Regulation of phagocyte triglyceride by a STAT-ATG2 pathway controls mycobacterial infection

Claire B. Péan1, Mark Schiebler2, Sharon W.S. Tan3, Jessica A. Sharrock1, Katrin Kierdorf1, Karen P. Brown2,4, M. Charlotte Maserumule2, Shinelle Menezes3, Martina Pilátová5, Kévin Bronda3, Pierre Guermonprez3, Brian M. Stramer5, R. Andres Floto2,4 & Marc S. Dionne1

*Mycobacterium tuberculosis* remains a global threat to human health, yet the molecular mechanisms regulating immunity remain poorly understood. Cytokines can promote or inhibit mycobacterial survival inside macrophages and the underlying mechanisms represent potential targets for host-directed therapies. Here we show that cytokine-STAT signalling promotes mycobacterial survival within macrophages by deregulating lipid droplets via ATG2 repression. In *Drosophila* infected with *Mycobacterium marinum*, mycobacterium-induced STAT activity triggered by *unpaired*-family cytokines reduces *Atg2* expression, permitting deregulation of lipid droplets. Increased *Atg2* expression or reduced macrophage triglyceride biosynthesis, normalizes lipid deposition in infected phagocytes and reduces numbers of viable intracellular mycobacteria. In human macrophages, addition of IL-6 promotes mycobacterial survival and BCG-induced lipid accumulation by a similar, but probably not identical, mechanism. Our results reveal *Atg2* regulation as a mechanism by which cytokines can control lipid droplet homeostasis and consequently resistance to mycobacterial infection in *Drosophila*. 

1. MRC Centre for Molecular Bacteriology and Infection, and Department of Life Sciences, Imperial College London, Ground Floor, Flowers Building, South Kensington Campus, London SW7 2AZ, UK. 2. Molecular Immunity Unit, MRC Laboratory of Molecular Biology, University of Cambridge Department of Medicine, Cambridge CB2 0QH, UK. 3. Centre for Molecular and Cellular Biology of Inflammation, School of Medicine, King’s College London, London SE1 1UL, UK. 4. Cambridge Centre for Lung Infection, Papworth Hospital, Cambridge CB23 3RE, UK. 5. Randall Division of Cell and Molecular Biophysics, King’s College London, London SE1 1UL, UK. Correspondence and requests for materials should be addressed to M.S.D. (email: m.dionne@imperial.ac.uk).
Virulent mycobacteria are critically dependent on their ability to survive within macrophages to cause disease. The tendency of macrophages to support or kill mycobacteria is directly regulated by distinct cytokine-JAK–STAT pathways: for example, the Th1 cytokine interferon-γ improves the ability of the macrophage to kill mycobacteria, whereas interleukin (IL)-10 and the Th2-derived cytokines IL-4 and IL-13 can inhibit killing of intracellular mycobacteria1–3. Our knowledge of the in vivo functions of these signals in mycobacterial infection, and of their transcriptional targets and the associated physiologies, remains incomplete, especially for those signals that inhibit killing of intracellular bacteria4,5. The genetic and cell-biological mechanisms that underlie these responses are potentially fertile ground for host-targeted therapies for tuberculosis6,7.

The fruitfly Drosophila melanogaster is a well-established model for the study of innate immunity8. Many aspects of the innate immune response to microbes are conserved between flies and vertebrates, including the central role of nuclear factor-kB family transcription factors and Toll-like receptors, anti-inflammatory effects of transforming growth factor-β family signals and the presence of bactericidal phagocytes homologous to vertebrate macrophages9–11. We have developed a system in which Drosophila are infected with Mycobacterium marinum, which allows us to identify host mechanisms involved in mycobacterial infection12,13. As in mammals, cytokine production by phagocytes is a critical component of the cellular response to infection. Flies have three known genes encoding IL-like signals, upd1, upd2 and upd3 (ref. 14). These proteins have structural and functional similarity to the four-helix-bundle cytokines of mammals15. They bind a GP130-like receptor, encoded by domeless, to activate a conserved JAK–STAT signalling cassette16. Upon bacterial infection in adult flies, upd3 is expressed by phagocytes and activates the JAK–STAT pathway in the fat body, where it is required for infection-induced expression of the stress peptide TotA14. This signalling pathway is critically required for antiviral defense17, it regulates larval haematoopoiesis18 and it is important in maintaining gut integrity in response to bacterial infection and other stresses19–23. Other roles in immune defense against bacteria are unknown.

Here we show that unpaired-family cytokines are critical regulators of defense against intracellular virulent mycobacteria. We show that M. marinum infection drives production of upd3 in phagocytes, and that reception of this signal by phagocytes is detrimental to the host. JAK–STAT blockade increased resistance to infection: it prolonged survival of the host, reduced mycobacterial numbers and delayed immune cell death, with similar effects observed in vitro. This effect was associated with overexpression of the autophagy-related gene Atg2 and not other autophagy genes, in vivo and in vitro. M. marinum infection of cultured phagocytes increased intracellular neutral lipids and drove accumulation of unusually large lipid droplets (LDs). Atg2 overexpression or JAK–STAT inhibition reduced intracellular mycobacterial number and partially normalized phagocyte lipid droplet size without significantly changing bulk autophagy. Direct inhibition of triglyceride synthesis prevented the infection-induced alteration in LDs, reduced mycobacteria–LD association and reduced intracellular viable mycobacteria. We then tested these findings in human cells. Similar to Drosophila, IL-6 signalling in macrophages increased intracellular Bacillus Calmette–Guérin (BCG) and Mycobacterium tuberculosis numbers in a dose-dependent manner; ATG2A expression was also reduced, although only to a very small extent. Importantly, the ability of IL-6 to increase the number of viable intracellular mycobacteria was abolished by inhibition of macrophage triglyceride synthesis. Together, these data indicate that loss of a STAT-activating cytokine pathway can reduce survival of intracellular mycobacteria via effects on cellular lipid deposition.

