Crohn’s disease-associated adherent-invasive *E. coli* are selectively favoured by impaired autophagy to replicate intracellularly

Pierre Lapaquette,1 Anne-Lise Glasser,1,2 Alan Huett,3 Ramnik J. Xavier3 and Arlette Darfeuille-Michaud1,2*

1Univ Clermont1, Pathogénie Bactérienne Intestinale, JE2526, USC-INRA 2018, Clermont-Ferrand F-63001, France.
2Institut Universitaire de Technologie en Génie Biologique, Aubière F-63172, France.
3Gastrointestinal Unit and Center for Computational and Integrative Biology, Massachusetts General Hospital, Harvard Medical School, Boston 02141 USA.

**Summary**

Ileal lesions in Crohn’s disease (CD) patients are colonized by pathogenic adherent-invasive *Escherichia coli* (AIEC) able to invade and to replicate within intestinal epithelial cells. Recent genome-wide association studies have highlighted the autophagy pathway as being associated with CD risk. In the present study we investigated whether defects in autophagy enhance replication of commensal and pathogenic *Escherichia coli* and CD-associated AIEC. We show that functional autophagy limits intracellular AIEC replication and that a subpopulation of the intracellular bacteria is located within LC3-positive autophagosomes. In IRGM and ATG16L1 deficient cells intracellular AIEC LF82 bacteria have enhanced replication. Surprisingly autophagy deficiency did not interfere with the ability of intracellular bacteria to survive and/or replicate for any other *E. coli* strains tested, including non-pathogenic, environmental, commensal, or pathogenic strains involved in gastroenteritis. Together these findings demonstrate a central role for autophagy restraining Adherent-Invasive *E. coli* strains associated with ileal CD. AIEC infection in patients with polymorphisms in autophagy genes may have a significant impact on the outcome of intestinal inflammation.

**Introduction**

Crohn’s disease (CD) and ulcerative colitis (UC) are two major forms of idiopathic inflammatory bowel disease (IBD), with a combined prevalence of about 150–200 cases per 100 000 in Western countries. They are multifactorial diseases, occurring in individuals with genetic predisposition in whom an environmental or microbial trigger causes an abnormal immune response (Strober et al., 2007; Xavier and Podolsky, 2007). Several lines of evidence suggest that bacteria play a role in the onset and perpetuation of IBD (Sartor, 2008). Intestinal bacteria are essential for the development of intestinal inflammation. In patients with CD, exposure of the terminal ileum postsurgically to luminal contents is associated with increased inflammation, and diversion of the faecal stream is associated with improvement (Rutgeerts et al., 1991).

Studies in CD patients have reported high concentrations of bacteria forming a biofilm on the surface of the gut mucosa (Swidsinski et al., 2002) and low numbers of anti-inflammatory bacteria (Sokol et al., 2008), and showed that high numbers of *Escherichia coli* colonize the epithelial intestinal layer (Darfeuille-Michaud et al., 1998; Martin et al., 2004; Conte et al., 2006; Kollahwski et al., 2006). As a commensal, *E. coli* coexists with its mammalian host in good harmony and rarely causes disease except in immunocompromised hosts or when the normal gastrointestinal barriers are breached. However, some *E. coli* strains have acquired specific virulence factors that increase their ability to adapt to new niches and allow them to cause a broad spectrum of diseases. Among the *E. coli* strains that can cause intestinal gastroenteritis in humans, there are six well-characterized pathotypes: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteraggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli*.
(DAEC) (for review Kaper et al., 2004). Genotypic characterization of CD-associated E. coli showed that they do not possess the virulence factors of any of the above pathogenic E. coli strains. However, they are fully virulent, able to adhere to and to invade/replicate within intestinal epithelial cells, and also survive and replicate within macrophages thereby producing large amounts of TNF-α (Boudeau et al., 1999; Glasser et al., 2001). These pathogenic E. coli are called AIEC for adherent-invasive Escherichia coli. Their association with CD has been reported in independent studies performed in Europe and the United States (Darfeuille-Michaud et al., 2004; Martin et al., 2004; Baumgart et al., 2007; Eaves-Pyles et al., 2007; Sasaki et al., 2007; Martinez-Medina et al., 2009) and particularly with ileal CD owing to abnormal expression of the specific host receptor CEACAM6 (Darfeuille-Michaud et al., 2004; Barnich et al., 2007).

