GSDME maintains hematopoietic stem cells by balancing pyroptosis and apoptosis

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Abstract
GSDME contains a pore-forming domain at its N-terminal region to execute pyroptosis. Our previous study has reported that forced expression of Gsdme impairs the reconstitution capacity of hematopoietic stem cells (HSCs). While, how GSDME-mediated pyroptosis regulates HSCs remains unknown. Here, we show that hematopoietic stem and progenitor cells are capable to undergo pyroptosis in response to cisplatin treatment and GSDME is one of the genes mediating such process. Gsdme−/− mice revealed no difference in the steady state of blood system while Gsdme−/− HSCs exhibited compromised reconstitution capacity due to increased apoptosis. Briefly, this study reveals that GSDME modulates HSC function by coordinating pyroptosis and apoptosis.

Keywords: Apoptosis, GSDME, Hematopoietic stem cell, Programmed cell death, Pyroptosis

1. INTRODUCTION
Hematopoietic stem cells (HSCs) are the source of all the blood cells. Based on their self-renewal and differentiation ability, HSCs maintain the life-long homeostasis of the blood system. Accumulating evidences have suggested that programmed cell death (PCD) plays an essential role in HSC maintenance.1-4 Apoptosis, necroptosis, and pyroptosis are the most intensively investigated PCD forms in recent years.6,7,8 Inhibition of HSCs’ apoptosis enhances their repopulation potential or resistance to lethal challenges.1,8,9 Meanwhile, several studies have shown that apoptosis serves to clear senescent or damaged HSCs.5,10 In addition to apoptosis, necroptosis is a form of programmed necrosis,6 which is triggered by TNF super-families and executed by the pore-forming protein MLKL.11,12 It has been reported that necroptosis is involved in HSC death or reconstitution defects.5,13 In addition to mediating cell death, PCD-related genes perform other functions in HSCs. For example, the apoptosis executor Caspase-3 and the phosphorylated form of pro-apoptotic protein BID maintain the quiescence of HSCs by regulating responsiveness to environmental cytokines or oxidative stress respectively.14,15

Pyroptosis is another type of programmed necrotic cell death and is executed by gasdermin family proteins, including GSDMA, GSDMB, GSDMC, GSDMD, and GSDME.16 Pyroptotic cell death has caused lots of concerns in recent years,17-19 while its role in HSCs remains poorly understood. One study has ever reported that the activating mutation of NLRP1a, which is a scaffold of the inflammasome,20 drives hematopoietic progenitor cells depletion via Caspase-1 dependent pyroptosis,21 wherein GSDMD plays a key role.20 GSDME-mediated pyroptosis is activated by caspase-3 or killer cell-derived Granzyme B. The high expression of GSDME in hematopoietic and stem cells (HSPCs)21 implies that GSDME may play an essential role in HSC maintenance, while how GSDME modulates HSC function remains unknown.

Our previous work has found that forced expression of Gsdme impairs the reconstitution capacity of HSCs.21 In this study, we employed Gsdme−/− mice to investigate the function of GSDME in HSC maintenance and we observed that GSDME has no significant effect on steady-state hematopoiesis. However, Gsdme−/− HSCs displayed compromised reconstitution capacity due to increased apoptotic activity. Moreover, pyroptosis happens in cisplatin-treated HSPCs, wherein GSDME is one of the mediators. In brief, this study reveals the important role of GSDME in HSC maintenance.

2. RESULTS
2.1. GSDME is dispensable for the homeostasis maintenance of blood system
To clarify whether the deletion of Gsdme affects the steady state maintenance of the blood system, we performed the complete blood cell count. The results revealed that the numbers of red blood cells, white blood cells, neutrophils, lymphocytes, and platelets hold static (Fig. 1A). Next, we analyzed the lineage distribution in peripheral blood (PB) and bone marrow (BM) of Gsdme−/− and WT mice (Fig. 1B, C). We then investigated the HSCs and progenitors of Gsdme−/− mice and observed that both the percentage and the total cell number of LT-HSCs (long-term HSCs), ST-HSCs (short-term HSCs), LSKs (Lin⁻/Sca-1⁻/c-Kit⁺), MPPs (multipotent progenitor), CLPs (common lymphoid progenitors), GMPs

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GSDME is indispensable for HSCs rebuilding blood system.