In Drosophila, one responsible effector may be ATG2.

Results
unpaired signalling decreases resistance to M. marinum.

The IL-6-like cytokine upd3 can be induced in haemocytes by infection with non-pathogenic bacteria; however, the ability of bacterial pathogens to induce its expression has not been previously explored14. This was particularly in question with M. marinum, which is not a strong agonist of known pattern-recognition pathways in Drosophila12,13. We thus examined the in vivo expression of upd3 during the initial phase of M. marinum infection using an upd3 green fluorescent protein (GFP) reporter and a haemocyte-specific dsRed nuclear marker10,14. We observed strong GFP induction in adult Drosophila haemocytes after mycobacterial infection (Fig. 1a), indicating that mycobacterial infection was indeed sufficient to drive upd3 production from macrophages in vivo. This effect was also visible by quantitative reverse transcriptase–PCR (qRT–PCR) in samples from whole flies (Fig. 1b).

To test the role of upd3 in mycobacterial infection in Drosophila, we infected flies carrying the os mutation with M. marinum. os affects expression of the cytokines upd1 and upd3, and impairs induction of upd3 by infection with non-pathogenic bacteria14,24. We observed that os mutants did not activate upd3 expression to the same degree as wild-type flies after M marinum infection, whereas upd1 expression was not induced by M. marinum infection in wild-type flies or os mutants (Fig. 1b). Despite identical starting inocula, os mutants carried a much lower mycobacterial burden at late stages of infection and survived longer than wild-type controls (Fig. 1c,d).

Although haemocytes were the only visible site of upd3 induction in M. marinum-infected upd3 > GFP flies, it remained possible that some other tissue was contributing to the relevant UPD3 pool. Moreover, although we saw no significant induction of upd1 by infection, changes in UPD1 might still contribute to the os phenotype. To verify that haemocyte-derived UPD3 was responsible for the mycobacterial resistance phenotype of os mutants, we knocked down upd3 with the haemocyte-specific crq-Gal4 driver and examined mycobacterial pathogenesis25. Haemocyte upd3 knockdown gave strong lifespan extension after infection and increased mycobacterial number (Fig. 1e,f). Together, these experiments indicate that haemocytes produce upd3 upon mycobacterial infection and haemocyte-derived upd3 shortens the infected lifespan of the host and impairs resistance against M. marinum.

Haemocyte STAT activity decreases resistance to mycobacteria.

To identify relevant UPD3 target tissues, we used RNA interference (RNAi) knockdown of Stat92E (the sole Drosophila STAT). Prior work has suggested that immune-induced STAT signalling is important in regulating humoral immune responses via effects on the Drosophila fat body26. However, Stat92E knockdown in the fat body had no effect on survival after M. marinum infection (Supplementary Fig. 1a). The primary direct immune effect of the fat body is the immune-inducible expression of antimicrobial peptides; we found that os mutant flies exhibited no consistent differences in antimicrobial peptide expression before haemocyte death (Supplementary Fig. 1b–e). We thus concluded that the effect of upd3 on mycobacterial infection was independent of the fat body.

We next tested the effect of cytokine signalling on haemocytes themselves. As the JAK–STAT pathway regulates larval haematoipoiesis, we combined our haemocyte drivers with Gal80TS9,10,
making their effect temperature sensitive, to enable adult-specific Stat92E knockdown18,27,28. Adult-specific Stat92E knockdown or expression of a dominant-negative form of the upd3 receptor dome in haemocytes significantly improved resistance to infection (Fig. 2a,b and Supplementary Fig. 2). These results together indicate that haemocyte-derived upd3 activates STAT signalling in haemocytes, decreasing resistance to mycobacterial infection and impairing host survival.

Cell death via necrosis or apoptosis is commonly observed in vertebrate macrophages infected with M. tuberculosis or M. marinum29–31. We observed marked cytotoxicity due to vertebrate macrophages infected with M. marinum in Drosophila S2R+ cells, an embryonic-derived macrophage cell line, and progressive loss of haemocytes during the last few days of M. marinum infection in vivo (Supplementary Fig. 3a–d). Just before dying, flies harboured no visible haemocytes and dramatically reduced expression of the haemocyte marker Hml was detected by qRT–PCR on the whole fly (Supplementary Fig. 3c–e). The os2 mutation improved haemocyte survival in infected flies (Supplementary Fig. 3b–d). Importantly, this was not a result of differential microbial internalization, as the fraction of haemocytes containing M. marinum was not different between control and upd3-IR flies 16 h after infection (Supplementary Fig. 4a,b). The difference in haemocyte survival, such as the difference in haemocyte number, was also seen in flies with adult-specific haemocyte Stat92E knockdown (Supplementary Fig. 4c–e). We thus conclude that upd3-STAT signalling alters the interaction between mycobacteria and phagocytes so as to impair the survival of infected cells.

