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Bid is a positive regulator for donor-derived lymphoid cell regeneration in γ -irradiated recipients

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Abstract

Objective—Hematopoietic regeneration is regulated by cell survival proteins such as the Bcl-2 family. Bid, a BH3-only protein of the Bcl-2 family, has multiple cellular functions and is involved in a variety of physiological or pathological conditions. We attempted to define its role in hematopoietic cell repopulation under the stress condition of bone marrow transplantation (BMT).

Materials and Methods—We performed conventional or competitive BMT with donor hematopoietic cells from Bid^{-/-} or Bid^{+/+} mice. Flow Cytometry was used for quantification of hematopoietic stem cells (HSCs), hematopoietic progenitor cells (HPCs) and differentiated cells in different lineages (T, B and Myeloid cells). Single cell culture and homing assays were performed to further evaluate HSC functions. HPCs were also measured by the colony-forming cell (CFC) culture.

Results—Contrary to the widely-recognized role of Bid as a pro-apoptotic protein, the absence of Bid significantly reduced the reconstitution of donor hematopoietic cells in γ -irradiated recipients. Interestingly, however, numbers of HSCs and HPCs, and their functions were not overtly altered. Instead, the regeneration of donor T and B cells was significantly impaired in the absence of Bid. Further analysis indicated an accumulation of the triple negative T cell population in the thymus, and pro-B cells in the bone marrow.

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Conflict of Interest Disclosure

The authors state that they have no conflict of interest.

Conclusion—Our current study demonstrates a positive impact of Bid on hematopoietic regeneration mainly due to its unique effects on donor lymphopoiesis in the transplant recipients.

Keywords

Bid; apoptosis; transplantation; hematopoietic stem and progenitor cells; T cells; B cells

Introduction

Hematopoietic stem cell (HSC) transplantation (HSCT) provides a cure for many hematological as well as some non-hematological disorders. However, its clinical efficacy needs to be further improved by enhancing the hematopoietic engraftment. Many critical factors contribute to blood cell regeneration after HSCT. The quality and quantity of donor stem cells, and the host conditions that have direct effects on HSC homing, lodgment, survival and differentiation all determine the outcome of hematopoietic engraftment. Under homeostasis conditions, different fate choices of HSC, including self-renewal, quiescence, migration, differentiation and cell death are balanced to meet physiological needs [1]. Under stress conditions, such as HSCT in recipients pre-conditioned by γ -irradiation, however, the fate choices of HSC could be potentially altered through extrinsic cues and intrinsic mediators. Therefore, identification of the key molecular events that can impact the function of transplanted hematopoietic cells would potentially improve the current clinical regimens of HSCT. For instance, targeting a specific cell cycle inhibitor, namely p18^{INK4C}, has been shown to overcome the exhaustion of transplanted HSCs in serial transplant recipients [2]. Yet transplanted hematopoietic cells must function via tight cell cycle control coupled with cell survival mechanisms to efficiently produce all the blood cell types in transplant recipients after bone marrow ablation. Thus, we have also begun to examine cell death proteins in HSCs under stress or damage conditions such as γ -irradiation [3].

The Bcl-2 family is composed of anti-apoptosis and pro-apoptosis members. The majority of the anti-apoptosis members contain four distinct Bcl-2 homology (BH) domains (BH1, BH2, BH3 and BH4). The pro-apoptosis members can be divided into two sub-groups: the BH3-only proteins and the multi-domain proteins with BH1, BH2 and BH3 domains. At least 11 members of the BH3-only group, such as Bid and Puma, have been identified in mammals, and many are important in regulating cellular functions such as immune-responses [4–6]. We have recently reported that the absence of Puma confers a striking survival advantage on HSCs and hematopoietic progenitor cells (HPCs) during hematopoietic regeneration after radiation damage [3]. Bid, another BH3-only protein, can be activated by different proteases through a variety of pathways, such as the death receptor pathway, the lysosomal pathway and the cytotoxic T cell-granzyme B pathway [7–10]. The truncated Bid can in turn inactivate Bcl-2 or activate two multi-domain pro-apoptosis Bcl-2 family proteins, Bax and Bak, to trigger mitochondria-mediated apoptosis. In addition to its pro-apoptotic activity, Bid also regulates cell proliferation via its function in maintaining calcium homeostasis in the endoplasmic reticulum and can be involved in cell cycle arrest and DNA repair through the ATM/ATR pathway during DNA damage or replicative stress conditions [11–14]. Given these varied effects, Bid is thus involved in multiple pathological conditions, contributing to chemoresistance [15, 16], liver injury [8], ischemic neuronal cell damage [17, 18], renal injury induced by ischemia-perfusion [19, 20], and tumorigenesis [12, 21].

Because of the diverse roles of Bid in cellular functions, we attempted to define its physiological role in hematopoietic cell repopulation under the stress condition of bone marrow transplantation (BMT). We have documented for the first time that Bid serves as a positive regulator for hematopoietic reconstitution in irradiated recipients at key maturation steps for both T and B cells without overt effects on HSCs and HPCs. These findings expand

the functional repertoire of the Bcl-2 family proteins in general, and Bid in specific, beyond their widely recognized roles in apoptosis, and strengthen the notion that hematopoietic reconstitution is regulated by different Bcl-2 family proteins at multiple differentiation stages and cell types in the hematopoietic system.