The theory of dysregulated host responses to intracellular microorganisms is emerging as a contributing factor in CD pathogenesis. Many IBD susceptibility loci have been identified. The most well-replicated IBD genetic association is the NOD2 (nucleotide-binding oligomerization domain 2) gene occurring in CD (Hugot et al., 2001; Ogura et al., 2001). Recent genome-wide association (GWA) studies, in addition to identifying new susceptibility genes, have firmly established that some of them contribute to IBD, and in particular to CD (Duerre et al., 2006; Hampe et al., 2007; Massey and Parkes, 2007; Rioux et al., 2007; Wellcome, 2007). A highly significant and replicated association between CD and variants in two separate autophagy genes (ATG16L1 and IRGM) has been shown. Thr300Ala substitution within the ATG16L1 (Autophagy-related like 1) gene is mostly associated with ileal CD (Hampe et al., 2007; Rioux et al., 2007). A second autophagic gene has been identified in CD susceptibility: the IRGM (Immunity-related GTPase family M) (Parkes et al., 2007; Wellcome, 2007). Autophagy is a process by which eukaryotic cells maintain homeostasis by degrading damaged organelles via the lysosomal pathway (Mizushima, 2007). In addition autophagy protects the cell by eliminating or limiting the growth of intracellular bacteria (Gutierrez et al., 2004; Birmingham et al., 2006). Thus a dysfunction in autophagy leads to persistent infection, as seen in Salmonella Typhimurium, Streptococcus pyogenes, and Mycobacterium tuberculosis (Nakagawa et al., 2004; Birmingham et al., 2006; Singh et al., 2006; Kuballa et al., 2008).

As defects in autophagy could predispose patients to CD by promoting prolonged survival and/or replication of intracellular microorganisms within host cells, the aim of the present study was to analyse whether the cellular autophagy mechanism can control bacterial survival and/or replication of various E. coli strains, including CD-associated strains, commensal, non-pathogenic or pathogenic strains involved in gastroenteritis. We provide here the first evidence that intracellular replication of CD-associated AIEC bacteria is correlated with a loss of autophagy function mediated by either of the two CD-associated autophagy genes; ATG16L1 and IRGM.

**Results**

Intracellular behaviour of commensal, environmental, enteropathogenic and CD-associated E. coli strains within wild-type and autophagy deficient mouse embryonic fibroblasts

To test the role of autophagy in its ability to limit intracellular E. coli replication we performed time-course invasion assays in wild-type (wt) mouse embryonic fibroblasts (MEFs) versus atg5−/− MEFs harbouring a knockout of the atg5 locus (atg5−/−). The ability of all the E. coli strains tested to invade wt MEFs or atg5−/− MEFs was compared (Fig. 1A). As expected, the CD-associated AIEC strain LF82 was the most invasive strain with a percentage of intracellular bacteria at 1 h post infection in wt MEFs and atg5−/− MEFs corresponding, respectively, to 3.93 ± 1.47% in 2.77 ± 0.73% of the inoculum. ETEC strain H10407 and the DAEC strain C1845, known to be invasive, also invaded MEFs but with lesser efficiency than AIEC strain LF82. Surprisingly, the EIEC strain E12860/0 did not efficiently invade MEFs but showed a high replication once internalized (Fig. 1B). The non-pathogenic E. coli K-12 strain MG1655, which is unable to replicate intracellularly, was similar in its behaviour to wt or atg5−/− MEFs (Fig. 1B and Fig. S1). Similarly, the numbers of intracellular bacteria for the environmental E. coli strain SMS 3.5 and the commensal E. coli strain HS were identical within wt and atg5−/− MEFs even though bacteria were able to replicate. Among the pathogenic E. coli strains responsible for gastroenteritis, only the AIEC strain LF82 showed a significant (P < 0.01) increase in the numbers of intracellular bacteria at 6 h post infection in atg5−/− MEFs compared with wt MEFs. These findings demonstrate that the survival and/or replication of most of the E. coli strains, including non-pathogenic K-12, commensal, environmental or pathogenic E. coli (ETEC, EPEC, EIEC and DAEC) is independent of the autophagy pathway. Confocal analysis showed the presence of large clusters of intracellular AIEC LF82 bacteria in atg5−/− MEFs, whereas only a few, mostly individual, bacteria were seen in wt MEFs (Fig. 1C). Most of the LF82-containing phagosomes in wt or atg5−/− MEFs stained positive with both LAMP-1 antibody and Lysotracker, indicating that the bacteria clusters were located in mature acidic phagolysosomes (Fig. 1D and Fig. S2). Moreover to assess if a part of AIEC LF82 bacteria was in contact with cytosol, we permeabilized...
MEFs with digitonin prior to staining bacteria using an antibody to lipopolysaccharide (LPS) O83. Both in MEFs wt and atg5−/−, a subpopulation of AIEC LF82 bacteria were positive for O83 staining, indicating that some bacteria are cytosolic or in damaged vacuoles (Fig. S3), a prerequisite for intracellular control by autophagy.