2.2. GSDME is indispensable for HSCs rebuilding blood system

To evaluate the impact of Gsdme deficiency on the long-term hematopoietic reconstitution capacity of HSCs, we performed the competitive HSC transplantation assay. 50 HSCs were freshly isolated from either Gsdme+/- mice, including RBC (red blood cell), WBC (white blood cell), Neu (neutrophil), Lym (lymphocyte) and PLT (platelet). Data are shown as mean ± SD. n = 14 mice for WT and 9 mice for Gsdme+/- group. (B, C) This histogram shows the lineage distribution of PB (B) and bone marrow (BM) (C) for WT and Gsdme+/- mice, including T cells (CD3+), B cells (B220+), and myeloid cells (CD11b+). Data are shown as mean ± SD. n = 6 mice for WT and 4 mice for Gsdme+/- group. (D–G) The histograms display the frequency (D, E) and absolute number (F, G) of HSPCs in the femur of WT and Gsdme+/- mice, including LT-HSC (long term-HSC, CD34+/Flt3+/LSK), ST-HSC (short term-HSC, CD34+/Flt3/-LSK), LSK (Lin-/Sca1+/c-Kit+), MPP (multipotent progenitor, CD34+/Flt3+/LSK), CLP (common lymphoid progenitor, CD127+/Flt3+/LSK), CMP (common myeloid progenitor, Lin-/Sca1+/c-Kit+), GMP (granulocyte/macrophage progenitor, Lin-/c-Kit+/Sca1+/CD34+/CD16/32K+), and MEP (megakaryocyte/erythroid progenitor, Lin-/c-Kit+/Sca1+/CD34+/CD16/32K+). Data are shown as mean ± SD. n = 6 mice for WT and 4 mice for Gsdme+/- group. Adult mice of 2- to 3-month old were analyzed for (A-G).

Figure 1. GSDME is dispensable for the homeostasis maintenance of blood system. (A) The histogram exhibits the complete blood cell counts (CBC) of peripheral blood (PB) from WT or Gsdme+/- mice, including red blood cell, white blood cell, neutrophil, lymphocyte and platelet. The result showed that the knockdown of GSDME severely impaired HSC reconstitution capacity, with reduced overall, T-lymphoid, and B-lymphoid chimerism. (B) This histogram shows the lineage distribution of PB (B) and bone marrow (BM) (C) for WT and Gsdme+/- mice, including T cells (CD3+), B cells (B220+), and myeloid cells (CD11b+). The result showed that the knockdown of GSDME severely impaired the long-term reconstitution capacity of HSCs, with reduced overall, lymphoid, and myeloid reconstitution (Fig. 2F). Moreover, HSCs caring GSDME shRNA displayed increased differentiation skewing toward T and B lymphocytes but reduced myeloid differentiation by the end of the third month (Fig. 2H). Given that the knockdown of Gsdme had a more severe effect than the knockout of GSDME on the reconstitution capacity of HSCs (Fig. 2B, G). The possible explanation is that a genetic compensation response may occur in Gsdme-/- HSCs, since the nonsense codons generated by truncated mutations can upregulate the transcription of related compensation genes.22 Taken together, these results suggest that GSDME is essential for HSC reconstitution.
Figure 2. Gsdme deficiency deteriorates the reconstitution ability of HSCs. (A-C) Freshly isolated 50 HSCs from WT or Gsdme<sup>−/−</sup> mice were transplanted into lethally irradiated recipients together with 5 x 10<sup>5</sup> competitor cells. Chimerism in PB 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<sup>+</sup>), T (CD3<sup>+</sup>), B (CD19<sup>+</sup>) and myeloid (CD11b<sup>+</sup>) cells every month after HSC transplantation (HSCT) (n = 5 for WT and 7 for Gsdme<sup>−/−</sup> group). (C) This histogram displays the lineage distribution of donor-derived PB at the fourth month after transplantation (n = 5 for WT and 7 for Gsdme<sup>−/−</sup> group). Data are shown as mean ± SD. (D) These scatter plots depict donor-derived HSC (left) and LSK (right) engraftment in recipient BM by the end of the fourth month post HSC transplantation (n = 5 for WT and 7 for Gsdme<sup>−/−</sup> group). Data are shown as mean ± SD. The gating strategy for donor chimerism and lineage distribution is in Sup. Fig. 1A and B, http://links.lww.com/BS/A26. (E) Representative Western blot showing knockdown efficiency of GSDME in LSKs. Freshly isolated 10<sup>5</sup> LSKs were infected with the shRNA targeting GSDME or the none target control (NTC) vector for 3 days. The whole cell lysates were subjected to Western blot using the antibody against GSDME. (F-H) 25,000 GFP<sup>+</sup> cells were isolated from GSDME-shRNA or shNTC (the non-target control shRNA) transduced LSKs at day 3 post infection and transplanted into lethally irradiated recipients together with 3.125 x 10<sup>5</sup> competitor cells. Chimerism in PB was evaluated every month until the third month. (F) The schematic diagram showing the experimental design for the transplantation of GSDME-shRNA transduced LSKs. (G) The line plots depict changes in PB chimerism of donor-derived cells (GFP<sup>+</sup>) in recipients at the indicated time.
2.3. *Gsdme* deficiency augments the apoptotic activity and apoptotic sensitivity of proliferative HSCs

