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# Atg5 Is essential for the development and survival of innate lymphocytes

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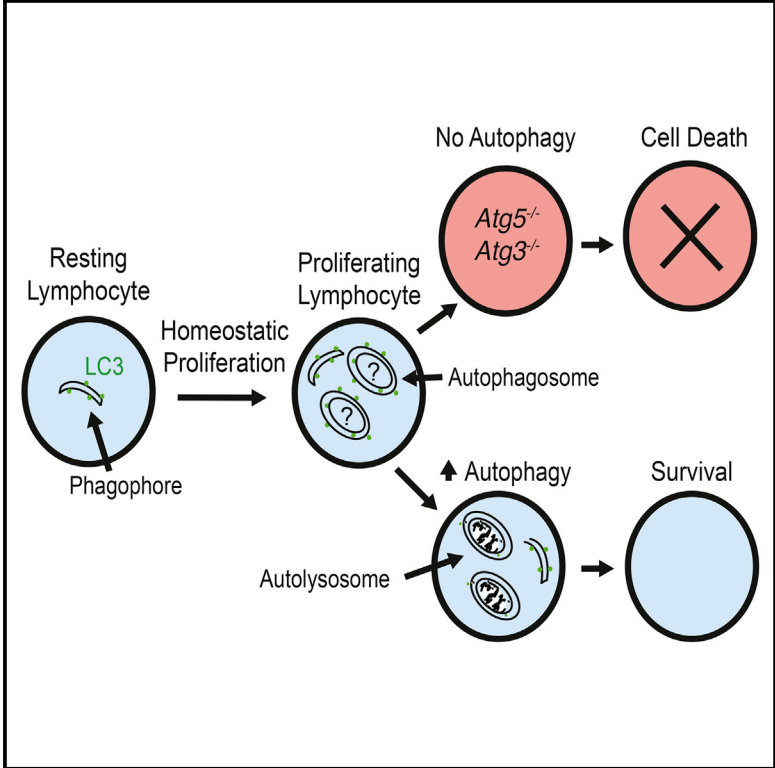
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## Atg5 Is Essential for the Development and Survival of Innate Lymphocytes

### Graphical Abstract



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### In Brief

Autophagy is an essential cellular survival mechanism induced during periods of stress. O’Sullivan et al. demonstrate that the essential autophagy gene *Atg5* is required for the development and survival of all ILC lineages by limiting cell-intrinsic apoptosis.

### Highlights

- *Atg5* is required for innate lymphocyte development
- Adaptive and innate lymphocytes induce autophagy during homeostatic expansion
- Autophagy is essential for lymphocyte survival during lymphopenia
- Metformin increases autophagy to enhance lymphocyte survival in lymphopenic hosts



# *Atg5* Is Essential for the Development and Survival of Innate Lymphocytes

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## SUMMARY

Autophagy is an essential cellular survival mechanism that is required for adaptive lymphocyte development; however, its role in innate lymphoid cell (ILC) development remains unknown. Furthermore, the conditions that promote lymphocyte autophagy during homeostasis are poorly understood. Here, we demonstrate that *Atg5*, an essential component of the autophagy machinery, is required for the development of mature natural killer (NK) cells and group 1, 2, and 3 innate ILCs. Although inducible ablation of *Atg5* was dispensable for the homeostasis of lymphocyte precursors and mature lymphocytes in lymphoreplete mice, we found that autophagy is induced in both adaptive and innate lymphocytes during homeostatic proliferation in lymphopenic hosts to promote their survival by limiting cell-intrinsic apoptosis. Induction of autophagy through metformin treatment following homeostatic proliferation increased lymphocyte numbers through an *Atg5*-dependent mechanism. These findings highlight the essential role for autophagy in ILC development and lymphocyte survival during lymphopenia.

## INTRODUCTION

Apoptosis and autophagy are evolutionarily conserved pathways that often elicit contrasting cellular outcomes in response to cellular stress (Mariño et al., 2014; Reed and Kroemer, 2000). Autophagy is a process in which cytosolic contents are engulfed into double-membrane vacuoles, or autophagosomes, and delivered to the lysosome for degradation (Levine et al., 2011; Mariño et al., 2014). Whereas apoptosis executes cell death programs during periods of metabolic starvation or stress, autophagy can serve as an essential cellular survival mechanism by maintaining energy homeostasis through its self-catabolic activity (Levine et al., 2011; Mariño et al., 2014).

Mice with conditional deletion of the gene encoding the essential autophagy machinery component *Atg7* in the hematopoietic system exhibit a drastic loss of self-renewing hematopoietic

