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The Crohn's disease: associated ATG16L1 variant and *Salmonella* invasion

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ABSTRACT

Objective: A common genetic coding variant in the core autophagy gene ATG16L1 is associated with increased susceptibility to Crohn's disease (CD). The variant encodes an amino acid change in ATG16L1 such that the threonine at position 300 is substituted with an alanine (ATG16L1 T300A). How this variant contributes to increased risk of CD is not known, but studies with transfected cell lines and gene-targeted mice have demonstrated that ATG16L1 is required for autophagy, control of interleukin-1-β and autophagic clearance of intracellular microbes. In addition, studies with human cells expressing ATG16L1 T300A indicate that this variant reduces the autophagic clearance of intracellular microbes.

Design/Results: We demonstrate, using somatically gene-targeted human cells that the ATG16L1 T300A variant confers protection from cellular invasion by *Salmonella*. In addition, we show that ATG16L1-deficient cells are resistant to bacterial invasion.

Conclusions: These results suggest that cellular expression of ATG16L1 facilitates bacterial invasion and that the CD-associated ATG16L1 T300A variant may confer protection from bacterial infection.

ARTICLE SUMMARY

Article focus

- Genome-wide association studies have implicated the T300A variant of ATG16L1 with the pathogenesis of Crohn's disease (CD).
- This T300A variant does not affect starvation-induced autophagy, but does reduce the clearance of intracellular microbes.
- Herein, we investigate whether the ATG16L1 T300A variant alters cellular invasion by intracellular microbes.

Key messages

- Intestinal epithelial cells (IEC) somatically targeted to express the ATG16L1 T300A variant show protection against invasion by *Salmonella*.
- ATG16L1-deficient cells are also resistant to bacterial invasion in vitro.
- This suggests that the CD-risk-associated variant in ATG16L1 may also be protective against bacterial invasion.

Strengths and limitations of this study

- Use of gene targeting in human cell line addresses the role of a point mutation in ATG16L1 in human cells with appropriate control cells (non-targeted parental cell lines).
- Focus on bacterial invasion adds new insight into the role of ATG16L1 in host–microbial interaction.
- The study does not address the role of ATG16L1 in infection of primary IEC or other cell types. Further studies are required to determine whether the CD-associated ATG16L1 variant impacts bacterial infection in the human population.

INTRODUCTION

Genome-wide association studies have implicated the core autophagy protein ATG16L1 in susceptibility to Crohn's disease (CD).[1–3] Autophagy is a cellular process that advances through the encapsulation of cytosolic material to form an autophagosome, which fuses to the lysosome to be degraded. This process involves the conjugation of a protein, LC3, to phosphatidylethanolamine (PE) in the forming membrane and this conjugation requires ATG16L1.[4 5] ATG16$^{-/-}$ yeast or ATG16L1$^{-/-}$ mouse cells do not conjugate LC3 to PE and are unable to perform autophagy.[5–7] Human cells rendered deficient for ATG16L1 by siRNA display the same phenotype.[3 8] ATG16L1 is required to survive periods of nutrient deprivation to control toll-like receptor-induced IL-1β production and for the clearance of intracellular microbes.[6–8] ATG16L1 also participates in cellular functions apart from autophagy, including interferon-γ-induced clearance of murine norovirus (MNV), hormone secretion from PC12 cells and the regulation of interferon production in response to VSV.[9–11] Mice hypomorphic for ATG16L1 display altered

Paneth cell gene expression and altered the regulation of granule exocytosis.[7] This phenotype is dependent on MNV infection of non-Paneth cells and the presence of normal commensal microbes.[12] Whether this phenotype relates to autophagy or a distinct role for ATG16L1 in the regulation of granule exocytosis is not clear. ATG16L1 is expressed widely and likely has unique cellular functions that relate to both autophagy-dependent and autophagy-independent roles for this protein.

A common non-synonymous variant in ATG16L1 resulting in the amino acid change at position 300 of the full-length protein from threonine to alanine (T300A) confers risk for CD, but does not affect the ability of ATG16L1 to perform autophagy in response to starvation.[8] However, cells from patients homozygous for the T300A variant, or epithelial cells transduced with this variant, fail to clear intracellular microbes indicating that reduced pathogen clearance in patients with this variant might contribute to risk of CD.[8] Conversely, ATG16L1$^{-/-}$ fibroblasts transduced with the ATG16L1 T300A variant do not display impaired autophagy of intracellular microbes.[13] ATG16L1-deficient cells accumulate intracellular microbes, such as *Salmonella* and *Shigella* over time, suggesting that ATG16L1 suppresses intracellular proliferation of these pathogens.[8 14] This may occur in the cytosol, where ATG16L1 participates in the encapsulation of microbes within LC3-positive membranes targeted for autophagic degradation.[8] During *Shigella* infection, ATG16L1 translocates to the cell membrane at the site of invasion where it is believed to be involved in encapsulating the invading microbe within autophagosomes.[14] Whether suppression of microbial infection is a function of ATG16L1 in vivo is not clear. ATG16L1 hypomorphic mice are not more susceptible to oral infection with *Listeria monocytogenes* and in fact are protected from uropathogenic *Escherichia coli* infections.[7 15] Thus, the role of ATG16L1 in microbial infection is not clear and may depend on the type of invading microbe and the cell type being examined.

