**BECN1 modulates hematopoietic stem cells by targeting Caspase-3-GSDME-mediated pyroptosis**

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**Abstract**

Hematopoietic stem cells (HSCs) maintain the blood system throughout the lifespan. However, the molecular mechanism maintaining HSC character remains not fully understood. In this study, we observed that the targeted deletion of Becn1 disrupts the blood system and impairs the reconstitution capacity of HSCs. Interestingly, Becn1 deletion did not lead to dysfunction of autophagy in HSCs, indicating a non-classical role of BECN1 in regulating HSCs function. While we observed the increase of Caspase-3-GSDME-mediated pyroptosis in Becn1 deficient hematopoietic stem and progenitor cells. Forced expression of the full-length GSDME compromises the function of HSCs. In brief, we identified a novel role of Becn1 in modulating HSCs by regulating pyroptosis, but not through autophagy. This study provides a new link between BECN1-Caspase-3-GSDME signaling and HSC maintenance.

**Keywords:** Autophagy, Becn1, Cell death, Hematopoietic stem cell, Pyroptosis

1. **INTRODUCTION**

Hematopoietic stem cells (HSCs) are a rare but long-lived population of blood cells.\(^1\) HSCs lie on the top of the blood system and give rise to all lineages of blood cells throughout the lifespan. Since the life-span of most mature blood cells are limited, maintenance of blood homeostasis almost relies on the self-renewal and differentiation ability of HSCs.\(^1\) HSCs locate far from the blood vessel,\(^2,3\) a niche relative shortage of nutrient and oxygen, and are metabolically inactive, which is considered to be indispensable for maintaining of HSCs.\(^4,8\) Disrupting the inactive metabolic state by targeting pyruvate dehydrogenase kinase (Pdk) reduced glycolysis, quiescence and reconstitution ability of HSC.\(^7\) While Pdk deletion was reported to inhibit the Acetyl-coenzyme A (AcCoA) depletion, both of which were involved in starvation-induced macroautophagy (hereafter called autophagy).\(^3\) Transcription factor FOXO3A protected HSCs to survive from metabolic stress via autophagy promotion. Autophagy gene Atg12 helped HSC to stay an inactive metabolic and functional state by metabolically activated mitochondria removing.\(^8\) Thus, under a specific niche environment, autophagy plays an important role in keeping the stemness of HSCs by metabolic modulation.

Autophagy is a highly conserved process of catabolism induced by various cellular stress,\(^10\) and plays an important role in maintaining the function of lots of stem cells, including HSCs.\(^1\) Autophagy is mediated by series of evolution-conserved autophagy-related (ATG) genes, such as Ulk1, FIP200, Becn1, Atg9a, Atg14, Atg5, Atg7, and Atg12.\(^11\) Previous reports showed that FIP200, which participates in autophagy initiation, was required for fetal HSCs maintenance.\(^12\) ATG5, ATG7, and ATG12, which are involved in autophagy vesicle elongation, are essential in maintaining adult HSCs.\(^8,13,14\) Becn1, one of the first identified autophagy genes found in mammalian and an ortholog of Atg6 in yeast, participates in phagophore nucleation, expansion, and also the non-autophagic process which is endosome maturation.\(^11,15,16\) Becn1 is unique among the ATGs because of its autophagy-independent function.\(^17\) Becn1 contains a Bcl-2-homology-3 (BH3)-only domain,\(^18\) a domain that usually plays a role in mediating cell death and recent studies revealed that Becn1 is involved in programmed cell death.\(^19,22\) while the function of Becn1 in HSCs has not yet been investigated.

In this study, we investigated the function of BECN1 in HSC maintenance by specifically knocking out Becn1 in hematopoietic cells, and we observed that specific deficiency of Becn1 resulted in disturbed homeostasis of the blood system and Becn1 deficient HSCs displayed compromised reconstitution capacity by activating Caspase-3-GSDME signaling, but not through regulating autophagy. Together, this study elucidates the mechanism of BECN1 in modulating HSC function and raises an intriguing connection between BECN1 and GSDME-mediated pyroptosis. It serves as a reference for future research on how autophagy-related genes regulate stem cell function.
2. RESULTS