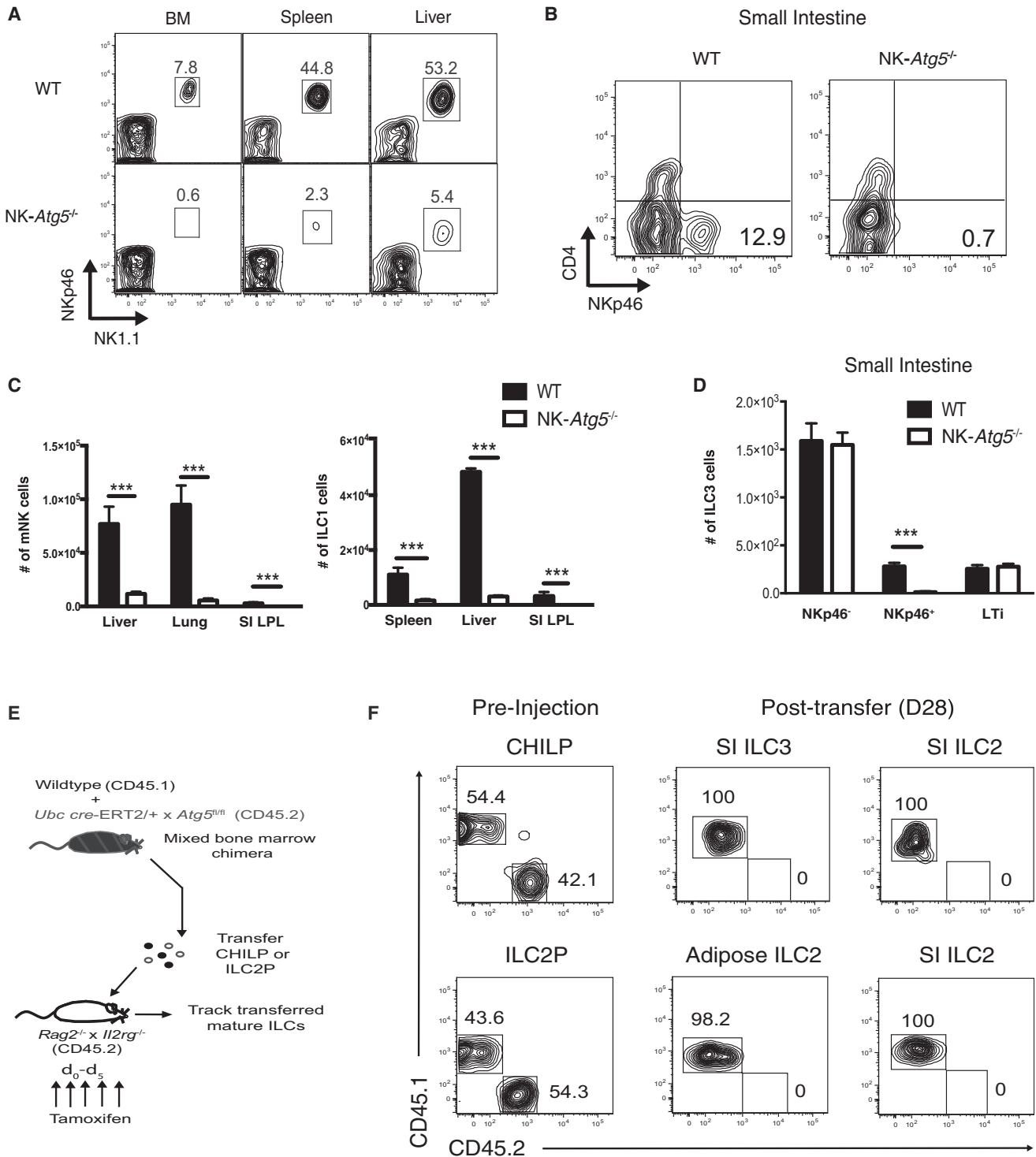
stem cells (HSCs) (Mortensen et al., 2011), which utilize *Atg12*-dependent autophagy during cellular stress for their survival (Warr et al., 2013). In the immune system, conditional gene deletion of *Atg7*, *Atg5*, or *Atg3* during early T cell development (Jia and He, 2011; Pua et al., 2007, 2009), *Atg7* or *Atg5* in the hematopoietic system (Miller et al., 2008; Mortensen et al., 2010), and *Atg5* or *Atg7* during invariant natural killer T (iNKT) cell development (Pei et al., 2015; Salio et al., 2014) results in diminished numbers of mature peripheral T, B, and iNKT cells that display increased apoptosis and dysfunctional organelle homeostasis, suggesting that autophagy is continuously utilized as a maintenance mechanism to promote adaptive lymphocyte development and homeostasis. Although these studies underpin the pro-survival role of autophagy during HSC and adaptive lymphocyte development and homeostasis, deletion of *Atg7* in the hematopoietic system, or conditional deletion of *Atg7* in the myeloid compartment does not negatively impact the development of innate immune cells such as macrophages, dendritic cells, or neutrophils (Bhattacharya et al., 2015; Mortensen et al., 2010), challenging the hypothesized role of autophagy as a constitutive pro-survival intracellular quality control mechanism in all leukocytes.

The family of innate lymphocytes consist of mature NK (mNK) cells, group 1 innate lymphoid cells (ILC1s), group 2 ILCs (ILC2s), and group 3 ILCs (ILC3s) including lymphoid-tissue inducer (LTI) cells (Artis and Spits, 2015). Although ILCs do not undergo RAG-dependent somatic rearrangement of antigen receptors (Spits et al., 2013), they share similar cytokine signaling and transcription factor requirements for their development, as well as functional attributes, with their adaptive T helper cell counterparts (Artis and Spits, 2015; De Obaldia and Bhandoola, 2015; Sun and Lanier, 2011; Verykokakis et al., 2014). Because it is not known whether autophagy is induced or necessary for the development of the ILC lineage, we investigated the impact of *Atg5* in regulating the development and homeostasis of innate lymphocyte populations using genetic ablation of *Atg5* at distinct developmental checkpoints.

## RESULTS AND DISCUSSION

### *Atg5* Is Required for ILC Development

In order to demonstrate the physiological importance of autophagy in ILC1s and certain ILC3s, we generated mice with



**Figure 1. Atg5 Is Essential for Innate Lymphocyte Development**

(A and B) Representative flow cytometric plots of lineage marker negative (Lin<sup>-</sup>) NKp46<sup>+</sup>NK1.1<sup>+</sup> cells (A) and Lin<sup>-</sup>NK1.1<sup>-</sup>CD127<sup>+</sup>CD90.2<sup>+</sup>Rorγt<sup>+</sup> (ILC3s) in indicated peripheral organs of NK-Atg5<sup>-/-</sup> mice or littermate Atg5<sup>fl/fl</sup> controls (called WT) (B).

(C and D) Absolute numbers of mNK cells (Lin<sup>-</sup>NK1.1<sup>+</sup>NKp46<sup>+</sup>DX5<sup>+</sup>Eomes<sup>+</sup>), ILC1 (Lin<sup>-</sup>NK1.1<sup>+</sup>NKp46<sup>+</sup>DX5<sup>-</sup>Eomes<sup>-</sup>) (C) and ILC3s in indicated peripheral organs of WT or littermate NK-Atg5<sup>-/-</sup> mice (D).

(E and F) WT mice (CD45.1 × CD45.2) were lethally irradiated and reconstituted with an equal number of bone marrow cells from WT (CD45.1) and *Ubc<sup>Cre-ERT2</sup> × Atg5<sup>fl/fl</sup>* (CD45.2) mice.

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NKp46<sup>+</sup> cell-specific deletion of the essential autophagy machinery component *Atg5* (*NKp46<sup>iCre</sup> × Atg5<sup>fl/fl</sup>*), hereafter referred to as “NK-*Atg5*<sup>-/-</sup> mice”. Analysis of the peripheral organs of NK-*Atg5*<sup>-/-</sup> mice revealed a pronounced reduction in the frequency (Figures 1A and 1B) and absolute numbers (Figures 1C and 1D) of mNK, ILC1, and NKp46<sup>+</sup> ILC3 cells compared to iCre-negative *Atg5<sup>fl/fl</sup>* littermate mice (called “WT” in this figure). Peripheral T and B cells were found at normal numbers in NK-*Atg5*<sup>-/-</sup> mice (data not shown). We found a marked reduction in the percentage (Figure S1A) and number (Figure S1B) of both iNK (NKp46<sup>+</sup>DX5<sup>-</sup> NK1.1<sup>+</sup> Eomes<sup>+</sup>) and NKp46<sup>+</sup>DX5<sup>-</sup> NK1.1<sup>+</sup> Eomes<sup>-</sup> cells in the bone marrow of NK-*Atg5*<sup>-/-</sup> mice compared to littermate controls, whereas the numbers of “refined” NK progenitor (rNKP) cells were unaffected, consistent with the lack of NKp46 surface expression on these early precursor cells (Narni-Mancinelli et al., 2011). Because of the drastically reduced peripheral mNK cell numbers, NK-*Atg5*<sup>-/-</sup> mice quickly succumbed to mouse cytomegalovirus (MCMV) infection (Figure S1C) and displayed higher viral titers when compared to littermate controls (Figure S1D). Together these results demonstrate the critical role for *Atg5* in the development of NKp46<sup>+</sup> ILCs in the bone marrow and periphery.