**AIEC LF82 bacteria induced autophagy in MEFs and were located within autophagosomes**

We next investigated whether autophagy is induced during infection of wt MEFs by AIEC strain LF82. We monitored autophagosome formation, as evidenced by
the recruitment of LC3 protein (microtubule-associated protein light chain 3) (in its active form, LC3 II) to the autophagosome. We analysed the shift of free cytosolic LC3 I towards the autophagosomal LC3 II by immunoblotting using an antibody raised against LC3 isoform B. In comparison to non-infected corresponding MEFs, the amount of LC3 conjugated form (LC3 II), which migrated slightly faster than the LC3 I, rapidly increased as of 30 min post infection, with a ratio LC3 II/LC3 I of 0.9 for LF82-infected MEFs compared with 0.4 for uninfected MEFs (Fig. 2A). At 1 h of infection, this ratio was 1.8. Analysis of protein extracts from 1 h to 6 h post infection revealed the presence of LC3 II, indicating an activation of the autophagic process during AIEC LF82 infection. Confocal analysis of immunostaining for LC3 protein indicated LC3 colocalization with AIEC LF82-containing vacuoles, indicating that bacteria were sequestred in autophagosomal structures (Fig. 2B). The percentages of colocalization of LC3 with intracellular bacteria increased from 5% at 1 h post infection to 16.8% at 4 h post infection (Fig. 2C). Of note, the LC3-colocalized bacteria were mostly observed as single bacteria in a vacuole. Together, these findings indicate that the replication of a subpopulation of intracellular AIEC LF82 bacteria could be hindered by the autophagy pathway in non-phagocytic cells.

**AIEC LF82 bacteria interact with autophagy in epithelial cell lines**

We next determined the intracellular behaviour of the reference AIEC strain LF82 in epithelial cell lines HeLa, Hep-2 and Intestine-407. Strain LF82 is able to strongly invade the three cell lines (Fig. 3A) and, as previously described for Hep-2 cells (Boudeau et al., 2001), AIEC strain LF82 is also able to replicate within HeLa and Intestine-407 cells. Analysis of LF82 infected-epithelial cells by transmission electron microscopy showed various intracellular compartments for intracellular LF82 bacteria at 6 h infection. A subpopulation of bacteria was observed as single bacteria in single membrane vacuoles (Fig. 3B, 1 and 5). LF82 bacteria were also observed as several bacteria in a single membrane vacuole (Fig. 3B, 2 and 6). As observed in MEFs, most of vacuoles were both LAMP-1 and lysotracker positive (46.3 ± 6.8%) (Fig. 3C and Fig. S4). In addition, some bacteria were observed by TEM in damaged vacuoles (Fig. 3B, 3 and 7) and confocal analysis after permeabilization with digitonin indicated the presence of bacteria positive for O83 labelling in contact with cytosol (Fig. 3D). Interestingly, other individual bacteria were enclosed in a multi-lamellar membrane vacuole that also contained sequestered cytoplasm, the main morphological evidence of autophagosomes (Fig. 3B, 4 and 8). These results suggest that autophagy could be involved in handling a subpopulation of intracellular AIEC LF82 bacteria.

**Fig. 2.** AIEC LF82 bacteria induced autophagy in MEF and were located within autophagosomes. A. Immunoblot analysis using antibodies against LC3 and actin to analyse the LC3 II/LC3 I ratio at early times of infection or post infection with AIEC LF82. B. Confocal microscopic examinations of AIEC LF82-GFP infected wt MEFs and atg5−/− MEFs at 6 h post infection to analyse LC3 colocalization using monoclonal antibody to LC3 (red). Nuclei are in blue. C. Percentage of LC3-positive vacuole containing LF82-GFP bacteria determined by confocal microscopy at various times post infection. Data shown represent means ± SEM of three independent experiments, counting 50 cells per experiment.

To provide further evidence of autophagic handling of internalized AIEC bacteria, we assessed endogenous LC3 I and LC3 II levels by immunoblotting. We first checked that LC3 II accumulated in cells treated with rapamycin, a drug known to induce autophagy (Fig. 4A). LC3 II levels also greatly increased in AIEC LF82-infected cells, with ratios of LC3 II/LC3 I forms ranging from 0.6 to 2.5 at 2 h and 6 h post infection respectively (Fig. 4A). To
investigate whether the active LC3 II generated during infection was located on the vacuole surrounding intracellular LF82 bacteria, we performed immunofluorescence assays using a polyclonal antibody raised against LC3 protein. We observed endogenous LC3-positive labelling of a subpopulation of AIEC LF82-containing vacuoles (Fig. 4B), indicating that autophagy plays a part in sensing AIEC LF82 bacteria. Thus, the autophagy machinery of epithelial cells is able to target a subpopulation of intracellular AIEC bacteria.
or 6 h post infection. In contrast, when the autophagy induction was performed after infection by AIEC LF82 bacteria, we observed a pronounced effect at 6 h post infection, in Atg5K130R expressing transfected HeLa cells compared with those transfected with pEGFP-Atg5 encoding wt Atg5 (Fig. 5E). Thus, by modulating autophagy, our experiments confirm that autophagy limits the number of intracellular AIEC LF82 bacteria in human epithelial cells.