Given that both forced or inadequate expression of GSDME impairs the long-term reconstitution potential of HSCs, and that the reconstitution ability of HSCs declines in aged mice, we wonder whether GSDME correlates with HSC aging. To address this question, we performed Western blot on HSCs (CD48-LSK) and HPCs (CD48+LSK) from aged (28 months) and young mice (3 months). The result showed that neither the expression nor activity of GSDME was changed in the aged hematopoietic system (Fig. 3A). Activated GSDME, the N-terminal GSDME, is not detectable in HSCs or HPCs of both aged and young mice (Fig. 3A). Thus, GSDME-mediated pyroptosis may not play a role in HSC aging.

Next, we aimed to investigate the role of GSDME in maintaining HSC viability. By Annexin-V binding and Propidium iodide (PI) uptake, we can distinguish and quantify apoptotic and necrotic cells. 8000 LSKs isolated from WT and *Gsdme*−/− mice were seeded and analyzed 24 hours later. The data showed that the frequency of total dead (Annexin-V+) cells and apoptotic cells (Annexin-V+/PI-) increased in *Gsdme*−/− LSKs (Fig. 3B), indicating that cell viability of Gsdme-deficient HSPCs decreases upon proliferation. Given that activated Caspase-3 (cleaved-CASP3) is considered as the hallmark of apoptosis, we then sought to evaluate the activation of Caspase-3 in *Gsdme*−/− LSKs after 24 hours of cultivation. The result showed that cleaved-CASP3 was elevated in *Gsdme*−/− LSKs (Fig. 3C), which confirms the higher apoptotic activity of Gsdme-deficient HSPCs upon proliferation.

Figure 3. *Gsdme* deficiency augments the apoptotic activity and apoptotic sensitivity of proliferative HSCs. (A) Representative Western blot showing the expression of GSDME in HSPCs from young and old mice. 20,000 freshly isolated HSCs (CD48 LSK) and HPCs (CD48+ LSK) were subjected to Western blot using indicated antibodies. (B) Representative flow cytometry plots (top) and histograms (bottom) showing cell viability of LSKs from WT and *Gsdme*−/− mice. Freshly isolated LSKs were cultured for 24 h before the cell viability analysis by Annexin-V and PI staining. n = 3 technical repeats per group, data are shown as mean ± SD. (C and D) Representative Western blot showing the activity of Caspase-3 in the LSKs (C) and LKs (D) from WT and *Gsdme*−/− mice. Cell lysates were subjected to Western blot using indicated antibodies. Fresh isolated LSKs in (C) were cultured for 24 h and the LKs in (D) were cultured overnight and treated with 40 μg/ml cisplatin for 5 h before the Western blot assay.
We then set out to evaluate the apoptotic sensitivity of Gsdme-deficient hematopoietic progenitor cells in response to apoptotic stimuli. We treated Gsdme−/− LK (Lin−/Sca-1−/c-Kit+) cells with cisplatin, which is a well-known drug to induce mitochondrial apoptosis by generating DNA lesions.27 We observed the activation of Caspase-3 in WT LK cells upon cisplatin treatment, while more Caspase-3 was activated in Gsdme−/− LK cells (Fig. 3D), indicating that the apoptotic sensitivity is increased in Gsdme−/− hematopoietic progenitor cells.