The ATG16L1 T300A variant (rs2241880) is common in the human population representing approximately 55% of alleles in the European population, and 20% and 40% of alleles in other populations.[1–3] The high frequency of this allele has facilitated studies of the human ATG16L1 T300A variant in primary human cells. However, these studies are hampered by a lack of appropriate controls, given the highly diverse genetic background of patients. In addition, although some human cells are readily acquired for study, such as haematopoietic cells, other cell types such as intestinal epithelial cells (IEC) are not readily maintained in culture ex vivo, thus hampering the analysis of ATG16L1 functions in diverse human cell types. To investigate the effects of the ATG16L1 T300A variant in human IEC, we used homologous recombination to generate gene-targeted human cell lines deficient in ATG16L1 (ATG16L1$^{\Delta/\Delta}$) or knocked in for the T300A variant (ATG16L1$^{300A/300A}$) and studied infection of these cells by *Salmonella typhimurium*. Here, we find that ATG16L1 facilitates bacterial invasion and that the ATG16L1 T300A variant reduces invasion of human cells by *Salmonella*.

METHODS AND MATERIALS

Generation of gene-targeted HCT116 cells and ATG16L1-complemented cells

HCT116 cells were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). The ATG16L1 locus was targeted through homologous recombination using adeno-associated virus (AAV)-delivered targeting vectors, as described.[9] The non-synonomous single-nucleotide polymorphism (SNP) associated with CD is located in exon 9 of full-length ATG16L1. Two targeting vectors were created, the first to create cells deficient in ATG16L1 (ATG16L1$^{\Delta/\Delta}$) and the second to knock-in the 300A variant in ATG16L1 (ATG16L1$^{300A/300A}$). In both cases, 1 kB of homology was amplified from HCT116 cDNA corresponding to regions 5′ and 3′ to the SNP in exon 9. Vector 1 was designed to delete approximately 1 kB of intronic DNA 5′ to exon 9. In vector 2, the whole sequence was preserved. The homologous DNA was cloned into the pSEPT vector (gift from F. Bunz, John's Hopkins, Baltimore, Maryland, USA) on the 5′ and 3′ side, respectively, of a floxed promoter-less neomycin resistance gene with a synthetic exon acceptor site. The 5′ region of homology, neomycin cassette and 3′ region of homology were then cloned into the NOTI sites of the pAAV-hrGFP vector (Agilent technologies, Santa Clara, California, USA) which was used to generate AAV to deliver the targeting construct to cells as per the manufacturer's instructions. Recombinant cell clones were selected based on resistance to neomycin (G418; 0.5 mg/ml; Life Technologies, Grand Island, New York, USA), and homologous recombinants were identified by PCR screening and confirmed by direct DNA sequencing. The neomycin cassette was then removed with Cre recombinase and the second allele was targeted using the same vector, as described.[9] The ATG16L1$^{\Delta/\Delta}$-targeting strategy removed a splice acceptor site and created a premature stop codon in exon 9 resulting in complete loss of full-length ATG16L1. The ATG16L1$^{300A/300A}$-targeting construct inserted a point mutation in exon 9 resulting in the codon change ACT (threonine) to GCT (alanine).

To generate PWPI 300T or PWPI 300A, cDNAs encoding isoform-001 (full-length ATG16L1) containing either A (300T) or G (300A) at position 898 were cloned into a lentiviral vector with an IRES-GFP (PWPI; gift of D. Trono, Ecole Polytechnique Federale de Lausanne, Switzerland), virus was produced and used to infect ATG16L1$^{\Delta/\Delta}$ HCT116. Pools of these cells were then sorted by flow cytometry to collect GFP+ cells to a purity of 90–95% before use in experiments. The assessment of ATG16L1 expression was performed by Northern blot for mRNA using RNA isolated with TRIzol Reagent (Life Technologies) and biotinylated probe generated using a full-length human ATG16L1 cDNA (Biotin

Decalabel DNA labelling kit, Fermentas). For protein expression, lysates of cells were generated with 1% Triton lysis buffer (1% Triton, 50 mM HEPES, 150 mM NaCl,1.5 mM $MgCl_2$, 1 mM ethylene glycol tetra-acetic acid, 10% glycerol, 1X protease inhibitor cocktail (Roche Diagnostics)), resolved by SDS-PAGE and immunoblotted with antibodies against ATG16L1 (MBL 040 and AbCam 47946-100).

