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MYELOID NEOPLASIA

Cytoprotective autophagy maintains leukemia-initiating cells in murine myeloid leukemia

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Key Points

- Autophagy is required for maintenance of AML-initiating cells and peripheral myeloblast survival.
- Loss of autophagy potentiates the therapeutic effects of AraC in vivo.

Despite advances in the treatment of acute myeloid leukemia (AML), relapse and drug resistance frequently occur. Therefore, detailed mechanisms of refractoriness, including leukemia-initiating cell (LIC) biology, should be elucidated to treat AML. The self-degradative property of cytosolic macromolecules is central to autophagy and can contribute to homeostasis and stress response. Recent reports suggest the importance of autophagy in hematopoietic stem cells and various tumors. Thus, this study investigated the functional role of autophagy in AML maintenance and drug resistance using tamoxifen-inducible conditional knockout mice of *Atg5* or *Atg7*, which are essential genes for autophagy, combined with an mixed lineage leukemia–eleven nineteen leukemia–induced murine AML model. Inactivation of autophagy by deletion of *Atg5* or *Atg7* prolonged survival in leukemic mice and reduced functional LICs. *Atg7*-deficient LICs displayed enhanced mitochondrial activity and reactive oxygen species production together with increased cell death. In addition, *Atg7* deletion markedly decreased peripheral blood leukemia cells, concurrent with increased apoptosis, suggesting a higher dependency on autophagy compared with bone marrow leukemia cells. Finally, cytarabine (AraC) treatment activated autophagy in LICs, and *Atg7* deletion potentiated the therapeutic effects of AraC, which included decreased LICs and prolonged survival, suggesting that autophagy contributes to AraC resistance. Our results highlight the intratumoral heterogeneity related to autophagy in AML and the unique role of autophagy in leukemia development and drug resistance. (*Blood*. 2016;128(12):1614-1624)

Introduction

The prognosis of acute myeloid leukemia (AML) remains dismal in the majority of patients even after recent advances in the pathophysiology in AML. One reason for this is the acquisition of resistance to key chemotherapeutic drugs such as cytarabine (AraC) and anthracyclines, resulting in relapse, which is mediated by a small subset of self-renewing cells called leukemia-initiating cells (LICs). Thus, further studies on the biological features of LICs and their mechanisms of drug resistance are essential to treat AML patients.

Macroautophagy (hereafter referred to as autophagy) is a process used for the degradation of cytosolic macromolecules by lysosomal enzymes.[1,2] Autophagy has various physiological roles through the maintenance of cellular homeostasis by degrading damaged or excess cytosolic components and supplying nutrients to starved cells.[3,4] Through autophagic processes, double membrane vesicles, called autophagosomes, engulf cytosolic components and transport their cargo to lysosomes. A group of proteins including autophagy–related 5 (ATG5) and ATG7 are indispensable for autophagy.[1,5,6]

Based on the complicated relationship between autophagy and tumors, it is unknown if inhibition of autophagy could be a beneficial approach to treat cancer.[7,8] In general, autophagy contributes to cell survival in established tumors.[9,10] Accordingly, inactivation of autophagy, through *Atg3* deletion, in breakpoint cluster region–Abelson murine leukemia viral oncogene homolog 1 (BCR-ABL1)–transduced murine bone marrow (BM) cells diminishes their capacity to produce chronic myeloid leukemia (CML) in transplanted mice because of p53-dependent growth arrest.[11] As such, the efficacy of the autophagy inhibitor chloroquine and its derivatives is currently being tested in clinical trials for the treatment of various types of cancer.[12] In contrast, it has also been reported that autophagy genes function as tumor suppressors in normal tissues.[13] Additionally, even in established tumors, it has been reported that inhibition of autophagy promotes cancer progression in a murine *TP53*-deficient pancreatic cancer model and in *EGFR*-mutated lung cancer.[14,15] Considering these facts, the dependency on autophagy could be strongly affected by biological context such as gene mutations and physiological conditions of the tumor microenvironment.

In addition, its association with drug resistance makes autophagy an attractive drug target. Autophagy is recognized as a nonapoptotic mechanism of programmed cell death and is required for cell death after chemotherapy in some cases.[16,17] However, combination treatment using an autophagy inhibitor with chemotherapy, irradiation, or targeted therapy has demonstrated efficacy in various cancers

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including hematological malignancies.[8,18,19] Considering the fact that the function of autophagy is context-dependent, it is worthwhile to evaluate the impact of inhibition of autophagy in vivo in different types of tumors, using animal models that can recapitulate physiological conditions and tumor microenvironments.

The role of autophagy in AML progression, including LIC maintenance, is not yet fully understood. Moreover, the exact function of autophagy in drug resistance in vivo needs to be elucidated; however, some studies have revealed that autophagy is required for drug resistance.[20-24] In the present study, to investigate the role of autophagy in AML progression and drug resistance in vivo, we inhibited autophagy in murine mixed lineage leukemia–eleven nineteen leukemia (MLL-ENL) leukemia and CML blast crisis (CML-BC).

Methods

Mice

Atg5$^{flox/flox}$ mice,[25] *Atg7*$^{flox/flox}$ mice,[5] and *GFP-LC3* mice[26] were obtained from RIKEN BRC (Tsukuba, Japan). *Cre-ER*^{T2} mice[27] were obtained from JAX (Bar Harbor, ME). *Atg5*$^{flox/flox}$ or *Atg7*$^{flox/flox}$ mice were crossed with *Cre-ER*^{T2} mice. Genotyping was performed with primers shown in supplemental Table 1 (available on the *Blood* Web site). Wild-type C57BL/6J (8- to 12-week-old) mice were purchased from Sankyo Laboratory Service (Tokyo, Japan). These mice were kept at the Center for Disease Biology and Integrative Medicine at the University of Tokyo, according to institutional guidelines. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Tokyo.

Generation of murine leukemia model

The procedure for developing murine bone marrow transplantation (BMT) leukemia models was derived from previous reports.[28,29] For producing retrovirus, MSCV-MLL-ENL-IRES-GFP, MSCV-MLL-ENL-IRES-Puro,[28] pGCDNsam-BCR-ABL1-IRES-GFP, or pGCDNsam-NUP98-HOXA9-IRES-KusabiraOrange were transfected into Plat-E packaging cells[30] using polyethylenimine.[31] The detailed protocol is described in the supplemental Methods.

Drug administration

For excising the loxP-flanked region in vivo, 1 mg/mouse tamoxifen (TAM; Cayman Chemical Company, Ann Arbor, MI) dissolved in peanut oil (Sigma, St. Louis, MO) was intraperitoneally administrated once daily for 5 consecutive days. In leukemia mouse models, TAM treatment started from day 7 after BMT. AraC (Tokyo Chemical Industry, Tokyo, Japan) was dissolved in phosphate-buffered saline (PBS) and intraperitoneally administrated to leukemic mice at a dose of 1 mg/mouse once daily.

Flow cytometry analyses and sorting

For staining, isolated mononulcear cells were incubated with 3% fetal calf serum/PBS containing diluted antibody as listed in supplemental Table 2. Stained cells were washed once with 3% fetal calf serum/PBS and analyzed. For apoptosis analysis, stained cells were washed once and subsequently incubated with annexin-V-allophycocyanin (BioLegend, San Diego, CA) and 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/mL) in accordance with the manufacturers' protocols. Mitochondrial activity was detected by MitoTrackerOrange CMTMRos (Life Technologies, Waltham, MA), and reactive oxygen species (ROS) levels were determined by CellRox DeepRed (Life Technologies). Cells (2×10^5) that were stained with antibodies against surface markers were washed once and incubated with 1× Hanks balanced salt solution containing a 1:2000 dilution of MitoTracker or a 1:500 dilution of CellRox at 37°C for 15 minutes. Stained cells were analyzed using an LSRII (BD Biosciences, San Jose, CA) and sorted with a BD FACSAria (BD Biosciences). Data were analyzed by FlowJo software (TreeStar, Ashland, OR).

Electron micrograph

Sorted c-Kit$^-$CD11b$^+$ BM or peripheral blood (PB) cells from MLL-ENL leukemic mice were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 50 mM phosphate buffer (pH 7.4) for 10 minutes at 4°C. The buffer was then replaced with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and incubated at 4°C overnight. Thereafter, sample preparation and imaging were conducted at Tokai Electron Microscopy Inc. (Nagoya, Japan).

Statistics

P values, for comparison of 2 different groups, were calculated using an unpaired 2-tailed Student *t* test unless otherwise mentioned. LIC frequency was assessed using the Poisson distribution. To analyze the survival curves, a log-rank test was used. *P* values $<.05$ were considered significant. Data analysis was performed using R software (http://www.R-project.org).

