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Defects in autophagy favour adherent-invasive *Escherichia coli* persistence within macrophages leading to increased pro-inflammatory response

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Summary

Ileal lesions in Crohn's disease (CD) patients are abnormally colonized by pathogenic adherent-invasive *Escherichia coli* (AIEC). AIEC bacteria are able to replicate within epithelial cells after lysis of the endocytic vacuole and within macrophages in a large vacuole. CD-associated polymorphisms in *NOD2*, *ATG16L1* and *IRGM* affect bacterial autophagy, a crucial innate immunity mechanism. We previously determined that defects in autophagy impaired the ability of epithelial cells to control AIEC replication. AIEC behave differently within epithelial cells and macrophages and so we investigated the impact of defects in autophagy on AIEC intramacrophagic replication and pro-inflammatory cytokine response. AIEC bacteria induced the recruitment of the autophagy machinery at the site of phagocytosis, and functional autophagy limited AIEC intramacrophagic replication. Impaired *ATG16L1*, *IRGM* or *NOD2* expression induced increased intramacrophagic AIEC and increased secretion of IL-6 and TNF- α in response to AIEC infection. In contrast, forced induction of autophagy decreased the numbers of intramacrophagic AIEC and pro-inflammatory cytokine release, even in a *NOD2*-deficient context. On the basis of our findings, we speculate that stimulating autophagy in CD patients would be a powerful therapeutic strategy to concomitantly restrain

intracellular AIEC replication and slow down the inflammatory response.

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are two major forms of idiopathic inflammatory bowel disease (IBD) affecting 1.4 million individuals in the USA and 2.2 million in Europe (Economou and Pappas, 2008; Shanahan and Bernstein, 2009). CD is now widely accepted as a genetically determined overactive immune response to the intestinal microbiota (Strober *et al.*, 2007; Kaser *et al.*, 2010; Cho and Brant, 2011). Immunologically, CD patients produce high amounts of IFN- γ in the inflamed lamina propria (Breese *et al.*, 1993; Fuss *et al.*, 1996) as the result of abnormal Th1- and Th17-mediated immune responses. Pro-inflammatory cytokines such as TNF- α and IL-6 play a pivotal role in inflammation-related tissue destruction in CD, and are involved in the differentiation of Th1 and Th17 T cells from naïve T CD4+ cells and their maintenance (Strober *et al.*, 2010; Strober and Fuss, 2011).

There is increasing evidence that *Escherichia coli* play a prominent part in CD pathogenesis. Dysbiosis of the lumenal- and mucosal-associated microbiome has been observed in IBD patients (Packey and Sartor, 2009; Sokol and Seksik, 2010), and independent studies have reported the presence of increased numbers of bacteria with invasive properties belonging to the *E. coli* species that abnormally colonize the ileal mucosa of CD patients (Darfeuille-Michaud *et al.*, 1998; 2004; Martin *et al.*, 2004; Baumgart *et al.*, 2007; Eaves-Pyles *et al.*, 2007; Sasaki *et al.*, 2007; Martinez-Medina *et al.*, 2009). These strains, termed adherent-invasive *E. coli* (AIEC), isolated from CD patients, form biofilm on the surface of the ileal mucosa owing to an abnormally increased expression of CEACAM6 receptor (Barnich *et al.*, 2007), induce epithelial injury and significantly increase erosive lesions and mucosal inflammation (Carvalho *et al.*, 2009). The presence of *E. coli* has been convincingly evidenced within macrophages in CD tissue. *E. coli* antigens have been identified in macrophages within the lamina propria and in

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the germinal centres of mesenteric lymph nodes in patients with CD (Ambrose *et al.*, 1984; Cartun *et al.*, 1993; Liu *et al.*, 1995) and *E. coli* DNA was detected in 80% of microdissected granulomas from CD patients (Ryan *et al.*, 2004). In addition, we recently reported that AIEC bacteria target M cells, which could allow them to interact with Peyer's patches and lamina propria macrophages (Chassaing *et al.*, 2011). *In vitro* studies have demonstrated that *E. coli* associated with CD are able to survive and replicate within macrophages and induce secretion of great amounts of the pro-inflammatory cytokine TNF α (Glasser *et al.*, 2001; Bringer *et al.*, 2006; Subramanian *et al.*, 2008) and to induce the formation of cell aggregates very similar to epithelioid granulomas (Meconi *et al.*, 2007).

Macrophages play a pivotal role in bacterial clearance, and autophagy is one of the main degradative pathway of the innate immune system responsible for the detection and elimination of intracellular bacteria (Nakagawa *et al.*, 2004; Birmingham *et al.*, 2006; Singh *et al.*, 2006). Genetic associations identified in CD have highlighted the key role of autophagy pathway in the disease. Various studies have reported a highly significant and replicated association between CD, variants of the intracellular bacteria sensing receptor Nod2 and variants in two separate autophagy genes (*ATG16L1* and *IRGM*) (Hugot *et al.*, 2001; Ogura *et al.*, 2001; Hampe *et al.*, 2007; Parkes *et al.*, 2007; Rioux *et al.*, 2007; Wellcome, 2007) and recent evidence has been shown of a genetic association of CD with a tSNP in the *ULK1* gene, which encodes a protein involved in autophagy initiation (Henckaerts *et al.*, 2011). Interestingly, a relation has been established between the autophagy machinery the intracellular bacteria sensing receptor Nod2 (Cooney *et al.*, 2010; Homer *et al.*, 2010; Travassos *et al.*, 2010) that recruits the critical autophagy protein ATG16L1 to the plasma membrane during bacterial invasion. We investigated the impact of a loss of autophagy function on the intramacrophagic persistence of CD-associated *E. coli* and pro-inflammatory cytokine response of infected-macrophages.

Results

AIEC induce recruitment of the autophagy machinery during phagocytosis at the bacteria entry site

Monocyte-differentiated THP-1 macrophages were infected with various AIEC strains and autophagy activation was monitored by detecting the conversion of free cytosolic LC3-I towards autophagosomal LC3-II by immunoblot using an antibody raised against LC3 isoform B (Fig. 1A). The amount of LC3-conjugated form (LC3-II) was greatly increased in AIEC-infected macrophages at

30 min post infection, with LC3-II/actin ratio ranging from 0.37 ± 0.09 for LF138-infected macrophages to 0.66 ± 0.19 for LF73-infected macrophages compared with 0.08 ± 0.04 for uninfected cells (Fig. 1B). In order to determine whether autophagy induced in macrophages in response to AIEC infection is functional, we analysed autophagy flux by comparing LC3-II turnover in presence or absence of Bafilomycin A1, a lysosomal inhibitor blocking autophagosome maturation, which leads to LC3-II accumulation and gives therefore information about the autophagic flux status (Klionsky *et al.*, 2008). A significant increase in LC3-II accumulation was observed at early time point post infection in Bafilomycin A1-treated THP-1 macrophages in response to infection compared with untreated cells, indicating a stimulation and not a blockade of the autophagic flux by AIEC bacteria (Fig. 1C and D). Induction of a functional and degradative autophagy flux in AIEC-infected THP-1 macrophages was confirmed by the concomitant decrease in p62, a cargo protein incorporated into the autophagosome and degraded inside autolysosomes (Fig. 1C and E). Altogether, these results indicated a functional and degradative autophagic flux in response to AIEC bacteria infection. Confocal analysis of LC3-immunolabelled macrophages indicated that AIEC LF82 bacteria localized in autophagosomal vacuoles soon after infection (Fig. 1F and G). This is in good accordance with LC3-II accumulation observed in autophagy flux analysis on AIEC-infected THP-1 cells (Fig. 1C). The autophagic nature of these compartments was confirmed by colocalization of ATG16L1 protein with AIEC LF82-containing vacuoles (Fig. 1H and I). Of interest, ATG16L1- and LC3-positive AIEC-containing vacuoles were closely associated with actin ruffles induced for bacteria engulfment (Fig. 1F and H), which indicates that the autophagic proteins were recruited at the AIEC bacteria entry site in order to immediately deliver AIEC bacteria to the autophagy machinery.