2.4. GSDME is involved in cisplatin-induced pyroptosis in HSPCs

Cisplatin has been reported to induce pyroptosis in some GSDME-expressing cancer cells.24 Given the expression of GSDME in HSCs and progenitor cells (Fig. 3A), we sought out to investigate whether GSDME-mediated pyroptosis happens in HSPCs. We treated WT LSK cells with cisplatin and examined the morphology of cell death by bright-field images. The result showed that cisplatin-treated LSK cells exhibited the morphology of pyroptosis, which is characterized by cell swelling and large bubbles from the plasma membrane (Fig. 4A).24 The cell viability result showed that WT LSKs shifted to Annexin-V+/PI+ upon cisplatin treatment (Fig. 4B), indicating that pyroptosis may be a form of LSK cell death. Given that the N-terminus of GSDME (GSDME-N) was responsible for the execution of GSDME-dependent pyroptosis, we then evaluated GSDME and found that GSDME-N was produced in LSKs upon cisplatin treatment (Fig. 4C). Taken together, these data imply that cisplatin-treated LSKs undergo GSDME-mediated pyroptosis. Next, we sought out to understand whether GSDME is the only mediator in this process, we then treated LSKs from WT and Gsdme−/− mice with cisplatin for 6 hours and subsequently evaluated the percentage of pyroptotic cells indicated by the black arrow in the image (Fig. 4D). We observed that the percentage of pyroptotic cells in the WT group increased to 30% upon cisplatin treatment, while it was 22% in Gsdme−/− group, which is significantly decreased than the WT group (Fig. 4E), indicating that GSDME does participate in cisplatin-induced pyroptosis in HSPCs. However, the pyroptotic morphology still existed in Gsdme−/− LSK cells (Fig. 4D), suggesting that GSDME is not the only mediator of cisplatin-induced cell death in HSPCs. The involvement of GSDME in cisplatin-induced pyroptosis was further confirmed by the reduced PI uptake in Gsdme−/− LSK cells (Fig. 4F).

Cisplatin-treated LK cells also presented extensive pyroptotic morphology and the production of N-terminal GSDME (Fig. 4G, H), and only an increase of necrotic cell death can be detected for WT LKs by Annexin-V and PI analysis (Fig. 4L). The pyroptotic morphology was further confirmed by the chemotherapy drug doxorubicin and etoposide (Sup. Fig. S2, http://links.lww.com/BS/A26), both of which were confirmed to induce GSDME-mediated pyroptosis in GSDME-expressing tumor cells.24 Given the higher activity of Caspase-3 induced in Gsdme−/− LKs (Fig. 3D), it is important to investigate the influence of Gsme deficiency on cell death of HPCs in response to cisplatin. To address this question, we performed the cell viability assay using Gsdme−/− LKs (Fig. 4I) and observed that Gsdme−/− LKs displayed reduced necrosis (Annexin-V+/PI+, Fig. 4J-left) and increased apoptosis (Annexin-V-/PI+, Fig. 4J-middle) after cisplatin treatment, but their viability was still lower than that of WT LKs according to the higher frequency of Annexin-V cells for Gsdme−/− LKs (Fig. 4J-right). Moreover, Gsdme−/− LKs slightly enhanced the apoptotic cell death but did not switch to apoptosis after cisplatin treatment (Annexin-V+/PI−, Fig. 4J-middle). The results suggest that GSDME plays a role in leading to cisplatin-induced necrotic cell death in HPCs but seems to have little effect on cisplatin-induced cell viability loss, and that other forms of necrosis play a dominant role in leading to the death of Gsdme−/− HPCs after cisplatin treatment instead of apoptosis.