Assessment of autophagy

To examine autophagy in HCT116 cells, we performed immunoblotting for LC3B (Cell Signalling 2775) and p62 (MBL 162-3), with actin (Santa Cruz C-11, sc-1615) and tubulin (Santa Cruz E-19, sc-12462) as controls, from cell lysates collected in 1% Triton lysis buffer, or Laemmli buffer where indicated, and resolved by SDS-PAGE. Reduced autophagy was indicated by decreased levels of lipidated LC3 (LC3 II) and by the accumulation of p62. In some cases, ammonium chloride (NH_4Cl; 50 mM) was added to the cell culture media for 2 h prior to lysis to inhibit lysosomal degradation of LC3 and allow accumulation of LC3-II.

Infection assays with *Salmonella typhimurium*

Salmonella enterica serovar typhimurium (*S typhimurium*; ATCC, Manassas, Virginia, USA) cultures were maintained in Luria Bertani (LB) broth and used for infecting cells following stationary culture overnight, as described.[16] For gentamycin protection assays, cultured HCT116 cells were exposed to *S typhimurium* for 10 min, followed by washing and addition of culture media containing 50 ug/ml gentamycin (Life Technologies, Grand Island, New York, USA). After 1 h of incubation, cells were lysed in 1% Triton lysis buffer and the lysates were serially diluted and plated on LB-Agar to count the colony forming units (CFU). In other experiments, cells infected with *Salmonella* as described were left for 1, 2, 4, 8 or 12 h before lysis and counting of CFUs.[8]

To identify intracellular *Salmonella* by epifluorescence microscopy in flow, cells were infected as described with green fluorescent protein positive (GFP+) *S typhimurium* and then stained with tetramethyl rhodamine (TmR)-conjugated wheat germ agglutinin (WGA) (molecular probes) to delineate the cell surface. Single cell suspensions were made using 5 ml polystyrene round bottomed tubes fitted with a cell strainer cap (BD Falcon) and the samples reconstituted in PBS. Samples were run on ImageStream X (Amnis) using masks to determine individual GFP peaks indicating individual bacterial cells and a second mask to identify the cells surface. GFP+ cells were counted as were the numbers of peaks inside the cell membrane mask. Statistical analyses were performed on exported data using Prism. Over 10 000 cells were run and analysed per sample per experiment.

Statistical analysis

Data represent the mean±SD of three independent experiments. Data were analysed using Prism software by ANOVA with post hoc Tukey's and significance was inferred at $p<0.05$.

RESULTS

Gene-targeted human colorectal cancer cell lines (HCT116) were generated as described.[17] Gene targeting was confirmed by direct genomic sequencing and testing for ATG16L1 mRNA and protein expression (figure 1A). The targeting that generated ATG16L1$^{\Delta/\Delta}$ cells deleted approximately 1 kB of intronic sequence 5′ to exon 9 of the coding sequence. This deleted an exon splice acceptor site, generated a premature stop codon in exon 9 and loss of ATG16L1 mRNA and the full-length isoforms of ATG16L1 protein (figure 1B,C). ATG16L1 has many splice isoforms and a small number of these do not utilise the intronic sequence deleted in this gene targeting. We prepared 96 cDNA clones from ATG16L1$^{\Delta/\Delta}$ cells and did not find any examples where a cDNA encoding a full length ATG16L1 isoform was generated. We cannot definitively state that there are no full-length isoforms or smaller isoforms of ATG16L1 in these cells. Therefore, we have generated a gene-targeted line that is deficient in ATG16L1 that we have designated ATG16L1$^{\Delta/\Delta}$. Cells expressing the CD-associated T300A variant were created using a targeting strategy that preserved all intronic sequence and changed a single base pair in exon 9 (A–G) resulting a change from alanine to threonine at position 300. The ATG16L1 T300A targeting did not alter the expression of the ATG16L1 gene or protein, as assessed by Northern and Western blotting (figure 1B,C). ATG16L1$^{\Delta/\Delta}$ and ATG16L1$^{300A/300A}$ cells were assessed for defects in autophagy by analysing the formation of lipid-conjugated LC3 (LC3-II). As expected, ATG16L1$^{\Delta/\Delta}$ cells failed to produce LC3-II and accumulated p62, a protein degraded by autophagy, indicating that ATG16L1$^{\Delta/\Delta}$ HCT116 cells are defective in autophagy (figure 1D,E). These phenotypes were reversed in ATG16L1$^{\Delta/\Delta}$ cells reconstituted with ATG16L1 (figure 1D,E). Consistent with previous studies using heterologous expression or cells from patients with the 300A variant, ATG16L1$^{300A/300A}$ cells displayed normal levels of LC3-II and p62, indicating that these cells do not exhibit overt defects in autophagy[8] (figure 1D,E). Inhibition of lysosomal degradation with ammonium chloride enhanced the accumulation of LC3-II in parental HCT116 cells and ATG16L1$^{300A/300A}$ cells, but not ATG16L1$^{\Delta/\Delta}$ cells, consistent with unimpaired autophagy in ATG16L1$^{300A/300A}$ cells.