AIEC bacteria targeted by the autophagic machinery are rapidly degraded

The LC3-II conversion and the percentage of AIEC LF82 bacteria enclosed in LC3-positive vacuoles were analysed from early (30 min) to late (24 h) time post infection in THP-1 macrophages, to determine whether AIEC bacteria persist within autophagosomes at late time post infection. We observed a peak in the amount of LC3-II at 1 h post infection that was then followed by a decrease in the LC3-II/actin ratio (Fig. 2A) reflecting a probable resolution of the autophagolysosomes and subsequent recycling of the LC3-II form. In parallel we observed that the number of AIEC LF82 bacteria localized in LC3-positive compartments decreased in a time-dependent manner with $12.2\% \pm 1.4\%$ of LF82-containing LC3-positive vacuoles

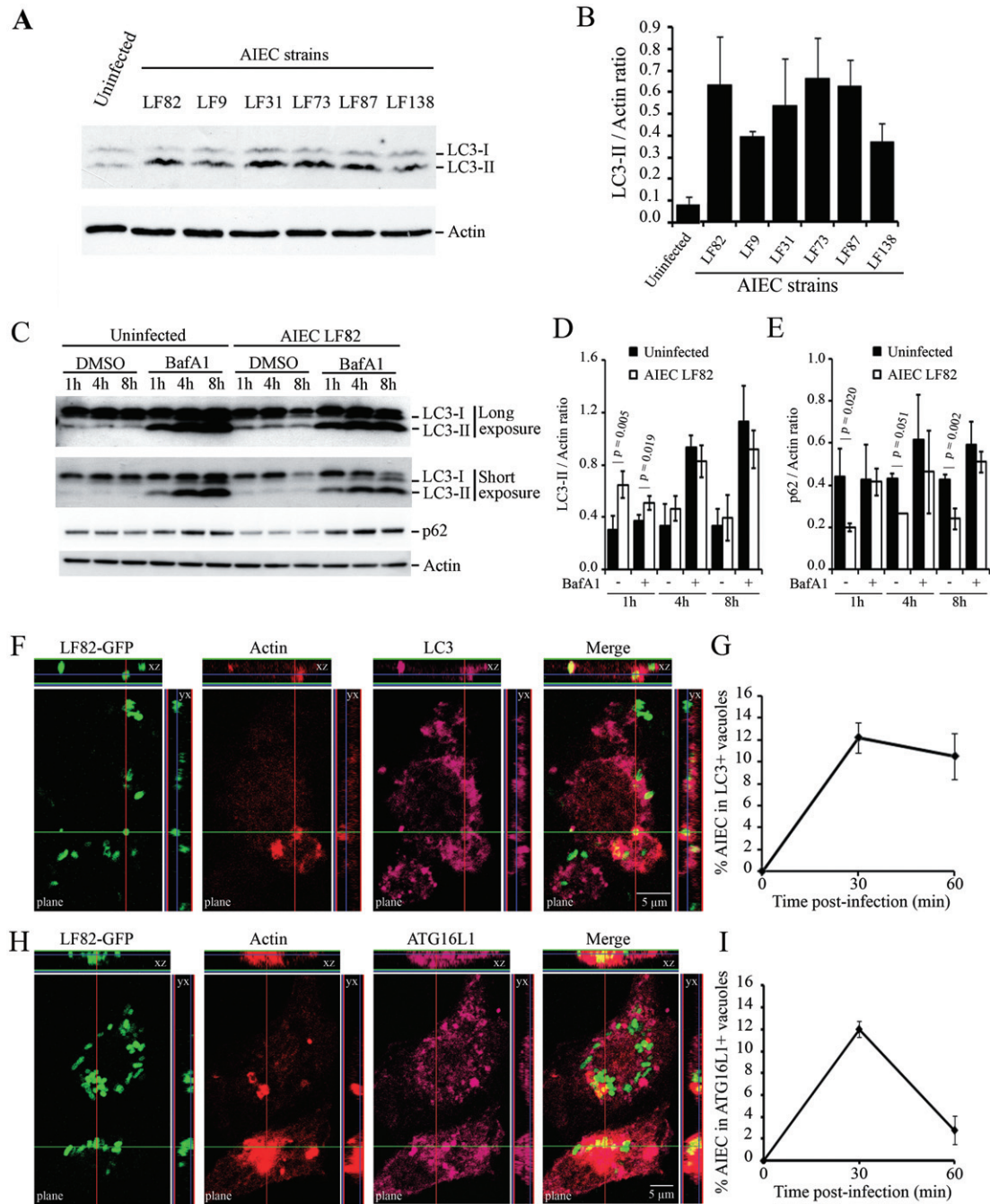


Fig. 1. The autophagy machinery is recruited at the site of entry of AIEC bacteria in human THP-1 macrophages.

A. THP-1 macrophages were infected with AIEC reference strain LF82 and AIEC strains LF9, LF31, LF73, LF87 and LF138. Protein extracts from uninfected and infected cells were processed for immunoblotting with anti-LC3 and anti-actin antibodies at 30 min post infection.

B. Quantification of LC3-II accumulation relative to actin was done.

C. THP-1 macrophages were treated with DMSO or Bafilomycin A1 (BafA1) at 150 nM for 30 min prior to AIEC LF82 infection and treatment was maintained during infection and post-infection periods. At 1 h, 4 h and 8 h post infection protein extracts from uninfected and AIEC-infected THP-1 macrophages were processed for immunoblotting with anti-LC3, anti-p62 and anti-actin antibodies. A long and a short exposure of LC3 immunoblotting are presented to allow quantification of LC3-II signal in protein extracts from untreated cells and Bafilomycin A1-treated cells respectively.

D and E. Quantification of LC3-II (D) or p62 (E) accumulation relative to actin was done.

F and H. Confocal microscopy examinations of colocalization between LC3 (purple, F) or ATG16L1 (purple, H) and GFP-expressing AIEC bacteria in infected-THP-1 macrophages at 30 min post infection. Nuclei and actin cytoskeleton were respectively stained with Hoescht (blue) and TRITC-labelled phalloidin (red). Z-stacks are presented.

G and I. Percentage of LC3 (G) or ATG16L1 (I) positive vacuoles containing GFP-expressing LF82 bacteria at 0, 30 and 60 min post infection. At least 100 cells were analysed for each experiment. For all experiments, data are means \pm SEM of three independent experiments.

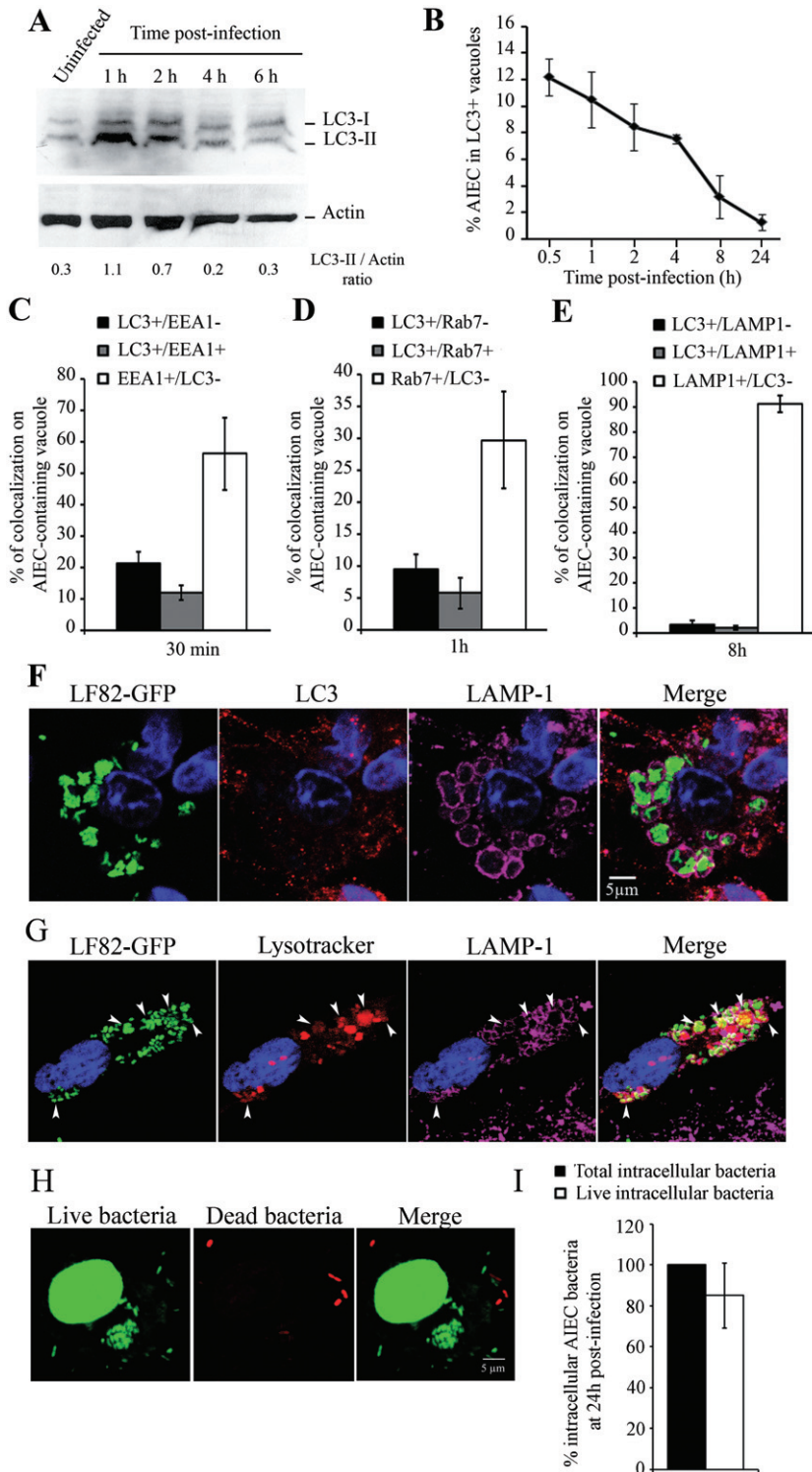


Fig. 2. AIEC bacteria are unable to persist within autophagic vacuoles.

