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Temporal Regulation of Intracellular Organelle Homeostasis in T Lymphocytes by Autophagy

Wei Jia and You-Wen He

The highly conserved self-degradation pathway known as autophagy plays important roles in regulating T lymphocyte homeostasis. Recently, we found that T lymphocytes lacking the autophagy-related gene Atg5 or Atg7 have defective survival and contain expanded mitochondria and endoplasmic reticulum (ER); however, whether these defects are caused by impaired autophagy or by defects in their autophagy-independent signaling capacity of Atg5 or Atg7 in T lymphocytes remains unknown. Furthermore, the function of the microtubule-associated protein L chain 3 (LC3) conjugation system in T lymphocytes remains unclear. To address these questions, we generated conditional knockout mice with specific deletion of Atg3, a ubiquitin enzyme E2-like molecule involved in the LC3 conjugation system, in T lymphocytes. Atg3-deficient T lymphocytes displayed a phenotype similar to those of Atg7- and Atg5-deficient T cells. The survival of Atg3-deficient naive CD4+ and CD8+ T cells was defective. Furthermore, the mitochondria and ER were expanded in Atg3-deficient T cells. Interestingly, mitochondrial and ER content did not change instantly upon inducible deletion of Atg3 in mature T lymphocytes in vitro. Instead, it began to expand 10 d after inducible deletion of Atg3 in mature T lymphocytes, and mitochondrial content continued to increase on day 18. Cell death began to increase 24 d after inducible deletion of Atg3. These data show that the LC3 conjugation system is essential for autophagy in T lymphocytes. Our data suggest that autophagy promotes T lymphocyte survival by regulating organelle homeostasis and that the decreased survival of autophagy-deficient T cells is due to the temporal accumulation of these autophagy-related defects. The Journal of Immunology, 2011, 186: 5313–5322.

The intracellular self-degradation homeostasis pathway macroautophagy (hereafter referred to as autophagy) is evolutionarily conserved from yeast to mammals (1–5). Generally, amino acid starvation, growth factor withdrawal, and certain stress conditions activate the autophagy pathway. Autophagy is initiated by a membrane structure termed an isolation membrane or autophore. Through an elongation step, the autophore forms a characteristic double-membrane structure called the autophagosome to surround long-lived cytosolic proteins, damaged or excess organelles, or invading intracellular pathogens. Finally, the autophagosome matures by fusing with the lysosome to form the autolysosome, and the materials inside the autolysosome are degraded by lysosomal proteases. The elongation step is generally directed by autophagy-related (Atg) molecules. To date, 33 different autophagy-related genes have been identified in yeast (6), and most of the corresponding homologous molecules have been confirmed in mammalian cells (7). Two ubiquitin-like conjugation systems, the Atg12 conjugation system and the microtubule-associated protein L chain 3 (LC3; Atg8 in yeast) conjugation system, control the elongation of the autophore to form the autophagosome. The molecular mechanisms of the autophagy-processing pathway have been reviewed in detail (8). In both conjugation systems, Atg7 serves as a ubiquitin enzyme E1-like molecule. Atg10 serves as an E2-like molecule in the ATG12 conjugation system. Atg7 and Atg10 catalyze Atg12 and Atg5 to form an Atg12–Atg5–Atg16L complex. LC3 is cleaved by the protease Atg4 to generate the cytosolic LC3-I. Atg7 and the other E2-like molecule, Atg3, then catalyze the conjugation of LC3-I to generate the membrane-bound lipid form, LC3-phosphatidylethanolamine, which is also called LC3-II (9). The Atg12 and LC3 conjugation systems interact and regulate each other. The Atg5–Atg12–Atg16L complex functions as an E3-like molecule for LC3 localization and lipidation (10, 11). Overexpression of both Atg5 and Atg12 inhibits the lipidation of LC3 (12).

Atg3 interacts with Atg12 (10), and in Atg3-deficient mice, the formation of Atg12–Atg5 conjugates is dramatically decreased. Furthermore, the elongation and closure of autophagosomes are defective in the absence of Atg3 (13). Therefore, both conjugation systems are essential for autophagy in mammalian cells. Interestingly, an Atg7/Atg5-independent alternative autophagy pathway has been identified in mouse cells (14). In Atg7- or Atg5-deficient cells, autophagosomes and autolysosomes formed and mediated protein degradation (14).

Autophagy has critical physiological functions in the immune system. Autophagy is the mechanism of clearance of intracellular pathogen infections (15), presentation of exogenous Ags (16), cross-presentation of endogenous Ags by MHC class II molecules (17, 18), and recognition of ssRNA viruses and production of IFN-α in plasmacytoid dendritic cells (19). Our group and others have shown that autophagy is essential for organelle homeostasis in lymphocytes (20, 21) and erythroid cells (22–24). Autophagy developmentally regulates mitochondria (20) and endoplasmic reticulum (ER) (21). In an Atg5 knockout fetal liver transfer system and an Atg7<sup>fl/fl</sup>Lck-Cre T cell-specific deletion system, we have demonstrated that autophagy is essential for T lymphocyte survival (25). To determine whether the phenotypes observed in

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; Atg, autophagy-related; DHE, dihydroethidium; DN, double-negative; EM, electron microscopy; ER, endoplasmic reticulum; ES, embryonic stem; Etox, exon fragment; FLICA, fluorochrome inhibitor of caspases; LA, long arm; LC3, microtubule-associated protein L chain 3; LN, lymph node; MFI, mean fluorescence intensity; 4-OHT, 4-hydroxytamoxifen; ROS, reactive oxygen species; SA, short arm.

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Atg5- and Atg7-deficient T cells are due to impairments in autophagy machinery or in the signaling capacity of the Atg5/Atg7 molecules and to further investigate the functions of the LC3 conjugation system in T lymphocytes, we generated Atg3 conditional knockout mice.

