Unique role for ATG5 in neutrophil-mediated immunopathology during M. tuberculosis infection

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Mycobacterium tuberculosis, a major global health threat, replicates in macrophages in part by inhibiting phagosome–lysosome fusion, until interferon-γ (IFN-γ) activates the macrophage to traffic M. tuberculosis to the lysosome. How IFN-γ elicits this effect is unknown, but many studies suggest a role for macroautophagy (herein termed autophagy), a process by which cytoplasmic contents are targeted for lysosomal degradation1. The involvement of autophagy has been defined based on studies in cultured cells where M. tuberculosis co-localizes with autophagy factors ATG5, ATG12, ATG16L1, p62, NDP52, BECN1 and LC3 (refs 2–6), stimulation of autophagy increases bacterial killing6–8, and inhibition of autophagy increases bacterial survival1,4,6,9,10,11. Notably, these studies reveal modest (~1.5–3-fold change) effects on M. tuberculosis replication. By contrast, mice lacking ATG5 in monocyte-derived cells and neutrophils (polymorphonuclear cells, PMNs) succumb to M. tuberculosis within 30 days4,9, an extremely severe phenotype similar to mice lacking IFN-γ signalling10,11. Importantly, ATG5 is the only autophagy factor that has been studied during M. tuberculosis infection in vivo and autophagy-independent functions of ATG5 have been described12–18. For this reason, we used a genetic approach to elucidate the role for multiple autophagy-related genes and the requirement for autophagy in resistance to M. tuberculosis infection in vivo. Here we show that, contrary to expectation, autophagic capacity does not correlate with the outcome of M. tuberculosis infection. Instead, ATG5 plays a unique role in protection against M. tuberculosis by preventing PMN-mediated immunopathology. Furthermore, while Atg5 is dispensable in alveolar macrophages during M. tuberculosis infection, loss of Atg5 in PMNs can sensitize mice to M. tuberculosis. These findings shift our understanding of the role of ATG5 during M. tuberculosis infection, reveal new outcomes of ATG5 activity, and shed light on early events in innate immunity that are required to regulate disease pathology and bacterial replication.

We first replicated the finding that Atg5 is critical in myeloid-derived cells for resistance to M. tuberculosis by infecting Atg5−/−; Lysm-cre (Lysm is also known as Lyz2) mice4,9, LysM-promoter-driven expression of Cre recombinase (Lysm-cre) results in deletion of a floxed gene in alveolar macrophages, recruited macrophages, inflammatory monocytes, monocyte-derived dendritic cells, and PMNs19,20. Following aerosol inoculation of M. tuberculosis into wild-type C57Bl/6 mice, bacteria replicate in innate immune cells until IFN-γ-producing T cells are recruited to the lungs between 18–20 days post-infection (d.p.i.), resulting in control of bacterial burden and survival21. Consistent with previous publications4,9, Atg5−/−; Lysm-cre mice lost 23% of their weight by 20 d.p.i. and succumbed to M. tuberculosis between 30 and 40 d.p.i. (Fig. 1a, b). In contrast, Atg5−/− control mice showed no signs of sickness or weight loss. Bacterial titres in Atg5−/−; Lysm-cre mice were significantly higher at 3 weeks post-infection (w.p.i.) than those in Atg5+/+ mice (Fig. 1c, d). By 5 w.p.i., Atg5+/+ mice had controlled pulmonary burden while Atg5−/−; Lysm-cre mice rapidly succumbed to infection (Fig. 1b, c).

In cultured cells, Atg5, p62 (also known as Sqstm1) and Ulk1 have similar roles in controlling M. tuberculosis survival and replication1,4,5,22. We therefore explored the role of these and other genes involved in autophagy in vivo, by infecting mice with germline deletions of Ulk1, Ulk2 (autophagy induction), Atg4b (isolation membrane elongation), or p62 (substrate targeting to autophagosomes). Surprisingly, mice lacking Ulk1, Ulk2, Atg4b or p62 showed no signs of sickness during infection, efficiently controlled bacterial burden, and survived over 80 days with M. tuberculosis (Fig. 1e–h, and Extended Data Fig. 1a). Potential redundancy may explain the lack of a phenotype in Ulk1−/−, Ulk2−/−, Atg4b−/−, and p62−/− mice during M. tuberculosis infection. However, loss of either Ulk1 or Ulk2 results in clear autophagy defects in cultured cells23, and Atg4b−/− mice have dramatic autophagy defects in many tissues, including a nearly complete loss of LC3-II (the lipidated form of microtubule-associated protein 1A/1B-light chain 3 (LC3), which localizes to autophagosome membranes) formation in the lungs, kidney and liver24. Regardless of issues with redundancy, these data indicate a lack of correlation between in vitro and in vivo findings of the role of these genes in controlling M. tuberculosis replication.

