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Impact of T300A Variant of *ATG16L1* on Antibacterial Response, Risk of Culture Positive Infections, and Clinical Course of Crohn's Disease

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OBJECTIVES: Autophagy-related 16-like 1 (ATG16L1) deficiency leads to impaired cellular autophagy and bacterial degradation as well as an altered cytokine production. The single-nucleotide polymorphism rs2241880 (T300A) is associated with an increased risk for Crohn's disease (CD). ATG16L1 polymorphisms could therefore have an impact on the risk of infectious complications and disease course in CD. We examined the impact of the T300A genotype on the antibacterial response toward a panel of pathogenic bacteria *in vitro*, as well as clinical infectious complications *in vivo* and the disease course in a Danish cohort of patients with CD.
METHODS: A total of 236 CD patients were genotyped for *ATG16L1*^{T300A}; their clinical records were reviewed, and microbial, radiological, and surgical data were scrutinized. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy controls and CD patients carrying the different *ATG16L1* genotypes, and the production of tumor necrosis factor (TNF)-α and interleukin (IL)-1β was measured by enzyme-linked immunosorbent assay after stimulation with a panel of pathogenic bacteria of clinical relevance for the gastrointestinal tract, e.g., enteroinvasive *Escherichia coli* (EIEC), *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, or *Mycobacterium avium paratuberculosis*.
RESULTS: Fifty-seven healthy controls (15, 29, 13) and 236 patients with CD (50, 108, 78) were genotyped for the T300A *ATG16L1* polymorphism (AA homozygous, GG homozygous risk variant, AG heterozygous variant, respectively). The median duration of disease was 128 months (range, 30–175). The cumulative follow-up of this cohort was 2,366 patient-years. *ATG16L1* gene variations interfered with the production of IL-1β, which was significantly increased in PBMCs from GG patients in response to all tested bacteria, whereas the TNF-α production was decreased in PBMCs from GG patients stimulated with EIEC, *L. monocytogenes*, and *S. typhimurium*, but unaffected by the other bacteria tested. Moreover, the GG variant showed a nonsignificant increase in the risk of bowel resections ($P = 0.07$) and postsurgical infections ($P = 0.08$), whereas the risk of non-disease-related infections was unaffected by genotype in the observation period. In addition, patients with AA and AG variants had a higher frequency of complicated fistulizing disease ($P = 0.03$) with an overall more aggravated disease course with an increased number of surgical procedures for fistulous disease from a median 6.5 operations (2.0 in GG patients; $P = 0.002$). This risk was independent on disease phenotype (penetrating vs. non-penetrating) and immunomodulating medication.
CONCLUSIONS: The T300A variant in patients with CD strongly increases the risk for complicated fistulizing disease, and significantly affects antibacterial responses *in vitro*, but the latter effect seems to have a minor role for the infectious risk in CD.
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INTRODUCTION

The cellular cytoplasmic process, autophagy, has a crucial role for the constitutive homeostasis by degradation of damaged cellular organelles and invasive pathogens through the cellular lysosomal machinery. Recent developments indicate an elementary function of the autophagy pathway and specific proteins in immunity and inflammation.[1,2] Accordingly, several genetic links have been identified between autophagy-related genes and the susceptibility to infectious and inflammatory diseases, including inflammatory bowel disease.[3] Differences in inflammatory reactions caused by changes in autophagy-related genes are also likely to affect tissue modulation and regeneration, and could thus be of significant importance for the risk of penetrating disease in inflammatory bowel disease.[4,5]

Single-nucleotide polymorphisms in genes involved in autophagy (e.g., unc-51-like autophagy-activating kinase 1, immunity-related p47 guanosine triphosphatase M protein, and autophagy-related 16-like 1 (ATG16L1)) have all been associated with an increased risk of Crohn's disease (CD).[6–10] ATG16L1 encodes a protein essential for canonical autophagy in eukaryotic cells.[11] Induction of autophagy leads to formation of double-membrane autophagosomes around an autophagic cargo followed by fusion with lysosomes. The

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formation of an autophagasome requires a network of protein interactions.[12–14] In this context, ATG16L1 is involved in the formation of ATG5–ATG12–ATG16L1 conjugate and elongation of the autophagic membrane.[11,15] In addition, the expansion of the phagophore during autophagosome formation depends in part on the trafficking of ATG16L1-containing vesicles from multiple membrane sources.[16–18]

The cellular autophagy pathway acts as a survival mechanism challenged by a number of environmental factors, including stress, starvation, and intracellular bacteria.[13,19] Several studies have reported a strong association between defect autophagy and an impaired bacterial handling.[20–23] Of interest, ATG16L1 deficiency abrogates the ability of cells to form autophagosomes,[24] which leads to mutilation of antigen uptake[25] as well as an insufficient enteric bacterial clearance.[26,27] The CD-associated *ATG16L1* variant rs2241880, which is a nonsynonymous A → G polymorphism encoding a threonine-to-alanine substitution at amino-acid position 300 (T300A),[28] has shown a strong impact on bacterial handling and generation of antigen-specific $CD4^+$ T-cell responses due to an impaired innate immune function.[29] In addition, activation of the CD-associated cytosolic pathogen receptor, nucleotide-binding oligomerization domain-containing protein 2 (NOD2), leads to the induction of the autophagic pathway through downstream interaction with ATG16L1 (refs 29–31), reinforcing the importance of this pathway in the pathogenesis of CD.