Results

Autophagy deficiency in MLL-ENL AML mice retarded leukemia progression

To investigate the importance of autophagy in AML progression, we developed a leukemia mouse model through BMT with *Atg5*- or *Atg7*-floxed BM cells transduced with MLL-ENL. To delete the *Atg5* or *Atg7* gene in vivo, *Atg5*$^{flox/flox}$ or *Atg7*$^{flox/flox}$ mice were crossed with TAM-inducible *Cre-ER*^{T2} mice.[27] Green fluorescent protein (GFP)-labeled MLL-ENL-driven AML cells, from primary recipients, were serially transplanted into secondary recipients, and secondary recipients received TAM treatment to delete *Atg5* or *Atg7* (supplemental Figure 1A). Genomic polymerase chain reaction and western blots of BM cells from TAM-treated, *Atg5*$^{flox/flox}$*:Cre-ER*^{T2} or *Atg7*$^{flox/flox}$*:Cre-ER*^{T2} MLL-ENL leukemic mice confirmed the in vivo deletion of *Atg5* or *Atg7* (supplemental Figure 2A; Figure 1A). Unconjugated microtubule-associated protein 1A/1B-light chain 3A (LC3A, or LC3A-I) covalently binds to phosphatidylethanolamine to form LC3A-II to induce autophagy through associations with several ATG molecules including ATG5 and ATG7.[1] Thus, LC3A-II is commonly used as an indicator of autophagy. We confirmed diminished conversion of LC3A-I to LC3A-II in TAM-treated *Atg5*$^{\Delta/\Delta}$ and *Atg7*$^{\Delta/\Delta}$ leukemic cells, revealing that genetic deletion of Atg5 or Atg7 can efficiently suppress autophagy in MLL-ENL leukemic cells (Figure 1A). In terms of disease progression in MLL-ENL mice, *Atg5*$^{\Delta/\Delta}$ and *Atg7*$^{\Delta/\Delta}$ animals displayed significantly longer survival than *Atg5*$^{flox/flox}$ and *Atg7*$^{flox/flox}$ controls (*Atg5*$^{\Delta/\Delta}$: median = 29 days vs *Atg5*$^{flox/flox}$: median = 25 days; *Atg7*$^{\Delta/\Delta}$: median = 23 days vs *Atg7*$^{flox/flox}$: median = 20 days) (Figure 1B-C). *Atg7*$^{\Delta/\Delta}$ MLL-ENL mice showed lower BM cellularity than control mice (Figure 1D), although the frequency of GFP$^+$ MLL-ENL cells in BM and spleen weight were not different between vehicle- and TAM-treated mice (supplemental Figure 2B-C).

Atg7 deletion in MLL-ENL leukemic mice decreased LIC frequency via induction of cell death

To further examine the role of autophagy in MLL-ENL leukemic mice, LIC frequency was evaluated in Atg7-deficient leukemic mice; MLL-ENL LICs are enriched in lineage$^-$c-Kit$^+$Sca1$^-$CD34$^+$CD16/32$^+$ (L-GMP) or lineage$^-$c-Kit$^+$ (LK) cells.[32,33] Flow cytometric analysis revealed that LK and L-GMP frequency were decreased in Atg7-deleted leukemic mice (Figure 2A; supplemental Figure 3A-B). Additionally, a similar reduction in LICs was observed in *Atg5*$^{\Delta/\Delta}$ leukemic mice, suggesting that the decreased frequency of LICs was caused by autophagy-related mechanisms (Figure 2B; supplemental Figure 3C). Hereafter, we mainly focused on the *Atg7* conditional

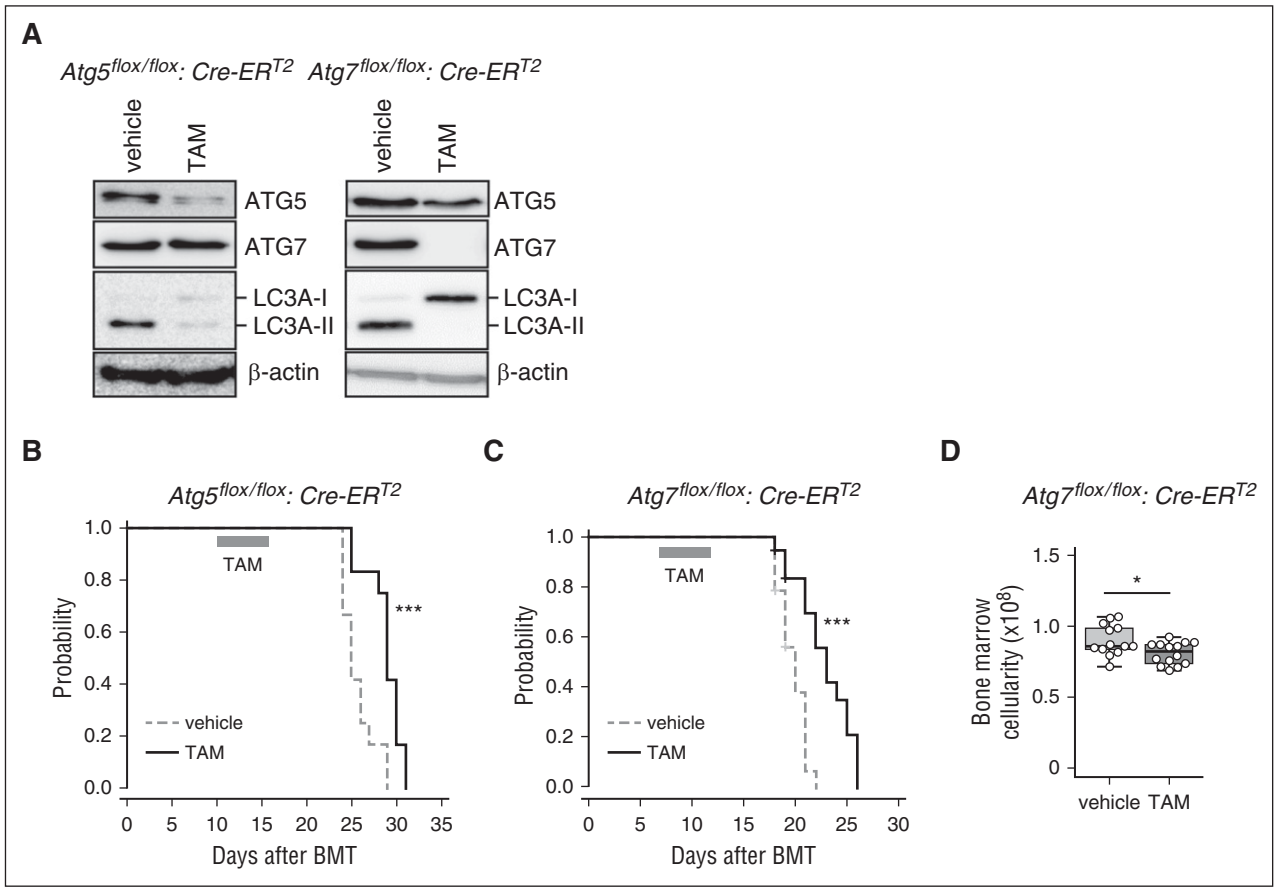

Figure 1. Retarded progression of MLL-ENL–induced murine leukemia caused by autophagy deficiency. (A) Protein levels of ATG5, ATG7, and LC3A were assessed by western blot analysis in BM cells isolated from MLL-ENL leukemic mice at day 18 post-BMT. β-actin was used as a loading control. (B-C) Kaplan-Meier curves represent vehicle- or TAM-treated *Atg5*$^{flox/flox}$:*Cre-ER*^{T2} (n = 12 each) and *Atg7*$^{flox/flox}$:*Cre-ER*^{T2} (n = 15 each) MLL-ENL leukemic mice. The *P* value was calculated by a log-rank test. (D) BM cellularities of vehicle- and TAM-treated *Atg7*$^{flox/flox}$:*Cre-ER*^{T2} leukemic mice are shown (n = 13). The box depicted in the upper and lower quartiles, and the median, are signified by thick lines within the box. The whiskers represent maximum or minimum data within the 1.5× interquartile range from the top or the bottom of the box, respectively. Each circle represents an individual animal. *$P < .05$; ***$P < .001$. All results except for western blotting are pooled data from at least 3 independent experiments with 1 to 4 animals per experiment.

knockout model to study the role of autophagy, as phenotypes were identical after knocking out *Atg5* or *Atg7*. In normal hematopoietic stem cells (HSCs), an *Atg7* deletion causes accumulation and increased activity of mitochondria, resulting in increased ROS production.[34,35] Similarly, *Atg7*$^{\Delta/\Delta}$ MLL-ENL LK cells demonstrated enhanced mitochondrial activity and increased ROS production compared with those of their *Atg7*$^{flox/flox}$ counterpart (Figure 2C-F). In lineage$^+$ cells, a population rich in differentiated cells, the Atg7 deletion had no effect on mitochondrial activity or ROS production (Figure 2C-F). When we assessed the apoptotic status of *Atg7*$^{\Delta/\Delta}$ and *Atg7*$^{flox/flox}$ MLL-ENL mice, annexin-V$^+$/DAPI$^+$ dead cells increased only in the *Atg7*$^{\Delta/\Delta}$ LK fraction (Figure 2G; supplemental Figure 3D). We confirmed that cell death in the LK fraction was also induced in TAM-treated *Atg5*$^{flox/flox}$ MLL-ENL leukemic mice (Figure 2H).