After observing a near complete loss of NKp46<sup>+</sup> ILCs in NK-*Atg5*<sup>-/-</sup> mice, we investigated whether *Atg5* may also regulate the development or homeostasis of additional ILC populations. Mature ILC2s and ILC3s can be derived from a common helper ILC progenitor (CHILP) in the bone marrow (Constantinides et al., 2014; Klose et al., 2014; Yang et al., 2015), whereas mature ILC2 cells can be exclusively derived from a ILC2 progenitor (ILC2P) population in the bone marrow upon adoptive transfer into lymphopenic hosts (Hoyle et al., 2012). To test if *Atg5*-deficiency also impacted the development of mature ILC2 and ILC3s, specifically from their respective committed progenitors, we generated mice with inducible deletion of *Atg5* (*Ubc<sup>Cre-ERT2</sup> × Atg5<sup>fl/fl</sup>*) upon administration of 4-hydroxytamoxifen. We then generated mixed bone marrow chimeric (mBMC) mice by co-adoptively transferring equal numbers of wild-type (WT) (CD45.1) and *Ubc<sup>Cre-ERT2</sup> × Atg5<sup>fl/fl</sup>* (CD45.2) mouse bone marrow cells into lethally irradiated WT (CD45.1 × CD45.2) hosts (hereafter referred to as WT:i-*Atg5* mBMC) (Figure 1E). At 8 weeks following bone marrow transplantation, bone marrow from WT:i-*Atg5* mBMC was harvested and CHILP or ILC2P populations were sorted to high purity (Figure S1E) and adoptively transferred into irradiated *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> hosts (Figure 1E). At the time of sorting, reconstitution by donor populations in WT:i-*Atg5* mBMC was comparable (Figure 1F and data not shown). Following tamoxifen treatment of recipient mice, we observed that adoptively transferred *Atg5*-deficient CHILPs failed to generate mature ILC2 and ILC3s in the small intestine, while *Atg5*-deficient ILC2Ps failed to generate mature ILC2s in peripheral

organs (Figure 1F). In contrast, mature WT ILC2 and ILC3 populations (CD45.1) were observed in peripheral organs of recipient mice 4 weeks after transfer (Figure 1F), highlighting the importance of *Atg5* in both ILC2 and ILC3 development in vivo.

### Lymphocytes Induce Autophagy in Lymphopenic Hosts

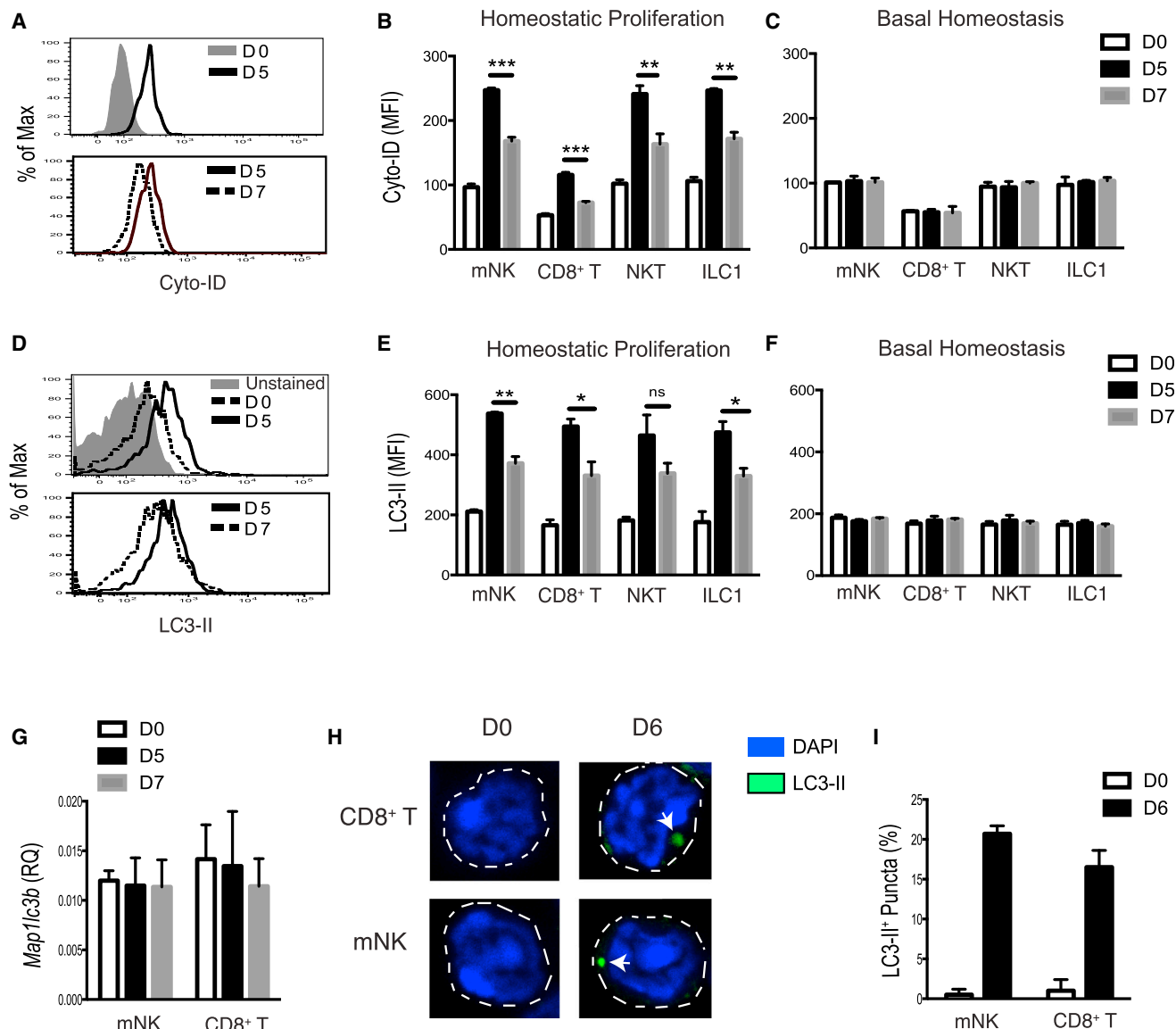
Recent studies have shown that autophagy is induced in antigen-specific T, B, and NK cells following clonal proliferation in response to viral infection to mediate cell survival (Chen et al., 2014; O’Sullivan et al., 2015; Puleston et al., 2014; Xu et al., 2014). In mice devoid of mature lymphocytes, adoptively transferred T and NK cells rapidly proliferate and persist long-term in peripheral organs in a process known as homeostatic proliferation (Jamieson et al., 2004; Pric et al., 2003; Ranson et al., 2003; Sun et al., 2011). Similar to splenic NK cells, we observed that liver ILC1s could also undergo homeostatic proliferation and persist for at least a month following transfer into *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> hosts (Figures S2A–S2C). Since immature adaptive and innate lymphocytes undergo proliferation during development to generate a mature compartment in the periphery (Hoyle et al., 2012; Kim et al., 2002; Koch and Radtke, 2011; Yang et al., 2015), we hypothesized that autophagy could also be induced following homeostatic proliferation during lymphopenia.

