The autophagy gene Atg16l1 differentially regulates T_reg and TH2 cells to control intestinal inflammation

Agnieszka M Kabat1, Oliver J Harrison1,2‡, Thomas Riffelmacher3,4‡, Amin E Moghaddam1, Claire F Pearson5, Adam Laing6, Lucie Abeler-Dörner6, Simon P Forman7, Richard K Grencis7, Quentin Sattentau1, Anna Katharina Simon3,4, Johanna Pott1*†, Kevin J Maloy1*†

1Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom; 2Immunity at Barrier Sites Initiative, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, United States; 3MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom; 4John Radcliffe Hospital, University of Oxford, Oxford, United Kingdom; 5Kennedy Institute of Rheumatology, University of Oxford, Oxford, United Kingdom; 6Peter Gorer Department of Immunobiology, King’s College London, London, United Kingdom; 7Faculty of Life Sciences, The University of Manchester, Manchester, United Kingdom

Abstract A polymorphism in the autophagy gene Atg16l1 is associated with susceptibility to inflammatory bowel disease (IBD); however, it remains unclear how autophagy contributes to intestinal immune homeostasis. Here, we demonstrate that autophagy is essential for maintenance of balanced CD4+ T cell responses in the intestine. Selective deletion of Atg16l1 in T cells in mice resulted in spontaneous intestinal inflammation that was characterized by aberrant type 2 responses to dietary and microbiota antigens, and by a loss of Foxp3+ T_reg cells. Specific ablation of Atg16l1 in Foxp3+ T_reg cells in mice demonstrated that autophagy directly promotes their survival and metabolic adaptation in the intestine. Moreover, we also identify an unexpected role for autophagy in directly limiting mucosal TH2 cell expansion. These findings provide new insights into the reciprocal control of distinct intestinal TH cell responses by autophagy, with important implications for understanding and treatment of chronic inflammatory disorders.

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Introduction Crohn’s disease (CD) and ulcerative colitis (UC) are the two most common forms of inflammatory bowel disease (IBD), characterized by chronic inflammation of the gastrointestinal tract. IBD is a complex multifactorial disease that emerges on a background of many genetic and environmental factors (Maloy and Powrie, 2011). In recent years, tremendous efforts have been undertaken to identify the genetic factors that influence susceptibility to IBD. In particular, genome-wide association studies (GWAS) and subsequent meta-analyses have identified over 150 distinct loci that influence IBD susceptibility, many of which have revealed novel pathways in disease pathogenesis (Van Limbergen et al., 2014). Among these, a single-nucleotide polymorphism (SNP) in the essential macroautophagy (hereafter called ‘autophagy’) gene ATG16L1 was associated with an increased risk of CD (Hampe et al., 2007; Rioux et al., 2007). A recent study showed that the IBD-predisposing T300A mutation in the coding region of ATG16L1 led to increased degradation of ATG16L1 protein and reduced autophagy (Murthy et al., 2014), indicating that decreased autophagy may contribute to
IBD development. Polymorphisms in several other autophagy-related genes, including IRGM, LRRK2 and SMURF1, are also linked to IBD susceptibility (Van Limbergen et al., 2014), suggesting that changes in the autophagy pathway alter intestinal homeostasis and predispose to chronic intestinal inflammation.

Autophagy is a highly conserved cellular process that targets cytoplasmic components for lysosomal degradation and maintains homeostasis by recycling damaged organelles and large cytoplasmic protein aggregates. Autophagy becomes particularly important during metabolic or infectious stress (Mizushima, 2007). Atg16l1 forms an essential autophagy complex with Atg5 and Atg12 that facilitates elongation of the initial isolation membrane that results in engulfment of the cargo and formation of the autophagosome. Subsequent fusion with the lysosome facilitates degradation and allows nutrient recycling (Mizushima et al., 2003). To identify the mechanisms through which autophagy may regulate intestinal tissue homeostasis, it is essential to understand the functional consequences of alterations in autophagy on both immune and tissue cells present in the gut.

To date, several studies have examined the role of autophagy and Atg16l1 in intestinal epithelial cells and myeloid cells for intestinal homeostasis. In these studies, Atg16l1 was shown to play a role in Paneth cell physiology, as well as in bacterial handling and regulation of inflammatory IL-1β secretion by myeloid cells (Cadwell et al., 2008; Kuballa et al., 2008; Saitoh et al., 2008; Plantinga et al., 2011). However, the role of Atg16l1 in intestinal adaptive immune responses has not yet been addressed.

CD4+ T cells constitute the largest population of intestinal lymphocytes and are central mediators of host protective and tolerogenic responses in the gut (Shale et al., 2013). In particular, thymus-derived and peripherally induced Foxp3+ CD4+ regulatory T cells (Treg and pTreg cells, respectively) are indispensable in promoting tolerance toward commensal and dietary antigens and for the prevention of aberrant effector T cell responses, including Th1, Th2 and Th17 cell responses (Izcue et al., 2009). An imbalance between effector and regulatory CD4+ T cells can promote chronic intestinal inflammation and accumulation of effector CD4+ T cells in the inflamed mucosa is a cardinal feature of IBD (Abraham and Cho, 2009; Maloy and Powrie, 2011; Shale et al., 2013).
Therefore, it is important to define factors that regulate aberrant CD4+ T cell responses in the gastrointestinal tract.

Previous studies utilizing mice with T-cell-specific deletion of essential autophagy genes (Atg3, Atg5, Atg7, Beclin1) pointed to a key role of autophagy in T cell homeostasis, as these mice exhibited decreased frequencies and numbers of CD4+ and CD8+ T cells and defects in T cell proliferation in vitro (Pua et al., 2009; Stephenson et al., 2009; Jia and He, 2011; Kovacs et al., 2012). In addition, recent studies highlighted the importance of autophagy in the development of memory CD8+ T cells (Puleston et al., 2014; Xu et al., 2014; Schlie et al., 2015). However, the exact requirements for autophagy during different stages of T cell activation and differentiation remain poorly understood (Xu et al., 2014). Given that the gastrointestinal tract is a site of continuous immune activation by external antigens and is therefore a challenging environment for the adaptive immune system, we hypothesized that a selective defect in autophagy may affect intestinal T cell homeostasis.

We investigated the role of Atg16l1 in intestinal CD4+ T cells by generating mice that selectively lack Atg16l1 in T cells. Here, we show that T-cell-specific deletion of Atg16l1 results in chronic intestinal inflammation accompanied by increased humoral responses toward commensal and dietary antigens. We further demonstrate that Atg16l1-deficiency has opposing effects on intestinal CD4+ T cells subsets; markedly enhancing T_{reg}2 responses whilst decreasing T_{reg} cell numbers. Through selective ablation of Atg16l1 in T_{reg} cells, we established the importance of cell-intrinsic autophagy for intestinal T_{reg} cell homeostasis. Furthermore, through complementary in vivo approaches we show that autophagy controls T_{reg}2 responses through two distinct mechanisms; through a cell-intrinsic pathway and by promoting extrinsic regulation by T_{reg} cells.

**Results**

**Selective deletion of Atg16l1 in T cells results in spontaneous intestinal pathology**

To investigate the role of autophagy in intestinal T cell homeostasis, mice carrying loxP-flanked alleles of the essential autophagy gene Atg16l1 (Atg16l1fl/fl) (Hwang et al., 2012) were crossed with CD4-Cre mice, generating Atg16l1fl/fl::CD4-Cre mice (hereafter denoted as Atg16l1^CD4^) in which Atg16l1 is selectively ablated in T cells from the double-positive stage of thymic development. To verify functional deletion of Atg16l1 autophagy levels were analyzed by autophagosome formation and LC3 lipidation. CD4+ T cells isolated from control Atg16l1^fl/fl^ mice exhibited increased LC3+ autophagosome formation after activation, as measured by intracellular LC3 accumulation in the presence of a lysosomal inhibitor (Figure 1A). In contrast, there was no increase in intracellular LC3 accumulation in CD4+ T cells from Atg16l1^CD4^ mice (Figure 1A). To verify this finding using another method, we assessed LC3 lipidation by Western blot analysis (Klionsky et al., 2012). Activated control Atg16l1^fl/fl^ CD4+ T cells exhibited increased lipidated LC3 II levels in the presence of chloroquine, indicative of autophagy-mediated turnover of LC3 II after T cell activation (Figure 1B). However, LC3 II levels in CD4+ T cells from Atg16l1^CD4^ mice were barely affected by activation (Figure 1B), confirming a block in autophagy.

Young Atg16l1^CD4^ mice appeared normal, initially gained weight in a manner comparable to Atg16l1^fl/fl^ littermates and exhibited normal intestinal morphology (Figure 1C,F-H). However, from around 5 months of age, Atg16l1^CD4^ mice stopped gaining weight (Figure 1C), developed splenomegaly and lymphadenopathy (Figure 1D,E) and chronic intestinal pathology that progressed with age (Figure 1F–I). Atg16l1^CD4^ mice exhibited significant inflammation of both the small intestine (SI) and colon, characterized by increased SI length, marked lengthening of crypts, shortening of villi and epithelial hyperplasia (Figure 1F–I). Thus, T-cell-specific Atg16l1 deletion resulted in spontaneous intestinal inflammation and systemic immune activation.

