



Figures and figure supplements

The autophagy gene *Atg16l1* differentially regulates T_{reg} and T_{H2} cells to control intestinal inflammation

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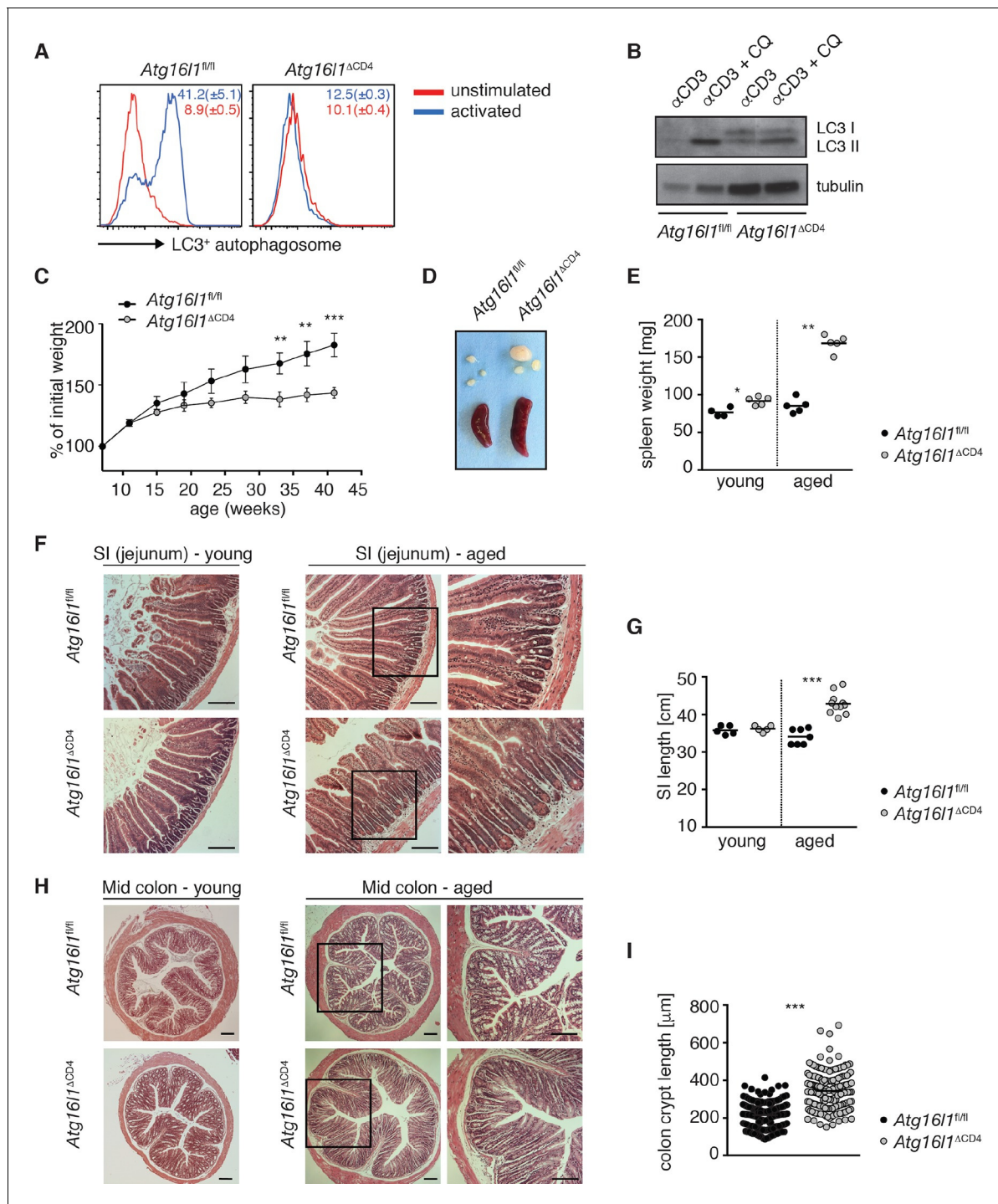


Figure 1. Aged *Atg16l1 Δ CD4* mice develop intestinal inflammation. (A) FACS analysis of LC3⁺ autophagosome formation in CD4⁺ T cells from cLP of *Atg16l1 Δ CD4* and *Atg16l1^{fl/fl}* mice after overnight activation with or without α -CD3 (5 μ g/ml) and α -CD28 (1 μ g/ml). (B) Western blot analysis of LC3 lipidation in naïve splenic CD4⁺ T cells isolated from *Atg16l1 Δ CD4* mice and *Atg16l1^{fl/fl}* mice after 3hr activation with α -CD3 (5 μ g/ml) and α -CD28 (1 μ g/ml) with or without chloroquine (CQ, inhibitor of lysosomal degradation, 50 μ M). (C) Weight curves of *Atg16l1 Δ CD4* and *Atg16l1^{fl/fl}* littermates. (D) Representative images of spleens and mesenteric lymph nodes (mLN) from aged *Atg16l1 Δ CD4* and *Atg16l1^{fl/fl}* littermates and (E) spleen weights of young and aged *Atg16l1 Δ CD4* and *Atg16l1^{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 Δ CD4* and *Atg16l1^{fl/fl}* littermates, scale bar 150 μ m. (G,I) Quantification of (G) SI lengths and (I) mid-colon crypt lengths in aged *Atg16l1 Δ CD4* and *Atg16l1^{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.

Figure 1 continued

an individual mouse and horizontal bars denote means (E,G). In (I) each dot represents an individual crypt measurement and horizontal bars denote means. Statistical significance was determined using two-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons (C) or the Mann-Whitney test (E,G,I), ** $p < 0.01$; *** $p < 0.001$. SI LP– small intestine lamina propria, cLP – colonic lamina propria. Young mice: 8–12 weeks old, aged mice >5 months old.

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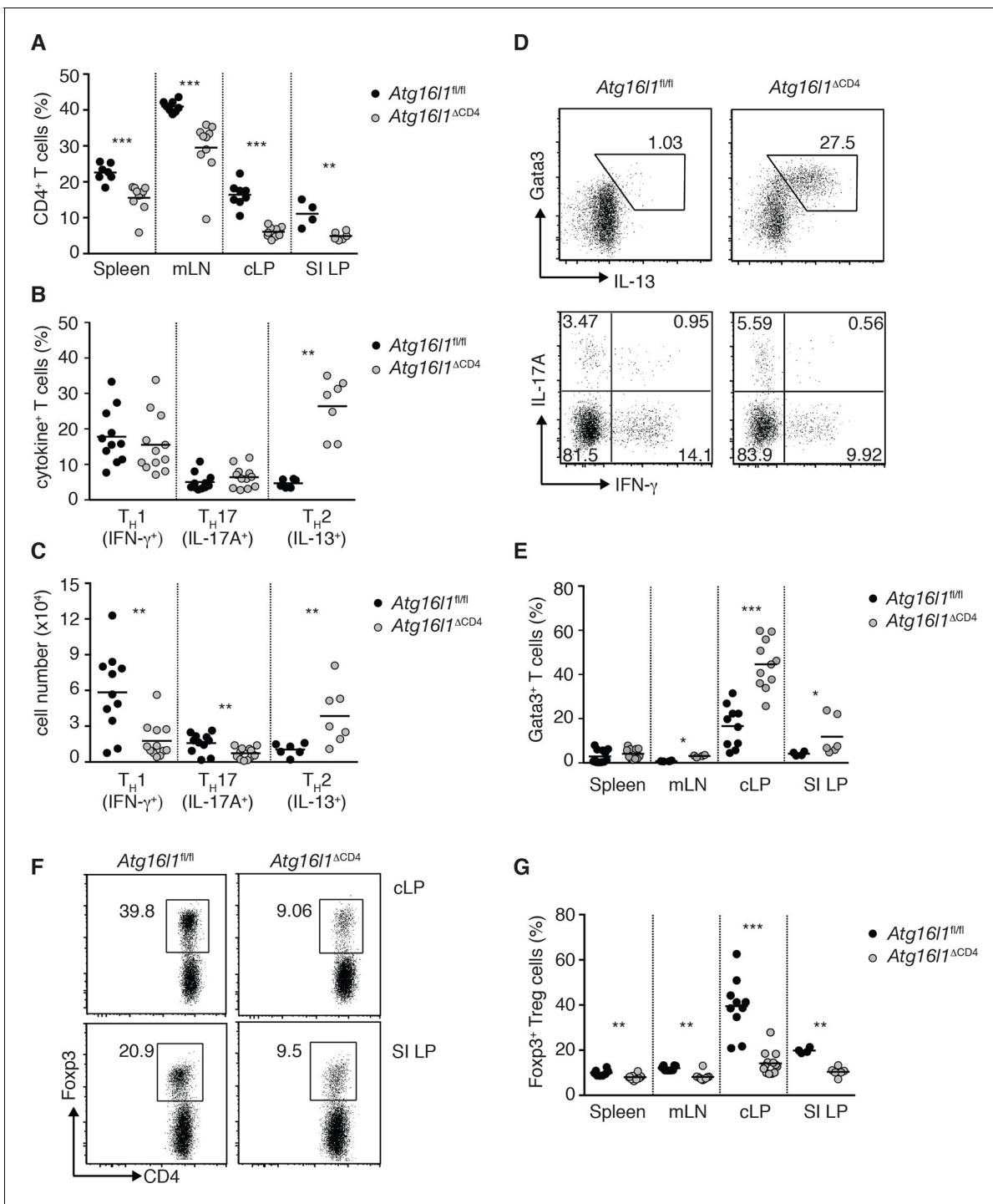


Figure 2. *Atg16l1^{ΔCD4}* mice exhibit reciprocal dysregulation of intestinal T_H2 and T_{reg} cells before the onset of intestinal inflammation. (A) Frequencies of CD4⁺ T cells as a proportion of live cells in young *Atg16l1^{ΔCD4}* and *Atg16l1^{fl/fl}* littermates. (B) Frequencies and (C) total numbers of IFN-γ⁺ T_H1, IL-17A⁺ T_H17 and IL-13⁺ T_H2 cells isolated from cLP of young *Atg16l1^{ΔCD4}* and *Atg16l1^{fl/fl}* littermates (gated on CD4⁺ T cells). (D) Representative FACS plots of Gata3 and IL-13 (top) or IFN-γ and IL-17A (bottom) expression by cLP CD4⁺ T cells isolated from young *Atg16l1^{ΔCD4}* and *Atg16l1^{fl/fl}* littermates (gated on CD4⁺ TCRβ⁺ Foxp3⁻ live cells). (E) Frequencies of Gata3⁺ CD4⁺ T cells in young *Atg16l1^{ΔCD4}* and *Atg16l1^{fl/fl}* littermates (gated on CD4⁺ TCRβ⁺ Foxp3⁻ cells). (F) Representative FACS plots and (G) frequencies of Foxp3⁺ T_{reg} cells in young *Atg16l1^{ΔCD4}* and *Atg16l1^{fl/fl}* littermates (gated on CD4⁺ TCRβ⁺ 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

Figure 2 continued on next page

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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.