Autophagy is activated by the infection of cells by microbes and autophagic membranes form around intracellular pathogens. In some cases, this activation of autophagy machinery facilitates microbial replication and release; whereas in other cases, autophagy induction is essential to suppress the growth of intracellular pathogens.[18–23] ATG16L1-deficient cells fail to suppress the intracellular growth of bacterial pathogens.[3 8 24] The effect of the ATG16L1 T300A variant on intracellular bacterial growth is controversial having been reported to impair or have no effect on control of intracellular

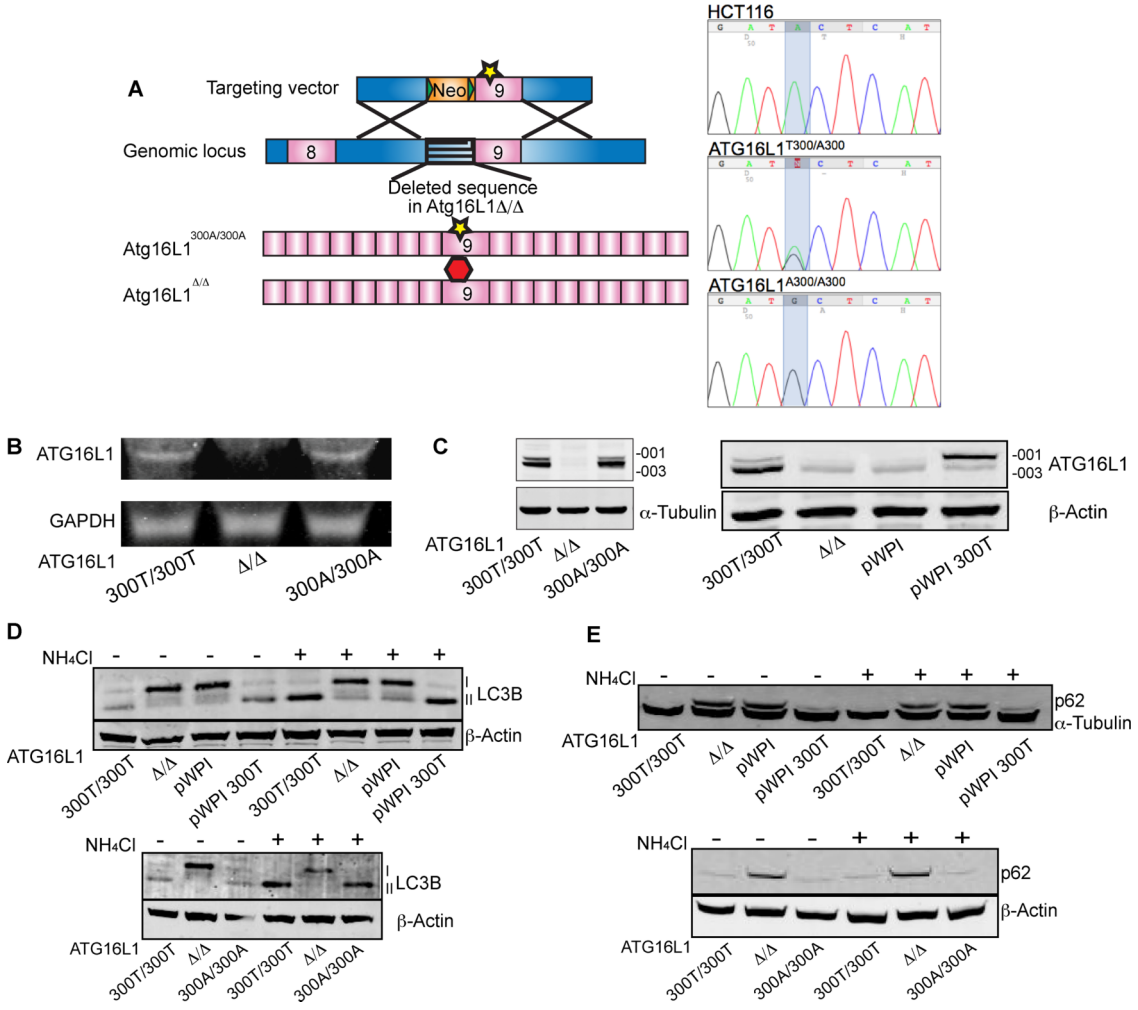

Figure 1 Generation and characterisation of ATG16L1$^{\Delta/\Delta}$ and ATG16L1$^{300A/300A}$ HCT116 cells. (A) Targeting strategy to generate ATG16L1$^{\Delta/\Delta}$ cells. A floxed-promoterless-neomycin resistance cassette was cloned into 2 kB of homology around exon 9. The construct deleted a splice acceptor site and generated unstable mRNA with a stop codon in the middle of exon 9. The neo cassette was removed by Cre recombinase and targeting was repeated for the second allele. For the generation of the ATG16L1$^{300A/300A}$ cells, a different construct was generated that targeted the 3′ side of exon 9, did not delete any intronic sequence and changed the nucleotide sequence from ACT (threonine) to GCT (alanine), as shown in the dendrogram of DNA sequence from these cells. (B) Northern blot showing lower expression of ATG16L1 mRNA in ATG16L1$^{\Delta/\Delta}$ cells compared to both ATG16L1$^{300T/300T}$ and ATG16L1$^{300A/300A}$ HCT116s. (C) Immunoblot for ATG16L1 protein (full-length isoform-001 and splice isoform-003 lacking exon 8) in (left panel; whole cell lysates collected in Laemmli buffer) lysates from ATG16L1$^{300T/300T}$, ATG16L1$^{\Delta/\Delta}$ or ATG16L1$^{300A/300A}$ cells; or (right panel) ATG16L1$^{300T/300T}$, ATG 16L1$^{\Delta/\Delta}$, ATG16L1$^{\Delta/\Delta}$ cells complemented with control PWPI lentivirus (pWPI) or lentivirus encoding ATG16L1 300T (pWPI 300T). (D) Immunoblot for LC3B showing conversion of LC3I (upper band) to LC3II (lower band) indicative of LC3 lipidation in; (upper panel) ATG16L1$^{300T/300T}$, ATG16L1$^{\Delta/\Delta}$, pWPI complemented and pWPI 300T -complemented cellular lysates or; (lower panel) ATG16L1$^{300T/300T}$, ATG16L1$^{\Delta/\Delta}$ or ATG16L1$^{300A/300A}$ HCT116 cellular lysates from standard cell culture media or in media containing the lysosome inhibitor NH_4Cl (50 mM) to allow the accumulation of LC3II. (E) Immunoblots showing accumulation of p62 in these cell types under the same conditions.

bacteria.[8 13 14] The role of ATG16L1 in cellular invasion by bacteria is less well known. To examine the role of ATG16L1 in microbial invasion of cells, we focused on the earliest time points of the interaction between *Salmonella* and human IECs in culture. Cells were exposed for 10 min to *Salmonella* followed by antibiotic treatment for 45 min to clear extracellular microbes. Cell lysates were then analysed for the presence of *Salmonella* by measuring colony forming units (CFUs) of the bacteria as an index of bacterial invasion into cells. HCT116 cells (homozygous for the 300T allele) displayed increasing CFUs of bacteria with increasing MOI (figure 2A). This was significantly reduced in ATG16L1$^{\Delta/\Delta}$ cells, indicating that ATG16L1-deficient cells are less prone to invasion by *Salmonella* (figure 2A). ATG16L1$^{300A/300A}$ cells also displayed significantly reduced CFUs compared to HCT116 cells indicating that the ATG16L1 T300A variant may reduce bacterial