**Atg16l1 deficiency has opposing effects on intestinal T_{reg} and T_{H}2 cells**

To characterize the effects of Atg16l1 on intestinal and systemic T cell homeostasis independently from any confounding effects of ongoing tissue inflammation, we analyzed young (8–12 weeks old) Atg16l1^CD4^ mice before the onset of inflammatory pathology or systemic symptoms. Whilst thymic T cell production was unperturbed in Atg16l1^CD4^ mice (Figure 2—figure supplement 1A,B), frequencies of CD4+ and CD8+ T cells in peripheral lymphoid organs were significantly decreased.
Figure 1. Aged Atg16l1\textsuperscript{ACD4} mice develop intestinal inflammation. (A) FACS analysis of LC3\textsuperscript{+} autophagosome formation in CD4\textsuperscript{+} T cells from cLP of Atg16l1\textsuperscript{ACD4} and Atg16l1\textsuperscript{fl/fl} mice after overnight activation with or without \(\alpha\)-CD3 (5 \(\mu\)g/ml) and \(\alpha\)-CD28 (1 \(\mu\)g/ml). (B) Western blot analysis of LC3 lipidation in naïve splenic CD4\textsuperscript{+} T cells isolated from Atg16l1\textsuperscript{ACD4} mice and Atg16l1\textsuperscript{fl/fl} mice after 3hr activation with \(\alpha\)-CD3 (5 \(\mu\)g/ml) and \(\alpha\)-CD28 (1 \(\mu\)g/ml) with or without chloroquine (CQ, inhibitor of lysosomal degradation, 50 \(\mu\)M). (C) Weight curves of Atg16l1\textsuperscript{ACD4} and Atg16l1\textsuperscript{fl/fl} littermates. (D) Representative images of spleens and mesenteric lymph nodes (mLN) from aged Atg16l1\textsuperscript{ACD4} and Atg16l1\textsuperscript{fl/fl} littermates and (E) spleen weights of young and aged Atg16l1\textsuperscript{ACD4} and Atg16l1\textsuperscript{fl/fl} littermates. (F,H) Representative photomicrographs of haemotoxilin and eosin (H&E) stained sections of (F) jejunum and (H) mid-colon from young and aged Atg16l1\textsuperscript{ACD4} and Atg16l1\textsuperscript{fl/fl} littermates, scale bar 150 \(\mu\)m. (G,I) Quantification of (G) SI lengths and (I) mid-colon crypt lengths in aged Atg16l1\textsuperscript{ACD4} and Atg16l1\textsuperscript{fl/fl} littermates. Data are representative of at least three independent experiments (A-E, F, H) or combined from two (G) or three (I) independent experiments, with at least 3 mice per group. Data shown as mean \pm s.e.m (A,C). Each dot represents an individual mouse and horizontal bars denote means (E,G). In (I) each dot represents an individual crypt measurement and horizontal bars denote mean.
compared to Atg16l1fl/fl littermates (Figure 2A and Figure 2—figure supplement 1C). Furthermore, we observed significant decreases in intestinal T cell frequencies and numbers in the clp and SI LP of Atg16l1ACD4 mice (Figure 2A and Figure 2—figure supplement 1D). As CD4+ T cells are the main drivers and regulators of chronic intestinal inflammation (Shale et al., 2013), we focused subsequent analyses on CD4+ T cells.

Despite reduced numbers of T cells, Atg16l1ACD4 mice developed exacerbated disease in a CD4+ T cell-mediated model of IBD, indicating that Atg16l1-deficient CD4+ T cells are capable of driving intestinal inflammation (Figure 2—figure supplement 2). Analysis of the effector CD4+ T cell compartment in Atg16l1ACD4 mice revealed that frequencies of colonic T H1 (IFN-γ) and T H17 (IL-17A) populations were comparable in young Atg16l1ACD4 mice and Atg16l1fl/fl littermates (Figure 2B, D), although, due to decreased colonic CD4+ T cell numbers, total T H1 and T H17 numbers were significantly decreased (Figure 2C). Conversely, both frequencies and total numbers of T H2 (IL-13+) cells were significantly increased in clp of young Atg16l1ACD4 mice (Figure 2B–D). These IL-13-producing cells were bona fide TH2 cells, as they co-expressed the lineage-specifying transcription factor Gata3 (Figure 2D,E). Interestingly, TH2 cell accumulation was primarily observed in the intestinal mucosa of Atg16l1ACD4 mice, as T H2 cell frequencies were only marginally increased in the mLN and remained unchanged in the spleen (Figure 2E). However, the functional consequences of TH2 expansion extended beyond the intestine, as Atg16l1ACD4 mice had increased frequencies of eosinophils in both the spleen and mLN and elevated serum levels of mast cell protease 1 (MCPT-1), a marker of intestinal mast cell activation (Figure 2—figure supplement 3A, B).

As Foxp3+ T reg cells play a non-redundant role in control of effector T cells and the development of intestinal inflammation (Izcue et al., 2009), we hypothesized that alterations in T reg might underlie the spontaneous intestinal pathology that developed in aged Atg16l1ACD4 mice. Indeed, we found that the frequencies of intestinal Foxp3+ T reg cells in young Atg16l1ACD4 mice were severely reduced, both in the SI and clp (Figure 2F,G). Taking into account the decreased frequencies of CD4+ T cells in Atg16l1ACD4 mice (Figure 2A), this equated to a reduction in T reg cell numbers by around 10-fold in the colonic LP and 4-fold in SI LP (Figure 2—figure supplement 4A). In contrast, thymic development of Foxp3+ T reg cells was not diminished in young Atg16l1ACD4 mice (Figure 2—figure supplement 4B), and we observed only minor, though significant, reductions in the frequencies and absolute numbers of Foxp3+ T reg cells in the spleen and mLN of Atg16l1ACD4 mice compared with Atg16l1fl/fl littermates (Figure 2F and Figure 2—figure supplement 4A). Thus, Atg16l1-deficiency profoundly affected the maintenance of Foxp3+ T reg cells in the periphery, particularly within the intestinal mucosa. Expression of neuropilin-1 (Nrp1) and Helios, putative markers proposed to distinguish pT reg and tT reg cells, were found at comparable levels on intestinal Foxp3+ T reg cells from Atg16l1fl/fl and Atg16l1ACD4 mice, suggesting that the local environment, rather than site of T reg induction, primarily dictates the requirement for autophagy in T reg cells (Figure 2—figure supplement 4C, D). Assessment of how Atg16l1-deficiency affected intestinal Foxp3+ T reg cell phenotype showed that impaired autophagy significantly increased expression of effector T H1 cytokines in T reg cells from clp and SI LP (Figure 2—figure supplement 4E). We also found that clp T reg cells from young Atg16l1ACD4 mice showed higher expression of CD103 and CTLA-4, but showed decreased expression of the activation markers CD25, CD69, and the terminal differentiation marker KLRG-1 (Cheng et al., 2012) (Figure 2—figure supplement 4F). In addition, intestinal T reg cells from young Atg16l1ACD4 mice had significantly increased expression of Ki67 and higher levels of phosphorylated S6, suggesting that the majority were in cell cycle (Figure 2—figure supplement 4G–I). Taken together, these results identify a crucial role for autophagy in the maintenance and functional regulation of intestinal T reg cells.

Overall, these results demonstrate that selective ablation of Atg16l1 in T cells led to a decrease in Foxp3+ T reg cells and selective expansion of T H2 cells that preceded the onset of overt pathology. In addition, these perturbations in T H1 cell subsets were largely limited to the mucosal environment.
Figure 2. *Atg16l1<sup>ACD4</sup>* mice exhibit reciprocal dysregulation of intestinal T<sub>H</sub>2 and T<sub>reg</sub> cells before the onset of intestinal inflammation. (A) Frequencies of CD4<sup>+</sup> T cells as a proportion of live cells in young *Atg16l1<sup>ACD4</sup>* and *Atg16l1<sup>fl/fl</sup>* littermates. (B) Frequencies and (C) total numbers of IFN-γ<sup>+</sup> T<sub>H</sub>1, IL-17A<sup>+</sup> T<sub>H</sub>17 and IL-13<sup>+</sup> T<sub>H</sub>2 cells isolated from cLP of young *Atg16l1<sup>ACD4</sup>* and *Atg16l1<sup>fl/fl</sup>* littermates (gated on CD4<sup>+</sup> T cells). (D) Representative FACS plots of Gata3 and IL-13 (top) or IFN-γ and IL-17A (bottom) expression by cLP CD4<sup>+</sup> T cells isolated from young *Atg16l1<sup>ACD4</sup>* and *Atg16l1<sup>fl/fl</sup>* littermates (gated on CD4<sup>+</sup> TCRβ<sup>+</sup> Foxp3<sup>+</sup> live cells). (E) Frequencies of Gata3<sup>+</sup> CD4<sup>+</sup> T cells in young *Atg16l1<sup>ACD4</sup>* and *Atg16l1<sup>fl/fl</sup>* littermates (gated on CD4<sup>+</sup> TCRβ<sup>+</sup> Foxp3<sup>+</sup> cells). Data are combined from three or more independent experiments with at least two mice per group (A, B, D, E, G) or are representative of four independent experiments with at least four mice per group (D, F). Each dot represents an individual mouse and horizontal bars denote means. Numbers indicate percentage of cells in gates or quadrants. Statistical significance was determined using the Mann–Whitney test, *p<0.05; **p<0.01; ***p<0.001. SI LP– small intestine lamina propria, cLP – colonic lamina propria. Young mice: 8–12 weeks old. Figure 2 continued on next page.
**Atg16l1<sup>fl/fl</sup>** mice exhibit elevated type 2 humoral responses to environmental antigens

We next assessed whether dysregulation in the intestinal T<sub>reg</sub> and T<sub>h</sub>2 compartment in Atg16l1<sup>fl/fl</sup> mice affected humoral responses. While at the limit of detection in Atg16l1<sup>fl/fl</sup> controls, serum IgE concentrations were significantly elevated in young Atg16l1<sup>ACD4</sup> mice and increased further as the mice aged (Figure 3A). Furthermore, levels of serum IgA and IgG<sub>1</sub> in young Atg16l1<sup>ACD4</sup> mice were also significantly elevated relative to Atg16l1<sup>fl/fl</sup> littermates (Figure 3—figure supplement 1A) and again increased as the Atg16l1<sup>ACD4</sup> mice aged (Figure 3B). In contrast, levels of isotypes not associated with T<sub>h</sub>2 help were identical in aged Atg16l1<sup>ACD4</sup> mice and Atg16l1<sup>fl/fl</sup> littermates (Figure 3B). Thus, there was a progressive dysregulation of T<sub>h</sub>2-associated antibody responses in Atg16l1<sup>ACD4</sup> mice. Consistent with these elevated humoral responses, young Atg16l1<sup>ACD4</sup> mice had higher frequencies of germinal center (GC), memory B cells and plasma cells in the spleen and mLN compared to Atg16l1<sup>fl/fl</sup> littermates (Figure 3—figure supplement 1B) and markedly enlarged Peyer’s patches were observed in aged Atg16l1<sup>ACD4</sup> mice (Figure 3C).

