Autophagy proteins suppress protective type I interferon signalling in response to the murine gut microbiota

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As a conserved pathway that lies at the intersection between host defence and cellular homeostasis, autophagy serves as a rheostat for immune reactions. In particular, autophagy suppresses excess type I interferon (IFN-I) production in response to viral nucleic acids. It is unknown how this function of autophagy relates to the intestinal barrier where host–microbe interactions are pervasive and perpetual. Here, we demonstrate that mice deficient in autophagy proteins are protected from the intestinal bacterial pathogen Citrobacter rodentium in a manner dependent on IFN-I signalling and nucleic acid sensing pathways. Enhanced IFN-stimulated gene expression in intestinal tissue of autophagy-deficient mice in the absence of infection was mediated by the gut microbiota. Additionally, monocytes infiltrating into the autophagy-deficient intestinal microenvironment displayed an enhanced inflammatory profile and were necessary for protection against C. rodentium. Finally, we demonstrate that the microbiota-dependent IFN-I production that occurs in the autophagy-deficient host also protects against chemical injury of the intestine. Thus, autophagy proteins prevent a spontaneous IFN-I response to microbiota that is beneficial in the presence of infectious and non-infectious intestinal hazards. These results identify a role for autophagy proteins in controlling the magnitude of IFN-I signalling at the intestinal barrier.

The cellular pathway of autophagy (macroautophagy) is mediated by the coordinated action of conserved autophagy proteins and involves the sequestration of cytosolic material within a double membrane-bound vesicle termed the autophagosome. The contents, which can include organelles and protein aggregates, are degraded and recycled following fusion of the autophagosome with the lysosome. Increasing evidence indicates that autophagy dampens inflammatory reactions by targeting immune activators and signalling molecules for degradation. For instance, autophagy inhibits type I interferon (IFN-I) production in response to sensing of viral RNA and DNA in the cytosol by RIG-I (retinoic-acid inducible gene I) and cGAS (cyclic GMP–AMP synthase), respectively1-4. It is unknown whether autophagy inhibits IFN-I signalling in response to the microbiota. However, an important role for autophagy in suppressing reactions towards the gut microbiota is suggested by the association between a gene variant of ATG16L1 and IBD susceptibility5.

ATG16L1 forms a complex that mediates the attachment of phosphatidylethanolamine (PE) to the ubiquitin-like molecule LC3, a step that is essential for the proper formation and function of the autophagosome5. We have previously generated mice with a germ-line gene-trap mutation that leads to decreased Atg16L1 expression and reduced autophagy6. These Atg16L1 hypomorph (Atg16L1HM) mice develop intestinal abnormalities related to IBD on infection with murine norovirus (MNV)7-9, an otherwise beneficial virus that persistently infects the intestine10. In contrast to this pathological response to a commensal-like virus, we found that Atg16L1HM mice are remarkably resistant to intestinal infection by the model Gram-negative bacterial pathogen Citrobacter rodentium11. This increased protection was dependent on macrophage cell types and not lymphocytes, suggesting that autophagy has a critical role in limiting innate immune responses to intestinal bacteria. Here, we show that autophagy inhibition in the intestinal epithelium confers resistance to C. rodentium through an enhanced IFN-I response to the microbiota.

Results
Resistance conferred by Atg16L1 mutation is dependent on IFN-I. Our previous RNA deep-sequencing (RNA–seq) experiment showed that transcripts associated with innate immunity were enriched in intestinal samples collected from Atg16L1HM mice compared with wild-type (WT) controls11. Among these transcripts were IFN-I stimulated genes (ISGs), reminiscent of observations made in autophagy-deficient tumour cells grown in culture12,13. To test whether this increase in IFN-I signalling mediates resistance to infection, we crossed Atg16L1HM mice with mice deficient in...
Colonic crypt hyperplasia is associated with successful infection (Fig. 1a,b), and protection from morbidity observed ifnar–/– mice was similar to WT and Atg16L1HMifnar–/– mice (Supplementary Fig. 1a-k). We also did not observe differences among genotypes in macrophages and dendritic cells, and neutrophils displayed a modest increase in the proportion of cytokine-producing lymphoid subsets when comparing infected WT, ifnar−/−, Atg16L1HM and Atg16L1HMifnar−/− mice (Supplementary Fig. 2a-g). Because these results did not reveal

the IFN-I receptor (Ifnar−/−). Atg16L1HM mice displayed >100-fold reductions in the number of C. rodentium recovered in stool following oral inoculation compared with WT controls starting around day 9 post infection, with the greatest difference occurring at day 15 (Fig. 1a,b). In contrast, bacterial burden in Atg16L1HMifnar−/− mice was similar to WT and Ifnar−/− mice throughout the course of infection (Fig. 1a,b), and protection from morbidity observed in Atg16L1HM mice was lost in Atg16L1HMifnar−/− mice (Fig. 1c). Colonic crypt hyperplasia is associated with successful C. rodentium colonization. Atg16L1HM mice showed reduced levels of crypt hyperplasia and overall a lower intestinal pathology score, whereas the colons of Atg16L1HMifnar−/− mice appeared similar to WT mice (Fig. 1d-f). Atg16L1HM, but not Atg16L1HMifnar−/−, mice also displayed decreased C. rodentium dissemination to the liver (Fig. 1g).

These results indicate that the benefit conferred by Atg16L1 mutation during C. rodentium infection is dependent on IFN-I signaling. Notably, ifnar−/− mice displayed similar bacterial burden and modestly reduced pathology compared to WT mice (Fig. 1f). This indicates that IFN-I is typically dispensable or deleterious in a WT setting, but is selectively important in Atg16L1HM mice.