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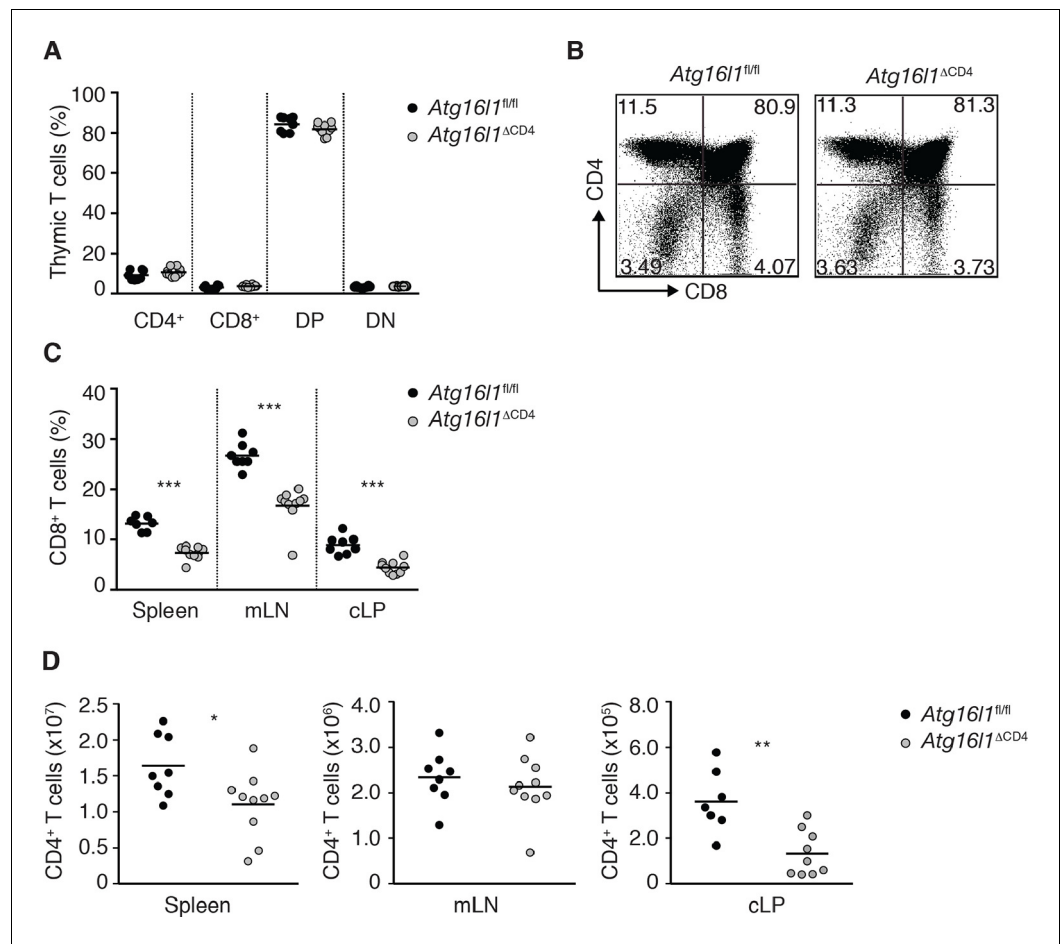


Figure 2—figure supplement 1. Characterization of immune cell compartments in young *Atg16l1^{ΔCD4}* mice. (A) Frequencies and (B) representative FACS plots of single positive CD4⁺, single positive CD8⁺, double positive (DP) CD4⁺ CD8⁺ and double negative (DN) CD4⁻ CD8⁻ thymocytes in young *Atg16l1^{ΔCD4}* and *Atg16l1^{fl/fl}* littermates. (C) Frequencies of CD8⁺ T cells in young *Atg16l1^{ΔCD4}* and *Atg16l1^{fl/fl}* littermates. (D) Total numbers of CD4⁺ T cells in spleen, mLN, and cLP of young *Atg16l1^{ΔCD4}* and *Atg16l1^{fl/fl}* littermates. Data are combined from (A,C,D) or representative of (B) two or three independent experiments with at least 4 mice per group. Each dot represents an individual mouse and horizontal bars denote means. Numbers indicate percentage of cells in quadrants. Statistical significance was determined using the Mann–Whitney test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. cLP – colonic lamina propria, mLN - mesenteric lymph nodes. Young mice: 8–12 weeks old.

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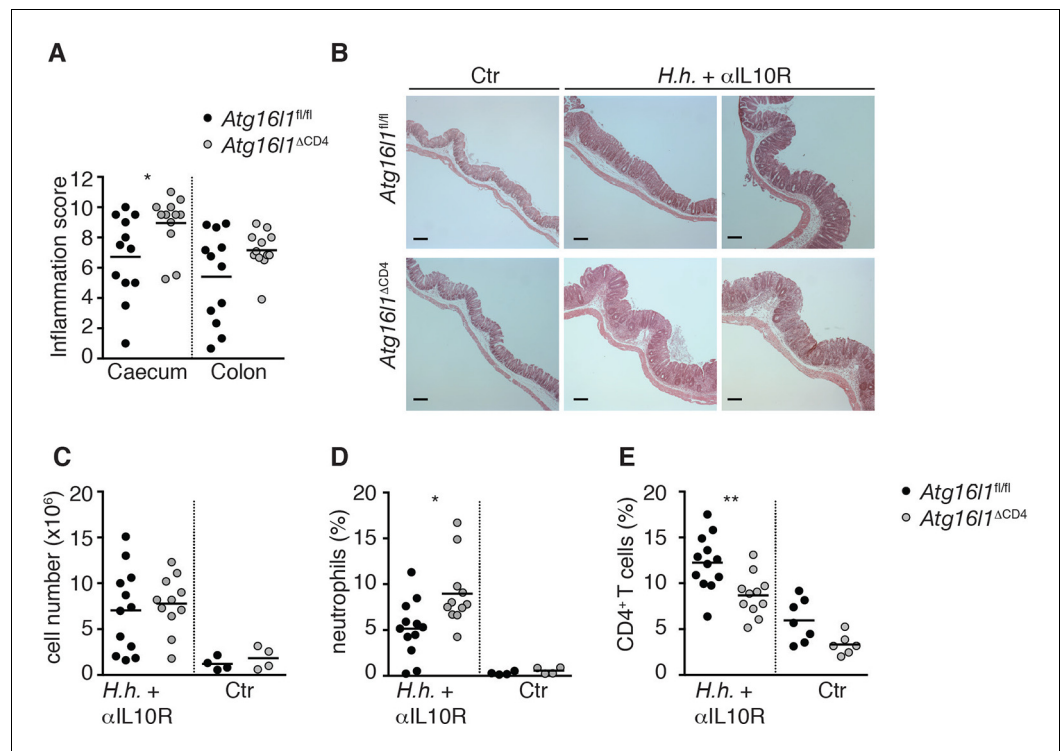


Figure 2—figure supplement 2. *Atg16l1*^{ΔCD4} mice have increased susceptibility to T-cell-mediated experimental IBD. Cohorts of young *Atg16l1*^{ΔCD4} and *Atg16l1*^{fl/fl} littermates were infected with *Helicobacter hepaticus* by oral gavage (three feeds of 1×10^8 CFU) and treated with anti-IL-10R mAb (1mg/mouse *i.p.* given weekly; *H.h.* + αIL10R) or left untreated (Ctr). Two weeks post-infection mice were sacrificed for analyses. (A) Caecum and colon histopathology scores in *H.h.* + αIL10R-treated *Atg16l1*^{ΔCD4} and *Atg16l1*^{fl/fl} littermates. (B) Representative photomicrographs of H&E stained caecum of untreated Ctr (left panels) or *H.h.* + αIL10R-treated (middle and right panels) *Atg16l1*^{ΔCD4} and *Atg16l1*^{fl/fl} littermates, scale bar 150 μm. (C) Total lamina propria leukocyte numbers and (D,E) frequencies of (D) neutrophils (Gr1^{hi} CD11b⁺) and (E) CD4⁺ T cells in cLP isolated from untreated Ctr or *H.h.* + αIL10R-treated *Atg16l1*^{ΔCD4} and *Atg16l1*^{fl/fl} littermates. Data are combined from two or three independent experiments with at least two mice per group. Each dot represents an individual mouse and horizontal bars denote means. Statistical significance was determined using the Mann-Whitney test, * $p < 0.05$; ** $p < 0.01$.

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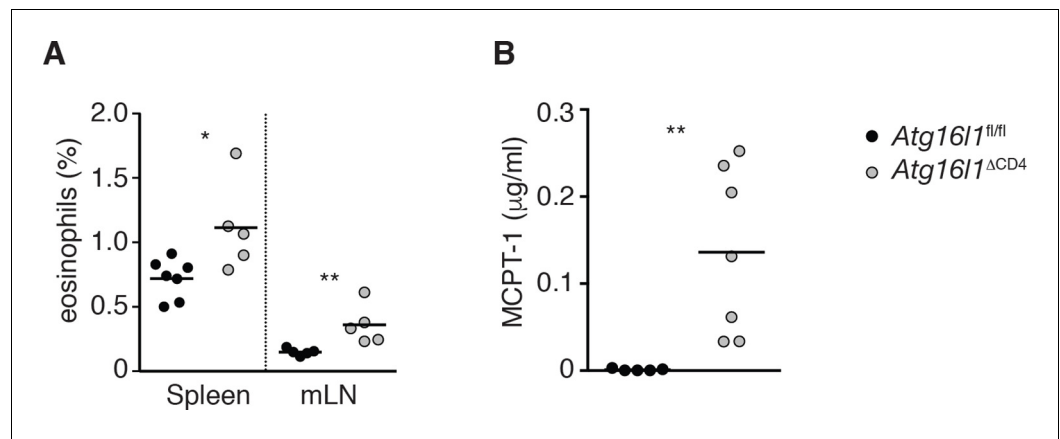


Figure 2—figure supplement 3. Elevated type 2 innate responses in *Atg16l1^{ΔCD4}* mice. (A) Frequencies of eosinophils (Ly6C^{low} Ly6G^{low} CD11b⁺ F4/80⁺) in spleen and mLN of young *Atg16l1^{ΔCD4}* and *Atg16l1^{fl/fl}* littermates. (B) Serum MCPT-1 levels in young *Atg16l1^{ΔCD4}* and *Atg16l1^{fl/fl}* littermates were measured by ELISA. Data are combined from (A) or representative of (B) two or three independent experiments with at least four mice per group. Each dot represents an individual mouse and horizontal bars denote means. Statistical significance was determined using the Mann–Whitney test, * $p < 0.05$; ** $p < 0.01$. mLN - mesenteric lymph nodes. Young mice: 8–12 weeks old.

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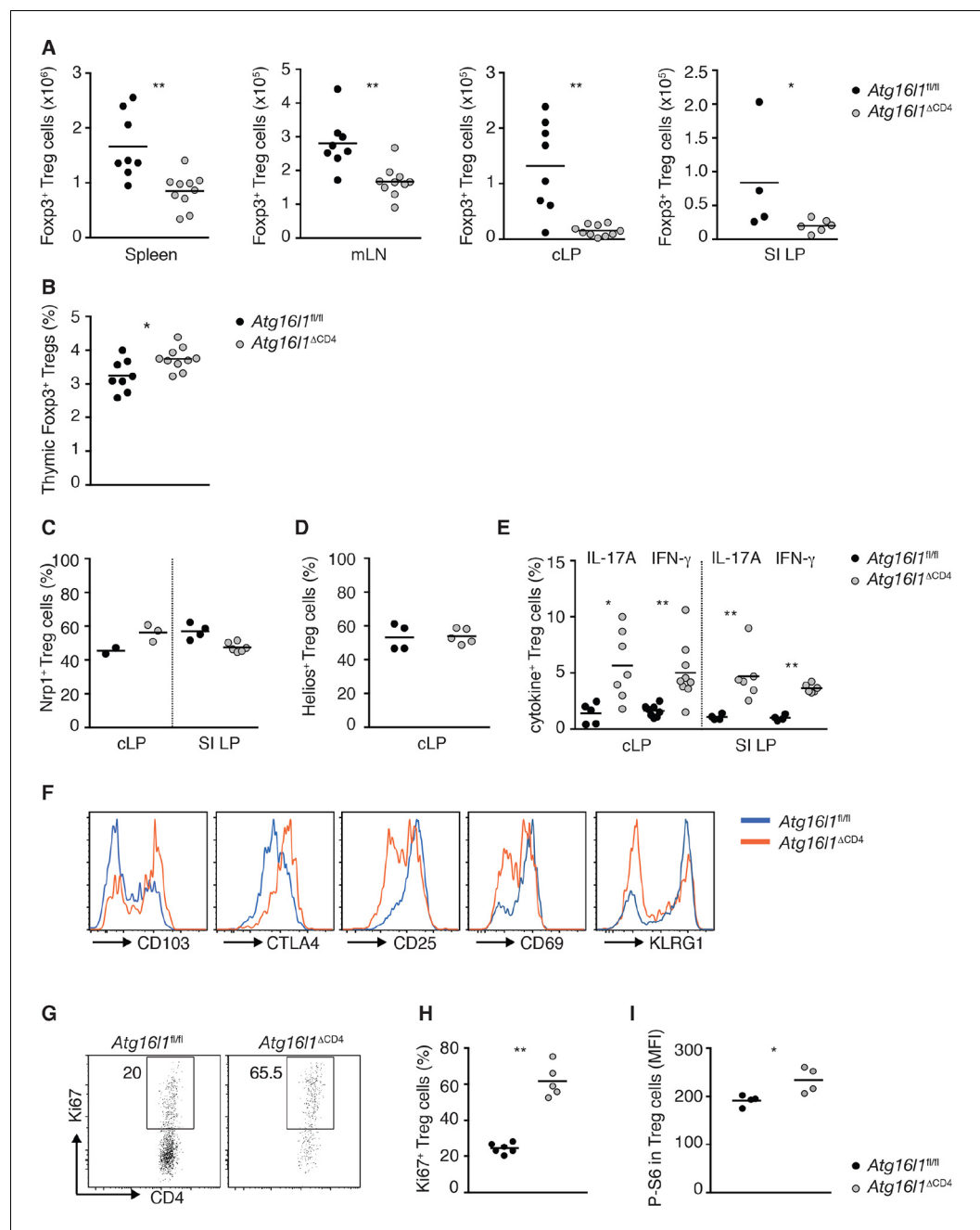