Multiple studies have demonstrated the critical role played by Foxp3<sup>+</sup> T<sub>reg</sub> cells in immune tolerance to dietary and microbial antigens within the intestine. Furthermore, changes in intestinal T<sub>reg</sub> and T<sub>h</sub>2 responses are associated with food hypersensitivities (Berin and Sampson, 2013). We hypothesized that the aberrant humoral responses in Atg16l1<sup>ACD4</sup> mice might be directed against luminal antigens. Soy is the main protein source in chow, and we detected high levels of anti-soy IgG<sub>1</sub> and IgA in sera from aged Atg16l1<sup>ACD4</sup> mice, whereas these responses were undetectable in control Atg16l1<sup>fl/fl</sup> littermates (Figure 3D). By contrast, we only detected marginal levels of soy-specific IgG<sub>2b</sub> or IgG<sub>2c</sub> in aged Atg16l1<sup>ACD4</sup> sera (Figure 3D). Importantly, elevated anti-soy IgG<sub>1</sub> and IgA antibodies were already present in sera from young Atg16l1<sup>ACD4</sup> mice, before the onset of intestinal inflammation (Figure 3—figure supplement 1C). Despite the very high levels of total serum IgE in aged Atg16l1<sup>ACD4</sup> mice, we did not detect elevated levels of anti-soy IgE (data not shown).

The absence of soy-specific IgE could be due to the inhibiting effects of persistent exposure to high-dose antigens on IgE responses (Sudowe et al., 1997; Riedl et al., 2005). Therefore, to test whether an IgE response was mounted during transient exposure to low-dose dietary antigens, we fed young Atg16l1<sup>ACD4</sup> and Atg16l1<sup>fl/fl</sup> mice with ovalbumin (OVA), either alone or in combination with the mucosal adjuvant cholera toxin (CT). As expected, anti-OVA IgE responses were undetectable in control Atg16l1<sup>fl/fl</sup> mice fed OVA alone and were only marginally increased by co-administration of CT (Figure 3E). In contrast, Atg16l1<sup>ACD4</sup> mice exhibited significantly elevated levels of anti-OVA IgE after being fed OVA alone and developed >10-fold higher levels of OVA-specific IgE after feeding of OVA with CT (Figure 3E). Together, these results indicate that Atg16l1<sup>ACD4</sup> mice displayed aberrant T<sub>h</sub>2-associated antibody responses towards otherwise innocuous dietary protein antigens.

Besides food antigens, the intestinal lumen harbors vast quantities of commensal-derived antigens. Thus, we measured antibodies directed against the flagellin antigen CBir1, produced by commensal bacteria belonging to Clostridia cluster XIVa, as antibodies against flagellin are readily detected in sera of IBD patients (Lodes et al., 2004). We detected significantly higher levels of CBir1-specific IgG<sub>1</sub> and IgA in the serum of aged Atg16l1<sup>ACD4</sup> mice compared to control Atg16l1<sup>fl/fl</sup> littermates, whereas anti-CBir1 IgG<sub>2b</sub> and IgG<sub>2c</sub> levels were comparable (Figure 3F). Furthermore, CBir1-specific IgG<sub>1</sub> and IgA were already detectable in young Atg16l1<sup>ACD4</sup> mice (data not shown).
contrast, increased T\textsubscript{H}2 cell-associated antibody responses were not mounted in young Atg16l1\textsuperscript{ACD4} mice following oral infection either with the Gram-negative bacterium Helicobacter hepaticus or with the nematode parasite Trichuris muris (Figure 3—figure supplement 1D,E). Taken together, these results indicate that the abnormal T\textsubscript{H}2-associated antibody responses observed in Atg16l1\textsuperscript{ACD4} mice preceded the development of overt inflammation and were selectively induced towards commensal microbiota and dietary antigens.
Figure 4. **Atg16l1** promotes survival of **T**\(_{\text{reg}}\) cells and limits **T**\(_{\text{H}2}\) cell survival. (A,B) **Atg16l1**\(^{+/+}\) or **Atg16l1**\(^{+/−}\) naive CD4\(^{+}\) T cells were cultured in **T**\(_{\text{H}0}\), **T**\(_{\text{reg}}\), or **T**\(_{\text{H}2}\) polarizing conditions for 48 hr and analyzed by FACS. Representative FACS plots show (A) Foxp3 and (B) Gata3 expression (gated on CD4\(^{+}\) TCR\(\beta\)\(^{+}\)T cells). (C) Frequencies of **T**\(_{\text{reg}}\) cells (Foxp3\(^{+}\)) and **T**\(_{\text{H}2}\) cells (Gata3\(^{+}\)) arising from **Atg16l1**\(^{+/+}\) or **Atg16l1**\(^{+/−}\) naive CD4\(^{+}\) T cells cultured in **T**\(_{\text{reg}}\) or **T**\(_{\text{H}2}\) polarizing conditions for 5 days. (D) **Atg16l1**\(^{+/+}\) or **Atg16l1**\(^{+/−}\) **T**\(_{\text{reg}}\) cells were cultured with anti-CD3 (3 mg/ml) and anti-CD28 (1 mg/ml) for 48 hr, then maintained in the presence of IL-4 and IL-13 for a further 5 days before FACS analysis of Foxp3 and CD25 expression of live CD4\(^{+}\) T cells. (E,F) Naive **Atg16l1**\(^{+/+}\) or **Atg16l1**\(^{+/−}\) CD4\(^{+}\) T cells were cultured with (E) 1 mg/ml or (F) 5 mg/ml anti-CD3 plus anti-CD28 (1 mg/ml) for 48 hr in **T**\(_{\text{reg}}\) or **T**\(_{\text{H}2}\) polarizing conditions, then maintained in polarizing conditions for a further 5 days before FACS analysis of cell survival. Histograms show gates and frequencies of live CD4\(^{+}\) T cells. (G) Representative FACS plots of viability dye and Annexin V staining of **T**\(_{\text{reg}}\) cells and **T**\(_{\text{H}2}\) cells from the cLP of young **Atg16l1**\(^{+/+}\) and **Atg16l1**\(^{+/−}\) littersmates, gated on CD4\(^{+}\) TCR\(\beta\)\(^{+}\)Foxp3\(^{+}\) (left panel), or CD4\(^{+}\) TCR\(\beta\)\(^{+}\)Gata3\(^{+}\) (right panel). Data are representative from two (D,G) or three independent experiments (A,B,E,F), or are combined from three independent experiments (C). Each dot represents an individual cell culture (C) or data are shown as mean ± s.e.m (A,B,D-F). Numbers indicate percentage of cells in quadrants (G). cLP – colonic lamina propria. Young mice: 8–12 weeks old.

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Figure 5. Autophagy contributes to the elevated Th2 responses in Atg16l1CD4 mice in a cell-intrinsic manner. Young Atg16l1CD4 mice (CD45.2+) were adoptively transferred with 4.5x10⁶ naive WT CD4+ T cells (CD45.1+) and analyzed 3 months later. (A) Frequencies of WT (CD45.1+) and Atg16l1-deficient (CD45.2+) CD4+ T cells in the spleen, mLN and cLP. (B) Frequencies of WT (CD45.1+) and Atg16l1-deficient (CD45.2+) Foxp3+ Treg cells in the spleen, mLN and cLP (gated on CD4+ TCRβ+ T cells). (C) Representative FACS plots showing gating of WT (CD45.1+) and Atg16l1-deficient (CD45.1+) CD4+ T cells and expression of IL-13 (Th2), IFN-γ (Th1) and IL-17A (Th17) in the cLP (gated on CD4+ TCRβ+ Foxp3+ T cells). (D) Frequencies of WT (CD45.1+) and Atg16l1-deficient (CD45.2+) Th2 (IL-13+), Th1 (IFN-γ+) and Th17 (IL-17A+) cells among CD4+ TCRβ+ Foxp3+ T cells in the cLP. (E) Frequencies of WT (CD45.1+) and Atg16l1-deficient (CD45.2+) Gata3+ CD4+ T cells in the spleen, mLN and cLP (gated on CD4+ TCRβ+ Foxp3+ T cells). (F) SI lengths and (G) representative photomicrographs of jejunum of control untreated Atg16l1fl/fl or Atg16l1CD4 littermates and reconstituted Atg16l1CD4 mice, scale bar 150 μm. (H) Serum IgE concentrations in control untreated Atg16l1fl/fl or Atg16l1CD4 littermates and adoptively transferred Figure 5 continued on next page.
Atg16l1 differentially regulates survival of Th2 and Treg cells

Given apparent opposing effects of Atg16l1 deficiency on Th2 and Treg cells, we questioned whether the disruption of autophagy pathway affects the differentiation of these T cell subsets. We found that, under Th2 or Treg polarizing conditions, differentiation of naïve CD4+ T cells isolated from Atg16l1<sup>ACD4</sup> or Atg16l1<sup>f/f</sup> littermates toward the Gata3<sup>+</sup> Th2 or Foxp3<sup>+</sup> Treg cell phenotype was comparable (Figure 4A–C). As Th2 cytokines can negatively affect Treg differentiation and stability (Dardalhon et al., 2008; Feng et al., 2014), it was possible that outgrowth of Th2 cells may also have contributed to the loss of intestinal Treg in Atg16l1<sup>ACD4</sup> mice. We therefore isolated Foxp3<sup>+</sup> Treg cells from Atg16l1<sup>ACD4</sup> and Atg16l1<sup>f/f</sup> littermates and activated them in vitro in the presence of IL-4 and IL-13. However, we did not find any evidence of Treg instability, as expression of Foxp3 and CD25 remained equally high in Atg16l1-deficient and WT Treg cells (Figure 4D).