We next tested the role of essential Atg genes other than Atg5 in resistance to M. tuberculosis. If ATG5 is required in vivo due to its role in canonical autophagy, then Lysm-cre deletion of other essential autophagy genes would result in a similar phenotype as observed in Atg5−/−; Lysm-cre mice. Contrary to expectation, Atg14−/−; Lysm-cre, Atg12−/−; Lysm-cre, Atg16L1−/−; Lysm-cre, Atg7−/−; Lysm-cre and Atg3−/−; Lysm-cre mice did not show any signs of sickness or weight loss following infection with M. tuberculosis and all survived over 80 d.p.i. (Fig. 1i and Extended Data Fig. 1b). In addition, these mice were all able to control M. tuberculosis burden in a manner similar to C57Bl/6 mice (Fig. 1j, k). These findings were particularly notable as these same Atg16L1−/−; Lysm-cre, Atg12−/−; Lysm-cre and Atg3−/−; Lysm-cre mice are dramatically more susceptible to Toxoplasma gondii, another pathogen for which IFN-γ plays a key role in resistance to infection12,14. Nevertheless, to compare the relative efficacy of conditional deletion of each essential autophagy factor, LC3 lipidation and p62 degradation were measured ex vivo in peritoneal exudate macrophages (Fig. 1l) and bronchoalveolar lavage macrophages (Extended Data Fig. 2). Consistent with previous publications using these mouse strains12,14, the floxed alleles in Atg5−/−; Lysm-cre, Atg14−/−; Lysm-cre, Atg12−/−; Lysm-cre and Atg3−/−; Lysm-cre mice were effectively targeted in vivo resulting in similar increases in the amounts of LC3-I (the non-lipidated form of LC3, which does not participate in autophagy) and p62, which indicate a defect in autophagy. Peritoneal macrophage and bronchoalveolar macrophages from Atg14−/−; Lysm-cre mice also

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accumulated p62 while, consistent with previous findings, the levels of LC3 were largely unaffected.

At 3 w.p.i., Atg5fl/fl mice have higher bacterial titres as compared to C57Bl/6 mice (Fig. 1d, k), which we attribute to hypomorphic expression of Atg5 from the Atg5fl/fl allele (Extended Data Fig. 3 and ref. 25). To determine if germine hypomorphism for an essential ATG factor other than ATG5 interferes with control of M. tuberculosis, we infected mice that are hypomorphic for ATG16L1 (Atg16l1fl/fl) (Fig. 1m). Atg16l1fl/fl mice showed no signs of sickness or weight loss following M. tuberculosis infection and controlled M. tuberculosis burden in a manner similar to C57Bl/6 mice (Fig. 1n–p and Extended Data Fig. 1c). Together, these data demonstrate that the loss of genes essential for canonical autophagy in LysM+ cells does not correlate with susceptibility to M. tuberculosis and suggest that ATG5 participates in a unique function not served by other essential ATG proteins. While autophagy-independent functions of ATG5 have been described12–18, this is the first example of ATG5 being important for a response to an infection independent of ATG16L1 and ATG12.

To further explore how ATG5 functions during M. tuberculosis infection, we next investigated the reports that Atg5fl/fl-Lysm-cre mice develop more severe inflammation following M. tuberculosis infection9,10. Various studies have demonstrated that myeloid-specific defects in components of the membrane elongation complex (ATG5, ATG7 or ATG16L1) can cause increased inflammation in vivo27–29. To distinguish between ATG16L1-dependent versus independent roles for ATG5 in regulating inflammation we measured immune responses to M. tuberculosis in the lungs of Atg5fl/fl-Lysm-cre, Atg16l1fl/fl-Lysm-cre and control mice. Phenotypes specific to loss of Atg5 might be responsible for susceptibility to M. tuberculosis since Atg16l1fl/fl-Lysm-cre mice control M. tuberculosis infection similarly to wild-type C57Bl/6 mice (Fig. 1). At 2 w.p.i., Atg5fl/fl-Lysm-cre lungs contained larger lesions than those in C57Bl/6, Atg16l1fl/fl-Lysm-cre and Atg16l1fl/fl mice (Fig. 2a), even though bacterial burdens were similar in each strain at this time point (Extended Data Fig. 4). By 3 w.p.i., Atg5fl/fl-Lysm-cre lungs were severely inflamed with large lesions and extensive consolidation, while Atg5fl/fl and Atg16l1fl/fl-Lysm-cre lungs showed only moderate increases in inflammation (Fig. 2a). Consistent with this, the lungs of Atg5fl/fl-Lysm-cre mice at 3 w.p.i. contained higher levels of pro-inflammatory cytokines than Atg16l1fl/fl-Lysm-cre or control mice (Fig. 2b). At 80 d.p.i., PMN-depleted Atg5fl/fl-Lysm-cre mice were monitored for their lost weight and survive over 80 d.p.i. (Fig. 3a, b). To survive M. tuberculosis infection independent of ATG16L1 and ATG12.