**Figure 1** | **The unpaired cytokines reduce resistance to M. marinum infection.** (a) upd3 expression in haemocytes after M. marinum infection. Left, intravital imaging of eGFP expressed under the control of upd3-Gal4 in flies injected with PBS or M. marinum (5,000 CFU). Haemocytes are labelled with nuclear dsRed directly driven by the Hml.I promoter. This image was taken 48 h after infection. Scale bar, 10 μm. Right, quantification of the fraction of GFP-positive cells from seven flies based on images similar to the one shown. Error bars show s.d. Values are statistically different (**P < 0.001**) by Mann-Whitney test. (b) mRNA expression of upd1 and upd3 in wild-type (w1118) and os2 flies, uninfected or 72 h after infection with M. marinum. Assayed by qRT–PCR and normalized to Rp11 (as a loading control), and then to uninfected wild-type flies for each gene. n = 4 for each condition. Data were log-transformed before statistical comparison by Student’s t-test (**P < 0.001**). (c) M. marinum burden in wild-type and os2 mutant flies, assayed by qRT–PCR. Flies were injected with 500 CFU M. marinum and kept at 25°C. P-value for differences in bacterial numbers by two-way analysis of variance (ANOVA), n = 6 per time point except for day 9 where n = 3 for os2 flies. (d) Survival of wild-type and os2-mutant flies, injected with PBS or infected with 500 CFUs M. marinum, as indicated. Flies were kept at 25°C. P-value for difference between infected survival curves by log-rank test. (e) M. marinum burden in control (w1118; crq-Gal4/+ ) and haemocyte-specific upd3-knockdown (w1118; UAS-upd3-IR/; crq-Gal4/+ ) flies, assayed by qRT–PCR. Flies were injected with 500 CFU M. marinum and kept at 25°C. Here and elsewhere, ‘crq-upd3-IR’ is used as an abbreviation for ‘crq-Gal4, UAS-upd3-IR’. P-value for differences in bacterial numbers by one-way ANOVA, n = 3 per time point (**P < 0.05**). (f) Survival of control (w1118; crq-Gal4/+ ) and haemocyte-specific upd3-knockdown (w1118; UAS-upd3-IR/; crq-Gal4/+ ) flies, injected with PBS or 500 CFU M. marinum. Flies were kept at 25°C. P-value for difference between infected survival curves by log-rank test.
Figure 2 | STAT activation in haemocytes impairs Atg2 expression to reduce mycobactericidal activity. (a) *M. marinum* burden in control (w1118; tub-Gal80ts/+; crq-Gal4/+) and inducible haemocyte-specific Stat92E-knockdown (w1118, UAS-Stat92E-IR/+; UAS-Stat92E-IR/tub-Gal80ts; crq-Gal4/+) flies, assayed by qRT–PCR. Flies were initially infected with 500 CFU *M. marinum* and cultured at 29 °C. *P*-value for difference in bacterial numbers by two-way analysis of variance (ANOVA), *n* = 5 per timepoint. (b) Survival of control (w1118; tub-Gal80ts/+; crq-Gal4/+) and haemocyte-specific Stat92E-knockdown (w1118, UAS-Stat92E-IR/+; UAS-Stat92E-IR/tub-Gal80ts; crq-Gal4/+) flies, injected with PBS or infected with 500 CFU *M. marinum*, as indicated. Infectious dose and temperature as in a. *P*-value for difference between infected survival curves by log-rank test. (c) Fold change in expression of several autophagy genes in Stat92E-IR (w1118, UAS-Stat92E-IR; UAS-Stat92E-IR/tub-Gal80ts; crq-Gal4/+) flies compared with controls (w1118; tub-Gal80ts/+; crq-Gal4/+) 5 days after injection with 500 CFU *M. marinum*. The value shown is the geometric mean of two ratios (knockdown/driver-only control and knockdown/STAT-IR only control). Atg2 fold change is statistically different from other autophagy genes (**P < 0.01 by Mann–Whitney test, *n* = 14). (d) Atg2 expression by qRT–PCR in S2R+ cells overexpressing HA (control) or upd3. Normalized to Rpl1. Values are statistically different (**P < 0.01 by Mann–Whitney test, *n* = 6). (e) Expression of Atg2 by qRT–PCR in S2R+ cells with Luciferase or Stat92E knocked down, with or without *M. marinum* infection. Normalized to Rpl1 and then to the uninfected Luciferase control. Values are statistically different as indicated (***P < 0.001 by Mann–Whitney test, *n* = 7). (f) Intracellular dsRed-expressing *M. marinum* in S2R+ cells overexpressing HA (control) or Atg2. Picture is a representative; graph shows normalized *M. marinum* fluorescence from 37 (Atg2 O/E) or 45 (control) cells. Values are statistically different (*P < 0.05 by Mann–Whitney test). (g) Intracellular dsRed-expressing *M. marinum* in S2R+ cells with Luciferase or Stat92E knocked down by RNAi. The graph shows normalized *M. marinum* fluorescence from 44 (Stat dsRNA) or 76 (Luc dsRNA) cells. Values are statistically different (***P < 0.01 by Mann–Whitney test).
distribution, both processes associated with intracellular survival or killing of mycobacteria.33–36.

