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1 **Intestinal epithelial cell autophagy is required to protect against TNF-**
2 **induced apoptosis during chronic colitis in mice**

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29 Running title: Epithelial autophagy dampens chronic colitis.

30

31 **Summary**

32 Genome-wide association studies have linked polymorphisms in the autophagy
33 gene *ATG16L1* with susceptibility to inflammatory bowel disease (IBD).
34 However, the cell-type specific effects of autophagy on the regulation of chronic
35 intestinal inflammation have not been investigated. Here, we assessed the effect
36 of myeloid-specific or intestinal epithelial cell (IEC)-specific deletion of *Atg16l1*
37 on chronic colitis triggered by the intestinal opportunistic pathogen *Helicobacter*
38 *hepaticus* in mice. Although *Atg16l1*-deficiency in myeloid cells had little effect
39 on disease, mice selectively lacking *Atg16l1* in IEC (*Atg16l1^{VC}*) developed
40 severely exacerbated pathology, accompanied by elevated pro-inflammatory
41 cytokine secretion and increased IEC apoptosis. Using *ex vivo* IEC organoids, we
42 demonstrate that autophagy intrinsically controls TNF-induced apoptosis and *in*
43 *vivo* blockade of TNF attenuated the exacerbated pathology in *Atg16l1^{VC}* mice.
44 These findings suggest that the IBD susceptibility gene *ATG16L1* and the process
45 of autophagy within the epithelium controls inflammation-induced apoptosis
46 and barrier integrity to limit chronic intestinal inflammation.

47 **Introduction**

48 Inflammatory bowel disease (IBD) is a chronic inflammatory disorder with
49 unknown etiology, with two main clinical forms – Crohn’s disease (CD) and
50 ulcerative colitis (UC). Although there are broadly conserved features of
51 intestinal immunopathology that present in IBD patients, including dysregulated
52 immune responses, aberrant cytokine secretion, and alterations in barrier
53 function and intestinal microbiota (Maloy and Powrie, 2011; Neurath, 2014), the
54 clinical manifestations are heterogeneous (Lonnfors et al., 2014). The
55 heterogeneous nature of IBD is further emphasized by genome wide association
56 studies (GWAS) that have identified many pathways that potentially contribute
57 to the pathogenesis of IBD (Jostins et al., 2012; Liu et al., 2015), and which allow
58 further stratification of patients, besides disease manifestations and
59 immunological profiling (de Souza et al., 2017). Much current IBD research
60 focuses on unraveling the mechanistic effects of genes and pathways that have
61 been implicated by GWAS. A better understanding of how host genetics control
62 disease development and progression should enable treatments to be utilized in
63 more effective and cost-efficient manner. Current treatment options for IBD
64 include, anti-inflammatory and immune-suppressive drugs, surgery, and
65 biologics that specifically target the dysregulated immune response (Chang and
66 Hanauer, 2017). In the latter category, anti-TNF treatment has high efficacy and
67 has been increasingly employed, however 40 % of IBD patients do not respond
68 to this therapy and many become refractory to treatment (Cohen and Sachar,
69 2017; Hendy et al., 2016). As it is not fully understood how anti-TNF treatment
70 acts and which cell types are targeted, it is difficult to predict which patients are
71 most likely to benefit and what mechanisms prevent responsiveness.

72 Polymorphisms in *ATG16L1* and other autophagy genes suggest an important
73 role of autophagy in IBD pathogenesis (Hampe et al., 2007; Rioux et al., 2007).
74 *ATG16L1* is an essential autophagy gene and the *T300A* polymorphism that
75 shows the strongest link to IBD development results in destabilization of the
76 protein, facilitating caspase 3 dependent degradation during cellular stress
77 (Mizushima et al., 2011; Murthy et al., 2014). Autophagy is a conserved
78 intracellular degradation pathway that facilitates maintenance of cellular
79 homeostasis during periods of stress or malnutrition, but it also impacts on
80 many pathways of cellular immune defense (Kabat et al., 2016b). The ubiquitous
81 nature of autophagy and its interactions with many other essential homeostatic
82 cellular processes makes it difficult to unravel precisely how disease-associated
83 polymorphisms can predispose to IBD. Nevertheless, several studies have
84 described cell-type specific functions of autophagy in the context of mucosal
85 homeostasis (Adolph et al., 2013; Cadwell et al., 2008; Kabat et al., 2016a; Kabat
86 et al., 2016b; Saitoh et al., 2008). In myeloid cells autophagy is implicated in
87 regulation of the proinflammatory cytokine response, particularly secretion of
88 inflammasome dependent cytokines and ROS levels are increased in autophagy-
89 deficient macrophages (Lassen et al., 2014; Saitoh et al., 2008; Zhang et al.,
90 2017). Bone-marrow chimeric mice with autophagy-deficient haematopoietic
91 cells, *Atg16l1*-hypomorphic mice or mice lacking *Atg16l1* in the myeloid
92 compartment all show increased pathology in the chemically induced model of
93 DSS colitis (Cadwell et al., 2010; Saitoh et al., 2008; Zhang et al., 2017). Although
94 autophagy enhances killing of *Salmonella* by DCs and macrophages *in vitro*, how
95 much this impacts on disease development during infection is less clear (Conway
96 et al., 2013; Thurston et al., 2012; Zhang et al., 2017). Recent studies also

97 revealed that autophagy in T cells is required for the maintenance of intestinal
98 homeostasis, as Treg cells rely on autophagy for their survival and function in
99 the gut (Kabat et al., 2016a; Wei et al., 2016). Autophagy has also been reported
100 to regulate many key functions of intestinal epithelial cells (IEC). Autophagy has
101 been shown to influence granule structure of goblet and Paneth cells (secretory
102 cells of the intestinal epithelium) under stressed conditions, such as during
103 norovirus infection or ER stress (Adolph et al., 2013; Cadwell et al., 2010; Lassen
104 et al., 2014; Patel et al., 2013). Epithelial autophagy has also been implicated in
105 barrier enforcement during *Salmonella* infection, as autophagy-deficiency of IEC
106 led to increased bacterial dissemination and inflammation (Benjamin et al.,
107 2013; Conway et al., 2013; Lassen et al., 2014).

108 Taken together, these studies indicate that autophagy has wide-ranging
109 functional effects on various cell types that could potentially regulate
110 inflammatory responses. However, thus far, there have been few comparative
111 studies of the cell-type specific effects of autophagy on the regulation of chronic
112 intestinal inflammation. Here, we have undertaken a comprehensive analysis of
113 the consequences of autophagy deficiency within different cellular
114 compartments on chronic intestinal pathology. We have utilized a well-
115 characterized mouse IBD model in which chronic intestinal inflammation is
116 induced by infection with the gram negative enteric bacterium *Helicobacter*
117 *hepaticus* together with concomitant blockade of immune regulatory circuits
118 using anti-IL10R (Kullberg et al., 2006). Our previous work has shown that this
119 model recapitulates several features of chronic intestinal pathology found in IBD
120 patients, that pathology is driven by similar excessive innate and adaptive
121 immune responses, and that disease is controlled by the same key pro-

122 inflammatory mediators (Hue et al., 2006; Kullberg et al., 2006; Maloy et al.,
123 2003; Schiering et al., 2014; West et al., 2017). We find that selective autophagy
124 deficiency in myeloid cells only marginally affects colitis development, whereas
125 autophagy deficiency in IEC results in severely exacerbated pathology. We
126 further show that autophagy regulates cytokine-induced apoptosis in IEC and
127 blockade of TNF attenuates chronic colitis in IEC-specific *Atg16l1*-deficient mice
128 by ameliorating epithelial apoptosis.

129 **Results**

130 ***Autophagy-deficiency in intestinal epithelial cells (IEC) predisposes to***
131 ***chronic colitis***

132 To analyze the cell-type specific role of autophagy in chronic intestinal
133 inflammation, we crossed *Atg16l1^{fl/fl}* mice (Hwang et al., 2012) with strains
134 expressing Cre recombinase under the control of various cell-type specific
135 promoters. Thus, we generated transgenic mice lacking *Atg16l1* in
136 neutrophils/macrophages (*Atg16l1^{LysM}*), in dendritic cells (*Atg16l1^{CD11c}*) or in IEC
137 (*Atg16l1^{VC}*). Analyses of *Atg16l1* expression by quantitative PCR confirmed that
138 LysM-Cre and CD11c-Cre driven recombination selectively reduced expression of
139 *Atg16l1* in myeloid cell compartments (CD11c⁺, MHC2⁺, CD45⁺ DC and CD64⁺,
140 CD11b⁺, CD45⁺ macrophages), whereas Villin-Cre driven recombination
141 selectively ablated *Atg16l1* expression in IEC (Fig. S1 A-C).

142 Intestinal inflammation was induced by oral infection with *Helicobacter*
143 *hepaticus* and blockade of the regulatory response with anti-IL10R antibody
144 (Kullberg et al., 2006)(Fig. 1 A). To exclude any potential microbiota differences,
145 the transgenic lines were bred as heterozygous for the *Cre* allele, allowing
146 experimental groups to be set up using littermate controls (*Atg16l1^{fl/fl}*) that were
147 co-housed throughout the experiment.

148 At two weeks after colitis induction, assessment of histopathology of the caecum
149 and colon revealed slightly elevated pathology in *Atg16l1^{CD11c}* and *Atg16l1^{LysM}*
150 mice compared to *Atg16l1^{fl/fl}* mice, but these differences were not statistically
151 significant (Fig. 1 B, C). Comparison with the concurrent littermate controls
152 confirmed that there was only a minor increase in pathology and revealed no

153 significant alterations within the lamina propria CD4⁺ T cell compartments in
154 either *Atg16l1*^{CD11c} or *Atg16l1*^{LysM} mice (Fig. S1 D-K).

155 In contrast, *Atg16l1*^{VC} mice showed significantly elevated histopathology in both
156 the caecum and colon at 2 weeks and 4 weeks after colitis induction (Fig. 1 B - G).

157 Although overall disease severity varied slightly between experiments, we
158 consistently observed significantly increased histopathology in *Atg16l1*^{VC} mice
159 compared to their *Atg16l1*^{fl/fl} littermates. This was in accordance with increased
160 weight loss in the *Atg16l1*^{VC} mice (Fig. 1 H) and increased numbers of CD4⁺ T
161 cells in the lamina propria compared to *Atg16l1*^{fl/fl} littermates (Fig. 1 I).

162 Furthermore, secretion of inflammatory cytokines, such as TNF, IFN- γ and IL1- β ,
163 by colon explants isolated from *Atg16l1*^{VC} mice were significantly elevated at 2
164 weeks after colitis induction compared to explants obtained from *Atg16l1*^{fl/fl}
165 littermates (Fig. 1 J-L). However, frequencies of CD4⁺ T effector and Treg subsets
166 in the lamina propria and *H.h.* colonization levels were not altered in the
167 *Atg16l1*^{VC} mice relative to their *Atg16l1*^{fl/fl} littermates (Fig. S2). We also tested
168 the role of intestinal epithelial autophagy in a model of acute intestinal infection
169 using oral infection with *Citrobacter rodentium* (Collins et al., 2014). We found
170 that *Citrobacter rodentium* infection induced comparable levels of intestinal
171 pathology in *Atg16l1*^{VC} mice and *Atg16l1*^{fl/fl} mice (Fig. S3 A, B, E, F), which was in
172 accordance with similar bacterial colonization levels (Fig. S3 C, D). Taken
173 together, these data reveal that autophagy within IEC exerts a protective effect
174 during *Helicobacter hepaticus*-triggered chronic colitis, whereas autophagy in
175 myeloid cell compartments (CD11c⁺ or LysM⁺ cells) plays only a marginal role.
176 Therefore, we focused our further analyses on the consequences of autophagy

177 impairment in IEC and the impact that it has on the development of chronic
178 colitis.

179

180 ***Autophagy does not regulate chemokine expression or ER stress induction in***
181 ***IEC***

182 We next sought to analyze how autophagy-deficiency within the epithelium
183 influences colitis development. First, we noticed that *in vivo* IEC from *Atg16l1^{VC}*
184 mice expressed elevated levels of chemokines *Cxcl2* and *Cxcl5* at 2 weeks and 4
185 weeks after colitis induction (Fig. 2 A, B).

186 To analyze whether the chemokine response of the epithelium is intrinsically
187 controlled by autophagy, we employed the *ex vivo* organoid system to grow
188 primary IEC from either *Atg16l1^{fl/fl}* or *Atg16l1^{VC}* mice (Heijmans et al., 2013).
189 Western blot analysis of LC3 lipidation confirmed autophagy deficiency in IEC
190 organoids derived from *Atg16l1^{VC}* mice (Fig. 2 D). In the presence of complete
191 medium containing the essential IEC growth factors (Wnt3a, R-spondin and
192 noggin), we found that autophagy-deficient colonic IEC proliferated and formed
193 organoids that were morphologically indistinguishable from wild type
194 organoids.

195 To mimic the conditions present in the inflamed intestine, we isolated lamina
196 propria leucocytes (LPL) from colitic mice (WT mice subjected to *H.h.* + α IL10R
197 treatment for 2 weeks) and cultured them overnight to generate inflammatory
198 conditioned medium (iCM) that was harvested from the culture supernatant. We
199 then stimulated *Atg16l1^{fl/fl}* or *Atg16l1^{VC}* IEC organoids with 10% iCM to assess
200 IEC responses to the colitic microenvironment. We found that both *Atg16l1^{fl/fl}*
201 and *Atg16l1^{VC}* IEC responded to stimulation with iCM, as evidenced by rapid

202 phosphorylation and nuclear translocation of Stat3 (Fig. 2 C, D). Western blot
203 analysis also confirmed robust and equivalent activation of the intracellular Map
204 kinase pathway in response to iCM stimulation in both *Atg16l1^{fl/fl}* and *Atg16l1^{VC}*
205 IEC (Fig. 2 D). In addition, *Cxcl2* and *Cxcl5* expression were induced to
206 comparable levels in *Atg16l1^{fl/fl}* and *Atg16l1^{VC}* organoids after 6h of stimulation
207 with iCM (Fig. 2 E, F).