**Intracellular AIEC LF82 bacteria replicate within cells having impaired ATG16L1 expression**

Since a coding polymorphism in the autophagy gene ATG16L1 was identified as a risk factor in CD, we assessed the effect of endogenous ATG16L1 knock-down in HeLa cells by specific siRNA. The efficiency of knock-down was evaluated by real-time RT-PCR at 48 h post transfection. Compared with control siRNA (siC), ATG16L1 siRNA (siATG16L1) yielded a 71% reduction in ATG16L1 transcripts (Fig. 6A). Following a reduction in ATG16L1 expression, we observed significant ($P < 0.04$) increases in the numbers of intracellular AIEC LF82 bacteria at 1 and 6 h post infection (Fig. 6B). To confirm these results we performed a quantitative microscopic analysis to determine the percentage of LC3-positive vacuoles containing intracellular AIEC bacteria. In LC3-GFP-expressing HeLa cells transfected with control
siRNA a percentage of 6.4 (±0.9) of internalized AIEC was observed within LC3-positive vacuoles after 3 h of infection (Fig. 6C and D). This was reduced to 0.9% (±0.4%) in cells treated with siRNA targeting ATG16L1. Rescue of ATG16L1 expression using constructs containing synonymous base changes resulted in complete rescue in the case of the *300T variant with 6.5% (±1%) of AIEC within LC3-positive compartments. However, the CD-associated allele *300A was unable to mediate full phenotypic rescue, despite equal levels of protein expression (Fig. 6E), with only 2% (±0.6%) of AIEC within LC3-positive vacuoles. Thus, as in the Salmonella Typhi-murium model, the CD-associated ATG16L1 variant is unable to mediate fully effective antibacterial autophagy (Kuballa et al., 2008).

IRGM, a CD-associated genetic marker, controls the fate of intracellular AIEC LF82 bacteria

The human IRGM gene product stimulates the early stages of autophagy, and we therefore performed siRNA experiments against endogenous IRGM transcripts in

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Fig. 6. Intracellular AIEC LF82 bacteria replicate within cells impaired in ATG16L1 expression. The siRNA experiments against endogeneous ATG16L1 transcripts were performed in HeLa cells transfected with ATG16L1 specific siRNA oligonucleotides.

A. Endogenous ATG16L1 mRNA knock-down by specific siRNA in HeLa cells after 48 h of transfection was assessed by real-time quantitative RT-PCR normalized to GAPDH, compared with control duplexes. RT-PCR was performed in triplicate. Results represent independent experiments. Statistical significance: ***P < 0.001.

B. ATG16L1 knock-down increases the ability of the AIEC LF82 bacteria to replicate intracellularly. HeLa cells transfected with control (black bar) or ATG16L1 (white bar) -directed siRNA oligos were infected for 3 h. Results are expressed as mean numbers ± SEM of colony-forming units (cfu) per well. Each point is the mean of at least three separate experiments. Statistical significance: *P < 0.05.

C. ATG16L1 knock-down and allele-specific rescue reveals compromised autophagy of AIEC mediated by the CD-associated *300A allele. HeLa-GFP-LC3 cells were transfected with control (siC) or ATG16L1-targeting siRNA duplexes, along with empty plasmid (siATG16L1), or allele-specific rescue constructs prior to infection with AIEC. After 3 h of infection, cells were fixed and stained for microscopic analysis. Data shown represent means ± SEM of three independent experiments, counting 50 cells per experiment. Significance was determined using two-tailed Student’s t-tests with Bonferroni correction for multiple comparisons.

D. Confocal microscopic examination of colocalization of intracellular AIEC LF82 bacteria with GFP-LC3 in HeLa-GFP-LC3 cells transfected with control or ATG16L1-targeting siRNA duplexes, along with empty plasmid, or allele-specific rescue constructs prior to infection with AIEC.