In this study, we show that the autophagy pathway is impaired in Atg3-deficient T lymphocytes. Survival is defective and organellar homeostasis is abnormal in Atg3-deficient T cells. Furthermore, we use an inducible deletion system to demonstrate a temporal accumulation of mitochondria and ER beginning 10 d after the deletion of Atg3. Accordingly, we show that cell death does not begin to increase until >3 wk after deletion of Atg3. Our results demonstrate that the LC3 conjugation system is dispensable for autophagy in T lymphocytes. We also show that autophagy regulates organellar homeostasis and is essential for T cell survival. Importantly, our findings provide clear evidence that the phenotype of autophagy-deficient T cells is due to the temporal accumulation of autophagy-related defects.

Materials and Methods

Generation of Atg3 conditional knockout mice

The bacterial artificial chromosome clone RP23-444N1, which contains the Atg3 genomic sequence, was obtained from BACPAC Resources Center at Children’s Hospital Oakland Research Institute (Oakland, CA) and was used as a template to clone the genomic fragments long arm (LA), short arm (SA), and exon fragment (Exon) by PCR. The exon fragment is flanked by two loxP sites, allowing for deletion in Cre-expressing cells at the genomic level. The primer sequences are as follows: LA forward primer with SacI restriction site, 5′-GCCCGGCGCTTGTAACTCCA-TACTGTTGACACACACCAAGGT-3′; LA reverse primer with NotI restriction site, 5′-GCCCGGCGCCGACCCCTTGTCCATCCATAGAACCT-CTACACCCCCCGG-3′; Exon forward primer with Smal restriction site, 5′-GCCCGGGTTTATTTATCCAGAGAAGAAAAGTACGCTACTTG-3′; Exon reverse primer with Smal restriction site, 5′-GCCCGGGAATTCATCAGATCCAGGCAAACAGACAGACA-3′; SA forward primer with SalI restriction site, 5′-CGTGCAAGCTTAAAGAATTTTACTTATAAGAAAGAAGAACATG-3′; SA reverse primer with SalI restriction site, 5′-CGTGCAACGGGTGCTATTTCAGTTCTTATCA-ACAATCTCTTAC-3′. PCR products were performed using BIO-X-ACT Long DNA Polymerase (Bioline, Taunton, MA). All cloned fragments were inserted into the pGKnneoF2L2DTA targeting vector (26). The resulting pGKnneoF2L2DTA-Atg3 construct was sequenced. Three mutations were found in the LA fragment in the intron region between exon 7 and exon 8. No mutations were found in the exon or SA fragments. EF1 embryonic stem (ES) cells were transfected with linearized pGK-Neo-FRT-Atg3. The targeted ES cell clones were screened for homologous recombination at the Atg3 genomic locus by PCR. The primers used for screening are as follows: forward ES cell screening primer Neo F2 (corresponding to the Neo region in pGKnneoF2L2DTA), 5′-CTCTATG6CTTCTGAAGGCGGAAGGAAG-3′; reverse ES cell screening primer R2 (corresponding to the sequence downstream of the SA fragment in the Atg3 genomic locus), 5′-TCTGCTGAAAA TAGAGTGAATGCTAAGGAGG-3′. The positive ES clones were further confirmed by PCR amplification of the region containing the other LoxP site between the LA fragment and the Exon fragment. The primers used for screening are as follows: forward Atg3 screening primer F450, 5′-AACCATTTGGGTCGTCTGTA-3′; and reverse Atg3 screening primer R450, 5′-CGATGGCATTCTTGCTGACAGAATG-3′. These primers were used to generate the Atg3f/+ or Atg3f/f mice. The targeted ES clone 3D11 was injected into B6 black mice blastocysts, and the injected blastocysts were transferred into the uterus of pseudopregnant female recipient mice. The germline transmitted transgenic mice were screened and mated. The germline transmitted type Atg3 is 748 bp. The PCR product of floxed Atg3 is 866 bp, and the PCR product of wild-type Atg3 is 748 bp. C. Deletion efficiency in Atg3f/Lck-Cre mice. CD4+ and CD8+ cells were sorted by flow cytometry to >98% purity, and genomic DNA was extracted. The fragment from Atg3 exon 8 and exon 9 was amplified by PCR using primers F6 and R6. Atg7 exon 14 PCR was used as a control. D. The expression of Atg3 in T cells was analyzed by Western blot. Samples of thymocytes or enriched T cells from Atg3f/Lck-Cre and Atg3f/ER-Cre control mice are shown in the left panel. The right panel shows the PCR screening results using genomic DNA from mouse tails as templates (left panel). The right panel shows the PCR screening results using genomic DNA from mouse tails as templates. The PCR product of floxed Atg3 is 866 bp, and the PCR product of wild-type Atg3 is 748 bp. C. Deletion efficiency in Atg3f/Lck-Cre mice. CD4+ and CD8+ cells were sorted by flow cytometry to >98% purity, and genomic DNA was extracted. The fragment from Atg3 exon 8 and exon 9 was amplified by PCR using primers F6 and R6. Atg7 exon 14 PCR was used as a control. D. The expression of Atg3 in T cells was analyzed by Western blot. Samples of thymocytes or enriched T cells from Atg3f/Lck-Cre and Atg3f/ER-Cre control mice are shown in the left panel. The right panel shows the PCR screening results using genomic DNA from mouse tails as templates. The PCR product of floxed Atg3 is 866 bp, and the PCR product of wild-type Atg3 is 748 bp. C. Deletion efficiency in Atg3f/Lck-Cre mice. CD4+ and CD8+ cells were sorted by flow cytometry to >98% purity, and genomic DNA was extracted. 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cell suspensions were prepared. RBCs were lysed with ACK buffer. Thymocytes were suspended in anti-Fc receptor Ab supernatant (2.4G2) and stained with anti-CD4–PE, anti-CD8–FITC, and 7-aminomethylcoumarin D (7-AAD) (BD Pharmingen, San Jose, CA). For analysis of double-negative (DN) thymocytes, thymocytes were stained with anti-CD4–allophycocyanin, anti-CD8–allophycocyanin, anti-CD3–allophycocyanin, anti-CD44–FITC, anti-CD25–PE, and 7-AAD. Splenocytes and LN cells were suspended in 2×2 supernatant and stained with anti-CD4–PE, anti-CD8–FITC, and 7-AAD. Alternatively, splenocytes or LN cells were stained with anti-CD4–allophycocyanin (or anti-CD8–allophycocyanin), anti-CD62L–PE, anti-CD44–FITC, and 7-AAD on ice for 15 min. Cells were then washed, and the samples were analyzed by flow cytometry using FACScan, FACStar Plus, or FACSCanto flow cytometers (BD Biosciences). All Abs were from Biolegend (San Diego, CA) or eBioscience (San Diego, CA).