2.1. Beclin deficiency results in a disturbed hematopoietic system

We crossed Beclin<sup>Becn1<sup>flox/flox</sup></sup> mice with Vav-iCre mice and obtained the mice with the specific deficiency of Beclin in the blood system: Beclin<sup>Becn1<sup>flox/flox;Vav-iCre</sup></sup> (hereafter named: Beclin<sup>Becn1<sup>ko</sup></sup>). Beclin<sup>Becn1<sup>ko</sup></sup> mice exhibited a significantly shorter lifespan compared to WT control mice in the same cohort (Fig. 1A). Moreover, there were more red blood cells (RBC) with a reduction of mean corpuscular volume (MCV) and an elevation of red blood cell distribution width (RDW-CV) in Beclin<sup>Becn1<sup>ko</sup></sup> mice (Fig. 1B and C), but the amount of hemoglobin in Beclin<sup>Becn1<sup>ko</sup></sup> mice decreased when it was compared with that in WT mice (Fig. 1C), indicating that Beclin deficiency results in microcytic hypochromic anemia even though it had enhanced erythropoiesis in Beclin<sup>Becn1<sup>ko</sup></sup> mice. Meanwhile, the deficiency of Beclin affected the production of platelets (Fig. 1B). Next, we evaluated the lineage distribution in peripheral blood and bone marrow of Beclin<sup>Becn1<sup>ko</sup></sup> mice and observed no difference in peripheral blood (Fig. 1D), but less myeloid cells in the bone marrow of Beclin<sup>Becn1<sup>ko</sup></sup> mice compared to control mice (Fig. 1E).

Given that hematopoietic stem and progenitor cells (HSPCs) are the sources of all blood cells, we then investigated the HSPCs in bone marrow (gating strategy was shown in Sup. Fig. 1A, http://links.lww.com/BS/A10). The total cell number of bone marrow did not change (Sup. Fig. 1B, http://links.lww.com/BS/A10), while the number of hematopoietic stem and progenitor cells (HSCs: LT-HSC) and MEP cells were increased in the spleen of Beclin<sup>Becn1<sup>ko</sup></sup> mice (Fig. 1B and C), but the percentage of hematopoietic stem cells (long-term HSC: LT-HSC), HSPCs (Lin<sup>-</sup>/Sca-1<sup>-</sup>/c-Kit<sup>-</sup>): LSK) and common lymphoid progenitors (CLP) were decreased in Beclin<sup>Becn1<sup>ko</sup></sup> mice (Fig. 1B and C), and the number of megakaryocyte-erythroid progenitor (MEP) cells were increased in the spleen of Beclin<sup>Becn1<sup>ko</sup></sup> mice (Fig. 1B and C). The number of LT-HSCs in Beclin<sup>Becn1<sup>ko</sup></sup> mice was reduced, while the number of ST-HSCs (short-term HSCs) did not show any obvious change (Fig. 1H), indicating that the LT-HSC is disturbed by Beclin<sup>Becn1</sup> loss.

Given that spleen is the primary site of stress erythropoiesis in mice with anemia, we then investigated the spleen and found that spleens were significantly larger with an increase of mean corpuscular volume (MCV) and an elevation of red blood cell distribution width (RDW-CV) in Beclin<sup>Becn1<sup>ko</sup></sup> mice (Fig. 1B and C), but the percentage of hematopoietic stem cells (long-term HSC: LT-HSC), HSPCs (Lin<sup>-</sup>/Sca-1<sup>-</sup>/c-Kit<sup>-</sup>): LSK) and common lymphoid progenitors (CLP) were decreased in Beclin<sup>Becn1<sup>ko</sup></sup> mice (Fig. 1F and G), the percentage of megakaryocyte-erythroid progenitor (MEP) cells were increased and the percentage of other progenitors remained static (Fig. 1F and G). The increase of MEP cells was consistent with the increase of RBC in peripheral blood of Beclin<sup>Becn1<sup>ko</sup></sup> mice (Fig. 1B and C). The number of LT-HSCs in Beclin<sup>Becn1<sup>ko</sup></sup> mice was reduced, while the number of ST-HSCs (short-term HSCs) did not show any obvious change (Fig. 1H), indicating that the LT-HSC is disturbed by Beclin<sup>Becn1</sup> loss.

2.2. Beclin deficiency impairs HSCs

Given that HSCs generate all blood cells throughout the lifespan, we then start to investigate the function of Beclin deficient HSCs. HSCs freshly isolated from Beclin<sup>Becn1<sup>ko</sup></sup> mice were competitively transplanted into lethally irradiated recipient mice together with 2.5 × 10<sup>7</sup> competitor cells and the chimerism of peripheral blood of recipients was evaluated every 4 weeks until the 4th month (Fig. 2A). The result showed that the reconstitution capacity of Beclin<sup>Becn1<sup>ko</sup></sup> HSCs was severely impaired (Fig. 2B), which was manifested by the reduction of all three lineages (Fig. 2B; gating strategy was shown in Sup. Fig. 2A, http://links.lww.com/BS/A11). The donor-derived lineage distribution in peripheral blood revealed that Beclin<sup>Becn1<sup>ko</sup></sup> HSCs display significantly differentiation bias towards T lineage and reduced myeloid potential at the end of the 4th month after transplantation (Fig. 2C; gating strategy was shown in Sup. Fig. 2B, http://links.lww.com/BS/A11). Moreover, analysis of the bone marrow of recipients revealed a striking decrease in the number of Beclin<sup>Becn1<sup>ko</sup></sup>-derived HSCs and LSK cells (Fig. 2D and E; gating strategy was shown in Sup. Fig. 2C, http://links.lww.com/BS/A11), indicating that Beclin<sup>Becn1</sup> deficiency results in the loss of self-renewal capacity of HSCs.