To clarify how cytokines interacted with other inflammatory cues to regulate phagocyte Atg2, we measured expression of Atg2 in S2R+ cells with or without Stat92E knockdown and with or without M. marinum infection. Stat92E knockdown or M. marinum infection gave mild or no increase in Atg2 expression but, when combined, these two stimuli strongly induced Atg2 expression, suggesting that upd3 limits infection-induced Atg2 overexpression (Fig. 2e). The difference observed between the in vivo context (where infection is not required for increased Atg2 expression in Stat92E knockdowns) and these in vitro observations may be the result of the different cytokine milieu experienced by cells in culture, compared with those in the animal, or to the contribution of other tissues.

Increased Atg2 impairs intracellular M. marinum survival. As Atg2 was specifically affected by loss of upd3-Stat92E signalling, we assayed the effect of Atg2 overexpression in infected phagocytes. Overexpression of GFP-ATG2 or RNAi of Stat92E reduced by 50% the number of intracellular mycobacteria per cell and eliminated mycobacterium-induced phagocyte death (Fig. 2f,g and Supplementary Fig. 5e,f). The effect of Atg2 on resistance to infection was not due to general changes in particle internalization, as Atg2 overexpression did not impair uptake of pHrodo-labelled beads (Supplementary Fig. 5g).

Atg2 knockdown has separable effects on autophagy and lipid deposition in HeLa cells. We assayed the effect of Atg2 overexpression on autophagy and found no clear differences in autophagosome number, processing of ATG8 or association of autophagosomes with mycobacteria (Supplementary Fig. 6).

M. marinum benefits from neutral lipids in phagocytes. In M. tuberculosis infection, some macrophages accumulate LDs, which has been suggested to promote mycobacterial persistence within the host.37,38. Indeed, intracellular survival of M. tuberculosis depends on its ability to recalibrate macrophage lipid homeostasis.34,39. M. marinum infection, similar to M. tuberculosis infection, induces lipid accumulation in Drosophila cells (Fig. 3a). As we had seen little effect on general autophagy from Atg2 overexpression, we explored the interaction of Atg2 with LDs. GFP-ATG2 associated with many LDs, as previously seen in HeLa cells, and sometimes appeared to coat intracellular mycobacteria (Fig. 3b and Supplementary Fig. 6a).35

Quantitative analysis of lipid deposition in infected cells revealed a mild increase in total intracellular neutral lipid, but a

Figure 3 | M. marinum driven lipid accumulation is required for intracellular growth or survival of mycobacteria. (a) Neutral lipids (stained with Oil Red O) and intracellular mycobacteria (stained with fluorescein isothiocyanate-anti-M tuberculosi) in cells variously uninfected, infected with live M. marinum or infected with heat-killed (H/K) M. marinum. (b) Co-localization of ATG2-GFP with LipidTox-stained intracellular neutral lipids. Cells were observed 48 h after transfection. Scale bars, 5 μm. (c) Quantification of neutral lipid volume per cell and total number of LDs with at least one axis >1 μm in length in cells treated as in b. Values are statistically different (***P < 0.01 and ****P < 0.001) by Mann-Whitney test. n = 130 for non-infected cells, n = 110 for M.m.-infected cells and n = 96 for cells in contact with heat-killed M.m. (d) Number of LDs (stained with BODIPY 500/510) of volume ≥1.5 μm3 in cells treated with dsRNA for mdy or Luc, and infected with dsRed M. marinum. Values are statistically different (***P < 0.001 by Mann-Whitney test, n = 71 for mdy dsRNA, n = 135 for Luc dsRNA). (e) Number of LDs (stained with BODIPY 500/510) at a distance ≤1 μm from bacteria and measured per unit of bacterial volume. Cells were treated with dsRNA for mdy or Luc and infected with dsRed M. marinum. Values are statistically different (***P < 0.001 by Mann-Whitney test, n = 64 for mdy dsRNA, n = 119 for Luc dsRNA). (f) Co-localization of BODIPY-stained LDs with dsRed M. marinum in S2R+ cells with or without mdy knockdown. Scale bar, 5 μm. (g) Quantification of bacterial volume per cells in S2R+ cells treated with dsRNA for mdy or Luc and infected with dsRed M. marinum. Values are statistically different (***P < 0.001 by Mann-Whitney test, n = 64 for mdy dsRNA, n = 119 for Luc dsRNA).
Increased Atg2 expression reduces large LDs. As preventing LD accumulation during infection reduced mycobacterial numbers and as ATG2 associated with LDs and reduced bacterial load, we hypothesized that ATG2 affected resistance to *M. marinum* by altering lipid deposition in immune cells. We tested this by infecting Atg2-transfected and control cells with *M. marinum* and observing LD volume (Fig. 4a–c). *M. marinum*-infected cells overexpressing Atg2 exhibited a marked reduction in the number of large LDs compared with infected controls. Stat92E knockdown, which induced Atg2 expression (Fig. 2e), also decreased large LD size in infected cells (Fig. 4d). Stat92E knockdown did not affect LD size in uninfected cells, in keeping with our observation that this knockdown did not affect Atg2 expression in the absence of infection (Fig. 2e). These data together suggest that overexpression of Atg2 observed in upd3/Stat92E knockdown flies renders phagocytes inhospitable to mycobacteria via effects on lipid deposition.