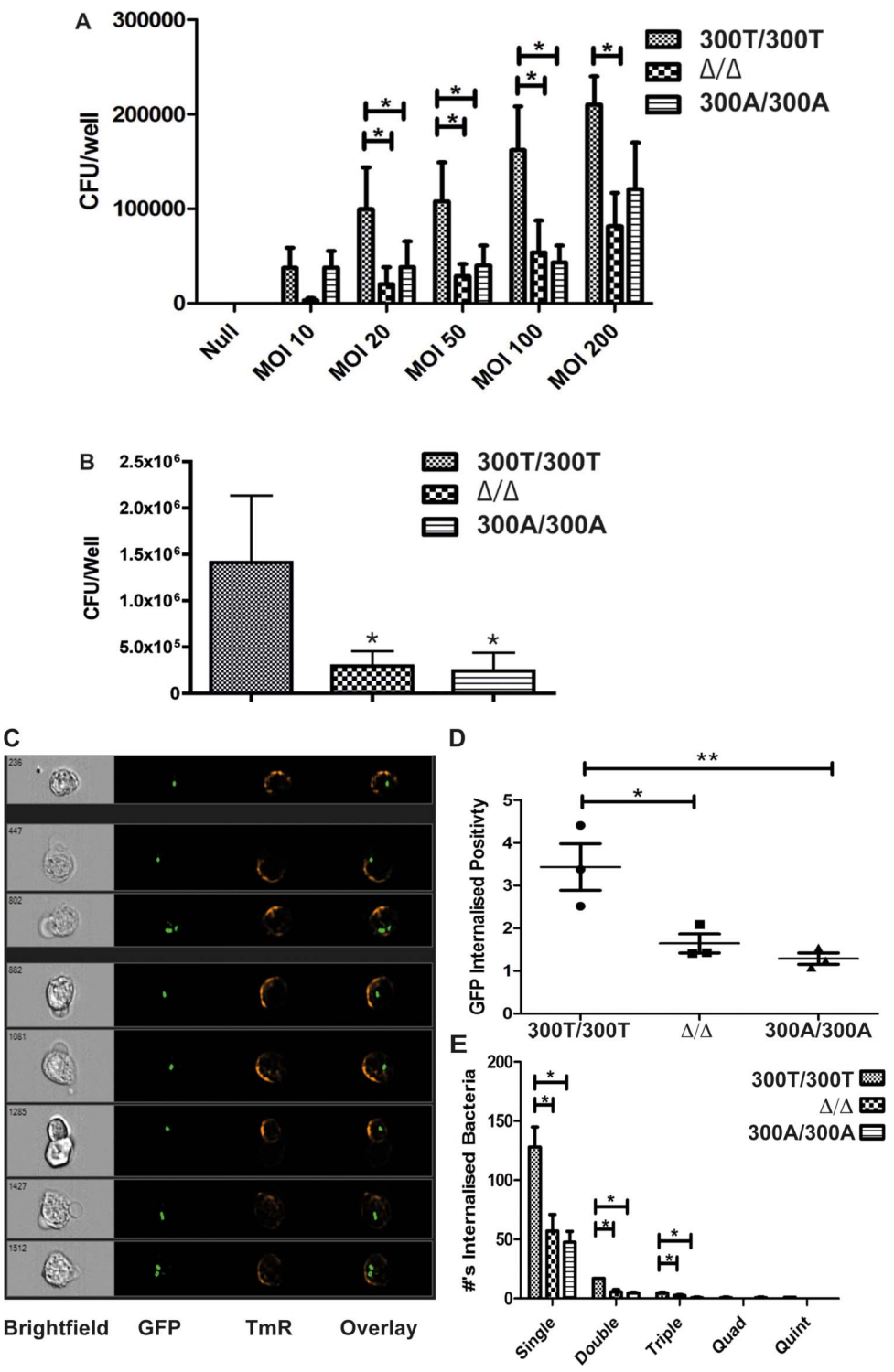

Figure 2 CD-associated ATG16L1$^{300A/300A}$ variant limits *Salmonella typhimurium* infection in human IECs. (A) ATG16L1$^{300T/300T}$ (WT), or gene-targeted ATG16L1$^{\Delta/\Delta}$ and ATG16L1$^{300A/300A}$ HCT116 cells were exposed in culture to increasing MOI of *S typhimurium* for 10 min, followed by gentamycin treatment to kill extracellular microbes and infecting cytosolic bacteria collected and counted as colony forming units. (B) CD-associated ATG16L1$^{300A/300A}$ variant limits *S typhimurium* infection in human IECs grown in transwell culture. ATG16L1$^{300T/300T}$, ATG16L1$^{\Delta/\Delta}$ or ATG16L1$^{300A/300A}$ HCT116 cells were grown on Transwell filters and exposed in culture, at 100 MOI, to *S typhimurium* for 10 min, followed by gentamycin treatment to kill extracellular microbes. Infecting cytosolic bacteria were collected and counted as colony forming units. (C) Representative images, using epifluorescence microscopy in flow (ImageStream), to identify cell surfaces (TmR-WGA) and internalised *Salmonella* (GFP) in 10 000 cells per sample. (D) Quantification of the number of ATG16L1$^{300T/300T}$, ATG16L1$^{\Delta/\Delta}$ and ATG16L1$^{300A/300A}$ cells with internalised *S typhimurium*. (E) Count of the number of *S typhimurium* per cell per sample.

invasion into these cells. This reduced infection of ATG16L1$^{\Delta/\Delta}$ and ATG16L1$^{300A/300A}$ cells was also observed in cells grown on Transwell filters, which more closely recapitulate the polarised in vivo characteristics of IECs (figure 2B). The decreased CFUs recovered from ATG16L1$^{\Delta/\Delta}$ and ATG16L1$^{300A/300A}$ cell cultures could reflect a decreased frequency of the infection of these cells or altered numbers of *Salmonella* infections per cell. Using epifluorescence microscopy in flow (ImageStream), we confirmed that ATG16L1$^{\Delta/\Delta}$ and ATG16L1$^{300A/300A}$ cells were less susceptible to infection by *Salmonella* (figure 2C). In addition, we found that there were fewer ATG16L1$^{\Delta/\Delta}$ and ATG16L1$^{300A/300A}$ cells infected with 1, 2 or 3 *Salmonella* per cell, supporting the observation that microbial infection is disrupted in ATG16L1$^{\Delta/\Delta}$ or ATG16L1$^{300A/300A}$ cells (figure 2C). Thus, the decreased infection of ATG16L1$^{\Delta/\Delta}$ or ATG16L1$^{300A/300A}$ cells reflects the fact that ATG16L1$^{300T/300T}$ cells are more frequently infected by *Salmonella*. Thus, cellular expression of ATG16L1 facilitates *Salmonella* invasion into cells and the CD-associated T300A variant in ATG16L1 impairs bacterial invasion.