There is accumulating evidence that the pathogenesis of CD involves an impaired innate bacterial handling, and CD indeed shares several features with chronic gastrointestinal infections.[32,33] Further, there are some evidences that bacterial handling is impaired not only in the gastrointestinal tract of CD patients, but systemically.[34] We therefore investigated how the CD-associated *ATG16L1* T300A variant affects the inflammatory response to an array of pathogenic bacteria of clinical relevance for abdominal infections related to this disorder by evaluating the secretion of the pro-inflammatory cytokines: tumor necrosis factor (TNF)-α; and interleukin (IL)-1β. These cytokines are key players in the host immune response toward bacterial infections,[35,36] and have further been associated with the disease activity in CD.[37] The bacteria used in this study included enteroinvasive *Escherichia coli* (EIEC), *Listeria monocytogenes* (*L. monocytogenes*), *Salmonella enterica serovar typhimurium* (*S. typhimurium*), *Staphylococcus aureus* (*S. aureus*), and *Mycobacterium avium paratuberculosis* (*M. avium paratuberculosis*). We further evaluated whether changes in the innate bacterial handling could affect the risk of clinical infections in general, but also infections associated with CD, including intra-abdominal abscess formation and post-surgical infectious complications. Finally, we wanted to explore whether the immunological changes associated with the T300A gene variant could influence the CD phenotype.

MATERIALS AND METHODS

Study population and genotyping. The study population comprised a total of 57 healthy controls and 236 patients with CD attending the East Danish IBD Centre at Herlev Hospital, Copenhagen, Denmark. The diagnostic criteria for CD were based on established guidelines using clinical, radiologic, endoscopic, and histologic criteria.[38] The charts of all patients were reviewed for microbiology and surgical data between 1 January 1999 and 1 June 2013. All participants included into the study were screened for the T300A polymorphism using TaqMan single-nucleotide polymorphism genotyping (Applied Biosystems, Foster City, CA, USA). Patients were classified in to three groups based on the T300A single-nucleotide polymorphism status: (1) the homozygous AA; (2) the homozygous GG (risk variant); or (3) the heterozygous AG.

Patients with CD were classified as patients with penetrating or non-penetrating disease (perianal fistulas and/or abscesses) in accordance with the Montreal classification.[39] Intra-abdominal infections had to be identified using computed tomography scan, ultrasound scan, and/or magnetic resonance imaging, and during surgical procedures with or without material from the fluid collections revealing positive cultures. Surgical infectious complications were defined as postoperative intra-abdominal fluid collections or surgical wound infections within 2 weeks from the surgical procedure with or without positive cultures from the isolated material if material was collected leading to renewed surgery, drainage, or start of antibiotics. Complicated fistulizing disease was defined as perianal fistulas needing three or more surgical interventions during the observation period. All positive bacterial cultures during the observation period were identified. These included cultures of blood, urine, feces, cerebrospinal fluid, nasopharyngeal-, conjunctival-, and skin swap cultures as well as cultures from infected tissue and fluid collections. Thus, results of all microbiology tests are stored in a central database covering the Capital Region of Copenhagen regardless whether they have been ordered by general practitioners or hospitals (MiBa, the Danish Microbiology Database), and data from this database and the microbiology database (OPUS) from the hospitals of the Capital Region of Copenhagen were applied. The use of microbiology databases to identify infections may result in an underestimation of the number of non-serious infections treated with antibiotics without microbial diagnostics in outpatient clinics such as upper respiratory infections, non-severe pneumonias, and cellulitis.

Patients included in the bacterial infection study were in complete clinical remission for at least 4 weeks (Harvey–Bradshaw index <5; ref. 40) and did not receive glucocorticoids or biologicals during minimum three preceding months. Long-term treatment with thiopurines was allowed if dosing had been stable for more than 2 months.

In the bacterial infection study, healthy human volunteers with AA genotype ($n=8$), CD patients with AA genotype ($n=9$), CD patients with homozygous GG ($n=7$), and CD patients with AG genotype ($n=8$) were included. All patients were screened for *NOD2* gene variants and were only included if they were *NOD2* wild type.