We therefore examined whether autophagy deficiency influenced the survival of Th2 or Foxp3<sup>+</sup> Treg cells. Thus, naïve CD4<sup>+</sup> T cells isolated from Atg16l1<sup>ACD4</sup> or Atg16l1<sup>f/f</sup> littermates were activated for 48 hr with anti-CD3 and anti-CD28 antibodies and then rested for 5 days. Cells were kept in Th2 or Treg polarizing conditions throughout the experiment. Following activation with different concentrations of anti-CD3 antibody, Atg16l1-deficient Th2 cells exhibited comparable or improved survival relative to WT Th2 cells (Figure 4E,F). In contrast, there was a 50–75% decrease in survival of Atg16l1-deficient Treg cells when compared to Atg16l1-sufficient Treg cells activated under the same conditions (Figure 4E,F). To establish whether autophagy-deficient Treg and Th2 cells exhibited similarly distinct survival profiles in vivo CD4<sup>+</sup> T cells isolated from cLP of Atg16l1<sup>ACD4</sup> or Atg16l1<sup>f/f</sup> littermates were stained with a viability dye and Annexin V. We observed that an increased proportion of Atg16l1-deficient intestinal Treg cells were dead or dying compared to WT Treg cells (Figure 4G). In contrast, Atg16l1-deficiency had no negative effect on the viability of intestinal Th2 cells, which was comparable to WT controls (Figure 4G). Together, these results indicate that Atg16l1-deficiency does not impair the differentiation or stability of Treg cells and does not promote differentiation towards the Th2 lineage. However, autophagy differentially impacts on the survival of mucosal Treg and Th2 cells.

Autophagy regulates intestinal Th2 responses in a cell-intrinsic manner

As pTreg cells are required to control Th2 responses at mucosal sites (Mucida et al., 2005; Curotto de Lafaille et al., 2008; Josefowicz et al., 2012), we examined whether the enhanced Th2 phenotype in Atg16l1<sup>ACD4</sup> mice could be corrected by reconstitution of the intestinal Foxp3<sup>+</sup> Treg compartment. We restored the pTreg population in young Atg16l1<sup>ACD4</sup> mice at the age of 10–12 weeks, before the onset of intestinal pathology, through adoptive transfer of congenic WT naïve CD45.1<sup>+</sup> CD4<sup>+</sup> T cells. Recipients were sacrificed 3 months later, when control Atg16l1<sup>ACD4</sup> littermates had developed intestinal inflammation. We detected CD45.1<sup>+</sup> donor CD4<sup>+</sup> T cells in all adoptively transferred Atg16l1<sup>ACD4</sup> mice, but the level of reconstitution varied by the organ examined. In reconstituted Atg16l1<sup>ACD4</sup> mice, donor WT CD4<sup>+</sup> T cells accounted for 37 ± 5% of total CD4<sup>+</sup> T cells in spleen and 18 ± 2% in mLN, whereas in the cLP they represented 56 ± 4% (Figure 5A). Thus, autophagy-deficient CD4<sup>+</sup> T cells had a survival disadvantage when compared to WT CD4<sup>+</sup> T cells within the intestinal mucosa. Overall, adoptive transfer of WT naïve CD4<sup>+</sup> T cells restored the total...
frequencies of CD4+ T cells in the cLP to levels comparable to control Atg16l1+/mice (Figure 5—
figure supplement 1A).

When we examined Foxp3+ Treg cells, the survival advantage conferred by autophagy was even more apparent, with around 50% of the donor WT naive CD45.1+ T cells developing into Foxp3+ pTreg cells in spleen, mLN and cLP of Atg16l1+CD4 recipients. As a result, the majority of Foxp3+ Treg cells in Atg16l1+CD4 mice were of WT donor origin (67 ± 5% in spleen, 59 ± 4% in mLN and 80 ± 5% in cLP) (Figure 5B).

Thus, adoptive transfer of WT-naïve CD4+ T cells resulted in efficient reconstitution of Foxp3+ pTreg cells in Atg16l1+CD4 mice; the total frequencies and numbers of Treg cells within the cLP of transferred mice were comparable with control Atg16l1+/mice (Figure 5—figure supplement 1B). As such, we could utilize this system to determine whether excessive Tn2 cell accumulation in Atg16l1+CD4 mice was due to impaired mucosal pTreg cells or to a cell-intrinsic effect of Atg16l1-deficiency in Tn2 cells.

When we analyzed the frequencies of Tn2 cells in the cLP of reconstituted Atg16l1+CD4 mice, we observed significantly higher frequencies of Gata3+ IL-13+ Tn2 cells among Atg16l1-deficient CD45.2+ CD4+ T cells compared with the WT donor CD45.1+ CD4+ T cells (Figure 5C–E). Indeed, frequencies of IL-13+ Atg16l1-deficient CD4+ T cells in the cLP of pTreg-reconstituted mice were comparable to those found in untreated Atg16l1+CD4 littermates (Figure 5C,D). In contrast, there was no difference in Tn17 cell frequencies between Atg16l1-deficient CD45.2+ and WT CD4+ T cells, and there was a significant decrease in Tn1 frequencies among Atg16l1-deficient CD4+ T cells (Figure 5C,D). In line with these observations, adoptively transferred Atg16l1+CD4 mice had comparable total frequencies and numbers of Tn2 cells as observed in untreated Atg16l1+CD4 mice (Figure 5—figure supplement 1C). Thus, provision of WT pTreg cells did not rescue the increased Tn2 phenotype of Atg16l1-deficient CD4+ T cells, indicating that autophagy directly regulates Tn2 cells through a cell-intrinsic mechanism.

Consistent with this finding, Atg16l1+CD4 mice reconstituted with WT pTreg cells still developed intestinal pathology and elevated serum IgE levels comparable to those present in untreated Atg16l1+CD4 littermates (Figure 5F–H).

**Autophagy is essential for Treg cell homeostasis and control of effector T cell responses in the gut**

Given that Atg16l1-deficiency significantly reduced the number of intestinal Treg cells in Atg16l1+CD4 mice, we hypothesized that Treg cells may be particularly reliant on autophagy compared to other subsets of CD4+ T cells. Indeed, in WT mice we found that levels of autophagy were significantly higher in Foxp3+ Treg cells compared to Foxp3+ CD4+ T cells, both constitutively and after TCR activation (Figure 6A,B). Together with our observations of impaired survival of Atg16l1-deficient Foxp3+ Treg cells (Figure 4E–G), this suggested an important cell-intrinsic role for autophagy in the maintenance of Treg cells. This hypothesis was further strengthened by analyses of mixed bone marrow (BM) chimeras where irradiated Rag1−/− mice were reconstituted with a 1:1 mixture of BM cells from Atg16l1+CD4 mice and congenic WT C57BL/6 mice (Figure 6—figure supplement 1A). In this setting, the reconstitution of CD4+ T cells was severely hampered in the absence of functional autophagy and this deficiency was most pronounced in the Treg compartment of the spleen and cLP (Figure 6—figure supplement 1B–E), confirming that Atg16l1-deficiency decreases the ability of Foxp3+ Treg cells to compete with WT Treg cells in a cell-intrinsic manner.

To definitively assess the cell-intrinsic requirement for autophagy in Foxp3+ Treg cells we crossed Atg16l1+/mice with mice expressing a YFP-Cre from the Foxp3 locus (Foxp3Cre mice) (Rubtsov et al., 2008), generating Atg16l1+/Foxp3Cre mice (hereafter denoted as Atg16l1Fopx3) in which Atg16l1 is selectively ablated in Foxp3+ Treg cells. These mice allowed us to analyze the consequences of a lack of autophagy in Treg cells in the context of autophagy-competent CD4+ T effector cells. As expected, Atg16l1Fopx3 mice showed a significant reduction of Atg16l1 expression in Foxp3+ Treg cells, but not in CD4+ Foxp3+ T cells (Figure 6—figure supplement 2A). Although Atg16l1Fopx3 mice appeared normal in early life, at around 5 months of age they developed a severe spontaneous inflammatory disease characterized by progressive weight loss, splenomegaly, lymphadenopathy and leukocyte infiltration in multiple tissues (Figure 6C–E). The gastrointestinal tract was particularly affected in aged Atg16l1Fopx3 mice, with marked inflammation in the SI and colon (Figure 6E,F). Intestinal inflammation in aged Atg16l1Fopx3 mice was characterized by massive accumulation of activated CD4+ T cells in the intestinal LP and mLN (Figure 7A–D and Figure 7—figure supplement 1A). The cLP infiltrate in aged Atg16l1Fopx3 mice contained a mixed...
population of T_{H}1, T_{H}17 and T_{H}2 effector cells, with a significant increase in the frequencies of IL-13^{+} CD4^{+} T_{H}2 cells (Figure 7E,F), although this T_{H}2 bias was not present in young Atg16l1^{D Foxp3} mice (Figure 7—figure supplement 1B). In addition, we observed increased frequencies of Gata3^{+} CD4^{+} T_{H}2 cells.
T cells in the spleen, mLN and cLP of aged Atg16l1<sup>D</sup>Foxp3<sup>m</sup> mice (Figure 7G). Analyses of humoral responses in aged Atg16l1<sup>D</sup>Foxp3<sup>m</sup> mice revealed significantly elevated levels of circulating IgE and IgA, however IgG<sub>1</sub> levels were not increased (Figure 7H and Figure 7—figure supplement 1C). Thus, selective ablation of Atg16l1 in Foxp3<sup>+</sup> T<sup>reg</sup> cells led to intestinal inflammation that was characterized by accumulation of all T<sub>h</sub> effector types, with a disproportionate increase in T<sub>h</sub>2 responses in
Figure 8. Cell-intrinsic autophagy is required for metabolic adaptation and survival of intestinal Foxp3⁺ T<sub>reg</sub> cells.