To investigate whether mature adaptive and innate lymphocytes induce autophagy during homeostatic expansion, we utilized Cyto-ID staining (which labels both autophagosomes and autolysosomes) (O’Sullivan et al., 2015; Puleston et al., 2014) in parallel with LC3-GFP transgenic mice (Mizushima et al., 2004) to assess autophagic activity by measuring the degradation of autophagosomes and LC3-II by flow cytometric analysis (LC3-II is the lipidated form of LC3-I that is selectively incorporated into the elongation membrane of early autophagosomes and subsequently degraded following lysosomal fusion with autophagosomes) (Eng et al., 2010; Klionsky et al., 2016). We found that Cyto-ID and LC3-II staining were both increased in adoptively transferred lymphocytes on d5 following transfer into *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> hosts (homeostatic proliferation) compared to resting lymphocytes (Figures 2A, 2B, 2D, and 2E), whereas adoptively transferred lymphocytes recovered from WT lymphoreplete hosts (basal homeostasis) did not display increased staining of LC3-II or Cyto-ID (Figures 2C and 2F). Accumulation of LC3-II and autophagosomes/autolysosomes during the first 5 days would be consistent with either increased production of LC3 protein, induction of autophagy, or the inhibition of autophagosome degradation by lysosomes. However, we did not observe an increase in *Map1lc3* (LC3) transcripts on d5 following homeostatic proliferation in sorted mNK or CD8<sup>+</sup> T cells compared to resting cells (Figure 2G), suggesting that increased LC3 protein is not due to elevated transcription in

(E) Schematic of experiment.

(F) Both WT (CD45.1) and *Ubc<sup>Cre-ERT2</sup> × Atg5<sup>fl/fl</sup>* (CD45.2) CHILP (Lin<sup>-</sup>NK1.1<sup>-</sup>DX5<sup>-</sup>FLT3<sup>-</sup>CD127<sup>+</sup>α<sub>4</sub>β<sub>7</sub><sup>+</sup>CD25<sup>-</sup>) and ILC2P (Lin<sup>-</sup>NK1.1<sup>-</sup>DX5<sup>-</sup>FLT3<sup>-</sup>CD127<sup>+</sup>α<sub>4</sub>β<sub>7</sub><sup>+</sup>CD25<sup>+</sup>) cells were adoptively transferred into sublethally irradiated *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> hosts and injected i.p. with 1 mg of tamoxifen daily for 5 days. After 28 days, the SI and adipose tissue were harvested from recipient mice and analyzed for mature ILC2 (Lin<sup>-</sup>NK1.1<sup>-</sup>DX5<sup>-</sup>CD127<sup>+</sup>CD90.2<sup>+</sup>GATA3<sup>+</sup>KLRG1<sup>+</sup>) and ILC3 cells (Lin<sup>-</sup> refers to TCRβ<sup>-</sup>CD19<sup>-</sup>CD3ε<sup>-</sup>Ly6G<sup>-</sup>TER119<sup>-</sup>TCRγδ<sup>-</sup> cells). The data are representative of two independent experiments, with n = 5 per time point. The samples were compared using an unpaired, two-tailed Student’s t test and data are presented as the mean ± SEM (\*\*p < 0.001).

See also Figure S1.



**Figure 2. Autophagy Is Induced in Lymphocytes following Homeostatic Expansion**

(A and B) WT (CD45.1) liver lymphocytes were adoptively transferred into *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> (homeostatic proliferation). Representative histograms (A) and quantification of Cyto-ID mean fluorescence intensity (MFI) in adoptively transferred NK, ILC1, NKT, and CD8<sup>+</sup> T cells harvested from livers of recipient mice on day 5 and 7 after transfer (B).

(C) WT (CD45.1) liver lymphocytes were adoptively transferred into WT (CD45.1 × CD45.2) recipients (basal homeostasis) and quantified for Cyto-ID staining on day 5 and 7 after transfer from recipient livers.

(D and E) LC3-GFP (CD45.2) transgenic liver lymphocytes were adoptively transferred into *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> recipients and livers were harvested on either day 5 or 7 post-transfer. Representative histograms (D) and quantification of LC3-II MFI for indicated adoptively transferred cell subsets (E).

(F) LC3-GFP transgenic liver lymphocytes were adoptively transferred into WT (CD45.1 × CD45.2) recipients and quantified for LC3-II staining for each indicated cell subset on day 5 and 7 after transfer from recipient livers.

(G–I) LC3-GFP transgenic splenocytes were adoptively transferred into *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> recipients.

(G) mNK and CD8<sup>+</sup> T cells were sorted at the indicated time points from recipient mice and analyzed for *Map1lc3b* (LC3) mRNA levels by quantitative real-time PCR (n = 3 for each time point).