Quantification of colonic lamina propria cells by flow cytometry at day 9 post infection did not reveal significant differences in the proportion of cytokine-producing lymphoid subsets when comparing infected WT, Ifnar−/−, Atg16L1HM and Atg16L1HMifnar−/− mice (Supplementary Fig. 1a-k). We also did not observe differences among genotypes in macrophages and dendritic cells, and neutrophils displayed a modest increase in Atg16L1HMifnar−/− mice (Supplementary Fig. 2a-g).
an obvious shift in leukocyte populations that explains the IFN-dependent protection observed in Atg16L1 HM mice, we focused on other aspects of immunity such as the microbiota.

We incorporated littermate controls in the above experiments, but this approach does not rule out the possibility that Ifnar deficiency ablates the enhanced resistance conferred by Atg16L1 mutation by reverting the microbiota of Atg16L1 HM mice to a WT-like state. We performed 16S rRNA sequencing of faecal microbiota isolated from mice representing the different genotypes used in this study, and included samples that were collected longitudinally from WT and Atg16L1 HM mice infected by C. rodentium to serve as a positive control for dysbiosis. Principal component analysis (PCA) and examination of the relative abundance of various taxa showed that samples from infected WT mice diverge from the other samples that cluster together (Supplementary Fig. 3a,c), similar to previous studies.14–16. The abundance of Enterobacteriaceae (a family that includes C. rodentium) increased in WT mice during infection, and as expected based on the lower C. rodentium burden and crypt hyperplasia,17 was less severe in Atg16L1 HM mice (Supplementary Fig. 3b). A comparison of uninfected Atg16L1 HM and Atg16L1 HM/Ifnar-/- mice indicated that Ifnar deletion does not significantly alter the microbiota of Atg16L1 HM mice. To examine how potential microbiota differences before C. rodentium infection impact the outcome of infection, germ-free (GF) mice were inoculated with stool isolated from uninfected WT, Atg16L1 HM, Ifnar-/- and Atg16L1 HM/Ifnar-/- mice, which were then infected with C. rodentium. We did not detect differences in bacterial burden or morbidity when comparing mice reconstituted with microbiota from different genotypes (Supplementary Fig. 3d-f). These results indicate that host genotype, and not initial differences in microbiota composition, is the dominant determinant of C. rodentium susceptibility.

Other models of autophagy deficiency reproduce resistance to C. rodentium infection. Next, we examined whether the effect of Atg16L1 mutation can be reproduced by deficiency in other autophagy genes. LC3b-/- mice that are deficient in one of several LC3 paralogues displayed reduced bacterial burden compared with WT controls (Fig. 2a,b). Recent studies have uncovered an innate immune function of ATG16L1 referred to as ‘targeting by autophagy’ proteins (TAG), which involves recruiting IFN-inducible GTPases to membranes associated with parasite replication and occurs independently of the autophagy protease ATG4B.22,23, Atg4b-/- mice displayed an immense reduction in bacterial burden and were resistant to weight loss and other signs of disease (Fig. 2c,d and Supplementary Fig. 4a,b). Therefore, susceptibility to C. rodentium is unlikely to be mediated by TAG. The accelerated reduction in bacteria burden may reflect the more severe reduction in autophagy displayed by Atg4b-/- mice than LC3b-/- mice.22–24.

Deletion of Atg7 or Atg16L1 in the intestinal epithelium has been reported to confer susceptibility to C. rodentium.25,26, which initially led us to investigate the role of ATG16L1 in the haematopoietic compartment. However, deletion of Atg16L1 in myeloid cells (Atg16L1 Ifnar-/-Lyz2Mcr) did not reproduce resistance to C. rodentium.24. Although we have shown that Atg16L1 deletion in dendritic cells increases T cell alloreactivity,24 the same Atg16L1 Ifnar-/-Cdl1Crl mice used in these studies displayed a similar response to C. rodentium infection as Atg16L1 Ifnar-/- control mice (Fig. 2c,f). In contrast, mice in which Atg16L1 is deleted in the intestinal epithelium (Atg16L1 Ifnar-/-VillinCre) showed a significant decrease in bacterial burden (Fig. 2g,h). It is possible that the discrepancy between our findings and the literature are due to differences in the health status of mice, particularly the presence of MNV, which we actively exclude from our mouse colonies and has been shown to alter immunity in Atg16L1 Ifnar-/-VillinCre mice.9,26.

The T300A variant of ATG16L1 associated with IBD introduces the equivalent variant (Atg16L1 T316A) a potential compensatory increase in ATG16L1 compared with WT. However, we observed that full-length ATG16L1 protein is decreased in Atg16L1 T316A knock-in mice orally gavaged with PAC-1, a chemical activator of caspase-3 (Supplementary Fig. 4c). Thus, this model represents an opportunity to compare the effect of chemically induced ATG16L1 inhibition on C. rodentium infection, which would avoid genotype-dependent effects on the microbiota that precede infection. We found that PAC-1-treated Atg16L1 T316A mice, but not untreated mice, phenocopied Atg16L1 HM mice by displaying decreased bacterial burden in the stool and disease compared to PAC-1 treated WT mice (Fig. 2i,j and Supplementary Fig. 4d). Thus, multiple animal models indicate that ATG16L1 and autophagy proteins mediate susceptibility to C. rodentium.