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To characterize cell populations contributing to the inflammation, flow cytometry was performed at 2 and 3 w.p.i. in Atg5fl/fl-Lysm-cre, Atg16l1fl/fl-Lysm-cre, and control mice. At 2 w.p.i., Atg5fl/fl-Lysm-cre lungs contained a significantly greater frequency of PMNs than Atg5fl/fl or C57Bl/6 mice (Fig. 2c and Extended Data Fig. 6). This difference was more pronounced at 3 w.p.i. and at this time point the frequency of PMNs in Atg5fl/fl-Lysm-cre lungs was also significantly higher than in Atg16l1fl/fl-Lysm-cre lungs (Fig. 2d). Atg5fl/fl-Lysm-cre lungs also contained a greater percentage of inflammatory monocytes than C57Bl/6 mice at 2 w.p.i., however this level was similar to Atg16l1fl/fl-Lysm-cre lungs and, by 3 w.p.i., was not significantly different from any other strain. The increased inflammation in Atg5fl/fl-Lysm-cre lungs likely contributes to the severe lung pathology and morbidity observed in these mice (Fig. 1a, b and Fig. 2a–d). In addition, the absence of higher bacterial burden at 2 w.p.i. (Extended Data Fig. 4) indicates that the increased inflammation in the M. tuberculosis infected Atg5fl/fl-Lysm-cre mice is a direct result of loss of Atg5 rather than a response to uncontrolled bacterial replication.

Excessive PMN recruitment is a hallmark of acute susceptibility to M. tuberculosis and is associated with uncontrolled tissue damage and progression of disease11. We hypothesized that the susceptibility of the Atg5fl/fl-Lysm-cre mice is related to the increased frequency of PMNs in these mice during M. tuberculosis infection and, therefore, sought to determine if depletion of PMNs would improve control of M. tuberculosis11. Antibody-mediated depletion of PMNs (anti-Ly6G, clone 1A8) from 10–28 d.p.i. allowed Atg5fl/fl-Lysm-cre mice to recover their lost weight and survive over 80 d.p.i. (Fig. 3a, b). To survive 80 d.p.i., PMN-depleted Atg5fl/fl-Lysm-cre mice must have functional IFNγ signalling and T-cell responses, since RAG−/− and PMN-depleted
alveolar macrophages, PMNs, recruited macrophages, and inflammatory monocytes as a percentage of all single cells in lungs at 2 w.p.i. (c) and 3 w.p.i. (d). Statistical differences were determined by one-way ANOVA and Bonferroni’s multiple comparison test (b–d). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; NS, not significant; error bars represent mean ± s.e.m. Samples represent biological replicates. See Supplementary Fig. 3 for sample sizes and results from all statistical comparisons. Extended Data Fig. 5 for cytokine levels in uninfected lungs, and Extended Data Fig. 6 for gating strategy and number of cells in lungs.

We next sought to determine in which cell type(s) Atg5 is required to control M. tuberculosis. Lysm-cre deletion occurs in PMNs, macrophages, inflammatory monocytes and myeloid-derived dendritic cells19,20, indicating that Atg5 plays a critical role in one or more of these populations during M. tuberculosis infection. Alveolar macrophages are the first cells infected upon inhalation of M. tuberculosis and are required for the establishment of infection20. Furthermore, previous in vitro studies suggested that a predominant role for ATG5 in PMNs20 (Fig. 4e). We therefore investigated whether ATG5 is required in alveolar macrophage to control M. tuberculosis by infecting Atg5fl/fl-Cd11c-cre mice, which lack ATG5 in alveolar macrophages and dendritic cells20. In contrast to Atg5fl/fl-Lysm-cre mice, Atg5fl/fl-Cd11c-cre mice did not lose weight during M. tuberculosis infection, were able to control bacterial burden, and survived over 80 d.p.i. (Fig. 4a–c). Alveolar macrophage from Atg5fl/fl-Cd11c-cre and Atg5fl/fl-Lysm-cre mice displayed similar autophagy defects (Fig. 4d and Extended Data Fig. 2), indicating that resistance to M. tuberculosis is neither dependent on nor correlated with autophagic capacity in alveolar macrophages. Furthermore, this suggests ATG5 plays an essential role within other cells targeted by Lysm-cre-mediated gene deletion, such as PMNs, recruited macrophages and/or inflammatory monocytes, to control M. tuberculosis infection.

We have shown that excessive PMN-dominated inflammation leads to the susceptibility of Atg5fl/fl-Lysm-cre mice. To determine whether loss of Atg5 from PMN is sufficient to cause susceptibility to M. tuberculosis, we next used Atg5fl/fl-MRP8-cre (MRP8 also known as S100a8) mice, which delete Atg5 in PMNs20 (Fig. 4e). Atg5fl/fl-MRP8-cre...
bone marrow PMNs from multiple comparison test (differences were determined by one-way ANOVA and Bonferroni’s and actin in bronchoalveolar macrophages (BAL) from striped bars), and 568 | NATURE | VOL 528 | 24/31 DECEMBER 2015 reducible across multiple experiments, and was independent of differences exhibited an average 2% weight gain. This split phenotype was repro-