Isolation of PBMCs. Whole blood was collected from the cubital vein. In brief, the collected blood was drawn in anticoagulant tubes containing EDTA (BD Diagnostics,

Franklin Lakes, NJ, USA) for immediate transfer to the laboratory in <10 min. Then the whole blood was diluted 1:2 with phosphate-buffered saline (Gibco, Invitrogen Ltd., Paisley, UK) containing EDTA. PBMCs were subsequently separated from whole blood by Ficoll-Paque density gradient centrifugation according the manufacturer's instructions (GE Healthcare, Uppsala, Sweden).

***Ex vivo* PBMC stimulation and TNF-α measurements.** After PBMC isolation, 1×10^6 cells were plated in 24-well plates (TPP, Trasadingen, Switzerland) in 450 µl culture medium (RPMI-1640 medium containing 10% fetal calf serum, 50 IU/ml penicillin, 50 µg/ml streptomycin, and 0.5 mg/ml gentamycin) at 37 °C in an atmosphere of 5% CO_2 and a relative humidity of 90%. The cells rested overnight and were then exposed to either (2×10^8 colony-forming units/ml) of EIEC, *L. monocytogenes*, *S. typhimurium*, *S. aureus*, or *M. avium paratuberculosis* for 4 h. The EIEC-prototype strain, derived from a patient with CD,[41] was kindly provided by Dr Arlette Darfeuille-Michaud, Université d'Auvergne, France. Other bacteria were generously provided by the Danish Technical University, Copenhagen, Denmark. The panel of bacteria were selected on the criteria that they were all (1) pathogenic bacteria, (2) of potential relevance to CD pathogenesis (EIEC, *M. avium paratuberculosis*, and *S. typhimurium*), and (3) represented both intracellular (*M. avium paratuberculosis* and *L. monocytogenes*) and extracellular (EIEC, *S. aureus*, *S. typhimurium*) as well as Gram-negative and Gram-positive bacteria. The bacteria were grown at standard conditions to 2×10^8 colony-forming units/ml and were heat inactivated 30 min at 95 °C. Cytokine production by PBMCs was assessed in culture supernatants using enzyme-linked immunosorbent assay (Diaclone Research, Besancon, France) for TNF-α and (eBioscience, San Diego, CA, USA) for IL-1β.

Statistics. Measurements of cytokines were expressed as median (interquartile ranges). Groups were compared using Mann–Whitney *U*-test. The number of surgical procedures for perianal fistulous disease in CD-patient groups, AA+AG and GG genotypes, was shown as interquartile ranges, and the difference between the groups was assessed by the Mann–Whitney *U*-test. The correlations between the frequencies of *ATG16L1* genotypes (AA+AG and GG) and the postsurgical serious infections were analyzed using 2×2 contingency tables (Fisher's exact test). The logistic regression (SPSS, IBM Corporation, Armonk, NY) was applied to examine any association between *ATG16L1* genotypes and other clinical data obtained from patient charts and to calculate age, gender, phenotype, and duration-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. The *Z*-test was used as a statistical tool for comparison of infectious risk by per patient-year between patient groups of CD (i.e., AA+AG and GG). If not directly specified, no statistical significant differences between AA and AG were revealed. In addition, multivariable logistic regression analyses were also performed to identify the independent impact of age, gender, and immunomodulating medications on infections. Results were expressed as ORs and their (95% CI). A two-tailed *P* value <0.05 was considered significant.

Ethics statement. The study has been approved by the Scientific Ethics Committee of the Copenhagen Capital Region. Written informed consents from all included patients were obtained before participation, and the project fulfilled the Helsinki V Declaration.

RESULTS

Patient characteristics. The patient characteristics were derived as shown in (Table 1). The studied cohort constituted of 57 healthy controls (15 AA, 13 AG, and 29 GG) and 236 CD patients (50 AA, 78 AG, and 108 GG). The median duration of disease was 128 months (range, 30–175). The cumulative follow-up of this cohort was 2,366 patient-years.

ATG16L1-mediated innate inflammatory response. We aimed to assess the effect of genetic variations of single-nucleotide polymorphism rs2241880 (AA, AG, and GG; G results in the T300A transition) on the release of pro-inflammatory cytokines TNF-α and IL-1β against a panel of pathogenic bacteria. The production of TNF-α (Figure 1a1–e1) and IL-1β (Figure 1a2–e2) by PBMCs isolated from healthy subjects with homozygous AA ($n=8$), CD patients with homozygous AA ($n=9$), CD patients with GG risk variant ($n=7$), or CD patients with AG genotype ($n=8$) was measured after stimulation (4 h) with either EIEC, *L. monocytogenes*, *S. typhimurium*, *S. aureus*, or *M. avium paratuberculosis.* These patient groups were chosen to identify variations in bacterial response determined by the disease (i.e., AA control vs. AA CD) and variations in bacterial response determined by the genotype (i.e., AA CD vs. AG CD vs. GG CD). PBMCs with the GG risk genotype released significantly less TNF-α after stimulation with EIEC, *L. monocytogenes*, or *S. typhimurium* (Figure 1a1–c1) as compared with patients with an AA or AG genotype. On the other hand, *ATG16L1* genotype had no effect on the TNF-α response after stimulation with *S. aureus* or *M. avium paratuberculosis* where differences between groups were negligible (Figure 1d1 or e1). For IL-1β, the GG risk genotype significantly increased the production of IL-1β as compared with other genotypes (Figure 1a2–e2). The levels of IL-1β produced by patients with the AG and AA genotypes were