To exclude the possibility that TAM treatment alone or activation of Cre-estrogen receptor fusion protein (Cre-ER) could cause artificial effects in hematopoietic cells,[36,37] we used *Cre-ER* negative (Cre$^-$) *Atg7*$^{flox/flox}$ leukemic mice and *Cre-ER* wild-type leukemic mice as controls. TAM treatment did not prolong survival in *Atg5*$^{flox/flox}$:Cre$^-$ or *Atg7*$^{flox/flox}$:Cre$^-$ leukemic mice and did not reduce the frequency of L-GMPs in *Atg7*$^{flox/flox}$:Cre$^-$ leukemic mice (supplemental Figure 4A-C). Moreover, the frequency of L-GMP cells in *Cre-ER* wild-type leukemic mice did not change after TAM treatment (supplemental Figure 4D).

These results suggest that autophagy is required for LIC maintenance by preventing cell death associated with accumulation of mitochondria and induced ROS production.

Atg7-deficient MLL-ENL LICs possessed decreased tumorigenic capacity

To characterize the defects of LICs in autophagy-deficient leukemic mice, functional analyses of *Atg7*$^{\Delta/\Delta}$ leukemic cells were performed. GFP$^+$ *Atg7*$^{\Delta/\Delta}$ BM cells had decreased in vitro colony-forming capacity compared with GFP$^+$ *Atg7*$^{flox/flox}$ BM cells (supplemental Figure 5A-B). When sorted GFP$^+$ BM cells from *Atg7*$^{flox/flox}$ or *Atg7*$^{\Delta/\Delta}$ MLL-ENL leukemic mice were serially transplanted into tertiary recipient mice, it was confirmed that the *Atg7* deletion had no impact on homing ability (Figure 3A-B; supplemental Figure 1B). Limiting dilution transplantation analysis revealed that the frequency of functional LICs decreased in autophagy-inactivated *Atg7*$^{\Delta/\Delta}$ mice (*Atg7*$^{flox/flox}$ vs *Atg7*$^{\Delta/\Delta}$, 1:46 vs 1:301, respectively) (Figure 3C-D). Taken together, *Atg7* is required for maintenance of functional LICs in MLL-ENL leukemia.

PB MLL-ENL leukemia cells had a high dependency on autophagy for survival

Intriguingly, both *Atg5*$^{\Delta/\Delta}$ and *Atg7*$^{\Delta/\Delta}$ MLL-ENL leukemic mice had significantly lower peripheral white blood cell (WBC) counts compared

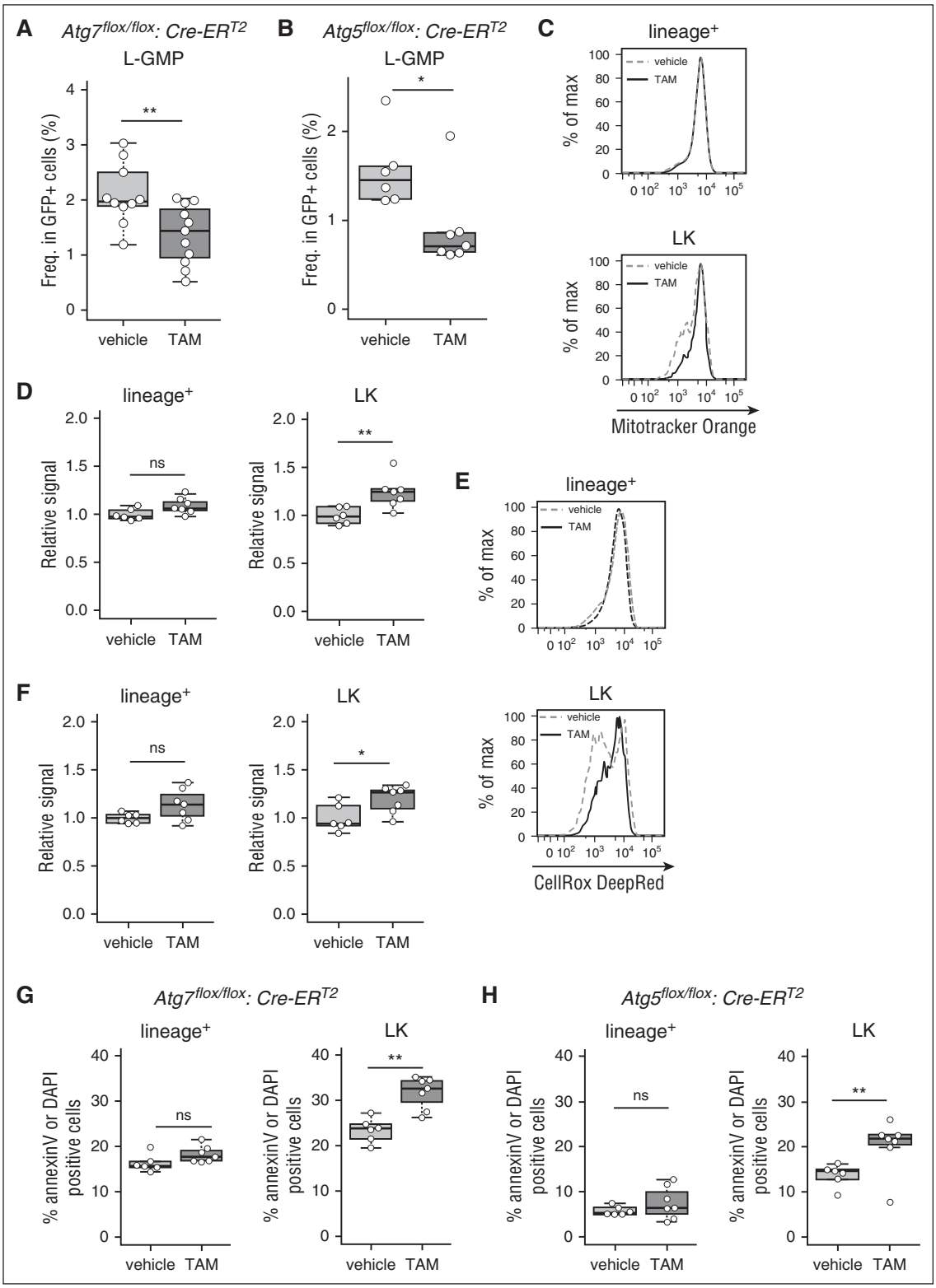

Figure 2. Induction of cell death, associated with activated mitochondria and ROS production, is reduced in LICs by *Atg7* deletion. Collective data indicating the frequencies of L-GMP cells from vehicle-treated (n = 10) or TAM-treated (n = 11) *Atg7*$^{flox/flox}$:*Cre-ER*^{T2} MLL-ENL leukemic mice (A) and vehicle-treated (n = 6) or TAM-treated (n = 7) *Atg5*$^{flox/flox}$:*Cre-ER*^{T2} MLL-ENL leukemic mice (B). (C) Representative flow cytometric analysis of mitochondrial activity in lineage$^+$ and LK cells from vehicle- or TAM-treated *Atg7*$^{flox/flox}$:*Cre-ER*^{T2} MLL-ENL leukemic mice. (D) Collective data of relative signal level of Mitotracker Orange in lineage$^+$ and LK cells from vehicle-treated (n = 6) or TAM-treated (n = 7) *Atg7*$^{flox/flox}$:*Cre-ER*^{T2} MLL-ENL leukemic mice. Relative signal represents geometric mean fluorescent intensity (MFI) of each sample divided by the average MFI from all vehicle samples. (E) Representative flow cytometric analysis of ROS levels in lineage$^+$ and LK cells from vehicle- or TAM-treated *Atg7*$^{flox/flox}$:*Cre-ER*^{T2} MLL-ENL leukemic mice. (F) Collective data of relative signal levels of CellRox DeepRed in lineage$^+$ and LK cells from vehicle-treated (n = 6) or TAM-treated (n = 7) *Atg7*$^{flox/flox}$:*Cre-ER*^{T2} MLL-ENL leukemic mice. (G) Boxplot of the frequencies of annexin-V$^+$ or DAPI$^+$ cells from vehicle-treated (n = 6) or TAM-treated (n = 7) *Atg7*$^{flox/flox}$:*Cre-ER*^{T2} MLL-ENL leukemic mice. (H) Boxplot of the frequencies of annexin-V$^+$ or DAPI$^+$ cells from vehicle-treated (n = 6) or TAM-treated (n = 7) *Atg5*$^{flox/flox}$:*Cre-ER*^{T2} MLL-ENL leukemic mice. **P* < .05; ***P* < .01. All results represent pooled data from at least 3 independent experiments with 2 to 4 animals per experiment.