A. THP-1 macrophages were infected with AIEC strain LF82 and were processed from 1 h to 6 h post infection for immunoblotting with anti-LC3 antibody. Quantification of LC3-II signal relative to actin is displayed below the representative immunoblot. **B.** Confocal microscopy analysis of the percentage of LC3-positive vacuoles containing GFP expressing-LF82 bacteria from 30 min to 24 h post infection. Data are means \pm SEM of three independent experiments. At least 100 cells were analysed at each post-infection time.

C–E. Confocal microscopy analysis of percentages of LC3-positive or -negative/EEA1 (C), Rab7 (D) or LAMP-1 (E)-positive or -negative vacuoles containing GFP expressing-LF82 bacteria at indicated time post infection. Data are means \pm SEM of three independent experiments. At least 100 cells were analysed at each post-infection time.

F. Representative confocal micrographs of colocalization between LC3 (red), LAMP-1 (purple) and GFP-expressing AIEC bacteria in infected-THP-1 macrophages at 24 h post infection.

G. Representative confocal micrographs of colocalization between Lysotracker Red DND-99 (acid vacuoles, red), LAMP-1 (purple) and GFP-expressing AIEC bacteria in infected-THP-1 macrophages at 24 h post infection. Nuclei were stained with Hoescht.

H. Representative confocal micrographs of intracellular AIEC bacteria observed within THP-1 macrophages at 24 h post infection. In order to distinguish live and dead intracellular AIEC bacteria, infected THP-1 macrophages were processed for a viability assay (see *Experimental procedures*).

I. Confocal microscopy analysis of the percentage of live intracellular AIEC bacteria relative to the total number of intracellular bacteria taken as 100%, observed within THP-1 macrophages at 24 h post infection. Data are means \pm SEM. At least 50 cells were analysed.

at 30 min post infection, $3.1\% \pm 1.6\%$ at 8 h post infection and $1.2\% \pm 0.6\%$ at 24 h post infection (Fig. 2B). To better characterize the intracellular traffic of AIEC bacteria in THP-1 macrophages, we analysed the number of AIEC LF82 bacteria located in LC3- and/or EEA1- and/or

Rab7- and/or LAMP1-positive vacuoles from 30 min to 8 h post infection. Results showed that a subpopulation of AIEC bacteria are sequentially localized within vacuoles that harboured EEA1 marker of early endosomes and Rab7 and LAMP-1 markers of late endosomes/

phagolysosomes but devoid of the LC3 autophagy marker (Fig. 2C–E). Of interest, we observed that intracellular AIEC bacteria observed within macrophages at 24 h post infection were mostly enclosed within LC3-negative, LAMP-1-positive and acid vacuoles (Fig. 2F and G) and that more than 80% of these intracellular bacteria were alive (Fig. 2H and I). Thus, LF82 bacteria targeted early by the autophagic machinery are likely to be rapidly degraded whereas AIEC bacteria that escape early uptake by autophagy at the site of entry resist in macrophages within LC3-negative vacuoles possessing classical phagolysosomal traits.

Impaired ATG16L1, IRGM or NOD2 expression favours persistence of AIEC bacteria within macrophages

Impairment of autophagy in THP-1 macrophages treated with wortmannin, an inhibitor of the autophagic process that interferes with PI3K protein networks, significantly increased in a dose-dependent manner the percentage of intramacrophagic AIEC LF82 bacteria (Fig. 3A). To mimic more specifically defects in autophagy associated with CD, due to polymorphisms in the autophagy-related genes, we used specific siRNA to analyse the effects of decreased ATG16L1 expression and over- or down-expression of IRGM on the control of the number of intramacrophagic AIEC bacteria. The efficiency of knock-down was checked by immunoblotting (Fig. 3B). Impaired autophagy in THP-1 macrophages transfected with siATG16L1 and siIRGM was confirmed by a decrease in LC3-II accumulation in Bafilomycin A1-treated macrophages compared with untreated cells (Fig. 3B). Decreased expression of ATG16L1 resulted in significant increases in the numbers of intramacrophagic LF82 bacteria at 1 h and 8 h post infection (Fig. 3C). Similarly, IRGM-decreased expression induced a significant increase in the percentage of intracellular LF82 bacteria at 8 h post infection compared with that observed in control siRNA-treated cells or untransfected cells (Fig. 3C). Confocal microscopy examinations revealed that, at 8 h post infection, AIEC LF82-infected macrophages transfected with ATG16L1 or IRGM siRNA showed no significant difference in the proportion of AIEC bacteria located in LAMP-1-positive/LC3-negative vacuoles, compared with control siRNA-treated cells (Fig. 3D and E). We also analysed the impact of IRGM overexpression on AIEC intramacrophagic persistence since we previously showed that both decreased and increased IRGM expression lead to a loss of control of intracellular replication of AIEC bacteria within epithelial cells (Brest *et al.*, 2011). IRGM overexpression in THP-1 macrophages led to an increase in LC3-II accumulation, which was in good agreement with previous data (Singh *et al.*, 2010), and indicates increased activity of the autophagic process (Fig. 4A and

B), and significant decreases in the number of intramacrophagic AIEC bacteria (Fig. 4C). However, confocal microscopy examination of macrophage nuclei (Fig. 4D and E) and analysis of caspase-3 cleavage (Fig. 4F and G) showed that overexpression of IRGM in macrophages induced cell death in a dose-dependent manner, leading to the release of bacteria in the extracellular cell culture medium containing gentamicin and making the quantification of AIEC intracellular persistence in such conditions undeterminable.

To address whether Nod2-mediated autophagy is critical for AIEC persistence and/or replication in macrophages, in a way similar to previous observations in dendritic cells (Cooney *et al.*, 2010), we first analysed by immunoblot LC3-II conversion in peritoneal macrophages isolated from wild-type or *NOD2* knockout (*NOD2*^{-/-}) mice in response to AIEC infection (Fig. 5A). LF82 infection of wild-type peritoneal macrophages induced an increase in the amount of LC3-II compared with uninfected cells (Fig. 5A and C). In contrast, in LF82-infected *NOD2*^{-/-} peritoneal macrophages, no significant increase in the amount of LC3-II was observed at 1 h or 2 h post infection compared with uninfected cells (Fig. 5A and C). Of note, the basal level of LC3-II was higher in *NOD2*^{-/-} macrophages than in wild-type macrophages (Fig. 5B). Confocal microscopy examinations showed that at 1 h post infection a significantly higher number of AIEC bacteria were located within LC3-positive compartments in peritoneal macrophages isolated from wild-type mice than in those isolated from *NOD2* knockout mice (Fig. 5D and E). We compared the ability of AIEC LF82 bacteria to survive intracellularly in wild-type and *NOD2*^{-/-} peritoneal macrophages. The percentage of intracellular AIEC at 4 h post infection relative to that obtained at 1 h post infection was significantly higher in *NOD2*^{-/-} peritoneal macrophages than in wild-type macrophages (Fig. 5F) and as previously seen with AIEC-infected dendritic cells (Cooney *et al.*, 2010), we also observed delayed AIEC bacteria clearance in *NOD2*^{-/-} macrophages.

Enhanced pro-inflammatory cytokine secretion in AIEC-infected macrophages displaying autophagy deficiency

We investigated whether the pro-inflammatory cytokine response of macrophages to AIEC infection is modulated according to the activity state of the autophagy machinery. Infection of human THP-1 macrophages with AIEC bacteria induced the secretion of high amounts of TNF- α and IL-6, compared with the basal secretion of uninfected cells (Fig. 6A and B). Knock-down of the autophagy-related genes ATG16L1 and IRGM in human THP-1 macrophages by siRNA resulted in significantly increased secretions of both TNF- α and IL-6 (Fig. 6C and D). In line with