mice were more susceptible to M. tuberculosis infection, as indicated by an average increase in weight loss compared to Atg5fl/ fl controls (Fig. 4f). However, analysis of individual mice revealed that only half of the Atg5fl/ fl-MRP8-cre mice lost weight following M. tuberculosis infection (between 10–20% of their starting weight); the remaining mice exhibited an average 2% weight gain. This split phenotype was repro-
ducible across multiple experiments, and was independent of differences in age, sex or litter of the mice, suggesting a threshold effect in the susceptibility of the Atg5fl/ fl-MRP8-cre mice. To study these two distinct outcomes, we compared responses in mice that lost over 5% of their starting weight at 20 d.p.i. (‘susceptible’) with the remaining mice (‘healthy’) (Fig. 4g). At 3 w.p.i., lungs from susceptible Atg5fl/ fl-MRP8-cre mice exhibited higher bacterial burden, cytokine responses, and frequency of PMNs (Fig. 4h–j and Extended Data Fig. 7). The susceptible Atg5fl/ fl-MRP8-cre mice displayed the same phenotypes as the Atg5fl/ fl-Lysm-cre mice, demonstrating a PMN-intrinsic role for ATG5 during acute M. tuberculosis infection. However, the incomplete penetrance of susceptibility in Atg5fl/ fl-MRP8-cre mice suggests that the extreme sensitivity of Atg5fl/ fl-Lysm-cre mice to M. tuberculosis results from the loss of Atg5 in macrophage and monocytes, as well as PMNs. Notably, Atg16l1fl/ fl-Lysm-cre mice are not susceptible to M. tuberculosis infection, even though PMNs (Extended Data Fig. 8), in addition to macrophages (Fig. 1l and Extended Data Fig. 2), from Atg16l1fl/ fl-Lysm-cre and Atg5fl/ fl-Lysm-cre mice have a similar defect in autophagy. This further supports that ATG5 functions, at least in part, independently of ATG16L1 to protect mice from M. tuberculosis infection.

Despite numerous in vitro studies emphasizing a role for autophagy in macrophages during M. tuberculosis infection (including, but not limited to, refs 1–8), our data show that loss of genes essential for canonical autophagy does not correlate with susceptibility to M. tuberculosis in the context of a complete immune response in the host. Importantly, mice used in our studies have similar autophagy defects and have been used in prior publications to investigate the function of individual ATG factors12–15, validating these mice as suitable genetic models to study autophagy in vivo. Our studies indicate that prior reports analysing the role of only a single autophagy gene to conclude that canonical autophagy is responsible for the phenotypes observed need to be re-examined. The observation that the Atg5fl/ fl and Atg5fl/ fl-Lysm-cre mice have only small differences in M. tuberculosis burden supports the other data presented here that the dramatic difference in the inflammatory response is the predominant driver of susceptibility in Atg5fl/ fl-Lysm-cre mice during M. tuberculosis infection. The apparent insignificance of autophagy for controlling M. tuberculosis replication may reflect the fact that M. tuberculosis encodes highly effective inhibitors of canonical autophagy; however, these mechanisms have yet to be described. Furthermore, studies investigating loss of autophagy, including this one, do not address whether activation of autophagy could enhance restriction of M. tuberculosis replication.

By analysing different Cre-mediated deletion strains, we have found that loss of Atg5 in PMNs, but not alveolar macrophages or dendritic cells, can cause susceptibility to M. tuberculosis. a–c, Weight change (a) and log pulmonary c.f.u. (b, c) of Atg5fl/ fl (open circles) and Atg5fl/ fl-Cd11c-cre (closed circles). d, Western blot analysis of p62, LC3, and actin in bronchoalveolar macrophages (BAL) from Atg5fl/ fl and Atg5fl/ fl-Cd11c-cre mice. e, Western blot analysis of p62, LC3, and actin in bone marrow PMNs from Atg5fl/ fl, Atg5fl/ fl-Lysm-cre and Atg5fl/ fl-MRP8-cre mice. f, Weight change of Atg5fl/ fl (open blue circles), Atg5fl/ fl-Lysm-cre (closed blue circles), Atg5fl/ fl-MRP8-cre (closed black diamonds) mice following infection with M. tuberculosis. g, Weight change of mice following infection with M. tuberculosis. 50% of Atg5fl/ fl-MRP8-cre mice lost over 5% of their weight by 20 d.p.i. (‘susceptible’, closed purple triangles) while 50% of Atg5fl/ fl-MRP8-cre mice did not (‘healthy’, open black triangles). h, log pulmonary c.f.u. at 3 w.p.i. i, j, C57Bl/6 (grey solid bars), Atg5fl/ fl (blue striped bars), Atg5fl/ fl-Lysm-cre (blue solid bars), healthy Atg5fl/ fl-MRP8-cre (purple striped bars), and ‘susceptible’ Atg5fl/ fl-MRP8-cre (purple solid bars). i, Concentration of TNF-α, IL-10, IL-6, MIP-1α (CCL3), MIP-2 (CCL2), IL-17a, KC (CCL1), and G-CSF (CSF3) in lungs (homogenized in 5 ml PBS + 0.02% Tween 80) at 3 w.p.i. j, Frequency of alveolar macrophages, PMNs, recruited macrophages, and inflammatory monocytes as a percentage of single cells in lungs at 3 w.p.i. Statistical differences were determined by one-way ANOVA and Bonferroni’s multiple comparison test (h–j). *P < 0.05, ***P < 0.001, ****P < 0.0001; NS, not significant; error bars represent mean ± s.e.m. Samples represent biological replicates. See Supplementary Fig. 5 for sample sizes and results from all statistical comparisons, and Extended Data Fig. 7 for total numbers of cells in lungs.