E. ATG16L1 knock-down and rescue results in similar levels of allele-specific expression. Expression of both rescue constructs was equal, as determined by Western blotting with ATG16L1-specific antibodies, using actin as a loading control.
autophagy is an innate defence mechanism acting as a cell-autonomous system for elimination of intracellular pathogens, these findings lead weight to the notion that intracellular bacteria including AIEC might play a role in CD pathogenesis. Thus, the aim of the present study was to determine whether AIEC bacteria can take advantage of defects in autophagy to replicate within host epithelial cells, thereby establishing a link between genetic CD predisposition and the abnormal presence of AIEC colonizing ileal mucosa.

To test the hypothesis that ineffective autophagy may enable persistent intracellular survival, we compared AIEC infection of autophagy-deficient (atg5−/−) with wt MEFs. We observed that autophagy can restrict the replication of AIEC LF82 intracellular bacteria and therefore constitutes an efficient host innate defence mechanism. We next observed that, as in S. Typhimurium infection (Birmingham et al., 2006), a subpopulation of intracellular AIEC bacteria is targeted by autophagy. Interestingly, when we examined the behaviour of related E. coli strains, including non-pathogenic, environmental, commensal or pathogenic ETEC, EPEC, DAEC, EIEC bacteria with regard to autophagy, we found that functional autophagy limited AIEC bacterial replication and that there was no significant differences in survival/replication of all the other E. coli strains tested between wt MEFs and atg5−/− MEFs. The findings obtained in experiments using MEFS were confirmed in human epithelial cell lines. Confocal analysis of specific autophagosomal marker LC3 immunostaining showed that a subset of AIEC LF82 bacteria were in a LC3-positive vacuole, as reported for S. Typhimurium (Birmingham et al., 2006). Moreover, transmission electron microscopy of AIEC LF82-infected Hep-2 and Intestine-407 cells revealed that these bacteria were sequestered as single bacteria in multi-lamellar membrane vacuoles, corresponding to autophagosomes (Klionsky et al., 2008). TEM or confocal images suggested that the bacteria were not able to replicate in a LC3-positive compartment since we only observed clusters of bacteria in LC3-negative spacious phagosomes or in autophagy deficient epithelial cells. Furthermore, dominant negative of Atg5 enhanced bacterial replication whereas induction of autophagy attenuated replication. A fraction of intracellular AIEC is observed free in the cytosol or in damaged vacuoles and could represent the preferential target of autophagy machinery. Indeed, in addition to free bacteria in the cytosol-like group A Streptococci that are handled by the autophagy process (Nakagawa et al., 2004), endocytic phagosome-damaging by bacteria represents a strong signalling node to induce autophagic response as very recently reported for Shigella flexneri (Dupont et al., 2009). However, in contrast to Shigella flexneri (Ogawa et al., 2005), AIEC bacteria did not subvert early autophagy. While AIEC bacteria at late time post infection are mostly in vacuoles presenting

Discussion

IBD involves a dysregulation of the normal coevolved homeostatic relationship between the gut microbiota and the host immune system. Microorganisms, including pathogens and members of the indigenous microbiota, may participate in multiple roles, including initiation and/or propagation of the inflammatory processes. Recent studies have demonstrated that among the enteric microflora adherent-invasive E. coli (AIEC) strains are found more commonly in IBD patients than in control patients (Darfeuille-Michaud et al., 2004; Martin et al., 2004; Baumgart et al., 2007; Eaves-Pyles et al., 2007; Sasaki et al., 2007; Martinez-Medina et al., 2009); Genome-wide association studies have identified new susceptibility genes ATG16L1 and IRGM, which are involved in bacterial clearance and antigen processing via autophagy. As autophagy is an innate defence mechanism acting as a

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Fig. 7. IRGM, a CD-associated genetic marker, controls the number of intracellular AIEC LF82 bacteria within epithelial cells. The siRNA experiments against endogeneous IRGM transcripts were performed in HeLa cells cotransfected with the Flag-tagged IRGM expression plasmid pCMV-3xFlag and with IRGM specific siRNA oligonucleotides.

A. Immunoblot using anti-Flag antibody to analyse IRGM expression.

B. IRGM knock-down increases the ability of the AIEC LF82 bacteria to replicate intracellularly. HeLa cells transfected with control (black bar) or IRGM (white bar) directed siRNA oligos were infected for 3 h. Results are expressed as mean numbers ± SEM of colony-forming units (cfu) per well. Each point is the mean of at least three separate experiments.

C. IRGM knock-down does not modify the survival/replication of non-pathogenic E. coli. Results are expressed as the number of intracellular bacteria at 6 h post infection relative to that obtained at 1 h post infection, taken as 100%. Statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.

D. Confocal microscopic examination reveals an increased number of intracellular AIEC LF82 bacteria in IRGM knock-down cells. Control siRNA-treated (siControl) and IRGM siRNA-treated (siRNA IRGM) cells were infected with AIEC LF82-GFP bacteria and imaged by confocal microscopy. Control cells show intracellular GFP-bacteria (green) as small clusters enclosed in LC3-positive (red) vacuoles.