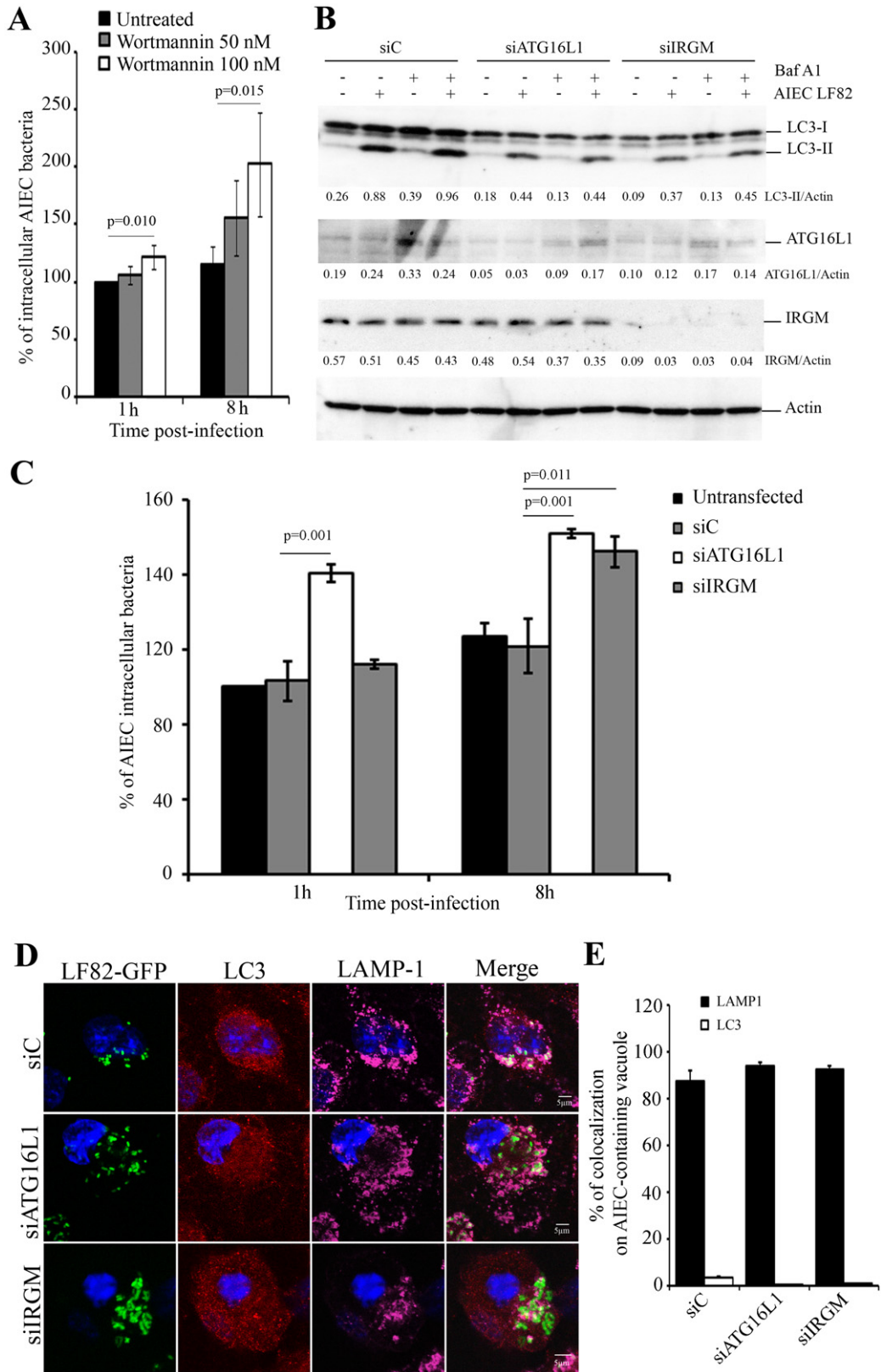


Fig. 3. ATG16L1 and IRGM, two autophagy-related proteins associated with CD, control the number of intracellular AIEC LF82 bacteria in human THP-1 macrophages.

A. THP-1 macrophages were treated with wortmannin at 50 nM (grey bar) or 100 nM (white bar) immediately after AIEC infection. Results are expressed as the number of intracellular bacteria at 1 h or 8 h post infection in treated cells relative to that obtained in untreated cells at 1 h post infection (black bar), taken as 100%. Data are means \pm SEM of at least three independent experiments.

B. THP-1 macrophages were transfected for 48 h with control (siC), ATG16L1 or IRGM-directed siRNA. When indicated, uninfected or AIEC-infected THP-1 macrophages were treated for 1 h with Bafilomycin A1 at 150 nM. Immunoblots using anti-LC3, anti-ATG16L1, anti-IRGM and anti-actin antibodies were performed. Quantification of LC3, ATG16L1 or IRGM relative to actin is displayed below immunoblots.

C. THP-1 macrophages untransfected or transfected for 48 h with control, ATG16L1 or IRGM-directed siRNA were infected with AIEC LF82 bacteria and the numbers of intracellular bacteria were determined by cfu quantification at 1 h and 8 h post infection using the gentamicin protection assay. Results are expressed as percentage \pm SEM of the number of intracellular bacteria at 1 h and 8 h post infection relative to the number of intracellular bacteria obtained at 1 h for untransfected cells, taken as 100%. Data are means \pm SEM of at least three independent experiments.

D. Representative confocal micrographs of colocalization between LC3 (red), LAMP-1 (purple) and GFP-expressing AIEC bacteria in infected-THP-1 macrophages treated with siC, siATG16L1 or siIRGM, at 8 h post infection. Nuclei were stained with Hoeschst.

E. Percentage of LC3 or LAMP1-positive vacuoles containing GFP-expressing LF82 bacteria at 8 h post infection. At least 100 cells were analysed for each experiment.

Data are means \pm SEM of three independent experiments.

what we observed in *ATG16L1* and *IRGM* knock-down THP-1 macrophages, AIEC LF82-infected peritoneal macrophages isolated from *NOD2*^{-/-} mice released significantly higher amounts of TNF- α than infected peritoneal macrophages from wild-type mice at 4 h post infection (Fig. 6E). We also observed increased but not significant IL-6 secretion by AIEC-infected *NOD2*^{-/-} macrophages (Fig. 6F). Together, these results indicate that macrophages displaying autophagy deficiency secrete larger amounts of pro-inflammatory cytokines in response to AIEC infection.

Activation of autophagy as a strategy to concomitantly restrain the number of intracellular AIEC bacteria and slow down inflammatory response

To assess the effect of autophagic process on AIEC bacteria that are not initially delivered to autophagosome or that are not targeted/uptake initially by the autophagy machinery, THP-1 cells were infected for 20 min, gentamicin was added to stop the infection and rapamycin or HBSS medium were added for a 2 h period, either immediately after cell infection or at 20 h post infection. Induction of autophagy immediately after infection increased the number of LC3-positive AIEC LF82-containing vacuoles (Fig. 7A) and resulted in a significant decrease in the number of intramacrophagic LF82 bacteria (Fig. 7B). In contrast, autophagy induction at 20 h post infection did not significantly modify either the number of LC3-positive LF82-containing vacuoles (data not shown) or the percentage of intracellular LF82 bacteria (Fig. 7C). As shown in Fig. 7D, this was not due to defective autophagy induction in AIEC LF82-infected macrophages treated with rapamycin at 20 h post infection. When we analysed pro-inflammatory cytokine secretion in response to AIEC infection, we observed that induction of autophagy, either by starvation or by treatment with rapamycin, led to a drastic significant decrease in the amount of TNF- α

secreted (Fig. 7E). In addition, we analysed the effect of forced autophagy activation in *NOD2*^{-/-} peritoneal macrophages either by starvation or by treatment with rapamycin on AIEC intramacrophagic persistence and secretion of pro-inflammatory cytokines. Very interestingly, we observed that induction of autophagy highly decrease the number of intracellular AIEC bacteria (Fig. 7F) as well as the amount of TNF- α and IL-6 secreted by both AIEC-infected wild-type and *NOD2*^{-/-} peritoneal macrophages compared with untreated cells at 4 h post infection (Fig. 7G and H). Thus, induction of autophagy allows macrophages to restrain the number of intracellular AIEC bacteria and to slow down the intensity of the associated pro-inflammatory response in both wild-type and *NOD2*-deficient macrophages.

Discussion

Among the large number of studies that have attempted to identify the infectious trigger involved in the abnormal immune response observed in CD there is increased evidence pointing to an abnormal colonization of the ileal mucosa by AIEC bacteria in CD 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). In parallel, several independent genome-wide association studies have linked defects in autophagy, a crucial element in the innate immune response to intracellular pathogens, to the pathogenesis of CD. Autophagy is a potent mechanism for restraining AIEC intracellular replication within epithelial cells (Lapaquette *et al.*, 2010; Brest *et al.*, 2011). However, its role in the control of AIEC multiplication within macrophages was still unknown and warranted investigation since the behaviour of intracellular AIEC bacteria is different in epithelial cells and in macrophages. Indeed, AIEC are able to moderately multiply within epithelial cells after lysis of the endocytic vacuole and within