Resistance conferred by Atg16L1 mutation is mediated by MAVS accumulation. Autophagy suppresses IFN-I signalling in part through the degradation of mitochondrial antiviral signalling protein (MAVS), a signalling intermediate that aggregates at the mitochondria to activate TBK-1 (tank-binding kinase 1) and IRF3 (interferon regulatory factor 3) downstream of the cytosolic RNA sensors RIG-I and MDA5.6–8. The ATG16L1 complex is recruited to mitochondria by NLRX1 and TUFM or COX5B.27. We found an increase in MAVS protein in whole colonic tissue collected from uninfected Atg16L1 HM mice compared with WT, and an increase in MAVS in whole colonic tissue from PAC-1-treated Atg16L1 T316A mice compared to PAC-1-treated WT, untreated Atg16L1 HM and untreated WT colonic tissues (Fig. 3a,b,d). In the presence of cytoplasmic DNA, cGAS activates stimulator of interferon genes (STING) to signal through TBK-1 and IRF3; autophagy proteins regulate STING trafficking and the availability of DNA in the cytolsol.28,29. We found that STING protein levels were similar in WT and Atg16L1 HM colonic tissues (Fig. 3a,c). These findings are consistent with a proteomics analysis showing that MAVS, MDA5 (melanoma differentiation associated protein 5), RIG-I, IRF3 and phospho-TBK-1 accumulate in Atg5-/- and Atg7-/- RAS-transformed cells, but STING does not.30,31. Also, we found that Atg16L1 HM and Atg16L1 T316A mice lose the superior protection observed in Atg16L1 HM mice (Fig. 3e-g). Although STING was not increased in colonic tissue from Atg16L1 HM mice, we hypothesized that cytosolic DNA sensing could contribute to enhanced resistance because MAVS and STING share downstream signalling factors (IRF3 and TBK-1) that are activated in autophagy-deficient cells.27. Indeed, Atg16L1 HM/STING-/- mice also lose the protection conferred by Atg16L1 mutation (Fig. 3h-j). These results indicate that sensing of cytosolic nucleic acids is necessary for the increased resistance to C. rodentium conferred by Atg16L1 mutation.

Increased IFN-I signalling in Atg16L1 HM mice is dependent on the microbiota. Increased ISG expression in Atg16L1 HM mice is observed both before and during C. rodentium infection.27 Such spontaneous activation could be explained by the microbiota. Although colons taken from conventional Atg16L1 HM and Atg4b-/- mice displayed increased expression of Mx2 and Oasl2 in a manner dependent on IFNAR, the expression of these representative ISGs was reduced to WT levels in GF Atg16L1 HM mice (Fig. 4a,b and Supplementary Fig. 4f). Mx2 expression was not increased in peripheral organs from Atg16L1 HM mice (Supplementary Fig. 4e), suggesting a local response. Immunohistochemistry (IHC) analyses confirmed that phospho-STAT1 (pSTAT1), another marker of IFN-I, is detected throughout the colonic epithelium and lamina propria dependent on IFNAR, the expression of these representative ISGs can also induce epithelial cell proliferation.36. These results indicate that sensing of cytosolic nuclear acids is necessary for the increased resistance to C. rodentium conferred by Atg16L1 mutation.

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and GF Atg16L1HM mice (Fig. 4e,f). These findings indicate that ATG16L1 suppresses IFN-I signalling downstream of local sensing of the microbiota.

Enhanced IFN-I response in Atg16L1 mutant mice protects against tissue injury. *C. rodentium* is avirulent in GF mice. Thus, we wished to use a non-infectious model of intestinal injury to investigate the functional consequence of microbiota-induced IFN-I signalling in the gut. We found that Atg16L1HM mice receiving the intestinal damaging agent dextran sodium sulfate (DSS) in drinking water displayed a striking reduction in lethality and weight loss compared with WT mice (Fig. 5a,b). In contrast, GF Atg16L1HM and GF WT mice were equally susceptible to DSS, even at a lower dose where survival is prolonged (Fig. 5a and Supplementary Fig. 4g). Atg16L1HM Mavs−/− mice displayed increased lethality and similar weight loss compared with Mavs−/− controls (Fig. 5c,d). Also, Atg16L1HMSting−/− mice displayed similar lethality compared with Sting−/− mice (Fig. 5e,f). Thus, Atg16L1 mutation protects from DSS-induced injury in a MAVS, STING and microbiota-dependent manner.

Resistance conferred by Atg16L1 mutation is dependent on CCR2. We previously demonstrated that depletion of myeloid cells with clodronate-loaded liposomes abrogates the enhanced resistance to *C. rodentium* displayed by Atg16L1HM mice11. This observation may reflect the essential role of CD11b+Ly6G+ monocytes recruited to the intestine through the chemokine receptor CCR238. However, Atg16L1HMflox/floxLyzMcre mice that are autophagy-deficient

![Fig. 2](image-url)
in myeloid cells are not resistant to *C. rodentium*[^3], and we did not detect a difference in the number of CD11b^+^Ly6C^hi^ monocytes in the colons of *Atg16L1^HM^* and WT mice (Supplementary Fig. 5a,b). Additionally, the IFN signature precedes monocyte recruitment because it is detectable in the absence of *C. rodentium* infection (Fig. 4a,b). Therefore, the key role of monocytes identified in our previous study appears to be at odds with the findings in this manuscript implicating ATG16L1 function in the intestinal epithelium.

One possible explanation is that properties of infiltrating monocytes, rather than their quantity, are altered on recruitment into the *Atg16L1* mutant colon. RNA-seq analysis of fluorescence-activated cell sorting (FACS)-purified CD11b^+^Ly6C^hi^ monocytes from the colon on day 9 post infection identified 812 genes with altered expression between *Atg16L1^HM^* and WT mice, many of which have known functions in myeloid cell differentiation and function (Fig. 6a, Supplementary Fig. 6 and Supplementary Table 1). Pathway analyses highlighted an enrichment in genes associated with metabolism, immune signalling and cell cycle regulation (Fig. 6b). The metabolic signatures and cell cycle regulation may reflect differences in macrophages involved in resolution of infection and tissue repair[^4]. Among the genes that were upregulated in monocytes collected from *Atg16L1^HM^* mice were several associated with antimicrobial responses (Supplementary Fig. 6). These results indicate that monocytes that migrate to the colon during *C. rodentium* infection acquire an altered gene expression profile in an *Atg16L1* mutant setting.