Table 1 Patient characteristics

	Healthy controls ($n=57$)	CD patients ($n=236$)
Gender (female/male)	43/13	141/95
Median age (years)	45 (29–70)	48 (21–94)
AA homozygous group	15	50
AG heterozygous group	13	78
GG homozygous risk variant	29	108
Median duration of disease (months)	—	128 (31–176)
Observation time (years)	—	2,366

CI, Chron's disease.

similar to infectious challenges. Thus, the pathogen-induced production of both TNF-α and IL-1β was revealed to be highly modulated in PBMCs from patients with CD and the mainly homozygous T300A variant, whereas PBMCs from heterozygous and wild-type patients were found to have overall similar responses when exposed to bacteria. In the subsequent clinical analyses, AA and AG patients were consequently combined and compared with GG patients.

Effect of *ATG16L1* polymorphisms on the risk of infectious complications and clinical course of Crohn's disease. Given the above-demonstrated substantial effect of genetic variations in *ATG16L1-related* genotypes in bacterial response, we next examined whether these genetic variants affected the risk of infectious complications and clinical course of CD patients by scrutinizing the medical, microbial, radiological, and surgical data of the clinical patient records.

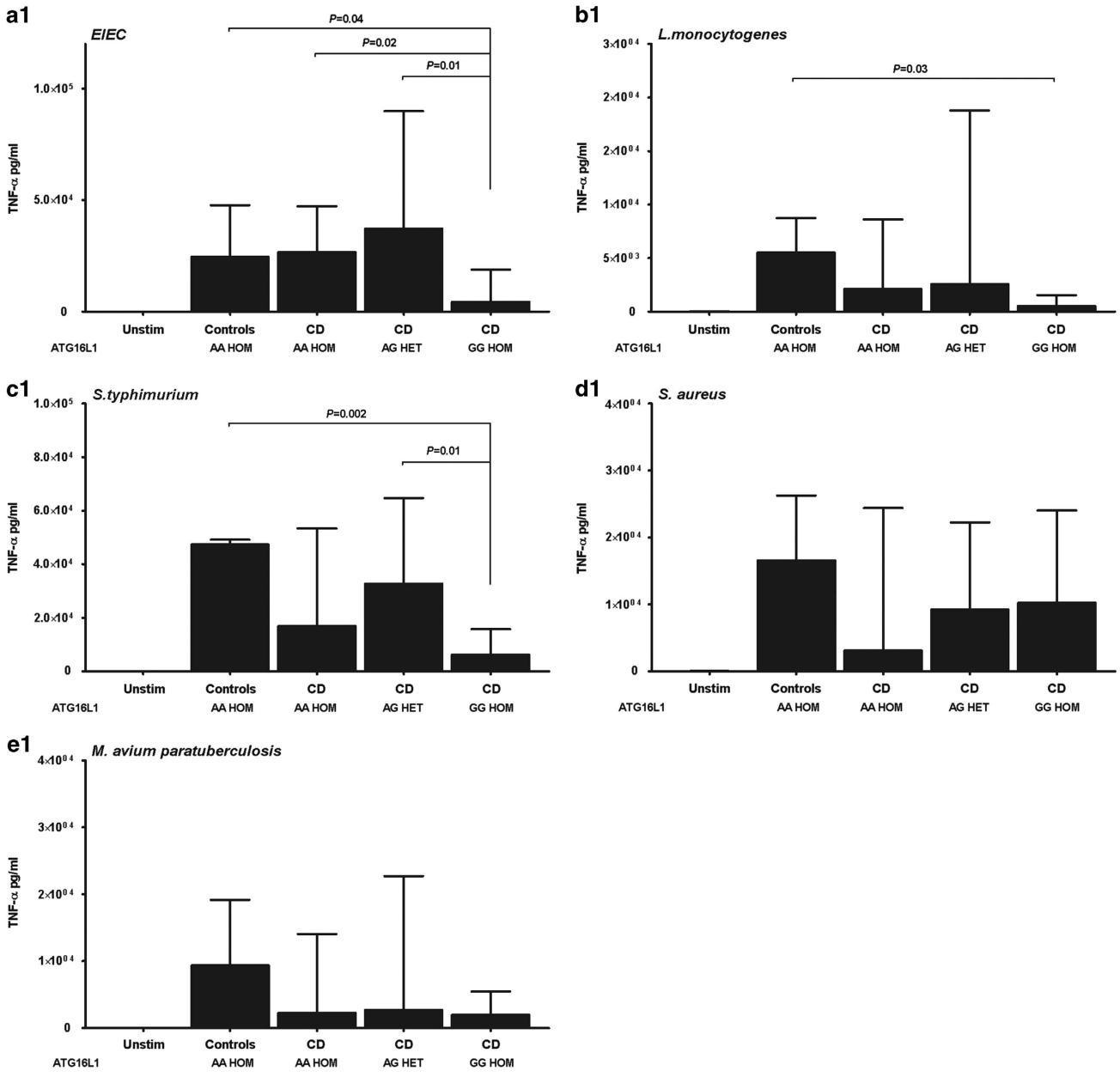

Figure 1 Tumor necrosis factor (TNF)-α and interleukin (IL)-1β production on bacterial stimulations. Peripheral blood mononuclear cells from controls with homozygous AA ($n = 8$), Crohn's disease (CD) with homozygous AA ($n = 9$), CD with AG genotype ($n = 8$), and CD with GG risk variant ($n = 7$) were isolated and stimulated with either (**a1** and **a2**) EIEC, (**b1** and **b2**) *L. monocytogenes*, (**c1** and **c2**) *S. typhimurium*, (**d1** and **d2**) *S. aureus*, or (**e1** and **e2**) *M. avium paratuberculosis*. After 4 h the (**1**) TNF-α and (**2**) IL-1β levels in cell supernatants were assessed by enzyme-linked immunosorbent assay. Data were given as medians, interquartile ranges. Comparisons between groups were done using Mann–Whitney *U*-test. owing to no differences in TNF-α or IL-1β release in the unstimulated cells, data from all groups were pooled together. *Significant TNF-α or IL-1β production compared with the unstimulated cells. A two-sided *P* value < 0.05 was considered significant.