**Autophagy analysis**

Splenocytes from Atg3<sup>fl/fl</sup>Lck-Cre and Atg3<sup>flu</sup> mice were stimulated with anti-CD3 mAb (2C11; 5 μg/ml) or cultured in RPMI 1640 medium overnight. The CD3 activated and unstimulated control T cells were stained with anti-CD4–FITC or anti-CD8–FITC. After washing, the cells were treated with 0.1% saponin (Sigma-Aldrich, St. Louis, MO) with 5% FBS containing 0.5% BSA for 10 min on ice and then intracellularly stained with rabbit anti-LC3 Ab (MBL International, Woburn, MA) for 30 min on ice. After washing, anti-rabbit–Cy3 Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) was added and incubated on ice for another 30 min. Z-stack images were acquired at 1 μm apart using 100× oil objective by a Zeiss Axios Observer D1-based imaging station (Zeiss) equipped with a CoolSNAP HQ CCD camera (Roper Scientific, Tucson, AZ) and recorded with MetaMorph 7.6 software (Universal Imaging, West Chester, PA). Three-dimensional deconvolution of the images was performed using AutoQuant X software (Media Cybernetics Bethesda, MD). LC3 puncta of three-dimensional deconvolution images was quantified by MetaMorph 7.6 software (Universal Imaging). A 1.5× background fluorescence intensity was used to set up the threshold for the images of each cells. Only the pixel of the LC3, which is >4, was considered as an LC3 punctum. The numbers of LC3 puncta per cell were counted, and a total of 30 cells from each group were analyzed. Starvation was induced by culturing the splenocytes in HBSS (Invitrogen, Carlsbad, CA) overnight. Then, T lymphocytes were purified using a mouse T cell negative enrichment kit (StemCell Technologies, Vancouver, BC, Canada), and cell lysates were prepared for LC3 Western blot analysis.

**Electron microscopy analysis**

FACS-sorted CD4<sup>+</sup> and CD8<sup>+</sup> T cells from spleen and LN of Atg3<sup>fl/fl</sup>Lck-Cre and Atg3<sup>flu</sup> mice were fixed in 4% glutaraldehyde/buffer overnight, and electron microscopy (EM) samples were prepared as described previously (20). Micrographs were taken with a Philips LS 410 electron microscope. The cytosol membrane structures were quantified and presented as the membrane score. The EM picture of each cell was divided into 12 equal sections according to a clock face. The membrane score was counted as each section containing membrane structure(s) for each cell.

**Apoptosis analysis**

The freshly isolated splenocytes were stained for surface markers using anti-CD4–allophycocyanin, anti-CD8–PE/Cy7, and anti-CD44–allophycocyanin Abs in 2.4G2 supernatant on ice for 15 min. Cells were then washed, resuspended in binding buffer, and stained with Annexin V-PE or Annexin V-Pacific Blue (Biologend) and 7-AAD (BD Biosciences). The samples were analyzed using an FACSCanto flow cytometer (BD Biosciences). Caspase-9 activity was detected by fluorochrome inhibitor of caspases

**FIGURE 2.** Impaired autophagy in Atg3-deficient T cells. A and B, LC3 puncta formation in Atg3-deficient T cells. Splenocytes from Atg3<sup>fl/flLck-Cre</sup> mice and Atg3<sup>flu</sup> control mice were stimulated with anti-CD3 Ab or cultured in complete RPMI 1640 medium overnight. LC3 puncta were intracellularly stained with rabbit anti-LC3 Ab and Cy3-labeled donkey anti-rabbit Ab. Z-stack images were captured and spaced at 1 μm apart using 100× oil objective in a Zeiss Axios Observer D1-based imaging station (Zeiss). A total of 30 cells in each group were analyzed. Representative images were shown in A, and the numbers of LC3 puncta were quantified (cut off at pixel >4). C, The level of LC3 II in Atg3-deficient T cells under starvation condition. Splenocytes from Atg3<sup>fl/flLck-Cre</sup> and Atg3<sup>flu</sup> mice were cultured in HBSS in starvation (Starv) condition or complete medium overnight. T lymphocytes were purified after culture, and cell lysates were prepared. The LC3-II was detected with anti-LC3 Ab by Western blot. D, The expression of p62 in Atg3-deficient T cells. The column figures are mean ± SD and from 50 cells of each group. *p = 1.46 × 10<sup>-21</sup>, **p = 1.23 × 10<sup>-22</sup>.