208 In order to test the response of primary IEC from *Atg16l1^{fl/fl}* or *Atg16l1^{VC}* mice to
209 bacterial stimulation, we adapted the primary IEC culture system to generate
210 polarized monolayers (Moon et al., 2013). Thus, IEC organoids from *Atg16l1^{fl/fl}* or
211 *Atg16l1^{VC}* mice were seeded as monolayers and subsequently infected with *H.*
212 *hepaticus* at the apical surface. Autophagy deficiency of the IEC monolayer
213 derived from *Atg16l1^{VC}* mice was again verified by blotting for lipidated LC3 (Fig.
214 2 G). We found that both WT and autophagy-deficient IEC rapidly responded to
215 *H. hepaticus* infection, with comparable phosphorylation of Erk1/2 and NFκB
216 p65 (Fig. 2 G). Furthermore, *Cxcl2* induction was also comparable between
217 *Atg16l1^{fl/fl}* and *Atg16l1^{VC}* IEC at 6h post *H. hepaticus* infection (Fig. 2 H).

218 Taken together, the *ex vivo* primary IEC stimulation experiments suggest that the
219 exacerbated chemokine production observed in *Atg16l1^{VC}* mice during colitis
220 (Fig. 2 A, B) is a consequence of the elevated inflammatory environment, rather
221 than any intrinsic hyperactivity caused by autophagy impairment in IEC.

222 Several recent reports have linked the ER stress pathway and autophagy in the
223 intestinal epithelium in the context of colitis (Adolph et al., 2013;
224 Tschurtschenthaler et al., 2017). Therefore, we also assessed ER stress induction
225 following iCM stimulation or *H. hepaticus* infection of primary IEC *ex vivo*. We
226 found that *Grp78* expression was not altered following iCM stimulation in either

227 *Atg16l1*^{fl/fl} or *Atg16l1*^{VC} IEC organoids (Fig. 2 I). In contrast, *H. hepaticus* infection
228 of IEC monolayers resulted in comparably increased expression of *Grp78* in both
229 *Atg16l1*^{fl/fl} and *Atg16l1*^{VC} IEC (Fig. 2 J). Moreover, phosphorylation of eIF2 α was
230 also increased following *H. hepaticus* infection; and again the levels were similar
231 in *Atg16l1*^{fl/fl} or *Atg16l1*^{VC} IEC monolayers (Fig. 2 K). We also analyzed markers
232 of ER stress in IEC isolated from *Atg16l1*^{fl/fl} or *Atg16l1*^{VC} mice after colitis
233 induction with *H. hepaticus* infection and α IL10R treatment. Consistent with the
234 *in vitro* data, *Grp78* expression was elevated to similar levels during colitis in
235 both *Atg16l1*^{fl/fl} and *Atg16l1*^{VC} mice, both in isolated IEC fractions, as well as in
236 whole colon tissue (Fig. 2 L, M).

237 Overall, these results suggest that, under these experimental conditions, ER
238 stress pathways in IEC do not seem to be influenced by the presence or absence
239 of the autophagy pathway.

240

241 ***Autophagy deficiency predisposes IEC to apoptosis***

242 We next hypothesized that the absence of a functional autophagy pathway might
243 predispose IEC to cell death during inflammatory conditions (Marino et al.,
244 2014). To assess whether autophagy regulated apoptosis in IEC during chronic
245 colitis, we assessed the number of apoptotic cells by TUNEL staining. We
246 observed increased numbers of TUNEL-positive IEC in both caecal and colonic
247 sections of *Atg16l1*^{VC} mice compared to *Atg16l1*^{fl/fl} littermates at 2 weeks after
248 colitis induction (Fig. 3 A). Moreover, western blot analyses of intestinal samples
249 revealed increased levels of cleaved caspase 8 in isolated IEC lysates and whole
250 colonic lysates obtained from *Atg16l1*^{VC} mice compared to those obtained from
251 *Atg16l1*^{fl/fl} littermates (Fig 3 B, C, D).

252 To determine whether the increased number of apoptotic IEC in *Atg16l1^{VC}* mice
253 was a consequence of the increased inflammation and elevated levels of pro-
254 inflammatory cytokines in *Atg16l1^{VC}* mice, or whether autophagy intrinsically
255 regulates apoptosis in IEC, we next assessed cytokine-induced apoptosis in
256 primary IEC *ex vivo*. Organoids generated from *Atg16l1^{fl/fl}* and *Atg16l1^{VC}* mice
257 were stimulated with 40% iCM for 24h and cell death and apoptosis were
258 assessed in parallel. We observed that cell death and caspase 3/7 activity was
259 induced by iCM stimulation in both *Atg16l1^{fl/fl}* and *Atg16l1^{VC}* IEC (Fig. 4 A, B),
260 with increased caspase activation detected in *Atg16l1*-deficient IEC (Fig. 4 B).
261 Furthermore, blockade of TNF and IFN- γ through addition of monoclonal
262 antibodies prevented cell death and apoptosis induction in IEC (Fig. 4 A, B),
263 suggesting that these cytokines were largely responsible for the apoptosis
264 induction in response to iCM. Indeed, stimulation of IEC organoids with TNF and
265 IFN- γ induced significantly higher levels of apoptosis (Fig. 4 A, B) and decreased
266 metabolic activity (Fig. 4 C, D) in *Atg16l1^{VC}* IEC organoids compared with
267 *Atg16l1^{fl/fl}* organoids. Western blot analysis confirmed increased apoptosis levels
268 in the *Atg16l1^{VC}* IEC organoids after stimulation with TNF and IFN- γ , as they
269 showed increased cleavage of caspases 8 and 3 (Fig. 4 E) and staining for cleaved
270 caspase 3 revealed increased numbers of apoptotic cells and disrupted cell
271 morphology (Fig. 4 F).

272 To confirm that TNF induced elevated IEC apoptosis in *Atg16l1^{VC}* mice, we
273 assessed small intestinal epithelial cell apoptosis in mice that received an
274 intraperitoneal injection of TNF (Vereecke et al., 2014) (Fig. 5A). We observed
275 that *Atg16l1^{VC}* mice lost significantly more weight than littermate controls
276 following TNF injection (Fig5 B). Furthermore, TUNEL staining of small intestinal

277 tissue sections revealed increased numbers of apoptotic IEC in *Atg16l1^{VC}* mice
278 compared to *Atg16l1^{fl/fl}* littermates (Fig. 5 C) and this correlated with increased
279 cleavage of caspase 8 in IEC lysates (Fig. 5 D,E).

280 Taken together, these results demonstrate that autophagy impairment renders
281 IEC more responsive to apoptosis induction following exposure to the pro-
282 inflammatory cytokine milieu present in the inflamed intestine.

283

284 ***TNF induced apoptosis drives exacerbated disease in Atg16l1^{VC} mice***

285 To functionally test whether TNF was the key driver of the increased IEC
286 apoptosis and exacerbated pathology in *Atg16l1^{VC}* mice in the complex setting of
287 chronic colitis, we induced colitis (*H. hepaticus* infection + anti-IL-10R
288 treatment) in *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* littermates and concomitantly treated a
289 group of mice with a blocking antibody against TNF (α TNF) (Fig. 6 A). We found
290 that, in contrast to their *Atg16l1^{fl/fl}* littermates, *Atg16l1^{VC}* mice exhibited
291 significant weight loss during the first two weeks after colitis induction and that
292 this was completely prevented by treatment with α TNF (Fig. 6 B).
293 Furthermore, α TNF treatment also reduced intestinal pathology in *Atg16l1^{VC}*
294 mice (Fig. 6 C; Fig. S4 A, B, C). Finally, in these experiments we also assessed the
295 influence of α TNF treatment on apoptosis levels in IEC. We found that α TNF
296 treatment of *Atg16l1^{VC}* mice led to a marked reduction of TUNEL positive IEC
297 (Fig. 6 D; Fig. S4 D), as well as decreased levels of cleaved caspase 8 in IEC lysates
298 (Fig. 6 E-G). Indeed, α TNF treatment reduced the exacerbated apoptosis
299 induction in IEC of *Atg16l1^{VC}* mice during chronic colitis to the levels present in
300 IEC isolated from *Atg16l1^{fl/fl}* littermates (Fig. 6 D-G). However, following TNF
301 blockade, some reduction of pathology was also observed in the *Atg16l1^{fl/fl}* mice

302 and pro-inflammatory mediators were reduced in both *Atg16l1*^{fl/fl} and *Atg16l1*^{VC}
303 littermates at two weeks of colitis (Fig. 6 C; Fig. S4 A, B, C, E, F), suggesting
304 several modes of action. In contrast, treating established colitis at day 6 post
305 colitis induction with α TNF resulted in reduction of pathology only in the
306 *Atg16l1*^{VC} mice (Fig. S5 A-C) and this correlated with reduced expression of pro-
307 inflammatory mediators (Fig. S5 D, E).
308 Overall, these findings confirm that TNF-induced apoptosis of autophagy-
309 deficient IEC is a key contributor to the exacerbated pathology, as blockade of
310 the TNF pathway attenuates the disease.

311 **Discussion**

312 GWAS studies linked polymorphisms in *ATG16L1* and other autophagy genes to
313 the development of IBD (Hampe et al., 2007; Rioux et al., 2007), suggesting an
314 important role of autophagy in IBD pathogenesis. However, despite extensive
315 investigation, the mechanistic relationship between autophagy and intestinal
316 inflammation is not fully understood. We performed comparative analyses to
317 assess the consequences of autophagy deficiency within different cellular
318 compartments of the intestinal mucosa. We found that autophagy deficiency in
319 LysM⁺ or CD11c⁺ cells only marginally affected chronic colitis development,
320 whereas autophagy deficiency in IEC resulted in severely exacerbated pathology.
321 This comparative approach, using tissue-specific *Atg16l1*-deficient mouse lines
322 in the same disease model, highlights the pivotal role of autophagy in
323 maintaining epithelial barrier function during inflammation.
324 Our results indicate that intestinal epithelial cells can function quite normally
325 without autophagy, as long as intestinal homeostasis is not perturbed. We could
326 not detect any abnormal growth pattern, differentiation or chemokine response
327 of *Atg16l1*-deficient IEC, nor altered susceptibility during acute *Citrobacter*
328 *rodentium* infection in *Atg16l1*^{VC} mice. *C. rodentium* infection induces an acute
329 and self-limiting epithelial hyperplasia, accompanied by mild inflammation, both
330 of which abate as the infection is cleared. In contrast, the *H. hepaticus* plus anti-
331 IL-10R model results in sustained activation of innate and adaptive immune
332 circuits that drive prolonged secretion of high concentrations of pro-
333 inflammatory cytokines. Under the severe stress conditions present during this
334 chronic colitis, *Atg16l1*-deficient IEC showed increased apoptosis, which
335 contributed to exacerbation of intestinal inflammation.

336 The effects of autophagy-deficiency in IEC have been studied in several different
337 models and mouse strains (Adolph et al., 2013; Benjamin et al., 2013; Cadwell et
338 al., 2008; Conway et al., 2013; Tschurtschenthaler et al., 2017). It has been
339 reported that autophagy deficiency in IEC resulted in enhanced bacterial
340 translocation and inflammation following acute *Salmonella* challenge (Benjamin
341 et al., 2013; Conway et al., 2013). However, when we evaluated *H. hepaticus* DNA
342 levels in MLNs of mice in the chronic colitis model (data not shown) as well as
343 systemic translocation of *C. rodentium* to liver and spleen following oral
344 infection, we did not observe elevated levels in the *Atg16l1^{VC}* mice compared to
345 *Atg16l1^{fl/fl}* mice. These results suggest that epithelial autophagy may be
346 important for containing invasive intestinal pathogens, but is not essential for
347 preventing systemic dissemination of non-invasive pathogens.

348 Several studies reported altered granule structure in autophagy-deficient
349 secretory IEC, such as Paneth cells and goblet cells (Adolph et al., 2013; Cadwell
350 et al., 2008; Lassen et al., 2014; Patel et al., 2013). This phenotype was linked to
351 increased pathology in norovirus infected *Atg16l1* hypomorphic mice during DSS
352 colitis (Cadwell et al., 2010). However, whether aberrant Paneth cell morphology
353 is a spontaneously occurring phenotype in autophagy-deficient IEC or requires
354 an additional trigger such as viral or bacterial infection or ER stress is not clear
355 (Adolph et al., 2013; Bel et al., 2017; Cadwell et al., 2008; Lassen et al., 2014;
356 Patel et al., 2013). Furthermore, in IBD patients, altered granule structure in
357 Paneth cells was associated with the *ATG16L1* T300A SNP (Cadwell et al., 2008).
358 How this morphologic phenotype in the small intestine might predispose to
359 colonic inflammation is poorly understood. Altered Paneth cell morphology
360 could lead to altered AMP secretion, which might impact on microbiota

361 composition, a plausible factor for disease susceptibility. However it was
362 recently described that *Atg16l1^{VC}* mice do not harbor an altered tissue-adherent
363 microbiota in the ileum compared to *Atg16l1^{fl/fl}* mice (Tschurtschenthaler et al.,
364 2017). In our study, we used *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* littermates to avoid any
365 potential microbiota effects and to ensure that any differences in disease
366 susceptibility were due to genotype.