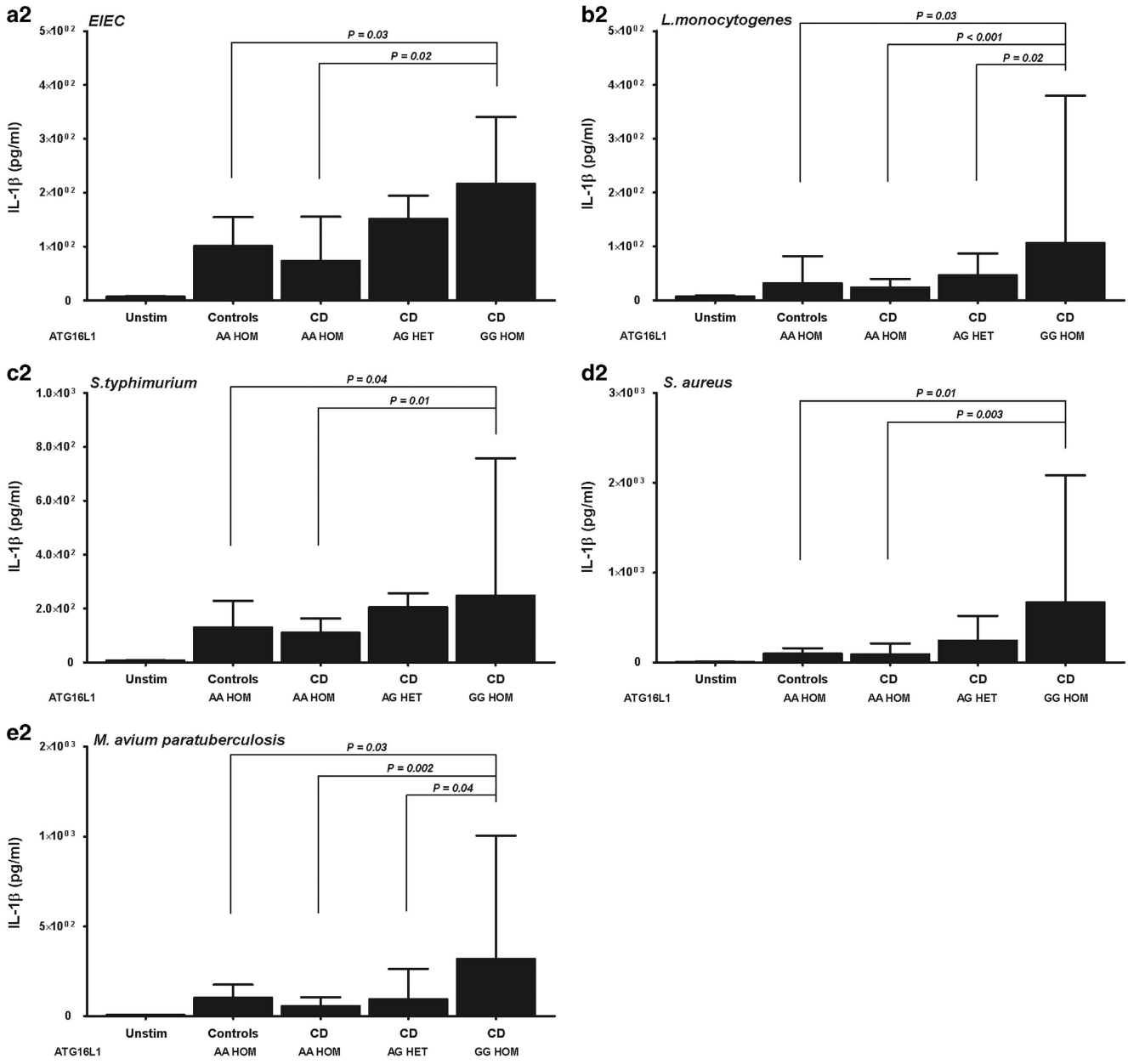

Figure 1 Continued.

Our bacterial experiments revealed that the AA and AG gene variations had similar profile of cytokine production in both TNF-α and IL-1β. On the basis of these data, CD with homozygous AA and CD with AG genotype were combined in the following analyses (AA+AG).

In general, there was no statistically significant association between disease-specific infectious complications and genotypes, or non-disease-specific infections and genotype. Thus, among the 108 CD patients with the GG genotype, 32 patients developed a spontaneous intra-abdominal abscess vs. 26 cases of 128 patients with AA+AG ($P = 0.29$, OR 0.71 (95% CI 0.38–1.34); Table 2). Likewise, postsurgical infectious complications defined as postsurgical intra-abdominal abscesses, anastomosis leaks and surgical wound infections were observed in 6/68 of intra-abdominal surgical procedures in the GG risk variant group, and 3/121 in the CD group with AA +AG genotypes ($P = 0.08$, OR 3.56 (95% CI 0.86–14.69); Table 2). No differences in immunosuppressive medication were identified between the groups (Table 2). Further, the risk of extraintestinal infections was similar in the two groups (Table 3). Regarding the impact of the genotype of *ATG16L1* on the clinical course of CD, the *ATG16L1* genotypes were not identified to affect the disease phenotype (penetrating vs. non-penetrating disease as defined in the Montreal classification; GG: 56/52, AA+AG: 52/76; $P = 0.18$, OR 0.70 (95% CI 0.40–1.20). Moreover, the number of patients with perianal fistulas

Table 2 The T300A variants of ATG16L1 association with penetrations, immunosuppressive medication, intra-abdominal abscess, and postsurgical infections

	GG risk variant	AA+AG genotype	*P*	OR (95% CI)