**FIGURE 3.** Thymocyte development in Atg3-deficient mice. A, Total of five Atg3<sup>fl/flLck-Cre</sup>, three Atg3<sup>fluLck-Cre</sup>, and four Atg3<sup>flu</sup> control mice. B, Thymocyte subpopulations in Atg3<sup>fl/flLck-Cre</sup> mice. Thymocytes from Atg3<sup>fl/flLck-Cre</sup> and Atg3<sup>fluLck-Cre</sup> mice were stained with anti-CD4–FITC, anti-CD8–PE, and 7-AAD. The cells were gated on the 7-AAD<sup>+</sup> population. Numbers represent the frequency of each gated subpopulation. C, DN subpopulations in Atg3-deficient mice. Thymocytes from Atg3<sup>fl/flLck-Cre</sup>, Atg3<sup>fluLck-Cre</sup>, and Atg3<sup>flu</sup> mice were stained with anti-CD4–allophycocyanin, anti-CD8–allophycocyanin, anti-CD3–allophycocyanin, anti-CD44–FITC, anti-CD25–PE, and 7-AAD. The samples were gated on CD4<sup>+</sup>, CD8<sup>+</sup>, CD<sup>-</sup>, and 7-AAD<sup>-</sup>negative cells. Numbers represent the frequency of each gated subpopulation. Both B and C flow cytometry analyses were repeated three times. *p = 0.018, **p = 0.031.
(FLICA) apoptosis detection kit (ImmunoChemistry Technologies, Bloomington, MN) according to the manufacturer’s instructions. Briefly, the splenocytes were incubated with caspase-9 FLICA (carboxyfluorescein [FAM]-LEHD-fluoromethyl ketone) at 37˚C for 30 min. After washing, the cells were further stained with surface markers as described above. The samples were analyzed by flow cytometry after another washing.

**In vitro inducible Atg3 deletion**

The splenocytes or LN cells from Atg3**f/f**ER-Cre or Atg3**f/f** control mice were cultured with 200 nM 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich) for 3 d with IL-7 (1 ng/ml; PeproTech, Rocky Hill, NJ). For longer cultures, 1 ng/ml IL-7 was added every other day, and the medium was changed when necessary.

**Mitochondria and ER staining**

Splenocytes from Atg3**f/f**Lck-Cre or 4-OHT–treated Atg3**f/f**ER-Cre spleenocytes and Atg3**f/f** control splenocytes were incubated with 100 nM MitoTracker Green (Invitrogen, Molecular Probes) or 1 μM ER-Tracker Blue-White DPX (Invitrogen, Molecular Probes) in RPMI 1640 medium at 37˚C for 30 min. Cells were washed with RPMI 1640 medium and then stained with anti-CD4–allophycocyanin, anti-CD8–PE/Cy7, anti-CD44–allophycocyanin/Cy7, and 7-AAD. The geometric mean intensity of fluorescence was used to represent the volume of mitochondria or ER. The mitochondria and ER of Atg3**f/f** ER-Cre splenocytes and Atg3**f/f** control splenocytes were analyzed on different days after 4-OHT treatment. The samples were analyzed using an FACSCanto flow cytometer (BD Biosciences).

**Reactive oxygen species production analysis**

Splenocytes from Atg3**f/f** Lck-Cre mice or Atg3**f/f** control mice were incubated with 2.5 μM dihydroethidium (DHE; Sigma-Aldrich) at 37˚C for 1 h. Cells were washed and stained with anti-CD4–allophycocyanin and anti-CD4–FITC or anti-CD8–FITC. The mean fluorescence intensity of the PE channel represented the DHE staining activity. The samples were analyzed using an FACStar flow cytometer (BD Biosciences).

**Western blot**

The T lymphocytes from Atg3**f/f** or Atg3**f/f**Lck-Cre mice were enriched using a mouse T cell enrichment kit (StemCell Technologies). Cell purity was determined by flow cytometry to be >95%. Total cell lysates were prepared in sample buffer (30 mM Tris-Cl [pH 6.8], 0.5% 2-ME, 2% SDS, 0.2% bromophenol blue, and 10% glycerol). The samples were separated by SDS-PAGE and then transferred to Immobilon-FL polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat milk and then incubated with primary Ab in PBS contain-

![FIGURE 4. Decreased mature T cells in Atg3**f/f**Lck-Cre mice. A, Total splenocytes in Atg3**f/f**Lck-Cre mice. Total cell numbers from four Atg3**f/f**Lck-Cre mice, three Atg3**f/f**Lck-Cre, and five Atg3**f/f** control mice (mean ± SD). B, Frequency of CD4* and CD8* subpopulations in Atg3**f/f**Lck-Cre mice. C, Numbers of CD4* and CD8* T cells in Atg3**f/f**Lck-Cre mice. Data are presented as mean ± SD from three mice of each group. D, Memory-like cell populations in Atg3**f/f**Lck-Cre mice. Splenocytes or LN cells were stained with anti-CD4–allophycocyanin (or anti-CD8–allophycocyanin), anti-CD44–FITC, anti-CD62L–PE, and 7-AAD. Samples were gated on 7-AAD– cells. Numbers represent the frequency of each gated subpopulation. E, Numbers of CD4**high** CD62L**low** population in Atg3-deficient mice. A total of four Atg3**f/f**, four Atg3**f/f**Lck-Cre, and five Atg3**f/+**Lck-Cre mice were analyzed. *p = 0.013, **p = 0.0067, ***p = 0.33 for CD4* cells and p = 0.15 for CD8* T cells.](http://www.jimmunol.org/)
ing 3% BSA (Sigma-Aldrich) and 0.5% Tween 20 at 4˚C overnight. The next day, the membranes were washed and incubated with Alexa Fluor 680-labeled anti-rabbit Ab (Invitrogen) or IRDye 800-labeled anti-goat Ab (Rockland Immunochemicals, Gilbertsville, PA) at room temperature for 1 h in PBS containing 3% BSA and 0.5% Tween 20. The blots were visualized using the Odyssey Infrared Imaging System and analyzed using Odyssey software (LI-COR Bioscience, Lincoln, NE). Atg3 rabbit polyclonal Ab was from Sigma-Aldrich. Anti-actin Ab was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-LC3 Ab (anti-LC3A/B, strong reactivity with LC3A/B-II) was from Cell Signaling Technology (Danvers, MA).

