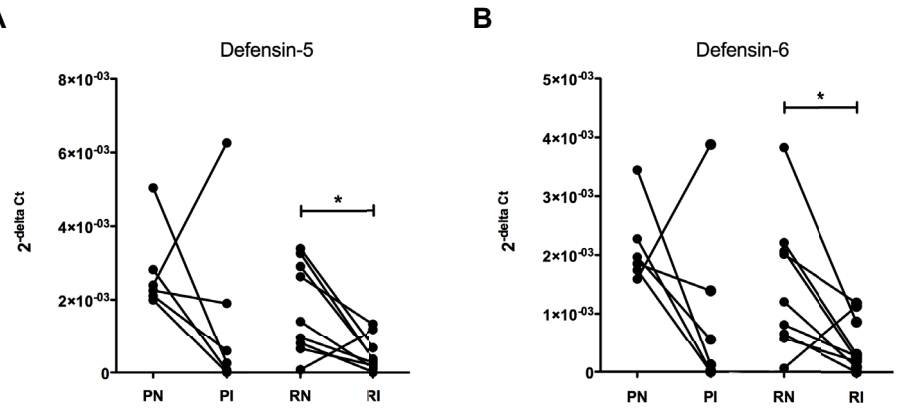


Figure 4. Differences in Defensin 5 and 6 gene expression between inflamed and non-inflamed paired samples of patients homozygous for *ATG16L1* protective allele (P) and risk (R). (A,B) There is no significant difference in the gene expressions between inflamed biopsies of both group of patients (PI and RI). *P<0.05.

was higher (average of 220 CFUs) than the one for monocytes homozygous for *ATG16L1* protective allele (average of 122 CFUs) (Figure 3B). There were no significant differences between the survival rate of AIEC in two types of monocytes with and without TNF-α stimulation. However, CFU numbers using monocytes homozygous for risk allele compared to the ones homozygous for protective allele were higher with an average of 96 vs. 41, respectively (Figure 3C).

Differences in inflammation-related gene expression

The NF-κB mediated inducible nitric oxide synthase (iNOS) was equally increased in the inflamed ileum of both *ATG16L1* genotypes (Supplementary figure S3A)[36]. Except for one patient homozygous for the *ATG16L1* protective allele, defensin 5 and 6 gene expression (involved in Paneth cell function) decreased upon inflammation in all other patients carrying the risk allele (P<0.05; Figure 4A and 4B).

The expression of various other cytokines; IL-10, COX2, IL-1β (related to pro-inflammatory type 1 macrophages), TGF-β gene (related to tissue repair type 2 macrophages) and MRC1 were not significantly different in the inflamed ileum of both patients groups (Supplementary figure S3B-F).

DISCUSSION

In this study, we show that CD patients homozygous for the *ATG16L1* risk allele are unable to adequately clear pathosymbionts, such as *Enterobacteriaceae*, *Bacteroidaceae* and *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 *ATG16L1* protective allele contain markedly less pathosymbionts and have relatively more *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 *in vitro* inflammation model for isolated monocytes, we furthermore demonstrate the *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[37,38]. This can include the uncontrolled immune response against a variety of either pathogenic or non-pathogenic bacteria in the gut[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 *Bacteroides fragilis* and Fusobacteria with regard to the recurrence of CD after ileocolonic resection have been described[24]. Additionally, it has been shown that the numbers of some non-pathogenic bacteria such as *F. prausnitzii* with beneficial anti-inflammatory effects on the epithelium, significantly decreases in CD patients[3]. Together with our findings, this suggest a difference in the immune response between patients homozygous for *ATG16L1* protective and risk allele with regard to killing invasive opportunistic pathogens, but only under inflammatory conditions. The higher abundance of *Lachnospiraceae* in biopsies from inflamed parts of CD patient's ileum homozygous for the protective allele could be a result of the immune system's inability 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 to those with the protective allele, is 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 there is a significant increase in the survival rate of AIEC in monocytes isolated from volunteers homozygous for the *ATG16L1* risk allele after stimulation with IL-1β and TNF-α, which corresponds with our findings in PMA-stimulated monocytes. This finding correspond well to the findings of Murthy *et al*. showing 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 agreement 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 are no differences in macrophages differentiation upon inflammation. However, the mechanism behind this inability and whether there is a difference in macrophages function upon activation needs to be further investigated.