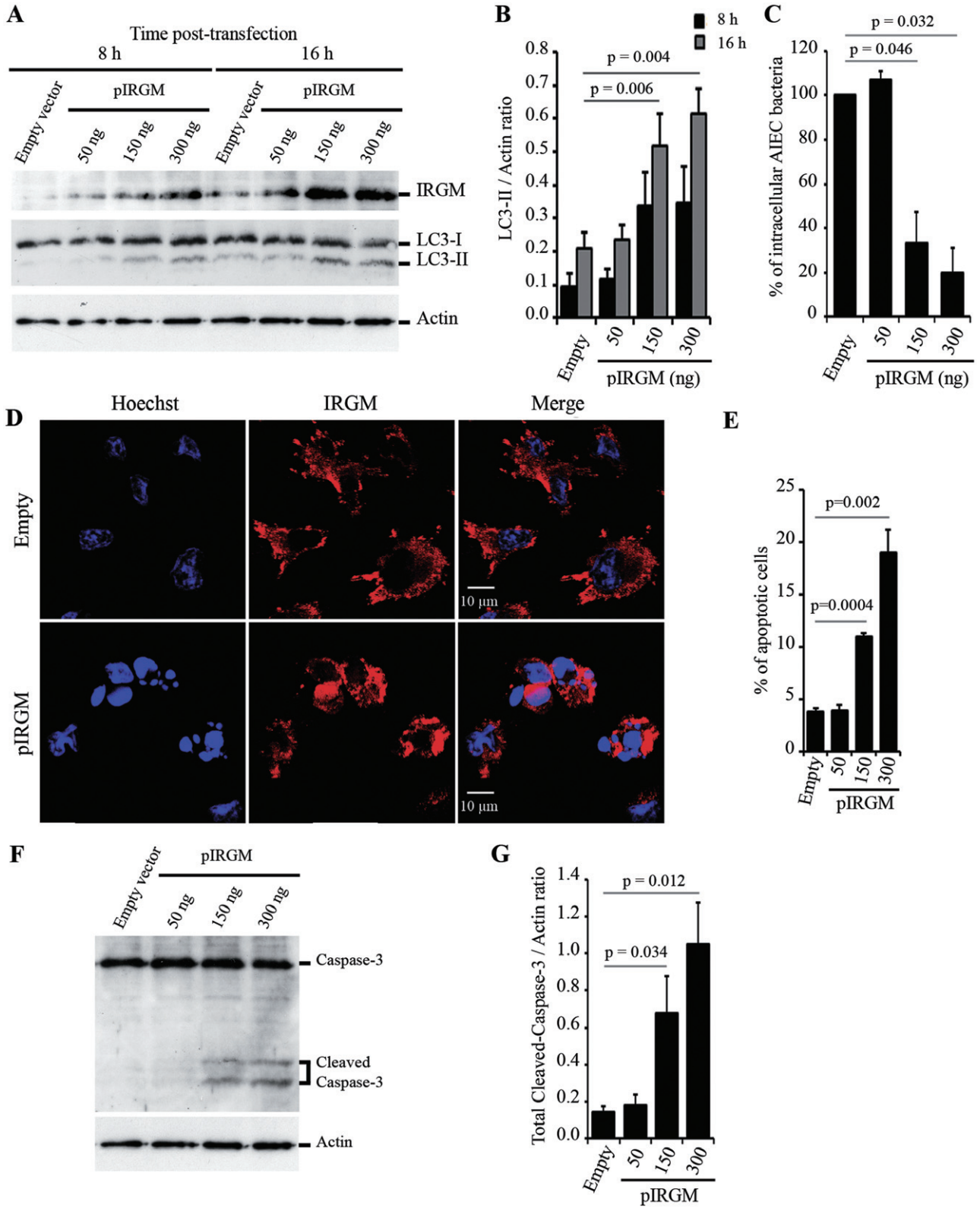


Fig. 4. Uncontrolled IRGM expression leads to macrophage cell death.

A. THP-1 macrophages were transfected with empty vector (300 ng) or with increasing amounts of IRGM-expressing vector (50–300 ng).

Protein extracts were processed for immunoblotting with anti-IRGM, anti-LC3 and anti-actin antibodies at 8 h and 16 h post transfection.

B. Quantification of LC3-II accumulation relative to actin was done, and data are means \pm SEM of three independent experiments.

C. THP-1 macrophages transfected with empty vector (300 ng) or with increasing amounts of IRGM-expressing vector (50–300 ng) were infected with AIEC strain LF82 at 8 h post transfection. The numbers of intracellular bacteria were determined by cfu quantification at 1 h and 8 h post infection. Results are expressed as the percentage \pm SD of intracellular bacteria at 8 h/1 h post infection relative to that obtained in empty vector transfected cells, taken as 100%.

D. Representative confocal micrographs showing apoptosis of IRGM-overexpressing macrophages (300 ng) at 16 h post transfection. IRGM was labelled with anti-IRGM antibody (red) and nuclei were stained with Hoescht (blue).

E. Percentage of macrophages presenting nucleus apoptotic features at 16 h post transfection. Data are means \pm SEM of four independent experiments. At least 100 cells were analysed for each condition.

F. Representative immunoblot using anti-Caspase-3 and anti-actin antibodies on protein extracts from THP-1 macrophages at 16 h post transfection.

G. Quantification of total cleaved Caspase-3 (15 and 17 kDa forms) relative to actin was done.

Data are means \pm SEM of four independent experiments.

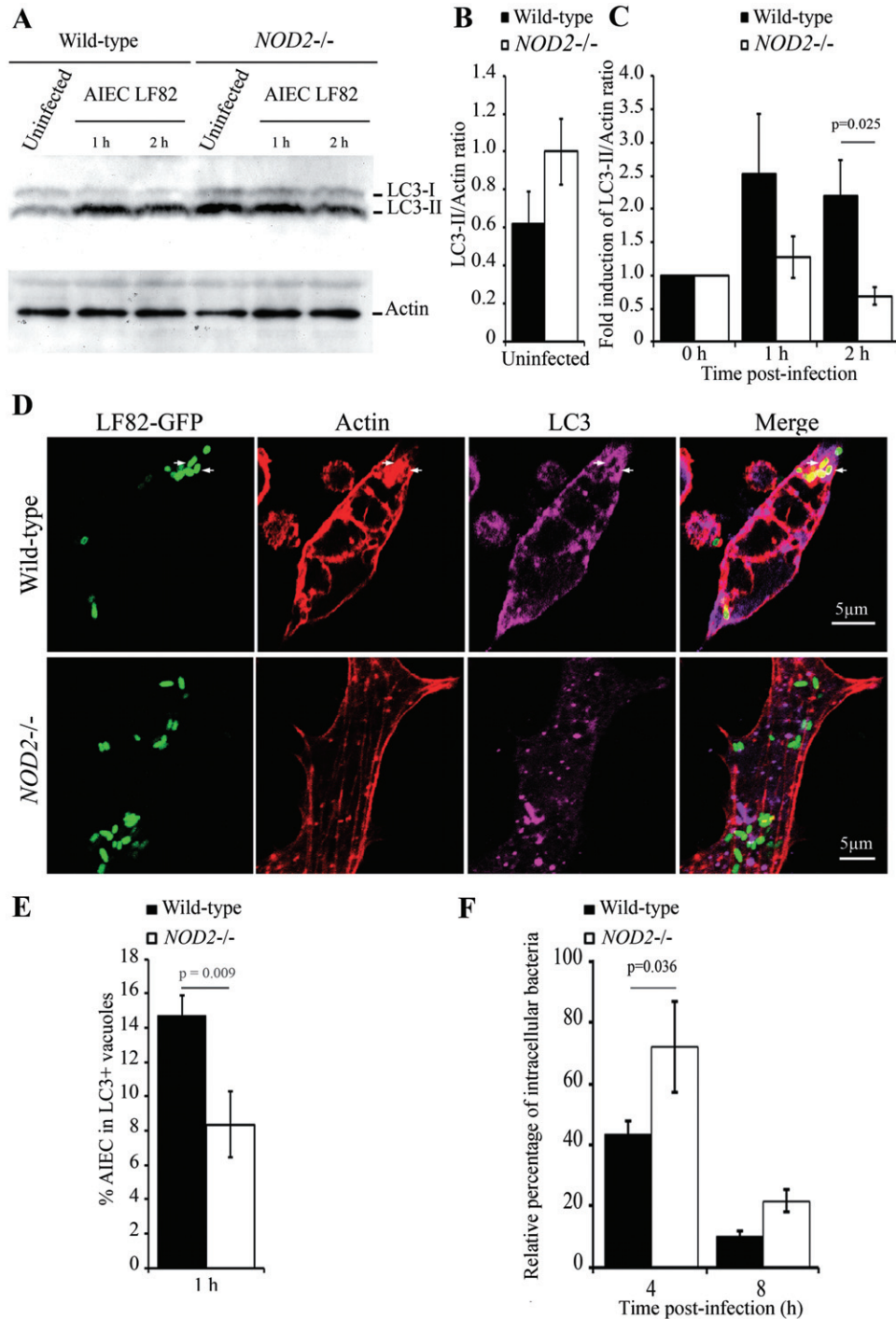
macrophages they highly replicate in a large vacuole with phagolysosomal traits, and AIEC-infected macrophages secrete large amounts of TNF- α . The aim of the present study was to analyse the impact of defects in autophagy on AIEC intramacrophagic survival and replication and on the outcome of inflammatory response.