The previous study revealed that loss of quiescence of HSCs resulted in the reduction of self-renewal capacity, we then sought to investigate the cell cycle status of HSCs and found that Beclin<sup>Becn1<sup>ko</sup></sup> HSCs were more active with an increased percentage at G<sub>1</sub> and S/G2/M stage but reduced percentage at G<sub>0</sub> stage (Fig. 2F and G), indicating that BECN1 might be a positive regulator of HSC quiescence.

The impairment of reconstitution and self-renewal ability of Beclin<sup>Becn1<sup>ko</sup></sup>-derived HSCs along with the reduction of HSC cell number in the steady state indicates that BECN1 plays an important role in maintaining HSCs.

2.3. Dysfunction of BECN1 in HSPCs results in activated pyroptosis

BECN1 is a BH3-only protein, and other BH3-containing proteins like Bid, Bad, Bim, Noxa, and PUMA play a role in programmed cell death, and that damaged HSCs underwent apoptosis or repaired once entering cell cycle, and that the frequency of HSCs in Beclin<sup>Becn1<sup>ko</sup></sup> mice was significantly decreased (Fig. 1F), and that Beclin deficient HSCs lost quiescence (Fig. 2F), it is conceivable that Beclin deficient HSCs may suffer from the stress of programmed cell death or relevant pathway(s). To test this hypothesis, we seeded 8000 LSK cells isolated from WT and Beclin<sup>Becn1<sup>ko</sup></sup> mice, and cell viability was evaluated 24h later by Fluorescence-activated cell sorting (FACS). The result showed that the percentage of apoptotic cells, which was represented as Annexin V<sup>-</sup>/PI<sup>-</sup>, remained static between WT and Beclin deficient LSK cells (Fig. 3A and B), but the percentage of necrotic cells, which was represented as Annexin V<sup>-</sup>/PI<sup>-</sup>, was significantly higher in Beclin deficient LSK cells (Fig. 3A and B), the increase of necrotic cell death in Beclin deficient LSK cells was also confirmed by DAPI uptake (Fig. 3C), suggesting that Beclin deficient HSPCs underwent cell death rapidly upon proliferation stress. To identify the gene(s) leading to the death of Beclin deficient HSPCs, we evaluated three classical cell death-related pathways, including apoptosis (wherein Caspase-3 is the main player), necroptosis (wherein RIPK1-RIPK3-MITF are the main players), and pyroptosis (wherein GSDMD and GSDME are the main well studied players). We conducted western blotting assays by using fresh Beclin<sup>Becn1<sup>ko</sup></sup> LSK cells and WT c-Kit<sup>+</sup> bone marrow cells to evaluate the expression of Caspase-3, RIPK3, MLKL, phosphorylated MLKL (hereafter named p-MLKL), GSDMD, and GSDME. The results revealed that RIPK3, MLKL, p-MLKL, and GSDMD remained static in Beclin<sup>Becn1<sup>ko</sup></sup> derived HSCs along with the reduction of HSC cell number in the steady state indicates that BECN1 plays an important role in maintaining HSCs.
Figure 1. Beclin 1 deficiency impairs homeostasis of the blood system. (A) Kaplan–Meier survival plot depicts survival curves for Beclin1	extsuperscript{−/−} mice (n = 53) and WT mice (n = 37). (B and C) These histograms exhibit the complete blood cell counts of peripheral blood samples from WT and Beclin1	extsuperscript{−/−} mice, including RBC (red blood cell), WBC (white blood cell), Neu (neutrophil), Lym (lymphoid cell), PLT (platelet) (B), HGB (hemoglobin), MCV (mean corpuscular volume), RDW (red cell distribution width) (C). Data are shown as mean ± SD, n = 5 mice per group. (D and E) This histogram shows the lineage distribution of peripheral blood (D) and bone marrow (E) for WT and Beclin1	extsuperscript{−/−} mice, including T cells (CD3	extsuperscript{+}), B cells (B220	extsuperscript{+}), and myeloid cells (CD11b	extsuperscript{+}). Data are shown as mean ± SD, n = 5 mice per group. (F and G) The histograms display the number of HSPCs per million bone marrow cells of WT and Beclin1	extsuperscript{−/−} mice, including LTHSC (long term-HSC, CD34	extsuperscript{−}/Flt3	extsuperscript{−}/LSK), ST-HSC (short term-HSC, CD34	extsuperscript{−}/Flt3	extsuperscript{−}/LSK), LSK (Lin	extsuperscript{−}/Sca1	extsuperscript{−}/c-Kit	extsuperscript{−}) (F), MPP (multipotent progenitor, CD34	extsuperscript{−}/Flt3	extsuperscript{−}/LSK), CLP (common lymphoid progenitor, CD127	extsuperscript{−}/Flt3	extsuperscript{−}/LSK), CMP (common myeloid progenitor, Lin	extsuperscript{−}/Sca1	extsuperscript{−}/c-Kit	extsuperscript{−}/CD34	extsuperscript{−}/CD16/32	extsuperscript{−}) and MEP (megakaryocyte/erythroid progenitor, Lin	extsuperscript{−}/c-Kit	extsuperscript{−}/Sca1	extsuperscript{−}/CD34	extsuperscript{−}/CD16/32	extsuperscript{−}) (G) (n = 5 mice per group). Data are shown as mean ± SD, n = 5 mice per group.
Figure 2. Becn1 deficiency reduces HSC reconstitution. (A–C) Freshly isolated 50 HSCs from WT or Becn1\textsuperscript{vKO} mice were transplanted into lethally irradiated recipients together with 2.5 × 10^5 competitor cells. Chimerism in peripheral blood was evaluated every month until the fourth month post transplantation (Tx). (A) The schematic diagram showing the experimental design for HSC competitive transplantation. (B) The line plots showing donor chimerism in overall (CD45.2\textsuperscript{+}), T (CD3\textsuperscript{+}), B (B220\textsuperscript{+}) and myeloid (CD11b\textsuperscript{+}) cell every month after HSC transplantation (HSCT) (n = 6 for WT and 5 for Becn1\textsuperscript{vKO} group). The gating strategy to generate these line plots is presented in sup. Fig. 2A, http://links.lww.com/BS/A11. (C) This histogram displays the lineage distribution of donor-derived peripheral blood at the fourth month after transplantation (n = 6 for WT and 5 for Becn1\textsuperscript{vKO} group). Donor chimerism in the cell recovery of HSCs is presented in sup. Fig. 2B, http://links.lww.com/BS/A11. (D and E) Representative flow cytometry plots (D) and scatter plot (E) showing donor-derived LSK and HSC engraftment in recipient bone marrow at the 4th month post-HSCT transplantation (n = 6 for WT and 5 for Becn1\textsuperscript{vKO} group). Data are shown as mean ± SD. The gating strategy of LSK and HSC engraftment is presented in sup. Fig. 2C, http://links.lww.com/BS/A11. (F and G) The histogram (F) and representative flow cytometry plots (G) display the cell cycle analysis of WT and Becn1\textsuperscript{vKO} HSCs. n = 5 mice per group, data are shown as mean ± SD.
Figure 3. Beclin 1-deficient HSCs show increased GSDME-mediated pyroptosis. (A–C) Representative flow cytometry plots (A) and histograms (B and C) showing cell viability of LSKs from WT and Beclin1+/− mice. Freshly isolated LSKs were cultured for 24 h before cell viability analysis by Annexin V (A and B) or DAPI (C). n = 3 repeats per group, data are shown as mean ± SD. (D) Representative western blot showing the level of RIPK3, MLKL, pMLKL, GSDMD, GSDME, and Caspase-3 (CASP3) in freshly isolated hematopoietic progenitor (c-Kit+) cells from WT and Beclin1+/− mice. Cell lysates were subjected to immunoblot analysis using indicated antibodies. pMLKL, phosphorylated MLKL; GSDME-FL, full-length GSDME; GSDME-N, the N-terminal product of GSDME. (E) Representative western blot showing the level of RIPK3, MLKL, GSDME, and Caspase-3 in HSCs (CD34+/c-Kit−) from WT and Beclin1+/− mice. Freshly isolated HSCs were cultured for 8 days. Cell lysates were subjected to immunoblot analysis using indicated antibodies. (F) Representative western blot showing the activation of Caspase-3 and GSDME.
respective points after transplantation. Data are shown as mean ± SD, n=5 mice per group. (K) Model for regulation of BECN1 in HSCs cell death. (L) Western blot showing GSDME overexpression in LSKs. Freshly isolated 10^5 LSKs were infected with the full-length GSDME-cDNA or control vector for 3 days. Cell lysates were subjected to western blot analysis without further isolation. Cell lysates were subjected to western blot using indicated antibodies. (M) The line plots depict changes in peripheral blood chimerism of donor-derived cells (CD45.2) in recipients at the indicated time points after transplantation. Data are shown as mean ± SD, n=5 mice per group. (N) Model for regulation of BECN1 in HSCs cell death.