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Fig. 3 Protection conferred by ATG16L1 inhibition is dependent on MAVS and STING. a-c, Representative western blot (a) and quantification of MAVS (b) and STING (c) from colonic tissue of uninfected *Atg16L1^HM^* (n = 18) and WT (n = 16) mice normalized to β-actin from three independent experiments. Relative intensity of MAVS protein was determined by complete lane analysis. Full-length original blots are provided in Supplementary Fig. 5a,b. d, Quantification of MAVS from colonic tissue of uninfected WT (n = 5), *Atg16L1^HM^* (n = 7), WT-Pac1 treated (n = 8) and *Atg16L1^HM^*-Pac1 treated (n = 9) mice. Protein is normalized to β-actin from two independent experiments. e, Bacteria in stool over time (e) and on day 15 (f) from WT (n = 13), *Atg16L1^HM^* (n = 19), Mavs−/− (n = 20) and *Atg16L1^HM^*/Mavs−/− (n = 26) mice inoculated with *C. rodentium*. Four independent experiments were performed. f, Quantification of disease over time for mice in e. h, Bacteria in stool over time (h) and on day 15 (i) from WT (n = 20), *Atg16L1^HM^* (n = 16), Sting−/− (n = 24) and *Atg16L1^HM^*/Sting−/− (n = 24) mice inoculated with *C. rodentium*. j, Quantification of disease over time for mice in h. Three independent experiments were performed. Data points in e, g, h and i and bars in b-d, f and i represent mean. Error bars represent s.e.m. Dots in b-d, f and i represent individual mice. An unpaired two-tailed t-test was used to evaluate differences between two groups (b,c). ANOVA with Holm–Sidak multiple comparisons test was used to evaluate significance for experiments involving multiple groups (d-j). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; NS, not significant (see Supplementary Table 2 for exact P values).
To validate gene expression data, we identified three surface receptors that were increased at the RNA level in monocytes derived from Atg16L1HM mice, and which were assigned to the pathway ‘inflammation and monocyte function’ (Supplementary Fig. 6): XCR1, Galectin-9 and major histocompatibility complex-class II (MHC-II). All three receptors were increased on the cell surface of CD11b+Ly6Chi monocytes from day 9 infected Atg16L1HM mice (Supplementary Fig. 5c). We next tested whether enhanced inflammasome activation leads to increased C. rodentium. ATG16L1 is a potent inhibitor of NLRP3 (NLR family pyrin-domain-containing 3) inflammasome activity in monocytic cells\(^4\), but we found NLRP3 to be dispensable for the protective effect of Atg16L1 mutation (Supplementary Fig. 5d,e). However, inflammasome complexes may function in a redundant manner during C. rodentium infection\(^4\), and the inflammasome gene Nlrc4 showed increased expression in infected Atg16L1HM mice (Supplementary Fig. 6 and Supplementary Table 1). Therefore, we crossed Atg16L1HM mice with Caspase1.11–/– double knockout mice to generally inhibit inflammasomes.

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**Fig. 4 | Type I IFN signature in colonic tissue of autophagy-deficient mice is dependent on the microbiota.**

**a, b.** Quantitative reverse transcription-polymerase chain reaction (qRT–PCR) analyses of Mx2 (a) and Oasl2 (b) expression relative to Gapdh in colonic tissue from uninfected Atg4b−/− (n = 10), Atg16L1HM (n = 12), WT (n = 15), GF WT (n = 15) and GF Atg16L1HM (n = 15) mice from four independent experiments. **c–f.** Representative IHC images and quantification of pStat1 (c,d) and Ki67 (e,f) staining in colonic tissue from Atg4b−/− (n = 7), Atg16L1HM (n = 7), WT (n = 6), GF WT (n = 7) and GF Atg16L1HM (n = 10) mice. Two independent experiments were performed. Bars in a, b, d and f represent mean. Error bars represent s.e.m. Scale bar, 100 μm. ANOVA with Holm–Sidak multiple comparisons test was used to evaluate significance in all graphs. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (see Supplementary Table 2 for exact P values).
We found that $\text{Atg16L1}^{\text{Hm}}$ $\text{Caspase1.11}^{--}$ mice have similar bacterial burdens and disease scores as WT and $\text{Caspase1.11}^{--}$ controls, and do not display the superior protection observed in $\text{Atg16L1}^{\text{Hm}}$ mice (Supplementary Fig. 5f–h). Thus, although we identified an epithelial-intrinsic function of ATG16L1 linked to IFN-I signalling, other cell types and immune processes also acquire a heightened state of activity during $\text{C. rodentium}$ infection.

To demonstrate that CD11b$^+$Ly6Chi monocytes contribute to the enhanced immunity displayed by $\text{Atg16L1}^{\text{Hm}}$ mice, we generated $\text{Atg16L1}^{\text{Hm}}\text{Ccr2}^{--}$ mice in which the chemokine receptor necessary for their recruitment during $\text{C. rodentium}$ infection is absent. WT, $\text{Ccr2}^{--}$ and $\text{Atg16L1}^{\text{Hm}}\text{Ccr2}^{--}$ mice all showed decreased colon length (Fig. 6f) and increased dissemination to the liver (Fig. 6g) compared to $\text{Atg16L1}^{\text{Hm}}$ mice at day 9 post infection. The intestinal inflammation at day 15 post infection in $\text{Ccr2}^{--}$ and $\text{Atg16L1}^{\text{Hm}}\text{Ccr2}^{--}$ mice was comparable to WT mice, with $\text{Atg16L1}^{\text{Hm}}$ mice showing both reduced levels of crypt hyperplasia and intestinal pathology score (Fig. 6h–j). These results support a model in which monocyte recruitment contributes to an antimicrobial and tissue regenerative response downstream of $\text{Atg16L1}$ mutation in the colon.