Our hypotheses that the *ATG16L1* risk allele impairs the autophagy process of pathogenic bacteria is in line with a previous study that showed that the *ATG16L1* risk allele increases susceptibility to *Helicobacter pylori* infection[41]. Moreover, *in vitro* studies revealed that the *ATG16L1* risk allele incapacitates the autophagy progress against *Salmonella* in human epithelial cells[42] and that siRNA knockdown of *ATG16L1* impairs the autophagy process of AIEC in HeLa cells[43]. In another study, the *ATG16L1-T300A* risk allele did not change the autophagy process against *Salmonella*

typhimurium in mouse embryonic fibroblasts[44], which is also in agreement with our findings since differences only occur under inflammatory conditions.

Paneth cells produce different antimicrobial peptides particularly the α-defensins 5 and 6. The expression of HD-5 and HD-6 in the paired (non-inflamed vs. inflamed) ileal biopsies from both genotypes was 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 result of an *ATG16L1*-related Paneth cell dysfunction.

Genotyping CD patients for genes that are involved in bacterial recognition and autophagy could reduce post-operational recurrence of CD after ileocecal resection. As a result, an appropriate antibiotic regimen or autophagy-stimulating drugs such as mTOR inhibitors could be prescribed, especially in CD patients homozygous for the *ATG16L1* risk allele prior and/or after surgery. Antibiotic propylaxis 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 *ATG16L1* risk allele[45].

ATG16L1 is a crucial factor in the autophagy pathway that is associated with the innate immune system. Mutations in this specific loci seems to affect the regulation of the immune response against the intestinal microbiota but only under conditions of inflammation. The *ATG16L1* risk allele in CD patients is associated with a higher abundance of pathosymbionts, such as *Enterobacteriaceae, Bacteroidaceae* and *Fusobacteriaceae* in the intestinal epithelial layer during inflammation. In contrast, patients with the protective allele have higher numbers of commensal bacteria such as *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[46].

In conclusion, this study shows that CD patients homozygous for the *ATG16L1–T300A* risk allele display impaired clearance of pathosymbionts in ileal inflammation and that 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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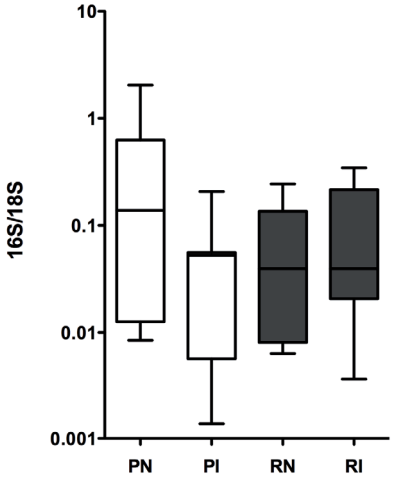
Supplementary table S1. List of CD patients used in this study, including their *ATG16L1, NOD2* and *IRGM* genotype, type of biopsy, age at visit date, medication used and smoking condition.