following apoptotic drug treatment. WT c-Kit+ cells were treated with ABT263 (10 μM) plus S63845 (10 μM) or mock treated for 5 h. Cell lysates were subjected to immunoblot analysis using indicated antibodies. (G) Representative western blot showing the level of Caspase-3 and GSDME in Caspase-3-shRNA or non-target control (NTC) shRNA infected c-Kit+ cells with or without apoptosis induction. WT c-Kit+ cells were infected with lentivirus carrying an NTC shRNA or a Caspase-3-shRNA lentivirus for 3 days and then treated with ABT263 (10 μM) plus S63845 (10 μM) or mock treated for 5 h. The infection rate was around 90% and the total cell population was collected for western blot analysis without further isolation. Cell lysates were subjected to immunoblot using indicated antibodies. (H) Representative western blot showing GSDME overexpression in LSKs. Freshly isolated 10^5 LSKs were infected with the full-length GSDME-cDNA or control vector for 3 days. Cell lysates of the total cell population were subjected to western blot using indicated antibodies. (I and J) 40,000 mCherry+ cells were isolated from full-length GSDME-flux of c-Kit+ cells from WT and BECN1vKO mice by using the conversion ratio of LC3-II using western blot, wherein LC3-II accumulates significantly on chloroquine treatment, which is a lysosome inhibitor, and the relative ratio of LC3-II to the loading controls such as actin between chloroquine treated and non treated group represents the autophagy flux of a sample.45 We then treated c-Kit+ bone marrow cells of WT and BECN1vKO mice with chloroquine and the transition of LC3-II was evaluated 4 h later. The result displayed that the transition of LC3-II was not reduced in BECN1vKO c-Kit+ cells compared to WT control (Fig. 4Aa). To further test the above result, we measured the autophagic activity of LSK cells from WT and BECN1vKO mice by using Cyto-ID dye, which was an amphilic tracer and a relative specific dye to detect autophagy level in live cells.50,51 This method was first validated on BM cells treated with the autophagy inducer rapamycin as well as the autophagy inhibitor chloroquine (Sup. Fig. 4A, http://links.lww.com/BloodA13), while the result on LSKs suggested that the transient autophagic level showed no difference between WT and BECN1vKO LSKs (Fig. 4B). To detect the autophagic degradation activity, LSKs were subjected to chloroquine and the autophagic flux was
measured. Consistently, the autophagic flux did not change in Beclin1 deficient LKSs (Fig. 4C).