Infection of human monocyte-derived THP-1 macrophages by AIEC bacteria rapidly activated autophagy and a subset population of AIEC bacteria was wrapped directly in autophagosomes at the site of phagocytosis by actin ruffles. This early autophagic response was transient since the number of AIEC bacteria that colocalized with ATG16L1 and LC3 markers reached a peak within the first 30 min of infection before decreasing, probably due to autophagolysosome resolution and degradation of their intraluminal content, as shown by the concomitant decrease in amount of cargo protein p62. In contrast to the autophagic response to AIEC bacteria observed in epithelial cells, which occurred several hours post infection (Lapaquette *et al.*, 2010), our findings show that in macrophages autophagy is an immediate response that achieves rapid efficient bacteria clearance, a trait that is expected for professional phagocytic cells.

AIEC bacteria that are not trapped within autophagosomes during phagocytosis are delivered inside vacuoles undergoing normal and sequential interaction with host endomembrane organelles and mature into phagolysosomes in an autophagy-independent manner. AIEC bacteria that persisted within THP-1 macrophages at late time post infection were localized within acid vacuoles that were LC3-negative/LAMP-1-positive and were alive. Thus, there has to be a rapid and extremely efficient autophagic response for macrophages to efficiently degrade AIEC. Interestingly, AIEC bacteria have the ability to survive and replicate in phagolysosomes in macrophages (Bringer *et al.*, 2006), but they are very sensitive to the autophagy-mediated degradative pathway. This suggests that AIEC bacteria are exposed within the autophagosome to bactericidal molecules that are absent

or non-activated within LC3-negative and LAMP-1-positive acid vacuoles in which AIEC bacteria replicate. This hypothesis is supported by studies that showed that the autophagy-related adaptor protein p62 delivered specific ribosomal and bulk ubiquitinated cytosolic proteins to autophagolysosomes, in which they are processed from innocuous precursors into potent neo-antimicrobial peptides, thereby explaining in part the potent bactericidal properties of autophagic organelles (Alonso *et al.*, 2007; Ponpuak *et al.*, 2010).

A highly significant and replicated association has been observed between CD, variants of the intracellular bacteria sensing receptor Nod2 and variants in two separate autophagy genes (*ATG16L1* and *IRGM*) (Hugot *et al.*, 2001; Ogura *et al.*, 2001; Hampe *et al.*, 2007; Parkes *et al.*, 2007; Rioux *et al.*, 2007; Wellcome, 2007). Interestingly, the autophagy machinery has also been linked to Nod2, which recruits the critical autophagy protein ATG16L1 to the plasma membrane during bacterial invasion (Cooney *et al.*, 2010; Homer *et al.*, 2010; Travassos *et al.*, 2010). We observed that impaired expression of ATG16L1, IRGM or NOD2 impaired AIEC bacteria targeting by the autophagy machinery and favoured their replication within macrophages. Similar findings were reported for other intracellular pathogens such as *Salmonella* serovar Typhimurium and *Mycobacterium tuberculosis* (Singh *et al.*, 2006; Rioux *et al.*, 2007; McCarroll *et al.*, 2008; Brooks *et al.*, 2011). We also observed reduced autophagy machinery activation following AIEC bacteria infection in *NOD2*^{-/-} macrophages, as evidenced by the lack of LC3-II conversion and the decrease in the number of AIEC bacteria located in LC3-positive vacuoles. These results are consistent with reduced localization of *Salmonella enterica* serovar Typhimurium and CD-associated adherent-invasive *E. coli* with autophagosomes reported in dendritic cells expressing CD-associated NOD2 variants (Cooney *et al.*, 2010; Travassos *et al.*, 2010). Thus, CD-associated common polymorphisms in autophagy-related *ATG16L1* or *IRGM* genes, which potentially alter directly the autophagy



machinery, or rare variants in *NOD2* gene, which impair the recruitment of the autophagy machinery at the bacteria entry site, may lead to a common defect, that of an uncontrolled replication of AIEC bacteria due to an inefficient autophagy degradative pathway within macrophages. Interestingly, we showed in the present study that forced induction of autophagy in a *NOD2*-deficient context allows to significantly decrease the number of

intracellular AIEC bacteria and the amount of TNF- α and IL-6.

Autophagy is involved in the regulation of inflammation, in particular by controlling inflammasome activation and thus limiting the production of inflammatory cytokines (Levine and Deretic, 2007; Saitoh *et al.*, 2008). Fetal liver-derived macrophages lacking ATG16L1 produce large amounts of IL-1 β and IL-18 in response to LPS, a ligand

Fig. 5. Nod2-dependent autophagy allows AIEC bacteria clearance in murine peritoneal macrophages.

- A. Uninfected or AIEC LF82-infected peritoneal macrophages isolated from wild-type or *NOD2*^{-/-} mice were processed for immunoblotting using anti-LC3 at 1 h or 2 h post infection.
- B. Quantification results of immunoblots are presented as LC3-II accumulation relative to actin for uninfected wild-type and *NOD2*^{-/-} peritoneal macrophages at 1 h post infection.
- C. Quantification results of immunoblots are presented as LC3-II accumulation relative to actin in AIEC LF82-infected wild-type versus *NOD2*^{-/-} peritoneal macrophages. The amount of LC3-II relative to actin quantified in uninfected cells was taken as 1. Data are means \pm SEM of at least four independent experiments.
- D. Confocal microscopy examinations of wild-type versus *NOD2*^{-/-} peritoneal macrophages infected with GFP-expressing AIEC LF82 bacteria, at 1 h post infection. Cells were labelled for actin cytoskeleton using TRITC-labelled phalloidin (red) and LC3 (purple).
- E. Confocal microscopy examinations of colocalization between LC3 (red) and GFP-expressing AIEC bacteria in wild-type and *NOD2*^{-/-} peritoneal macrophages at 1 h post infection. Data are means \pm SEM of three independent experiments. At least 100 cells were analysed for each experiment.
- F. Wild-type and *NOD2*^{-/-} peritoneal macrophages were infected with AIEC strain LF82 and the numbers of intracellular bacteria were determined by cfu quantification at 1 h, 4 h and 8 h post infection. Results are expressed as percentage \pm SEM of the number of intracellular bacteria at 4 h and 8 h post infection relative to the number of intracellular bacteria obtained at 1 h, taken as 100%. Data are mean of at least three independent experiments.

for TLR4. This phenotype was also observed in macrophages from *Atg7*-deficient mice, demonstrating the importance of the whole autophagy process in the regulation of inflammatory response. In addition, mice lacking ATG16L1 in haematopoietic cells are highly susceptible to DSS-induced acute colitis indicating the importance of autophagy to restrict intestinal inflammation (Saitoh *et al.*, 2008), and a recent study reported that CD-associated ATG16L1 polymorphism modulates pro-inflammatory cytokine response when cells are specifically triggered with a NOD2 ligand (Plantinga *et al.*, 2011). AIEC bacteria induced the release of large amounts of TNF- α by infected macrophages and strong gut inflammation in mouse models (Glasser *et al.*, 2001; Carvalho *et al.*, 2008; 2009). The present findings show that high autophagy activity correlates with significant decreases in TNF- α and IL-6 from human THP-1 macrophages in response to AIEC infection, whereas lack of autophagic response resulting from altered *ATG16L1*, *IRGM* or *NOD2* expression leads to amplified pro-inflammatory cytokine secretion. Thus, in CD patients, polymorphisms in *ATG16L1*, *IRGM* or *NOD2* leading to autophagy defects, could profoundly tip the balance towards a pro-inflammatory cytokine response state.

Our observations provide clues for a critical role of immediate xenophagy against CD-associated AIEC bacteria in macrophages. Due to the presence of risk alleles in autophagy related genes *ATG16L1*, *IRGM* and *NOD2* in CD-affected individuals, and considering the fact that autophagy is a vital process for cells, we could hypothesize that risk polymorphisms associate to CD have a minimal effect on the basal autophagic process, but in case of AIEC/bacteria infection, or environmental stress, it could lead to a less efficient autophagic response, allowing persistence of AIEC bacteria in replicative niche inside macrophages. Autophagy state can deeply impact on the outcome of the macrophage pro-inflammatory response to AIEC infection. Induction of autophagy, by the use of rapalogs for example, makes it possible to target AIEC

bacteria entering macrophages within autophagosomes (Dancey, 2010). As a consequence there is a significant decrease in the number of intramacrophagic AIEC bacteria and a drastic decrease in pro-inflammatory cytokine secretion. Restoring bacteria-induced autophagy in patients genotyped for risk alleles in *ATG16L1*, *IRGM* or *NOD2* (or composite) could be a powerful therapeutic strategy to concomitantly restrain the number of intracellular AIEC bacteria and to slow down inflammatory response.

Experimental procedures

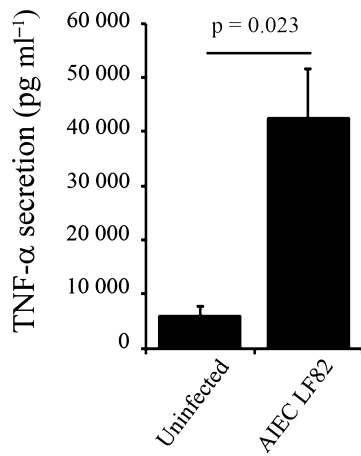
Bacterial strains

The six AIEC strains (AIEC reference strain LF82 and AIEC strains LF9, LF31, LF73, LF87 and LF138) were isolated from CD patients (Boudeau *et al.*, 1999; Darfeuille-Michaud *et al.*, 2004). The plasmid pFPV25.1, which harbours the green fluorescent protein (GFP), was used to visualize bacteria for confocal microscopy analysis (Valdivia and Falkow, 1997). Bacteria were grown routinely in Luria-Bertani (LB) broth or on LB agar plates overnight at 37°C.