**Fig. 5** | Autophagy deficiency protects against chemical injury of the intestine. a, b, Survival (a) and change in body weight (b) of WT ($n=38$), $\text{Atg16L1}^{\text{Hm}}$ ($n=35$), GF WT ($n=17$) and GF $\text{Atg16L1}^{\text{Hm}}$ ($n=16$) mice receiving 5% DSS in drinking water for 7 days. c, d, Survival (c) and change in body weight (d) of $\text{Mavs}^{--}$ ($n=15$) and $\text{Atg16L1}^{\text{Hm}}\text{Mavs}^{--}$ ($n=25$) mice receiving 5% DSS. e, f, Survival (e) and change in body weight (f) of $\text{Sting}^{--}$ ($n=12$) and $\text{Atg16L1}^{\text{Hm}}\text{Sting}^{--}$ ($n=10$) mice receiving 5% DSS. At least two independent experiments were performed. Data points represent mean $\pm$ s.e.m. in b, d and f. An unpaired two-tailed $t$-test was used to evaluate differences between two groups where data were distributed normally with equal variance between conditions in b, d and f. The log-rank Mantel–Cox test was used for comparison of mortality curves in a, c and e. **$P<0.01$, ***$P<0.001$, ****$P<0.0001$; NS, not significant (see Supplementary Table 2 for exact $P$ values).
Fig. 6 | Protection conferred by Atg16L1 mutation is associated with enhanced monocyte function. a, RNA-seq analysis was performed on monocytes taken from WT and Atg16L1HMc mice on day 9 post C. rodentium infection. Volcano plot of all transcripts that mapped to the murine transcriptome show 404 differentially upregulated genes in monocytes from Atg16L1HMc mice (labelled in red) and 402 downregulated genes (labelled in blue). n = 4 mice per group. b, Functional classification of differentially regulated genes from a by Ingenuity pathway analysis. The arrowed line marks where the P value becomes less than 0.05 and highlights the pathways that are significantly different between WT and Atg16L1HMc transcriptional profiles. Inositol BMD, inositol biosynthesis, metabolism and degradation; CGMS, cholecystokinin and gastrin-mediated signalling. c, d, Bacteria recovered from stools over time (c) and on day 15 (d) from WT (n = 19), Atg16L1HMc (n = 18), Ccr2−/− (n = 29) and Atg16L1HMc/Ccr2−/− (n = 22) mice inoculated with C. rodentium. Six independent experiments were performed. e, Quantification of disease over time for mice in c, f. Colon length of WT (n = 5), Atg16L1HMc (n = 4), Ccr2−/− (n = 5) and Atg16L1HMc/Ccr2−/− (n = 5) mice infected at day 9 with C. rodentium infection from one experiment. g, Bacterial burden in the liver measured by c.f.u. per gram tissue in WT (n = 7), Atg16L1HMc (n = 10), Ccr2−/− (n = 9) and Atg16L1HMc/Ccr2−/− (n = 9) mice at day 9 post C. rodentium infection from two experiments. h, j, Representative H&E-stained colonic sections (h), quantification of crypt length (i), and cumulative pathology score (j) on day 15 post infection from WT (n = 8), Atg16L1HMc (n = 9), Ccr2−/− (n = 8) and Atg16L1HMc/Ccr2−/− (n = 9) mice. Three independent experiments were performed. Scale bar = 100 μm. Data points in c and e and bars in dfgij represent mean. Data points in dfgi represent individual mice. Error bars represent s.e.m. An unpaired two-tailed t-test was used to evaluate differences between two groups (b). ANOVA with Holm–Sidak multiple comparisons test was used to evaluate significance for experiments involving multiple groups (c–j). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (see Supplementary Table 2 for exact P values).
NATURE MICROBIOLOGY

Discussion

Aberrant reactions to the microbiota are implicated in a range of disorders including IBD, yet a certain degree of responsiveness is necessary for host defence towards bona fide pathogens that access the gastrointestinal tract. We found that autophagy proteins in the colon suppress spontaneous activation of IFN-I signalling by the microbiota. Although our studies reveal a beneficial consequence of this enhanced activity, an IFN-I signature is a hallmark of several autoimmune diseases. Our findings indicate that mechanisms are in place to avoid spontaneous harmful IFN-I responses to the microbiota, which comes at the cost of antimicrobial immunity and tissue repair. Indeed, autophagy deficiency has been shown to cause inappropriate IFN-I signalling in the absence of DNAseII, a gene mutated in systemic lupus erythematosus. Given this relationship between IFN-I and autoimmune antibody responses, it will be interesting to examine how autophagy deficiency impacts the generation of anti-bacterial IgG, previously shown to promote killing by phagocytes.

We cannot rule out non-autophagy functions of ATG16L1, ATG4B and LC3B. However, our findings are consistent with the role of autophagy in mediating the degradation of MAVS. Previous studies examining autophagy-deficient cells showed accumulation of MAVS and downstream signalling molecules shared with STING. The observation that deletion of either MAVS or STING compromises the benefit of Atg16L1 mutation suggests that both DNA and RNA moieties from the microbiota function as triggers. Our observations are further supported by the finding that RNA derived from the microbiota is recognised by RIG-1. Recent studies indicate that metabolites from the microbiota potentiate IFN-I responses, and that autophagy inhibits TLR3 and TLR4 signalling by degrading the adaptor TRIF (TRIM-domain-containing adapter-inducing interferon-β), which can converge on a similar signalling pathway as cytosolic sensors. A future direction would be to examine how specific ligands gain access to the epithelium to trigger immune responses and identify the relevant members of the microbiota. Our conventional mice are free of known viruses, including MNV, but it will be important to determine whether viral members of the microbiota (including phages and endogenous retroviruses) contribute to the spontaneous IFN-I signalling we observe in autophagy-deficient mice.