367 Several studies have illustrated compensatory mechanisms of ER stress and
368 autophagy in intestinal epithelial cells. Indeed, transgenic mice lacking both the
369 ER stress effector *Xbp1* and *Atg16l1* selectively in IEC develop severely
370 exacerbated levels of ER stress and spontaneous pathology in the ileum (Adolph
371 et al., 2013; Tschurtschenthaler et al., 2017). Furthermore, these investigators
372 also reported spontaneous inflammation in the ileum of aged (>35weeks of age)
373 *Atg16l1^{VC}* mice, concomitant with enhanced ER stress levels (Tschurtschenthaler
374 et al., 2017). During chronic colitis induction we observed elevated levels of
375 *Grp78* in colonic IEC, indicative of enhanced ER stress, however we detected
376 similar levels of *Grp78* in IEC isolated from *Atg16l1^{fl/fl}* and *Atg16l1^{VC}* mice,
377 suggesting autophagy-independent regulation of ER stress during colonic
378 inflammation. Moreover, *ex vivo* primary colonic IEC organoids stimulated with
379 inflammatory cytokines or infected with *H. hepaticus* exhibited ER stress
380 responses that were independent of the *Atg16l1* genotype. These findings
381 suggest that autophagy and ER stress responses are only under certain
382 conditions compensatory and could have different regulatory functions in the
383 epithelium of the small intestine and colon.

384 Our study reveals a link between autophagy and apoptosis in IEC. We found that
385 autophagy-deficiency in IEC enhanced apoptosis induction during inflammatory

386 conditions, thereby potentially weakening the barrier integrity. Furthermore, we
387 observed that *Ag16l1*-deficient IEC organoids were hyper-susceptible to TNF-
388 induced apoptosis. Similarly, during chronic colitis we observed that high
389 intestinal TNF levels were associated with increased numbers of apoptotic IEC in
390 *Atg16l1^{VC}* mice, but not *Atg16l1^{fl/fl}* littermates. Of note, experimental
391 administration of TNF also induced elevated levels of apoptosis in small
392 intestinal epithelial cells of *Atg16l1^{VC}* mice, confirming an intrinsic control of IEC
393 apoptosis by autophagy. Indeed blocking TNF during chronic colitis resulted in a
394 specific and marked reduction of IEC apoptosis in *Atg16l1^{VC}* mice, confirming our
395 hypothesis that the exacerbated pathology was caused by increased apoptosis of
396 autophagy-deficient IEC under inflammatory conditions. However, continuous
397 blockade of TNF throughout chronic colitis resulted in reduced pro-
398 inflammatory chemokine levels and decreased intestinal pathology in both
399 *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* mice, which indicates that TNF also has additional roles
400 in chronic inflammation, such as immune cell activation and survival (Billmeier
401 et al., 2016). Furthermore, although treatment of already established colitis with
402 α TNF does not reduce pathology in WT (*Atg16l1^{fl/fl}*) mice (Kullberg et al., 2001;
403 West et al., 2017), we found that it attenuated colitis in *Atg16l1^{VC}* mice, indicating
404 that autophagy-deficiency in IEC renders the disease responsive towards α TNF
405 treatment.

406 In accordance with our results, a recent study reported that autophagy-
407 deficiency also renders small intestinal epithelial cells hyper susceptible to TNF-
408 induced cell death (Matsuzawa-Ishimoto et al., 2017). These investigators used a
409 model of intestinal inflammation triggered by infection with murine norovirus
410 and treatment with DSS, and primarily focused on the small intestine, where a

411 lack of autophagy led to increased susceptibility to necroptosis and a loss of
412 Paneth cells (Matsuzawa-Ishimoto et al., 2017). In contrast, we observed
413 exacerbated activation of caspase-8 and caspase-3 in *Atg16l1*-deficient colonic
414 IEC, as well as DNA fragmentation, all of which are hallmarks of apoptotic death.
415 Therefore, although autophagy appears to generally protect IEC from TNF-
416 induced cell death, there may be differences in the dominant death pathways
417 triggered in distinct compartments or cell types (eg. small intestine versus colon;
418 Paneth cells versus absorptive enterocytes), or during different types of
419 inflammatory challenge (eg. Norovirus + DSS versus *H. hepaticus* + anti-IL-10R).
420 These results are consistent with previous studies in which epithelial apoptosis
421 has been linked to IBD pathology, with IEC having been described to be
422 particularly susceptible to TNF induced apoptosis (Takahashi et al., 2014; Zeissig
423 et al., 2004). For example, in a mouse model of spontaneous ileitis (SAMP1/YitFc
424 model) a single shot of α TNF treatment ameliorated disease in the ileum
425 correlating with reduction of epithelial apoptosis (Marini et al., 2003). Similarly,
426 in the *Atg16l1/Xbp1^{VC}* model of spontaneous enteritis, the pathology scores
427 strongly correlated with the degree of epithelial apoptosis (Adolph et al., 2013).
428 Moreover, mice with hypomorphic expression of *Atg16l1* showed increased
429 pathology with transmural inflammation in chemically induced colitis, which
430 was reduced to wild-type levels by α TNF/ α IFN γ treatment (Cadwell et al.,
431 2010). Together with these studies, our findings emphasise the links between
432 autophagy in IEC and regulation of apoptosis in the inflamed intestine.
433
434 Of great interest is whether IBD patients bearing polymorphisms in autophagy
435 genes present with increased IEC apoptosis and a more severe disruption of the

436 epithelial barrier. Although the IBD associated *ATG16L1* T300A SNP does not
437 confer loss of function under homeostatic conditions, it results in a
438 destabilization of the protein by enhancing caspase 3 dependent degradation
439 (Murthy et al., 2014). Therefore, under inflammatory conditions the T300A SNP
440 will enhance degradation of *ATG16L1* and may thereby make the IEC more
441 susceptible to apoptosis. Interestingly, α TNF treatment of IBD patients has been
442 linked to reduction of IEC apoptosis (Zeissig et al., 2004), therefore a key future
443 issue is to determine whether patients that harbor genetic predisposition for IEC
444 apoptosis show better responsiveness to α TNF treatment. For example, it has
445 been shown that SNPs in *A20*, an inhibitory regulator of TNF signaling correlate
446 with α TNF responsiveness of patients (Vereecke et al., 2014). In this context, it is
447 striking that a recent study reported a correlation between the *ATG16L1* T300A
448 SNP and a beneficial outcome in colon cancer (Grimm et al., 2016), which might
449 be a result of increased apoptosis susceptibility of IEC harboring the SNP.

450 Overall our study proves that impaired autophagy in epithelial cells, but not in
451 myeloid cells, results in exacerbated chronic colonic inflammation. We showed
452 that autophagy-deficiency leads to enhanced cytokine-induced IEC apoptosis.
453 The potential to attenuate the exacerbated pathology in *Atg16l1^{VC}* mice by α TNF
454 treatment is of great clinical interest and may have implications for treatment of
455 IBD patients harboring SNPs in autophagy genes.

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468

469 **Author Contributions**

470 J.P. and A.M.K. designed and performed experiments. J.P. analyzed data. J.P. and
471 K.J.M. secured funding and wrote the manuscript. A.M.K edited the manuscript.

472

473 **Declaration of Interests**

474 The authors declare no competing interests.

475

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653
654

655 **Figure legend**

656 **Figure 1: Epithelial autophagy dampens chronic colitis**

657 *Atg16l1^{VC}*, *Atg16l1^{CD11c}*, *Atg16l1^{LysM}* or *Atg16l1^{fl/fl}* littermates were orally infected
658 with 10⁸ CFU *H.h.* on 3 consecutive days and injected with 1 mg anti-IL10R
659 weekly. **A)** Schematic of treatment protocol. **B, C)** Histopathology of the caecum
660 (B) or colon (C) was assessed on day 14 and presented relative to the respective
661 *Atg16l1^{fl/fl}* littermates. **D, E)** Representative H&E sections of caecum (D) or colon
662 (E) from *Atg16l1^{VC}* or *Atg16l1^{fl/fl}* littermates at day 14. **F, G)** Histopathology
663 scores of the caecum (F) and colon (G) of *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* littermates at
664 2 and 4 weeks. **H)** Weight curves of *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* littermates during
665 chronic colitis. **I)** Total numbers of CD4⁺TCRβ⁺ T cells in isolated colonic LPLs
666 from *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* littermates at steady-state and at day 14 after
667 colitis induction. **J-L)** TNF (J), IFNγ (K) and IL-1β (L) levels in colonic organ
668 culture supernatants from *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* littermates at steady-state
669 and at day 14 after colitis induction.

670

671 Data are combined from at least 3 independent experiments (B,C,F,G,H,I)
672 (representative D,E) or 2 independent experiments (J,K,L). Each dot represents
673 an individual mouse (B,C,F,G,I,J,K,L), or data are shown as mean ± s.e.m (H).
674 Horizontal bars denote medians. Scale bars are 100 μm. Statistical significance
675 was determined using Kruskal-Wallis test with correction for multiple
676 comparisons (B, C), two-way analysis of variance (ANOVA) with Bonferroni's
677 correction for multiple comparisons (H) or Mann Whitney test, * p<0.05; **
678 p<0.01; *** p<0.001. ns – not significant, *H.h.* – *Helicobacter hepaticus*, p.i. – post
679 infection.

680 **Figure 2: *Atg16l1*-deficient IEC do not exhibit enhanced inflammatory or**
681 **stress responses**

682 **A, B)** qPCR analysis of *Cxcl2* (A) and *Cxcl5* (B) expression in IEC isolated from
683 *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* littermates at day 14 after colitis induction. **C-K)** IEC
684 from *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* mice were grown *ex vivo* in organoids.
685 Inflammatory conditioned medium (iCM) was harvested from LPL cultures from
686 colitic mice. **C)** Organoids stimulated with 10% iCM for 30 min were stained for
687 pSTAT3 (red) and STAT3 (green) and DAPI (blue). **D)** *Atg16l1^{VC}* and *Atg16l1^{fl/fl}*
688 organoids were stimulated with 10% iCM for indicated times and lysates were
689 probed with antibodies directed against indicated proteins. **E, F)** qPCR analysis
690 of *Cxcl2* (E) and *Cxcl5* (F) expression by *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* organoids
691 treated with 10% iCM for 6h. **G, H)** Monolayers of *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* IEC
692 were infected with *H.h.* at a MOI of 100:1. **G)** Lysates were blotted after indicated
693 times for indicated proteins. **H)** qPCR analysis of *Cxcl2* expression by IEC
694 monolayers after 6h of *H.h.* infection. **I)** qPCR analysis of *Grp78* expression by
695 *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* organoids treated with 10% iCM for 12h. **J)** qPCR
696 analysis of *Grp78* expression by *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* monolayers infected
697 with *H.h.* (MOI 100:1) for 6h. **K)** Lysates of *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* monolayers
698 infected with *H.h.* (MOI 100:1) were blotted after indicated times for p ϵ IF2a and
699 ϵ IF2a. **L, M)** qPCR analysis of *Grp78* expression in whole colonic tissue (L) or IEC
700 (M) isolated from *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* littermates at day 14 after colitis
701 induction.

702

703 Data are representative of 2 (C, G, K) or 3 (A, B, D, L, M) independent
704 experiments or combined from at least 3 independent experiments (E, F, H, I, J).

705 Data are shown as medians \pm s.e.m (E, F, H, I, J), or each dot represents individual
706 mouse (A, B, L, M). Horizontal bars denote medians. Scale bar is 50 μ m. Statistical
707 significance was determined using Mann Whitney test, * $p < 0.05$; ** $p < 0.01$; ***
708 $p < 0.001$. iCM – inflammatory conditioned medium, *H.h.* – *Helicobacter hepaticus*.

709 **Figure 3: Autophagy deficiency increases IEC apoptosis during chronic**
710 **colitis**

711 *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* littermates were orally infected with 10⁸ CFU *H.h.* on 3
712 consecutive days and injected with 1 mg anti-IL10R weekly. **A)** TUNEL (red)
713 stainings of caecum and mid colon sections isolated at day 14 after colitis
714 induction. Counterstaining with wheat germ agglutinin (green) and DAPI (blue).
715 **B,C)** Lysates of IEC isolated from *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* littermates at 1 week
716 (B) and 2 weeks (C) after colitis induction were blotted for cleaved caspase 8. **D)**
717 Quantification of western blot bands of caspase 8 p43 (upper panel) and p18
718 (lower panel) in lysates of whole colon tissue isolated from *Atg16l1^{VC}* and
719 *Atg16l1^{fl/fl}* littermates at day 14 after colitis induction.

720

721 Data are representative of 3 independent experiments (A-D). Each dot
722 represents individual band (D). Horizontal bars denote median. Scale bar is 100
723 μ m. ctl- control, Atg- *Atg16l1*, *H.h.* - *Helicobacter hepaticus*.