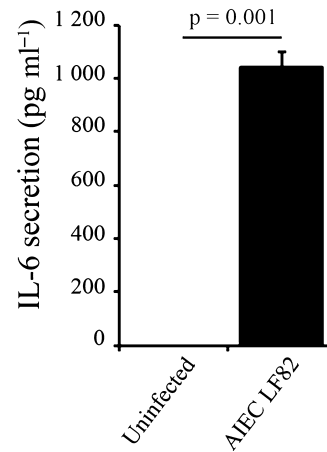
Cell culture

The human monocytic cell line THP-1 was maintained in an atmosphere containing 5% CO₂ at 37°C in the culture medium recommended by ATCC. THP-1 monocytes were differentiated in macrophages by an 18 h treatment with 20 ng ml⁻¹ phorbol myristate acetate (PMA). Primary cultures of peritoneal macrophages were established from resident cells of wild-type or *Nod2* knockout (KO) female FVB mice (Barreau *et al.*, 2007). Briefly, elicited macrophages were obtained by peritoneal lavage 4 days after injection of 1.5 ml of 4% thioglycollate broth in the peritoneal cavity. Mice were euthanized and 6 ml of cold HBSS medium (Lonza) was injected intraperitoneally. The peritoneal wash containing macrophages was collected and centrifuged. Cells were then washed in PBS and the cell pellet obtained after centrifugation was suspended in RPMI 1640 medium (Lonza) supplemented with L-glutamine (2 mM, Lonza), antibiotic (penicillin, 100 U ml⁻¹, Lonza) and 10% FCS (Lonza). Cells were seeded at

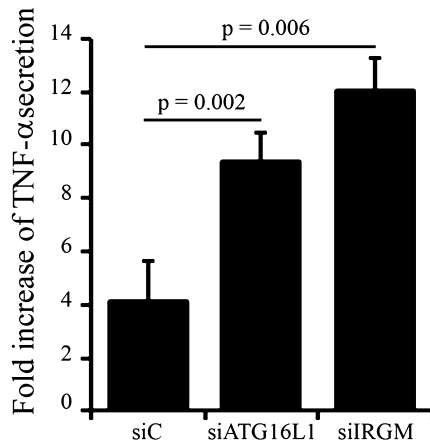
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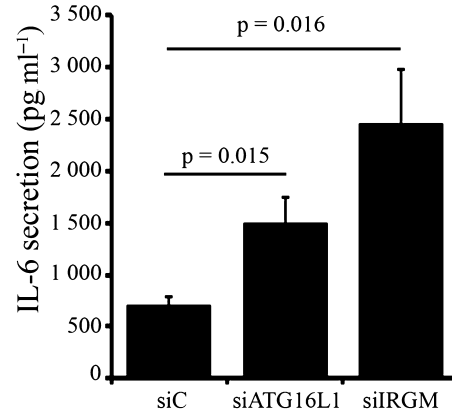
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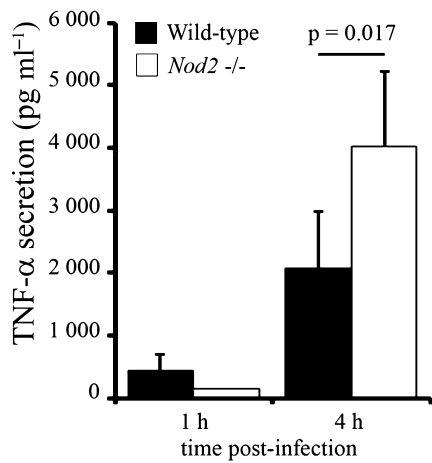
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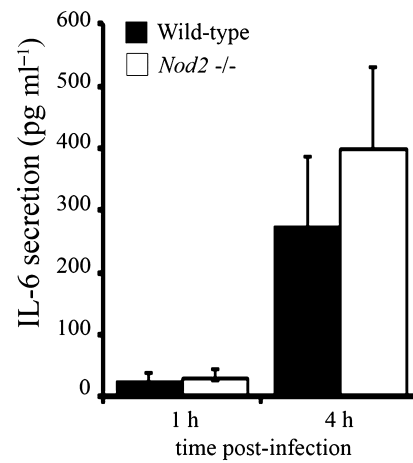


Fig. 6. Impaired autophagy results in an increased release of pro-inflammatory cytokines by AIEC-infected macrophages. Amounts of cytokines secreted by uninfected and AIEC-infected macrophages were quantified by ELISA.

A and B. Levels of TNF- α (A) and IL-6 (B) secreted by AIEC LF82-infected THP-1 macrophages compared with uninfected cells. Data are mean (pg ml⁻¹) \pm SEM of cytokine amounts released in the culture supernatants, at 8 h post infection.

C and D. Levels of cytokines secreted at 8 h post infection in response to AIEC infection were compared in untreated THP-1 macrophages and in macrophages for which autophagy was impaired by treating cells with siATG16L1 or siIRGM.

C. Results are expressed as fold increase in TNF- α level secreted in culture supernatants of AIEC-infected THP-1 cells treated with siRNA control (siC), siATG16L1 or siIRGM relative to corresponding uninfected cells.

D. Data are mean (pg ml⁻¹) \pm SEM of IL-6 released in culture supernatants of AIEC-infected cells treated with siC, siATG16L1 or siIRGM.

E and F. TNF- α (E) and IL-6 (F) levels secreted in response to AIEC infection by peritoneal macrophages isolated from wild-type mice or *NOD2*^{-/-} mice.

Data are means (pg ml⁻¹) \pm SEM of cytokine amounts released in the culture supernatants, at 4 h post infection. For all experiments, data are means of at least three independent experiments.

a concentration of 1×10^5 cells per cm². The non-adherent peritoneal cells were removed after 3 h of incubation at 37°C in a humidified 5% CO₂ atmosphere.

Antibodies and reagents

For Western blot analysis, rabbit anti-LC3 and anti-actin were purchased from Sigma, rabbit anti-Caspase-3 from Cell Signalling, rabbit anti-IRGM from ProSci and anti-p62 from BD Biosciences. For immunofluorescence analysis, rabbit anti-LC3 was purchased from MBL, rabbit anti-ATG16L1 from Abgent, mouse anti-LAMP-1 from DSBH Iowa, mouse anti-EEA1 from BD Biosciences, rabbit anti-Rab7 from Sigma and rabbit anti-IRGM from ProSci. Hoechst 33342 was purchased from Sigma. Rabbit anti-serum against *E. coli* LPS O83 was generously provided by Lothar Beutin (Department of Biological Safety, Robert Koch Institut, Berlin, Germany). Lysotracker probe DND-99 (100 nM, 1 h prior to fixation) was purchased from Invitrogen. Rapamycin (LC laboratories) and Hank's balanced salt solution (HBSS; Sigma) were used as autophagy inducers. Wortmannin was used to block autophagy (Sigma). Bafilomycin A1 (LC laboratories) was used to block the autophagy flux. Viability of intramacrophagic bacteria was assessed using the LIVE/DEAD^(R) *BadLight*TM viability kit from Molecular Probes.

Transfection of siRNA and plasmids

siRNA experiments directed against ATG16L1 or IRGM were performed using stealth RNAi (Invitrogen). The sequences used were as previously described (Rioux *et al.*, 2007; McCarroll *et al.*, 2008) and targeted all *ATG16L1* or *IRGM* mRNA variants. Transfections were performed using Lipofectamine RNAimax (Invitrogen). To evaluate knock-down efficiency, protein extracts from RNAi-treated THP-1 cells were analysed by immunoblot. The empty plasmid pCMV and plasmid pCMV-3xFlag-IRGM have been described previously (Brest *et al.*, 2011). THP-1 macrophages were transfected with plasmids and Lipofectamine LTX (Invitrogen) for 16 h according to the manufacturer's protocol for this cell line.

Macrophage survival assay

Internalization of bacteria within cells and the ability of bacteria to survive and replicate within macrophages were determined by the gentamicin protection assay (Bringer *et al.*, 2005). Briefly, THP-1 macrophages and peritoneal macrophages were infected

at a multiplicity of infection (moi) of 100 and 20 bacteria per macrophage respectively. After 10 min of centrifugation at 1000 *g* and a 10 min incubation period at 37°C with 5% CO₂, the infected macrophages were washed twice with PBS, and fresh cell culture medium containing 50 μ g of gentamicin ml⁻¹ was added for a 1 h, 4 h, 8 h or 24 h period. To determine the number of intracellular bacteria, the cell monolayers were washed once with PBS and lysed with 1% Triton X-100 (Sigma) in deionized water. This concentration of Triton X-100 had no effect on bacterial viability for at least 30 min. Samples were mixed, diluted and plated onto LB agar plates to determine the number of cfu recovered from the lysed monolayers.