3. DISCUSSION

Caspase-3 activation in HSCs has long been considered a hallmark of apoptosis. Here, we provide evidence that the activation of Caspase-3 in HSCs induced by cisplatin is able to lead to GSDME-mediated pyroptosis. Moreover, it is the first time to demonstrate that the pyroptosis executor GSDME is essential for HSC reconstitution, and we shed light on the interplay of apoptosis and GSDME-mediated pyroptosis in HSCs. Furthermore, we proved that GSDME is not the only mediator of HSC pyroptosis. Briefly, our study may help us to understand more about programmed death in HSCs as well as the role of GSDME in HSC maintenance.

Our study found that HSPCs underwent GSDME-dependent pyroptosis in response to the pro-apoptotic stimulus cisplatin (Fig. 4A-F). The choice of death forms for HSCs may be explained by the hypothesis we mentioned before.21 In brief, pyroptosis is a form of necrotic cell death and may save more energy than apoptosis, thus pyroptosis may fit better for HSCs with a low metabolic state. This study will change the concept that HSCs undergo apoptosis upon Caspase-3 activation induced by apoptotic stimuli. Cisplatin has been reported to induce pyroptosis in GSDME-expressing cell lines.24 It is the first time to prove that pyroptosis is able to happen in cisplatin-treated HSCs. However, GSDME is only responsible for partial cisplatin-induced necrotic cell death in HSPCs (Fig. 4D-F), which is similar to that in mouse macrophages.28 Given the pyroptotic morphology in Gsdme-deficient HSPCs (Fig. 4D), we assume that other gasdermin family members may also involve in cisplatin-induced pyroptosis. This may be true since GSDME is not the only gasdermin involved in cell death after apoptosis induction,16 for example Caspase-8 activation during TAK1 inhibition can activate both GSDME and GSDMD,29 and Caspase-8 was reported to be activated by cisplatin.30,31 Caspase-1 which can lead to GSDMD-dependent pyroptosis can be activated by cisplatin in mouse macrophages.28 Moreover, GSDMA was reported to be upregulated in TGF-β induced apoptosis.32 The roles of other gasdermin family members in HSCs are needed to be clarified in further studies. Furthermore, the energy may be not abundant for proliferative HSPCs and they would thus like to undergo necrotic cell death but not apoptosis even when Gsdme is deficient.

Our previous study demonstrated that overactive GSDME impaired the reconstitution capacity of HSCs,21 while here, we demonstrate that Gsdme deletion impairs HSC function (Fig. 2B, G). The present result seems to be conflict with our previous study, but it can be explained that a balanced cell death pathway is necessary for HSC maintenance. The increased apoptotic activity of cultured Gsdme−/− HSPCs indicates that GSDME connects apoptosis and pyroptosis in HSCs (Fig. 3B,C). Furthermore, the activation of Caspase-3 is enhanced in cultured Gsdme−/− HSPCs (Fig. 3C,D), which provides direct evidence that GSDME is essential to balance pyroptosis and apoptosis. Increased activity of Caspase-3 has also been reported in Gsdmd−/− bone marrow-derived macrophages (BMDMs) upon LPS plus nigericin treatment,17 thus a similar mechanism may be shared by gasdermins to balance pyroptosis and apoptosis. Since GSDME is a substrate of Caspase-3,24 we assume that the higher
apoptotic activity in Gsdme−/− HSPCs may be caused by a loss of competitive binding of GSDME with other substrates of Caspase-3 that can enhance apoptotic signals. For example, the C-terminus of BECN1 produced by the cleavage of caspases including Caspase-3 enhances apoptosis.33