Proliferation assay

The splenocytes from Atg3f/f or Atg3f/fLck-Cre mice were incubated with 5 μM CFSE (Invitrogen) in 5% FCS-PBS for 5 min at room temperature. Cells were washed with 5% FCS-PBS and 3 times and stimulated with soluble anti-CD3 (20 μg/ml) or anti-CD28 (1 μg/ml). The cells were then stained with anti-CD4–allophycocyanin, anti-CD8–FITC, anti-CD44–allophycocyanin/Cy7, and 7-AAD in 2.4G2 supernatant on ice for 15 min. After washing, the samples were analyzed by flow cytometry. The 7-AAD-negative cells were gated and analyzed. Unstimulated cells were used to gate the CFSE-diluted cells.

Statistical analysis

Two-tailed Student t tests were used to compare the means of different samples.

Results

Generation of Atg3 conditional knockout mice

Our previous data from Atg5 knockout fetal liver transfer experiments and Atg7f/fLck-Cre mice suggested that autophagy is essential for T lymphocyte survival (25) and that autophagy mediates developmental regulation of organelle homeostasis (20, 21). Two ubiquitin-like pathways direct the process of autophagy, and the results from studying Atg5- or Atg7-deficient T lymphocytes may only reflect the requirement of one of these pathways in T lymphocyte survival. To investigate the function of the LC3 pathway in T lymphocytes, an Atg3 conditional knockout mouse line was generated. The gene encoding Atg3, which is located on chromosome 16, is 30,895 bp long, and the mRNA transcript is 2014 bp. Atg3 consists of 12 exons encoding 315 aas. Because another gene, named Slc35a5, oriented in the opposite direction is located 200 bp upstream of Atg3 on chromosome 16, the deletion of exon 1 of Atg3 may affect the function of this upstream gene. Atg3 is an E2-like molecule, and its E2-like conjugating enzyme active site cysteine is encoded by exon 10 (30). We thus chose to target the 1345 bp region beginning upstream of exon 8 and ending in the intron region downstream of exon 10 (Fig. 1A). The deletion of exon 8 to exon 10 results in an out-of-frame mutation in exon 11 and the introduction of a stop codon in exon 11. In total, this targeting strategy results in the deletion of ~100 aa.

As shown in Fig. 1A, two loxP sites were inserted upstream of exon 8 and downstream of exon 10. As a result, we expected a genomic deletion of 1345 bp of Atg3 in Cre-expressing cells. EF1 ES cells in which positive homologous recombination occurred were selected by PCR screening for the loxP site downstream of exon 8 and the introduction of a stop codon in exon 11. The germline transmitted mice were bred with FLPeR mice to delete the neomycin resistance cassette. Mice homologous for the floxed Atg3 allele were then intercrossed with Lck-Cre mice to generate Atg3f/fLck-Cre mice, in which Atg3 is specifically deleted in T lymphocytes, or bred with ER-Cre mice to generate Atg3f/fER-Cre mice, in which Atg3 can be inducibly deleted. The results from studying Atg3f/fLck-Cre mice and Atg3f/fER-Cre mice, in which Atg3 can be inducibly deleted, further confirmed the deletion efficiency at the protein level by Western blot. Atg3 protein was absent in Atg3f/fLck-Cre thymocytes and purified Atg3f/fLck-Cre splenic naive T cells (Fig. 1D, left panel). Furthermore, in the Atg3f/fER-Cre inducible deletion model, Atg3 was efficiently deleted in Atg3f/fER-Cre splenocytes upon treatment with 4-OHT for 3 d (Fig. 1D, right panel). Together, these data demonstrate the successful generation of T lymphocyte-specific (Atg3f/fLck-Cre) and inducible (Atg3f/fER-Cre) models of deletion of Atg3.

Atg3 is essential for autophagy in T lymphocytes

Our previous data showed that TCR stimulation induces autophagy in T lymphocytes (20). To determine whether the autophagy pathway remains intact or is impaired in Atg3-deficient T cells, splenocytes from Atg3f/fLck-Cre and control mice were stimulated with anti-CD3, and LC3 puncta were quantified by intracellular staining using anti-LC3 Ab. The number of LC3 puncta in CD3-stimulated Atg3-deficient CD4+ T cells was much lower than that in control CD4+ T cells (Fig. 2A, 2B). Similar results were observed in Atg3-deficient CD8+ T lymphocytes (data not shown).

FIGURE 5. Survival defect in Atg3-deficient T cells. A, Freshly isolated splenocytes from Atg3f/fLck-Cre and Atg3f/f control mice were stained with anti-CD4–allophycocyanin, anti-CD8–PE/Cy7, anti-CD4–FITC, Annexin V, and 7-AAD. Annexin V and 7-AAD staining was analyzed in CD4+ and CD8+ subpopulations. Numbers represent the percentage of each gated population. Data are representative of three experiments. B, Increased caspase-9 activity in Atg3-deficient T cells. Splenocytes from Atg3f/fLck-Cre and Atg3f/f mice were incubated with caspase-9 FLICA and analyzed by flow cytometry. The cells were gated on CD4+ and CD8+ cells. Data are representative of three experiments. C, The survival defect in Atg3-deficient T cells is partially rescued by zVAD. Splenocytes from Atg3f/fLck-Cre and Atg3f/f mice were cultured in RPMI 1640 medium containing 10% FBS with or without IL-7 (1 ng/ml) or IL-7 in combination with zVAD (20 μM) for 48 h. The cells were stained with anti-CD4–allophycocyanin, anti-CD8–FITC, anti-CD4–allophycocyanin/Cy7, and 7-AAD. The 7-AAD+ cells are dead cells (*p < 0.05, zVAD treatment versus RPMI culture). CD44hi, CD44low; CD48hi, CD48low.
Consistent with the LC3 punctum formation results, the level of LC3 II was also reduced in Atg3-deficient T cells under starvation conditions (Fig. 2C).