(A) Foxp3⁺ T<sub>reg</sub> cell frequencies among CD4⁺ TCR<sup>β</sup>⁺ T cells in Atg16l1<sup>Flox</sup> and Atg16l1<sup>fl/fl</sup> littermates and (B) representative FACS plots of Foxp3 expression in cLP CD4⁺ T cells from young Atg16l1<sup>Flox</sup> and Atg16l1<sup>fl/fl</sup> littermates (gated on CD4⁺ TCR<sup>β</sup>⁺ T cells). (C) qPCR analysis of glycolytic gene levels in sorted Foxp3⁺ T<sub>reg</sub> cells from spleen and cLP of young Atg16l1<sup>Flox</sup> and Foxp3<sup>Cre</sup> mice (sorted for CD4⁺ TCR<sup>β</sup>⁺ YFP⁺). (D) qPCR analysis of FAS and FAO gene levels in Foxp3⁺ T<sub>reg</sub> cells from the spleen and cLP of young Atg16l1<sup>Flox</sup> and Foxp3<sup>Cre</sup> mice (sorted for CD4⁺ TCR<sup>β</sup>⁺ YFP⁺). FAS: fatty acid synthesis, FAO: fatty acid oxidation, Glut1: glucose transporter 1, Slc16a3: solute carrier family 16 member 3 (lactic acid and pyruvate transporter), Tpi1: triosephosphate isomerase 1, Aldo–a: aldolase a, Ldh–a: lactate dehydrogenase a, Gpi1: Glucose phosphate isomerase 1, Pgk1: Phosphoglycerate kinase 1, Acc1: acetyl-CoA carboxylase 1, Acc2: acetyl-CoA carboxylase 2, Sreb1: sterol regulatory element binding transcription factor 1, Sreb2: sterol regulatory element binding transcription factor 2, Fdft1: farnesyl-diphosphate farnesyltransferase 1, Fabp: Fatty acid-binding protein. Data are representative from Figure 8 continued on next page.
two (C,D) or three independent experiments (A,B). Each dot represents individual mouse (A) or data are shown as mean ± s.e.m (C,D). Gene expression levels are shown as mean ± s.e.m of three technical replicates (C,D). Numbers indicate percentage of cells in gates (B). cLP – colonic lamina propria. Young mice: 8–12 weeks old. DOI: 10.7554/eLife.12444.019

The following figure supplements are available for figure 8:

**Figure supplement 1.** Atg16l1-deficient colonic Treg cells exhibit increased cytokine secretion.
DOI: 10.7554/eLife.12444.020

**Figure supplement 2.** Increased lipid uptake by intestinal Treg cells.
DOI: 10.7554/eLife.12444.021

**Figure supplement 3.** T[b]2 cells exhibit an enhanced glycolytic metabolic profile that is independent of autophagy.
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Aged mice. However, the breadth and magnitude of T[b]2-associated responses were less pronounced in Atg16l1[b]Foxp3[b] mice compared to those observed in Atg16l1[b]CD4[b] mice.

When we examined the Treg cell compartment in Atg16l1[b]Foxp3[b] mice, we found significantly decreased frequencies of Foxp3[b] Treg cells in the spleen and mLN compared to Atg16l1[b]h2F[b] littermates, although thymic T[b]m cell frequencies were similar (Figure 8A). As found in Atg16l1[b]CD4[b] mice, intestinal LP Foxp3[b] Treg cells were severely depleted in Atg16l1[b]Foxp3[b] mice and those remaining exhibited significantly increased expression of effector T[b]h cytokines (Figure 8A,B and Figure 8—figure supplement 1A). Thus, Treg cell-specific deletion of Atg16l1 recapitulated the Treg cell deficits observed in Atg16l1[b]CD4[b] mice, showing that cell-intrinsic autophagy is essential for peripheral Treg cell homeostasis, especially in the intestine.

**Differential survival of autophagy-deficient Treg cells and T[b]h2 cells is associated with an altered metabolic profile**

Finally, we investigated mechanisms that might underlie the striking survival defect of Atg16l1[b]deficient intestinal Treg cells. Analyses of key regulators of apoptosis revealed that Atg16l1[b]deficient Treg cells isolated from spleen and cLP had comparable expression of pro-apoptotic (Bim, Bax) and anti-apoptotic (Bcl2) genes as those isolated from control mice (Figure 8—figure supplement 1B). As recent evidence suggests that tissue-resident Treg cell populations may exhibit specialized metabolic adaptations (Burzyn et al., 2013), we compared the expression of metabolic genes by WT and Atg16l1[b]deficient Treg cells. Analyses of genes involved in glycolysis, fatty acid synthesis (FAS) and fatty acid oxidation (FAO), revealed that Atg16l1[b]deficient Treg cells had higher expression of glycolytic genes, including Glut1, Scl16a3, Tpi1, Ldh-a, Aldo-a, Gpi1 and Pgk1, than control Treg cells (Figure 8C). Strikingly, this augmented glycolytic signature was much more pronounced in Atg16l1[b]deficient Treg cells isolated from cLP versus those from the spleen (Figure 8C). Conversely, expression of many key genes involved in FAS/FAO, including Acc1, Acc2, Srebfb1, Srebfb2, Fdft1, Fabp1, Fabp4 and Fabp5 was markedly decreased in Atg16l1[b]deficient Treg cells (Figure 8D). Again, these differences were most pronounced in the intestine; WT cLP Treg cells showed increased FAS/FAO gene expression compared to their spleen counterparts, whereas Atg16l1[b]deficient cLP Treg cells were not able to up-regulate the expression of FAS/FAO genes (Figure 8D). Thus, Atg16l1[b]deficiency profoundly influenced the expression of metabolic genes in intestinal Treg cells, with an altered balance of glycolytic and FAS/FAO gene expression. Further evidence of increased reliance on lipid metabolism by colonic Treg cells was provided by our observation that Treg cells isolated from the cLP showed markedly increased lipid uptake in comparison to mLN or spleen Treg cells (Figure 8—figure supplement 2A,B). A similar pattern was observed when we assayed expression of CD36, a fatty acid translocase that enhances FA uptake: colonic Treg cells showed increased expression of CD36 compared to splenic and mLN Treg cells (Figure 8—figure supplement 2C,D). Interestingly, we found that Atg16l1[b]deficient Treg cells showed comparable levels of lipid uptake and CD36 expression as their autophagy-sufficient counterparts (Figure 8—figure supplement 2A–D), suggesting that autophagy does not affect lipid uptake per se but rather affects lipid metabolism.

Together, these results demonstrate that cell-intrinsic autophagy is indispensable for Foxp3[b] Treg cell maintenance and function in peripheral tissues, particularly to suppress inflammatory responses.
within the gastrointestinal tract. Decreased survival of Atg16l1-deficient T<sub>reg</sub> cells was associated with an altered metabolic profile, suggesting that autophagy plays an integral role in facilitating the metabolic adaptions required for long-term T<sub>reg</sub> cell survival in the intestine.

We next explored whether autophagy had a general effect on T cell metabolic profile and whether this might explain the differential effects on T<sub>H</sub>2 and T<sub>reg</sub> cells. Evidence that this might be the case came from our observation that Atg16l1-deficient naïve CD4<sup>+</sup> T cells exhibited increased cell size compared with naïve CD4<sup>+</sup> T cells isolated from Atg16l1<sup>fl/fl</sup> littermates (Figure 8—figure supplement 3A). We therefore measured oxygen consumption rate (OCR), which is an indicator of oxidative phosphorylation (OXPHOS), and extracellular acidification rate (ECAR), an indirect indicator of aerobic glycolysis. We found that Atg16l1-deficient naïve CD4<sup>+</sup> T cells exhibited significantly increased OCR and ECAR, metabolic changes that are typically observed in activated CD4<sup>+</sup> T cells and are associated with increased aerobic glycolysis (Figure 8—figure supplement 3B).

As T<sub>H</sub>2 cells have previously been reported to display an increased glycolytic rate compared to other T<sub>H</sub> subsets (Michalek et al., 2011; Yang et al., 2013), we hypothesized that they may be more resistant to the increased glycolysis that is induced in the absence of autophagy. As it was not possible to sort T<sub>H</sub>2 cells from the cLP, we performed this analysis on in vitro cultures of T<sub>H</sub>2 and T<sub>reg</sub> cells. We found that T<sub>H</sub>2 cells were larger than T<sub>reg</sub> cells, expressed higher levels of c-Myc, a critical regulator of metabolic reprogramming in activated T cells, and had markedly higher ECAR, all indicative of enhanced aerobic glycolysis (Figure 8—figure supplement 3C–E). Furthermore, while Atg16l1-deficient T<sub>reg</sub> cells showed higher expression of c-Myc, significantly increased levels of ECAR and OCR, and were larger than their control Atg16l1-sufficient counterparts, we observed constitutively high and comparable levels of glycolysis in Atg16l1-deficient and Atg16l1-sufficient T<sub>H</sub>2 cells (Figure 8—figure supplement 3C–E). These patterns were recapitulated when expression of key metabolic genes were analyzed; T<sub>H</sub>2 cells showed high expression of a panel of glycolytic genes irrespective of their autophagy Atg16l1 genotype, whereas T<sub>reg</sub> cell expression of glycolytic genes was generally lower, unless the T<sub>reg</sub> cells were autophagy-deficient (Figure 8—figure supplement 3F). Taken together, these results suggest that the enhanced glycolytic metabolism constitutively employed by T<sub>H</sub>2 cells makes them more resistant to the metabolic changes that occur in the absence of autophagy.