Previous studies have found that ATG16L1-deficient cells transduced with the allele of ATG16L1 that is protective in CD (ATG16L1 300T) are able to control intracellular growth of *Salmonella*, whereas transduction with the CD-associated ATG16L1 T300A isoform impairs this suppression of microbial growth.[8] To test this in our invasion assay, we transduced ATG16L1$^{\Delta/\Delta}$ cells with the protective (ATG16L1 300T) and risk-associated (ATG16L1 300A) alleles of ATG16L1 and measured *Salmonella* invasion in these cells. The number of CFUs recovered after *Salmonella* infection in ATG16L1$^{\Delta/\Delta}$ cells was increased by lentiviral complementation with ATG16L1 300T, indicating that the full length isoform of ATG16L1 is sufficient to restore *Salmonella* invasion in ATG16L1$^{\Delta/\Delta}$ cells (figure 3). The number of recovered CFUs in cells transduced with the ATG16L1 300A variant was significantly reduced compared to that recovered from ATG16L1 300T-expressing cells (figure 3). These results indicate that complementation with the allele of ATG16L1 that is protective in CD restores the ability of these cells to be infected by *Salmonella*, whereas complementation with the CD-associated allele significantly attenuates bacterial invasion of cells.

Studies using siRNA knockdown and heterologous complementation have found increased titres of intracellular *Salmonella* at later time points after infection in cells deficient in ATG16L1 or expressing the 300A variant.[8] In order to assess this in our model, we evaluated growth of *Salmonella* inside HCT116 cells following 1–12 h of infection. ATG16L1$^{\Delta/\Delta}$ and ATG16L1$^{300A/300A}$ cells contained significantly higher titres of intracellular *Salmonella*, compared to ATG16L1$^{300T/300T}$ cells, 8 h after infection (figure 4). This confirms previous observations that ATG16L1 deficiency or the CD-associated 300A variant decreases intracellular clearance of *Salmonella*.[2 8] Thus, cells lacking ATG16L1 or expressing the CD-associated ATG16L1 T300A allele are not only less susceptible to invasion by *Salmonella* but also less capable of clearing these bacteria once they become infected.

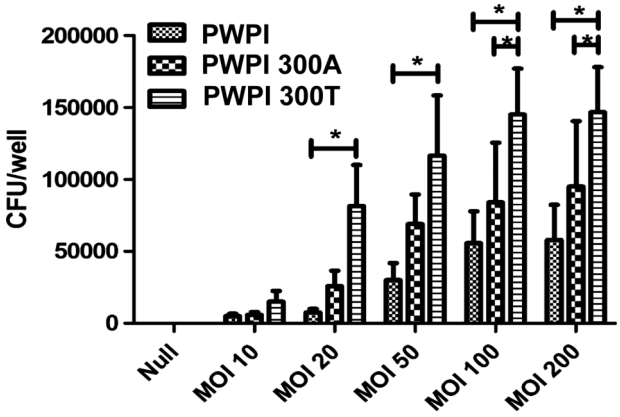

Figure 3 Complementation with CD-associated ATG16L1$^{300A/300A}$ variant limits *Salmonella typhimurium* infection in ATG16L1$^{\Delta/\Delta}$ cells. ATG16L1$^{\Delta/\Delta}$ cells complemented with lentiviral expression vectors encoding ATG16L1 300T (PWPI 300T), the CD-associated ATG16L1 (PWPI 300A) variant, or empty vector (PWPI) were examined for rates of *S typhimurium* infection as described.

DISCUSSION

In the present study, we found that the CD-associated ATG16L1 T300A variant decreased the invasion of human cells by *Salmonella*. We also observed that ATG16L1-deficient cells were resistant to invasion, suggesting that ATG16L1 facilitates the invasion of cells and that this is hampered in cells with the T300A genetic variant. In the reduced number of cells that became infected in our studies, *Salmonella* proliferated

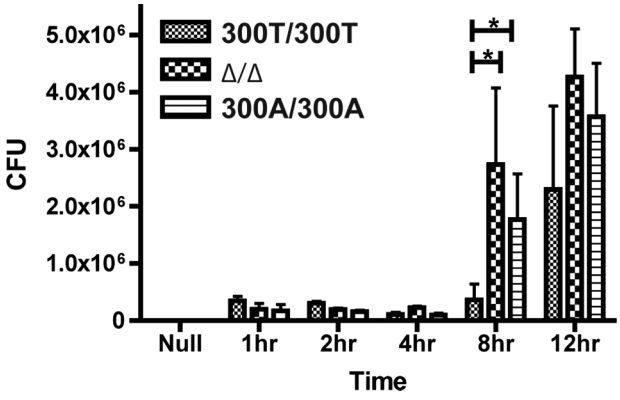

Figure 4 CD-associated ATG16L1$^{300A/300A}$ variant limits clearance of intracellular *Salmonella typhimurium*. Cells were exposed in culture to *S typhimurium* for 10 min, washed in gentamycin to kill extracellular microbes and then left in culture for indicated times. Intracellular *S typhimurium* were collected by cell lysis and further washing and quantified by colony formation assays, *p<0.05.