Penetrating disease	56	52	0.18	0.70 (0.40–1.20)
Non-penetrating disease	52	76		
Immunosuppressive medication	17	11	0.15	0.58 (0.25–1.31)
No immunosuppressive medication	91	117		
Intra-abdominal abscess	32	26	0.29	0.71 (0.38–1.34)
No intra-abdominal abscess	76	102		
Postsurgical serious infections	6	3	0.08	3.56 (0.86–14.69)
No postsurgical serious infections	68	121		

ATG16L1, autophagy-related 16-like 1; CI, confidence interval; OR, odds ratio.
AA, AG, and GG represent the genotype of *ATG16L1*.
The correlations between the frequencies of *ATG16L1* genotypes (AA+AG and GG) and postsurgical serious infections were analyzed using 2x2 contingency tables (Fisher's exact test). The logistic regression was used to examine any association between *ATG16L1* genotypes and other clinical data obtained from patient charts and to calculate age, gender, phenotype, and duration-adjusted ORs and 95% CIs. A two-tailed *P* value <0.05 was considered significant.
The denominator is different in the individual 2×2 tables, as postsurgical infections were related to the total number of intra-abdominal surgical procures performed within the group, whereas the other categories were related to the total number of patients with the specific gene variants.

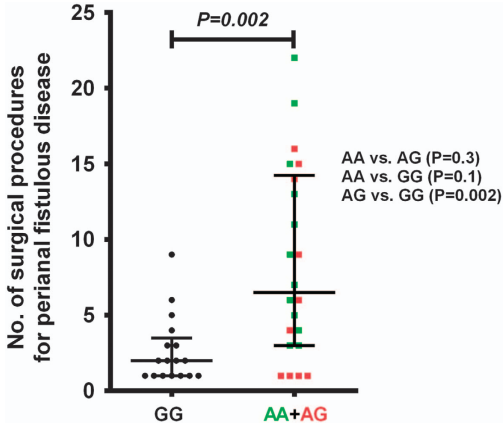

Figure 2 The effect of ATG16L1 T300A genotypes on the perianal fistulous disease. The CD-patient groups; AA, AG, and GG genotypes of ATG16L1; were compared together with respect to the number of surgical procedures for perianal fistulous disease (AA vs. AG ($P=0.3$), AA vs. GG ($P=0.1$), and AG vs. GG ($P=0.002$)). AA and AG are pooled together. Comparisons between groups were done using Mann–Whitney *U*-test. A two-sided *P* value <0.05 was considered significant.