Discussion

The unique challenges of the intestine necessitate complex mechanisms of tolerance and immune regulation to maintain homeostasis (Izcue et al., 2009). As altered mucosal CD4<sup>+</sup> T cell responses are implicated in intestinal diseases of increasing prevalence, including food allergies and IBD (Maloy and Powrie, 2011; Berin and Sampson, 2013), it is important to understand the factors that control effector and regulatory T cell homeostasis in the gut. Here, we identify Atg16l1 and autophagy as a new critical pathway regulating intestinal T<sub>reg</sub> and T<sub>H</sub>2 responses.

Recent studies addressing the role of autophagy in distinct leukocyte populations have highlighted T cells as being very sensitive to perturbations in the autophagy pathway (Ma et al., 2013). Our data extend these findings by showing that autophagy is particularly important for the survival of CD4<sup>+</sup> T cells within the gut environment, as Atg16l1 deletion in T cells led to a severe reduction of CD4<sup>+</sup> T cell numbers in the intestinal LP. This deficit was confirmed in mixed bone marrow chimeras, where Atg16l1-deficient CD4<sup>+</sup> T cells failed to reconstitute the intestinal LP compartment, and by the rapid outgrowth of adoptively transferred WT CD4<sup>+</sup> T cells in the colonic LP of Atg16l1<sup>−/−</sup>CD4<sup>+</sup> recipients. However, despite the reduction in intestinal CD4<sup>+</sup> T cells, Atg16l1<sup>−/−</sup>CD4<sup>+</sup> mice spontaneously developed progressive, chronic intestinal inflammation. To confirm their increased predisposition to develop intestinal pathology, we used an experimental model of IBD triggered by infection with Helicobacter hepaticus and concomitant treatment with anti-IL-10R mAbs (Song-Zhao and Maloy, 2014). This model induces severe typhocolitis that is T cell dependent and displays several features of human IBD pathology and does not require any specific genetic manipulation or chemical barrier disruption. We found increased intestinal pathology in Atg16l1<sup>−/−</sup>CD4<sup>+</sup> mice, confirming that Atg16l1-deficient T cells could mediate potent inflammatory responses in the gut. Thus, selective autophagy deficiency within T cells decreases the competitiveness of these cells and simultaneously predisposes to intestinal inflammation.
We found that Atg16l1<sup>ACD4</sup> mice exhibited a drastic reduction in Foxp3<sup>+</sup> T<sub>reg</sub> populations in the cLP and SI LP, together with marked changes in intestinal T<sub>reg</sub> phenotype, including increased cell cycling and aberrant production of T<sub>H</sub> effector cytokines. The role of autophagy in Foxp3<sup>+</sup> T<sub>reg</sub> cells is not well defined. T cell-specific ablation of Vps34, which encodes a class III phosphatidylinositol 3-kinase that promotes autophagy, resulted in decreased frequencies of T<sub>reg</sub> cells in the thymus, spleen and lymph nodes (Parekh et al., 2013). However, as Vps34 also has autophagy-independent functions (Backer, 2008), it was unclear as to what extent these changes were due to impaired autophagy. Furthermore, we did not find any deficit in thymic T<sub>reg</sub> cell development in Atg16l1-deficient T cells. However, we observed that T<sub>reg</sub> cells isolated from the mLN and colonic LP had increased levels of autophagy compared to effector T cells, suggesting that autophagy is particularly important for the maintenance of T<sub>reg</sub> cells in the periphery. Indeed, we demonstrated that cell-intrinsic autophagy is indispensable for the maintenance and function of Foxp3<sup>+</sup> T<sub>reg</sub> cells in the gastrointestinal tract, as selective deletion of Atg16l1 in the Foxp3<sup>+</sup> T<sub>reg</sub> compartment in Atg16l1<sup>ACFoxp3</sup> mice led to a loss of intestinal Foxp3<sup>+</sup> T<sub>reg</sub> cells and to severe inflammation of the small intestine and colon. In this context, it is pertinent that rapamycin, which induces autophagy through its inhibitory activity on mTOR, has been shown to promote expansion of T<sub>reg</sub> cells in vitro and in vivo (Pollizzi and Powell, 2015). Similarly, several small-molecule inducers of autophagy were shown to selectively promote the development of T<sub>reg</sub> cells in vitro (Shaw et al., 2013). Taken together with our findings, these observations suggest that boosting autophagy may represent a rational therapeutic approach to enhance T<sub>reg</sub> responses in the intestine.

How does autophagy intrinsically regulate T<sub>reg</sub> cell homeostasis? Our data indicate that autophagy is not required for the differentiation of Foxp3<sup>+</sup> T<sub>reg</sub> cells in vitro or in vivo for thymic generation of T<sub>reg</sub> cells in vivo. However, we found that Atg16l1-deficient T<sub>reg</sub> cells showed significantly decreased survival compared to WT T<sub>reg</sub> cells both in vitro and in vivo. As recent evidence indicates that T<sub>reg</sub> cells utilize a distinct metabolic program that favors lipid oxidation for energy provision (Maclver et al., 2013), one potential explanation is that autophagy regulates T<sub>reg</sub> cell metabolism and thereby their survival. Indeed, we found that Atg16l1-deficient T<sub>reg</sub> cells expressed a distinct metabolic profile to their WT counterparts, exhibiting increased expression of genes involved in glycolysis and reduced expression of genes involved in FAS/FAO. Fatty acid metabolism is emerging as a potent regulator of T cell responses and preferential utilization of FAO has been linked to T<sub>reg</sub> cell function (Tu et al., 2009). O’Sullivan et al., 2014) Thus, autophagy could play a similar survival role in T<sub>reg</sub> cells, by facilitating the degradation of intracellular lipid stores to release FAs that fuel FAO. Additionally, as degradation of intracellular lipids by autophagy is important to avoid lipotoxicity (Galluzzi et al., 2014), defective autophagy could lead to a toxic build up of intracellular lipids in intestinal T<sub>reg</sub> cells.

The imbalance between glycolysis and FAS/FAO observed in autophagy-deficient T<sub>reg</sub> cells could indicate that these cells have stalled in the activated/effector state and are unable to make the metabolic adaptations necessary for long-term survival. This is supported by our data showing that a higher proportion of autophagy-deficient T<sub>reg</sub> cells appear to be in cell cycle, but they have reduced expression of terminal differentiation markers. Consistent with our findings, a recent study reported that autophagy deficiency in T<sub>reg</sub> cells resulted in increased mTORC1 activation and glycolysis, leading to phenotypic instability, including expression of pro-inflammatory cytokines (Wei et al., 2016). However, the molecular mechanism behind decreased survival of autophagy-deficient T<sub>reg</sub> cells was not elucidated (Wei et al., 2016). It is striking that autophagy deficiency had a more detrimental effect on intestinal T<sub>reg</sub> cells than on those found in secondary lymphoid organs. Recent evidence suggests that tissue-resident T<sub>reg</sub> cells undergo tissue-specific adaptations, and metabolic changes are emerging as an important facet of such reprogramming (Burzyn et al., 2013; Liston and Gray, 2014). Taken together, our results suggest that autophagy is intrinsic to the survival of intestinal T<sub>reg</sub> cells, and that autophagy deficiency could induce the phenotype of such cell. As such, metabolic reprogramming may represent a rational approach to enhance T<sub>reg</sub> responses in the intestine.
Paralleling decreased T<sub>reg</sub> responses in Atg16<sup>L1</sup><sub>CD4</sub> mice, we observed a selective expansion of T<sub>H2</sub> cells in the intestinal LP that was already present in young mice and preceded the onset of overt pathology. Our subsequent analyses indicated that autophagy limits mucosal T<sub>H2</sub> cells through both cell-intrinsic and cell-extrinsic (T<sub>reg</sub>-mediated) regulation. One possibility is that Atg16<sup>L1</sup>-deficient T<sub>H2</sub> cells may be somewhat resistant to T<sub>reg</sub> suppression. However, when we reconstituted pT<sub>reg</sub> cells in Atg16<sup>L1</sup><sub>CD4</sub> mice we observed a negative correlation between the numbers of intestinal T<sub>reg</sub> cells and T<sub>H2</sub> cells (data not shown), suggesting that autophagy-deficient T<sub>H2</sub> cells are partially controlled by T<sub>reg</sub> cells. Our data strongly suggest that the intrinsic survival advantage of Atg16<sup>L1</sup>-deficient T<sub>H2</sub> cells is primarily responsible for their outgrowth in the intestine. Indeed, we observed increased survival of Atg16<sup>L1</sup>-deficient T<sub>H2</sub> cells in vitro, suggesting that autophagy might directly inhibit T<sub>H2</sub> cell expansion. This concept is consistent with a previous study that reported enhanced survival of T<sub>H2</sub> cells in vitro when autophagy was inhibited and that autophagy mediated death of T<sub>H2</sub> cells during growth-factor withdrawal (Li et al., 2006). However, we provide evidence for an additional mechanism that could explain the preferential expansion of Atg16<sup>L1</sup>-deficient T<sub>H2</sub> cells in the intestine, related to the unique ability of T<sub>H2</sub> cells to cope with prolonged high levels of glycolysis. Our data contribute to accumulating evidence that a shift toward glycolysis is a general phenomenon observed when the autophagy pathway is perturbed in T cells. We observed characteristic signs of increased glycolysis in Atg16<sup>L1</sup>-deficient naïve CD4<sup>+</sup> T cells and T<sub>reg</sub> cells, such as increases in cell size, c-Myc levels and expression of glycolytic genes, as well as elevated ECAR. Others have reported a similar glycolytic shift in autophagy-deficient CD8<sup>+</sup> memory T cells (Puleston et al., 2014) and T<sub>reg</sub> cells (Wei et al., 2016). Interestingly, T<sub>H2</sub> cells have previously been shown to display an increased glycolytic rate compared to other T<sub>H</sub> subsets (Michalek et al., 2011; Yang et al., 2013). We confirmed the high levels of constitutive glycolysis in T<sub>H2</sub> cells and showed that these were comparable in Atg16<sup>L1</sup>-deficient and control T<sub>H2</sub> cells. Moreover, Gata3 activation was previously linked to induction of glycolysis after TCR activation in T cells, through induction of c-Myc, a critical regulator of metabolic reprogramming (Wang et al., 2011; Wang et al., 2013; Wan, 2014). We therefore propose that in T<sub>H2</sub> cells Gata3 orchestrates metabolic adaptations that enable these cells to cope with prolonged high levels of glycolysis, thus making them resistant to metabolic changes enforced by autophagy deficiency. Overall, our results indicate that autophagy is a key pathway through which T<sub>H2</sub> responses are restrained in vivo. A lack of this restraint leads to a gradual loss of tolerance to intestinal antigens, as the excessive T<sub>H2</sub> responses in Atg16<sup>L1</sup><sub>CD4</sub> mice led to production of IgG<sub>1</sub> and IgA antibodies toward commensal microbiota and dietary antigens that increased with age. Furthermore, Atg16<sup>L1</sup><sub>CD4</sub> mice developed very high levels of circulating IgE, and mounted de novo IgE antibody responses toward introduced dietary antigen.