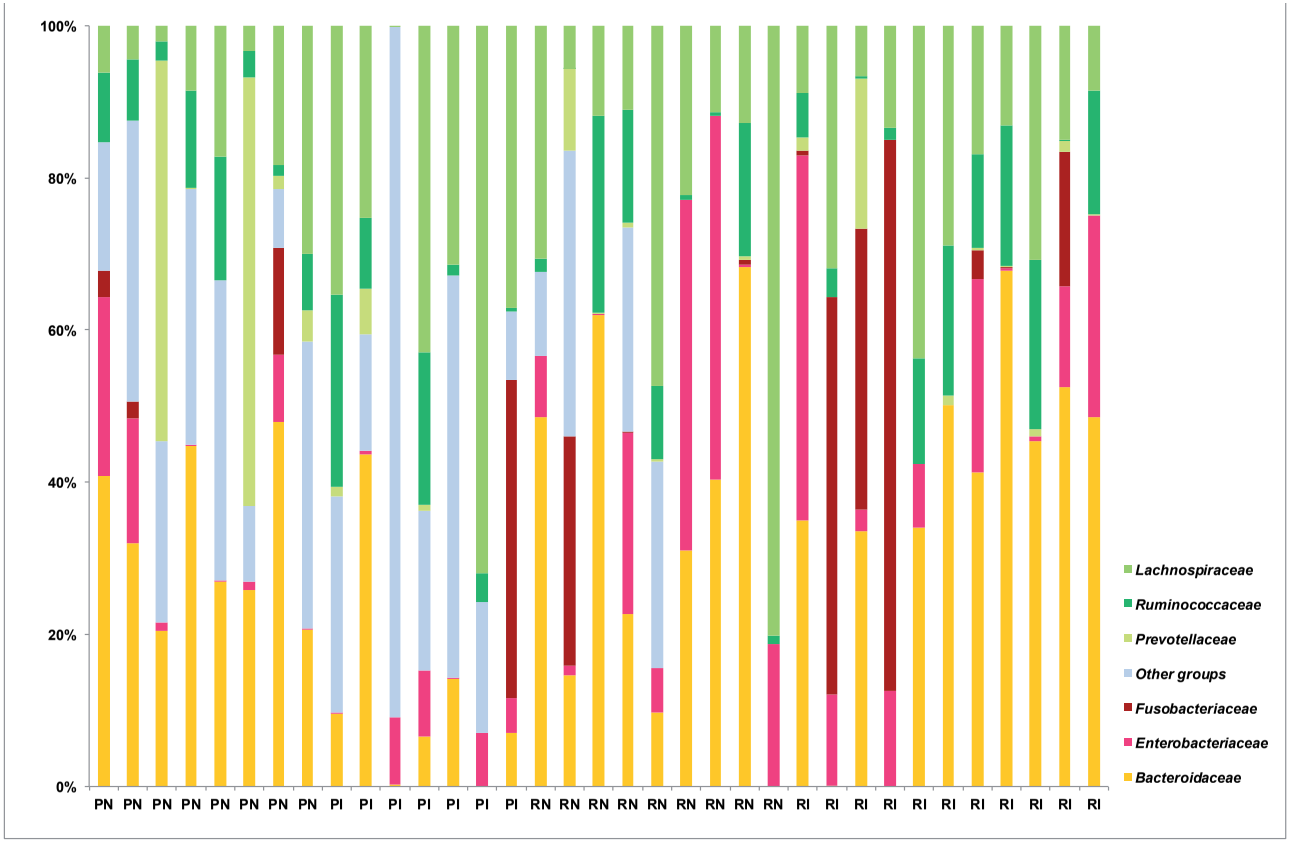
Patient number	*ATG16L1* (rs2241880)	*NOD 2* (R702W)	*NOD2* (G908R)	*IRGM* (rs13361189)	Inflamed biopsy	Non-inflamed biopsy	Age at the visit date	Medication	Smoking
1	*ATG16L1-T300*	Wildtype	Wildtype	Wildtype		•	31	None	Non-smoker
2	*ATG16L1-T300*	Wildtype	Wildtype	Wildtype		•	38	Azathioprine	Smoker
3	*ATG16L1-T300*	Wildtype	Heterozygote	Wildtype	•	•	47	Budesonide	Smoker
4	*ATG16L1-T300*	Wildtype	Wildtype	Wildtype	•	•	70	Budesonide	Non-smoker
5	*ATG16L1-T300*	Wildtype	Wildtype	Heterozygote	•	•	24	None	Non-smoker
6	*ATG16L1-T300*	Wildtype	Wildtype	Heterozygote	•		64	None	No data
7	*ATG16L1-T300*	Wildtype	Wildtype	Wildtype	•	•	26	None	Non-smoker
8	*ATG16L1-T300*	Heterozygote	Wildtype	Wildtype	•	•	44	None	Non-smoker
9	*ATG16L1-T300*	Wildtype	Wildtype	Wildtype	•	•	48	Azathioprine	Non-smoker
10	*ATG16L1-T300A*	Heterozygote	Wildtype	Wildtype	•		30	Prednisolone/ Azathioprine	Non-smoker
11	*ATG16L1-T300A*	Wildtype	Wildtype	Wildtype	•	•	56	Sulfasalazine	Smoker
12	*ATG16L1-T300A*	Wildtype	Wildtype	Wildtype	•	•	26	Azathioprine	Non-smoker
13	*ATG16L1-T300A*	Wildtype	Wildtype	Heterozygote	•	•	49	Budesonide	Non-smoker
14	*ATG16L1-T300A*	Heterozygote	Wildtype	Wildtype	•	•	31	Azathioprine	Smoker
15	*ATG16L1-T300A*	Wildtype	Wildtype	Wildtype	•	•	19	Azathioprine / Mesalazine	Non-smoker
16	*ATG16L1-T300A*	Wildtype	Wildtype	Heterozygote	•	•	59	Budesonide	Smoker
17	*ATG16L1-T300A*	Wildtype	Wildtype	Heterozygote	•		28	Methotrexate	Non-smoker
18	*ATG16L1-T300A*	Heterozygote	Wildtype	Wildtype	•	•	43	Infliximab	Smoker
19	*ATG16L1-T300A*	Wildtype	Wildtype	Wildtype	•	•	60	Mesalazine	Smoker
20	*ATG16L1-T300A*	Wildtype	Wildtype	Wildtype	•	•	25	Methotrexate	Non-smoker

Supplementary Table S2. Primers and probes used in this study.