It was reported that the LC3-binding protein p62/SQSTM1 is constantly degraded by the autophagy pathway and accumulates in autophagy-deficient cells (31, 32). p62 was accumulated in Atg3-deficient T lymphocytes, whereas Atg3 wild-type naive T cells expressed background levels of p62 (Fig. 2D). The ultrastructure of Atg3-deficient T cells was further analyzed by EM. EM pictures show that some membrane structures that are not characteristic of autophagosomes accumulated in Atg3-deficient T cells (Fig. 2E, 2F). The above results demonstrate that Atg3 is required for autophagy in T lymphocytes.

Atg3 is essential for T lymphocyte survival

Atg3fr/lck-Cre mice appear similar to Atg3fr/littermates. No obvious abnormalities were found in Atg3fr mice, suggesting that the mutations introduced in the targeting construct in the intron region between exon 7 and exon 8 do not affect the function of Atg3. The thymic cellularity of Atg3fr/lck-Cre mice was ~50% of that of Atg3fr and Atg3fr/lck-Cre mice (Fig. 3A); however, the frequencies of DN, double-positive, and single-positive thymocytes in Atg3fr lck-Cre mice were comparable to those in Atg3fr control mice (Fig. 3B, 3C). These results suggest that the loss of Atg3 did not obviously alter thymocyte development. Together with our previous findings in Atg7fr/lck-Cre mice (20) and Atg5 knockout fetal liver transfer experiments (25), these results are

**FIGURE 6.** Abnormal organelle homeostasis in Atg3-deficient T cells. A–C, Mitochondria in Atg3-deficient T cells. Splenocytes from Atg3fl/fl/lck-Cre and Atg3fl/fl control mice were incubated with 100 nM Mito-Tracker Green at 37°C for 30 min. After washing, the splenocytes were stained with anti-CD4–allophycocyanin, anti-CD8–PE/Cy7, anti-CD44–allophycocyanin/Cy7, and 7-AAD. Each subpopulation was gated on 7-AAD+ cells. A, FACS profiles of Mito-Tracker Green staining of Atg3-deficient T cells. Shaded profiles, Atg3fl/fl T cells; open profiles, Atg3fl/fl/lck-Cre T cells. B, The mean fluorescence intensity (MFI) of Mito-Tracker Green from 3 pairs of Atg3fl/fl/lck-Cre and Atg3fl/fl mice. C, The ratio of Mito Tracker MFIs in Atg3-deficient cell populations to those in corresponding wild-type cell populations. CD19+ cells were used as a negative control. The bar figures are mean ± SD from six pairs of Atg3fl/fl and Atg3fl/fl/lck-Cre mice. D–F, Atg3-deficient T cells produce elevated levels of ROS. Splenocytes from Atg3fl/fl/lck-Cre and Atg3fl/fl control mice were incubated with DHE for 1 h at 37°C. After washing, the cells were stained with Abs described above. The fluorescence intensity of the PE channel represents ROS production. All samples were gated on 7-AAD+ cells. D, FACS profiles of DHE staining. Shaded profiles, Atg3fl/fl/lck-Cre T cells; open profiles, Atg3fl/fl T cells. E, MFIs of DHE staining. Data are mean ± SD from three pairs of Atg3fl/fl/lck-Cre and Atg3fl/fl mice. F, The ratio of DHE MFIs in Atg3-deficient cells to those in corresponding wild-type cells. Data are mean ± SD from five pairs of Atg3fl/fl/lck-Cre and Atg3fl/fl mice. CD19+ cell population was used as a negative control. *p < 0.01, **p < 0.05, ***p > 0.05, Atg3fr/lck-Cre to Atg3fr.
consistent with the observations that thymocyte development is largely normal in the absence of autophagy.

Spleenic cellularity was decreased in Atg3\textsuperscript{f/f}Lck-Cre mice. The total splenic cellularity of Atg3\textsuperscript{f/f}Lck-Cre mice was ~60% of that of control Atg3\textsuperscript{f/+} mice (Fig. 4A). Furthermore, the frequency and absolute number of both CD4\textsuperscript{+} and CD8\textsuperscript{+} splenic T cells were decreased in Atg3\textsuperscript{f/f}Lck-Cre mice. The frequency and number of CD4\textsuperscript{+} T cells in Atg3\textsuperscript{f/f}Lck-Cre mice were 50 and 30%, respectively, of those in the control mice. The frequency and number of CD8\textsuperscript{+} T cells in Atg3\textsuperscript{f/f}Lck-Cre mice were 20 and 10%, respectively, of those in the control mice (Fig. 4B, 4C). The splenic cellularity and the number of CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells in Atg3\textsuperscript{f/f}Lck-Cre mice were not significantly different from those in Atg3\textsuperscript{f/+} mice (Fig. 4A, 4C).

We next examined the CD44 and CD62L expression patterns in Atg3\textsuperscript{f/f}Lck-Cre splenocytes and LN cells. The frequency of memory-like T cells (CD62L\textsuperscript{low} and CD44\textsuperscript{high}) among splenocytes and LN cells was increased within both the CD4\textsuperscript{+} and CD8\textsuperscript{+} compartment of Atg3\textsuperscript{f/+}Lck-Cre mice but not significantly changed in Atg3\textsuperscript{f/f}Lck-Cre mice (Fig. 4D, 4E). These results were consistent with the lymphopenic environment in Atg3\textsuperscript{f/f}Lck-Cre mice.