A recent study developed a sensitive approach to measure autophagy flux in primary cells.52 In this system, GFP-LC3-RFP-LC3D-G protein can be cleaved into GFP-LC3 and RFP-LC3D-G fragments, in which GFP-LC3 can participate and be degraded in normal autophagy process, but RFP-LC3D-G stays in the cytosol as a relatively stable internal control, thus the mean fluorescence intensity (MFI) of RFP to GFP ratio represents the autophagic flux.52 We produced lentivirus expressing GFP-LC3-RFP-LC3D-G and infected target cells for autophagic flux measurement. First of all, we validate the sensitivity of this approach on 293T and EL4 cells by rapamycin treatment (Sup. Fig. 4B, http://links.lww.com/BS/A13). Then we infected WT and Beclin1vKO LSK cells. Three days later, the autophagic flux was measured by FACS and the result shows that no difference was observed between WT and Beclin1 deficient HSCs (CD48+/c-Kit+Sca-1+) (Fig. 4D and E, the calculation method was shown in Sup. Fig. 4C, http://links.lww.com/BS/A13).

Taken together, our study shows that Beclin1 regulates the function of HSCs through Caspase-3-GSDME signaling, but not autophagy. Loss of BECN1 in HSCs leads to the activation of GSDME-mediated pyroptosis which directly results in HSC death and finally results in HSC dysfunction. Our study uncovered that BECN1 maintains HSC by acting as a negative regulator of GSDME-mediated pyroptosis.

3. DISCUSSION

Although Beclin1 is a classic gene in the field of autophagy, its role in autophagy is controversial. Some studies have reported that Beclin1 is necessary for autophagy,53,54 but different studies were showing that some specific substances, such as cis-saturated,46 arsenic trioxide, and resveratrol,44,55 were able to induce autophagy in Beclin1 dysfunctional cells. In this study, we investigated the role of Beclin1 in HSC maintenance and found that targeted deletion of Beclin1 impaired HSCs by activating CASP3-GSDME-mediated pyroptosis, but not through autophagy. In addition to direct evidence supporting this conclusion (Fig. 4A-D), two previous reports revealed that the lifespan of mice with targeted deletion of Atg5 or Atg7 in the blood system was only about 3 months.13,14 However, our data shows that the lifespan of hematopoietic-specific deletion of Beclin1 mice is much longer (Fig. 1A). These data may suggest that Beclin1 plays a role...
in the blood system different from that of other canonical autophagy genes such as Atg5 and Atg7. Moreover, we observed that Becn1 deficient HSCs lost quiescence, and underwent GSDME-mediated pyroptosis. The possible explanation could be that Becn1 deficient HSCs are activated to replenish the loss caused by pyroptosis, eventually leading to the disturbed homeostasis of the blood system (Fig. 1B–K). Both CASP3 and GSDME were activated in Becn1 deficient HSPCs (Fig. 3D and E), but pyroptosis rather than apoptosis was activated (Fig. 3A–C). The possible reason is that HSC’s characteristics determine the occurrence of pyroptosis rather than apoptosis in response to Becn1 dysfunction. HSCs are at a low metabolic state and mainly rely on glycolysis for energy supply, which is a quick but inefficient manner even though it can offer a survival advantage under hypoxic conditions. Activated HSCs increase the demands for ATP and further decrease their ATP level. Apoptosis is a form of well-programmed cell death by forming apoptotic bodies, and it requires energy to complete this process. GSDME-mediated pyroptosis is a kind of necrosis, and might demand little or no energy. Earlier studies showed that cells with a high ATP level died of apoptosis while necrosis with ATP depletion, thus it is the ATP level inside a cell that matters for cell death decision. Therefore, damaged HSCs with low energy inside would prefer to die in an energy-saving way. Since more HSCs are activated and enter cell cycle upon Becn1 loss, which increases the ATP demands and decreases the internal ATP level of HSCs, these damaged and activated HSCs would probably prefer to die of necrosis under the proliferation stress since the energy shortage. GSDME-mediated pyroptosis might fit better for Becn1 deficient HSCs than apoptosis in terms of energy. A previous study has shown that cells with a high GSDME level underwent pyroptosis but not apoptosis after chemotherapy drug treatment since pyroptosis was “faster” than apoptosis, which correlated well with our discovery on the death mode preference. This death preference might also be controlled by cell energy levels inside.