(H and I) Confocal microscopy (original magnification, 63×) of sort-purified mNK or CD8<sup>+</sup> T cells (H) and quantification of LC3-II<sup>+</sup> puncta on either day 0 or 6 post-transfer (presented as the frequency of cells with more than one LC3-II<sup>+</sup> punctum) (I). The data are representative of two independent experiments, with n = 3–4 per time point. The samples were compared using an unpaired, two-tailed Student's t test and data presented as the mean ± SEM (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, and not significant: ns).

lymphocytes during early homeostatic proliferation. In contrast, we observed that adaptive and innate lymphocytes collectively displayed a reduction in Cyto-ID staining (Figures 2A and 2B) with a concomitant loss of LC3-II fluorescence (Figures 2D and 2E) between day 5 and 7 after transfer into *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup>, but not WT hosts (Figures 2C and 2F), indicating that lymphocytes may induce autophagy following homeostatic proliferation, but not during basal homeostasis. In support of this hypothesis, adoptively transferred LC3-GFP transgenic lymphocytes did not alter the amounts of LC3 transcripts at these time points (Figure 2G), and we observed the presence (Figure 2H) and higher frequency (Figure 2I) of LC3-II puncta by confocal microscopy in lymphocyte populations following homeostatic proliferation compared to resting cells. Together, these results suggest that lymphocytes induce autophagy following homeostatic proliferation.

### Autophagy Is Required for Lymphocyte Survival following Homeostatic Proliferation but Is Dispensable in Lymphoreplete Mice

To study the functional relevance of increased autophagic activity in lymphocytes during lymphopenia without the confounding developmental defects, we utilized mice with either inducible deletion of the essential autophagosome machinery component *Atg5* (*Ubc*<sup>Cre-ERT2</sup> × *Atg5*<sup>fl/fl</sup>) or *Atg3* (*Ubc*<sup>Cre-ERT2</sup> × *Atg3*<sup>fl/fl</sup>) (Jia and He, 2011). WT (CD45.1) and *Ubc*<sup>Cre-ERT2</sup> × *Atg3*<sup>fl/fl</sup> (CD45.2) mBMC mice were generated and treated with tamoxifen for 5 days prior to adoptive transfer of splenocytes into *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> hosts (Figure 3A). Whereas inducible deletion of *Atg3* (*i-Atg3*<sup>-/-</sup>) resulted in similar expansion of adoptively transferred *i-Atg3*<sup>-/-</sup> and WT NK and CD8<sup>+</sup> T cells after transfer, we observed a drastic reduction in the percentage of *i-Atg3*<sup>-/-</sup> NK and CD8<sup>+</sup> T cells during later time points (Figures 3B and S2D) with a significant loss in the absolute numbers of indicated *i-Atg3*<sup>-/-</sup> lymphocytes in peripheral organs compared to WT controls on day 28 after transfer (Figures 3C and S2E). Inducible deletion of either *Atg3* or *Atg5* resulted in significant reductions in percentage (Figure 3D) and absolute number (Figure 3E) of liver lymphocyte lineages on day 28 after adoptive transfer. Furthermore, the persistence of both *i-Atg5*<sup>-/-</sup> ILC3 and ILC2s was greatly diminished compared to WT controls following either interleukin (IL)-7 or IL-33-induced proliferation in WT:*i-Atg5* mBMC mice, respectively, although cytokine treatment resulted in similar expansion of these populations (Figures S3A–S3I).

Innate and adaptive lymphocytes require autophagy only during development, homeostatic, or cytokine-induced proliferation, because we did not observe a loss of *Atg5*-deficient lymphocyte precursors in the bone marrow (Figure S4A) or mature lymphocytes in the periphery (Figures S4B and S4C) in tamoxifen treated WT:*i-Atg5* mBMC mice after 3 weeks of basal homeostasis. Furthermore, these results were not due to decreased proliferation of *Atg5*-deficient lymphocytes, because similar Ki67 staining was observed between adoptively transferred *i-Atg5*<sup>-/-</sup> and WT mNK and CD8<sup>+</sup> T cells during homeostatic proliferation and both ILC2/ILC3 populations during cytokine-induced proliferation (Figures S4D and S4E and data not shown). Instead, the observed survival defect in *Atg5*-deficient lymphocytes was likely due to enhanced apoptosis following

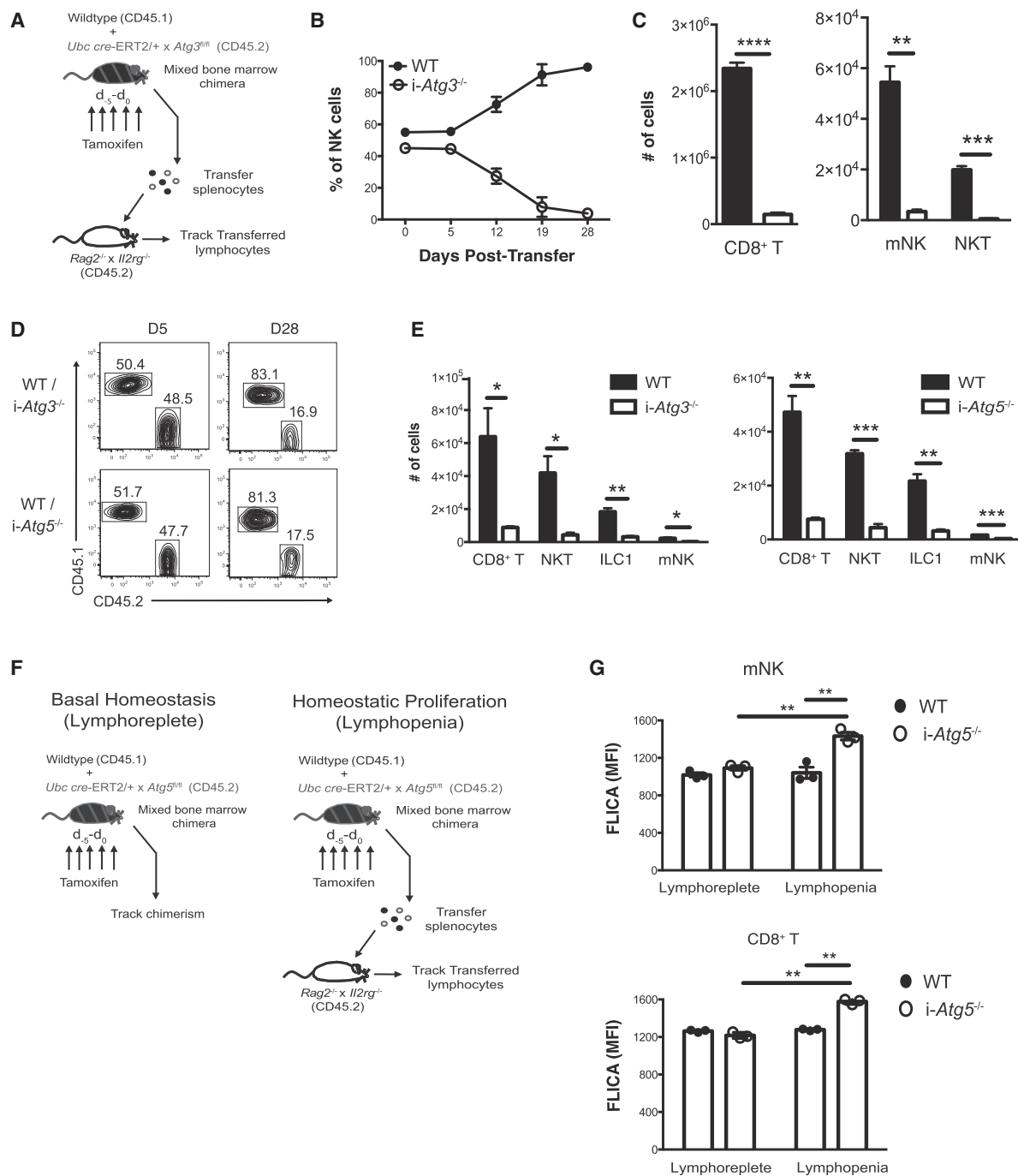
homeostatic expansion, as *i-Atg5*<sup>-/-</sup> mNK and CD8<sup>+</sup> T cells both displayed increased FLICA staining on d9 following homeostatic proliferation compared to co-transferred WT controls that was not observed in lymphoreplete hosts (Figures 3F and 3G). Together, these data indicate that autophagy is essential for the survival of lymphocytes by shielding them against apoptosis following homeostatic proliferation, but not during basal homeostasis in lymphoreplete mice.