was similar in both groups, while patients with the GG variant showed a statistically nonsignificant increase in the rate of intestinal resections ($P=0.07$, OR 0.59 (95% CI 0.39–1.05); Table 4). On the other hand, the rate of complicated fistulizing disease was more frequent among patients with AA+AG than among CD patients with the GG risk variant ($P=0.03$, OR 3.01 (95% CI 1.13–8.02); Table 4). Accordingly, fistulizing disease was significantly more severe for AA and AG patients with a median number of surgical perianal fistula procedures of 6.5 (3.0–14.25 interquartile ranges) as compared with 2.0 (1.0–3.5 interquartile ranges) among GG patients ($P=0.002$; AA vs. AG ($P=0.3$), AA vs. GG ($P=0.1$), and AG vs. GG ($P=0.002$); Figure 2). Any impact of disease and treatment characteristics, or demographic factors predisposing to infections was, however, not observed in this study (multivariable analyses not shown).

DISCUSSION

In the present study we revealed that the gene variations of *ATG16L1* aggravate the production of key pro-inflammatory cytokines against several pathogenic bacteria, although the overall cytokine profile indicates that the GG risk variant might have disparate effects on the secretion of TNF-α and IL-1β. Although the production of TNF-α is decreased in PBMCs with the GG risk variant in response to a range of bacteria, such as EIEC, *L. monocytogenes*, or *S. typhimurium*, the production of IL-1β in these cells is markedly increased in response to other bacteria, including EIEC, *L. monocytogenes*, *S. typhimurium*, *S. aureus*, *or M. avium paratuberculosis.* These data suggest that the antibacterial response depends on both the species of bacteria and on the genotype of the *ATG16L1* gene, and suggest that specific bacterial infections might be of importance for patients with CD bearing the disease-associated *ATG16L1* gene variant. Earlier studies from mainly animal models have additionally reported a decreased bacterial clearance due to deficiencies in autophagic pathway.[20–23,42] The key effect of the GG risk variant confirms previous results in which inhibition of autophagy in human PBMCs with 3MA, a blocker of the Beclin-1 complex that regulates the initiation of autophagy, leads to a decreased TNF-α production and an elevated IL-1β secretion after activation of TLR2 and TLR4 (ref. 43). This regulation was seen on the transcription level. It was in addition inflammasome independent, and probably involved the inhibition of p38 mitogen-activated protein kinase phosphorylation.[43] On the other hand, *Atg16l1*-deficient murine macrophages produced higher amounts of IL-1β after stimulation with lipopolysaccharide due to an increased inflammasome-mediated processing of pro-IL-1β.[24] Moreover, recent studies have shown increased ATG16L1 cleavage by caspase-3 and -7, and caspases have therefore been proposed as a possible explanation for the T300A-related effects.[24,42,44] Further studies are, however, needed to elucidate the regulatory role of ATG16L1 in the activation of TNF-α and IL-1β synthesis.

Taken together, these data might suggest that *ATG16L1* gene variations affect the production of TNF-α and IL-1β in response to common pathogenic bacteria, and they not only

affect cytokines important for CD but also impair appropriate innate responses for specific pathogens. However, other genetic differences between groups could also be involved in these variations, although the influence of another important gene for bacterial recognition and CD risk, *NOD2*, was excluded in this study by only including wild-type *NOD2* patients in the *in vitro* experiments.

Therefore, we attempted to translate these *ex vivo* findings into the clinical setting of CD. Although we did find a numerical trend toward the risk of common infections in GG patients, this trend was surprisingly weak both in terms of infections of non-gastrointestinal organs and in terms of CD-specific infections (Table 3). This could be due to under-reporting, as our definition of infections required a positive bacterial culture to be present. However, this would affect patients equally, regardless of their genotype. Another more likely explanation is that infectious diseases are handled by the various immunological mechanisms engaged by a complex immune system, including ATG16L1-independent innate responses and adaptive responses in the host, which could make up for the defects in immune responses caused by autophagy deficiency.[45–47] Thus, earlier studies have indicated a correlation between the T300A gene variant and the clinical course of ileal CD.[48,49] However, disease activity in patients with the GG genotype correlated with the presence of bacterial DNA in blood samples, indicating that the *ATG16L1* gene function could affect bacterial handling in the gut.[50] Remarkably, in our study patients with AA+AG alleles suffered more of perianal CD complications as compared with those with the GG allele. As the GG gene variant is rather common in the CD population, it could be assumed that the GG genotype should be correlated with more common clinical presentations of the disease unlike complicated perianal disease. Nevertheless, these findings are in accordance with the notion that the functionally ATG16L1-related NOD2 is also associated with ileocecal disease among patients with *NOD2* variations,[51] and do not seem to dispose patients for fistulizing perianal disease. Therefore, the homozygous GG variant might primarily be related to ileocecal disease because of an impaired handling of bacteria in this part of the intestine, and might not confer risk of fistulizing disease that could involve unrelated pathogenetic pathways.