intracellularly over time. This confirms prior work showing that cells expressing the ATG16L1 T300A variant fail to clear intracellular pathogens.[8] Thus, the CD-associated variant in ATG16L1 may both reduce bacterial invasion and reduce clearance of microbes that successfully invade the cell. The ATG16L1 300A allele is present in approximately half of people of European ancestry and confers an OR for CD of approximately 1.8 in homozygous individuals.[1–3] It is possible that this allele may confer a benefit to carriers, namely resistance to infection by facultative intracellular pathogens. As is the case with other variants associated with autoimmune disorders, the benefit conferred by this allele also may also independently confer increased risk for disease.[25 26] It is possible that the low OR (for CD susceptibility) and high prevalence of the ATG16L1 T300A variant may reflect the combined beneficial effects of protection from bacterial invasion and detrimental effects of susceptibility for intracellular microbial growth. Mice hypomorphic for ATG16L1 are protected from uropathogenic *E coli* infections and also clear *Listeria* infections as effectively as WT mice.[7 15] It is not known whether the ATG16L1 T300A variant confers risk or protection from *Salmonella* infection in the human population. A small study of *Helicobacter pylori* infections in the European population suggested that the ATG16L1 T300A variant associates with the increased infection with *H pylori*.[27] Whether the ATG16L1 T300A variant confers risk or protection from infection in the human population may vary depending on the nature of the pathogen and typical duration of infection.

Although reduced numbers of ATG16L1 mutant cells were infected with *Salmonella*, over time, these cells accumulated more intracellular *Salmonella*. This has been associated with a lack of autophagic clearance through defective bacterial capture in these and other cell types.[38] Our results therefore not only support previous studies showing decreased intracellular clearance of microbes in cells having the CD-associated ATG16L1 variant but also extend this observation to include the fact that this genetic variant also reduces bacterial invasion. Although our results confirm several prior studies in IEC, Fujita *et al* found that ATG16L1-deficient fibroblasts transduced with the ATG16L1 T300A variant displayed no difference in *Salmonella* infection compared to cells transduced with wild-type ATG16L1. This may reflect a difference in the cell types studied, as it is known that *Salmonella* invasion of fibroblasts occurs through distinct mechanisms compared to epithelial cells.[28] For example, type-III secretion-deficient *Salmonella* readily invades fibroblasts, but not epithelial cells. The use of alternative entry mechanisms by *Salmonella* invading fibroblasts may explain why Fujita *et al*[13] did not observe the effect of the ATG16L1 T300A variant on intracellular *Salmonella* proliferation that has been reported in epithelial cells.[28] What remains to be determined is whether bacterial invasion is fundamentally altered in IEC having the ATG16L1 T300A mutation. It is possible that the rapid type-III-mediated invasion of cells by *Salmonella* requires wild-type ATG16L1 and that in its absence, invasion occurs through alternate routes such as adhesins or bulk cellular uptake.[28 29] Microbes taken up by these potentially non-physiological routes of infection might not be recognised by the autophagic machinery, and may therefore proliferate intracellularly. Whether *Salmonella* infection can occur through its natural routes independently of ATG16L1, in vivo, remains to be determined.

ATG16L1 localises to the cell membrane and this is increased at the sites of entry of *Shigella* by a NOD2-dependent mechanism.[14] It has been posited that the translocation of ATG16L1 to sites of pathogen contact with the membrane is a cellular response to initiate autophagic clearance at the invasion site.[14] Another possibility is that ATG16L1 may be recruited to the membrane by the pathogen, in order to facilitate invasion. The ATG16L1 T300A variant may therefore reduce *Shigella* invasion because it is not efficiently recruited to the site of microbial invasion. How ATG16L1 is recruited to the membrane is not known, but this may involve the association of ATG16L1 with NOD1 or NOD2, which translocates to the membrane.[14 30] The CD-associated NOD2 variant L1007fsinsC impairs ATG16L1 recruitment to the cell membrane and it will be of interest to determine whether that variant also decreases *Salmonella* invasion in human cells.[14] Lastly, we cannot exclude the possibility that the ATG16L1 T300A variant or lack of ATG16L1 alters cellular functions in such a way that cells become resistant to microbial invasion through a mechanism that is indirectly related to ATG16L1.

We studied *Salmonella* invasion in our system in order to compare our findings with previously reported studies of the effects of the ATG16L1 polymorphism on bacterial infection in human epithelial cells. It is not clear whether *Salmonella* invasion in human IEC is relevant to human CD. Gastrointestinal infections with pathogens like *Salmonella* can trigger or increase the likelihood that a patient will subsequently develop inflammatory bowel disease (IBD).[16] It will be of interest to examine whether the ATG16L1 T300A variant alters invasion of human cells by pathogens implicated in IBD, such as adherent invasive *E coli*. A role for any pathogen in human CD is not established, but there is substantial evidence that the intestinal microbiota and defects in host responses to microbes play a key role in the etiopathogenesis of CD. Whether the ATG16L1 T300A polymorphism associates with altered handling of intestinal microbes or a particular microbiome profile remains to be determined.

Contributors SFM, JSM and DLB designed the study, performed and analysed the experiments and wrote the manuscript. MFL, WAG, SJB, JPL, TPV and MB generated key reagents, intellectual content and provided technical support. JSM, MFL, JPL, TPV, MB and DLB conceived of and initiated the study. *SFM and JSM are co-first authors. All authors have read and approved the final manuscript.

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