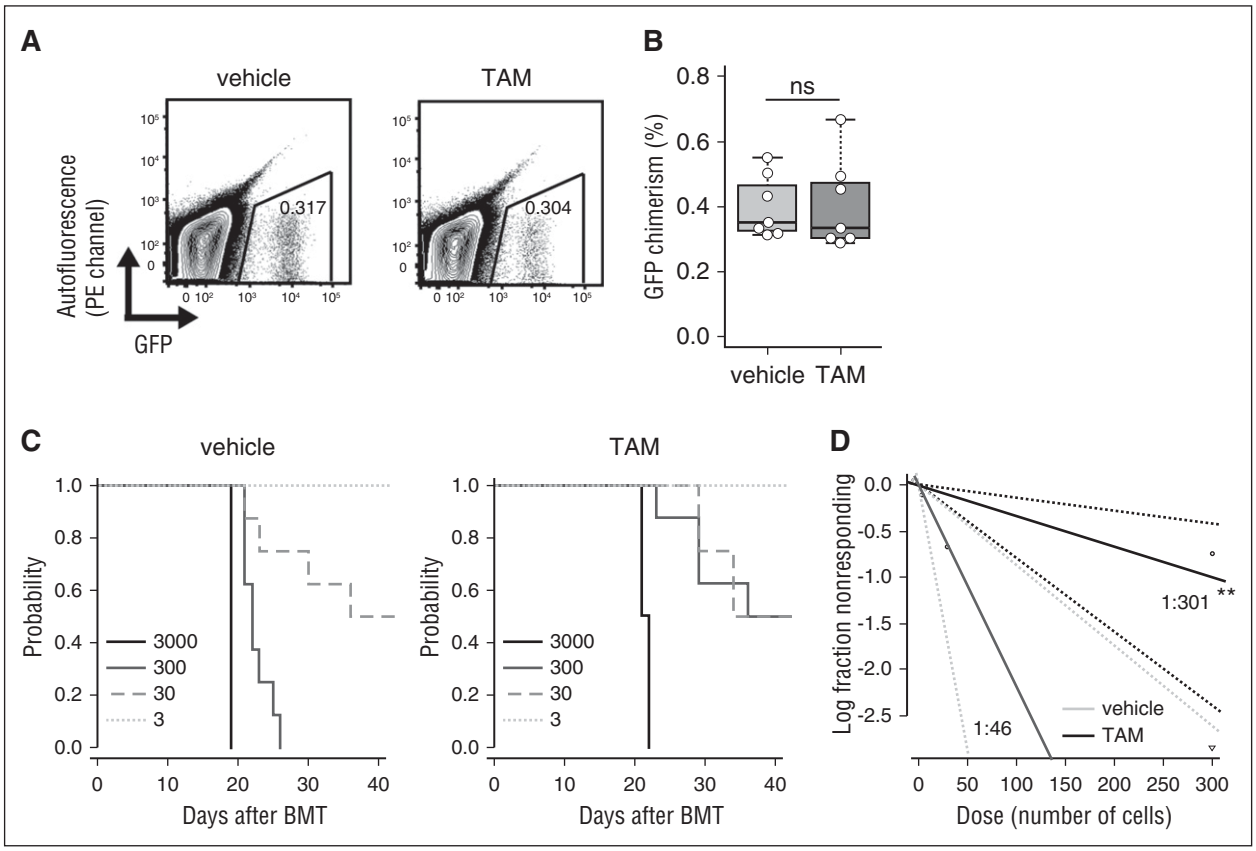

Figure 3. Decreased frequency of functional LICs in *Atg7*-deleted MLL-ENL mice. (A) Representative flow cytometric plot of donor cell engraftment 16 hours after BMT with 1×10^6 GFP$^+$ *Atg7*$^{flox/flox}$ or *Atg7*$^{\Delta/\Delta}$ cells. (B) Collective data of donor cell engraftment at 16 hours after BMT with 1×10^6 GFP$^+$ *Atg7*$^{flox/flox}$ or *Atg7*$^{\Delta/\Delta}$ cells (n = 7 per group). (C) Kaplan-Meier curves represent survival of tertiary recipients with 3, 30, 300, or 3000 GFP$^+$ BM cells from vehicle- or TAM-treated leukemic mice (n = 4-8 per group). (D) Poisson statistical analysis of limiting dilution transplantation assay. Plots were generated for estimation of the frequency of functional LICs in GFP$^+$ BM cells from vehicle- or TAM-treated leukemic mice (n = 4-8 at each cell dose). The plot represents the logarithm of the frequency of living mice at 42 days after injection for the indicated number of cells. LIC frequency was 1:46 for vehicle-treated (95% confidence interval, 1:18-115) and 1:301 for TAM-treated (95% confidence interval, 1:126-718) leukemic mice. $P = .0002$.

with those of their respective counterparts (Figure 4A-B). This contrasted the minimal contribution of *Atg5*/*Atg7* deletion in reducing AML burden in BM (Figure 1D; supplemental Figure 2B-C). These results led us to hypothesize that various extracellular factors in PB can result in autophagy dependence in peripheral leukemia cells. We thus evaluated the apoptotic status of PB *Atg5*$^{\Delta/\Delta}$ and *Atg7*$^{\Delta/\Delta}$ MLL-ENL leukemia cells. To minimize possible bias derived from differential frequencies of c-Kit$^+$ cells between BM and PB, CD11b$^+$ cells, in which MLL-ENL (GFP$^+$) leukemia cells accumulate in both BM and PB, were subdivided into c-Kit$^-$ and c-Kit$^+$ fractions for further analysis (supplemental Figure 6A). Strikingly, a TAM-induced *Atg5*/*Atg7* deletion caused increased apoptosis only in PB CD11b$^+$ leukemia cells and not in similar cells of BM origin, irrespective of c-Kit expression (Figure 4B-C; supplemental Figure 6B).

To further analyze autophagy-related functions in PB MLL-ENL leukemia cells, in vitro colony formation assays and in vivo serial BMT assays with PB leukemia cells were performed. PB *Atg7*$^{\Delta/\Delta}$ leukemia cells showed decreased colony forming capacity in vitro (Figure 4D), and recipients of PB *Atg7*$^{\Delta/\Delta}$ leukemia cells displayed marginal but significantly prolonged survival in vivo (Figure 4E; supplemental Figure 1C). This demonstrated that PB MLL-ENL leukemia cells also had leukemia-initiating capacity, which was partly regulated by autophagy.

Additionally, we evaluated the apoptotic status of PB cells from *Atg7*$^{flox/flox}$:Cre$^-$ or *Cre-ER* leukemic mice with wild-type Atg genes as controls. TAM treatment did not cause reduction in WBCs or induction of apoptosis in PB. Therefore, the possibility of a cytotoxic effect on PB leukemic cells mediated by the Cre-ER system was excluded (supplemental Figure 4E-I).

Taken together, autophagy protects PB leukemia cells from apoptosis irrespective of their immunophenotypic maturity.

PB MLL-ENL leukemia cells had an increased number of autophagosomes

We next investigated whether PB leukemia cells have enhanced autophagic activity. For this, BM and PB c-Kit$^-$CD11b$^+$ MLL-ENL leukemia cells were subjected to western blotting to quantify LC3A conversion. Protein analysis revealed that PB leukemia cells had a higher level of LC3A-II than BM leukemia cells, indicating that PB leukemia cells were rich in autophagosomes (Figure 5A-B). In addition, electron microscopic analysis confirmed that PB leukemia cells contained an increased number of autophagosomes compared with BM cells (Figure 5C-D). We next used *GFP-LC3* transgenic mice to evaluate autophagy.[26] When autophagy is activated in *GFP-LC3* mice, GFP-LC3 is degraded resulting in decreased GFP intensity.[38] We developed *GFP-LC3* transgenic MLL-ENL leukemic mice (supplemental Figure 1D), and GFP expression in BM and PB leukemia cells was measured by flow cytometry (supplemental Figure 7A). In both