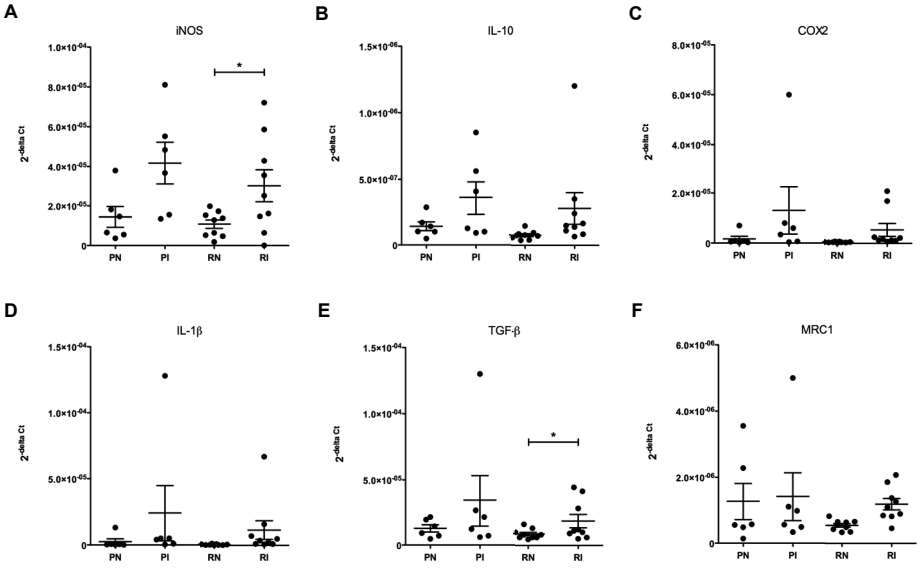
Gene	Sense	Anti-sense	Probe
16S	5′-CGG TGA ATA CGT TCC CGG-3′	5′-TAC GGC TAC CTT GTT ACG ACT T-3′	5′ FAM-CTT GTA CAC ACC GCC CGT C-TAMRA 3′
18S	5′-CGG CTA CCA CAT CCA AGG A-3′	5′-CCA ATT ACA GGG CCT CGA AA-3′	5′ FAM-CGC GCA AAT TAC CCA CTC CCG A-TAMRA 3′
IL-1β	5′-ACA GAT GAA GTG CTC CTT CCA-3′	5′-GTC GGA GAT TCG TAG CTG GAT-3′	5′ FAM-CTC TGC CCT CTG GAT GGC GG-TAMRA 3′
iNOS	5′-GGC TCA AAT CTC GGC AGA ATC-3′	GGC CAT CCT CAC AGG AGA GTT-3′	5′ FAM-TCC GAC ATC CAG CCG TGC CAC-TAMRA 3′
TGF-β	5′-GGC CCT GCC CCT ACA TTT-3′	5′-CCG GGT TAT GCT GGT TGT ACA-3′	5′ FAM-ACA CGC AGT ACA GCA AGG TCC TGG C-TAMRA 3′
IL-10	5′-GCC GTG GAG CAG GTG AAG-3′	5′-GAA GAT GTC AAA CTC ACT CAT GGC T-3′	5′ FAM-TGC CTT TAA TAA GCT CCA AGA GAA AGG CAT C-TAMRA 3′
COX2	5′-GTT GAA TCA TTC ACC AGG CAA A-3′	5′-CTG TAC TGC GGG TGG AAC ATT-3′	5′ FAM-CCA CCA GCA ACC CTG CCA GCA-TAMRA 3′
MRC1	5′-CTC CTA CTG GAC ACC AGG CAA T-3′	5′-CGG CAC TGG GAC TCA CTG-3′	5′ FAM-CCA CGC AGC GCT TGT GAT CTT CA-TAMRA 3′
DEFA5 (HD-5)	TaqMan® Gene Expression Assay: Hs00360716_m1		
DEFA6 (HD-6)	TaqMan® Gene Expression Assay: Hs00427001_m1		



Supplementary figure S1. The 16S/18S enumeration ratios in the biopsies. There are no significant differences between biopsies of patients homozygous for *ATG16L1* protective and risk allele and 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 S2. The percentages of abundance of the major bacterial groups in inflamed and non-inflamed ileal biopsies from patients homozygous for *ATG16L1* protective or risk allele. Most abundant bacterial families are *Bacteroidaceae* (25%), *Lachnospiraceae* (18%), *Enterobacteriaceae* (9.4%), *Ruminococcaceae* (7.3%) and *Fusobacteriaceae* (6.4%).



Supplementary figure S3. Differences in inflammation-related gene expression. The differences between iNOS, IL-10 and COX2 (upper panel) and IL-1β, TGF-β and MRC1 (lower panel) 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).