Figure 2—figure supplement 4. Characterization of *Atg16l1*-deficient T_{reg} cells. (A) $Foxp3^{+}$ T_{reg} cell numbers in spleen, mLN, cLP and SI LP of young *Atg16l1*^{ΔCD4} and *Atg16l1*^{fl/fl} littermates. (B) Frequencies of $Foxp3^{+}$ T_{reg} in the thymus of young *Atg16l1*^{ΔCD4} and *Atg16l1*^{fl/fl} littermates (gated on single positive $CD4^{+}$ $TCR\beta^{+}$ cells). (C) Frequencies of Neuropilin-1⁺ ($Nrp1^{+}$) $Foxp3^{+}$ T_{reg} cells in the cLP and SI LP of young *Atg16l1*^{ΔCD4} and *Atg16l1*^{fl/fl} littermates (gated on $CD4^{+}$ $TCR\beta^{+}$ $Foxp3^{+}$ T cells). (D) Frequencies of Helios⁺ $Foxp3^{+}$ T_{reg} cells in the cLP of young *Atg16l1*^{ΔCD4} and *Atg16l1*^{fl/fl} littermates (gated on $CD4^{+}$ $TCR\beta^{+}$ $Foxp3^{+}$ T cells). (E) Frequencies of IL-17A⁺ or IFN- γ^{+} of $Foxp3^{+}$ T_{reg} cells in the cLP and SI LP of young *Atg16l1*^{ΔCD4} and *Atg16l1*^{fl/fl} littermates (gated on $Foxp3^{+}$ $CD4^{+}$ $TCR\beta^{+}$ T cells). (F) Expression of CD103, CTLA4, CD25, CD69 and KLRG1 by cLP $Foxp3^{+}$ T_{reg} cells from young *Atg16l1*^{ΔCD4} and *Atg16l1*^{fl/fl} littermates (gated on $Foxp3^{+}$ $CD4^{+}$ $TCR\beta^{+}$ T cells). (G) Representative FACS plots and (H) frequencies of Ki67⁺ $Foxp3^{+}$ T_{reg} cells in cLP of young *Atg16l1*^{ΔCD4} and *Atg16l1*^{fl/fl} littermates (gated on $Foxp3^{+}$ $CD4^{+}$ $TCR\beta^{+}$ T cells). (I) Mean fluorescence intensity (MFI) of phospho-S6 (P-S6) in $Foxp3^{+}$ T_{reg} cells in cLP of young *Atg16l1*^{ΔCD4} and *Atg16l1*^{fl/fl} littermates. Data are combined from two independent experiments with at least four mice per group (A,B,E), are representative from two independent experiments with Figure 2—figure supplement 4 continued on next page

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at least four mice per group (C,F-H), or are from one experiment (D,I). Each dot represents an individual mouse and horizontal bars denote means. Numbers indicate percentage of cells in gates. Statistical significance was determined using the Mann–Whitney test, * $p < 0.05$; ** $p < 0.01$. mLN - mesenteric lymph nodes, SI LP- small intestine lamina propria, cLP - colonic lamina propria. Young mice: 8–12 weeks old.

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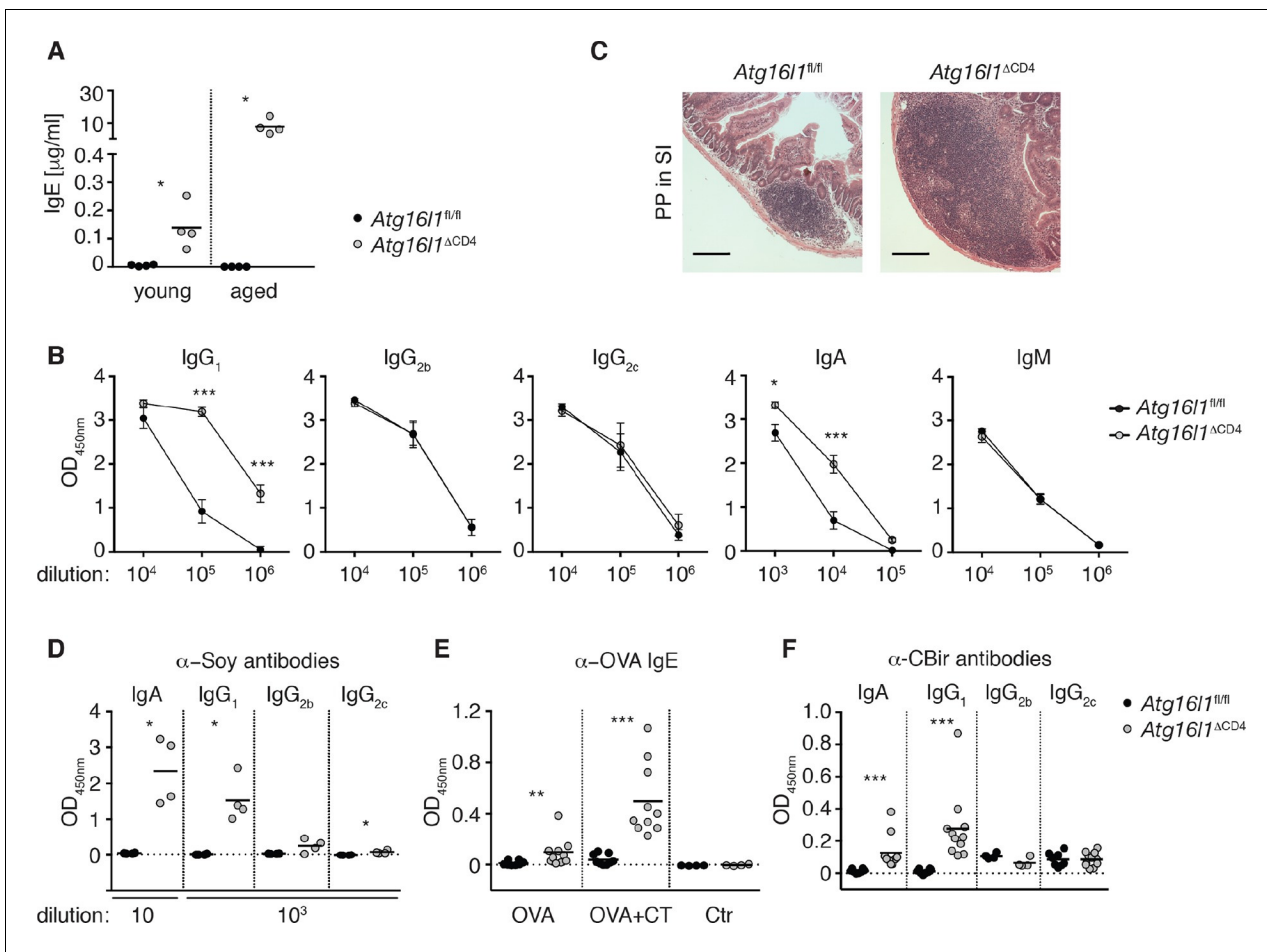


Figure 3. *Atg16l1*^{ACD4} mice develop elevated T_H2-associated antibodies against intestinal luminal antigens. (A) Serum IgE concentrations in cohorts of young and aged *Atg16l1*^{ACD4} and *Atg16l1*^{fl/fl} littermates were measured by ELISA. (B) Serum antibody IgG₁, IgG_{2b}, IgG_{2c}, IgA and IgM isotype levels in aged *Atg16l1*^{ACD4} and *Atg16l1*^{fl/fl} littermates were measured by ELISA. (C) Representative photomicrographs of H&E stained sections of Peyer's patch (PP) in the SI (jejunum) of aged *Atg16l1*^{ACD4} and *Atg16l1*^{fl/fl} littermates, scale bar 150 μm . (D) Serum levels of Soy-specific IgA, IgG₁, IgG_{2b}, IgG_{2c} antibodies in aged *Atg16l1*^{ACD4} and *Atg16l1*^{fl/fl} littermates were measured by ELISA. (E) Young *Atg16l1*^{ACD4} and *Atg16l1*^{fl/fl} littermates were fed with ovalbumin (OVA) alone or with cholera toxin (CT) as described in methods and levels of OVA-specific serum IgE were measured 8 weeks after first challenge by ELISA. (F) Levels of CBir1-specific IgA, IgG₁, IgG_{2b} and IgG_{2c} antibodies in serum of aged *Atg16l1*^{ACD4} and *Atg16l1*^{fl/fl} littermates were measured by ELISA, serum was diluted 50x. Data are representative from at least two independent experiments with at least three mice per group (A-D) or combined from two (E) or three (F) independent experiments with at least three mice per group. Each dot represents an individual mouse and horizontal bars denote means (A,D,E,F). Serum isotype levels are shown as mean \pm s.e.m (B). Statistical significance was determined using the Mann-Whitney test (A,D-F) or two-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons (B), * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. SI – small intestine. Young mice: 8–12 weeks old, aged mice > 5 months old.

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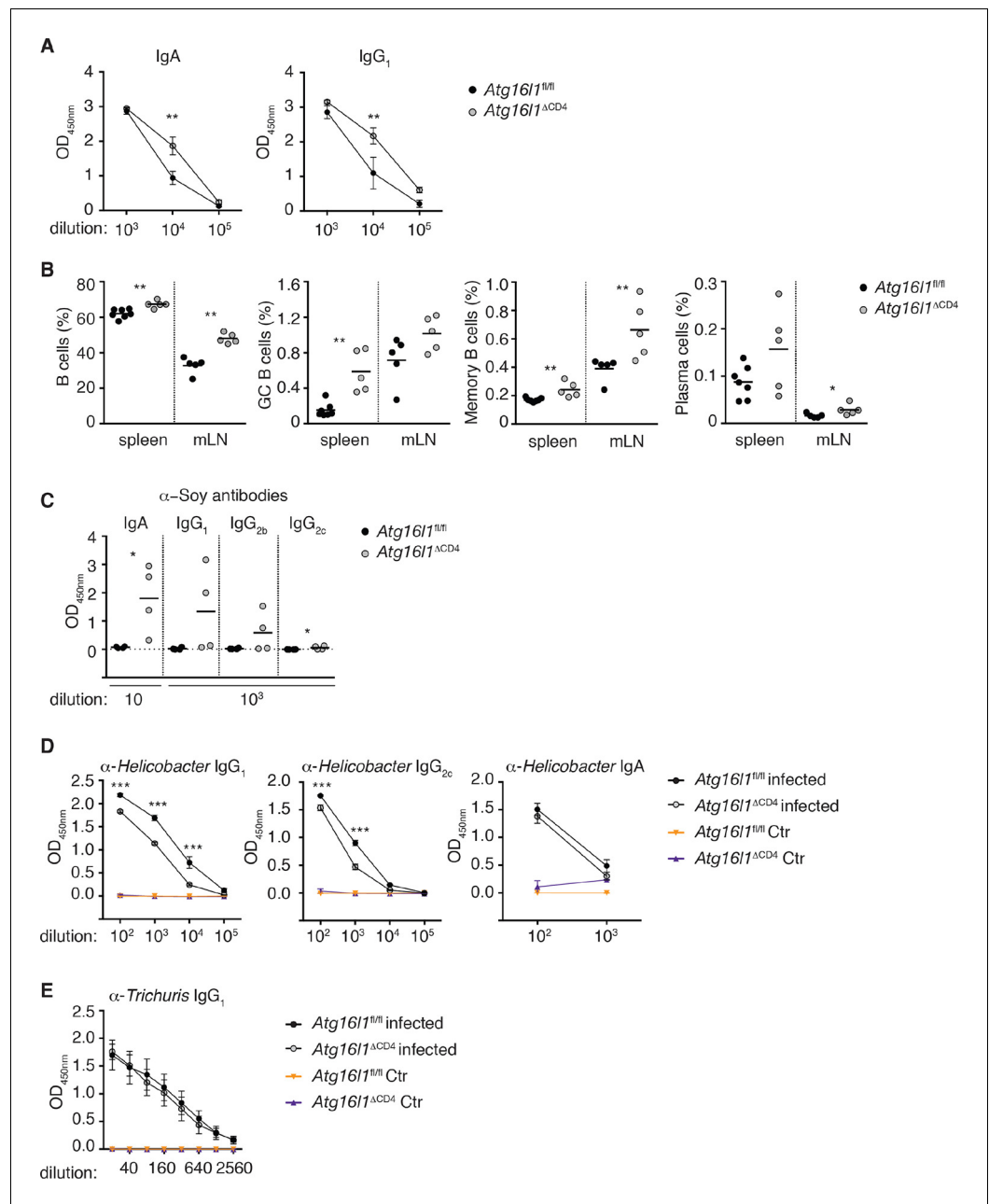