724 **Figure 4: Autophagy deficiency predisposes IEC to apoptosis**

725 *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* IEC were grown *ex vivo* in organoids. Inflammatory
726 conditioned medium (iCM) was harvested from LPL cultures from colitic mice. **A,**
727 **B)** *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* organoids were stimulated for 18h with 40% iCM
728 (iCM); iCM plus anti-TNF (10µg/ml) and anti-IFN γ (10µg/ml) (iCM+ $\alpha\alpha$); or with
729 TNF (100ng/ml) and IFN γ (20ng/ml) (IFN γ + TNF). Cytotoxicity (A) and
730 apoptosis (B) were assessed with Apotox-Glo (Promega kit). **C, D)** *Atg16l1^{VC}* and
731 *Atg16l1^{fl/fl}* IEC organoids were stimulated with TNF (100ng/ml) and IFN γ
732 (20ng/ml) for 24h prior to incubation with alamarBlue for 3h. C) Fluorescence
733 intensity was measured at 540nm/ 590nm, normalized to values prior to
734 treatment and plotted relative to the untreated genotype control. D) Picture of
735 the 96-well plate after further 24h incubation with alamarBlue. **E)** *Atg16l1^{VC}* and
736 *Atg16l1^{fl/fl}* IEC organoids were stimulated with TNF (100ng/ml) and IFN γ
737 (20ng/ml) for indicated times and caspase 8 and 3 cleavage detected by western
738 blot. **F)** *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* IEC organoids were stimulated with TNF
739 (100ng/ml) and IFN γ (20ng/ml) for 18h and cells were stained for cleaved
740 caspase 3 (red), the epithelial marker E-cadherin (green) and DAPI (blue).

741

742 Data are representative of 3 independent experiments (A-F). Data are shown as
743 mean \pm s.e.m (A-C). Scale bar is 50 μ m. Statistical significance was determined
744 using students t test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. iCM – inflammatory
745 conditioned medium, $\alpha\alpha$ – anti-TNF and anti-IFN γ , T – TNF, γ - IFN γ .

746 **Figure 5: TNF induces epithelial apoptosis in *Atg16l1*^{VC} mice**

747 *Atg16l1*^{VC} and *Atg16l1*^{fl/fl} littermates were injected intraperitoneally with 10 µg of
748 TNF and sacrificed 6h later. **A)** Schematic of the treatment protocol. **B)** Body
749 weight at 6h post-injection as percentage of initial weight. **C)** TUNEL staining
750 (red) of small intestinal sections; counterstaining with wheat germ agglutinin
751 (green) and DAPI (blue). **D, E)** Representative western blot (D) and
752 quantification (E) of cleaved caspase 8 in isolated IEC.

753

754 Data are combined (B, E) or representative (C, D) of 3 independent experiments.

755 Each dot represents individual mouse (B, E). Scale bar is 100 µm. Statistical

756 significance was determined using Mann Whitney test, * p<0.05; ** p<0.01.

757 Horizontal bars denote medians. i.p.- intraperitoneal, WGA – wheat germ

758 agglutinin, cl. casp8- cleaved caspase 8, ctl- control, Atg - *Atg16l1*.

759 **Figure 6: TNF blockade attenuates colitis and IEC apoptosis in *Atg16l1*^{VC}**
760 **mice**

761 *Atg16l1*^{VC} and *Atg16l1*^{fl/fl} littermates were orally infected with 10⁸ CFU *H.h.* on 3
762 consecutive days and injected with 1 mg anti-IL10R i.p., with some cohorts also
763 receiving 1 mg anti-TNF i.p. at d0 and d6. **A)** Schematic of the treatment protocol.
764 **B)** Weight curves and **C)** representative micrographs of H&E stained colonic
765 sections from *Atg16l1*^{VC} and *Atg16l1*^{fl/fl} littermates at day 14 after colitis
766 induction. **D)** Representative images of TUNEL (red) staining of caecal sections
767 at day 14 after colitis induction; counterstaining with WGA (green) and DAPI
768 (blue). **E, F, G)** Western blot analysis for cleaved caspase 8 in colonic IEC lysates
769 isolated from *Atg16l1*^{VC} and *Atg16l1*^{fl/fl} littermates at day 14 after colitis
770 induction. Representative blot (E) and quantification of caspase 8 p43 (F) and
771 p18 (G) relative to tubulin.

772

773 Data are combined (B, F, G) or representative (C, D, E) from 3 independent
774 experiments. Each dot represents an individual mouse (F, G), or data are shown
775 as mean ± s.e.m (B). Scale bars are 100 µm. Statistical significance was
776 determined using two-way analysis of variance (ANOVA) with Bonferroni's
777 correction for multiple comparisons. Results shown for comparison between
778 *Atg16l1*^{VC} and *Atg16l1*^{VC} + αTNF group (B) or Mann Whitney test, ** p<0.01; ***
779 p<0.001, ****p<0.0001. *H.h.* – *Helicobacter hepaticus*, pi – post infection.

780 **STAR Methods**

781 **Contact for Reagent and Resource Sharing**

782 Further information and requests for resources and reagents should be directed
783 to and will be fulfilled by the Lead Contact, Dr. Kevin Maloy
784 (kevin.maloy@path.ox.ac.uk).

785

786 **Experimental model and subject details**

787 ***Mice***

788 *Atg16l1*^{fl/fl} mice and *Atg16l1*^{fl/fl} Villin-Cre (*Atg16l1*^{VC}) mice were generated and
789 provided by the H. Virgin laboratory (Washington University, Saint Louis, MO,
790 USA), as described (Hwang et al., 2012). *Atg16l1*^{fl/fl} mice were crossed to
791 C57BL/6J-Tg (Itgax-cre,-EGFP) and *Lyz2*^{tm1(cre)} to generate *Atg16l1*^{CD11c} and
792 *Atg16l1*^{LysM} mice. All above strains were bred and maintained under specific
793 pathogen-free conditions and tested negative for *Helicobacter* species. Mice were
794 housed in groups of 2-5 in individually ventilated cages (IVC) and were 6-12
795 weeks old when used. All procedures on mice were conducted in accredited
796 animal facilities at the University of Oxford (Oxford, UK) in accordance with the
797 UK Scientific Procedures Act (1986) under a project license (PPL 30/3423)
798 authorized by the UK Home Office Animal Procedures Committee and approved
799 by the local ethical review panel. Mice were bred hemizygous for the Cre allele,
800 resulting in *Atg16l1*^{fl/fl} Cre⁻ and *Atg16l1*^{fl/fl} Cre⁺ littermates. The colitis models
801 show some variability over time and between cages, which suggest a strong
802 contribution by the microbiota. Therefore, in all experiments age- and
803 sex-matched littermates were used that were kept co-housed throughout the
804 experiments. Mice were allocated to experimental groups according to genotype,

805 litter and cage, ensuring that in every cage and from every litter both genotypes
806 were present and treatment regimes were evenly distributed over cages.

807

808 ***Organoid culture***

809 Organoids were cultured as previously described by van den Brink and
810 colleagues (Heijmans et al., 2013). Colonic crypts were isolated from *Atg16l1^{fl/fl}*
811 mice or *Atg16l1^{vc}* mice and seeded in matrigel (Corning, Wiesbaden, Germany)
812 and cultured in the presence of N2 and B27 supplements (Fisher Scientific- UK
813 Ltd, Loughborough, UK), mEGF (Fisher Scientific; 50ng/ml), Y-27632 (Sigma-
814 Aldrich, St. Louis, US; 10uM), N-Acetylcysteine (Sigma-Aldrich, 1mM) and
815 conditioned medium containing Wnt3a (20%), Noggin (20%) and R-Spondin
816 (10%). The conditioned media were generated from Rspol-Fc Hek 293T cells
817 (kind gift from Dr. Calvin Kuo, Stanford) and Nog-Fc Hek 293T cells (kind gift
818 from Dr. Gjis van den Brink, Amsterdam) and Wnt3A L cells (ATCC® CRL-2647™).
819 After expansion by several passages organoids were seeded in 24 well plates for
820 experiments with indicated stimuli.

821 For bacterial infection experiments, organoids were trypsinized, filtered and
822 about 4 wells (from a 24-well plate) of organoids were seeded into one collagen-
823 coated 24-well plate well (Collagen-I, A1048301, ThermoFisher Scientific). The
824 resulting IEC monolayers were cultured in complete organoid medium for 2 days
825 prior to infection.

826

827

828

829

830 **Method details**

831 ***Colitis models***

832 *H. hepaticus* (NCI-Frederick isolate 1A) (Ward et al., 1994) was cultured in TSB
833 media supplemented with 10% FCS and trimethoprim (5µg/ml), vancomycin (10
834 µg/ml) and polymyxin B (25 IU/ml) (TVP, Oxoid) under microaerophilic
835 conditions (10% CO₂, 10% H₂, 80% N₂) (Song-Zhao and Maloy, 2014). Chronic
836 colitis was induced by infecting mice intragastrically with 10⁸ CFU *H. hepaticus* at
837 3 consecutive days and injecting intraperitoneally 1 mg of mAb anti-IL10R (clone
838 1B1.2) weekly. For protection experiments mice were treated with 1 mg of mAb
839 anti-TNF (XT3.11) weekly.

840 For infection experiments *C. rodentium* (ICC169) was grown in Luria Broth
841 supplemented with nalidixic acid in log phase to an OD of 1. Mice were infected
842 intragastrically with 5x10⁹ CFU *C. rodentium* (Song-Zhao et al., 2014).

843

844 ***TNF-induced enteropathy***

845 Mice were injected intraperitoneally with 10 µg of murine TNF (Peprotech) and
846 scarified after 6h for tissue isolation.

847

848 ***Histological assessment of intestinal inflammation***

849 Mice were sacrificed at indicated time points whereupon tissue sections were
850 fixed in buffered 10% formalin and paraffin-embedded. Histological analysis of
851 H&E stained sections for intestinal inflammation was performed as described
852 (Song-Zhao and Maloy, 2014). Briefly, inflammation was graded semi-
853 quantitatively on a scale from 0 to 3, for four criteria; (a) epithelial hyperplasia
854 and goblet cell depletion, (b) lamina propria leukocyte infiltration, (c) area of

855 tissue affected, and (d) markers of severe inflammation, including crypt
856 abscesses, sub- mucosal inflammation, and ulceration. Scores for individual
857 criteria were added up for an overall inflammation score between 0 and 12.
858 Scoring was performed by two scientists in a blinded fashion.

859

860 ***Isolation of cells and flow cytometry analysis and sorting***

861 For IEC preparations colons were isolated and digested in 5mM EDTA in RPMI
862 (5% FCS) for 10 min. Digests were vigorously shaken and cell suspension
863 filtered. Cell pellets were lysed in RLT (Qiagen, Manchester, UK) for RNA
864 preparation or RIPA buffer containing proteinase inhibitors (Roche) for western
865 blot analysis. Cell suspensions from spleen, mLN, and intestinal lamina propria
866 were prepared as previously described (Uhlig et al., 2006). For intracellular
867 cytokine staining cells were stimulated for 3h with PMA (100 ng/ml) and
868 Ionomycin (1 µg/ml) in the presence of Brefeldin A (10 µg/ml). For FACS
869 analysis the following antibodies from eBioscience (Hatfield, UK) were used:
870 anti-CD4 (GK1.5), anti-TCRβ (H57-597), anti-CD45 (30-F11), anti-GR.1 (RB6-
871 8C5), anti-Foxp3 (FJK-16s), anti-IFN-γ (XMG1.2), anti-IL-17A (eBio17B7). The
872 following antibodies were from BioLegend (San Diego, USA): anti-F4/80 (BMB),
873 anti-CD11b (M1/70). Fixable Viability Dye from eBioscience was used to stain
874 dead cells. For data acquisition a Cyan ADP was used (Beckman Coulter, High
875 Wycombe, UK) and analyzed using FlowJo software (Tree Star, Ashland, USA). To
876 verify excision efficiency of Atg16l1 in distinct cellular population of the
877 intestinal mucosa, cells were sorted with a MoFlo Astrios Sorter (Beckman
878 Coulter). LPL were stained with viability dye (eFluor 780), anti-CD45-APC, anti-
879 CD11c-Fitc, anti-MHC2-eFluor450, anti-CD11b-PerCP Cy5.5, anti-CD64-PE and

880 two populations were collected in RLT buffer: DC defined as live, CD45⁺, CD11c⁺
881 and MHC2⁺ and macrophages as live, CD45⁺, CD11b⁺ and CD64⁺.

882

883 ***Immunofluorescent stainings***

884 Colonic tissue samples were formalin-fixed and paraffin-embedded. Sections
885 were deparaffinized, rehydrated, proteinase k treated and stained according to
886 the *in situ* cell death detection kit, Fluorescein (Sigma-Aldrich). Counterstaining
887 was performed with Wheat Germ Agglutinin, Alexa Fluor 647 Conjugate (Fisher
888 Scientific) and DAPI (Sigma-Aldrich). Slides were mounted with ProLong Gold
889 Antifade Mountant (Fisher Scientific).

890 For immunofluorescent staining of organoids, cells were grown on cover slips.
891 Following stimulation cells were fixed with 4% paraformaldehyde at room
892 temperature followed by methanol treatment at -20C. Cells were blocked in 5%
893 goat serum, 5% BSA and 0.5% saponine. Stainings were performed with pAb
894 mouse-anti-Ecadherin (BD Bioscience), rabbit-anti-phospho Stat 3, mouse-anti
895 Stat 3 and rabbit-anti-cleaved caspase 3 from Cell Signalling (Danvers, USA) and
896 goat-anti-rabbit Alexa Fluor 555 or goat-anti-mouse Alexa Fluor 488 (Fisher
897 Scientific). Images were acquired with an Olympus Fluoview FV1000 confocal
898 microscope and Olympus Fluoview Software (Olympus, Tokyo, Japan). One
899 representative image of the TUNEL stained sections per mouse was taken after
900 careful screening of all sections while blinded for the genotype. We then grouped
901 the images according to the treatment/ genotype and a different still blinded
902 scientist in terms of genotype or treatment chose the representative image per
903 group.