Pyroptosis is a kind of lytic cell death and can release immunogenic cell contents, including damage-associated molecular patterns (DAMPs), which could prevent the malignant transformation of HSCs. Both Becn1 and GSDME are reported tumor suppressors. Becn1 heterozygous mice show an increased incidence of lymphomas, leukemias, and liver cancers, and 40% to 75% of prostate, breast, and ovarian cancers bear the missense deletion of Becn1. Gsdme silencing was found in cancers of the breast, gastro, and colorectum. The loss of function of Becn1 alone in HSC might be tumorigenic. To avoid tumor, GSDME-mediated pyroptosis was activated in Becn1 deficient HSCs and led to rapid cell death with the release of DAMPs. Thus, the activation of GSDME-mediated pyroptosis may be a tissue-protective mechanism used by HSPCs to prevent malignancy after Becn1 loss. This hypothesis may also be true for other tissues, for example, the prostate or breast does not express GSDME, while tumor in these tissues is highly associated with Becn1 monoallelic deletion.

While there are some limitations to this study. First, autophagy is sensitive to environmental stimulation, since the technical limitation, we could not detect autophagy activity of fresh HSC in a relatively short time from mice, even by using the most sensitive method in measuring basal autophagic activity at present. The transgenic mice expressing GFP-LC3-RFP-LC3 and G combined with other HSC-specific reporter mice are necessary to be done to settle this problem. Second, since the low percentage of cells suffering from pyroptosis at one time, we could not provide a photograph to see the HSCs form Becn1flox/flox mice undergoing pyroptosis directly. Third, we only showed loss of function of HSCs with more GSDME activation, the detailed mechanism of GSDME-mediated pyroptosis pathway in HSC modulation needs to be studied further. Finally, as a well-studied autophagic related gene, we have only focused on the autophagic roles of Becn1, while its functions in the endocytic process of HSC is worth studying further.

In conclusion, our study raises the connection between GSDME-mediated pyroptosis and HSCs cell fate modulation, which gives us a new sight for understanding Becn1 in the homeostasis of the blood system. While how does the GSDME pathway modulate HSC cell fate in detail, and whether is activated Caspase-3 the only proteinase to cleave and activate GSDME upon Becn1 loss? How does Becn1 deficiency lead to Caspase-3 activation and GSDME-mediated pyroptosis? Is it shared by other autophagy genes or Becn1-specific? These questions need to be studied further. The non-canonical autophagy machinery and its physiological function are also worth discussion in future investigation.

4. MATERIALS AND METHODS

4.1. Animals

Becn1lox/lox (CD45.2, #028794), Vav-iCre (CD45.2), C57BL/6 (CD45.2), C57BL/6-JL (CD45.1), and CD45.1/2 mice. Becn1lox/lox mice were purchased from Jackson Laboratory and were crossed with Vav-iCre mice to obtain Becn1 specific deleted mice in the blood system. CD45.1/2 mice were obtained by crossing CD45.1 and CD45.2 mice. Mice were all C57BL/6 background and maintained in specific pathogen-free (SPF) animal facility. Mice of both genders were used and all experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Tsinghua University.