The above results suggest that survival is defective in Atg3-deficient T cells. To test this hypothesis, freshly isolated splenocytes were stained with Annexin V and 7-AAD. As shown in Fig. 5A, the frequency of Annexin V\textsuperscript{+} cells among naive (CD44\textsuperscript{low}) CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells was two times higher in Atg3\textsuperscript{f/f}Lck-Cre mice than that in Atg3\textsuperscript{f/+} mice. To further examine the apoptotic defect in Atg3-deficient T cells, the active form of caspase-9 was analyzed in freshly isolated T cells from Atg3\textsuperscript{f/f}Lck-Cre and Atg3\textsuperscript{f/+} mice. Increased active caspase-9 was found in Atg3\textsuperscript{f/f}Lck-Cre T cells (Fig. 5B). These data suggest that the impaired survival of Atg3-deficient T cells is related to caspase activation.

The impaired survival in Atg3-deficient T cells was further characterized in vitro. To decrease the spontaneous T lymphocyte death, IL-7 was added in the culture. IL-7 greatly enhanced the survival of Atg3\textsuperscript{f/f} wild-type T cells in vitro compared with RPMI 1640 medium only culture (Fig. 5C). More dead cells were found in Atg3-deficient T cells in in vitro culture. The frequency of apoptosis among Atg3-deficient T cells was greater than that among wild-type cells even in the presence of IL-7 (Fig. 5C). To further characterize the cell death of Atg3-deficient T cells, the pan-caspase inhibitor zVAD was used in cell survival assay. As shown in Fig. 5C, zVAD partially rescued the survival defect in Atg3-deficient T cells in the presence of IL-7, suggesting that the death of Atg3-deficient T cells may be mediated by the apoptosis pathway (Fig. 5C). Collectively, these results demonstrate that Atg3 and autophagy are essential for the survival of T lymphocytes.

Atg3 regulate organelle homeostasis in T lymphocytes

Using a T cell-specific Atg7 deletion model, we previously showed that autophagy mediates the developmental regulation of mitochondrial content (20) and ER homeostasis (21). We thus analyzed mitochondrial and ER homeostasis in the Atg3 T cell-specific deletion system. Splenocytes from Atg3\textsuperscript{f/f}Lck-Cre mice were stained with the cell permeable mitochondria- or ER-specific staining reagents Mito-Tracker Green or ER-Tracker Blue-White, respectively. As shown in Fig. 6A, 6B, 6G, and 6H, both mitochondria and ER were expanded in Atg3-deficient mature CD4\textsuperscript{+} or CD8\textsuperscript{+} naive T lymphocytes. The mitochondria and ER in Atg3-deficient naive T cells were increased to >150% of wild-type control cells as shown in Fig. 6C and 6F. These data suggest that inhibition of autophagy in these cells affected mitochondrial and ER homeostasis. Because the mitochondria were expanded in Atg3-deficient T cells, we next analyzed reactive oxygen species (ROS) production in Atg3\textsuperscript{f/f}Lck-Cre mice. Naive Atg3-deficient CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells produced more ROS than corresponding wild-type control cells (Fig. 6D, 6F), suggesting that the abnormal expansion of mitochondria in the absence of Atg3 may drive overproduction of ROS.

Atg3-deficient T cells cannot proliferate efficiently

To further assess the function of Atg3-deficient T cells, splenocytes from Atg3\textsuperscript{f/f}Lck-Cre and Atg3\textsuperscript{f/+} control mice were stimulated with anti-CD3 Ab. All analyzed cell populations were gated on 7-AAD-negative cells to exclude dead cells. CFSE dilution was only analyzed in gated live cells. Both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells from wild-type Atg3\textsuperscript{f/+} mice proliferated efficiently (Fig. 7A). The frequency of proliferating cells was >90% when wild-type cells were stimulated with soluble anti-CD3 Ab alone or in combination with anti-CD28 Ab. In contrast, Atg3-deficient CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells did not proliferate efficiently; the frequency of proliferating cells among these populations was <60% (Fig. 7A). Although Atg3-deficient T cells did
not proliferate as well as wild-type T cells, Atg3-deficient CD4+ and CD8+ T cells expressed CD69 and CD25 after anti-CD3 Ab stimulation (Fig. 7B). These data indicate that autophagy may regulate T lymphocyte proliferation but that it does not affect their activation.

**Cumulative defects in autophagy-deficient T cells**

Our previous data and the above results indicate that autophagy is essential for T cell survival and that autophagy mediates the regulation of organelle homeostasis. These data were all obtained using T cell-specific deletion models or Atg5 knockout fetal liver transfer experiments. To further investigate the functions of autophagy in mature T cells, Atg3f/f mice were bred with ER-Cre mice to generate Atg3f/fER-Cre mice, in which Atg3 can be inducibly deleted. As shown in Fig. 1D (right panel), Atg3 was deleted in ER-Cre+ cells upon in vitro treatment with 200 nM 4-OH tamoxifen for 3 d. After 4-OHT treatment, apoptosis was analyzed in Atg3-deficient naive CD4+CD44low or CD8+CD44low T cells. On day 3 after 4-OHT treatment, the majority of naive T cells from both Atg3f/f and Atg3f/fER-Cre mice were apoptotic. More than 50% of the naive CD4+ or CD8+ T cells were Annexin V+, and ~50% of the naive CD4+ cells and 60% of the naive CD8+ T cells were 7-AAD+ (Fig. 8A, upper left panel). No significant differences were found between 4-OHT–treated Atg3f/f cells and Atg3f/fER-Cre cells (Fig. 8A). This spontaneous death of naive T cells during the 3-d culture period was rescued by the addition of 1 ng/ml IL-7 (Fig. 8A, upper right panels). The results of carrier control ethanol treatment were shown in Fig. 8A, lower panels. Analysis of apoptosis on days 7, 10, and 18 after 4-OHT–mediated deletion of Atg3 in the presence of IL-7 showed no difference in Annexin V and 7-AAD staining between CD4+ and CD8+ T cells from Atg3f/f and Atg3f/fER-Cre mice (data not shown). In contrast, when the cells were cultured for >24 d, ~13% of Atg3-deficient CD4+ T cells and 25% of Atg3-deficient CD8+ T cells were 7-AAD+, whereas <3% of control CD4+ and 5% of CD8+ T cells were 7-AAD+ (Fig. 8B). These results suggest that although acute loss of Atg3 does not affect T cell survival, accumulation of defects during long-term cultures may result in the death of autophagy-deficient T cells.