Figure 3—figure supplement 1. Dysregulated humoral responses in young *Atg16l1*^{ACD4} mice. (A) Serum antibody IgA and IgG₁ isotype levels in young *Atg16l1*^{ACD4} and *Atg16l1*^{fl/fl} littermates were measured by ELISA. (B) Frequencies of B cells (B220⁺), germinal center B cells (GC: B220⁺ GL7⁺ CD95⁺), memory B cells (B220⁺ GL7⁺ IgM⁺ IgG⁺) and plasma cells (CD138⁺) in the spleen and mLN of young *Atg16l1*^{ACD4} and *Atg16l1*^{fl/fl} littermates (gated on live CD45⁺ cells). (C) Serum levels of Soy-specific IgA, IgG₁, IgG_{2b}, IgG_{2c} antibodies in young *Atg16l1*^{ACD4} and *Atg16l1*^{fl/fl} littermates were measured by ELISA. (D) Cohorts of young *Atg16l1*^{ACD4} and *Atg16l1*^{fl/fl} littermates were infected with *Helicobacter hepaticus* by oral gavage (three feeds of 1×10⁸ CFU) and levels of serum *Helicobacter*-specific IgG₁, IgG_{2c} and IgA antibodies were determined three weeks later by ELISA. (E) Cohorts of young *Atg16l1*^{ACD4} and *Atg16l1*^{fl/fl} littermates were orally infected with *Trichuris muris* (200 eggs) and levels of *T. muris*-specific IgG₁ in serum were determined 34 days later by ELISA. Data are from one experiment with at least three mice per group (A–D) or representative from two independent experiments with five mice per group (E). Each dot represents an individual mouse and horizontal bars denote means (B,C) or data represent mean ± s.e.m (A,D,E). Statistical significance was determined using the Mann–Whitney test (A,C) or two-way analysis of variance (ANOVA) with Bonferroni’s correction for multiple comparisons (B,D,E): statistical significance was determined between infected groups (b,c), *p<0.05; **p<0.01. mLN - mesenteric lymph nodes. Young mice: 8–12 weeks old. *Figure 3—figure supplement 1 continued on next page*

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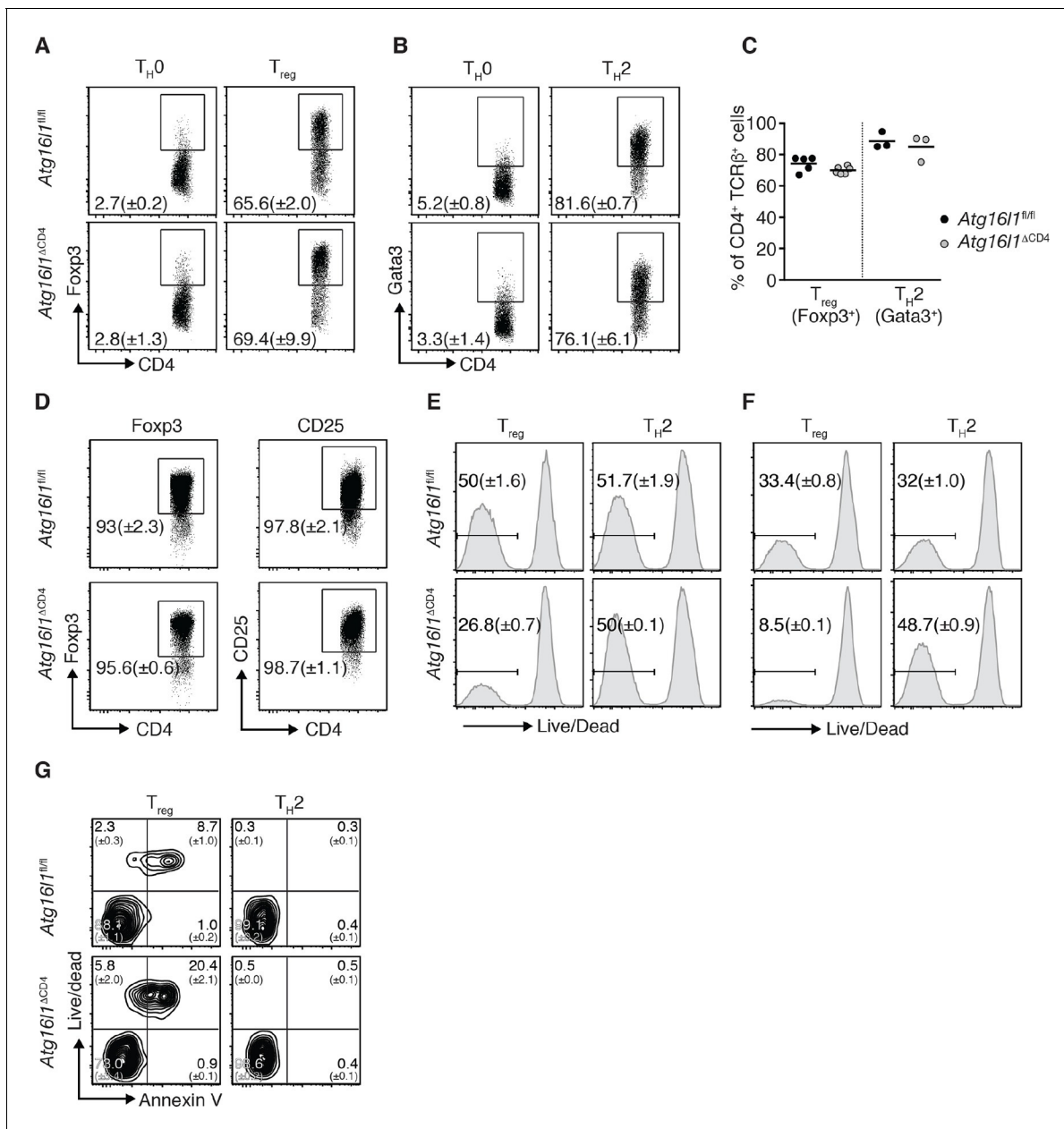


Figure 4. *Atg16l1* promotes survival of T_{reg} cells and limits T_{H2} cell survival. (A,B) $Atg16l1^{\Delta CD4}$ or $Atg16l1^{fl/fl}$ naïve $CD4^+$ T cells were cultured in T_{H0} , T_{reg} , or T_{H2} polarizing conditions for 48 hr and analyzed by FACS. Representative FACS plots show (A) Foxp3 and (B) Gata3 expression (gated on $CD4^+$ TCR β^+ T cells). (C) Frequencies of T_{reg} cells (Foxp3 $^+$) and T_{H2} cells (Gata3 $^+$) arising from $Atg16l1^{\Delta CD4}$ or $Atg16l1^{fl/fl}$ naïve $CD4^+$ T cells cultured in T_{reg} or T_{H2} polarizing conditions for 5 days. (D) $Atg16l1^{\Delta CD4}$ or $Atg16l1^{fl/fl}$ T_{reg} cells were cultured with anti-CD3 (3 μ g/ml) and anti-CD28 (1 μ g/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) Naïve $Atg16l1^{\Delta CD4}$ or $Atg16l1^{fl/fl}$ $CD4^+$ T cells were cultured with (E) 1 μ g/ml or (F) 5 μ g/ml anti-CD3 plus anti-CD28 (1 μ g/ml) for 48 hr in T_{reg} or T_{H2} 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_{reg} cells and T_{H2} cells from the cLP of young $Atg16l1^{\Delta CD4}$ and $Atg16l1^{fl/fl}$ littermates, gated on $CD4^+$ TCR β^+ Foxp3 $^+$ (left panel), or $CD4^+$ TCR β^+ 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 \pm 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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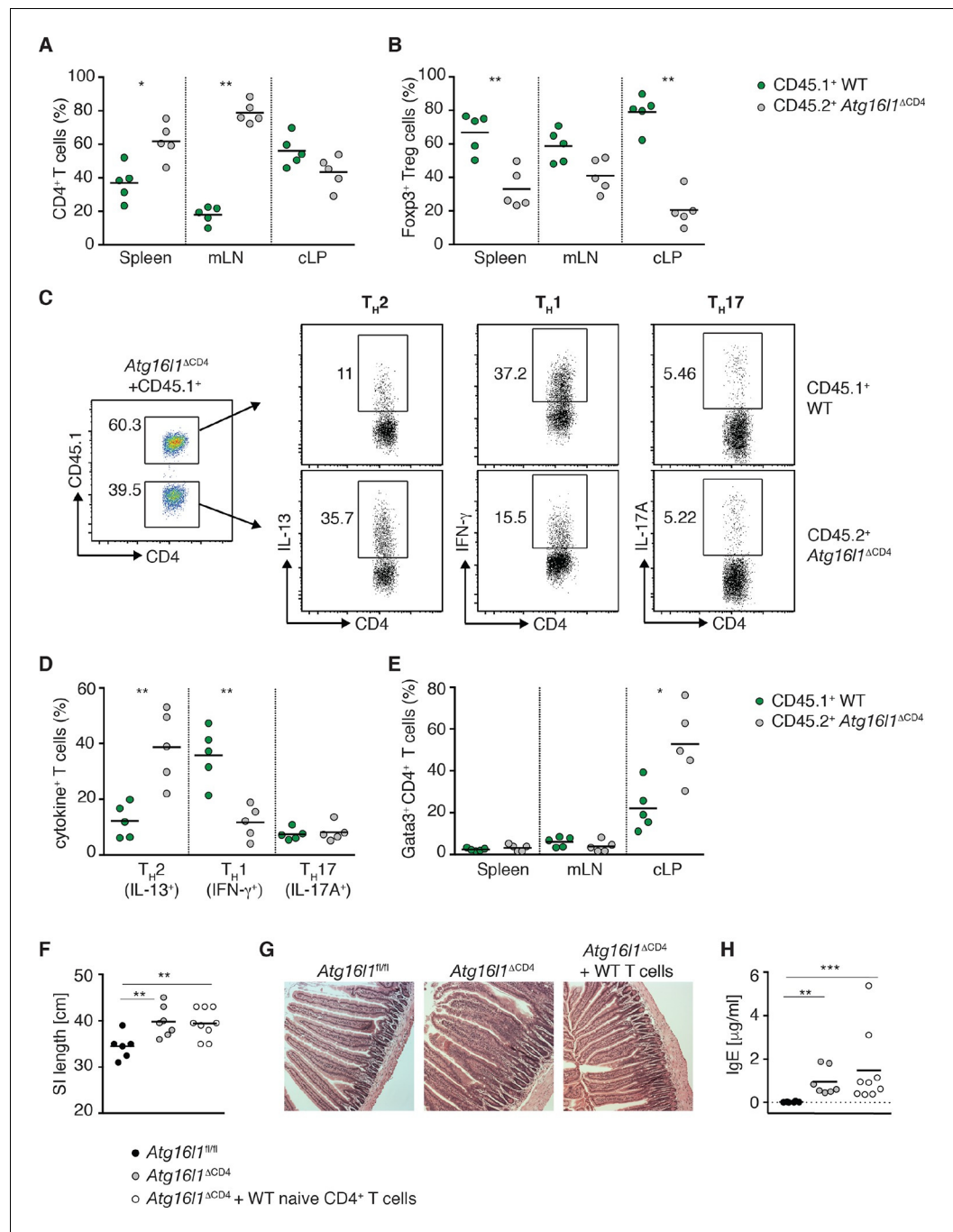