IL-6 impairs mycobacterial killing via effects on triglyceride. To see whether our results were relevant to human mycobacterial disease, we tested the ability of several JAK–STAT activating cytokines to impair killing of *Mycobacterium bovis* BCG by THP-1 cells. IL-6, added 2 h after infection, strongly inhibited the mycobacterial activity of these cells (Fig. 5a). To further clarify the timing of this effect, we measured viable intracellular BCG in cells treated before and/or after infection with IL-6; we observed that either pre-, post- or pre- and postinfection IL-6 caused a significant increase in viable intracellular BCG at 24 and 48 h.
We also observed that IL-6 caused a dose-dependent increase in survival of an auxotrophic mutant of *M. tuberculosis* within primary human macrophages (Fig. 5c). We then tested the effect of ATG2A overexpression on intracellular lipid stores in THP-1 cells and observed that overexpression of ATG2A was sufficient to markedly reduce neutral lipid stores in these cells (Fig. 5d). *upd3* has been suggested to be an evolutionary homologue of IL-6, and IL-6 is inducible by *M. tuberculosis* and can prevent killing of intracellular mycobacteria,

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**Figure 5 | IL-6 impairs intracellular killing of *M. tuberculosis* through altered LD homeostasis.** (a,b) Screening of the effect of cytokines signaling through JAK–STAT pathway on intracellular survival of mycobacteria in human macrophages. (a) Differentiated THP-1 cells were incubated with a luminescent strain of *M. bovis* BCG (BCG-lux) for 2 h, repeatedly washed, then treated with cytokines (20 ng/ml) for 24 h at 37°C. Cells were then lysed and luminescence measured. Viable intracellular mycobacteria were increased by IL-6 (red) and IL-5, and reduced by granulocyte–macrophage colony-stimulating factor and Leptin (green). Significance comparisons are between indicated treatment and untreated control. (b) Comparison of effects of treatment with IL-6 before and after infection in THP-1 cells. (c) Primary human macrophages were incubated with Bleupan *M. tuberculosis* for 2 h, repeatedly washed and then treated with IL-6 (red, at concentrations shown) for 24 h at 37°C before cells were lysed and plated to count CFUs. Significance comparisons are between indicated treatment and untreated control. (d) ATG2A-V5 overexpression reduces intracellular neutral lipids in THP-1 cells. Scale bars, 10 μm. (e) Treatment of primary human macrophages with IL-6 (80 ng/ml for 24 h) causes a small reduction in ATG2A protein abundance. Quantification is from three independent experiments. Error bars represent s.e.m. (f,g) BCG-induced LD accumulation is enhanced by IL-6 treatment. Representative images (f) and fluorescence quantification (g) of intracellular LDs (red), in primary human macrophages 24 h after infection with GFP-labelled *M. bovis* BCG (green). LDs are increased following infection, further increased in the presence of IL-6 treatment (80 ng/ml; red) and significantly reduced by pretreatment with the DGAT1 inhibitor T863 (10 μM, 48 h). Scale bars, 10 μm (f). (h) The increase in intracellular viable *M. bovis* BCG in primary human macrophages treated with IL-6 (red; 80 ng/ml for 24 h post infection) is blocked by pretreatment with the DGAT1 inhibitor T863 (as above). *P < 0.05, **P < 0.005 and ***P < 0.001. Figures are representative of at least three independent experiments with at least three replicates per data point.
We next tested the effect of IL-6 treatment on abundance of ATG2A protein. Although IL-6 treatment caused a statistically significant reduction in ATG2A protein abundance in monocyte-derived macrophages \((P=0.0384,\) unpaired Student’s \(t\)-test), this effect was extremely small (Fig. 5e). Thus, although the STAT-ATG2 transcriptional circuit may function in similar ways in human and fly macrophages, it is difficult to imagine that the effect of exogenous IL-6 on mycobacterial number is driven by reductions in total quantity of cellular ATG2A.

We then examined the effect of IL-6 on lipid accumulation in infected primary human macrophages. We treated macrophages with IL-6 with or without pharmacological inhibition of DGAT1. IL-6 treatment potentiated the increase in intracellular lipid deposition driven by mycobacterial infection (Fig. 5f–h). DGAT1 inhibition reduced intracellular triglyceride levels, eliminated the ability of mycobacteria to drive lipid accumulation and abolished the ability of IL-6 to promote intracellular mycobacterial survival (Fig. 5i–k), indicating that the ability of IL-6 to increase intracellular mycobacterial numbers depended on its ability to enhance bacterial access to host triglyceride.