904

905 ***Western blot analysis***

906 For immunoblot analysis cells were lysed after indicated times in RIPA buffer
907 and 1-5 ug total protein were analyzed per lane. For detection the following
908 primary antibodies were used: anti-LC3 antibody (L7543; Sigma-Aldrich) anti-
909 phospho-Stat3, anti-Stat3, anti-phospho-ERK1/2, anti-phospho-Mek, anti-
910 phospho-p65, anti-p65, anti-phospho-eIF2a, anti-eIF2a, anti-phospho-p38, anti-
911 cleaved caspase 8, anti-cleaved caspase 3, (all antibodies from Cell Signalling)
912 and anti-tubulin antibody (sc5286, Santa Cruz Biotechnology, Dallas, USA), and
913 secondary HRP conjugated anti-rabbit or anti-mouse antibody (Cell Signalling).

914

915 ***Cell death and metabolic assay***

916 For cell death detection the ApoTox Glo Assay from Promega (Southampton, UK)
917 was used. Organoids were seeded in 4ul matrigel in a 96 well pate; following
918 stimulation the ApoTox Glo Assay was used according to manufactures
919 instructions.

920 For the assessment of metabolic activity with AlamarBlue (Fisher Scientific) as a
921 correlate of cell viability organoids were seeded in 4ul matrigel in a 96 well
922 plate. Organoids were incubated with AlamarBlue for 3h prior to stimulation to
923 generate reference metabolic activity. Fluorescent intensity was measured at
924 540nm/ 590nm. Following stimulation same procedure was repeated and values
925 were normalized to untreated values.

926

927 ***RNA and qPCR***

928 For qPCR analysis cells were lysed in RLT and RNA purified using the RNeasy kit
929 from Qiagen. 1-2 µg of RNA were reverse transcribed with SuperScript III

930 reverse transcriptase (Fisher Scientific) and quantitative real-time PCR was
931 carried out with the StepOnePlus Real time system from Applied Biosystems.
932 RNA from sorted cell populations was purified with the RNeasy Micro kit
933 (Qiagen) and all RNA was transcribed. qPCR Mastermix (Eurogentec, Liege,
934 Belgium) was used for Taqman reactions and iQ Sybr Green Supermix (Biorad,
935 Kidlington, UK) for Sybr green reactions. Taqman probes for *Cxcl2*
936 (Mm_00436450_m1), *Atg16l1* (Mm_00513084_m1) and *Hprt*
937 (Mm_03024075_m1) were from Applied Biosystems and primers for *Grp78*
938 (forwards: acttggggaccacattctt; reverse: atcgcaatcagacgctcc) for Sybr green
939 PCR from Sigma-Aldrich. Relative expression was analysed according to the Pfaffl
940 method (Pfaffl, 2001).

941

942 ***Organ explants***

943 Pieces of 10-20 mg of colon or caecum were isolated and cultured in complete
944 RPMI (10% FCS, 5 μ M β -mercaptoethanol (Fisher Scientific), 1x
945 Penicillin/Streptomycin (Fisher Scientific), 1x Glutamine (Fisher Scientific)) for
946 20h. Supernatants were tested for cytokine levels with the Luminex Multiplex
947 Assay (Thermo Fisher Scientific) and normalised to input tissue weight.

948

949 ***Generation of inflammatory conditioned medium (iCM)***

950 Lamina propria leukocytes were isolated from C57Bl/6 mice at 2 weeks of *H.h.* +
951 α IL10R colitis and 2.3x10⁶ cell/ml were seeded in complete RPMI for 20h.
952 Supernatants from cells of 7 mice were pooled and frozen at -80C.

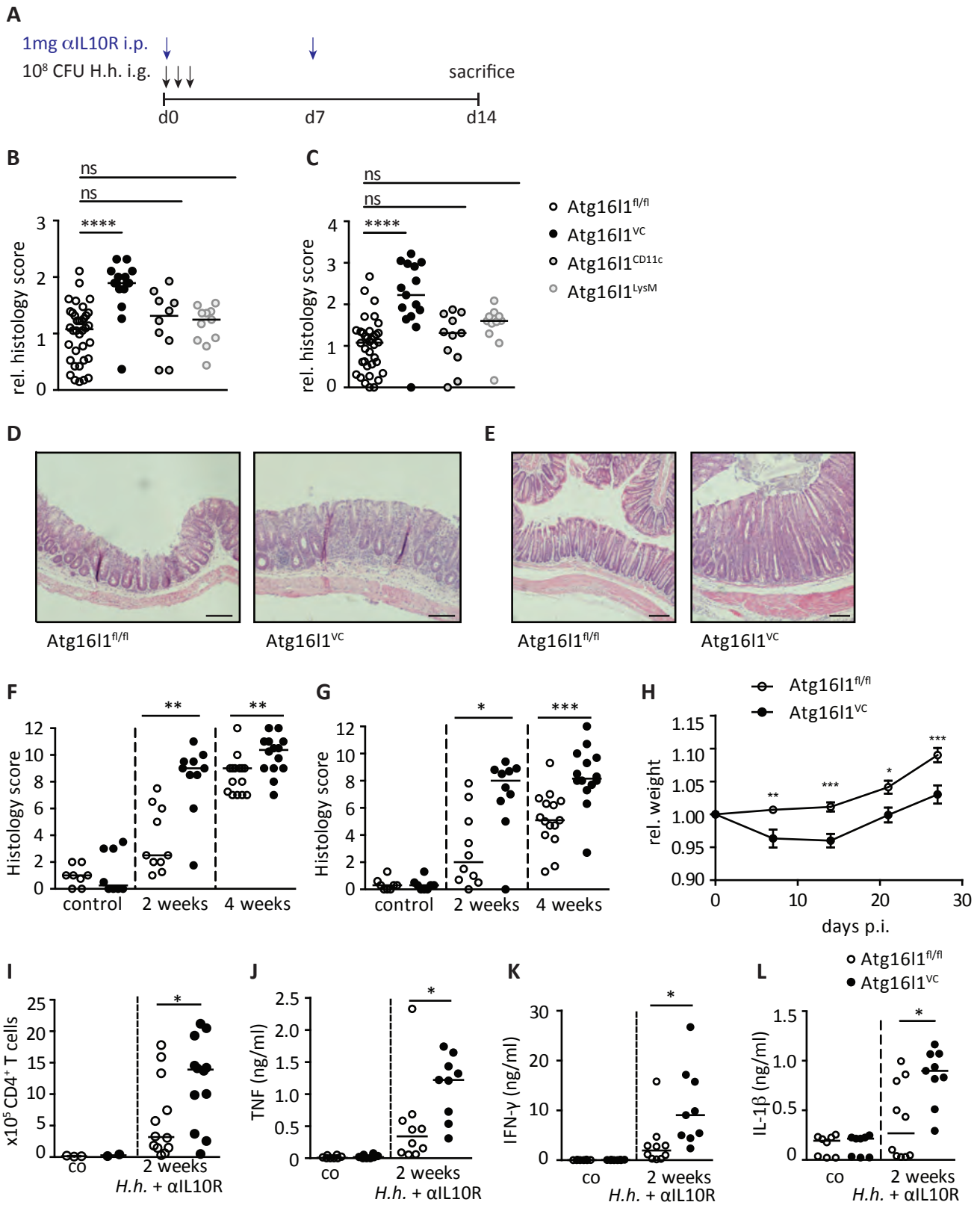
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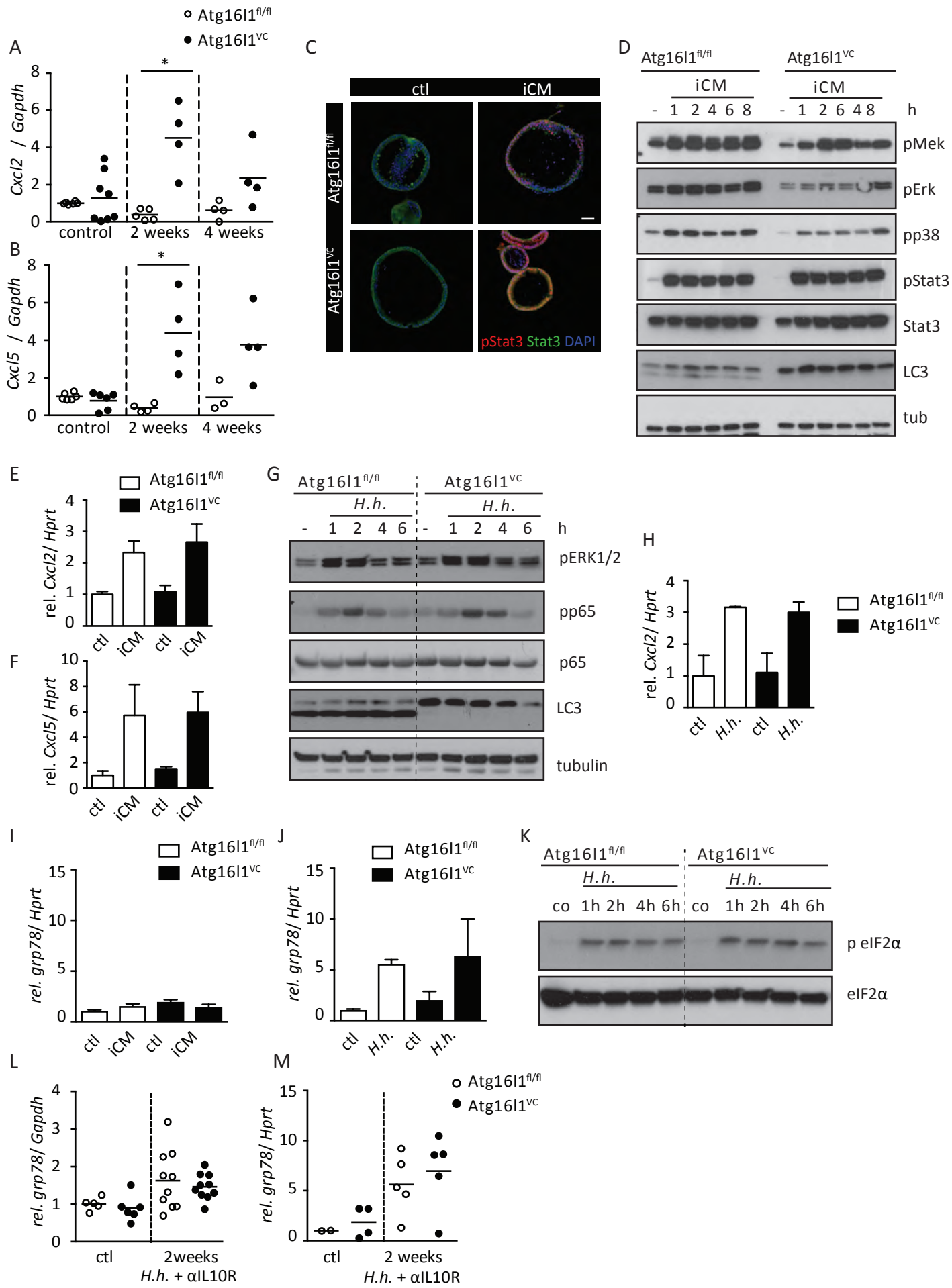
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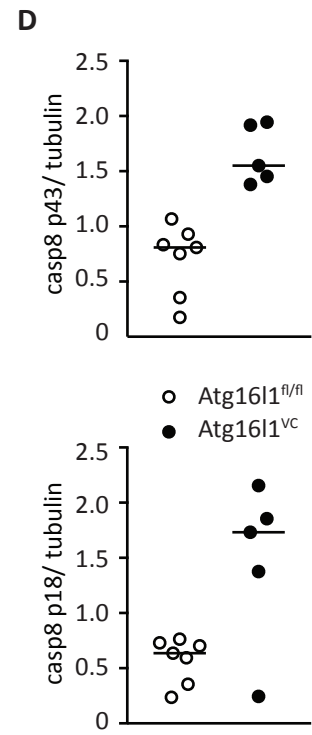
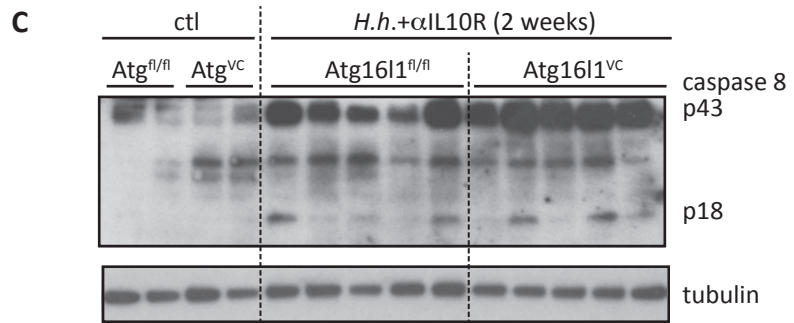
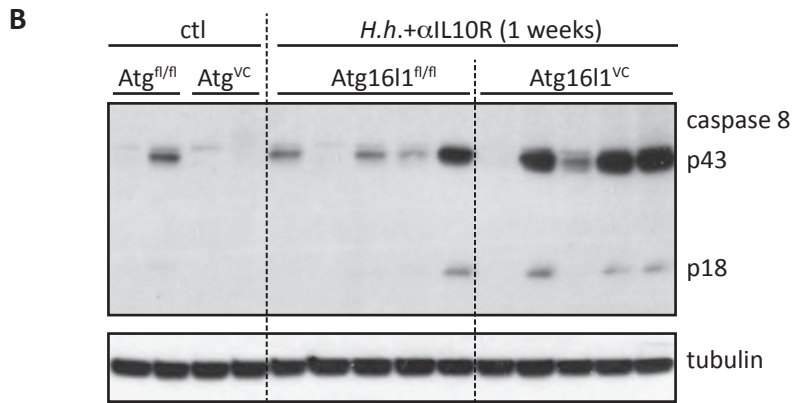
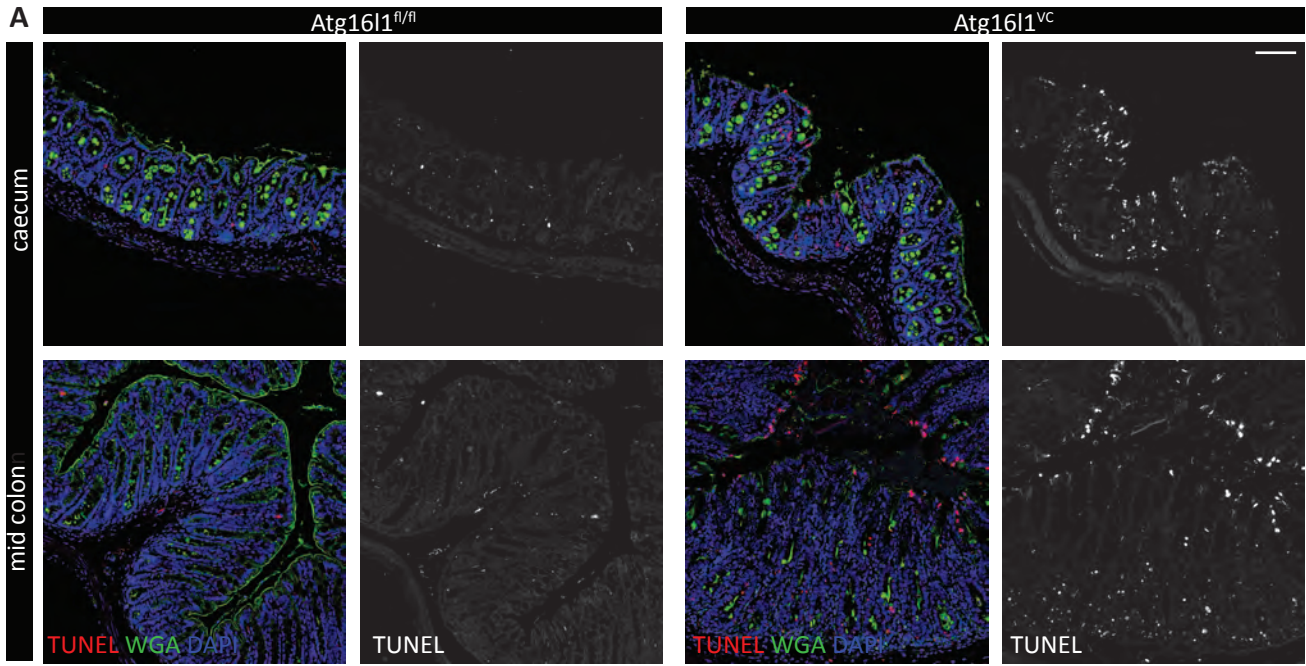
955 **Quantification and statistical analysis**