Figure 5. Autophagy contributes to the elevated T_H2 responses in *Atg16l1*^{ACD4} mice in a cell-intrinsic manner. Young *Atg16l1*^{ACD4} mice (CD45.2⁺) were adoptively transferred with 4-5 \times 10⁶ naive WT CD4⁺ T cells (CD45.1⁺) and analyzed 3 months later. (A) Frequencies of WT (CD45.1⁺) and *Atg16l1*-deficient (CD45.2⁺) CD4⁺ T cells in the spleen, mLN and cLP. (B) Frequencies of WT (CD45.1⁺) and *Atg16l1*-deficient (CD45.2⁺) Foxp3⁺ T_{reg} cells in the spleen, mLN and cLP (gated on CD4⁺ TCR β ⁺ 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 (T_H2), IFN- γ (T_H1) and IL-17A (T_H17) in the cLP (gated on CD4⁺ TCR β ⁺ Foxp3⁺ T cells). (D) Frequencies of WT (CD45.1⁺) and *Atg16l1*-deficient (CD45.2⁺) T_H2 (IL-13⁺), T_H1 (IFN- γ ⁺) and T_H17 (IL-17A⁺) cells among CD4⁺ TCR β ⁺ 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⁺ TCR β ⁺ Foxp3⁺ T cells). (F) SI lengths and (G) representative photomicrographs of jejunum of control untreated *Atg16l1*^{fl/fl} or *Atg16l1*^{ACD4} littermates and reconstituted *Atg16l1*^{ACD4} mice, scale bar 150 μ m. (H) Serum IgE concentrations in control untreated *Atg16l1*^{fl/fl} or *Atg16l1*^{ACD4} littermates and adoptively transferred

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Atg16l1^{ACD4} mice were measured by ELISA. Data are representative of two independent experiments with at least four mice per group (A-E,G) or combined from two independent experiments (F,H). Each dot represents cells coming from the donor or the hosts within the individual transferred mouse (A,B,D,E) or each dot represents an individual mouse (F,H), horizontal bars denote mean. Numbers indicate percentage of cells in gates. Statistical significance was determined using the Mann–Whitney test, * $p < 0.05$; ** $p < 0.01$. mLN - mesenteric lymph nodes, cLP – colonic lamina propria. Young mice: 10–12 weeks old.

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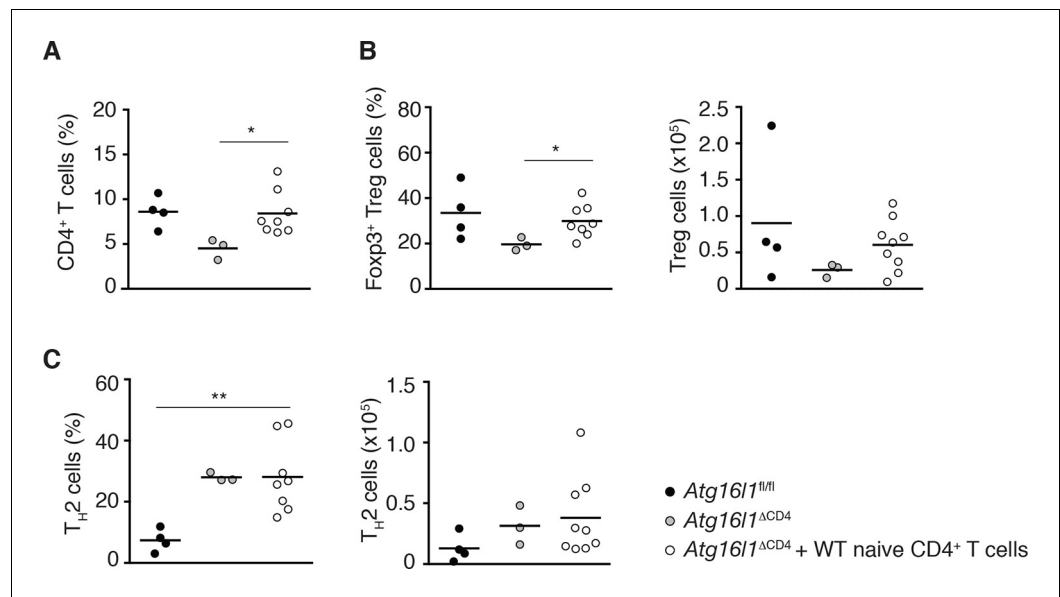


Figure 5—figure supplement 1. Reconstitution of intestinal CD4⁺ T cell compartments in adoptively transferred *Atg16l1*^{ΔCD4} mice. Young *Atg16l1*^{ΔCD4} mice (CD45.2⁺) were adoptively transferred with 4-5x10⁶ naïve WT CD4⁺ T cells (CD45.1⁺) *i.v.* and cLP populations analyzed 3 months later. **(A)** Frequencies of total CD4⁺ T cells. **(B)** Frequencies and total numbers of Foxp3⁺ T_{reg} cells (gated on CD4⁺ TCRβ⁺ T cells). **(C)** Frequencies and total numbers of T_{H2} (IL-13⁺) cells (gated on CD4⁺ TCRβ⁺ Foxp3⁻ T cells). Data are combined from two independent experiments with at least four mice per group. Each dot represents an individual mouse, horizontal bars denote mean. Statistical significance was determined using the Mann–Whitney test, *p<0.05; **p<0.01. cLP – colonic lamina propria. Young mice: 10–12 weeks old.

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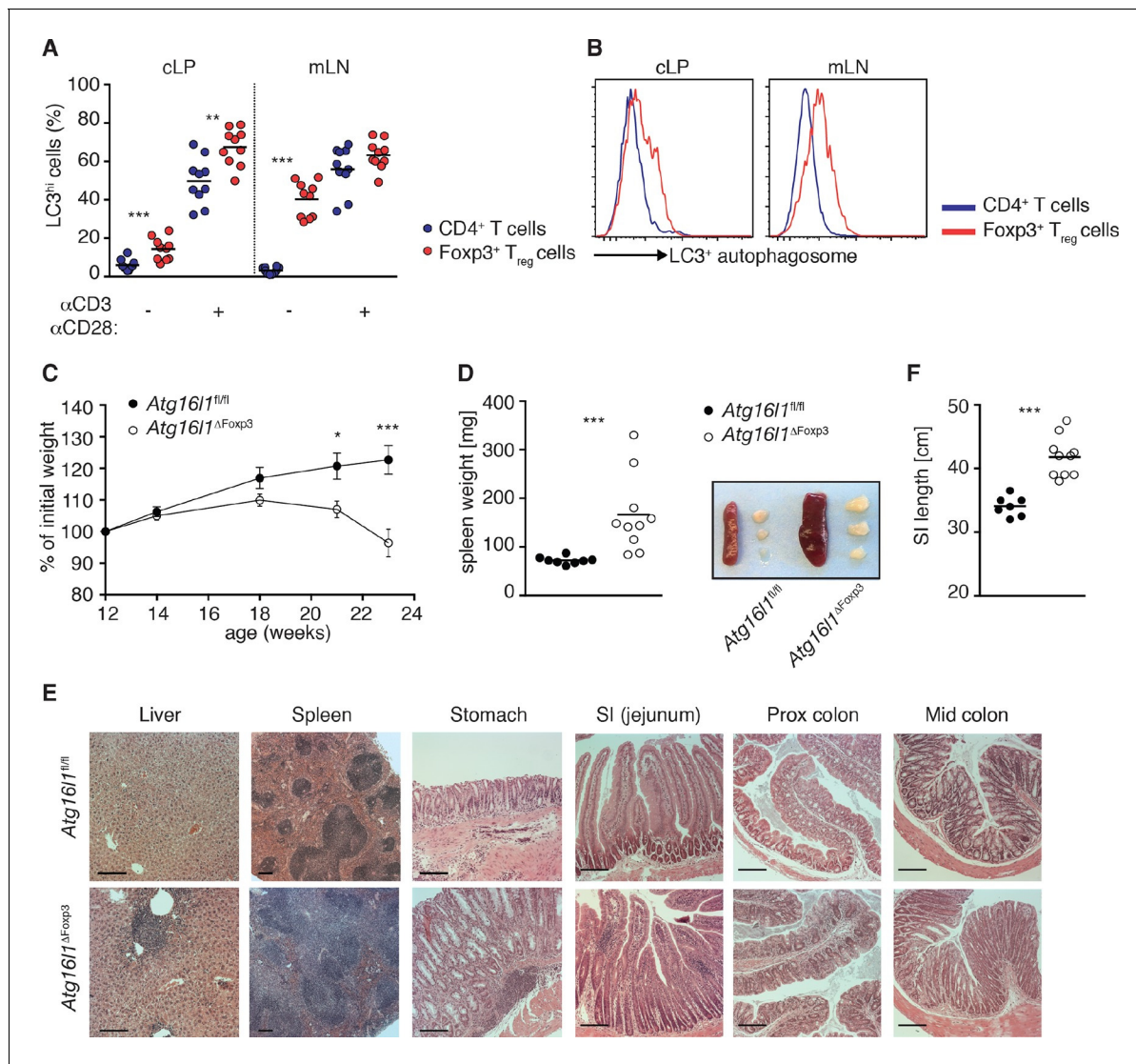


Figure 6. Aged *Atg16l1^{ΔFoxp3}* mice develop spontaneous multi-organ inflammation. (A) LC3⁺ autophagosome formation by Foxp3⁻ CD4⁺ T cells and Foxp3⁺ T_{reg} cells from cLP and mLN of WT mice in unstimulated cells or after overnight activation with α-CD3 (5 μg/ml) and α-CD28 (1 μg/ml). (B) Representative LC3 staining of unstimulated cells (gated on Foxp3⁺ CD4⁺ TCRβ⁺ T_{reg} cells or Foxp3⁻ CD4⁺ TCRβ⁺ T cells). (C) Weight curves and (D) spleen weights and representative images of spleen and mLN of aged *Atg16l1^{ΔFoxp3}* and *Atg16l1^{fl/fl}* littermates. (E) Representative photomicrographs of H&E stained sections of liver, spleen, stomach, SI (jejunum), proximal colon and mid-colon of aged *Atg16l1^{ΔFoxp3}* and *Atg16l1^{fl/fl}* littermates, scale bar 150 μm. (F) Quantification of SI length. Data are combined from two to four independent experiments with two to five mice per group (A,D,F) or are representative of two to three independent experiments with two to five mice per group (B,C,E). Each dot represents an individual mouse and horizontal bars denote means (A,D,F). Data shown as mean ± s.e.m (C). Statistical significance was determined using two-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons (C) or using the Mann–Whitney test (A,D,F), *p<0.05; **p<0.01; ***p<0.001. mLN - mesenteric lymph nodes, SI - small intestine lamina propria, cLP - colonic lamina propria. Aged mice >5 months old.