E. Confocal microscopic examinations of IRGM siRNA-treated Hela cells infected with AIEC LF82 GFP at 6 h post infection labelled with lysotracker (red) and for LAMP-1 (purple) to visualize the vacuoles.
features of mature phagolysosomes they are not targeted by the autophagy process. This finding is consistent with previous reports for S. Typhimurium and Streptococcus pyogenes (Nakagawa et al., 2004; Birmingham et al., 2006).

So far, the bacterial pathogens demonstrated to be controlled by autophagy exhibit significant adaptations to intracellular modes of survival and pathogenesis. Indeed, Shigella, Salmonella and AIEC all been characterized as intracellular pathogens, in contrast to EPEC, EHEC, DAEC and ETEC whose lifestyles are primarily extracellular, despite their ability to enter cells in limited numbers. Also recent data indicates that both the bacteria and their vacuolar remnants are targeted by autophagy upon vacuolar lysis and bacterial escape (Birmingham et al., 2006, 2007; Dupont et al., 2009). Since we found that pathogens without such direct intracellular adaptations (EPEC, ETEC, DAEC) or commensal bacteria, do not trigger autophagy this raises the question of whether it is the specific intracellular replication strategies of pathogens that renders them susceptible to autophagy. Bacteria which attempt vacuolar escape appear susceptible to autophagy when affecting exit of their entry vacuoles, and even those which attempt to maintain the vacuolar niche can fail and succumb to autophagy. Intracellular bacteria have conflicting needs, the vacuole is a secure environment, but both nutrition and space are extremely limited. They must either escape and replicate in the cytoplasm, or permeabilize/grow the vacuole to provide sufficient nutrition for replication. However, these routes to growth all expose them to autophagy, through signals yet to be defined. Thus vacuolar exit must be either rapid and complete, such as seen in Shigella or Listeria (High et al., 1992; Cossart and Lecuit, 1998), or significant effort must be expended to safely grow the vacuole to allow slow, but continued replication as shown for Salmonella and AIEC (Bruell et al., 2001; Glasser et al., 2001). Both approaches result in sufficient replication to yield a viable intracellular pathogenic strategy, but both require specific and elaborate mechanisms to be effective.

The host also is subject to strict requirements, signaling to trigger autophagy must be both specific and rapid, to avoid unnecessary degradation of organelles and cytoplasm, yet restrict pathogen escape and replication. To this end, any defects in autophagy or upstream signalling are likely to yield impaired control of infections, especially at sites where innate immune surveillance is restricted due to high bacterial load, as in the gut. In this report, we investigated the impact of expression alteration of the two autophagy-related genes identified as susceptibility genes for CD, ATG16L1 and IRGM. A functional knock-down of ATG16L1 by siRNA abrogates autophagy of AIEC strain LF82. This loss was fully rescued by expression of ATG16L1, but failed to be rescued by expression of the CD-associated ATG16L1 variant *300A. This result is similar to that observed in the S. Typhimurium model, where *300A is also less able to mediate antibacterial autophagy (Kuballa et al., 2008). This suggests that autophagy may be a general feature of the epithelial cell autophagic response to infection with an intracellular bacterium and that the CD-associated *300A ATG16L1 allele results in a diminished and ineffective autophagic response to intracellular pathogens. IRGM protein, which is highly expressed in response to infection, seems to be critical for clearance of intracellular bacteria such as Salmonella spp. by autophagy (Taylor, 2007). Mice rendered deficient in the homologue of IRGM gene have impaired ability to eliminate the intracellular pathogens Toxoplasma gondii, Listeria monocytogenes and S. Typhimurium (Collazo et al., 2001; Henry et al., 2007). In addition, experiments in murine macrophages showed that IRGM1-mediated autophagy generated large autolysosomal organelles as a mechanism for the elimination of intracellular Mycobacterium tuberculosis, and experiments in human macrophages reported that knock-down of IRGM leads to markedly prolonged survival of Mycobacterium tuberculosis (Singh et al., 2006). In the present study, we observed that when intracellular IRGM expression was reduced by specific siRNA there was a great increase in the number of AIEC LB82 intracellular bacteria, indicating a key role of IRGM in controlling intracellular AIEC replication. The absence of LC3 colocalization with bacteria was accompanied by huge clusters of intracellular AIEC LB82 bacteria mostly located in non-acidic compartments. Such huge intracellular microcolonies were not observed in LF82-infected atg5/−/− MEFs or in ATG16L1-depleted HeLa cells, indicating that the role of IRGM may not be restricted to autophagy since this protein has been described to be associated to the membrane of the phagosomes. In accordance, defective bacterial killing in LRG-47/−/− (a murine ortholog of IRGM) macrophages due to impaired maturation of bacteria-containing phagosomes was reported (MacMicking et al., 2003).