Experiments using the Lck-Cre system have demonstrated that autophagy is essential in maintaining the homeostasis of mitochondria and ER in T cells. As the Lck-Cre induced deletion of autophagy genes occurs at early stage of thymocyte development, the defects in autophagy-deficient T cells may be due to cumulative effects. To investigate the accumulation of autophagy defect, the organelle homeostasis was thus analyzed in the ER-Cre inducible deletion system. No change in mitochondrial volume was detected on day 3 after inducible deletion of Atg3; however, mitochondrial volume began to increase on day 10 and further expanded by days 18 and 28 after inducible deletion of Atg3 in naive CD4+ or CD8+ T cells. Similarly, no change in ER content was detected on day 3 after inducible deletion of Atg3; however, ER volume was expanded in Atg3f/fER-Cre CD4+ and CD8+ T cells on day 10 after inducible deletion of Atg3 (Fig. 9). These results demonstrate that excess mitochondria and ER accumulate over time in Atg3-deficient T cells and suggest that these cumulative defects in organelle homeostasis may cause cell death in Atg3- and autophagy-deficient naive T cells.

**FIGURE 8.** Cumulative defects in Atg3-deficient T cells. A, Survival of T lymphocytes in short-term culture after inducible deletion of Atg3. Splenocytes from Atg3f/fER-Cre and Atg3f/f control mice were treated with 200 nM 4-OHT or equal volume of ethanol (ETOH) in vitro for 3 d with or without IL-7 (1 ng/ml). The cells were then stained with anti-CD4-allophycocyanin, anti-CD8-PE/Cy7, and anti-CD44-APC/Cy7. After washing, the cells were resuspended in binding buffer and stained with Annexin V-PE and 7-AAD and analyzed by flow cytometry. B, Increased T cell death in long-term culture after inducible deletion of Atg3. 4-OHT–treated Atg3f/fER-Cre and control splenocytes were continually cultured in complete RPMI 1640 medium. IL-7 (1 ng/ml) was added every other day. On day 24, the cells were stained for surface markers and 7-AAD and analyzed by flow cytometry. B, Increased T cell death in long-term culture after inducible deletion of Atg3. 4-OHT–treated Atg3f/fER-Cre and control splenocytes were continually cultured in complete RPMI 1640 medium. IL-7 (1 ng/ml) was added every other day. On day 24, the cells were stained for surface markers and 7-AAD and analyzed by flow cytometry.
Atg12–Atg5 conjugates function as a ubiquitin-protein ligase (E3)-like enzyme and enhance the generation of LC3-phosphatidylethanolamine (10, 12). Atg3 interacts with Atg12 and facilitates the formation of the Atg12–Atg5 complex (30). Our data suggest that Atg3 deficiency impairs autophagy in T lymphocytes, resulting in dysregulation of mitochondrial and ER homeostasis. A recent publication showed that Atg12 conjugates with Atg3, and Atg12–Atg3 complex regulates mitochondrial homeostasis through a mitophagy-independent pathway in mouse embryonic fibroblasts (36). Our results reported in this study, together with our previous findings in Atg5- or Atg7-deficient T cells, indicate that autophagy pathways are essential for maintaining lymphocyte physiological function.

The Atg3ERCre inducible deletion system provides a novel method to further investigate the functions of autophagy in T lymphocytes. After inducible deletion of Atg3, no acute defects were observed in T cell function. T cells in which Atg3 had been inducibly deleted survived similar to wild-type T cells, and mitochondria and ER were not expanded at early time points; however, if the Atg3-deficient T lymphocytes were cultured for longer periods of time (>2 to 3 wk), the organelles were gradually expanded, and the frequency of cell death began to increase. These effects may be related to the basic functions of autophagy. Generally, the physiological function of autophagy is to provide cells with energy during starvation, growth factor withdrawal, or other stress conditions and to remove long-lived proteins or damaged, aged, or excess organelles. The temporal accumulation of mitochondria and ER after deletion of Atg3 suggests that the survival and proliferation defects observed in Atg5-, Atg7-, and Atg3-deficient T cells are indirect effects of impaired autophagy. After expanded organelles accumulate to a certain level, the cells may not be able to compensate for these abnormalities, and the survival defects become apparent.

The relationships among autophagy, cell death, and cell survival are complex (37). Our previous data and the results presented in this study demonstrate that autophagy is essential for the survival of T lymphocytes (20, 25). IL-7 upregulates the expression of Bcl-2 to promote the survival of T lymphocytes (38); however, IL-7 does not rescue the survival defect in Atg3-deficient T cells. The enhanced activation of caspase-9 and the fact that pan-caspase inhibitor zVAD partially rescues the survival defect in Atg3-deficient T cells suggest that the cell death in autophagy-deficient T cells is due to the abnormal activation of the apoptosis pathway; however, other factors related to impaired autophagy may also affect the survival of T lymphocytes. Interestingly, abolishing the Atg12–Atg3 conjugates inhibits cell death by increasing the expression of Bcl-2 member Bcl-xL in mouse embryonic fibroblasts (36). However, this is independent of autophagy because the disruption of the conjugates of the Atg12 and Atg3 complex does not affect the starvation-induced autophagy (36).

In summary, our results indicate that Atg3 and LC3 conjugation are essential for autophagy in T lymphocytes. The homeostasis of organelles in Atg3-deficient T cells is abnormal, and the survival of Atg3-deficient T cells is defective. By using an inducible deletion model, we have provided clear evidence that the survival defect and organelle expansion phenotypes in Atg3-deficient T cells are temporally accumulated effects of impaired autophagy.

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