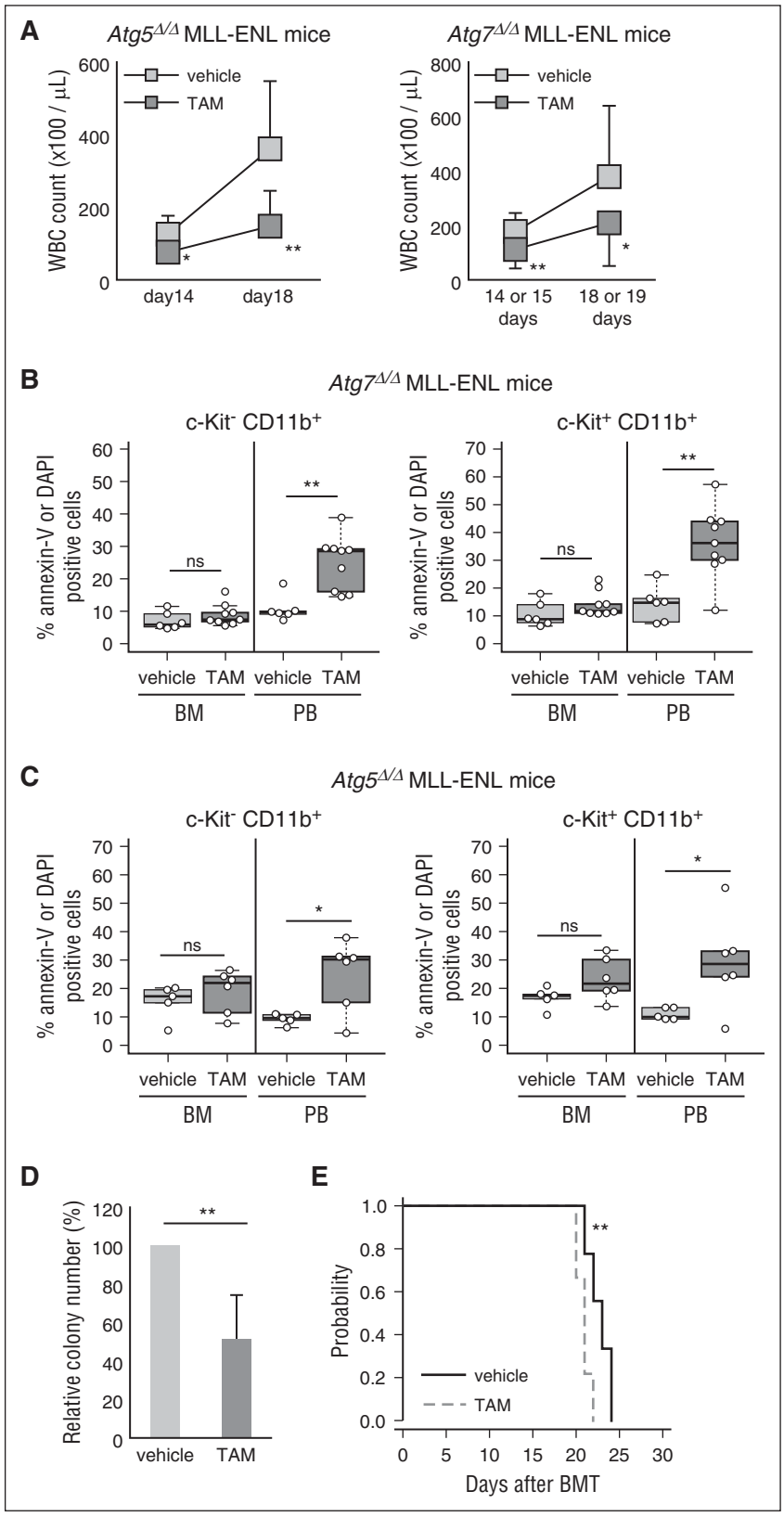

Figure 4. Dependence of PB leukemia cells on autophagy. (A) WBC counts of PB from *Atg5*$^{flox/flox}$*:Cre-ER*^{T2} or *Atg7*$^{flox/flox}$*:Cre-ER*^{T2} MLL-ENL leukemic mice were measured at indicated days after BMT (>7 mice per group, mean ± standard deviation [SD]). *P* values were calculated by a Welch *t* test. Collective data of frequencies of annexin-V$^+$ or DAPI$^+$ cells in c-Kit$^-$CD11b$^+$ or c-Kit$^+$CD11b$^+$ cells obtained from vehicle- or TAM-treated *Atg7*$^{flox/flox}$*:Cre-ER*^{T2} (B) and *Atg5*$^{flox/flox}$*:Cre-ER*^{T2} (C) mice (n = 6-9 per group). (D) Relative colony numbers from GFP$^+$ PB cells sorted from vehicle- or TAM-treated *Atg7*$^{flox/flox}$*:Cre-ER*^{T2} MLL-ENL leukemic mice are shown (n = 4 per group, mean ± SD). (E) Kaplan-Meier curves representing survival of tertiary recipients of GFP$^+$ PB cells from vehicle- or TAM-treated *Atg7*$^{flox/flox}$*:Cre-ER*^{T2} MLL-ENL leukemic mice (n = 9 per group). The *P* value was calculated by a log-rank test. $^*P < .05$; $^{**}P < .01$. All results represent pooled data from at least 3 independent experiments with 1 to 4 animals per experiment.

c-Kit$^-$CD11b$^+$ and c-Kit$^+$CD11b$^+$ fractions, GFP expression in PB leukemia cells was decreased compared with that in BM leukemia cells (Figure 5E-F). To examine if the accumulation of autophagosomes was cell autonomous, we performed ex vivo culture of BM and PB leukemia cells. The enhanced LC3A-II expression in PB cells that was detected in vivo immediately disappeared when BM and PB c-Kit$^-$CD11b$^+$ cells from MLL-ENL leukemic mice were cultured (supplemental Figure 8A). Moreover, the difference in GFP-LC3

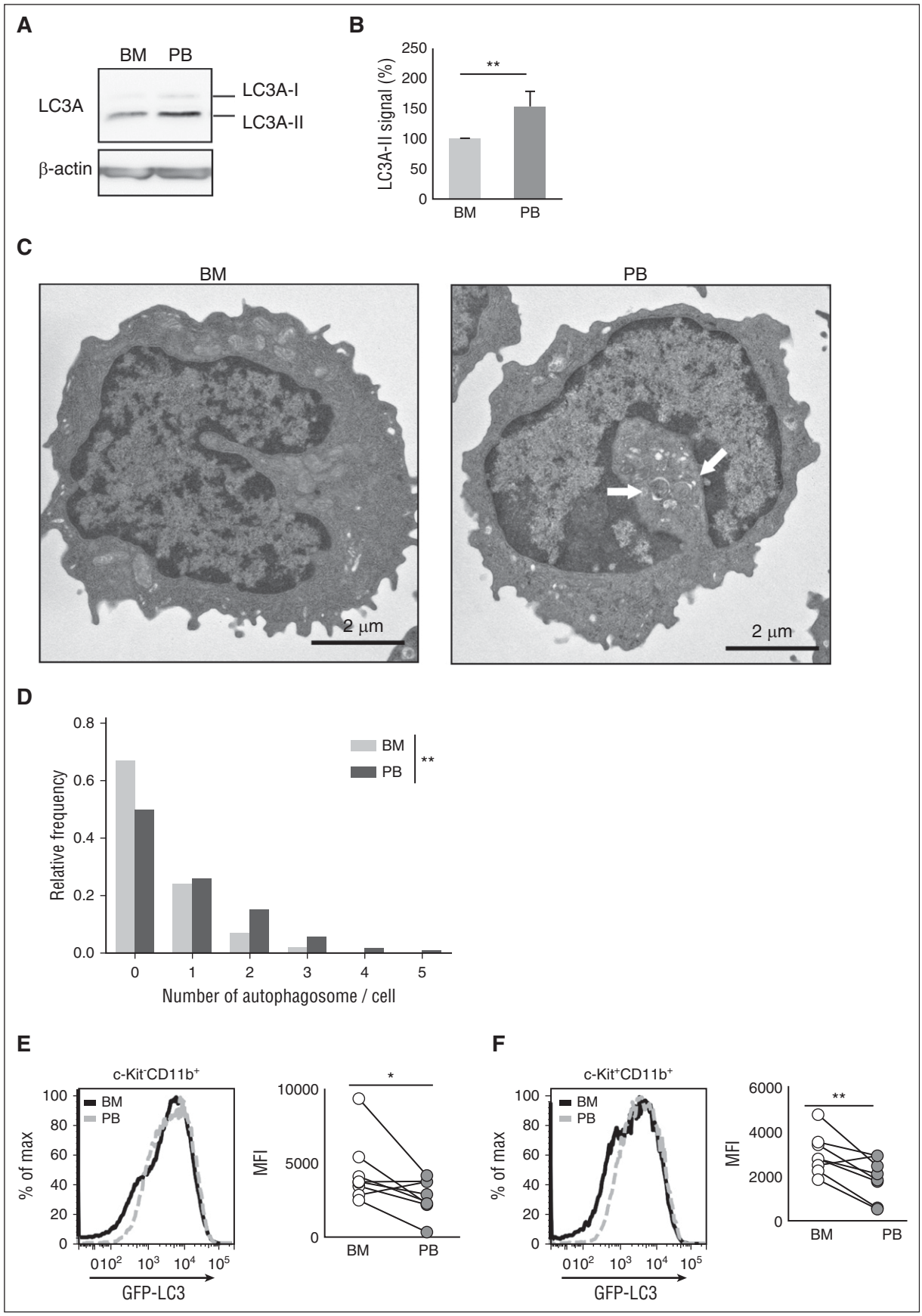

Figure 5. Increased number of autophagosomes and decreased GFP-LC3 expression were observed in PB leukemic cells. (A) Expression of LC3A-I and LC3A-II in BM and PB from *GFP-LC3* MLL-ENL leukemic mice. (B) Relative signal intensities of LC3A-II in BM and PB from *GFP-LC3* MLL-ENL leukemic mice are shown. Values were normalized to the intensity of LC3A-II/β-actin from BM samples (n = 5, mean ± SD). (C) Representative electron micrograph of c-Kit^{-}CD11b^{+} cells from BM or PB of

expression between BM and PB was maintained in leukemic mice after BMT of PB leukemic cells that expressed lower levels of GFP-LC3 (supplemental Figure 8B-C). These results suggest that the PB environment might enhance autophagy in PB leukemia cells.