Figure 4 | Loss of Atg5 in PMNs, but not alveolar macrophages or dendritic cells, can cause susceptibility to M. tuberculosis. a–c, Weight change (a) and log pulmonary c.f.u. (b, c) of Atg5fl/ fl (open circles) and Atg5fl/ fl-Cd11c-cre (closed circles). d, Western blot analysis of p62, LC3, and actin in bronchoalveolar macrophages (BAL) from Atg5fl/ fl and Atg5fl/ fl-Cd11c-cre mice. e, Western blot analysis of p62, LC3, and actin in bone marrow PMNs from Atg5fl/ fl, Atg5fl/ fl-Lysm-cre and Atg5fl/ fl-MRP8-cre mice. f, Weight change of Atg5fl/ fl (open blue circles), Atg5fl/ fl-Lysm-cre (closed blue circles), Atg5fl/ fl-MRP8-cre (closed black diamonds) mice following infection with M. tuberculosis. g, Weight change of mice following infection with M. tuberculosis. 50% of Atg5fl/ fl-MRP8-cre mice lost over 5% of their weight by 20 d.p.i. (‘susceptible’, closed purple triangles) while 50% of Atg5fl/ fl-MRP8-cre mice did not (‘healthy’, open black triangles). h, log pulmonary c.f.u. at 3 w.p.i. i, j, C57Bl/6 (grey solid bars), Atg5fl/ fl (blue striped bars), Atg5fl/ fl-Lysm-cre (blue solid bars), healthy Atg5fl/ fl-MRP8-cre (purple striped bars), and ‘susceptible’ Atg5fl/ fl-MRP8-cre (purple solid bars). i, Concentration of TNF-α, IL-10, IL-6, MIP-1α (CCL3), MIP-2 (CCL2), IL-17a, KC (CCL1), and G-CSF (CSF3) in lungs (homogenized in 5 ml PBS + 0.02% Tween 80) at 3 w.p.i. j, Frequency of alveolar macrophages, PMNs, recruited macrophages, and inflammatory monocytes as a percentage of single cells in lungs at 3 w.p.i. Statistical differences were determined by one-way ANOVA and Bonferroni’s multiple comparison test (h–j). *P < 0.05, ***P < 0.001, ****P < 0.0001; NS, not significant; error bars represent mean ± s.e.m. Samples represent biological replicates. See Supplementary Fig. 5 for sample sizes and results from all statistical comparisons, and Extended Data Fig. 7 for total numbers of cells in lungs.

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1. Deretic, V. Autophagy in tuberculosis. Cold Spring Harb. Persp. Med. 4, a018481 (2014).
2. Dutta, R. K., Kathania, M., Reje, M. & Majumdar, S. IL-6 inhibits IFN-γ-induced autophagy in Mycobacterium tuberculosis H37Rv infected macrophages. Int. J. Biochem. Cell Biol. 44, 942–954 (2012).
3. Juárez, E. et al. NOD2 enhances the innate response of alveolar macrophages to Mycobacterium tuberculosis in humans. Eur. J. Immunol. 42, 880–889 (2012).
4. Watson, R. O., Manzanillo, P. S. & Cox, J. S. Extracellular M. tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. Cell 150, 803–815 (2012).
5. Gutierrez, M. G., Seto, S., Tsujimura, K., Horii, T. & Koide, Y. Autophagy adaptor protein p62/ SQSTM1 and autophagy-related gene Atg5 mediate autophagosome formation in response to Mycobacterium tuberculosis infection in dendritic cells. PLoS ONE 8, e66017 (2013).
6. Sakowski, E. T. et al. Ubiquitin 1 promotes IFN-γ-induced xenophagy of Mycobacterium tuberculosis. PLoS Pathog. 11, e1005076 (2015).
7. Gutierrez, M. G. et al. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 119, 753–766 (2004).
8. Wang, J. et al. MicroRNA-155 promotes autophagy to eliminate intracellular mycobacteria by targeting Rho. PLoS Pathog. 9, e1003697 (2013).
9. Castillo, E. F. et al. Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation. Proc. Natl Acad. Sci. USA 109, E3168–E3176 (2012).
10. Cooper, A. M. et al. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J. Exp. Med. 178, 2243–2247 (1993).
11. Nandi, B. & Behar, S. M. Regulation of neutrophils by interferon-γ limits lung inflammation during tuberculosis infection. J. Exp. Med. 208, 2251–2262 (2011).
12. Choo, J. et al. The parasitophorous vacuole membrane of Toxoplasma gondii is targeted for disruption by ubiquitin-like conjugation systems of autophagy. Immunity 40, 924–935 (2014).
13. Hwang, S. et al. Nondegradative role of Atg5-Atg12/Atg16L1 autophagy protein complex in antiviral activity of interferon gamma. Cell Host Microbe 11, 397–409 (2012).
14. Zhao, Z. et al. Autophagosome-independent essential function for the autophagy protein Atg5 in cellular immunity to intracellular pathogens. Cell Host Microbe 4, 458–469 (2008).
15. Martinez, J. et al. Molecular characterization of LC3-associated phagocytosis reveals distinct roles for Rubicon, NOX2 and autophagy proteins. Nature Cell Biol. 17, 893–906 (2015).
16. Joulin, N. et al. The Atg5-Atg12 conjugate associates with innate antiviral immune responses. Proc. Natl Acad. Sci. USA 104, 14050–14055 (2007).
17. Youseli, S. et al. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nature Cell Biol. 8, 1124–1132 (2006).
18. Maskey, D. et al. Atg5 is induced by DNA-damaging agents and promotes mitotic catastrophe independent of autophagy. Nature Commun. 4, 2130 (2013).
19. Jakubzick, C. et al. Lymph-migrating, tissue-derived dendritic cells are minor constituents within steady-state lymph nodes. J. Exp. Med. 205, 2839–2850 (2008).
20. Abram, C. L., Roberge, G. L., Hu, Y. & Lowell, C. A. Comparative analysis of the efficiency and specificity of myeloid-Cre deleting strains using ROSA-EYFP reporter mice. J. Immunol. Methods 408, 89–100 (2014).
21. Flynn, J. L. & Chan, J. Immunology of tuberculosis. Annu. Rev. Immunol. 19, 93–129 (2001).
22. Jayaswal, S. et al. Identification of host-dependent survival factors for intracellular Mycobacterium tuberculosis through an siRNA Screen. PLoS Pathog. 6, e1000839 (2010).
23. Jung, C. H. et al. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol. Biol. Cell 20, 1992–2003 (2009).
24. Marinho, G. et al. Autophagy is essential for mouse sense of balance. J. Clin. Invest. 120, 2331–2344 (2010).
25. Lin, H. H. et al. Dynamic involvement of ATG5 in cellular stress responses. Cell Death Dis. 5, e1478 (2014).
26. Cadwell, K. et al. A key role for autophagy and the autophagy gene Atg16L1 in mouse and human intestinal Paneth cells. Nature 456, 259–263 (2008).
27. Abdel Fattah, E., Bhattacharya, A., Herron, A., Safdar, Z. & Eissa, N. T. Critical role for IL-18 in spontaneous lung inflammation caused by autophagy deficiency. J. Immunol. 194, 5407–5416 (2015).
28. Kanayama, M., He, Y.-W. & Shinohara, M. L. The lung is protected from spontaneous inflammation by autophagy in myeloid cells. J. Immunol. 194, 5465–5471 (2015).
29. Saitoh, T. et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1β production. Nature 456, 264–268 (2008).
30. Leemans, J. C. et al. Depletion of alveolar macrophages exerts protective effects in pulmonary tuberculosis in mice. J. Immunol. 166, 4604–4611 (2001).