### Induction of Autophagy by Metformin Enhances Lymphocyte Survival following Homeostatic Proliferation

In order to address whether augmenting autophagic activity in lymphocytes by pharmacological means could promote their survival during lymphopenia, we adoptively transferred lymphocytes from LC3-GFP mice into *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> hosts and treated recipient mice with metformin, an AMPK activator that can induce autophagy through inhibition of mTOR (Kim et al., 2011). Treatment of recipient mice with metformin dramatically increased the absolute numbers of CD8<sup>+</sup> T cells, NKT cells, mNK cells, and ILC1 on day 28 after transfer (Figure 4A). Metformin administration increased autophagic activity in these transferred lymphocytes following homeostatic proliferation, as we observed a reduction in LC3-II in indicated lymphocytes from metformin treated mice compared to PBS treated controls (Figure 4B). To test whether metformin treatment enhanced survival of lymphocytes in an autophagy-dependent mechanism, lymphocytes from tamoxifen-treated WT:*i-Atg5*<sup>-/-</sup> mBMC were transferred into *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> hosts and metformin was administered daily from day 5 to 28 after transfer (Figure 4C). Indeed, the increase in these lymphocytes from metformin-treated mice was *Atg5*-dependent, as *i-Atg5*<sup>-/-</sup> lymphocytes failed to demonstrate enhanced survival (Figure 4D). These results support the hypothesis that increasing autophagic activity through metformin treatment is sufficient to enhance both adaptive and innate lymphocyte survival following lymphopenia-driven expansion.

In summary, our study demonstrates a critical role for the essential autophagosome machinery component *Atg5* in the development of all ILC subsets. Although lymphocytes may block the degradation of autophagosomes early during homeostatic expansion and then transiently relieve this inhibition later during homeostatic proliferation, our evidence suggests that autophagy is induced during this process, and that autophagy is necessary and sufficient for both adaptive and innate lymphocyte survival following lymphopenia-driven proliferation by limiting cell-intrinsic apoptosis. These results provide a general molecular mechanism for how lymphocytes are able to persist long-term in lymphopenic hosts and support the hypothesis that autophagy is necessary for lymphocyte survival following proliferation during ontogeny. Although autophagy has been implicated in preventing mature T cell apoptosis by promoting organelle turnover in vitro (Jia and He, 2011), our results indicate that autophagy is not induced in mature lymphocytes during basal homeostasis to limit apoptosis, because *Atg5* is dispensable for the survival of mature lymphocytes and bone marrow precursors during basal homeostasis in lymphoreplete mice. These results suggest that autophagy is not used continuously





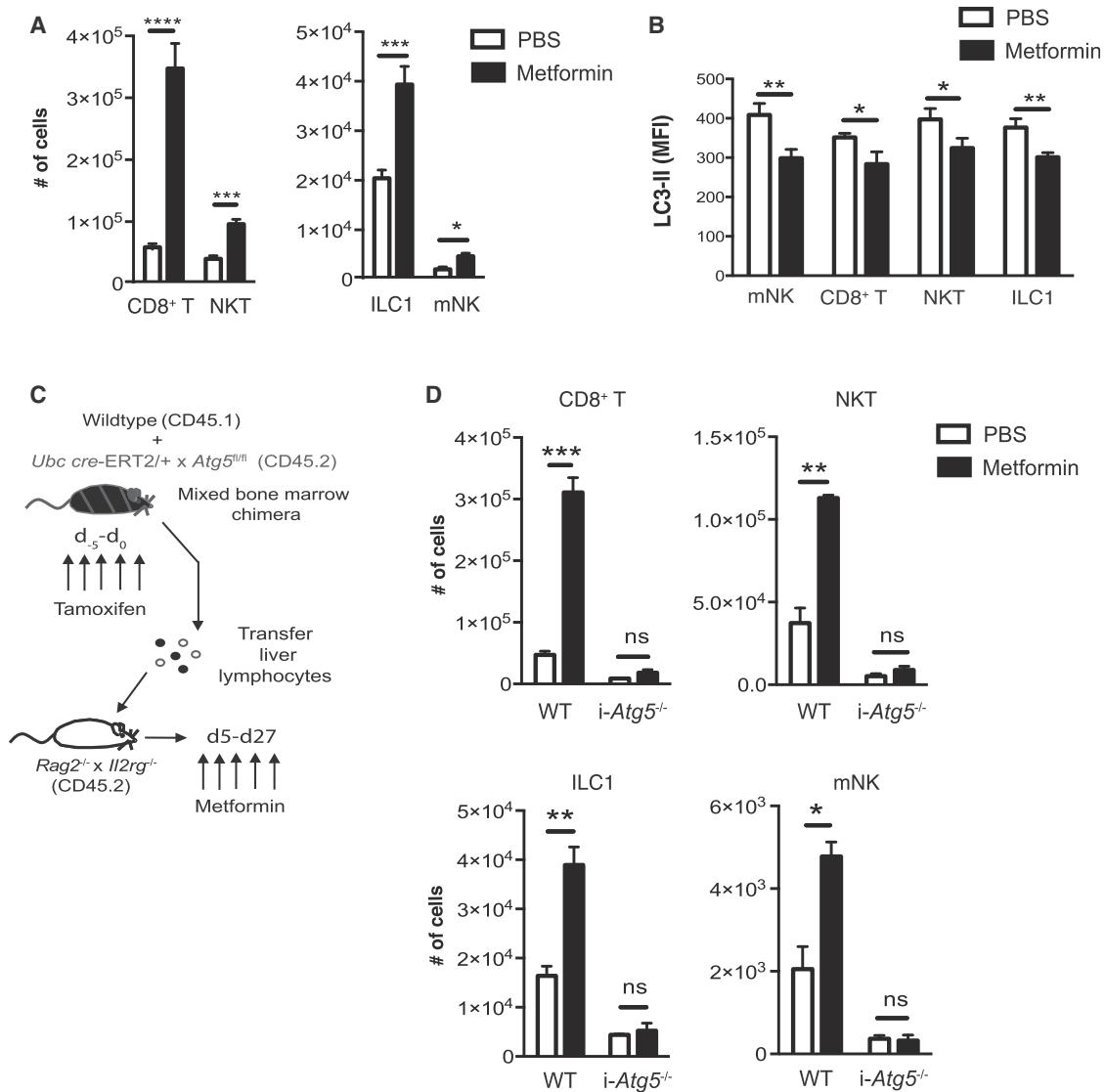
**Figure 3. Autophagy Is Critical for Lymphocyte Survival following Homeostatic Proliferation**