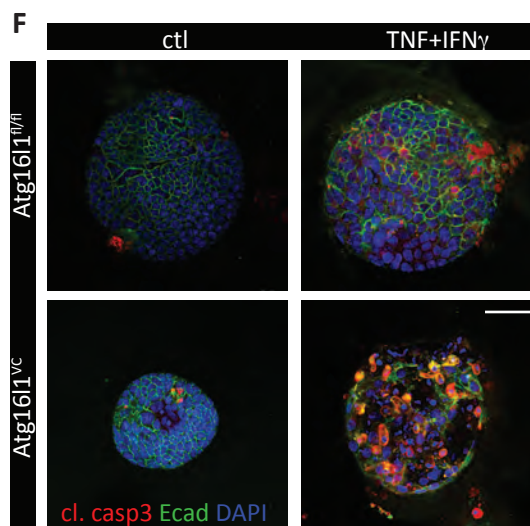
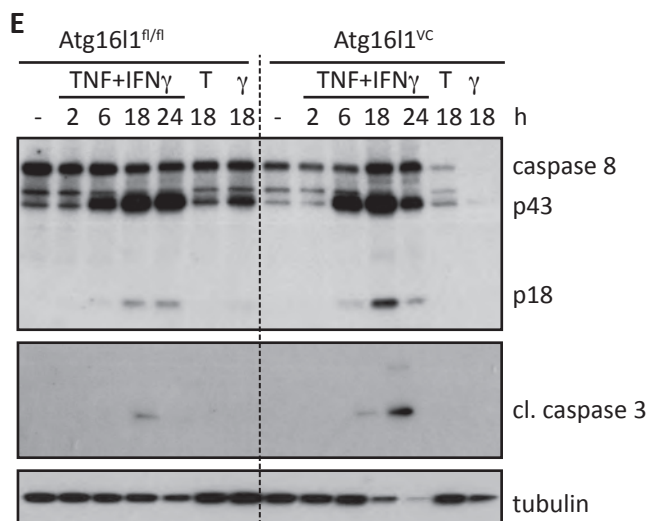
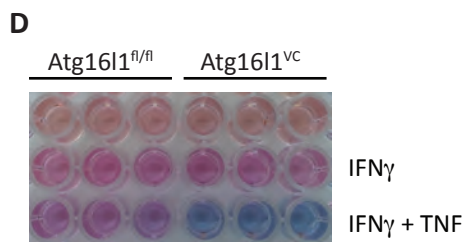
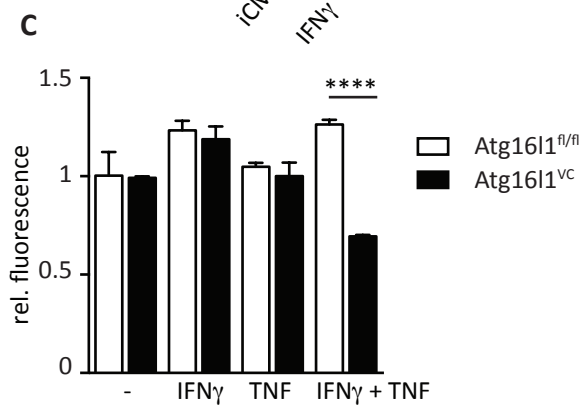
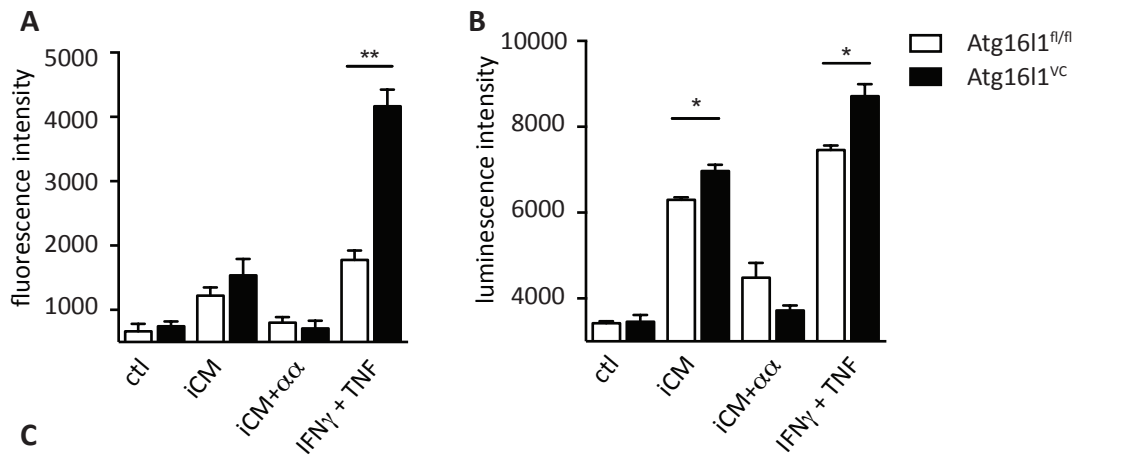
956 For weight curves p-values were determined by two-way ANOVA with
957 Bonferroni post-tests. For other experiments, p-values were determined by
958 nonparametric Mann–Whitney test, or by Kruskal-Wallis test with correction for
959 multiple comparisons. Differences were considered statistically significant when
960 $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Statistics were calculated using
961 GraphPad Prism 6 software. The statistical tests applied, definition of center,
962 dispersion and precision measures depicted, as well as the number of
963 experimental repeats are specified in the figure legends.

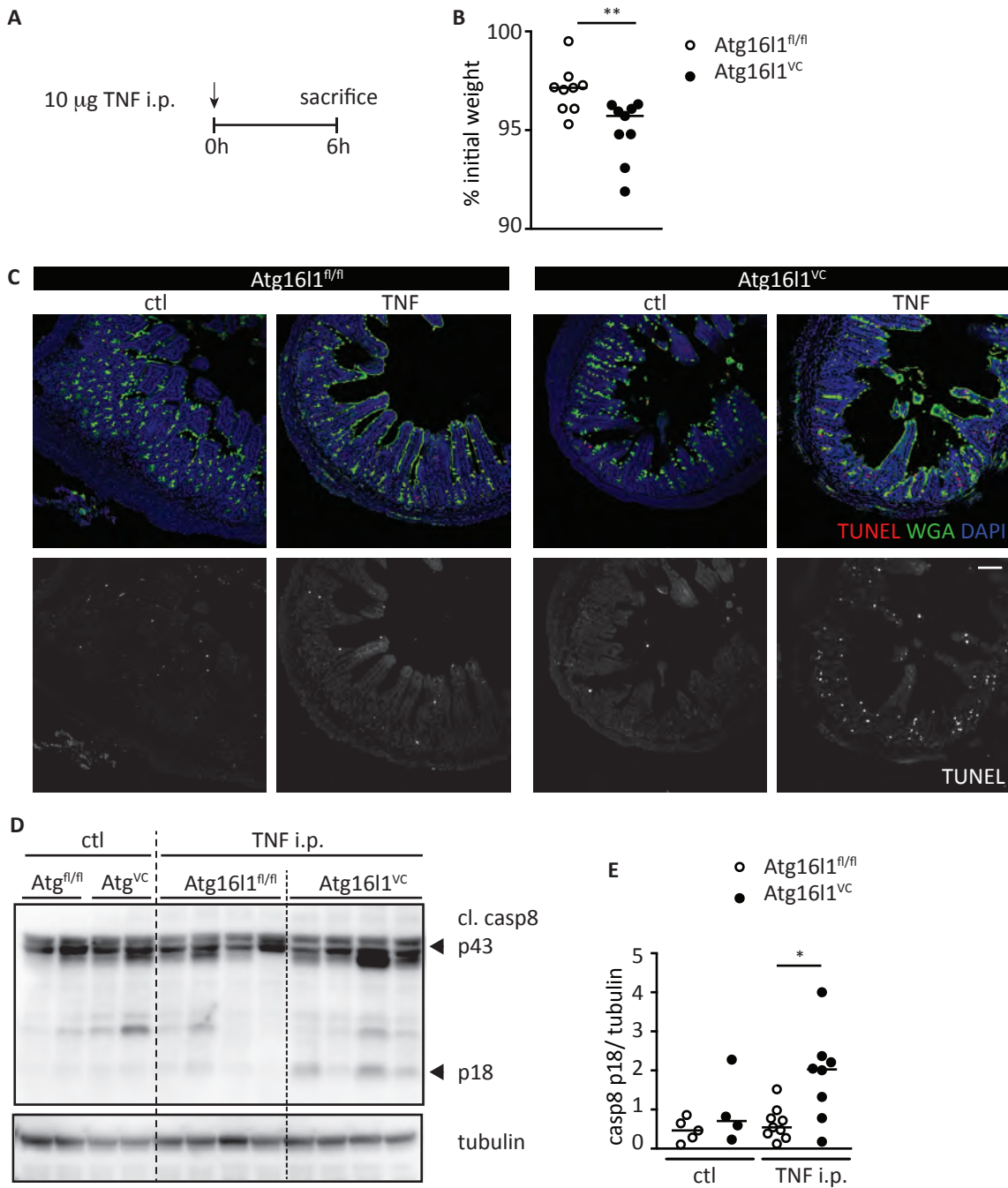
964 For *in vivo* experiments, sample size was determined by power analysis using
965 power of trial software, which calculates a power value based on X^2 test
966 statistics. Calculated required sample sizes were applied whenever possible.