Supplementary Information is available in the online version of the paper.

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Author Contributions J.M.K. designed and performed experiments, analysed data, and wrote the manuscript. J.P.H., S.P., A.K. and J.D. generated mouse strains. H.W.V. provided all mouse strains, analysed data and wrote the manuscript. C.L.S. designed experiments, analysed data and wrote the manuscript.

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METHODS

No statistical methods were used to predetermine sample size. **Cells and media.** *Mycobacterium tuberculosis* Erdman was cultured at 37°C in 7H9 (broth) or 7H10 (agar) (Difco) medium supplemented with 10% oleic acid/albumin/dextrose/catalase (OADC), 0.5% glycerol, and 0.05% Tween 80 (broth). *Ex vivo* macrophages were enriched from mice by bronchoalveolar lavage or peritoneal lavage with DMEM + 10% FBS + 1% MEM non-essential amino acids (Cellgro 25-025-CD) + 100 U ml⁻¹ penicillin + 100 μg ml⁻¹ streptomycin (Sigma, St. Louis). Lymphocytes were treated with ACK lysis buffer (10 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) to lyse red blood cells, plated in tissue-culture-treated plates, and incubated at 37°C in 5% CO₂ for at least 4 h to allow adherence of macrophages. Wells were washed vigorously with PBS to remove non-adherent cells and lysed in 2× Laemmli buffer for western blot analysis.

Bone-marrow-derived macrophages were isolated from femurs and tibias of mice, and cultured in DMEM + 20% FBS + 10% supernatant from 3T3 cells expressing M-CSF + 1% MEM non-essential amino acids (Cellgro 25-025-CD) + 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Sigma P4333) at 37°C in 5% CO₂.

PMNs for *ex vivo* western blotting analysis were purified from unfractionated bone marrow by negative selection via MACS column (Miltenyi Biotech, 130-097-658) according to the manufacturer’s guidelines and immediately lysed in 2× Laemmli buffer.

**Western blotting.** Protein samples were diluted in 2× Laemmli buffer, resolved using 4–20% polyacrylamide gels (BioRad no. 456-1096) transferred to PVDF membrane (GE Healthcare 10600023) and detected with the following antibodies: LC3b (Sigma L7543—detects LC3-I and LC3-II), p62/SQSTM1 (Sigma P0067), ATG5 (Sigma A2859), β-actin (Cell Signaling Technology no. 4970) and goat-anti-mouse horseradish peroxidase (HRP) and goat-anti-rabbit-HRP as appropriate. HRP was detected using Western Lightning Plus ECL (PerkinElmer no. 21162001). Actin (Sigma A2228) was detected using a Sypro Ruby Gold kit (Invitrogen). The β-actin antibody was used to normalize the signal variability.