Normal mature myeloid cells in PB did not depend on autophagy

The importance of autophagy in hematopoietic cells has been emphasized by several prior reports.[39-42] Considering the enhanced dependency of PB MLL-ENL leukemia cells on autophagy, we next elucidated the role of autophagy in normal PB myeloid cells. For this, PB cells obtained from vehicle- or TAM-treated MLL-ENL-free *Atg7$^{flox/flox}$:Cre-ER^{T2}* mice were analyzed. *Atg7$^{\Delta/\Delta}$* mice showed no change in PB WBC counts even after TAM administration (supplemental Figure 9A-B). Additionally, an Atg7 deletion had no impact on apoptosis in PB c-Kit$^-$CD11b$^+$ or c-Kit$^+$CD11b$^+$ cells (supplemental Figure 9C). Moreover, normal c-Kit$^-$CD11b$^+$ cells of the PB exhibited lower expression of LC3A-II and displayed enhanced expression of GFP-LC3 compared with those of their counterparts in BM (supplemental Figure 9D-E). Therefore, normal BM or PB myeloid cells have no dependency on autophagy for at least short-term survival, suggesting that enhanced autophagy dependency in PB is specific for MLL-ENL leukemia cells.

Role of autophagy in a murine CML–blast phase model

To investigate the role of autophagy in other AML models, we used BCR-ABL1 and NUP98-HOXA9–driven murine CML-BC models (supplementary Figure 1E).[43] Similar to the MLL-ENL model, *Atg7$^{\Delta/\Delta}$* CML-BC mice survived longer with no reduction in tumor burden in the BM or spleen (Figure 6A; supplemental Figure 10A-B). Moreover, *Atg7$^{\Delta/\Delta}$* CML-BC mice showed a significantly diminished LK fraction, which contains LICs in this model[44] (Figure 6B; supplemental Figure 10C). In addition, deletion of *Atg7* reduced the WBC count and induced apoptosis specifically in PB leukemia cells, which was similar to results observed for MLL-ENL leukemia (Figure 6C-D). The BM of deceased or moribund CML-BC mice was occupied by myeloblasts irrespective of *Atg7* deletion, suggesting leukemia development in these mice (supplemental Figure 10D). These results suggest that the leukemia promoting and PB cell supportive roles of autophagy are not restricted to MLL-ENL leukemia but are also present in other types of myeloid leukemia.

Inactivation of autophagy potentiated the therapeutic effects of AraC in vivo

Although the contribution of autophagy to drug resistance in AML has been investigated by in vitro culture, the exact role of autophagy in drug resistance in vivo remains elusive. Hence, the effect of autophagy inactivation in combination with AraC, one of the most common clinically used chemotherapeutic agents for AML, was investigated in MLL-ENL leukemic mice. First, the expression of GFP-LC3 in MLL-ENL leukemia cells after AraC treatment was measured. AraC treatment reduced GFP-LC3 expression in L-GMP cells but not in lineage$^+$ cells (supplemental Figure 11A-C). The overall survival of *Atg7$^{flox/flox}$:Cre-ER^{T2}* MLL-ENL mice was enhanced in the cohort treated with TAM and AraC, indicating that *Atg7* deletion potentiated the cytotoxic effect of AraC (Figure 7A). *Atg7* deletion showed an additive effect with AraC treatment on BM cell reduction (supplemental Figure 11D). Furthermore, *Atg7* deletion enhanced the efficacy of AraC in reducing the frequency and the absolute number of LICs (Figure 7B; supplemental Figure 11E). When the effect of TAM plus AraC therapy was assessed in PB from *Atg7$^{flox/flox}$:Cre-ER^{T2}* MLL-ENL mice, WBC counts in MLL-ENL mice treated with TAM and AraC were prominently reduced and were comparable to those of mice treated with AraC alone (Figure 7C). Of note, AraC treatment combined with *Atg7* deletion resulted in increased apoptosis in PB MLL-ENL leukemia cells compared with AraC treatment or *Atg7* deletion alone; however, the combinatorial effect on apoptosis in BM leukemia cells was not significant (Figure 7D; supplemental Figure 11F). In deceased or moribund TAM- and AraC-treated mice, the BM was occupied by myeloblasts similar to that observed in control mice, suggesting that these mice developed leukemia before death (supplemental Figure 11G). Altogether, *Atg7* deletion potentiated the efficacy of AraC treatment, and especially the capacity to target LICs, which highlights the important role of autophagy on AraC resistance in MLL-ENL leukemia.

Discussion

In this study, we evaluated the role of autophagy in leukemia progression, LIC maintenance, and drug resistance in vivo. Our results revealed that in the BM autophagy is specifically required for LICs, but not for differentiated leukemic blasts, to prevent oxidative stress; this should result in the maintenance of appropriate LIC frequency. In contrast, in the PB, autophagy supported survival of leukemia cells irrespective of their differentiation status. Furthermore, inactivation of autophagy potentiated the efficacy of AraC treatment in vivo.

The reason why in the BM LICs, but not other myeloblasts, required autophagy remains elusive. Our data support the hypothesis that accumulation of mitochondria and ROS production are possibly associated with the maintenance of LICs. In fact, normal HSCs have a fewer numbers of mitochondria than those of progenitor cells and use glycolytic metabolism in the hypoxic BM niche.[45] Although leukemia cells contain more numbers of mitochondria than those of normal counterparts,[46] fewer mitochondria might be required for maintenance of self-renewal capacity in LICs. These mechanisms are expected to be affected by the microenvironment, and this might account for inconsistencies in previous reports, specifically that pharmacological inhibition of autophagy did not inhibit proliferation in leukemic cell lines.[20,23] For comprehensive understanding of the mechanisms of autophagy in LIC maintenance, further investigation is warranted.

Our study could also help to characterize the impact of autophagy on AraC resistance in vivo. The relationship between autophagy and

Figure 5 (continued) MLL-ENL leukemic mice. The grids were observed by a transmission electron microscope (JEM-1400Plus; JEOL Ltd., Tokyo, Japan). Digital images were taken with a CCD camera (VELETA; Olympus Soft Imaging Solutions, Germany). White arrows indicate autophagosomes. (D) Frequency distribution of detectable autophagosomes per cell from electron micrograph analysis. The number of autophagosomes in 100 cells, derived from 2 independent mice, was counted. The *P* value was calculated with the Wilcoxon rank sum test. Representative histogram and collective data (n = 8) of GFP-LC3 expression in c-Kit$^-$CD11b$^+$ (E) or c-Kit$^+$CD11b$^+$ (F) cells in BM or PB from *GFP-LC3* MLL-ENL leukemic mice. Geometric MFIs were plotted. *P* values were calculated by a Student paired *t* test. $^*P < .05$; $^{**}P < .01$. All results are pooled data from at least 3 independent experiments with 1 to 3 animals per experiment.

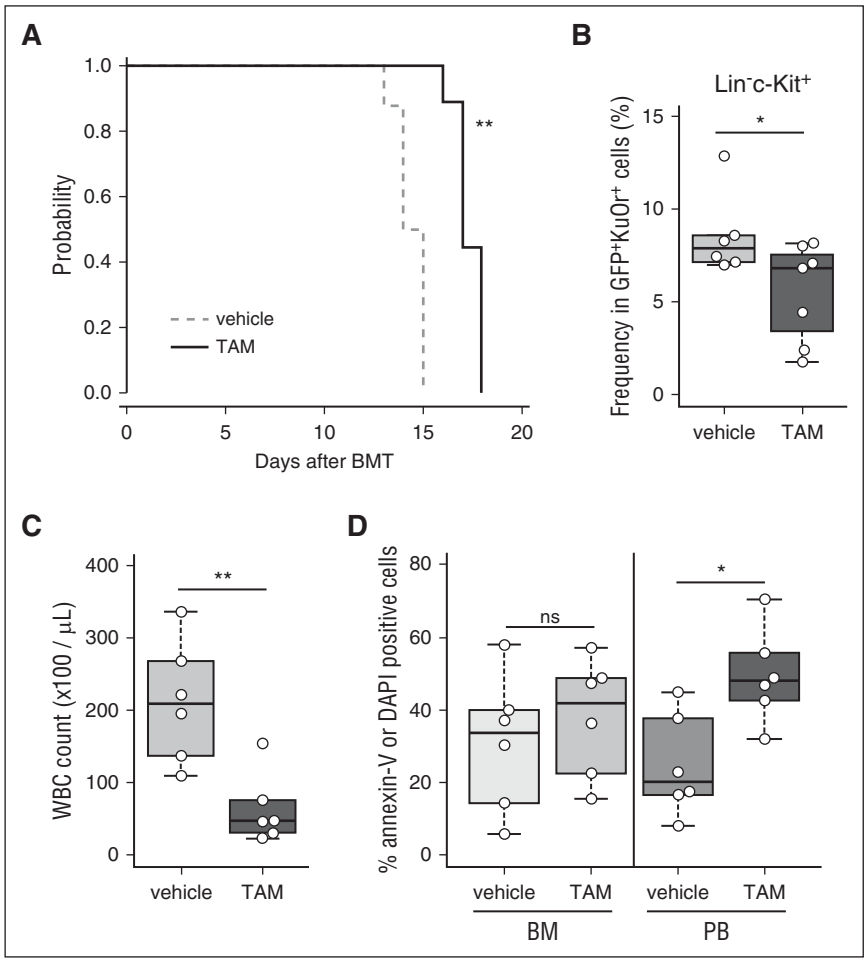

Figure 6. Requirement for autophagy in leukemia progression, stem cell maintenance, and PB leukemia cell survival in a murine CML-BC model. (A) Kaplan-Meier curves representing vehicle-treated (n = 9) and TAM-treated (n = 9) *Atg7*$^{flox/flox}$:*Cre-ER*^{T2} CML-BC mice. The *P* value was calculated by a log-rank test. (B) Frequencies of LIC fractions (GFP$^+$KusabiraOrange$^+$Lineage$^-$c-Kit$^+$) in BM from vehicle-treated (n = 6) or TAM-treated (n = 7) *Atg7*$^{flox/flox}$:*Cre-ER*^{T2} CML-BC mice were determined by flow cytometric analysis. (C) WBC counts in PB from vehicle- or TAM-treated *Atg7*$^{flox/flox}$:*Cre-ER*^{T2} CML-BC mice at day 14 after BMT were plotted (n = 6 per group). (D) Frequencies of annexin-V$^+$ or DAPI$^+$ cells in c-Kit$^-$CD11b$^+$ cells in BM or PB from vehicle- or TAM-treated *Atg7*$^{flox/flox}$:*Cre-ER*^{T2} CML-BC mice were plotted (n = 6 per group). **P* < .05; ***P* < .01. All results represent pooled data from at least 3 independent experiments with 2 to 3 animals per experiment.