4.2. Antibodies for flow cytometry

The fluorescence conjugated antibodies were used for flow cytometry: anti-c-Kit-APC (1:100, eBioscience, 17–1171–82), anti-APC-microbeads (1:50, Miltenyi Biotec, 130–090–855), Streptavidin-APC-Cy7 (1:100, BioLegend, 405208), Streptavidin-PerCP-Cy5.5 (1:100, BD Biosciences, 551419), anti-ScA-1-PE-Cy7 (1:100, BD Biosciences, 558162), anti-CD115-PE (1:100, BioLegend, 115904), anti-CD150-Brilliant Violet 605 (1:100, BioLegend, 115927), anti-CD34-FTTC (1:25, eBioscience, 11–0341–81), anti-CD34-AF700 (1:25, eBioscience, 56–0341–82), anti-Flt3-PE-CF594 (1:100, BD Biosciences, 562537), anti-CD16-32-FITC (1:100, eBioscience, 11–0161–85), anti-CD127-Brilliant Violet 421 (1:100, BioLegend, 135024), anti-CD48-PerCP-Cy5.5 (1:100, BioLegend, 103422), anti-CD3-APC (1:300, BioLegend, 100312), CD4-PerCP-Cy5.5 (1:300, BioLegend, 100434), anti-CD8-FTTC (1:300, BioLegend, 100706), anti-B220-V500 (1:300, BD Biosciences, 561226), anti-B220-FTTC (1:300, BioLegend, 103206), anti-B220-Fluor 700 (1:300, BioLegend, 103232), anti-B220-PE (1:700, BioLegend, 103227), anti-CD11b-PerCP-Cy5.5 (1:300, BioLegend, 101228), anti-CD11b-APC-eFluor780 (1:300, eBioscience, 47–0112–82), anti-CD11b-PE-Cy7 (1:300, BioLegend, 101216), anti-Ter119-FITC (1:250, BioLegend, 116215), anti-CD45.1-FTTC (1:300, BioLegend, 110706), anti-CD45.1-Alexa Fluor 700 (1:300, BD Biosciences, 561235), anti-CD45.2-PE (1:300, BioLegend, 109808) and anti-CD45.2-FTTC (1:300, BioLegend, 109806). The biotin labeled lineage antibodies were from BioLegend: anti-Ter-119 (116204), anti-CD3 (100244), anti-CD4 (100508), anti-CD8 (100704), anti-B220 (103204), anti-CD11b (101204), and anti-Gr-1 (108404).
4.3. Complete blood cell counts

Peripheral blood samples were obtained from the tail using EDTA-containing tubes and performed by an automatic hematology analyzer BC-5000 (Mindary).

4.4. Cell sorting and flow cytometry

Bone marrow cells were isolated by crushing tibia, femur and pelvic bones (with spines for some experiments) in D-HANK’s buffered saline solution containing 2% fetal bovine serum (FBS), 1% HEPES and 50 U/mL penicillin/streptomycin (HBSS+), and filtered through 100 μm nylon strainer. For hematopoietic stem and progenitor (c-Kit+) cells enrichment, bone marrow cells were stained with c-Kit-APC followed by anti-APC-microbeads and MACS Separation LS Columns from Miltenyi Biotec. For HSC sorting, c-Kit+ cells were stained with Streptavidin-APC-Cy7, Sca-1-PE-Cy7, c-Kit-APC, CD150-PE, CD34-FITC following the biotin-labeled lineage antibodies against Ter-119, CD3, CD4, CD8, B220, CD11b, and Gr-1. For LSK cells sorting, c-Kit+ cells were stained with Streptavidin-APC-Cy7, Sca-1-PE-Cy7, and c-Kit-APC following the biotin-labeled lineage antibodies. Cells were stained with 10 ng/mL DAPI before cell sorting by In

4.5. Transplantation

For HSCs transplantation, 30 HSCs (CD45.2) were freshly isolated and injected into lethal irradiated (10 Gy, delivered in two doses 3 hour apart) recipient mice (CD45.1/2) from the tail vein with 2.5 × 10^5 CD45.1-derived total bone marrow cells as competitors. The lethal radiation was taken using an X-ray irradiator (RS 2000, Radiant Source Technologies). For overexpression experiment, LSKs were isolated from lower libs and irradiator (RS 2000, Radiant Source Technologies). For overexpression experiment, LSKs were isolated from lower libs and irradiator (RS 2000, Radiant Source Technologies).

4.6. In vitro cell culture and apoptosis induction

293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), and EL4 cells were cultured in RPMI 1640 medium, supplied with 10% FBS. Primary hematopoietic cells were cultured in StemSpan serum-free medium (SFEM) (Stem Cell Technologies, #09650, Vancouver, Canada) supplied with 20 ng/mL mTPO (Peprotech, 315-14), 20 ng/mL mSCF (Peprotech, 250-03) and 50 U/mL penicillin/streptomycin (HyClone, SV30010). All cells were cultured at 37°C with 5% CO2. For apoptosis induction, 2 × 10^5 c-Kit+ cells were treated with 10 μM ABT263 (Selleck, S001) and 10 μM S63845 (Selleck, S8383) for 5 h before western blot procedure mentioned above.

4.7. Cell cycle and cell viability assay

For HSC cell cycle analysis, 3 × 10^5 c-Kit+ cells were enriched and stained with relative surface markers, then fixed for 15 min, washed and permed along with intracellular Ki67 staining for 30 min by using FIX and PERM Cell Fixation & Permeabilization Kit (Life Technologies, GA0004) and Ki67-FITC (BD Biosciences, 558616), then stained with 50 ng/mL DAPI (Sigma, D8417). For cell viability assay, 8000 LSKs were plated per well and cultured in SFEM for 24 h, then washed and stained with Annexin V-FITC (BD Biosciences, 556420) and Propidium iodide (MCE, #HY-D0815/CS-7538) according to the product introduction, DAPI (20 ng/mL) uptake was performed on LSKs after 24 h cultivation at an initial number of 2000 to 3000.