**Discussion**

Here we identify a molecular mechanism that can reduce mycobacterial viability in phagocytes and show how this mechanism is inhibited by cytokine signalling in vivo. We show that haemocyte-derived cytokine signals activate JAK–STAT signalling in haemocytes in the early phases of *M. marinum* infection. Inhibiting production or response to this signal in adult haemocytes results in enhanced resistance to mycobacterial infection. We then show that flies in which either *upd3* or JAK–STAT signalling is impaired in haemocytes exhibit increased expression of *Atg2*, but not other autophagy genes. *Atg2* overexpression is sufficient to reduce mycobacterial survival in cultured *Drosophila* phagocytes and to inhibit changes in neutral lipid metabolism observed in *M. marinum*-infected cells. In parallel, we demonstrate that disturbing pathogen-induced changes in triglyceride metabolism directly impairs bacterial survival. Finally, a similar mechanism appears to function in human macrophages in response to IL-6 signalling. Together, these data indicate that some STAT-activating cytokines increase intracellular mycobacterial survival via effects on triglyceride deposition, that ATG2 regulation is a potential effector and that inhibition of macrophage triglyceride accumulation may be a useful therapeutic approach in mycobacterial disease.

Mycobacterium-infected macrophages often show aberrant accumulation of neutral lipids, but the biological importance of this event is only partly understood. Intracellular mycobacteria depend on host lipids as a source of nutrients, but this is not necessarily the only important role of neutral lipids in promoting mycobacterial survival. The fact that we observe changes in mycobacterial numbers as a result of triglyceride perturbations as little as 24 h after infection implies that accumulation of triglycerides may also directly disrupt phagocyte killing mechanisms. In this context, it may be relevant that neutral lipids have recently been observed to be protective against oxidative stress in the developing *Drosophila* central nervous system, suggesting that lipid accumulation in macrophages might protect intracellular bacteria from oxidative killing mechanisms. Nonetheless, this effect will clearly not be universal: in some contexts, LDs contain large amounts of free histones that can exert direct antibacterial properties. Another possibility is that the effect on bacterial survival is due to changes in production of lipids used as signalling molecules, such as leukotrienes, lipoxins and other eicosanoids. Although the roles of these molecules in *Drosophila* immune responses has not been characterized, they play critical roles in regulation of vertebrate responses to mycobacterial infection and it is not difficult to imagine that their production would be altered by the effects on triglyceride deposition we document here. In either case, our observations suggest the possibility that fusion of LDs with bacteria-containing vesicles is normally a productive aspect of the host defense that has been co-opted by some intracellular pathogens to promote their own nutrition and/or protect themselves from intracellular killing—although other explanations for these phenomena cannot be excluded.

Although autophagy can be altered by pro- and anti-inflammatory cytokines and has been argued to be important in clearance of intracellular *M. tuberculosis*, the *in vivo* relevance of this effect is not clear. The role of autophagy in *M. marinum* infection is similarly unclear, though cytosolic *M. marinum* can be sequestered in double-membrane-derived vesicles in bone-marrow-derived macrophages independently of the classical autophagy pathway proteins ATG5 or LC3, suggesting an unusual autophagy-like mechanism may be involved in the response to this organism. Our work suggests that the effects of autophagy on infection and sequestration of mycobacteria and the effects of mycobacteria on intracellular lipid inclusions may be fundamentally linked by common underlying molecular mechanisms. Effects ascribed to autophagic mechanisms may,
in fact, be due to autophagy components—in the case of our work, ATG2—acting outside of the normal autophagy pathways to alter lipid accumulation. Future work, in particular to delineate the genetic requirements for lipophagy as opposed to general autophagy, will help resolve these effects in infection.

Data suggest a model in which STAT-driven ATG2 repression deregulates lipid storage upon infection to the advantage of mycobacteria (Fig. 6). Despite potent bactericidal mechanisms, macrophages are often unable to eradicate M. tuberculosis, which then persists in the organs of the infected individual53. Therefore, novel strategies are needed to counteract bacterial latency and permit killing. Our work suggests two strategies to target mycobacterial infection by altering lipid metabolism in macrophages. One such strategy is direct inhibition of DGAT1. The fact that ATG2A overexpression is sufficient to reduce intracellular triglyceride in cultured THP-1 cells, and that Atg2 overexpression is sufficient to reduce mycobacterial survival in fly cells, suggests another such strategy. The strong and specific repression of Atg2 by JAK-STAT signalling, at least in Drosophila, indicates one regulatory mechanism of this gene, but even in this comparatively simple system other regulators are critical. Identification of these other regulatory mechanisms may open new avenues for antimycobacterial therapies.

**Methods**

**Drosophila stocks.** The following stocks were used: from the Bloomington Drosophila Stock Center: az (BDSC 79), Gal80ts (BDSC 7017, 7018, 7019, 7108), c564 (BDSC 6982), Him-l-Ga4 (BDSC 30140) and UAS-Stat92E-IR (BDSC 26899); from the Vienna Drosophila Resource Center: UAS-upd3-IR (VDRC 108689) and UAS-Stat92E-IR (VDRC 106908); from Herve Aigaisse (Yale University): UAS-upd3-IR; from Nathalie Franc (The Scripps Research Institute): cry-Ga4; from James Castelli-Gair Hombria (Centro Andaluz de Biología del Desarrollo): UAS-done50.

**Survival analysis.** Mycobacterial and Drosophila culture and infections were carried out substantially as described, using 5- to 9-day-old male flies12. Injected flies were incubated 20 flies to a vial and the number dead was recorded twice a day; all survival experiments were repeated at least three times with qualitatively similar results. When using a tubulin-Gal80ts construct, crosses were kept at 18°C and flies were collected and switched to 29°C the day after eclosion; they were then maintained at 29°C for the duration of the experiment. For experiments not involving tub-Gal80ts, flies were kept at 25°C at all times.