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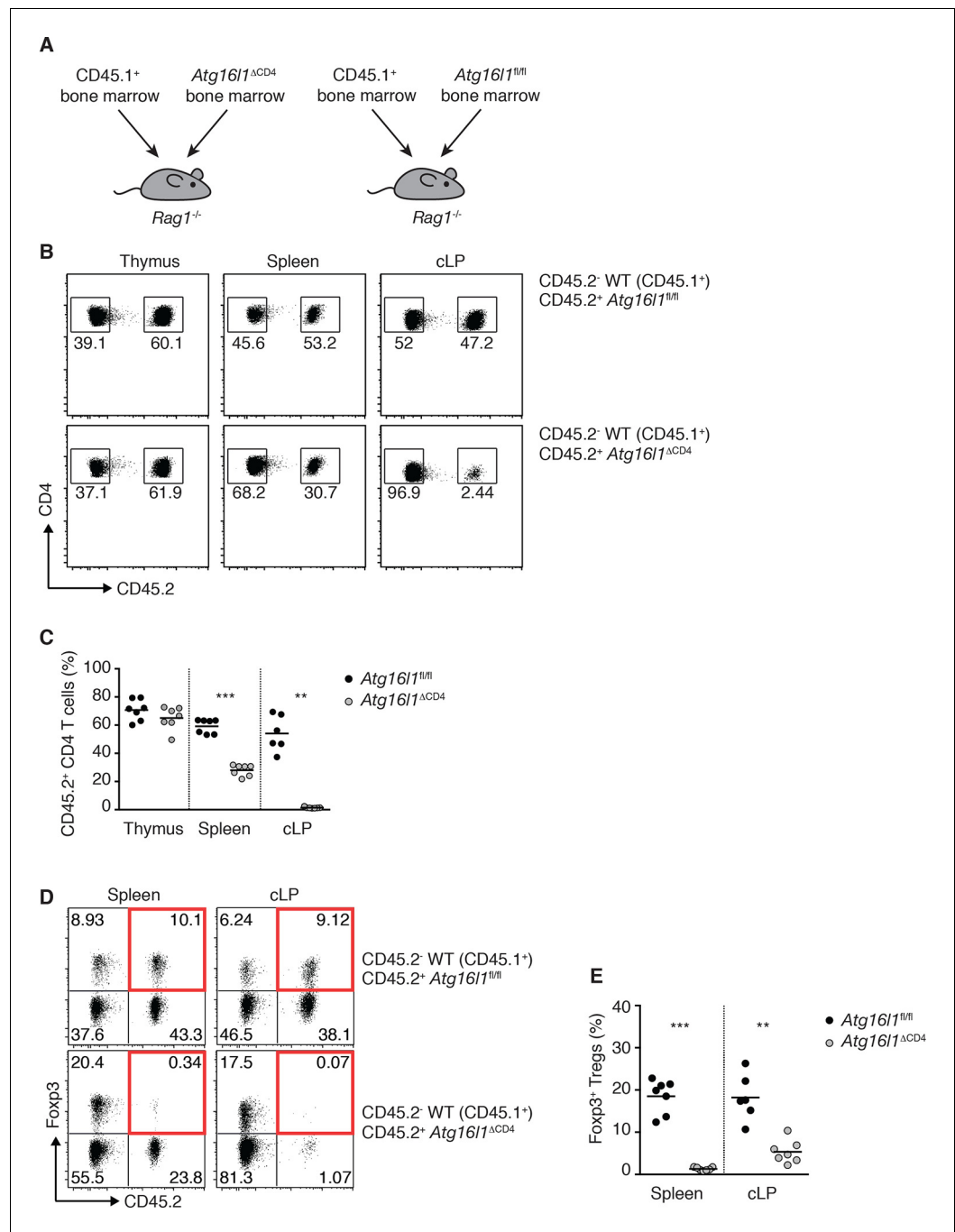


Figure 6—figure supplement 1. Impaired reconstitution of mixed bone marrow chimeras by *Atg16l1*-deficient T cells. (A) Experimental design for the generation of mixed bone marrow (BM) chimeras. BM cells isolated from WT (CD45.1⁺) and *Atg16l1*^{fl/fl} or *Atg16l1*^{ΔCD4} (CD45.2⁺) mice were injected at a 1:1 ratio into lethally irradiated (1100 Rad) *Rag1*^{-/-} recipients (total of 1x10⁷ cells per mouse). (B) Representative FACS plots showing frequencies of *Atg16l1*^{ΔCD4} or *Atg16l1*^{fl/fl} (CD45.2⁺) and WT (CD45.2⁻) CD4⁺ T cells in the thymus, spleen and cLP of mixed BM chimeras (gated on CD4⁺ TCRβ⁺ T cells). (C) Frequencies of *Atg16l1*^{ΔCD4} or *Atg16l1*^{fl/fl} (CD45.2⁺) CD4⁺ T cells in mixed BM chimeras (shown as percentage of total CD4⁺ TCRβ⁺ T cells). (D) Representative FACS plots and (E) frequencies of Foxp3⁺ T_{reg} cells derived from *Atg16l1*^{ΔCD4} or *Atg16l1*^{fl/fl} (CD45.2⁺) cells in mixed BM chimeras (gated on CD4⁺ TCRβ⁺ T cells). Highlighted top right quadrants indicate T_{reg} cells derived from *Atg16l1*^{fl/fl} or *Atg16l1*^{ΔCD4} BM. Data are representative from two independent experiments with at least seven mice per group. Each dot represents an individual mouse and horizontal bars denote means. Numbers indicate percentage of cells in gates. Statistical significance was determined using the Mann–Whitney test, **p<0.01; ***p<0.001.

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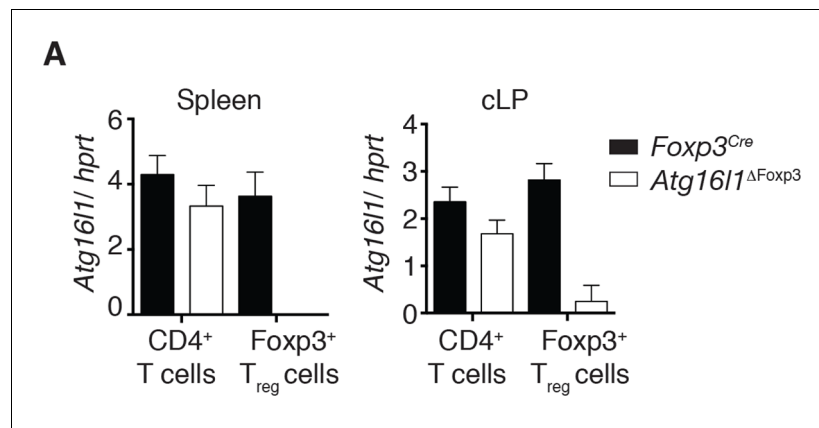


Figure 6—figure supplement 2. Analysis of *Atg16l1* expression in *Atg16l1*^{ΔFoxp3} mice. (A) qPCR analysis of *Atg16l1* exon 3 levels in sorted CD4⁺ Foxp3⁻ T cells and Foxp3⁺ T_{reg} cells from spleen and cLP of *Atg16l1*^{ΔFoxp3} and *Foxp3*^{Cre} mice. Data are representative from two independent experiments with 5 mice per group. *Atg16l1* exon 3 levels are shown as mean ± s.e.m of three technical replicates, normalised to expression of *hprt*. cLP – colonic lamina propria.

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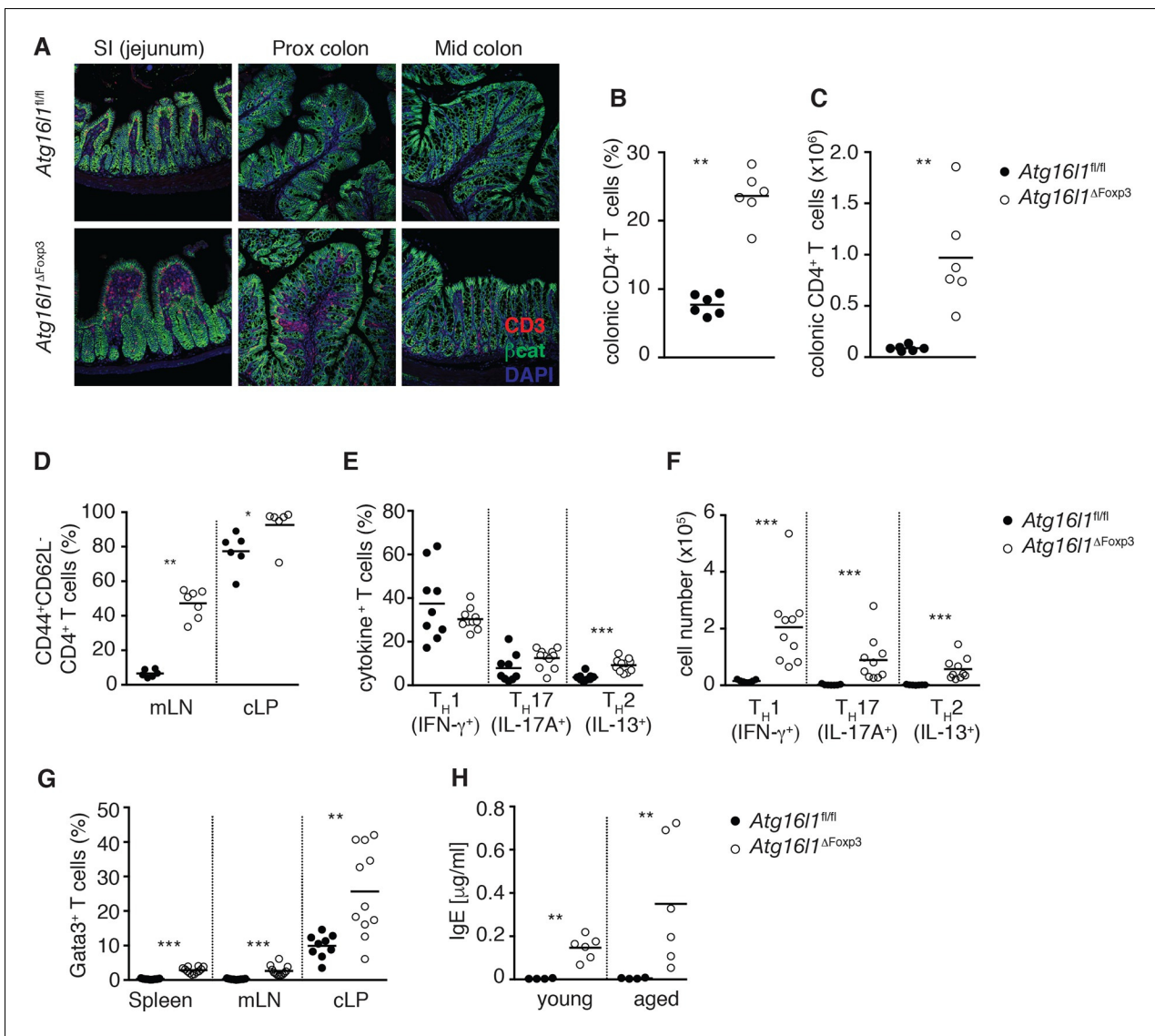


Figure 7. *Atg16l1^{ΔFoxp3}* mice cannot control pro-inflammatory T_H effector responses. (A) Representative immunofluorescence images of small intestine and proximal and mid colon of aged *Atg16l1^{ΔFoxp3}* and *Atg16l1^{fl/fl}* littermates stained for CD3 (red), β-catenin (green) and DAPI (blue). (B) Frequencies and (C) total numbers of cLP CD4⁺ TCRβ⁺ T cells in aged *Atg16l1^{ΔFoxp3}* and *Atg16l1^{fl/fl}* littermates. (D) Frequencies of effector (CD44⁺CD62L⁻) CD4⁺ T cells in the mLN and cLP of aged *Atg16l1^{ΔFoxp3}* and *Atg16l1^{fl/fl}* littermates (gated on CD4⁺ TCRβ⁺ Foxp3⁻ T cells). (E) Frequencies and (F) total numbers of T_H1 (IFN-γ⁺), T_H17 (IL-17A⁺), T_H2 (IL-13⁺) T cells in the cLP of aged *Atg16l1^{ΔFoxp3}* and *Atg16l1^{fl/fl}* littermates (gated on CD4⁺ TCRβ⁺ Foxp3⁻ T cells). (G) Frequencies of Gata3⁺ CD4⁺ T cells in aged *Atg16l1^{ΔFoxp3}* and *Atg16l1^{fl/fl}* littermates (gated on CD4⁺ TCRβ⁺ Foxp3⁻ T cells). (H) Serum IgE concentrations in *Atg16l1^{ΔFoxp3}* and *Atg16l1^{fl/fl}* littermates were measured by ELISA. Data are combined from two to four independent experiments with two to five mice per group (B-H) or are representative of two independent experiments with two to five mice per group (A). Each dot represents an individual mouse and horizontal bars denote means. Statistical significance was determined using the Mann-Whitney test *p<0.05; **p<0.01; ***p<0.001. mLN - mesenteric lymph nodes, cLP – colonic lamina propria. Young mice: 8–12 weeks old, aged mice >5 months old.