drug resistance has been vigorously investigated; histone deacetylase inhibitors, mammalian target of rapamycin inhibitors, and AraC were all affected by autophagy in AML cell lines.[20-24] However, considering that the dependency on autophagy in leukemia cells is expected to be highly affected the surroundings such as the BM or PB microenvironment, it is important to evaluate if autophagy has an essential role in vivo. As expected, we revealed that in vivo treatment of AraC activated autophagy specifically in LICs, and that *Atg7* deletion potentiated the therapeutic efficacy of AraC.

An excess of leukemic blasts in the PB is known to be a sign of overflow from the BM. Our unique findings could suggest that PB leukemia cells also have leukemia-initiating capacity, which is partly regulated by autophagy. Possible explanations for the dependency on autophagy in PB leukemia cells can be derived from several environmental differences between BM and PB. These include oxygen partial pressure, cytokine concentrations, and adhesion to stromal cells, among others. These factors might render PB leukemia cells more dependent on autophagy. Taking into account the recent finding that granulocyte colony-stimulating factor–mobilized PB HSCs have enhanced autophagy and dependency on autophagy,[47] it is suggested that undifferentiated cells, which normally reside in BM, might require autophagy to manage the cellular stress of the PB environment.

In this study, we detected an enhanced number of autophagosomes in PB leukemia cells through both electron microscopy and biochemical methods. At the same time, we confirmed that the degradation of autophagic substrates was induced in PB leukemia cells through measuring GFP-LC3 expression. These results support the notion that autophagy is activated in PB leukemia cells, although further experiments by using lysosome inhibitors are still required to confirm autophagic flux.[48] Molecular mechanisms underlying the possible activation of autophagy in PB leukemia cells were not revealed by this study. Semicomprehensive reverse transcription polymerase chain reaction analysis revealed that a small number of genes were upregulated or downregulated in PB cells (greater than twofold), although expression of the majority of autophagy-associated genes was not altered (data not shown). More precise investigation of the mechanisms of altered autophagy would provide further understanding of the intratumor heterogeneity of AML.

Some issues remain before autophagy inhibitors can be applied to human AML. First, we revealed that survival in leukemic mice was slightly but significantly prolonged after Atg5/7 deletion. To determine the clinical importance of this finding, similar studies using models that have high clinical predictability, such as human AML-transplanted immunodeficient mice, are required. Second, the fact that normal HSCs also depend on autophagy (shown in previous reports) raises concern about possible toxicity of autophagy inhibition.[34,40,49] However, defects in HSCs and myeloproliferation, specifically caused by autophagy deficiency, seem to be observed 6 to 9 weeks after autophagy inactivation. In contrast, the deficiencies in LICs were detectable 1 week after TAM treatment in our study, implying that LICs might be more susceptible to autophagy inhibition than HSCs. As our study did not evaluate the effects of systemic inhibition of

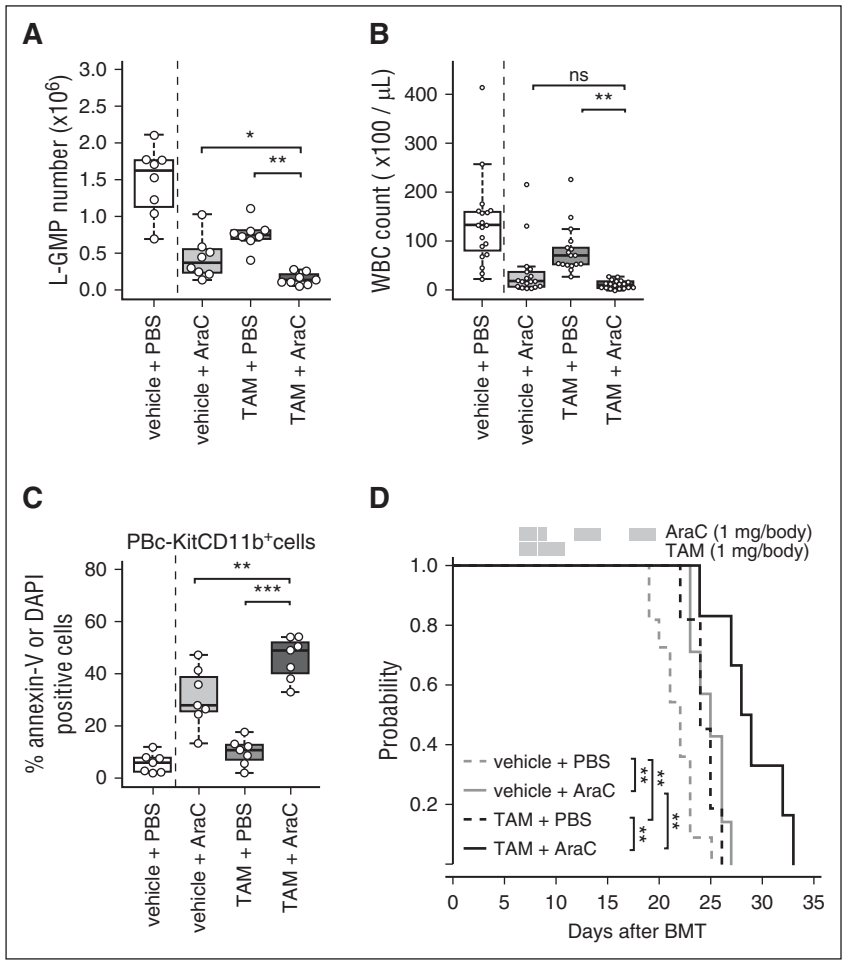

Figure 7. Combinatorial effect of AraC treatment and Atg7 deletion in MLL-ENL leukemic mice. (A) Kaplan-Meier curves of *Atg7*$^{flox/flox}$*:Cre-ER*^{T2} MLL-ENL leukemic mice treated with vehicle or TAM in combination with PBS or AraC (n = 6-11 mice per group). The *P* value was calculated by a log-rank test. (B) L-GMP numbers in GFP$^+$ BM MLL-ENL cells were plotted (n = 8 per group). (C) WBC counts in PB from *Atg7*$^{flox/flox}$*:Cre-ER*^{T2} MLL-ENL mice receiving vehicle + PBS (n = 19), vehicle + AraC (n = 18), TAM + PBS (n = 17), and TAM + AraC (n = 19) were plotted. (D) Frequencies of annexin-V$^+$ or DAPI$^+$ cells in c-Kit$^-$CD11b$^+$ PB from *Atg7*$^{flox/flox}$*:Cre-ER*^{T2} MLL-ENL mice receiving vehicle + PBS (n = 6), vehicle + AraC (n = 7), TAM + PBS (n = 7), and TAM + AraC (n = 7) were plotted. Multiple comparisons were performed by analysis of variance followed by a Student *t* test, adjusting for multiple testing using Holm's step-down method. $^{**}P < .01$; $^{***}P < .001$. All results represent pooled data from at least 2 independent experiments with 1 to 2 animals per experiment.

autophagy, further investigation is required. Third, it has recently been reported that heterozygous deletion of Atg5 results in increased leukemogenic potential in a murine BMT model with transduction of MLL-ENL.[50] In that study, it was also shown that homozygous deletion of *Atg5* induces substantial cell death in a MLL-ENL induced leukemic cell line. These results suggest that complete ablation of autophagy could inhibit tumor survival or self-renewal activity; however, mild inactivation of autophagy might enhance the malignant phenotype of MLL-ENL leukemia. Thus, small-molecule inhibitors with high efficacy might be required for successful autophagy inhibition therapy.

Despite these concerns, autophagy inactivation by small molecules including chloroquine is a promising concept for leukemia therapy. As autophagy inhibition reduced LIC frequency, with little effect on total tumor burden (as observed in this study), autophagy inhibitors might be suitable for use in combination with other conventional drugs such as AraC or anthracyclines. Furthermore, based on the higher dependency on autophagy of PB leukemia cells, treatment with autophagy inhibitors combined with stem cell mobilizer drugs like plerixafor could be a promising for antileukemia therapy.