(A–C) WT:*i-Atg3* mixed bone marrow chimeras were injected i.p. with 1 mg of tamoxifen (*i-Atg3<sup>-/-</sup>*) daily for 5 days and splenocytes were adoptively transferred into *Rag2<sup>-/-</sup> x Il2rg<sup>-/-</sup>* hosts. Percentages of adoptively transferred WT or *i-Atg3<sup>-/-</sup>* NK cells in the blood at indicated time points post-transfer (B) and absolute numbers of indicated lymphocytes from the spleen of recipients on day 28 post-transfer (C).

(D and E) WT:*i-Atg5* or WT:*i-Atg3* mixed bone marrow chimeras were injected i.p. with 1 mg of tamoxifen (*i-Atg3<sup>-/-</sup>* or *i-Atg5<sup>-/-</sup>*) daily for 5 days and liver lymphocytes were adoptively transferred into *Rag2<sup>-/-</sup> x Il2rg<sup>-/-</sup>* hosts. Representative plots show adoptively transferred ILC1 populations on day 5 and day 28 post-transfer from the liver (D) and absolute numbers of indicated lymphocyte populations harvested from the liver on day 28 post-transfer (E).

(F) Schematic of experiment. Briefly, WT:*i-Atg5* mixed bone marrow chimeras were injected i.p. with 1 mg of tamoxifen (*i-Atg5<sup>-/-</sup>*) daily for 5 days and lymphocyte chimerism was subsequently measured in the blood (basal homeostasis) or splenocytes were adoptively transferred into *Rag2<sup>-/-</sup> x Il2rg<sup>-/-</sup>* hosts (homeostatic proliferation).

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**Figure 4. Induction of Autophagy through Metformin Treatment Enhances Lymphocyte Survival following Homeostatic Proliferation in an *Atg5*-Dependent Manner**

(A and B) LC3-GFP transgenic liver lymphocytes were adoptively transferred into *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> (CD45.2) recipients and either treated with 200 μg of metformin or control PBS i.p. daily from day 5 to day 27 post-transfer. Absolute numbers on day 28 post-transfer (A) and LC3-II MFI of indicated lymphocytes on day 9 post-transfer from harvested recipient livers of each indicated cohort (B).

(C and D) WT:*i-Atg5* mixed bone marrow chimera were injected i.p. with 1 mg of tamoxifen (*i-Atg5*<sup>-/-</sup>) daily for 5 days and liver lymphocytes were adoptively transferred into *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> hosts that either received 200 μg of metformin or control PBS daily from day 5 to day 27 post-transfer. (D) Absolute numbers of indicated lymphocytes on day 28 post-transfer from livers of each indicated cohort. The data are representative of two independent experiments, with n = 3–4 per cohort. The samples were compared using an unpaired, two-tailed Student's t test and the data presented as the mean ± SEM (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, and \*\*\*\*p < 0.0001).

as a survival mechanism in mature lymphocytes and is only utilized to inhibit apoptosis following periods of cellular differentiation or stress, such as clonal proliferation in response to viral

infection (Chen et al., 2014; O'Sullivan et al., 2015; Puleston et al., 2014; Xu et al., 2014), cytokine deprivation (Warr et al., 2013), or lymphopenia and cytokine-driven expansion.

(G) Quantification of FLICA MFI of indicated lymphocytes in the blood on day 9 for each cohort. The data are representative of two independent experiments, with n = 3–4 per cohort. The samples were compared using an unpaired, two-tailed Student's t test and data presented as the mean ± SEM (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, and \*\*\*\*p < 0.0001).

See also Figures S2–S4.

How autophagy enables lymphocytes to evade apoptosis during development and following homeostatic proliferation remains unknown. *Atg3*-deficient T cells, *Atg7* and *Atg5*-deficient iNKT cells, and *Atg7*-deficient HSCs all displayed higher mitochondrial mass than littermate controls (Jia and He, 2011; Mortensen et al., 2011; Pei et al., 2015; Salio et al., 2014), suggesting that removal of damaged mitochondria through mitophagy may promote the survival of lymphocytes during development. Although we have previously demonstrated that antigen-specific NK cells rapidly remove dysfunctional mitochondria through BNIP3 and BNIP3L-mediated mitophagy following MCMV infection (O'Sullivan et al., 2015), *Bnip3* is not required for T, NKT, or mNK survival during development or following homeostatic expansion (T.E.O. and J.C.S., unpublished data). Furthermore, lymphocytes undergoing homeostatic expansion do not reduce their mitochondrial content 28 days after transfer into lymphopenic hosts (T.E.O. and J.C.S., unpublished data). Together, these observations suggest that mitophagy may be dispensable for lymphocyte development and survival during homeostasis. Future work will be necessary to elucidate the molecular mechanisms by which autophagy regulates lymphocyte survival during lymphopenia and whether these findings will have clinical significance during immune reconstitution following bone marrow transplantation.