4.8. Western blot analysis and related antibodies

Fresh isolated c-Kit+ cells were lysed with NETN buffer (0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris–HCl, pH 8.0, 100 mM NaCl, containing protease and phosphatase inhibitors cocktail) for 30 min on ice, then centrifuged at 13,000 rcf for 10 min, 4°C. The supernatant was collected and boiled with 1 x SDS loading (4% SDS, 30 mM Tris base pH 6.8, 20% glycerol, 0.4% Bromphenol-blue) at 100°C for 8 min. In vitro cultured c-Kit+ cells, LSKs and HSCs were collected and sonicated by Sonicator (Diagenode, Bioruptor plus) in 1 x SDS loading and then boiled before running the SDS-PAGE according to the regular procedure for western blot. Samples were subjected to 13.5% or 15% SDS-PAGE with rabbit-derived primary antibodies against Caspase-3 (1:800, CST, 9662S) and LC3 (1:3000, Sigma, L7543), 10% SDS-PAGE with antibodies against RIPK3 (1:1000, PROSCI, 2283), MLKL (1:1000, ANGENT, AP14272b), Phospho-MLKL (1:1000, Abcam, ab196436), GSDMD (1:1000, Abcam, ab209845), GSDME (1:1000, Abcam, ab215191) and BECN1 (1:700, Proteintech, 11306-1-AP). H4 (1:1000, Proteintech, 16047-1-AP) and Actin (1:1000, HuaBio, ET1701–80) were used as the internal reference. HRP-linked anti-rabbit IgG (1:10,000, Cell Signalling, 7074S) was used as the secondary antibody.

4.9. Plasmid construction and lentivirus packaging

Mouse cDNA for full-length GSDME (forward primer: GAGAAGTATGTGCAGCGATGGTTGCAAAAGCAACT-GG and reverse primer: GGAGGGAGAGGGCCGATCCCTAATTCCAGCATGTCGG-AGAAAGG), and full-length Caspase-3 (forward primer: GGGAGGGAGAGGGCGGATCCCTAATCCAGCATGTCGG-AGAAAGG) were cloned into pCMV-DEST and subjected to lentiviral production system. The pCMV-vector-resistant 293T cells were co-transfected with pCMV-Dux-DEST plasmid, pCMV-DEST plasmid and pMDL-gag-pol plasmid. The virus was purified and concentrated. The expression vectors were transduced into H9 cells with 5% FBS, 2% NIH-3T3 conditioned media, and 10 ng/mL VEGF, 2 h before western blot procedure mentioned above.
For autophagic activity measurement by western blot, 10^5 c-Kit+ cells were cultured overnight in SFEM medium and treated with or without 30 μM chloroquine for 4h, samples were sonicated and boiled in 1 × SDS loading. The autophagic activity of the sample was compared by the LC3-II/actin ratio between the chloroquine treated and none treated group. By using Cyto-ID dye (1:2000, Enzo, ENZ-51031-0050), 7500–9000 LSks were plate per well cultured for 24h, three duplicates were prepared, then washed with HBSS+ buffer and stained with Cyto-ID dye for 20min at cell culture condition, then washed and resuspended in HBSS+ buffer containing 10ng/mL DAPI and analyzed by flow cytometry. The transient autophagic level was indicated by MFI. For autophagic flux measurement, freshly isolated LSks were cultured for 3h and then were incubated with or without 15 μM chloroquine for another 21h. Autophagic flux was indicated by the increase of MFI (ΔMFI) between chloroquine treated and none treated group: ΔMFI Cyto-ID = MFI Cyto-ID (+Q) – MFI Cyto-ID (−Q). By using GFP-LC3-RFP-LC3ΔG plasmid, lentiviruses were prepared and infected 10,000 LSks, 3 duplicates were prepared and stained for Sca-1-PE-Cy7, c-Kit APC, and CD48-PerCP-Cy5.5 before flow cytometry analysis. Autophagic flux was indicated by the relative ratio of MFI (REP) to MFI (GFP), finally, the ratio was normalized to the WT group. For Cyto-ID method verification, BM cells were treated with 300 nM rapamycin and 15 μM chloroquine for 18h before flow cytometry analysis. For GFP-LC3-LC3ΔG plasmid verification, GFP and RFP double-positive 293T and EL4 cells were isolated after lentivirus infection and treated with 300nM Rapamycin for 13h before flow cytometry analysis.

4.11. Statistics

All data are presented as mean ± standard deviation (SD). Statistical significance was determined using a two-tailed unpaired Student's t test by GraphPad Prism 6.0 and was considered significant when <0.05. * P < 0.05, ** P < 0.01, *** P < 0.001; ns, not significant. The survival curve is analyzed using the log-rank test. All experiments were repeated twice or more times independently.

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