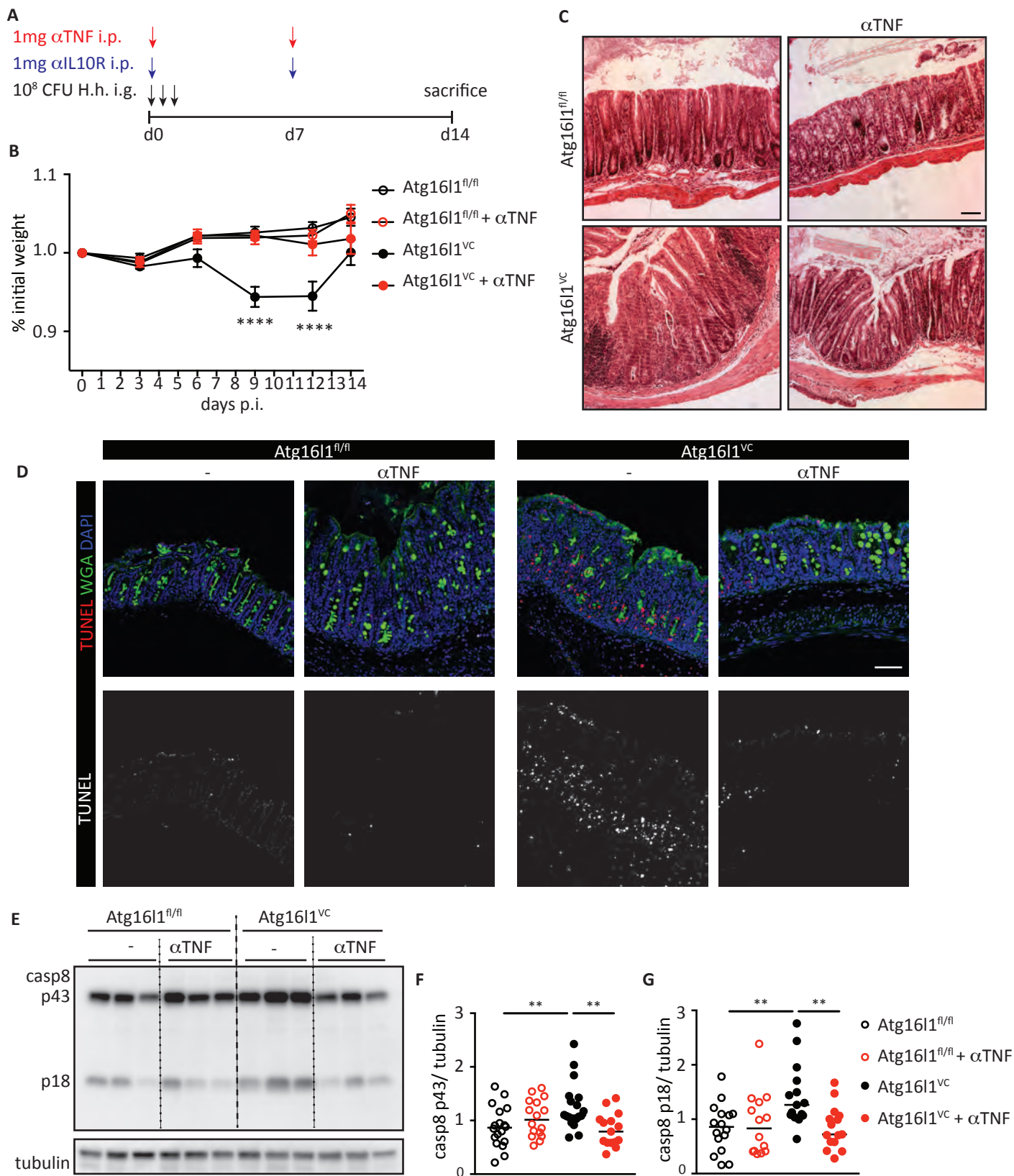












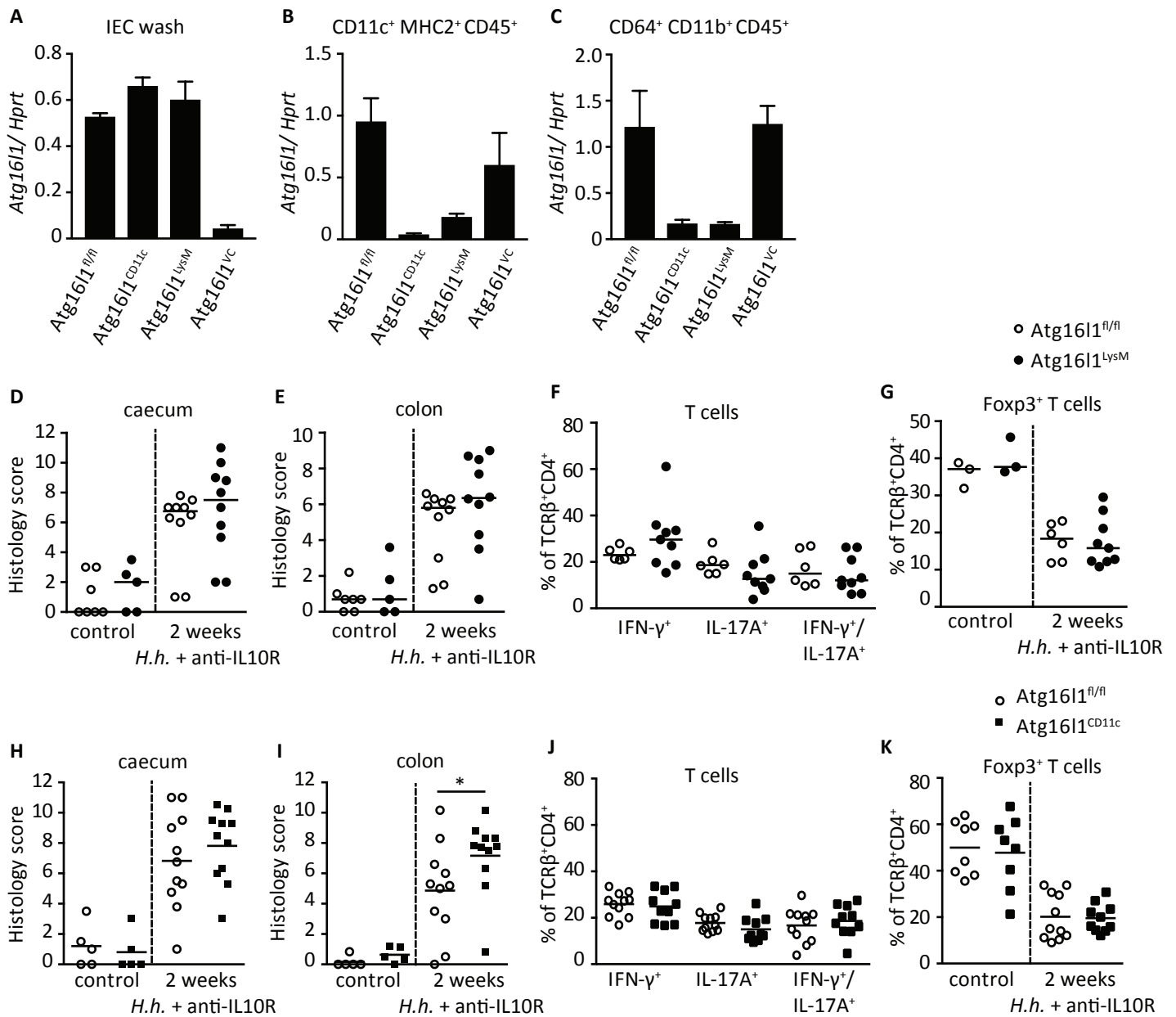


Figure S1. Related to Figure 1. Detailed analysis of *Atg16l1*^{LysM}, *Atg16l1*^{CD11c} and *Atg16l1*^{VC} mice.

A-C) Verification of *Atg16l1* deletion in distinct compartments of the intestinal mucosa in *Atg16l1*^{CD11c}, *Atg16l1*^{LysM}, *Atg16l1*^{VC} and *Atg16l1*^{fl/fl} mice. qPCR analysis of *Atg16l1* expression from IEC washes (A) or on sorted DC (B) and macrophage (C) populations from LPL. *Atg16l1*^{CD11c}, *Atg16l1*^{LysM} or *Atg16l1*^{fl/fl} mice were orally infected with 10⁸ CFU *H.h.* on 3 consecutive days and injected with 1 mg anti-IL10R weekly and sacrificed at day 14 after colitis induction. D, E) Histopathology scores of caecum (D) and colon (E) samples from *Atg16l1*^{LysM} and *Atg16l1*^{fl/fl} littermates. F) Frequencies of cytokine positive CD4⁺TCRβ⁺ T cells in PMA, Ionomycin re-stimulated colonic LPLs from *Atg16l1*^{LysM} and *Atg16l1*^{fl/fl} littermates. G) Frequencies of Foxp3⁺ CD4⁺TCRβ⁺ T cells in colonic LPLs from *Atg16l1*^{LysM} and *Atg16l1*^{fl/fl} littermates. H, I) Histopathology scores of caecum (H) and colon (I) samples from *Atg16l1*^{CD11c} and *Atg16l1*^{fl/fl} littermates. J) Frequencies of cytokine positive CD4⁺TCRβ⁺ T cells in PMA, Ionomycin re-stimulated colonic LPLs from *Atg16l1*^{CD11c} and *Atg16l1*^{fl/fl} littermates. K) Frequencies of Foxp3⁺ CD4⁺TCRβ⁺ T cells in colonic LPLs from *Atg16l1*^{CD11c} and *Atg16l1*^{fl/fl} littermates.

Data are combined from 2 independent experiments (A-K). Bars represent means with SEM (A-C) or each dot represents individual mouse (D-K). Horizontal bars denote medians. Statistical significance was determined using Mann Whitney test, * p<0.05; *H.h.* – *Helicobacter hepaticus*, pi – post infection.

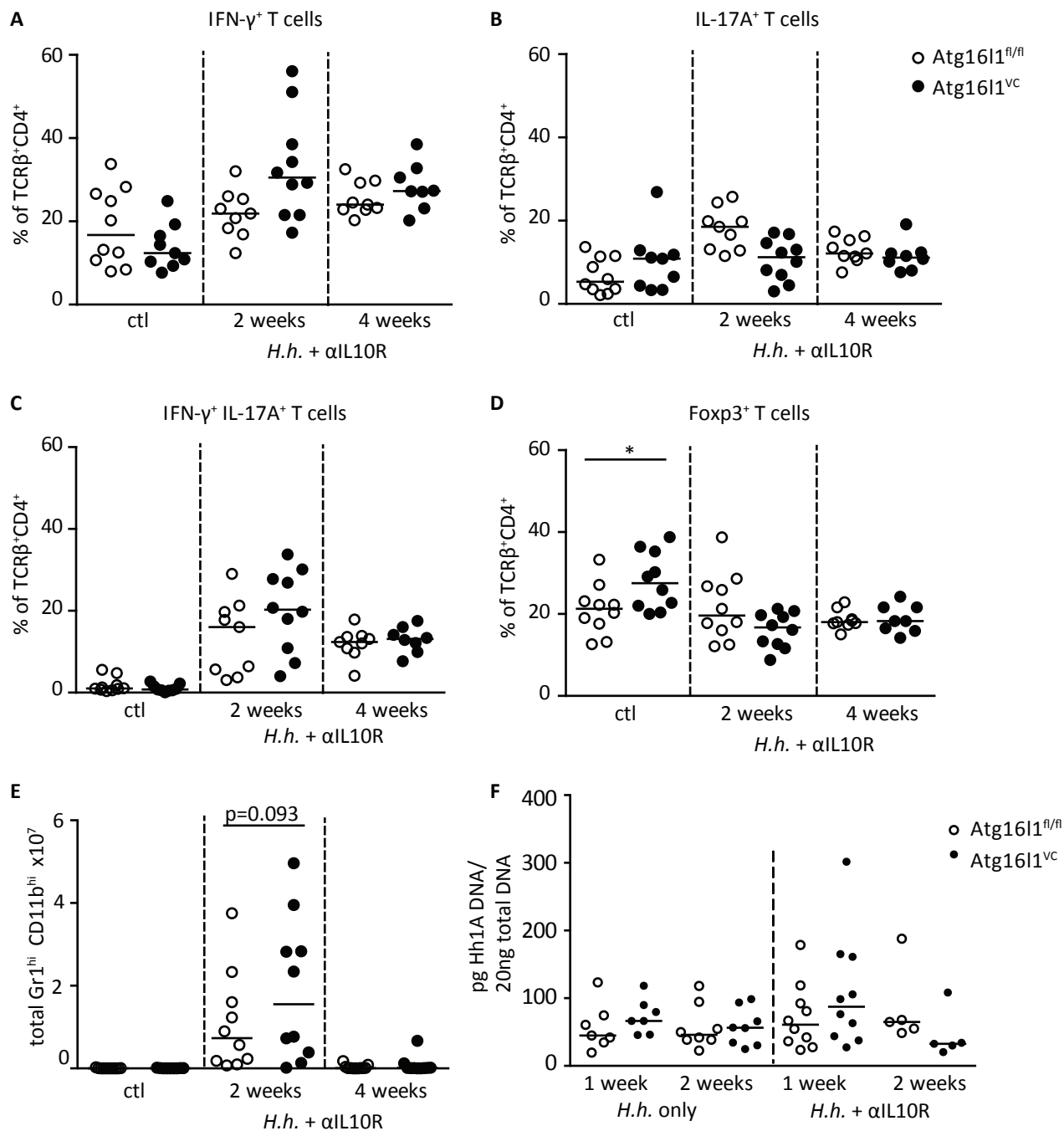


Figure S2. Related to Figure 1. Lamina propria leukocyte analysis of *Atg16l1*^{VC} mice.

Atg16l1^{VC} and *Atg16l1*^{fl/fl} littermates were orally infected with 10⁸ CFU *H.h.* on 3 consecutive days and injected with 1 mg anti-IL10R weekly. LPLs were analyzed by FACS at 2 weeks and 4 weeks after colitis induction. A-C) Prior to FACS staining LPLs were restimulated with PMA, Ionomycin and Brefeldin A for 3 h. Frequencies of IFN- γ ⁺ (A), IL-17A⁺ (B) and IFN- γ ⁺ IL17A⁺ cells of CD4⁺TCR β ⁺ T cells (C). D) Frequencies of Foxp3⁺ cells of CD4⁺TCR β ⁺ T cells. E) Total numbers of Gr1^{high} CD11b^{high} cells in LPLs. F) *H.h.* DNA quantities were determined by qPCR in caecal content of *Atg16l1*^{VC} and *Atg16l1*^{fl/fl} littermates infected with *H.h.* only or treated with anti-IL10R weekly.