Enzyme-linked immunosorbent assays for TNF- α and IL-6 quantifications

The amount of TNF- α and IL-6 released in the culture supernatant was determined by enzyme-linked immunosorbent assay (ELISA; R&D systems, Lille, France). Cytokine concentrations were assessed according to the manufacturer's instructions.

Autophagy modulation

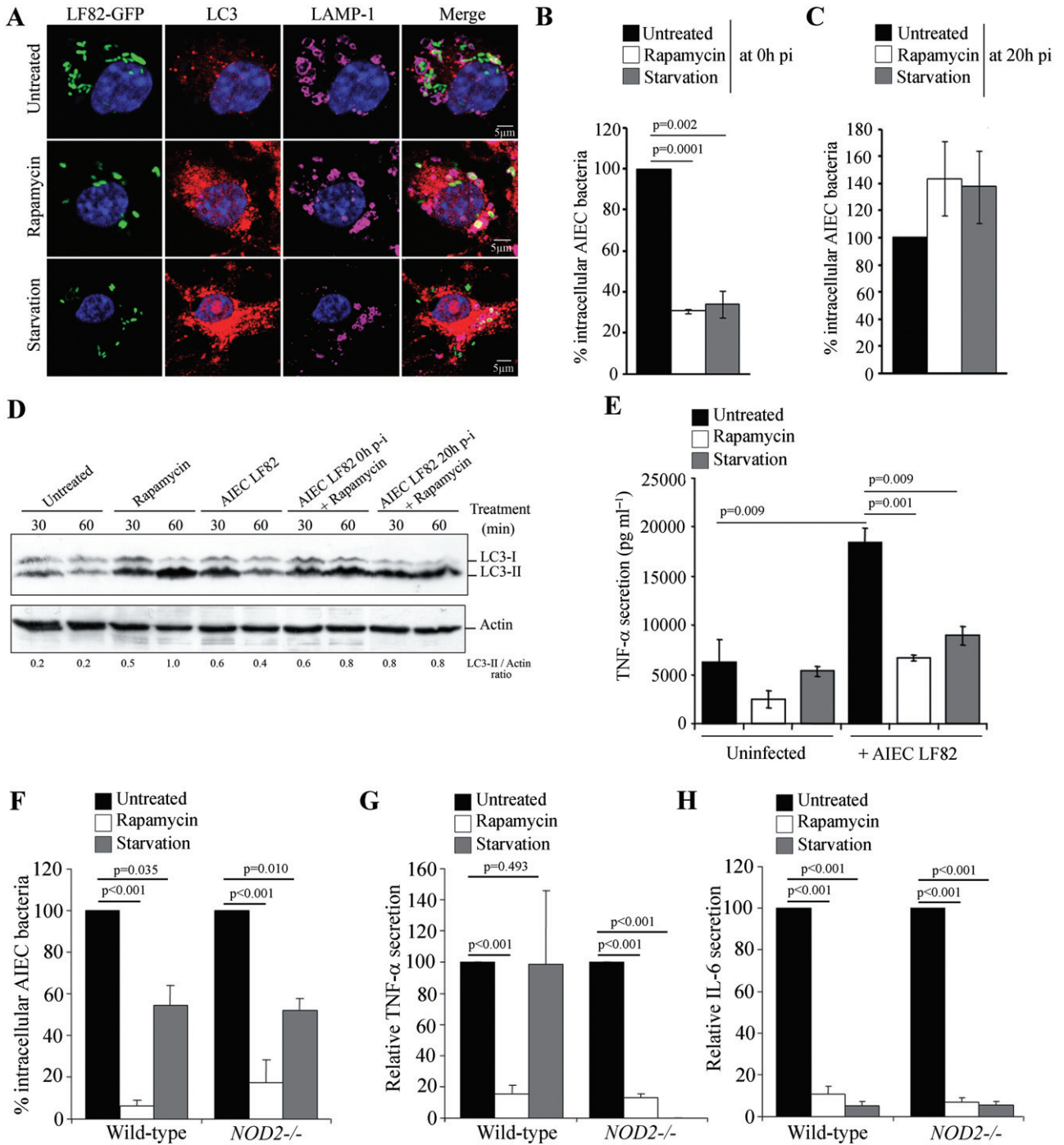
Autophagy was induced by treatment with 40 μ g ml⁻¹ rapamycin, immediately after infection (0 h post infection) or 20 h later (20 h post infection). Autophagy was also induced by the incubation of cells in HBSS minimum medium. Autophagy was blocked by wortmannin treatment, with doses of 50 and 100 nM immediately after infection.

Blockade of the autophagy flux

Macrophages were pre-treated with Bafilomycin A1 at 150 nM for 30 min prior to infection. Bafilomycin A1 at 150 nM was maintained in the cell culture medium during infection and gentamicin incubation period.

Immunoblot analysis

Whole-cell protein extracts were prepared by adding lysis buffer (2% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA). Proteins were separated on SDS/15% PAGE gels, transferred to nitrocellulose membrane, blocked 2 h in Tris-buffered saline (TBS) solution containing 2% BSA, probed overnight with primary antibodies and for 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 ImageJ software.

Fluorescence microscopy

Briefly, cells were fixed with 4% paraformaldehyde and immunostained overnight at 4°C with the indicated specific primary antibodies. They were then incubated for 1 h with secondary

antibodies. The slides were examined with a Zeiss LSM 510 Meta confocal microscope. To determine the percentage of positive AIEC LF82-containing phagosomes for a specific marker, at least 100 bacteria-containing phagosomes were counted and scored for the presence or absence of the marker protein. Each confocal microscopy image is representative of at least three independent experiments. To determine the percentage of apoptotic cells, at least 300 nuclei were counted and scored for morphological features of apoptosis (nucleus shrinkage, chromatin condensation and fragmentation).

Fig. 7. Pharmacological- and physiological-induced autophagy at early time post infection restrains the number of intramacrophagic AIEC LF82 bacteria and slows down pro-inflammatory response induced by bacteria.

A. Confocal microscopy examinations of colocalization between LC3 (red), LAMP-1 (purple) and GFP-expressing AIEC bacteria in infected-THP-1 macrophages at 2 h post infection. THP-1 macrophages were treated with rapamycin at $40 \mu\text{g ml}^{-1}$ or starved (HBSS medium) for a 2 h period in order to induce autophagy. Nuclei were stained with Hoescht.

B and C. After 20 min infection THP-1 macrophages were incubated with gentamicin-containing RPMI medium supplemented with rapamycin at $40 \mu\text{g ml}^{-1}$ (white bar) or gentamicin-containing HBSS medium (grey bar) for a 2 h period in order to induce autophagy, immediately after AIEC cell infection (B) or at 20 h post infection (C). The numbers of intracellular bacteria were determined by cfu quantification. Results are expressed as the number of intracellular bacteria after the 2 h period of autophagy induction in treated cells relative to that obtained in untreated cells, taken as 100%.

D. Protein extracts from uninfected and AIEC-infected cells that were treated or not with rapamycin at $40 \mu\text{g ml}^{-1}$ for 1 h were processed for immunoblotting with anti-LC3 and anti-actin. Quantification of LC3-II relative to actin is displayed below immunoblot.

E. The level of TNF- α secreted in response to AIEC infection was compared in untreated THP-1 macrophages and in macrophages for which autophagy was induced by treating cells with rapamycin at $40 \mu\text{g ml}^{-1}$ or by starving them (HBSS medium) for a 2 h period prior to infection. Data are means (pg ml^{-1}) \pm SEM of cytokine amounts released in cell culture supernatants at 24 h post infection.

F. Wild-type and *NOD2*^{-/-} peritoneal macrophages were treated with rapamycin at $40 \mu\text{g ml}^{-1}$ (white bar) or starved (HBSS medium, grey bar) during the gentamicin incubation period (1 h or 4 h post infection). The numbers of intracellular AIEC bacteria were determined by cfu quantification. Results are expressed as percentages of the number of intracellular AIEC bacteria at 4 h post infection relative to those obtained at 1 h post infection. Data are mean \pm SEM of five independent experiments.

G and H. The amounts of TNF- α (G) and IL-6 (H) secreted in response to AIEC infection were compared in untreated wild-type and *NOD2*^{-/-} peritoneal macrophages and in peritoneal macrophages that were treated with rapamycin at $40 \mu\text{g ml}^{-1}$ or starved (HBSS medium) during the gentamicin incubation period (4 h post infection) to induce autophagy. Results are expressed as cytokine amounts secreted by rapamycin or HBSS-treated macrophages relative to cytokine amounts secreted by untreated cells, taken as 100%.

Data are means \pm SEM of five independent experiments.

Statistical analysis

Student's *t*-test was used for comparison of the two groups of data. All experiments were performed at least three times. A *P*-value less than or equal to 0.05 was considered statistically significant.

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