As polymorphisms in autophagy genes are linked to IBD susceptibility, our results point towards a novel mechanism that links impaired autophagy to intestinal inflammation through dysregulation of mucosal T cell responses. Previous studies focused on the role of ATG16L1 and autophagy in myeloid cells and the intestinal epithelium. They suggested that impaired autophagy could result in reduced intestinal barrier integrity due to impaired Paneth cell function within the intestinal epithelial layer and elevated cytokine responses by macrophages and dendritic cells (Cadwell et al., 2008; Saitoh et al., 2008; Lassen et al., 2014). Our data add a further layer to the control of intestinal homeostasis by autophagy, by showing that autophagy impairment alters the local T cell compartment and promotes T cell driven intestinal pathology. We present compelling evidence that autophagy deficiency in T<sub>reg</sub> cells leads to a deficit in intestinal T<sub>reg</sub> cells and the development of severe intestinal pathology. Although the contribution of the T<sub>H2</sub> axis to IBD remains unclear (Strober et al., 2002; Shale et al., 2013), polymorphisms in IL-4, IL-5 and IL-13 have been implicated by GWAS in both CD and UC (Van Limbergen et al., 2014) and elevated levels of antibodies recognizing food and commensal antigens have been detected in IBD patients (Lodes et al., 2004; Cai et al., 2014). Moreover, as defective T<sub>reg</sub> and increased T<sub>H2</sub> responses at the mucosa are observed in food allergies and asthma, our findings might also have implications for these conditions. Indeed, epidemiological studies show an overlap between IBD and T<sub>H2</sub> driven diseases, such as atopic dermatitis and asthma (Lees et al., 2011). Furthermore, polymorphisms in the essential autophagy gene Atg5 have recently been implicated in asthma susceptibility (Martin et al., 2012; Poon et al., 2012). Autophagy is an attractive therapeutic target and several autophagy modulating compounds are already in clinical trials for the treatment of various disorders (Jiang and Mizushima, 2014). Furthermore, natural dietary-derived compounds, including retinoid acid (Isakson et al., 2014).
2010) and vitamin D (Yuk et al., 2009), have been shown to enhance autophagy. Taken together with our results, these findings raise the possibility that activation of autophagy through dietary or pharmacological modulation might have beneficial effects in disorders with a signature of decreased T<sub>reg</sub> and elevated T<sub>H2</sub> responses, including intestinal inflammation and various hypersensitivities.

Materials and methods

Mice

Atg16l1<sup>fl/fl</sup> mice were generated and provided by the H. Virgin laboratory (Washington University, Saint Louis, MO), as described (Hwang et al., 2012). Atg16l1<sup>fl/fl</sup> mice were crossed to B6.Cg-Tg(Cd4-cre)1Cwi/BfluJ (CD4-Cre mice) and B6.129(Cg)-Foxp3<sup>tm4(YFP/cre)Ayr</sup>/J (Foxp3<sup>Cre</sup> mice, Jackson Laboratory, Bar Harbor, ME) to generate Atg16l1<sup>DCD4</sup> and Atg16l1<sup>DFoxp3</sup> mice, respectively. All above strains, together with B6.SJL-CD45.1 (CD45.1<sup>+</sup> mice), B6.129(Cg)-Foxp3<sup>hCD2</sup> mice (Komatsu et al., 2009) were bred and maintained under specific pathogen-free conditions. Unless stated otherwise, mice were analyzed at 8–12 weeks (young mice) or > 5 months of age (aged mice). In the gene expression analysis Atg16l1<sup>DFoxp3</sup> mice and Foxp3<sup>Cre</sup> mice were co-housed and age- and sex- matched. In all other experiments mice used were age- and sex-matched littermates that were kept co-housed throughout the experiments.

T cell-mediated colitis

Experimental T cell-mediated colitis was induced by infection with Helicobacter hepaticus and concomitant IL-10R blockade as described (Song-Zhao and Maloy, 2014). Briefly, mice were infected with H.hepaticus (10<sup>8</sup> CFU per mouse) by oral gavage on three consecutive days and anti-IL-10R mAb (1B1.2) was administrated via i.p. injection (1 mg per mouse) on the first and seventh day of the infection. Mice were sacrificed 2 weeks after colitis induction.

Histological assessment of intestinal inflammation

Mice were euthanized at indicated time points whereupon tissue sections were fixed in buffered 10% formalin and paraffin-embedded. Sections were then cut and stained with hematoxylin and eosin. Histological analysis of intestinal inflammation was performed as described (Song-Zhao and Maloy, 2014). Briefly, inflammation was graded semi-quantitatively on a scale from 0 to 3, for four criteria; (a) epithelial hyperplasia and goblet cell depletion, (b) lamina propria leukocyte infiltration, (c) area of tissue affected, and (d) markers of severe inflammation, including crypt abscesses, submucosal inflammation, and ulceration. Scores for individual criteria were totaled for an overall inflammation score between 0 and 12.

Isolation of cells and flow cytometry analysis

Cell suspensions were prepared from the thymus, spleen, mLN, bone marrow and intestinal lamina propria as previously described (Uhlig et al., 2006). The following antibodies from eBioscience (Hatfield, UK) were used: anti-CD16/32 (93), anti-CD4 (GK1.5), anti-CD8α (53.6.7), anti-TCRβ (H57-597), anti-CD45 (30-F11), anti-CD44 (1M7), anti-CD62L (MEL-14), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD103 (2E7), anti-CD69 (H1.2F3), anti-KLRG1 (2F1), anti-CD25 (7D4), anti-CD36 (No.72–1), anti-hCD2 (2E7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD103 (2E7), anti-CD69 (H1.2F3), anti-KLRG1 (2F1), anti-CD25 (7D4), anti-CD36 (No.72–1), anti-hCD2 (RPA-2.10), anti-CTL4 (UC10-4B9), anti-GR.1 (RB6-8C5), anti-CD11b (M1/70), anti-Siglec F (E50-2440), anti-Gata3 (TWAJ), anti-Foxp3 (FJK-16s), anti-Ki67 (SolA15), anti-Helios (22F6), anti-Bcl2 (10C4), anti-PS6 (cupk43k), anti-IFN-γ (XMG1.2), anti-IL-17A (eBio17B7), anti-IL-13 (eBio13A). The following antibodies were from BioLegend (San Diego, CA): anti-CD138 (281–2), anti-CD161 (PK136), anti-F4/80 (BMB), anti-CD11b (M1/70). The following antibodies were from BD Biosciences (San Jose, CA): anti-CD45.1 (RA3 6B2), anti-GL7 (GL7), anti-CD95 (Jo2), anti-CD3 (145-2C11), anti-CD19 (1D3), anti-Ly6C (AL-21), anti-Ly6G (1A8), anti-IgM (R6-60.2), anti-IgG1 (A85-1). Anti-c-Myc antibody was from Cell Signaling Technology (D84C12, Danvers, MA). Anti-Neuropilin1 polyclonal antibody was from R&D Systems (FAB566A, Minneapolis, MN). Fixable Viability Dye from eBioscience was used to stain dead cells. Annexin V staining was performed using eBioscience kit (88–08006) according to manufacturer instructions. For intracellular cytokine staining cells were stimulated for 3h with PMA (100ng/ml) and lonomycin (1 μg/ml) in the presence of Brefeldin A (10 μg/ml).
Autophagosome formation detection by flow cytometry was performed using FlowCellect Autophagy LC3 Antibody-based Assay Kit (FCCH100171, Merk-Millipore, Billerica, MA) according to the manufacturer’s instructions and following cell surface markers staining. The Autophagy LC3 Antibody-Based Assay Kit involves a permeabilization step to wash out cytosolic LC3-I, allowing for antibody-based detection of membrane bound LC3-II. For autophagy detection in WT Treg cells B6 Foxp3<sup>Cre</sup> were used, as this allowed the detection of Foxp3<sup>+</sup> Treg cells on the basis of surface expression of hCD2 marker. All data were acquired using a Cyan ADP (Beckman Coulter, High Wycombe, UK) and analyzed using FlowJo software (Tree Star, Ashland, OR).