**Intravital imaging.** For haemocyte counts, 5-7-day-old flies carrying Him-l-Ga4, UAS-2×eGFP or cry-Ga4, UAS-2×eGFP were injected with 500 colony-forming unit (CFU) M. marinum and imaged at indicated times. Flies were glued to coverslips and imaged immediately using a confocal microscope (SP5, Leica) equipped with an environmental control chamber. The environmental chamber was set to 25°C or 29°C when tub-Gal80ts flies were used. Flies that died during the procedure were excluded from the analysis. GFP was excited at 488 nm with an argon laser and 5.6 μm sections were imaged through the coverslip, glue and dorsal cuticle. Flies carrying Him-l-dsRed.nls and upd3-Gal4, UAS-GFP were imaged 48 h after injection of 5,000 CFUs using the same procedure with sequential excitation at 488 and 561 nm, and with images taken every 0.6 μm.

**Drosophila cell culture and infection.** S2R+ cells (DGRC, Bloomington, Indiana) were maintained in M3 medium supplemented with 10% fetal bovine serum and 30 μM 1- penicillin-streptomycin. S2R+ cells were transfected using Effectene (Qiagen); 24 h later, they were washed and then infected with M. marinum (sus- pend M. marinum in medium supplemented with 10% FBS, 100 μg/ml penicillin-streptomycin) at a multiplicity of infection (MOI) of 10-20. Samples were collected after 24 or 48 h for western blotting, qRT-PCR or imaging.

For overexpression experiments, GFP-tagged human ATG2A (from plasmid pEGFP-C1-hAtg2A, Addgene, deposited by Noboru Mizushima) and Drosophila upd3 (by PCR from Drosophila complementary DNA) were cloned into pFAC-HA (a vector containing the Drosophila Actin SC promoter from Nic Tapon (Francis Crick Institute)). In all overexpression experiments, the displayed control is cells transfected in parallel with the empty vector, in which a 3×HA peptide is expressed. Phagocytosis assays on cultured cells were done as described31. For RNAi experiments, cells were bathed in double-stranded RNA (dsRNA) targeting Stat92E or, as a control, Renilla luciferase. RNAi constructs were designed using the SnapDrago web tool with no predicted off-targets; template amplicons were produced by PCR from plasmid or Drosophila genomic DNA with the following primers: 5′-TTA TAC GAC TCA CTA TAG GGA GAC TGA TCA AGA GCG AAG AAG G-3′; 5′-TTA TAC GAC TCA CTA TGA GGA TAC TCA CAT GGA TAC TCT CAC GAA CTC GGT GTT G3′-3′; 5′-Stat92E 5′-TAA GAC TCA CTA TGA GGA TAA AGC TGC TGG CCG AAA ACT A-3′; 5′-Stat92E-R, 5′-TAA GAC TCA CTA TGA GGA TAC TCA CAT GGA TAC TCT CAC GAA CTC GGT GTT G-3′; 5′-mdyL, 5′-TAA TAC GAC TCA CTA TAG GGA GAG CTA GGT CTA ATT CTT AAA CTT GGC G-3′; and 5′-mdyR, 5′-TAA TAC GAC TCA CTA TAG GGA AAA ACG CCG TGA AGG TGC TGG CCG AAA ACT A-3′.

**Flow cytometry of Drosophila haemocytes and S2R+ cells.** For cytotoxicity assays in uninfected and infected Drosophila S2R+ cells, the supernatant medium containing non-adherent cells was collected 24 h post infection and transferred directly into a FACs tube. The adherent S2R+ cells of the same sample were collected 2 h after 1 ml of ice-cold culture medium and pipetting up and down. The medium was then added to the previously collected supernatant sample. All samples were filtered with a 70 μm cell strainer filter (BD Biosciences) and kept on ice before FACs analysis. Five minutes after analysis, 5 μl of propidium iodide (PI) was added to 500 μl of samples to stain dead cells. Cells were analysed with a FACs Aria II (Becton Dickinson (BD)), using a 100 μm nozzle and the FACs-Diva software. All experiments included the following controls: M. marinum only samples with and without PI staining, and non-infected cells with and without PI.

For phagocytosis assays with phrodo-coated beads in uninfected and infected Drosophila S2R + cells, supernatant and adherent cells were collected as described above with post-transfection. Cells (20,000 in a 15-μl volume and 200 μl of 1% PMA 48 h before infection, inoculated with BCG-LR Fortessa). Samples were acquired with a BD Fortessa and analysed using the FlowJo analysis software. Four samples per genotype were analysed.