**Data and statistics.** When used, centre values and error bars represent the mean ± 1 s.e.m.

**Flow cytometry.** Lungs were perfused with sterile PBS and digested at 37°C for 1 h with 625 g ml⁻¹ collagenase D (Roche 1108887103) and 75 U ml⁻¹ DNase I (Sigma D4527). Single-cell suspensions were stained in PBS + 2% FBS + 0.1% sodium azide in the presence of Fc receptor blocking antibody (BD Pharmingen 553541) and stained with the antibodies against the following mouse markers: CD11b PerCP-Cy5.5 (BD Pharmingen 550993), CD11c APC-Cy7 (eBioscience 47-0114), Ly6C PE (BD Pharmingen 560592), Ly6G PE-Cy7 (BD Pharmingen 560601), and F4/80 APC (Invitrogen MF48005). The FITC channel was used to determine autofluorescence. Cells were stained for 20 min at 4°C and then fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 20 min at room temperature. Flow cytometry was performed on a FACSCanto II (BD Bioscience) and data was analysed with FlowJo (Tree Star Inc.). Gating strategies are depicted in Extended Data Fig. 6a.

**RNA extraction and quantification.** Following tissue disruption by bead-beating (MP Biosciences), RNA was extracted from *M. tuberculosis*-infected lungs using the RNeasy Kit according to the manufacturer’s guidelines (Qiagen 74106). cDNA was made with SuperScript III reverse transcriptase using oligo-dT primers (Life Technologies 18080-051). qRT–PCR was performed using iTAQ SYBR Green (BioRad 172–5121) and transcript levels were normalized to actin. The following primers were used: *Atg16l1* forward, 5′-CCGAATCTGGACTGTTGATG-3′; reverse, 5′-CGGAGATCCACAGGAGTAG-3′; *Atg4b* forward, 5′-ATTACCTTGGATGGCTACGTACATGG-3′; reverse, 5′-ACCTACTAATGACGCTTCCG-3′; *Atg5* forward, 5′-ATGAGCTCGAGATGATCCTGGA-3′; reverse, 5′-ATGATCGTTGATGATCCTGGA-3′; *Atg7* forward, 5′-ATGAGCTCGAGATGATCCTGGA-3′; reverse, 5′-ATGATCGTTGATGATCCTGGA-3′; *Atg14* forward, 5′-ATGAGCTCGAGATGATCCTGGA-3′; reverse, 5′-ATGATCGTTGATGATCCTGGA-3′.

**PMN depletion.** Mice were treated with 0.2 mg anti-Ly6G (clone 1A8) or 0.2 mg rat IgG (Sigma IB015) via intraperitoneal injection every 48 h between days 10 and 28 post infection. Efficacy of PMN depletion was confirmed by loss of CD11b⁺*Gr-1⁺* cells in lungs at 21 d.p.i. Anti-Ly6G was collected from IA8 hybridoma*®* grown in Serum Free Medium (Gibco no. 12045-076) in CL350 Bioreactor flasks (Argos Technologies no. 900 10).

**Data and statistics.** All experiments were performed at least twice. When shown, multiple samples represent biological (not technical) replicates of mice randomly sorted into each experimental group. No blinding was performed during animal experiments. Animals were only excluded when pathology unrelated to *M. tuberculosis* infection was present (that is, weight loss due to malocclusion).

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Determination of statistical differences was performed with Prism 5 (Graphpad Software, Inc.) using log-rank Mantel–Cox tests (survival), unpaired two-tailed *t*-tests (to compare two groups with similar variances), or one-way ANOVA with Bonferroni’s multiple comparison test (to compare more than two groups). Sample sizes were sufficient to detect differences as small as 10% using the statistical methods described. When used, centre values and error bars represent the mean ± s.e.m. Sample sizes and the results of all comparisons can be found in Supplementary Figs 2–10.