In summary, this study clarifies the autophagic modulation of pro-inflammatory cytokines, i.e., TNF-α and IL-1β, in patients with CD in response to bacteria. Specifically, the CD-associated *ATG16L1* variant T300A alters the secretion of TNF-α and IL-1β. However, this defect of bacterial handling on the cellular level translates poorly in to risk of clinical infections, e.g., intra-abdominal abscess' and postsurgical infections, and non-inflammatory bowel disease-related infections. However, the T300A genotype is shown to protect from complex fistulizing disease without affecting the overall phenotype of CD in terms of penetrating vs. non-penetrating disease. These translational findings further reveal the effect of genetic autophagy variants in inflammation and disease behavior, thereby highlighting the potential of addressing/targeting autophagy for pharmacogenomic and/or therapeutic approaches against CD.[52]

Table 3 The T300A variants of ATG16L1 association with extraintestinal infections

	GG risk variant	AA+AG genotype	*P*
Blood			
E. coli	0.0008	0.0040	0.6
S. aureus	0.0044	0.0088	0.7
Klebsiella species	0.0062	0.0016	0.6
Urine			
E. coli	0.0204	0.045	0.3
S. aureus	0.0177	0.0249	0.7
Klebsiella species	0.0088	0.0048	0.7
Enterococcus species	0.0151	0.0128	0.9
Streptococcus species	0.0071	0.008	0.9
Corynebacterium species	0.0008	0.0024	0.8
Feces			
Salmonella species	0.0053	0.0056	0.97
Clostridium difficile	0.0044	0.0152	0.4
Airway (sputum)			
S. aureus	0.0017	0.0016	0.98
Streptococcus species	0.0017	0	0.6
Haemophilus influenzae	0.0017	0.0064	0.6
Skin			
S. aureus	0.0177	0.0152	0.9
Enterococcus species	0.0017	0	0.6
Streptococcus species	0.0035	0.0056	0.8

ATG16L1, autophagy-related 16-like 1.
AA, AG, and GG represent the genotype of *ATG16L1*.
The *Z*-test was used as a statistical tool for comparison of infectious risk by per patient-year. A two-tailed *P* value <0.05 was considered significant.

Table 4 The T300A variants of ATG16L1 association with bowel resections, perianal fistulas, and complex fistulas

	GG risk variant	AA+AG genotype	*P*	OR (95% CI)
Bowel resections	36	30	0.07	0.59 (0.39–1.05)
No bowel resections	72	98		
Perianal fistulas	17	22	0.78	1.12 (0.55–2.25)
No fistulas	91	106		
Complex fistulas	6	18	0.03	3.01 (1.13–8.02)
Non complex fistulas	102	110		

ATG16L1, autophagy-related 16-like 1; CI, confidence interval; OR, odds ratio.
AA, AG, and GG represent the genotype of *ATG16L1*.
The frequencies of *ATG16L1* genotypes in clinical data obtained from patient charts were analyzed using logistic regression. The results are expressed as age, gender, phenotype, and duration-adjusted OR (95% CI). A two-tailed *P* value <0.05 was considered significant.

CONFLICT OF INTEREST

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Specific author contributions: Mohammad Salem: planning and conducting the study, collecting and interpreting the data, and drafting the manuscript; Ole Haagen Nielsen, Kris Nys, and Jakob Benedict Seidelin: planning the study, interpreting the data, and drafting the manuscript; Shiva Yazdanyar: collecting and interpreting the data, and revising the manuscript.
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Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ The nonsynonymous polymorphism 2241880 (T300A) in autophagy-related 16-like 1 (*ATG16L1*) has been associated with an increased risk of developing Crohn's disease (CD).
- ✓ In murine models, T300A alters the pathogen clearance and results in imbalanced CD-associated pro-inflammatory cytokines.

WHAT IS NEW HERE

- ✓ T300A leads to altered production pro-inflammatory cytokines toward bacterial infections in human peripheral blood mononuclear cells (PBMCs). We observed a decreased tumor necrosis factor-α production after stimulation with enteroinvasive *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella typhimurium*, whereas interleukin-1β release was increased in response to bacterial infections in PBMCs from patients with CD and homozygous for T300A.
- ✓ The homozygous T300A variant conferred a surprisingly non-relationship with infections related to CD including postsurgical infections.
- ✓ Patients with the homozygous T300A variant had a lower frequency of complicated fistulizing disease. Thus, these findings reveal the role of genetic autophagy variants in inflammation and disease behavior, thereby highlighting the autophagy as a strategy for the treatment of CD.

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