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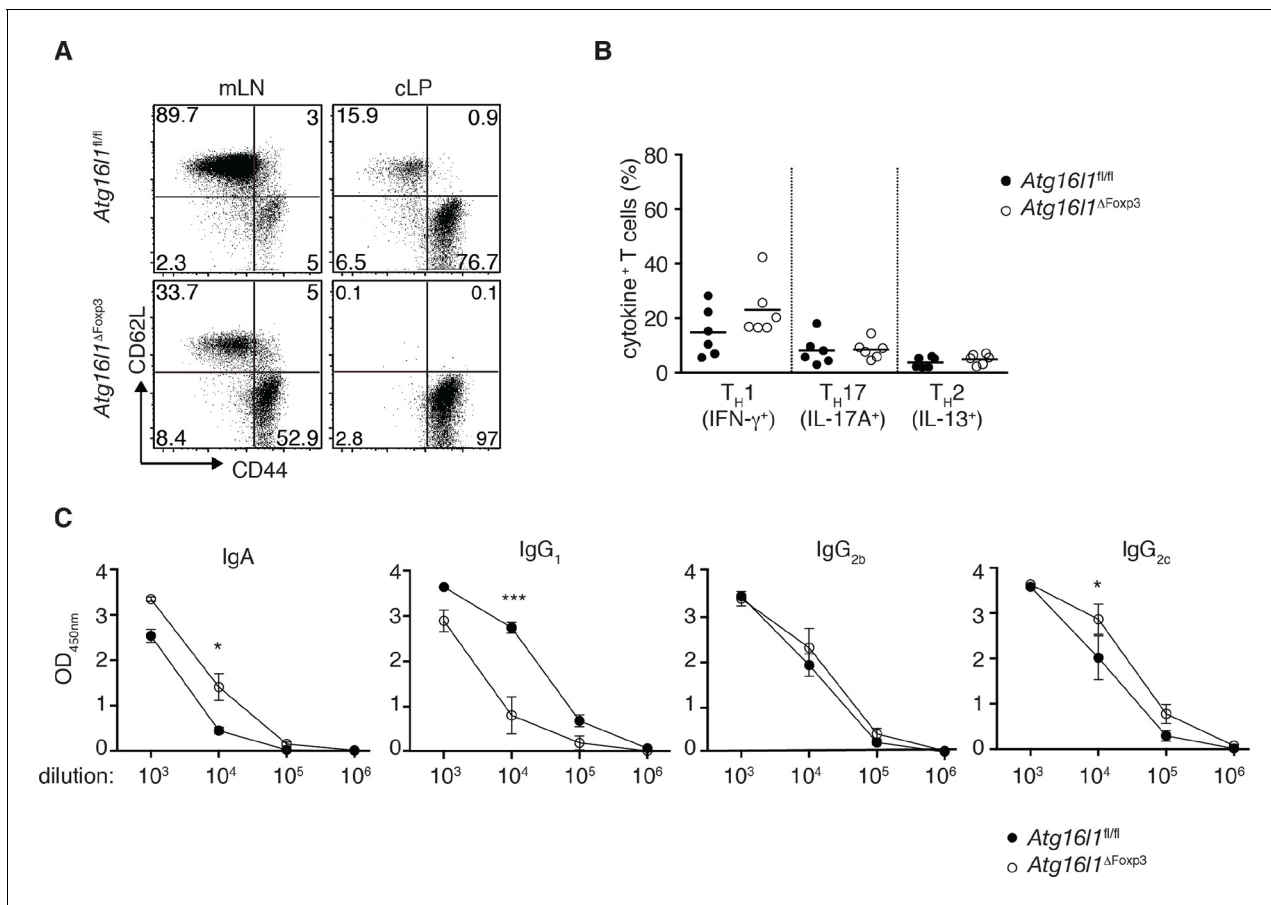


Figure 7—figure supplement 1. Additional characterization of *Atg16l1*^{ΔFoxp3} mice. (A) Representative FACS plots of CD44 and CD62L expression by CD4⁺ T cells from cLP of aged *Atg16l1*^{ΔFoxp3} and *Atg16l1*^{fl/fl} littermates (gated on CD4⁺ TCR β ⁺ Foxp3⁻ T cells). (B) Frequencies of T_H1 (IFN- γ ⁺), T_H17 (IL-17A⁺), T_H2 (IL-13⁺) T cells in the cLP of young *Atg16l1*^{ΔFoxp3} and *Atg16l1*^{fl/fl} littermates (gated on CD4⁺ TCR β ⁺ Foxp3⁻ T cells). (C) Serum antibody IgA, IgG₁, IgG_{2b}, IgG_{2c} isotype levels in aged *Atg16l1*^{ΔFoxp3} and *Atg16l1*^{fl/fl} littermates. Data are combined from three independent experiments with two to five mice per group (B) or are representative from two to three independent experiments with two to five mice per group (A,C). Each dot represents an individual mouse and horizontal bars denote means. Numbers indicate percentage of cells in gates. Data shown as mean \pm s.e.m (C). Statistical significance was determined using the Mann–Whitney test, *p<0.05; **p<0.01; ***p<0.001. mLN - mesenteric lymph nodes, cLP – colonic lamina propria. Young mice: 8–12 weeks old, aged mice >5 months old.

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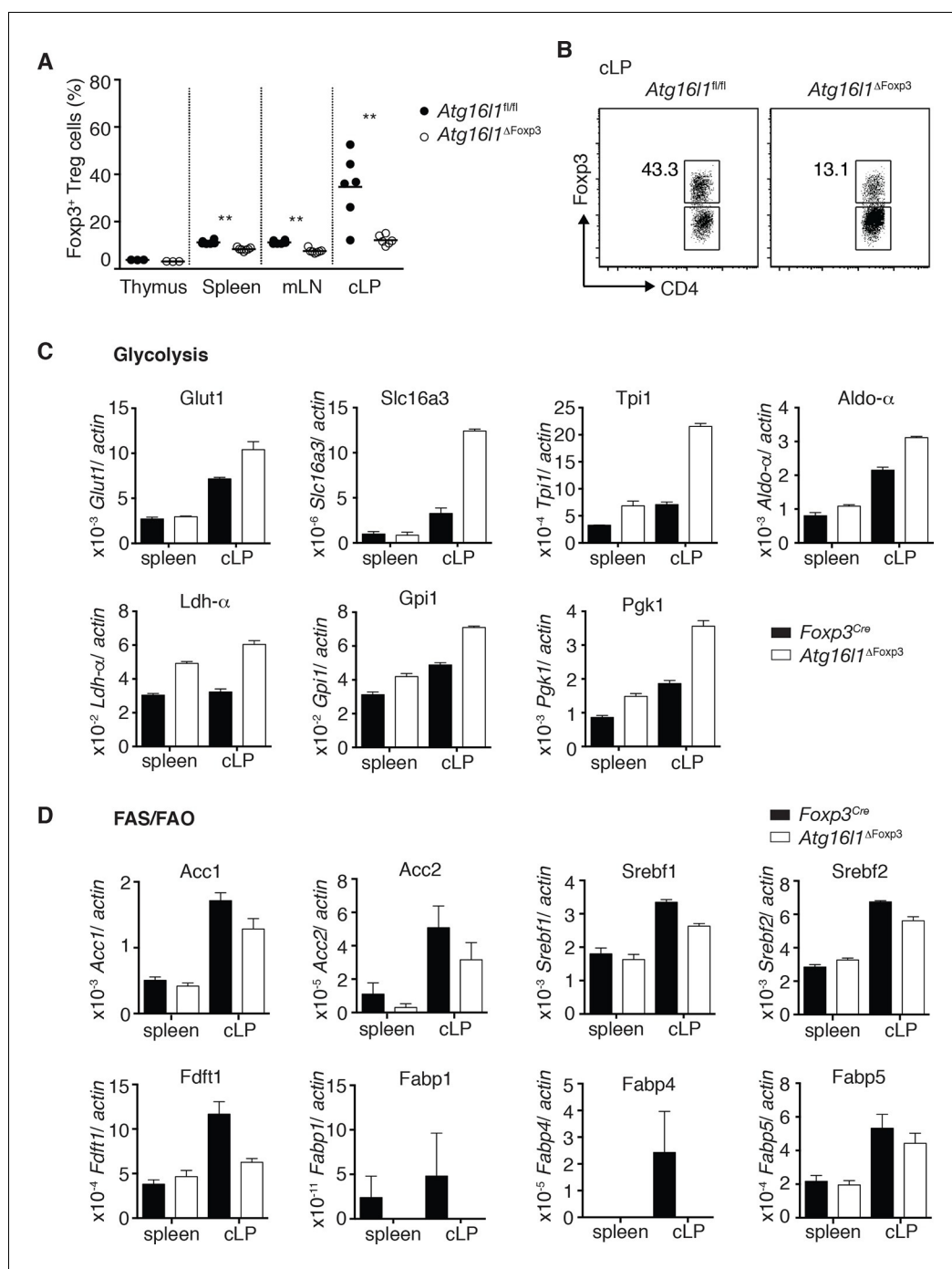


Figure 8. Cell-intrinsic autophagy is required for metabolic adaptation and survival of intestinal Foxp3⁺ T_{reg} cells. (A) Foxp3⁺ T_{reg} cell frequencies among CD4⁺ TCR β ⁺ T cells in *Atg16l1^{ΔFoxp3}* and *Atg16l1^{fl/fl}* littermates and (B) representative FACS plots of Foxp3 expression in cLP CD4⁺ T cells from young *Atg16l1^{ΔFoxp3}* and *Atg16l1^{fl/fl}* littermates (gated on CD4⁺ TCR β ⁺ T cells). (C) qPCR analysis of glycolytic gene levels in sorted Foxp3⁺ T_{reg} cells from spleen and cLP of young *Atg16l1^{ΔFoxp3}* and *Foxp3^{Cre}* mice (sorted for CD4⁺ TCR β ⁺ YFP⁺). (D) qPCR analysis of FAS and FAO gene levels in Foxp3⁺ T_{reg} cells from the spleen and cLP of young *Atg16l1^{ΔFoxp3}* and *Foxp3^{Cre}* mice (sorted for CD4⁺ TCR β ⁺ YFP⁺). FAS: fatty acid synthesis, FAO: fatty acid oxidation, Glut1: glucose transporter 1, Slc16a: solute carrier family 16 member 3 (lactic acid and pyruvate transporter), Tpi1: triosephosphate isomerase 1, Aldo- α : aldolase α , Ldh- α : lactate dehydrogenase α , Gpi1: Glucose phosphate isomerase 1, Pkg1: Phosphoglycerate kinase 1, Acc1: acetyl-CoA carboxylase 1, Acc2: acetyl-CoA carboxylase 2, Srebf1: sterol regulatory element binding transcription factor 1, Srebf2: sterol regulatory element binding transcription factor 2, Figure 8 continued on next page

Figure 8 continued

Fdft1: farnesyl-diphosphate farnesyltransferase 1, Fabp: Fatty acid-binding protein. Data are representative from two (C,D) or three independent experiments (A,B). Each dot represents individual mouse (A) or data are shown as mean \pm s.e.m (C,D). Gene expression levels are shown as mean \pm 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.
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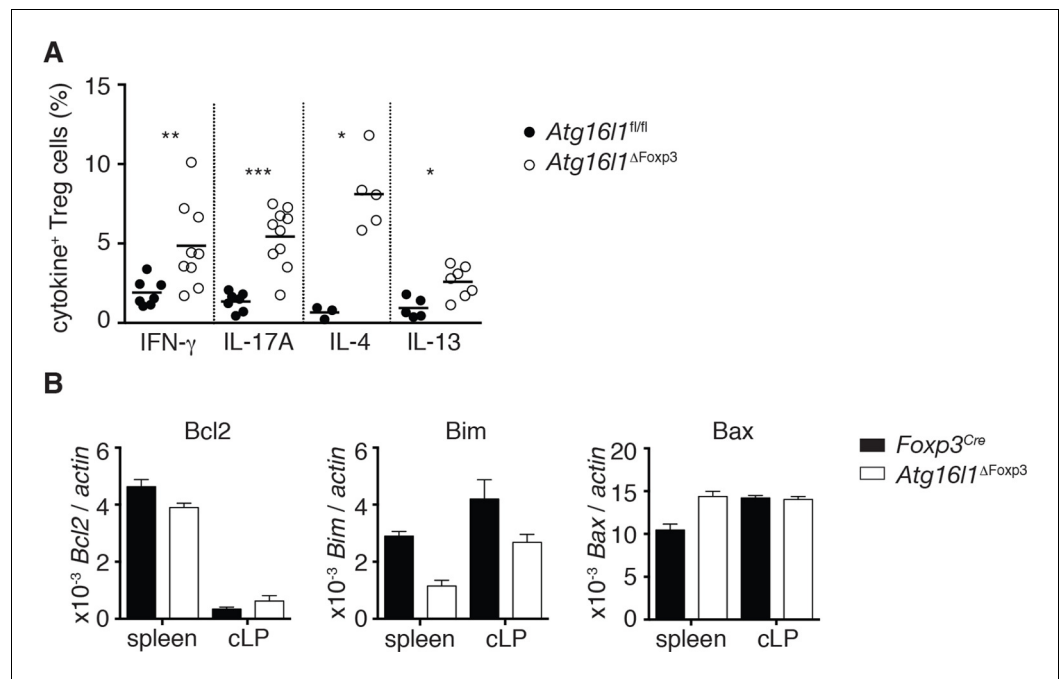