**REFERENCES**

31. Jia, W. & He, Y.-W. Temporal regulation of intracellular organelle homeostasis in T lymphocytes by autophagy. *J. Immunol.* 186, 5313–5322 (2011).
32. Malhotra, R., Warne, J. P., Salas, E., Xu, A. W. & Debnath, J. Loss of Atg12, but not Atg5, in pro-opiomelanocortin neurons exacerbates diet-induced obesity. *Autophagy* 11, 145–154 (2015).
33. Komatsu, M. et al. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. *Cell* 131, 1149–1163 (2007).
34. Lee, E.-J. & Tourrier, C. The requirement of uncoupled 51-like kinase 1 (ULK1) and ULK2 in the regulation of autophagy. *Autophagy* 7, 689–695 (2011).
Extended Data Figure 1 | Survival of mice with defects in autophagy genes other than Atg5. Per cent survival of mice following infection with 100 colony-forming units (c.f.u.) of aerosolized M. tuberculosis. a, Survival of C57Bl/6 (open squares), Ulk1−/− (blue triangles), Ulk2−/− (inverted pink triangles), Atg4b−/− (red diamonds), and p62−/− (green circles) mice. b, Survival of Atg14lfl/fl-Lysm-cre (purple diamonds), Atg12fl/fl-Lysm-cre (red inverted triangles), Atg16l1fl/fl-Lysm-cre (green triangles), Atg7fl/fl-Lysm-cre (pink diamonds), Atg3fl/fl-Lysm-cre (brown circles) and corresponding floxed control mice. Floxed control mice are shown in open shapes, LysM–Cre-expressing mice are shown in closed shapes. c, Survival of C57Bl/6 (open squares), Atg16l1Hsf1 (open circles). Samples represent biological replicates. See Supplementary Fig. 6 for sample sizes.
Extended Data Figure 2 | Analysis of autophagy in bronchoalveolar macrophages. Western blot analysis of p62, LC3, and actin levels in ex vivo macrophages isolated from bronchoalveolar lavages of uninfected mice. For gel source data, see Supplementary Fig. 1.
Extended Data Figure 3 | *Atg5*fl/fl bone-marrow-derived macrophages are hypomorphic for ATG5. Western blot analysis of ATG5 (ATG5–ATG12 conjugate, 56 kDa) and actin in uninfected-bone-marrow-derived macrophages. For gel source data, see Supplementary Fig. 1.
Extended Data Figure 4 | Loss of *Atg5* or *Atg16l1* in LysM^+^ cells does not lead to increased c.f.u. at 2 w.p.i. log pulmonary c.f.u. at 2 w.p.i.

Samples represent biological replicates; error bars represent mean ± s.e.m. See Supplementary Fig. 7 for sample sizes and results from all statistical comparisons.
Extended Data Figure 5 | Cytokine levels in uninfected lungs.
Concentration of cytokines in lungs (homogenized in 1 ml PBS plus 0.05% Tween 80) from uninfected mice. Levels of IFN-γ, IL-6, MIP-1α, IL-17, and G-CSF were below the limit of detection. C57Bl/6 (grey solid bars), *Atg5*fl/fl (blue striped bars), *Atg5*fl/fl-Lysm-cre (blue solid bars), *Atg16l1*fl/fl (green striped bars), *Atg16l1*fl/fl-Lysm-cre (green solid bars). Statistical differences were determined by one-way ANOVA and Bonferroni’s multiple comparison test. n.s., not significant. Samples represent biological replicates; error bars represent mean ± s.e.m. See Supplementary Fig. 8 for sample sizes and results from all statistical comparisons.
Extended Data Figure 6 | Number of inflammatory cells in lungs of mice at 2 and 3 w.p.i. (related to Fig. 2). a, Gating strategy for analysis of inflammatory cells in lungs at 2 and 3 w.p.i. Single lung cells were gated based on CD11b, CD11c, Ly6G, Ly6C and autofluorescence (auto). The parental gate is shown above each contour plot. Representative data are shown from an Atg5fl/fl mouse at 2 w.p.i. b, c, C57Bl/6 (grey solid bars), Atg5fl/fl (blue striped bars), Atg16l1fl/fl (green striped bars), Atg16l1fl/fl-Lysm-cre (green solid bars). Mean number of alveolar macrophages, PMNs, recruited macrophages, and inflammatory monocytes in lungs at 2 w.p.i. (b) and 3 w.p.i. (c). d, e, Flow cytometry data presented in b and c and in Fig. 2 are the compilation of results from five experiments. In some experiments, different amounts of lung were collected for analysis, making it difficult to compare the average number of each cell type between strains, unless the data are normalized (as done in Fig. 2c, d—percentage of total cells). Therefore, to compare the raw number of cells detected in each cell population, each mouse analysed at 2 w.p.i. (d) and 3 w.p.i. (e) has been graphed individually. Each line represents a different mouse, with dots indicating the number of total cells, alveolar macrophages, PMNs, recruited macrophages and inflammatory monocytes. Statistical differences were determined by one-way ANOVA and Bonferroni’s multiple comparison test (b, c); *P < 0.05; n.s., not significant. Samples represent biological replicates; error bars represent mean ± s.e.m. See Supplementary Fig. 9 for sample sizes and results from all statistical comparisons.
Extended Data Figure 7 | Number of inflammatory cells in lungs of mice at 3 w.p.i. (related to Fig. 4). Number of alveolar macrophages, PMNs, recruited macrophages, and inflammatory monocytes in lungs at 3 w.p.i. C57Bl/6 (grey solid bars), Atg5^fl/fl (blue striped bars), Atg5^fl/fl -Lysm-cre (blue solid bars), 'healthy' Atg5^fl/fl -MRP8-cre (purple striped bars), and 'susceptible' Atg5^fl/fl -MRP8-cre (purple solid bars). Statistical differences were determined by one-way ANOVA and Bonferroni's multiple comparison test; *P < 0.05; n.s., not significant. Samples represent biological replicates; error bars represent mean ± s.e.m. See Supplementary Fig. 10 for sample sizes and results from all statistical comparisons.
Extended Data Figure 8 | Analysis of autophagy in bone marrow PMNs.
Western blot analysis of p62, LC3, and actin in bone marrow PMNs from uninfected mice. Each lane represents an individual mouse. Two replicates of the Atg5flo^t-Lysm-cre and Atg16l1flo^t-Lysm-cre mice are shown. For gel source data, see Supplementary Fig. 1.