In vitro mycobacterial infection assays. Luminiscence reporter strain infection: a validated luminiscence reporter strain of M. bovis BCG (BCG-lux) encoding the Vibrio lux AB gene, generated as described60, was used to infect macrophages. Macrophages were co-cultured for 7 days in 2 ml of medium containing 1% L-leucine, 0.024 mg ml-1 calcium pantothenate and 0.2 mg ml-1 2-hydroxypropyl-β-cyclodextrin (4/EE/1187) or THP-1 cells (ATCC), were differentiated by incubation in 24-well plates in the presence of 9 μl of recombinant human IL-4 and 10 μl of recombinant human GM-CSF for 2 days. Macrophages were harvested at 7 days post infection and were incubated with 5 μl of 1× PMA 24 h before infection, inoculated with BCG-lux (at an MOI of 5:1) for 2 h at 37°C, washed, followed by incubation in 24 h at 37°C in the presence of compounds as indicated. Unless otherwise stated, recombinant cytokines (PeProtech) were used at 20 ng ml-1 and added to cells following 2h incubation with mycobacteria. Where indicated, cells were pretreated with DGAT1 inhibitor T863 (10 μM) for 48 h and then 24 h post infection, IL-6 was treated for 24 h post infection at 80 ng ml-1. Cells were lysed and luminescence measured as described35. Experiments were carried out in sextuplicate. Results are representative of at least three separate experiments.

M. tuberculosis infection: M. tuberculosis ΔαΔ3 ΔαΔ3 (Bleupan)26 stably transfected with μpSP12-GFP was grown in Middlebrook 7H9 broth (Difco) containing 0.5% glycerol, 0.5% glucose, 10% OADC (BD), 0.05 μg ml-1 l-arginine, 0.024 mg ml-1 calcium pantothenate and 0.2 mg ml-1 N-Hydroxymycin B in 250 ml sterile disposable Erlenmeyer flasks with mild agitation at 37°C. Bacterial cells were harvested at mid log phase by centrifugation at 3000 g for 7 min, pellets were resuspended in 7H9 broth containing leucine and pantethein supplements as above plus 18% glycerol and frozen at –80°C until use. Cells were infected at an MOI of 5:1, lysed at times indicated and CFUs enumerated as described35.

Confocal immunofluorescence on human cells. Primary human macrophages were grown on glass coverslips, infected with M. bovis BCG (BCG-lux) and treated as described, rinsed with PBS, fixed with methanol and permeabilized with 0.1% Triton X-100 (Sigma)26 before being stained with Nile red 1:10,000 (dissolved in...
isoopropanol), rinsed with water and then mounted with ProLong Gold antifade containing DAPI (Invitrogen). Images were acquired on a Zeiss LSM880 confocal microscope (Plan-Apochromat × 63/1.40 Oil-immersion lens) and analysed with Zen 2010 (Carl Zeiss), and fluorescence per cell measured by Volocity software.

**Antibodies and western blots.** For the anti-ATG2A blotting shown in Fig. 5, primary human macrophages were treated with IL-6 80 ng ml–1 for 24 h before being lysed. The primary antibody used was anti-ATG2A pAb (MBL Life Science) at 1:1000. Other western blots were performed with anti-Dro sophila Atg8 (courtesy of Kajsa Kóhler (University of Zurich))22 and G. Juhasz (Eotvos Loránd University)23, both used at 1:200 and anti-α-tubulin (Developmental Studies Hyridoma Bank 12G10, used 1:10,000). Immunofluorescence on fly cells was performed with fluorescein isothiocyanate-labelled anti-M tuberculos is (Invitrogen PA1-28997, used 1:50) and rabbit anti-GFP (Invitrogen A-11122, used 1:100).

Immunofluorescence on human cells was performed with anti-V5 from Novus Biologicals (NB 600-381), 1:400.

**M. marinum culture.** M. marinum strain M and fluorescent derivatives were grown stationing at 25°C in Middlebrook 7H9 media, supplemented with OADC, Tween-80, glycerol and antibiotics as necessary to maintain fluorescent plasmid(s). Single-cell bacterial suspensions were produced as follows. Cultures were pelleted by centrifugation at 4,000 g for 5 min, then resuspended in PBS + 0.2% Tween-80. Clumps were then separated by centrifugation at 200 g for 5 min. The supernatant from this centrifugation contained primarily single bacterial cells. M. marinum strains were all strain M carrying various fluorescent proteins under the control of the msp12 promoter26; all were courtesy of Lalita Ramakrishnan and Antonio Pagan (University of Cambridge).

**Statistics.** Statistical analyses were performed using GraphPad Prism or R. Two-way analysis of variance, Mann–Whitney test and Kolmogorov–Smirnov test were used in this study. Statistical analysis was only performed on experiments with a *P* value <0.05. Unless otherwise noted, error bars correspond to s.e.m.

**qRT–PCR analysis.** RNA was extracted from adult flies using TRIzol (Invitrogen) according to the manufacturer’s directions, except that samples from which mycobacterial RNA was amplified were first homogenized in 3:1 mixture of chloroform and methanol34. Mycobacterial quantification was performed using a standard curve which was generated on experiments performed on at least four samples per condition. *P*-values are as follows: *P*<0.05, **P**<0.01 and ***P**<0.001. Unless otherwise noted, error bars correspond to s.e.m.

**Data availability.** All relevant data are available from the authors, except where precluded by human subject confidentiality.

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Author contributions
Study conception and design: M.S.D. and C.B.P. Acquisition of data: C.B.P., M.S., S.W.S.T., J.A.S., K.K., K.P.B., M.C.M., S.M., M.P., K.P.B. and M.S.D. Analysis and interpretation of data: C.B.P., M.S., P.G., B.M.S., R.A.F. and M.S.D. Drafting of manuscript: C.B.P. and M.S.D. Critical revision: M.S.D., R.A.F. and C.B.P.

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