However, Gsdme deficiency does not impair the homeostasis of the hematopoietic system at steady-state conditions (Fig. 1A–G), but it reduces HSC regeneration (Fig. 2B,F). Given that most HSCs are quiescent at steady-state conditions but proliferate during transplantation,34 and that the cell viability of Gsdme-deficient HSPCs upon proliferation is reduced (Fig. 3B), we assume that the apoptosis signal may be elevated during HSC proliferation and may be even strengthened in Gsdme-deficient HSCs. A higher risk of apoptosis for proliferative HSCs may be true on account of the
increased DNA damage, which can be confirmed by the accumulation of γH2AX and DNA damage response in cycling HSCs.15,16 Given that some inflammatory factors can drive HSC proliferation,17 the inflammatory environment and danger-associated molecular patterns in the BM of irradiated recipients17 may also be insults that lead to the death of Gsdme−/− HSCs. Similar to GSDME which works as a switch between pyroptosis and apoptosis, the apoptosis mediator Caspase-8 works as a switch between apoptosis and necroptosis,12 the loss of function of Caspase-8 impairs the function of hematopoietic progenitors and causes monocytosis death.38 Collectively, different cell death pathways may be mutually restrained and the fine balance of them maintains HSC function.

However, since the necrotic cells are easily lost during HSC isolation, we could not provide direct evidence to prove whether GSDME-mediated pyroptosis mediated HSC death under physiological conditions, but the in vivo HSC transplantation experiments can provide evidence that GSDME functions during stress conditions. In summary, our study demonstrates that GSDME can mediate HSC pyroptosis upon apoptotic stimuli and that GSDME plays an essential role in HSC regeneration via the interplay of GSDME-mediated pyroptosis and apoptosis.

There are still lots of questions remaining to be settled. Further proofs should be provided to explain how GSDME functions to balance pyroptosis and apoptosis in HSCs. Moreover, the functions of other gsdmers in HSCs and how GSDME interplays with them, and the possible compensation genes of Gsdme in HSCs are worth studying in further investigations.

4. MATERIALS AND METHODS

4.1. Animals

Gsdme−/− (C57BL/6, CD45.2) mice24 were a gift from Dr Feng Shao. C57BL/6-SJL (CD45.1) (Stock No: 002014) and C57BL/6 WT (CD45.2) (Stock No: 000664) mice were from the Jackson Laboratory. CD45.1/2 mice were heterozygotes from CD45.1 and CD45.2 mice. All the mice were maintained under the SPF conditions at Tsinghua University according to the protocols approved by the IACUC.

4.2. Complete blood cell and bone marrow cell count

PB was collected with an EDTA-containing tube from the tail and performed by the automatic hematology analyzer (BC-5000, Mindray). Single-cell suspension samples from BM were analyzed by Vi-CELL XR cell viability analyzer (Beckman Coulter).

4.3. Flow cytometry

Flow cytometry analysis and cell sorting were performed as previously described.21 Briefly, bones from hindlimbs, pelvic bones (with spines for some experiments) were crushed and filtered for single-cell suspension. For HSC or LSK sorting, c-Kit+ cells were enriched and stained with biotin-labeled lineage antibodies (biotin-lineage mix) followed by Streptavidin-APC-Cy7, Sca-1-PE-Cy7, c-KiAPC for LSK and CD150-PE, CD34-APC-Cy7, CD127-Brilliant Violet 421 following the biotin-antibodies listed above. HSPCs chimerism was analyzed at the end of the fourth month.

For RNAi transduced HSC transplantation, 10^5 LSKs isolated from hind limbs and spines of WT mice (CD45.2) were infected with lentivirus carrying the shRNA targeting the gene or the non-target control shRNA. 2.5 × 10^6 GFP+ cells were isolated and co-transplanted with 3.125 × 10^5 competitors (CD45.1) into lethally irradiated recipients (CD45.2). Lineage analysis for GFP chimera was performed every month until the antibodies listed above. HSPCs chimera was analyzed at the end of the fourth month.