The enhanced antimicrobial activity displayed by Atg16L1tm1 mice is dependent on infiltrating monocytes, yet we show that ATG16L1 in the intestinal epithelium mediates this increased resistance. Our results are most consistent with a model in which a heightened state of immunity is established by microbiota-triggered IFN-I signalling due to the absence of autophagy in the intestinal epithelium, and then autophagy-sufficient monocytes that are recruited to this microenvironment in response to C. rodentium acquire enhanced activity that accelerates the resolution of infection. This model highlights how autophagy proteins in the parenchyma can affect the local environment in a way that alters activation of infiltrating immune cells.

The mechanism revealed by this current study supports the emerging theme that autophagy proteins have a central role in balancing host defence and inflammation. In the presence of a short-term threat to the gut, such as through C. rodentium infection or acute chemical injury, autophagy proteins have an adverse function. However, the benefit of autophagy inhibition is pathogen-specific, as demonstrated by the critical role of ATG5 and ATG16L1 in the epithelium during Salmonella enterica Typhimurium infection. Also, our previous study showing that a persistent strain of MNV, but not a strain that causes an acute infection, triggers IBD pathologies in Atg16L1tm1 mice suggests that there is a long-term cost to autophagy deficiency, and Atg16L1tm1 mice are not universally protected from acute bacterial infection, as evidenced by their susceptibility to Staphylococcal infection. In this context, it is notable that Atg16L1tm1 mice become resistant to C. rodentium upon caspase-3 activation, because we showed that the same Atg16L1tm1

Methods

Mice. Age- and gender-matched 8- to 12-week-old mice on the C57BL/6J background were used as WT controls unless stated otherwise and bred on site. Atg16L1tm1, Atg16L1tm1Mavs−/−, Atg16L1tm1Villimek−/−, Atg16L1tm1Casp3−/− and LC3B−/− mice have been described previously. Atg16L1tm1 mice were generously provided by Skip Virgin (Washington University School of Medicine). For single allele autophagy mutants, we used Atg16L1tm1Mavs−/− and Atg16L1tm1Villimek−/− and Atg16L1tm1Casp3−/− mice that with their respective homzygous WT littermate controls (Atg16L1tm1−/− and Atg16L1tm1−/−). Atg16L1tm1Villimek−/− and Atg16L1tm1Casp3−/− mice were generated along with Atg16L1tm1 littermates by breeding Cre-positive and negative Atg16L1tm1 mice, Ifnar−/−, Cer2−/−, Mavs−/−, Sting−/− and Caspase11−/− mice were purchased from Jackson Laboratory and crossed to Atg16L1tm1 mice to generate double mutants. For these compound mutants, we fixed the Atg16L1tm1 allelic state, then generated littermates that were homzygous knockout and wild type for the other allele. Using the Ifnar-deficient mice as an example, Atg16L1tm1Ifnar−/− mice were bred to each other through a trio mating scheme to generate Atg16L1tm1Ifnar−/− and Atg16L1tm1Ifnar−/− mice for experiments. To increase the number of mice analysed, experiments were supplemented with progeny generated from Atg16L1tm1Ifnar−/− and Atg16L1tm1Ifnar−/− breeder pairs. Mavs−/− mice were on a C57BL/6J background and were bred to generate Mavs−/− mice to generate Mavs−/− mice, which were crossed to each other to generate controls for Atg16L1tm1 and Mavs double mutants that were generated by breeding Atg16L1tm1Mavs−/− mice, similar to other compound mutants. Experimental groups were established based on genotypes and infection status. All other aspects were randomized. Blinding was used during data collection by assigning numbers in place of genotype and infection status to mice in the studies. The number of animals used in the experiments in this study was estimated based on a power analysis with the following assumptions: standard deviation will be ~20% of the mean, P value will be under 0.05 when the null hypothesis is false, the effect size (Cohen’s d) is between 1.0 and 2.0. The minimal number of mice required under these conditions ranges between 5 and 38 for in vivo experiments depending on the assay. Additionally, we have carefully chosen the sample size based on empirical evidence of what is necessary for interpretation of the data and statistical significance. All animal studies were performed according to approved protocols from the NYU School of Medicine Institutional Animal Care and Use Committee (IACUC).

Bacterial infection. C. rodentium strain DBS100 was grown overnight in Luria–Bertani broth with shaking at 37 °C and diluted 1:100 followed by an additional 3% of growth until bacteria were at an optical density of 2. Bacterial density was confirmed by dilution plating. Mice were inoculated by oral gavage with 2 × 10^7 c.f.u. resuspended in 100 μl PBS. Severity of disease was quantified as an scoring system in which a given individual mouse received a score of 0–4 for each of the following: hunched posture, inactivity, ruffled fur and diarrhea. Mice received an additional score between 0 and 2 for weight loss calculated as percent of initial body weight, with scores of 0–0.5, 0.5–6–10, 1–11–15, 1.5–16–20% and 2= greater than 20% loss. For quantification of bacterial shedding and dissemination, stool pellets or livers from individual mice were weighed, homogenized in PBS, and plated on serial dilutions on MacConkey agar. For caspase-3 activation, 50 mg kg−1 PAC-1 (APEXBio) was administered by oral gavage once a day for 3–5 days before C. rodentium infection.

Gnotobiotics. Previously described GF WT and Atg16L1tm1 mice were maintained in flexible film isolators, and absence of faecal bacteria and fungi was assessed by plating aerobic culture in brain heart infusion, sabaraud and intestinal broth (Sigma), and qPCR for bacterial 16S and eukaryotic 18S ribosomal RNA genes through sampling of stool from individual cages in each isolator on a monthly basis. Mice were transferred into individually ventilated Tecniplast ISOcages for DSS treatment to maintain sterility under positive air pressure. For microbiota recovery experiments, stool slurries were prepared by suspending 2–3 faecal pellets into 2 ml PBS and passing through a 40μm filter. GF mice were gavaged with 200 μl of stool slurries before transfer into ISOcages. Intestinal flora was allowed to stabilize for three weeks before mice were infected with C. rodentium.