ATG16L1 polymorphism T300A predisposes to ileal disease and polymorphisms of the IRGM locus might predispose to fistulizing behaviour in CD (Prescott et al., 2007; Rioux et al., 2007; Latiano et al., 2009). In parallel, AIEC are mainly associated with severe ileal CD, and high levels of antibodies against E. coli OmpC are present in patients with severe CD, characterized by small bowel involvement, frequent disease progression, longer disease duration and greater need for intestinal surgery (Landers et al., 2002; Mow et al., 2004). Thus, the CD risk-conferring large upstream deletion at the IRGM locus and the ATG16L1 mutation observed in a CD patient population associated with the presence of pathogenic AIEC bacteria colonizing the intestinal mucosa may lead...
to functional alterations in the ability of intestinal cells to initiate and sustain autophagy to control AIEC replication. AIEC infection in patients with polymorphisms in autophagy genes may have a significant impact on the outcome of intestinal inflammation. From the data presented here, we suggest that it would be of interest to investigate the presence of mucosa-associated AIEC bacteria and ATG16L1 or IRGM polymorphism in CD patients in order to define CD patients at high risk of developing structuring and fistulizing CD.

Experimental procedure

**Bacterial strains and cell culture**

*Escherichia coli* strain LF82 was isolated from a chronic ileal lesion of a patient with CD. Non-pathogenic *E. coli* K-12 strain MG1655, the environmental *E. coli* strain SMS 3.5, the commensal *E. coli* strain HS, enteropathogenic *E. coli* (EPEC) strain E2348/69 (D), the diffusely adhering *E. coli* strain E12860/0 were tested in parallel. All the strains were grown in Luria–Bertani (LB) broth and plated on LB agar.

Human epithelial cell lines HeLa, Hep-2 and Intestine-407 were obtained from ATCC and cultured as described elsewhere (Boudeau et al., 1999). Stable HeLa-GFP-LC3 cells were generated by lentiviral transduction were used for quantitative microscopy (Kuballa et al., 2008). MEFs deficient in atg5 and the corresponding wt cells, kindly provided by Dr Naboru Mizuschima, were maintained as previously described (Nakagawa et al., 2004).

**Antibodies and reagents**

Rabbit polyclonal anti-LC3B, anti-Flag and anti-Actin for Western blot experiments were purchased from Sigma, and anti-ATG16L1 from Affinity Bioreagents. Rabbit polyclonal anti-LC3 for immunofluorescence analysis was purchased from MBL. Mouse monoclonal anti-LAMP-1 human and Rat monoclonal anti-LAMP-1 mouse were from DSBH Iowa. Rabbit antibody raised against *E. coli* LPS O83 was generously provided by Lothar Beutin (Department of biological safety, Robert Koch Institut, Berlin, Germany). TRITC-labelled phalloidin for visualization of actin cytoskeleton in MEF and Hoechst 33424 for labelling nuclei were purchased from Sigma, and Lysotracker probe DND-99 was purchased from Invitrogen. Rapamycin (LC laboratories) and Hank’s balanced salt solution (Sigma), LPS (Calbiochem) and IFN-γ (1000 U ml⁻¹) stimulations, cells were treated for 48 h before infection.

**Differential permeabilization with digitonin**

This assay was performed as previously described by Checroun et al. (2006) (PNAS). Briefly, infected HeLa cells or MEFs were washed 2× with KHM buffer (110 mM potassium acetate, 20 mM Hepes and 2 mM MgCl₂), incubated with KHM containing 25 μg ml⁻¹ digitonin (Sigma) for 1 min at room temperature and then washed with KHM buffer. Cells were then incubated with O83 antibody diluted in KHM buffer for 12 min at 37°C, washed with PBS and fixed with 4% PFA.

**Autophagy induction**

Autophagy was induced by using rapamycin treatment, with dose ranging from 20 to 80 μg ml⁻¹, during 3 h. Autophagy was also induced by incubation of cells monolayer during 2 h in HBSS minimum medium before infection. For LPS (100 ng ml⁻¹) and IFN-γ (1000 U ml⁻¹) stimulations, cells were treated for 48 h before infection.