4.4. Transplantation

For HSC transplantation, 50 HSCs (CD34+CD150−LSK, CD45.2) were freshly isolated and transplanted with 5 × 10^5 complete BM cells (CD45.1) into lethally irradiated recipients (CD45.1/2). Lineage analysis for donor (CD45.2) chimera was performed every month until the fourth month according to the antibodies listed above. HSPCs chimera was analyzed at the end of the fourth month.

For RNAi transduced HSC transplantation, 10^5 LSKs isolated from hind limbs and spines of WT mice (CD45.2) were infected with lentivirus carrying the shRNA targeting the gene or the non-target control shRNA. 2.5 × 10^6 GFP+ cells were isolated and co-transplanted with 3.125 × 10^5 competitors (CD45.1) into lethally irradiated recipients (CD45.2). Lineage analysis for GFP chimera was performed every month until the third month according to the antibodies listed above.

4.5. In vitro cell culture and treatment

293T cells were maintained in DMEM supplied with 10% FBS and 50 U/ml penicillin/streptomycin (HyClone, SV30010). LSKs were cultured in SFEM (Stem Cell Technologies, #09650, Vancouver, Canada) supplied with 50 U/ml penicillin/streptomycin, 20 ng/ml mPTO (Peprotech, 315-14), and 20 ng/ml mSCF (Peprotech, 250-03). For cisplatin treatment, LSKs were cultured overnight and treated with cisplatin (Harvey, HZB0054-100) or saline. 4 to 5 × 10^5 LSKs cultured in 96-well plated were treated with 40 μg/ml cisplatin for 6 hours for cell morphology assay, and 6000 to 8000 cells cultured in 96-well plated were treated with 20 μg/ml cisplatin for 6.5 for Annexin-V and PI staining. 2 × 10^5 LK cells were treated with 40 μg/ml cisplatin for 5 hours for Western blot assay (24 well plate). For lentivirus infection, 10^5 freshly isolated LSKs were plated in the well of 96-well plate with 100 μl SFEM, the virus was added to the cells 3 to 4 hours later, and cells were washed and transplanted to 24-well plated 11 to 12 hours post the lentivirus infection.

4.6. Cell viability and morphology assay

Cell viability assay was performed by Annexin-V and PI staining as previously described.21 Cell morphology assay was performed by static bright-field images taken by a phase-contrast microscope (Olympus, CXX-41).

4.7. Western blot analysis and related antibodies

Cells were collected and sonicated by Sonicator (Diagenode, Bioruptor plus) in 1 × SDS loading (2% SDS, 25 mM Tris base/ pH 6.8, 10% glycerol, 0.02% Bromophenol blue). Cell lysates were boiled at 100°C for 5 min and subjected to 13% SDS-PAGE with the antibody against Caspase-3 (1:800, CST, 9662S), and 10% SDS-PAGE with the antibody against GSDME (1:1000, Abcam, ab15191). Actin (1:1000, HuaBio, ET1701-80) and H3 (1:1000, Proteintech, 17168-1-AP) were used as the internal reference. All the original Western blot data were presented in (Sup. Fig. 3, http://links.lww.com/BS/A26).
4.8. Plasmid construction

The shRNA sequence (TGCTGTTGACATGGACGCCCTC- GACAAAAATTGTTGGCAAATAGTGGAAGGCAAGATGTT- TTGGCAGAATTTGTCGTTGCGCTACTGCGCCTCGGA) targeting GSDME was cloned into SF-LV-miRE-EGFP plasmid.

4.9. Lentivirus production and concentration

Lentivirus was packaged and concentrated as previously described.21 Briefly, the shRNAs and helper plasmids were transfected to 293T cells using Polyethylenimine (Polysciences, 23966). Supernatants were collected at 48- and 72-hours post transfection and then concentrated (250×) by ultracentrifuge (Beckman, OPTIMA XL-90).

4.10. Statistics

GraphPad Prism 6.0 was used for statistical analyses. P values were calculated by 2-tailed Student’s t test (unpaired). Significance: *P < .05, **P < .01, ***P < .001. All experiments were repeated independently at least twice.

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