Tissue collection and microscopy. Haematoxylin and eosin (H&E)-stained colon sections were prepared as previously described from mice that were euthanized by cervical dislocation to preserve the integrity of intestinal tissue. Severity of tissue pathology was blindly quantified by a pathologist (R.X.). Mice received a pathology score between 0 and 3 for crypt abscess, hyperplasia, mucin...
depletion and for crypt loss and damage. A score of 0 = negative pathology, 1 = mild, 2 = moderate and 3 = severe pathology for the indicated fields. A length of at least 30 crypts per mouse was measured for crypt hyperplasia. Mean values were calculated for each mouse and used as individual data points. For IHC, formalin-fixed paraffin-embedded sections were deparaffinized online and antigen retrieved in Ventana Cell Conditioner 2 (Citrate). Endogenous peroxidase activity was blocked with hydrogen peroxide. Unconjugated polyclonal rabbit anti-mouse phospho-STAT1 (Cell Signaling Technology) and unconjugated rabbit anti-β-actin (abcam, ab19785) were used and detected with anti-rabbit, horseradish peroxidase (HRP) conjugated multimer and visualized with 3,3-diaminobenzidine and enhanced with copper sulfate. For Ki67 quantification, 10 images were collected per mouse, and from each image three crypts were quantified. Mean values were calculated from 30 images for each mouse and used as individual data points. For p-STAT1 quantification, the number of positive cells was quantified using ImageJ software. The whole colon was sectioned per mouse with mean values calculated for each mouse and used as individual data points. Imaging was performed on the Evos FL Color imaging microscope. All analyses of slides were performed blind and quantified using ImageJ software.

RNA isolation and qPCR. Colonic tissue (2 mm) and 25 mg heart, liver, lung and mesenteric lymph node (MLN) were washed and cut open (when appropriate), then suspended in 700 μl of RLT buffer (Mini RNeasy Kit) and homogenized using TissueRuptor (Qiagen). RNA was then isolated using the RNeasy Mini Kit (Qiagen). As per the manufacturer’s instructions, DNase treatment was performed using an Rnasy DNase kit (Qiagen) and protocol. cDNA synthesis was performed using a ProtoScript M-MuLV First Strand cDNA synthesis kit (New England Biolabs) and Random Primers. qPCR was performed on a Roche480 LightCycler using the following primers: Gapdh forward 5′tcgctccgggtctactac3′, Gapdh reverse 5′gaggctgctgtgaagcag3′, Ms2 forward 5′cagctgctctcaggaagt3′ and Ms2 reverse 5′tactggatgatcaagggaacgtgg3′, Osxl forward 5′ggatcctgggaaggaacctg3′ and Osxl reverse 5′tgctcgcctcttcgaaactg3′. Method: and values were expressed as fold change normalized to uninfected WT mice.

Flow cytometry and RNA purification. To isolate immune cells from the lamina propria of the large intestine, small pieces of colon were washed with Hank’s balanced salt solution (HBSS) and incubated at 37 °C with shaking in HBSS containing 1 mM dithiothreitol (DTT) and 1 μM EDTA for 15 min at 37 °C. Tissues were then suspended in 700 μl of HBSS, and then incubated in HBSS containing 0.1 U ml−1 dispase (Sigma), metronidazole (1 g l−1), vancomycin (0.5 g l−1), streptomycin (100 mg l−1), and gentamicin (100 mg l−1). Tissues were then pelleted twice at 10,000 × g for 5 min. Due to the similar molecular weights of MAVS, STING, ATG16L1 and β-actin, parallel gels were equally loaded, and equivalently processed. For gel electrophoresis, 20–30 μg of protein was run at 120V for 1 h using a 4–12% gradient protein gel (BioRad), electrophoresis chamber and running buffer. Protein was then detected using Thermo Super Signal West Pico Chemiluminescent Substrate and imaged using a BioRad ChemiDoc XRS imaging system. β-Actin, ATG16L1 and STING band intensity were quantified using single band analysis while MAVS protein intensity was quantified using complete lane analysis.

DSS treatment. Conventional mice were given 5% DSS (IBB Consultancy) for 7 days and both survival and weight loss was measured over time. GF mice were given 3% or 5% DSS and delivered in filter-sterilized water containing ampicillin (1 g l−1); American Bioanalytical), vancomycin (0.5 g l−1; MP biomedicals), neomycin (1 g l−1; Sigma), metronidazole (1 g l−1; Sigma) and 1% sucrose (Fisher). Antibiotic-containing water was replaced at least once a week. After DSS treatment, mice received regular or antibiotic-containing drinking water for the remainder of the experiment.

Statistical analysis. All analyses except for RNA-seq data used Graphpad Prism v7. An unpaired two-tailed t-test was used to evaluate differences between two groups where data was distributed normally with equal variance between conditions. An ANOVA with Holm–Sidak multiple comparisons test was used to evaluate experiments involving multiple groups. The log-rank Mantel–Cox test was used for comparison of mortality curves.

Data availability. The data that support the findings of this study are available from the corresponding author upon request. FASTQ files corresponding to the RNA-seq data have been deposited in a public database (RNA-seq GEO accession no. GSE115025, 16S GEO accession no. GSE116491).
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Author contributions

P.K.M. and K.C. formulated the original hypothesis, designed the study and analysed the results. E.R. assisted with transcriptomics analyses. R.X. performed histopathology analyses. E.K.M. assisted with experiments involving GF mice. S.L.S. and F.Y. assisted with analyses of microbial communities. P.K.M. and K.C. wrote the manuscript, and all authors commented on the manuscript, data and conclusions.