**CD4<sup>+</sup> T cell purification**

Bulk CD4<sup>+</sup> T cells were purified from the spleen and mLN by negative selection as previously described (Coccia et al., 2012). Naïve CD4<sup>+</sup> T cells were then sorted as CD4<sup>+</sup> CD25<sup>-</sup> CD44<sup>-</sup> CD62L<sup>+</sup> T<sub>reg</sub> cells were sorted as CD4<sup>+</sup> CD25<sup>+</sup> when sorted from Atg16l1<sup>Cre</sup> and Atg16l1<sup>fl/fl</sup> mice and as CD4<sup>+</sup> YFP<sup>+</sup> when sorted from Atg16l1<sup>fl/Cre</sup> and Foxp3<sup>Cre</sup> mice. Cells were sorted using an Astros, Beckman Coulter MoFlo XDP or AriaIII BD Bioscience. Post-sort flow cytometry analyses confirmed that the purity of sorted populations was >97%.

**Adoptive transfer of naïve CD4<sup>+</sup> T cells**

Naïve CD4<sup>+</sup> T cells from WT (CD45.1<sup>+</sup>) mice were sorted as described above and transferred to Atg16l1<sup>fl/fl</sup> recipient (CD45.2<sup>+</sup>) mice via intravenous injection (4-5x10<sup>6</sup> cells per mouse). Analysis of spleen, mLN and cLP CD4<sup>+</sup> T and T<sub>reg</sub> cells was performed 3 months after transfer.

**Generation of mixed bone marrow chimeras**

BM cells were isolated from the tibia and femur of WT (CD45.1<sup>+</sup>) mice and Atg16l1<sup>fl/fl</sup> or Atg16l1<sup>Cre</sup> (CD45.2<sup>+</sup>) mice and injected i.v. at 1:1 ratio (a total of 1x10<sup>7</sup> cells per mouse) into lethally irradiated (1100 Rad, split dose) Rag1<sup>-/-</sup> recipients. Mice were allowed to reconstitute for at least 8 weeks before analysis.

**Immunization with ovalbumin (OVA)**

For induction of OVA-specific IgE antibodies two treatment regimes were utilized. For OVA only immunization mice were fed three times by oral gavage with ovalbumin grade VII (5 mg per mouse, Sigma-Aldrich, St Louis, MO) with 21-day intervals between feeds. For adjuvanted immunization, mice were initially fed with OVA (5 mg per mouse) plus cholera toxin (10<sup>μg</sup> per mouse, Biological Compbell), after which they were fed twice with OVA only (5mg per mouse), with 21-day intervals between feeds.

**Infection with Trichuris muris and detection of T. muris-specific IgG<sub>1</sub>**

Mice were orally infected with ~200 Trichuris muris eggs. Serum was collected on day 34-post infection and assayed by ELISA for parasite-specific IgG<sub>1</sub>. Ninety-six-well plates were coated with 5 μg/ml T. muris excretory/secretory antigen and incubated with serial two-fold diluted serum. Bound IgG<sub>1</sub> was detected using biotinylated anti-murine IgG<sub>1</sub> (AbD Serotec, Kidlington, UK).

**Lipid uptake measurement**

Atg16l1<sup>fl/fl</sup> and Atg16l1<sup>Cre</sup> mice were injected i.p. with 50 μg of fluorescent 16-carbon fatty acid analog BODIPY C-16 (Molecular Probes) reconstituted in DMSO. Mice were culled 1 hr later and tissue collected for analysis by flow cytometry.

**Metabolic analysis using XF 96 extracellular flux analyzer**

The real-time extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using a XF 96 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA). Briefly, naïve (CD62L<sup>+</sup>CD4<sup>+</sup>) CD4<sup>+</sup> T cells, or in vitro polarized Th2 and T<sub>reg</sub> cells, were washed twice in assay medium (RPMI 1640 without sodium bicarbonate, 20 mM glucose, 1% FCS, 2mM pyruvate) and seeded at 3–4 x 10<sup>5</sup> cells per well in assay medium in a 96-well XF plate coated with poly-L-lysine (Sigma). T cells were rested for 1 hr at 37°C without CO<sub>2</sub> before analysis.
Polarization and stimulation of CD4+ T cell subsets

Naïve CD4+ T cells were cultured (3x10^5 cells/well) in 96-well plates coated with anti-CD3 mAb (5 µg/ml) and soluble anti-CD28 mAb (1 µg/ml) and kept in presence of IL-2 (100 U/ml). For T\(_{H0}\) conditions anti-IL-4 (10 µg/ml) and anti-IFN-γ (10 µg/ml) mAb were added. Cultures were supplemented with IL-12 (10 ng/ml) and anti-IL-4 mAb (10 µg/ml) for T\(_{H1}\) polarization; with IL-4 (20 ng/ml), anti-IFN-γ (20 µg/ml) and anti-IL-12 (10 µg/ml) for T\(_{H2}\) polarization; and with TGF-β (5 ng/ml), anti-IFN-γ, anti-IL-4 mAb and anti-IL-12 (all 10 µg/ml) for induced T\(_{reg}\) polarization. Sorted T\(_{reg}\) cells were activated for 48h with anti-CD3 mAb (5 µg/ml) and soluble anti-CD28 mAb (1 µg/ml) plus IL-2 (100 U/ml) and then cultured with IL-4 (10 ng/ml), IL-13 (10 ng/ml) and IL-2 (100 U/ml) for 5 days. All cytokines were from R&D Systems. Anti-CD3 (145-2C11), anti-CD28 (37.51), anti-IFN-γ (XMG1.2), anti-IL-12 (C17.8) and anti-IL-4 (11B11) mAb were from eBioscience. Cells were cultured in RPMI-1640 Medium, 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml of Penicillin/Streptomycin, and 0.05 mM 2-mercaptoethanol.

Measurement of serum antibodies and cytokines

All immunoglobulin isotypes except for IgE were measured by enzyme-linked immunosorbent assay (ELISA) using the SBA Clonotyping System (Southern Biotech, Birmingham, AL). IgE concentration was determined using an anti-mouse IgE ELISA (BioLegend), according to manufacturer’s instructions. For the detection of soy-specific, CBir-specific and Helicobacter-specific antibodies ELISA was performed with plates coated with purified soy antigen (5 µg/ml), CBir peptide (10 µg/ml) and soluble Helicobacter antigen (sHel antigen, 10 µg/ml) respectively. sHel antigen was prepared as previously described (Kullberg et al., 1998). For the detection of OVA-specific IgE, a sandwich ELISA was performed with biotinylated-OVA used for detection. MCPT-1 concentrations were measured by ELISA (eBioscience).

Immunofluorescence microscopy

Colonic and small intestine tissue samples were formalin-fixed, paraffin-embedded and sectioned as per histological analysis. Sections were deparaffinized, rehydrated, and subjected to sodium citrate-based antigen retrieval, then stained with mouse pAb anti-β-catenin (610153, BD Bioscience), rabbit pAb anti-CD3 (ab5690, Abcam, Cambridge, UK) and secondary goat antibodies conjugated to AlexaFluor488 or 555 (Life Technologies, Carlsbad, CA). Slides were mounted with DAPI-containing Vectashield (Vector Laboratories, Burlingame, CA). Images were acquired with an Olympus FluvioView FV1000 confocal microscope and Olympus FluvioView Software (Olympus, Tokyo, Japan).

Western blotting analysis

CD4+ T cells purified by negative selection were lysed in RIPA buffer containing protease inhibitor cocktail (Roche, Basel, Switzerland). Protein levels were normalized by Biorad DC protein assay (BioRad Laboratories, Hercules, CA), resolved by SDS-PAGE and, following transfer onto nitrocellulose membranes, were blotted with anti-LC3 antibody (L7543; Sigma-Aldrich) and anti-tubulin antibody (sc5286, Santa Cruz Biotechnology, Dallas, TX), and secondary HRP conjugated anti–rabbit antibody (7074S, Cell Signaling Technology).

Fluidigm gene expression analysis

CD4+ T cells and T\(_{reg}\) cells were sorted for each population based on surface marker and YFP expression from spleen and cLP of Atg16l1\(^{Flox}\) and Foxp3\(^{Cre}\) mice. Two hundred cells/population were sorted in triplicates from a total of four (spleen) or six (cLP) mice per group. Alternatively, 250 cells from in vitro polarized populations of T\(_{H1}\) and T\(_{reg}\) cells were sorted from triplicate culture wells. RNA was reverse transcribed and cDNA was pre-amplified using the CellsDirect OneStep qRT kit (Invitrogen). The selected autophagy, apoptotic and metabolic genes were amplified and analyzed for expression using a dynamic 48x48 array (Biomark Fluidigm) as previously described (Tehranchi et al., 2010). Data were analyzed using the 2\(^{-}\Delta\Delta Ct\) method, and the results were normalized to actin or HPRTp expression.
Statistical analysis
For weight curves and antibody titers, p-values were determined by two-way ANOVA with Bonferroni post-tests. For the metabolic analysis using XF 96 extracellular flux analyzer, p-values were determined using unpaired Student’s t-test. For all other experiments, p-values were determined by nonparametric Mann–Whitney test. Differences were considered statistically significant when p<0.05 (*<p<0.05, **p<0.01, ***p<0.001). Data are shown as mean ± s.e.m. Statistics were calculated using GraphPad Prism 6 software. For in vivo experiments, sample size was determined by power analysis using power of trial software, which calculates a power value based on X² test statistics. Calculated required sample sizes were applied whenever possible. No mouse was excluded from the analysis. With the exception of histological assessment of intestinal inflammation, experimenters were not ‘blinded’ to allocation of animals to experimental groups.

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Additional information

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Author contributions
AMK, OJH, TR, AEM, AL, LA-D, SPF, JP, Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article; CFP, Assisted in the in vivo experiments, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article; RKG, QS, AKS, KJM, Conception and design, Analysis and interpretation of data, Drafting or revising the article

Author ORCIDs
Kevin J Maloy, http://orcid.org/0000-0001-5795-7688

Ethics
Animal experimentation: All procedures on mice in this study were conducted in accordance with the UK Scientific Procedures Act (1986) under a project license (PPL 30/2872) authorized by the UK Home Office Animal Procedures Committee and approved by the Sir William Dunn School of Pathology Local Ethical Review Committee.
References