In summary, our findings demonstrate that leukemia cells use cytoprotective autophagy for LIC maintenance, survival in PB, and AraC resistance. Even considering that normal hematopoiesis requires autophagy, this function could be a possible therapeutic target in AML and other cancers.

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Authorship

Contributions: Y.S., J.K., K.N., K.K., T.S., and M.K. designed the research; Y.S., T.T.-K, and K.M. performed the experiments; and Y.S., J.K., K.N., T.S., and M.K. wrote the manuscript.

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References

1. Mizushima N. Autophagy: process and function. *Genes Dev.* 2007;21(22):2861-2873.
2. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet.* 2009;43:67-93.
3. Choi AMK, Ryter SW, Levine B. Autophagy in human health and disease. *N Engl J Med.* 2013; 368(7):651-662.
4. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature.* 2008;451(7182):1069-1075.
5. Komatsu M, Waguri S, Ueno T, et al. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol.* 2005;169(3): 425-434.
6. Kuma A, Hatano M, Matsui M, et al. The role of autophagy during the early neonatal starvation period. *Nature.* 2004;432(7020):1032-1036.
7. Cheong H, Lu C, Lindsten T, Thompson CB. Therapeutic targets in cancer cell metabolism and autophagy. *Nat Biotechnol.* 2012;30(7):671-678.
8. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. *Nat Rev Cancer.* 2007; 7(12):961-967.
9. White E. Deconvoluting the context-dependent role for autophagy in cancer. *Nat Rev Cancer.* 2012;12(6):401-410.
10. Yang S, Wang X, Contino G, et al. Pancreatic cancers require autophagy for tumor growth. *Genes Dev.* 2011;25(7):717-729.
11. Altman BJ, Jacobs SR, Mason EF, et al. Autophagy is essential to suppress cell stress and to allow BCR-Abl-mediated leukemogenesis. *Oncogene.* 2011;30(16):1855-1867.
12. Sehgal AR, Konig H, Johnson DE, et al. You eat what you are: autophagy inhibition as a therapeutic strategy in leukemia. *Leukemia.* 2015; 29(3):517-525.
13. Takamura A, Komatsu M, Hara T, et al. Autophagy-deficient mice develop multiple liver tumors. *Genes Dev.* 2011;25(8):795-800.
14. Rosenfeldt MT, O'Prey J, Morton JP, et al. p53 status determines the role of autophagy in pancreatic tumour development. *Nature.* 2013; 504(7479):296-300.
15. Wei Y, Zou Z, Becker N, et al. EGFR-mediated Beclin 1 phosphorylation in autophagy suppression, tumor progression, and tumor chemoresistance. *Cell.* 2013;154(6):1269-1284.
16. Kroemer G, Levine B. Autophagic cell death: the story of a misnomer. *Nat Rev Mol Cell Biol.* 2008; 9(12):1004-1010.
17. Mariño G, Niso-Santano M, Baehrecke EH, Kroemer G. Self-consumption: the interplay of autophagy and apoptosis. *Nat Rev Mol Cell Biol.* 2014;15(2):81-94.
18. Amaravadi RK, Yu D, Lum JJ, et al. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *J Clin Invest.* 2007;117(2):326-336.
19. Bellodi C, Lidonnici MR, Hamilton A, et al. Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in Philadelphia chromosome-positive cells, including primary CML stem cells. *J Clin Invest.* 2009;119(5): 1109-1123.
20. Altman JK, Szilard A, Goussetis DJ, et al. Autophagy is a survival mechanism of acute myelogenous leukemia precursors during dual mTORC2/mTORC1 targeting. *Clin Cancer Res.* 2014;20(9):2400-2409.
21. Torgersen ML, Engedal N, Bøe SO, Hokland P, Simonsen A. Targeting autophagy potentiates the apoptotic effect of histone deacetylase inhibitors in t(8;21) AML cells. *Blood.* 2013;122(14):2467-2476.
22. Bosnjak M, Ristic B, Arsikin K, et al. Inhibition of mTOR-dependent autophagy sensitizes leukemic cells to cytarabine-induced apoptotic death. *PLoS One.* 2014;9(4):e94374.
23. Palmeira-dos-Santos C, Pereira GJS, Barbosa CMV, Jurkiewicz A, Smaili SS, Bincoletto C. Comparative study of autophagy inhibition by 3MA and CQ on Cytarabine-induced death of leukaemia cells. *J Cancer Res Clin Oncol.* 2014; 140(6):909-920.
24. Zhao X, Liu J, Peng M, Liu J, Chen F. BMP4 is involved in the chemoresistance of myeloid leukemia cells through regulating autophagy-apoptosis balance. *Cancer Invest.* 2013;31(8): 555-562.
25. Hara T, Nakamura K, Matsui M, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature.* 2006;441(7095):885-889.
26. Mizushima N, Yamamoto A, Matsui M, Yoshimori T, Ohsumi Y. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol Biol Cell.* 2004;15(3):1101-1111.
27. Ruzankina Y, Pinzon-Guzman C, Asare A, et al. Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss. *Cell Stem Cell.* 2007;1(1):113-126.
28. Arai S, Yoshimi A, Shimabe M, et al. Evi-1 is a transcriptional target of mixed-lineage leukemia oncoproteins in hematopoietic stem cells. *Blood.* 2011;117(23):6304-6314.
29. Takeshita M, Ichikawa M, Nitta E, et al. AML1-Evi-1 specifically transforms hematopoietic stem cells through fusion of the entire Evi-1 sequence to AML1. *Leukemia.* 2008;22(6):1241-1249.
30. Kitamura T, Koshino Y, Shibata F, et al. Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. *Exp Hematol.* 2003;31(11):1007-1014.
31. Reed SE, Staley EM, Mayginnes JP, Pintel DJ, Tullis GE. Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors. *J Virol Methods.* 2006; 138(1-2):85-98.
32. Krivtsov AV, Twomey D, Feng Z, et al. Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature.* 2006;442(7104):818-822.
33. Somervaille TC, Cleary ML. Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. *Cancer Cell.* 2006;10(4):257-268.
34. Liu F, Lee JY, Wei H, et al. FIP200 is required for the cell-autonomous maintenance of fetal hematopoietic stem cells. *Blood.* 2010;116(23):4806-4814.
35. Mortensen M, Soilleux EJ, Djordjevic G, et al. The autophagy protein Atg7 is essential for hematopoietic stem cell maintenance. *J Exp Med.* 2011;208(3):455-467.
36. Sánchez-Aguilera A, Arranz L, Martín-Pérez D, et al. Estrogen signaling selectively induces apoptosis of hematopoietic progenitors and myeloid neoplasms without harming steady-state hematopoiesis. *Cell Stem Cell.* 2014;15(6):791-804.
37. Higashi AY, Ikawa T, Muramatsu M, et al. Direct hematological toxicity and illegitimate chromosomal recombination caused by the systemic activation of CreERT2. *J Immunol.* 2009; 182(9):5633-5640.
38. Shvets E, Fass E, Elazar Z. Utilizing flow cytometry to monitor autophagy in living mammalian cells. *Autophagy.* 2008;4(5):621-628.
39. Guan JL, Simon AK, Prescott M, et al. Autophagy in stem cells. *Autophagy.* 2013;9(6):830-849.
40. Mortensen M, Ferguson DJP, Edelmann M, et al. Loss of autophagy in erythroid cells leads to defective removal of mitochondria and severe anemia in vivo. *Proc Natl Acad Sci USA.* 2010; 107(2):832-837.
41. Pua HH, Dzhagalov I, Chuck M, Mizushima N, He Y-W. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. *J Exp Med.* 2007; 204(1):25-31.
42. Sandoval H, Thiagarajan P, Dasgupta SK, et al. Essential role for Nix in autophagic maturation of erythroid cells. *Nature.* 2008;454(7201):232-235.
43. Dash AB, Williams IR, Kutok JL, et al. A murine model of CML blast crisis induced by cooperation between BCR/ABL and NUP98/HOXA9. *Proc Natl Acad Sci USA.* 2002;99(11):7622-7627.
44. Sato T, Goyama S, Kataoka K, et al. Evi1 defines leukemia-initiating capacity and tyrosine kinase inhibitor resistance in chronic myeloid leukemia. *Oncogene.* 2014;33(42):5028-5038.
45. Simsek T, Kocabas F, Zheng J, et al. The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. *Cell Stem Cell.* 2010;7(3):380-390.
46. Škrtić M, Sriskanthadevan S, Jhas B, et al. Inhibition of mitochondrial translation as a therapeutic strategy for human acute myeloid leukemia. *Cancer Cell.* 2011;20(5):674-688.
47. Leveque-El Mouttie L, Vu T, Lineburg KE, et al. Autophagy is required for stem cell mobilization by G-CSF. *Blood.* 2015;125(19):2933-2936.
48. Klionsky DJ, Abdelmohsen K, Abe A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy.* 2016;12(1):1-222.
49. Warr MR, Binnewies M, Flach J, et al. FOXO3A directs a protective autophagy program in haematopoietic stem cells. *Nature.* 2013; 494(7437):323-327.
50. Watson AS, Riffelmacher T, Stranks A, et al. Autophagy limits proliferation and glycolytic metabolism in acute myeloid leukemia. *Cell Death Dis.* 2015;1:15008.