**Plasmids and transfection**

The pEGFP-Atg5 or pEGFP-Atg5K130R expression vectors were a kind gift from T. Yoshimori (Osaka University, Japan). Plasmid pCMV-3xFlag-IRGM and ATG16L1 rescue constructs have been previously described (Kuballa et al., 2008; McCarroll et al., 2008). HeLa cells were transfected with plasmids and Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

**siRNA directed against ATG16L1 or IRGM**

siRNA experiments were performed using stealth RNAi (Invitrogen). The sequences used were as previously described (Rioux et al., 2007; McCarroll et al., 2008). Transfections were performed using Lipofectamine 2000 (Invitrogen). To evaluate knock-down efficiency, total mRNA from RNAi-treated HeLa cells was isolated using TRIzol reagent (Invitrogen) and analysed by real-time quantitative RT-PCR assays. Analysis of protein extract from RNAi-treated HeLa cells by Western blot confirmed knock-down.

**Immunoblot analysis**

Whole-cell protein extracts were prepared by adding lysis buffer (2% Triton X-100, 50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA). Proteins were separated on SDS/15% PAGE gels, transferred to polyvinylidene difluoride membrane, blocked 2 h in tris-buffered saline solution containing 2% BSA, probed overnight with primary antibodies, and 2 h with secondary HRP-coupled antibodies. Anti-actin was used to normalize protein quantity. After membrane revelation using the ECL detection kit (Amersham), quantification was done with Phoretix 1D software.

**Electron microscopy**

Cross-sections of Hep2 or Intestine-407 were prepared as previously described (Boudeau et al., 1999). Ultrathin sections
stained with uranyl acetate and lead citrate were then processed for electron microscopy with a Hitachi H-7650 transmission electron microscope at 80 kV.

**Fluorescence microscopy**

After bacterial infection HeLa cells or MEFs were fixed with 3% paraformaldehyde and immunostained overnight at 4°C, with the indicated specific primary antibodies. A 1 h incubation with secondary antibodies was done. The slides were examined with a Zeiss LSM 510 Meta confocal microscope. For quantitative microscopy stable HeLa-GFP-LC3 cells were transfected with 20 pmol of siRNA duplex and 500 ng of appropriate plasmid using Lipofectamine 2000. Following a media change after 6 h, cells were allowed to grow for 48 h and subsequently infected or lysed for Western blotting.

**Statistical analysis**

Experiments were independently carried out at least three times and one representative data set out of the three independent experiments was presented where appropriate. The results were evaluated for statistical significance by student t-test. Error bars were marked as the standard deviation (SD) of the mean. P-values less than 0.05 were regarded as significant.

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and the CD-associated adherent-invasive E. coli atg5 performed with AIEC LF82-infected wt MEFs (black lines) and LAMP-1 and vacuole acidification in MEFs. Experiments were taken as 100%. Times of gentamycin treatment relative to that obtained at 1 h expressed as percentage of intracellular bacteria after various times post infection ranging from 1 to 6 h. Results are mean percentage of LC3 colocalization on AIEC LF82-containing phagosome or standard error of the mean (SEM). At least three independent experiments were performed.

Fig. S3. Representative confocal micrographs of AIEC LF82-GFP-infected wt MEFs or atg5−/− MEFs at 6 h post infection labelled free cytosolic AIEC LF82 with O83 antibodies (red) after permeabilization of plasma membrane with digitonin. Vacuoles were stained with LAMP-1 antibodies (purple).

Fig. S4. Kinetics of the acquisition of lysosomal marker LAMP-1 (black lines) and vacuole acidification (grey lines) in AIEC LF82-infected Hela cells. Cells were infected with GFP-expressing AIEC strain LF82 for 3 h at moi of 10 and then incubated for 1, 2, 4 or 6 h in cell culture medium containing gentamycin (80 μg ml⁻¹). Labelling of LAMP-1 was performed using mouse antibodies to the human LAMP-1. AIEC LF82 containing vacuole acidification was assessed by using Lysotracker Red DND-99 as described in Experimental procedure. For each point, at least 100 AIEC LF82 containing vacuoles were counted and scored for the presence or absence of the markers. The results shown are the mean percentages of marker colocalization on AIEC LF82-containing phagosome ± standard error of the mean (SEM). At least three independent experiments were performed.

Fig. S5. Confocal analysis of percentage of colocalization of AIEC LF82-containing vacuoles with autophagic marker LC3 either in untreated cells (black bars) or in cells treated during 3 h with rapamycin (40 μg ml⁻¹, white bars) to induce autophagy. Hela cells were infected with GFP-expressing AIEC strain LF82 for 3 h at moi of 10 and then incubated in gentamycin containing medium. Cells were treated for additional 3 h with rapamycin at 1 or 20 h post infection. At least 100 AIEC LF82 containing vacuoles were counted and scored for the presence or absence of endogenous LC3 immunostaining. The results shown are the mean percentage of LC3 colocalization on AIEC LF82-containing phagosomes ± standard error of the mean. At least three independent experiments were performed.

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