Data are combined from at least 3 independent experiments (A-E) or from 2 independent experiments (F). Each dot represents an individual mouse (A-F). Horizontal bars denote medians. Statistical significance was determined using Mann Whitney test, * p<0.05; ctl – control, *H.h.* – *Helicobacter hepaticus*, p.i. – post infection.

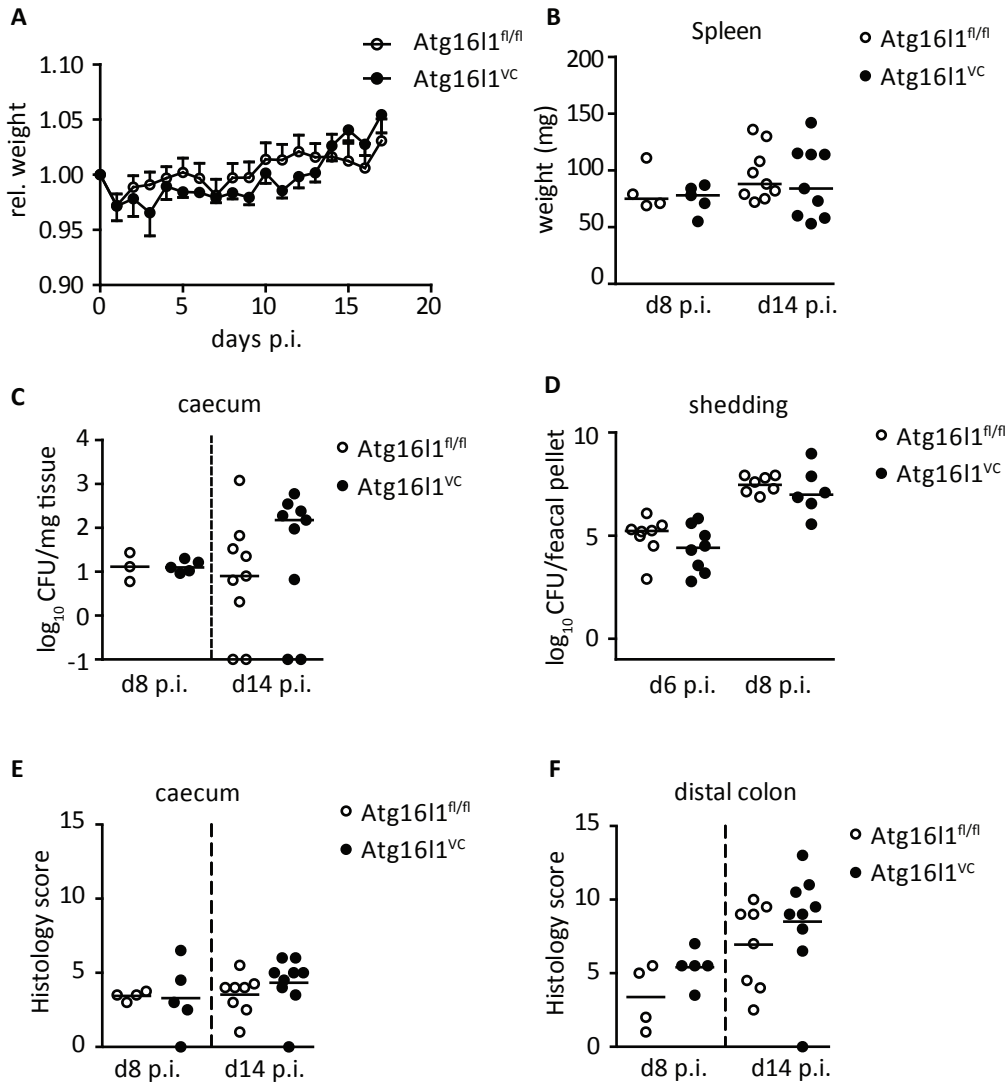


Figure S3. Related to Figure 1. *Atg16l1^{VC}* mice do not show increased susceptibility to *Citrobacter rodentium* infection. *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* littermates were orally infected with 10^9 CFU *C. rodentium*. A) Weight was followed over 18 days. B) Spleen weights at day 8 and day 14 p.i.. C) Tissue adherent CFU in caecal tissue and D) CFU in faecal pellets of *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* littermates at day 8 and day 14 p.i.. E, F) Histopathology scores of the caecum (E) and distal colon (F) of *Atg16l1^{VC}* and *Atg16l1^{fl/fl}* littermates at day 8 and day 14 p.i..

Data are combined from 2 independent experiments (A-F). Each dot represents an individual mouse (B-F) or mean \pm S.E.M. (A). Horizontal bars denote medians. Statistical significance was determined using Mann Whitney test. CFU- colony forming units, p.i. – post infection.

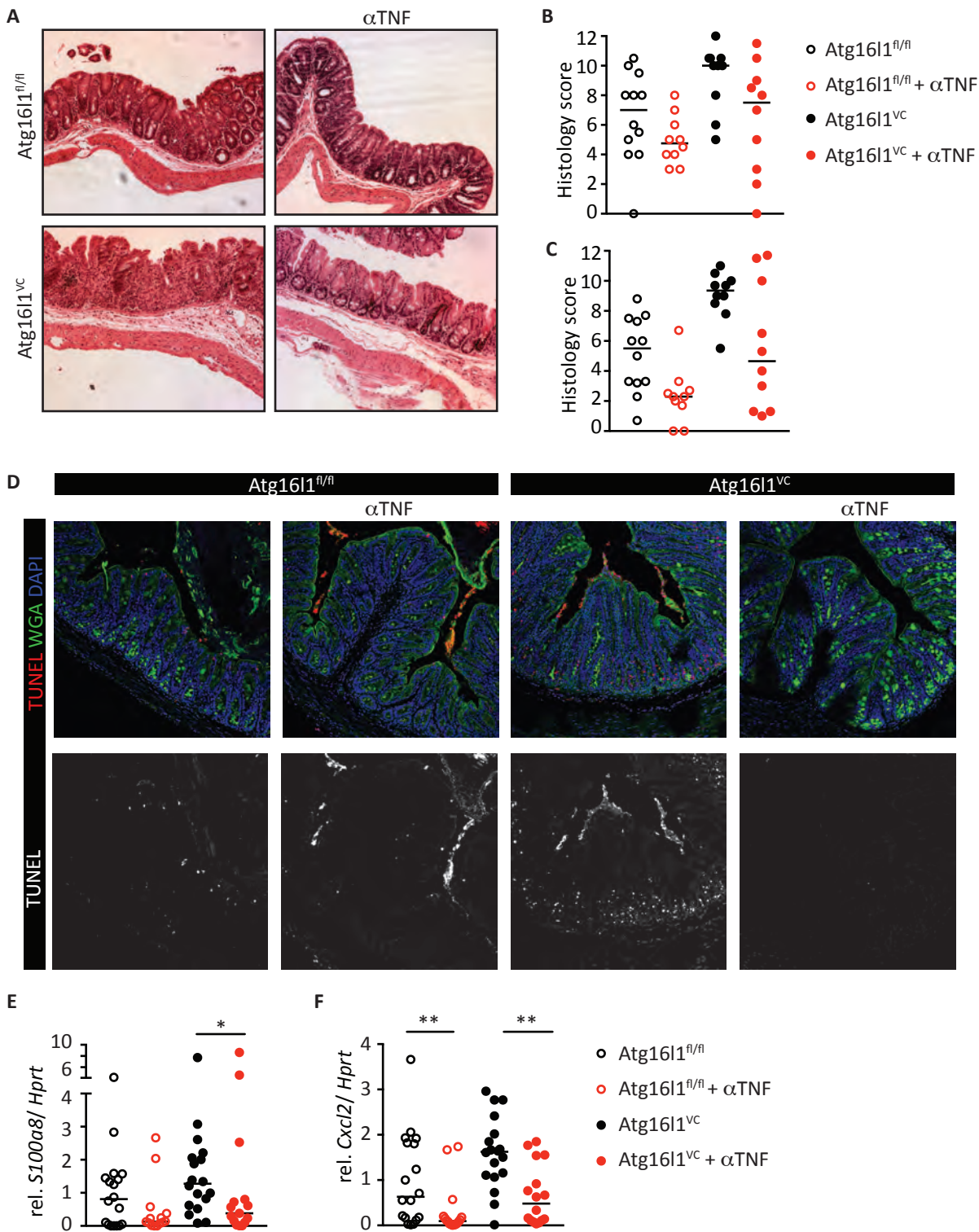


Figure S4. Related to Figure 6. TNF blockade attenuates colitis and epithelial apoptosis in *Atg161^{vc}* mice.

Atg161^{vc} and *Atg161^{fl/fl}* littermates were orally infected with 10^8 CFU *H.h.* on 3 consecutive days and treated weekly with 1 mg anti-IL10R i.p. with indicated groups also receiving 1 mg anti-TNF i.p. at day 0 and day 6. Mice were sacrificed at day 14 after colitis induction. A) Representative micrographs of H&E stained caecal sections of *Atg161^{vc}* and *Atg161^{fl/fl}* littermates. B) Histopathology scores of the caecum (B) and colon (C) of samples from *Atg161^{vc}* and *Atg161^{fl/fl}* littermates. D) Representative images of TUNEL (red) staining of colon sections, counterstaining with WGA (green) and DAPI (blue). E, F) qPCR analysis of expression of *S100a8* (E) and *Cxcl2* (F) in isolated IECs from *Atg161^{vc}* and *Atg161^{fl/fl}* littermates. Results were normalized to *Hprt* expression and expressed relative to the *Atg161^{fl/fl}* group.

Data are combined (B, C, E, F) or representative (A, D) of 3 independent experiments. Each dot represents an individual mouse (B, C, E, F). Horizontal bars denote medians. Statistical significance was determined using Mann Whitney test, * $p < 0.05$; ** $p < 0.01$. *H.h.* – *Helicobacter hepaticus*, p.i. – post infection.

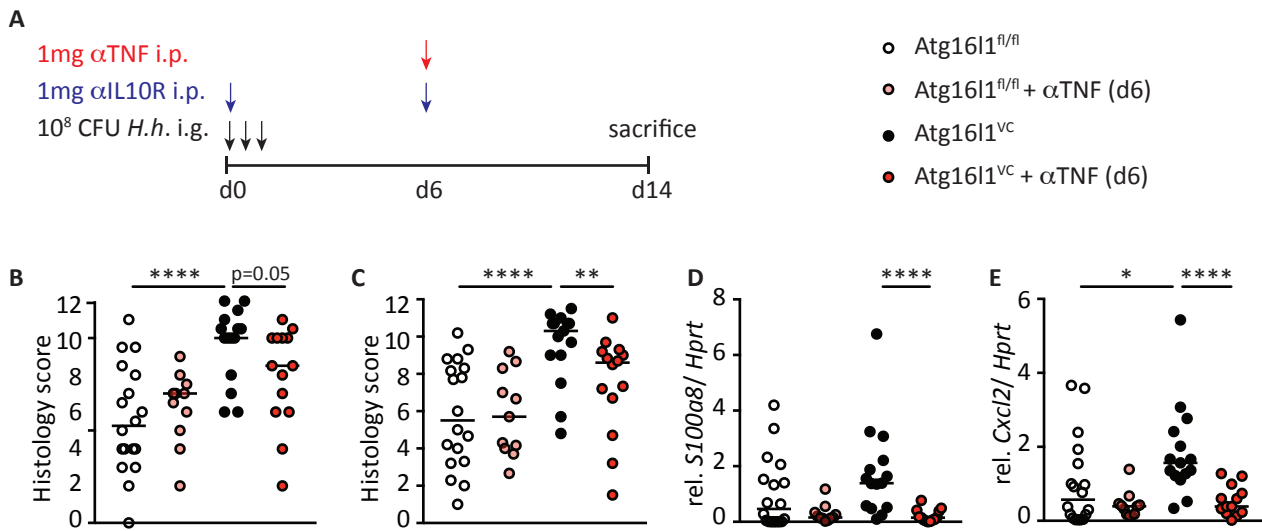


Figure S5. Related to Figure 6. α TNF therapy attenuates pathology in *Atg16l1*^{vc} mice.

Atg16l1^{vc} and *Atg16l1*^{fl/fl} littermates were orally infected with 10^8 CFU *H.h.* on 3 consecutive days and treated weekly with 1 mg anti-IL10R i.p. with indicated groups also receiving 1 mg anti-TNF i.p. at day 6 after colitis induction. Mice were sacrificed at day 14 after colitis induction. A) Schematic of the treatment protocol. B,C) Histopathology scores of the caecum (B) and colon (C) samples. D,E) Gene expression analysis of isolated IECs for *S100a8* (D) and *Cxcl2* (E). Results were normalized to *Hprt* expression and expressed relative to the *Atg16l1*^{fl/fl} group.

Data are combined from 3 independent experiments (B-E). Each dot represents an individual mouse (B-E). Horizontal bars denote medians. Statistical significance was determined using Mann Whitney test, * p<0.05; ** p<0.01; **** p<0.0001. *H.h.* – *Helicobacter hepaticus*.