Figure 8—figure supplement 1. *Atg16l1*-deficient colonic T_{reg} cells exhibit increased cytokine secretion. (A) Frequencies of IFN- γ ⁺, IL-17A⁺, IL-4⁺, IL-13⁺ Foxp3⁺ T_{reg} cells in the cLP of aged *Atg16l1*^{ΔFoxp3} and *Atg16l1*^{fl/fl} littermates (gated on Foxp3⁺ CD4⁺ TCR β ⁺ T cells). (B) qPCR analysis of *Bcl2*, *Bim* and *Bax* levels in Foxp3⁺ T_{reg} cells from young cLP of *Atg16l1*^{ΔFoxp3} and *Foxp3*^{Cre} mice (sorted for CD4⁺ TCR β ⁺ YFP⁺). Data are combined from three independent experiments with two to five mice per group (A) or are representative from two independent experiments (B). Each dot represents an individual mouse and horizontal bars denote means (A). Gene expression levels are shown as mean \pm s.e.m of three technical replicates (B). cLP – colonic lamina propria. Young mice: 8–12 weeks old.

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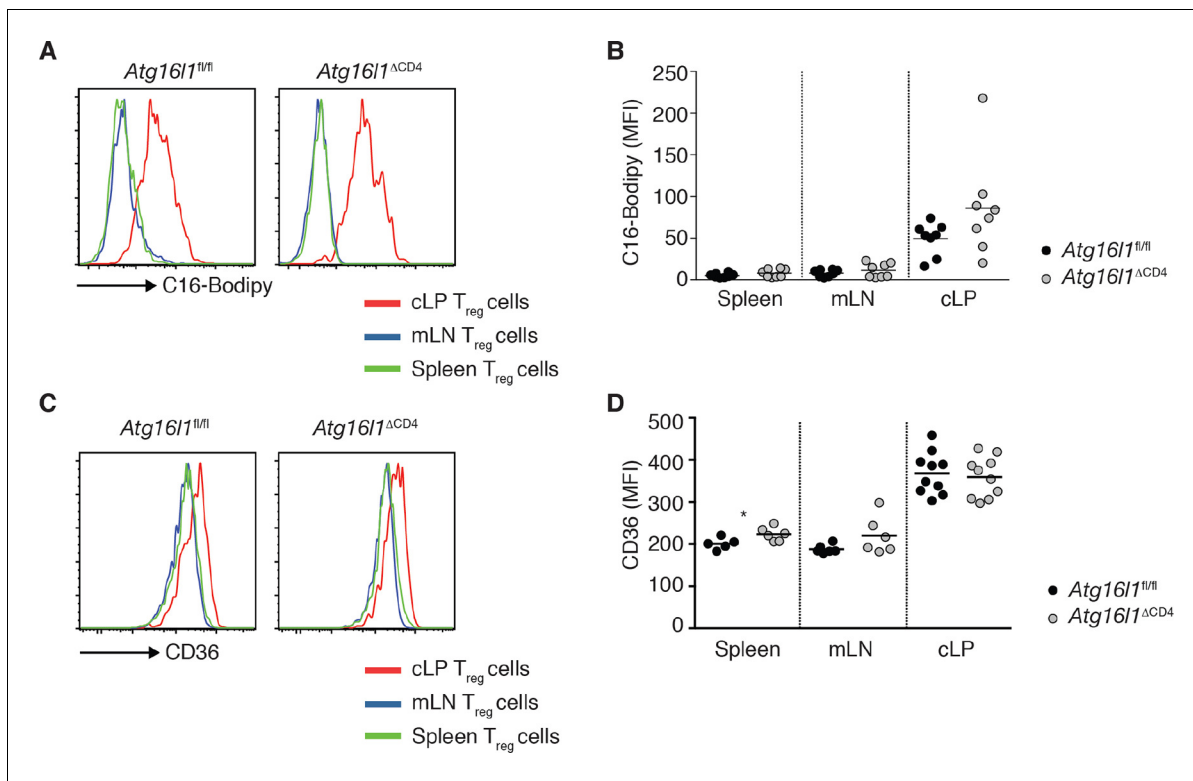


Figure 8—figure supplement 2. Increased lipid uptake by intestinal T_{reg} cells. (A,B) *Atg16l1^{fl/fl}* and *Atg16l1^{ΔCD4}* littermates were injected i.p. with 50 μg of fluorescent 16-carbon fatty acid analog BODIPY C-16 and culled 1 hr later and tissue was collected for analysis by flow cytometry. (A) Representative FACS plots and (B) quantification of C16-Bodipy uptake by Foxp3⁺ T_{reg} cells in the spleen, mLN and cLP (gated on Foxp3⁺ CD4⁺ TCRβ⁺ T cells). (C) Representative FACS plots and (D) quantification of CD36 expression by Foxp3⁺ T_{reg} cells in the spleen, mLN and cLP of *Atg16l1^{fl/fl}* and *Atg16l1^{ΔCD4}* littermates (gated on Foxp3⁺ CD4⁺ TCRβ⁺ T cells). Data are combined from (B,D) or are representative of (A,C) two independent experiments with 3–5 mice per group. Each dot represents an individual mouse and horizontal bars denote means. Statistical significance was determined using the Mann–Whitney test, *p<0.05. mLN - mesenteric lymph nodes, cLP – colonic lamina propria. Young mice: 8–12 weeks old.

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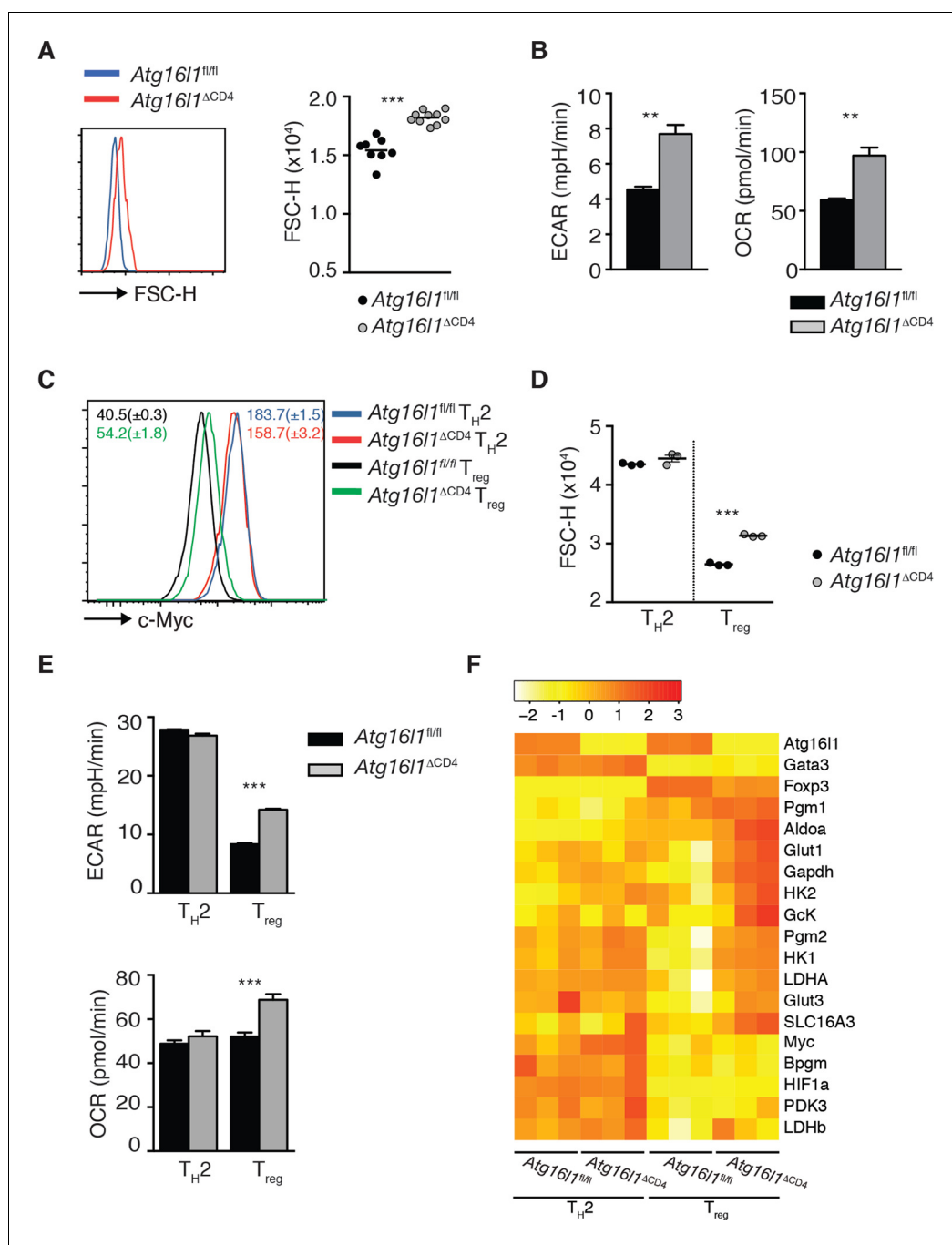


Figure 8—figure supplement 3. T_{H2} cells exhibit an enhanced glycolytic metabolic profile that is independent of autophagy. (A) Representative FACS plot and quantification of the cell size (FSC-H) of naïve (CD44⁻ CD62L⁺) CD4⁺ T cells from the spleen of *Atg16l1*^{ΔCD4} or *Atg16l1*^{fl/fl} littermates. (B) Basal level of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in naïve (CD44⁻ CD62L⁺) unstimulated CD4⁺ T cells isolated from the spleen of *Atg16l1*^{ΔCD4} or *Atg16l1*^{fl/fl} littermates measured using the Seahorse metabolic flux analyzer. (C,D) Expression of c-Myc (C) and cell size (D) was analyzed by FACS in *Atg16l1*^{ΔCD4} or *Atg16l1*^{fl/fl} CD4⁺ T cells that were cultured in T_{H2} or T_{reg}-polarizing conditions for 3 days and rested for one day in the presence of polarizing cytokines. (E) Basal level of OCR and ECAR were measured by Seahorse metabolic flux analyzer in *Atg16l1*^{ΔCD4} or *Atg16l1*^{fl/fl} CD4⁺ T cells cultured in T_{H2} or T_{reg}-polarizing conditions for 3 days and rested for 2 days in the presence of polarizing cytokines. (F) qPCR analysis of glycolytic gene levels in *Atg16l1*^{ΔCD4} or *Atg16l1*^{fl/fl} CD4⁺ T cells cultured in T_{H2} or T_{reg} polarizing conditions for 3 days and rested for 1 day in the presence of polarizing cytokines. Data are combined from two independent experiments (A), or are representative of two independent experiments (B-F). *** indicates statistical significance.

Figure 8—figure supplement 3 continued

experiments (B-D), or are from one experiment (E,F). Each dot represents an individual mouse (A) or individual cell culture (D). ECAR and OCAR data represent mean \pm s.e.m values of T cell populations that were assayed in triplicates or quadruplicates (B,E). Gene expression data of triplicate cultures represent normalized expression values for each gene that were scaled to a mean of 0 and a standard deviation of 1 (F). Statistical significance was determined using the Mann–Whitney test (A) or unpaired Student's t –test (B,D,E), **p<0.01; ***p<0.001.

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