## EXPERIMENTAL PROCEDURES

### Mice

Mice were bred at Memorial Sloan Kettering Cancer Center in accordance with the guidelines of the Institutional Animal Care and Use Committee. The following strains were used in this study: C57BL/6 (CD45.2), B6.SJL (CD45.1), *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup>, *Nkp46*<sup>Cre</sup> (Narni-Mancinelli et al., 2011), *Ub*<sup>ERT2-Cre</sup> × *Atg3*<sup>fl/fl</sup> (Jia and He, 2011), LC3-GFP transgenic (Mizushima et al., 2004), and *Atg5*<sup>fl/fl</sup>, *Ub*<sup>ERT2-Cre</sup>. mBMC mice were generated as previously described (Sun et al., 2009). Experiments were conducted using age- and gender-matched mice in accordance with approved institutional protocols.

### Virus Infection

MCMV (Smith strain) was passaged serially through BALB/c hosts two times, and viral stocks were prepared by using a dounce homogenizer to dissociate the salivary glands of infected mice 3 weeks after infection. Experimental mice were infected by intraperitoneal (i.p.) injection with  $2.5 \times 10^4$  PFU.

### Adoptive Transfer of Mature and Progenitor Innate Lymphocytes

In adoptive transfer experiments, splenocytes or liver lymphocytes from indicated mBMC mice or liver lymphocytes from LC3-GFP transgenic mice were injected intravenously (i.v.) into *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> mice. In certain experiments, lymphocyte populations were pre-incubated with CellTrace Violet (Life Technologies) according to the manufacturers instructions and analyzed on day 5 after transfer. In ILC progenitor adoptive transfer experiments, bone marrow from indicated mBMC mice was stained with surface antibodies and ~1,000 CHILP or ~3,000 ILC2P populations were sorted to >95% purity and adoptively transferred into individual irradiated *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> recipients. Mature ILC populations were harvested from these mice on day 28 after transfer. In some experiments, *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> recipient mice or mBMC mice were injected i.p. with 1 mg/day of hydroxytamoxifen (4-OHT) dissolved in corn oil (Sigma), or corn oil alone as a control for 5 consecutive days. In certain adoptive transfer experiments, recipient mice were injected i.p. with PBS or 200 μg/day metformin (Sigma) from day 5 to 9 or 5 to 27 after transfer and tamoxifen treatment. In certain experiments, mBMC mice were injected i.p. with 1 mg/day of tamoxifen or control corn oil for 5 consecutive days and then received three i.p. injections of either 15 μg anti-IL-7 complex (clone M25, Bio X Cell)/2.5 μg rhIL-7 (eBioscience) every other day to expand ILC3s

(Abt et al., 2015) or 500 ng rmlL-33 (BioLegend) every other day to expand ILC2s (Molofsky et al., 2015).

### Flow Cytometry and Cell Sorting

Cell surface staining was performed using the following fluorophore-conjugated antibodies (BD Biosciences, eBioscience, BioLegend, Tonbo, and R&D Systems): NK1.1 (PK136), CD11b (M1/70), CD19 (ID3), CD27 (LG.3A10), CD49b/DX5 (DX5), KLRG1 (2F1), Nkp46 (29A1.4), CD45.1 (A20), CD45.2 (104), CD8α (53-6.7), TCRβ (H57-597), Eomes (Dan11mag), FLT3 (A2F10), a4B7 (DATK32), CD127 (A7R34), CD3e (17A2), TCRγδ (GL3), Ly6G (1A8), CD25 (PC61), GATA3 (TWAJ), Rorγt (B2D), CD122 (TM-B1), CD90.2 (30-H12), and TER119 (TER-119). Intracellular staining was performed with the Foxp3 Transcription Factor Staining Buffer Set (eBioscience). Pan-caspase staining was carried out using the carboxyfluorescein FLICA in vitro Poly Caspase Kit (ImmunoChemistry Technologies). Liver lymphocytes were harvested and stained with cell surface antibodies and then incubated with 1:400 Cyto-ID Autophagy Detection Reagent (Enzo Life Sciences) for 30 min at 37°C to measure autophagosomes. LC3-GFP transgenic liver lymphocytes or splenocytes were harvested and stained with cell surface antibodies and then permeabilized using a selective permeabilization reagent (FlowCelect Autophagy LC3 Kit, Millipore) to remove cytosolic LC3-I. Autophagosome-associated LC3-II was then stained using an anti-GFP Alexa Fluor 488 Antibody (Invitrogen). Flow cytometry and cell sorting were performed on the LSR II and Aria II cytometers (BD Biosciences), respectively. Data were analyzed with FlowJo software (Tree Star).

### Cellular Imaging

LC3-GFP transgenic mNK or CD8<sup>+</sup> T cells were sort purified and then permeabilized using a selective permeabilization reagent (FlowCelect Autophagy LC3 Kit, Millipore) to remove cytosolic LC3-I. Autophagosome-associated LC3-II was then stained using an anti-GFP Alexa Fluor 488 antibody (Invitrogen). Cells were then analyzed on a Leica SP8 confocal microscope at the MSKCC Molecular Cytology Core. Images were acquired at a magnification of 63×. Spot-counting analysis was used for evaluation of LC3-II puncta<sup>+</sup> cells in each cohort.

### Quantitative Real-Time PCR

Sorted NK or CD8<sup>+</sup> T cells were lysed in Tri-Reagent (Ambion). RNA purification and cDNA synthesis were carried out with the QIAGEN RNeasy Kit (with on-column DNase I treatment) and MuLV reverse transcriptase and oligo(dT) 16 primers (Applied Biosystems). iQ Sybr Green SuperMix (BioRad) was used for quantitative real-time PCR. Data were normalized to that for *Actb* and expressed as relative target abundance via the  $\Delta\Delta C_t$  method, where  $C_t$  (threshold cycle) is the cycle number at which the amplification curve intersects the threshold value. *Map1c3b* forward primer: TTATAGAGCGATAC AAGGGGGAG and *Map1c3b* reverse primer: CGCCGTCTGATTATCTTGA TGAG. *Actb* forward primer: TGCGTGACATCAAAGAGAAG and *Actb* reverse primer: CGGATGTCAACGTCAACTT.

### Statistical Analyses

For graphs, data are shown as mean ± SEM and, unless otherwise indicated, statistical differences were evaluated using a two-tailed unpaired Student's t test, assuming equal sample variance.  $p < 0.05$  was considered significant. Graphs were produced and statistical analyses were performed using GraphPad Prism.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.04.082>.

### AUTHOR CONTRIBUTIONS

T.E.O. and J.C.S. designed the study. M.O. provided critical discussion and interpretation of results. G.W.D. provided mice. T.E.O. and C.D.G. performed cell sorting. M.R., O.-E.W., and T.L.G. aided with tissue processing. T.E.O.

performed all additional experiments. T.E.O. and J.C.S. analyzed the data and wrote the manuscript.

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