Competing interests

The authors declare no competing interests.

Additional information

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Experimental design

1. Sample size
   Describe how sample size was determined.
   The number of animals used in the experiments in this study is estimated based on a power analysis with the following assumptions: standard deviation will be ~20% of the mean, p-value will be under 0.05 when the null hypothesis is false, the effect size (Cohen’s d) is between 1.0-2.0. The minimal number of mice required under these conditions ranges between 5-38 for in vivo experiments. Additionally, we have carefully chosen the sample size listed below based on empirical evidence of what is necessary for interpretation of the data and statistical significance.

2. Data exclusions
   Describe any data exclusions.
   No data was excluded from the analysis.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   In all experiments, no attempts at replication failed.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Groups were established based off of genotype and infection status. All other aspects were randomized.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   Blinding was used during data collection by assigning numbers in place of genotype and infection status to mice in the studies. Samples were also blindly assessed by a trained pathologist (R.X).

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters
For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| Item                                                                 | Confirmed |
|---------------------------------------------------------------------|-----------|
| The exact sample size \( (n) \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |           |
| A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |           |
| A statement indicating how many times each experiment was replicated |           |
| The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |           |
| A description of any assumptions or corrections, such as an adjustment for multiple comparisons |           |
| The test results (e.g. \( P \) values) given as exact values whenever possible and with confidence intervals noted |           |
| A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |           |
| Clearly defined error bars                                           |           |

See the web collection on statistics for biologists for further resources and guidance.

7. Software
Policy information about availability of computer code

Describe the software used to analyze the data in this study.

All analysis except for RNA seq, flow cytometry, and western blot analysis were performed using Graphpad Prism v7. ImageJ 1.49v was used to histology measurements. Flojo v 10.2 was used to analyze all flow cytometry experiments. ImageLab software v 5.2 was used to analyze western blot experiments.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability
Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All unique materials used are readily available from the authors.

9. Antibodies
Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

IHC: Unconjugated polyclonal rabbit anti-mouse phospho-STAT1 (Clone: S8D6 Cell Signaling Technology), unconjugated rabbit anti-mouse Ki67 (Clone:M3062, Spring Biosciences).

Western Blot: rabbit anti-mouse MAVS(Cell signalling, cat #M9835), rabbit anti-mouse Sting (Clone D2P2F, Cell signalling cat# 13647), mouse anti-mouse beta-actin (Clone 8H1O0D10, Cell signalling cat 3700), anti-Atg16L1 (MBL cat# M150-3)

Flow Cytometry: CD4 (RM4-5), CD8a (S3-6-7), CD19 (6D5), CD11b (M1/70), TCRβ (H57-597), and CD90.2 (S3-2.1), and intracellular markers IFNy (XMG1.2) and IL-17a (TC11-18H10.1) from Biolegend, and IL-13 (ebio13A) and IL-22(1H8PWSR) from ebiosciences. A fixable live/dead stain from Biolegend was used to exclude dead cells. CD103(2E7), CD11c(N418), CD115(9-4D2-1E4), Ly6C(HK1.4), Ly6G(1A8) F4/80(BM8), MHC-II(M5/114.15.2), Galectin 9(108A2), Xcr1(zet) and live/dead viability stain. All antibodies were from Biolegend. TCRβ (H57-597), CD19(6D5), CD103 (2E5), Ly6G(1A8), Cd11b (M1/70), Ly6C (HK1.4), FC block, and Dapi for viability stain, with all antibodies were from Biolegend.
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used.
      No eukaryotic cell lines were used.
   b. Describe the method of cell line authentication used.
      No eukaryotic cell lines were used.
   c. Report whether the cell lines were tested for mycoplasma contamination.
      No eukaryotic cell lines were used.
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.
      No eukaryotic cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
   Provide details on animals and/or animal-derived materials used in the study.

   Age and gender matched 8-12 week old mice on the C57BL/6J background were used as WT controls unless stated otherwise and bred onsite. Atg16L1HM, Atg16L1f/f Villincre, Atg16L1f/f Cdlccre, and LC3b-/- mice were previously described. Atg4b-/- mice were generously provided by Skip Virgin (Washington University School of Medicine). For single allele autophagy mutants, we used Atg16L1HM/+, Atg4b+/–, and LC3b+/– breeders to generate Atg16L1HM/+/+, Atg4b+/–/–, and LC3b+/–/– mice with their respective homozygous WT littermate controls (Atg16L1+/+ and Atg4b+/–/–). Atg16L1f/f Villincre and Atg16L1f/f Cdlccre mice were generated along with Atg16L1f/f littermates by breeding Cre-positive and negative Atg16L1f/f mice. Ifnar-/-, Ccr2-/-, Mavs-/-, Sting-/-, and Caspase1.11-/- mice were purchased from Jackson Laboratory and crossed to Atg16L1HM mice to generate double mutants. For these compound mutants, we fixed the Atg16L1HM allele first, then generated littermates that were homozygous knockout and wild-type for the other allele. Using the Ifnar-deficient mice as an example, Atg16L1HM/HMIfnar+/– mice were bred to each other through a triating scheme to generate Atg16L1HM/HMIfnar+/+ and Atg16L1HM/HMIfnar+/– mice for experiments. To increase the number of mice analyzed, experiments were supplemented with progeny generated from Atg16L1HM/HMIfnar+/– breeder pairs. Mavs-/- mice are on a mixed C57BL/6 and 129/SvEv background and were bred to wild-type C57BL/6J mice to generate Mavs+/– mice, which were crossed to each other to generate controls for Atg16L1 and Mavs double mutants that were generated by breeding Atg16L1HM/Mavs+/- mice, similar to other compound mutants.

Policy information about studies involving human research participants

12. Description of human research participants
   Describe the covariate-relevant population characteristics of the human research participants.

   The study did not involve human research participants.