Methods

Mice

Bid^{-/-} mice were established as described previously [8] and had been backcrossed to C57BL/6 (CD45.2) mice for twelve generations. Bid^{-/-} mice were generated by mating Bid^{-/-} × Bid^{-/-} mice and C57BL/6 mice were used as Bid^{+/+} for the study. C57BL/6 (CD45.2⁺) and congenic B6.SJL (CD45.1⁺) mice were from either The Jackson Laboratory (Bar Harbor, ME) or Taconic (Hudson, NY). F1 mice (CD45.1⁺/CD45.2⁺) were generated by crossing C57BL/6 and B6.SJL breeders. All mice were maintained in a certified animal facility at the University of Pittsburgh Cancer Institute per procedures approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. Mice 8–20 weeks old were used in the study.

Bone marrow transplantation (BMT)

For competitive BMT (cBMT), equal numbers of donor bone marrow cells (CD45.2⁺) from Bid^{-/-} or Bid^{+/+} male mice and competitor bone marrow cells (CD45.1⁺CD45.2⁺) were mixed and transplanted intravenously through the tail into pre-irradiated (10 Gy) female recipients (CD45.1⁺). The efficiency of engraftment was measured every 4 weeks as the percentage of CD45.2⁺ cells or the ratio of CD45.2⁺ to CD45.1⁺CD45.2⁺. In some of the experiments, CD45.2⁺ donor bone marrow cells (5×10⁶) from Bid^{-/-} or Bid^{+/+} male mice were transplanted into pre-irradiated (10 Gy) female C57 BL/6 recipients. Mice were considered fully reconstituted 4 months after the transplantation.

Flow cytometric analysis and cell sorting

Markers used for identification of HSC and HPC populations were as described previously [22–24]: anti-mouse Sca-1, c-kit, CD34, Flt3, FcR II/II, IL-7R and lineage markers CD4, CD8, B220, Mac-1, Gr-1, and Ter119. T cell subset analysis used anti-mouse CD3, CD4, CD8, CD25, and CD44. B cell subset analysis used anti-mouse B220, CD43, and IgM. Anti-mouse CD45.1 and CD45.2 were used to distinguish donor, recipient and competitor cells. Anti-mouse CD16/32 was used to block non-specific binding of FcR during staining. All antibodies were from eBioscience (San Diego, CA). Flow cytometric-based proliferation assays using BrdU incorporation and the apoptosis assay using Annexin V were as previously described [25]. Propidium iodide (PI) or 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) were used to detect dead cells. A BD LSR II cytometer with 3-laser (488 nm, 633 nm and 405 nm), 12-parameter configuration and a BD FACSAria high-speed cell sorter with 5-laser (488nm, 632nm, 633nm, 405nm and 355nm), 16-parameter configuration (BD biosciences, San Jose, CA) were used for data analysis and cell sorting. A Coulter AC^T 10 (Beckman Coulter, Miami, FL) was used for white blood cell counts.

Single HSC in vitro assay

As previously described [26], single HSCs of lineage⁻Sca-1⁺c-kit⁺CD34⁻ were deposited at one cell per well in 96-well plates with medium containing 100 ng/ml of mouse stem cell factor (mSCF), 10 ng/ml of mouse interleukin 3 (mIL-3), 25 ng/ml of mouse thrombopoietin (mTPO) and 10 ng/ml of mouse erythropoietin (mEPO). Cells were cultured at 37 °C, 5% CO₂ incubator for 10 days. Colonies formed in individual wells were cytopinned and stained with May-Gruenwald-Giemsa solution. Neutrophils, macrophages, erythroblasts and

megakaryocytes were identified based on microscopically observed morphologies. The frequency of single HSCs in all lineages was calculated. For HSC proliferation assay, single HSCs of lineage⁻ Sca-1⁺c-kit⁺CD34⁻ were deposited at one cell per well in 96-well plates with medium containing 300ng/ml of mouse SCF, 2ng/ml Flt3L and 20ng/ml IL-11. Cells were cultured at 37°C, 5% CO₂ incubator. The number of cells in each well was counted at 24 hour intervals for 4 days.

HSC homing assay

Approximately 1×10^5 carboxyfluorescein succinimidyl ester (CFSE)-labeled long-term (LT)- and short-term (ST)-HSCs (Lineage⁻Sca-1⁺c-kit⁺Flt3⁻) from Bid^{-/-} or Bid^{+/+} mice were sorted and injected into lethally irradiated recipients. The next day, bone marrow cells were harvested and stained with anti-mouse CD45 antibody. The frequency of homed HSCs (percent CFSE⁺ cells in total CD45⁺ cells) was measured using flow cytometry. The absolute number of homed HSCs per harvested bone marrow was also calculated for comparison.

Colony forming unit (CFU) assay

A sample of $1-2 \times 10^4$ bone marrow cells was plated in methylcellulose medium M3434 (StemCell Technologies, Vancouver, BC) and cultured at 37 °C, in a 5% CO₂ incubator. Total colonies, as well as BFU-Es, CFU-GMs, and CFU-GEMMs, were counted on day 7.

Statistic analysis

Statistical analysis was done by *t*-test or χ^2 test. A p-value of 0.05 or less was considered significant.

Results

Reduced reconstitution of donor hematopoietic cells in the absence of Bid

To investigate the effect of Bid on hematopoietic regeneration after BMT, we performed the competitive BMT (cBMT), in which 2×10^6 donor bone marrow cells from Bid^{-/-} or Bid^{+/+} mice (CD45.2⁺) were mixed with an equal number of competitor bone marrow cells from the F1 mice of a C57BL/6 by B6.SJL cross (CD45.1⁺CD45.2⁺) and co-transplanted into pre-lethally irradiated (10 Gy) recipients (CD45.1⁺). The reconstitution of donor hematopoietic cells was monitored every 4 weeks by calculating the frequency of donor cells (percent CD45.2⁺) or the ratio of donor cells to competitors in peripheral blood. Unexpectedly, we found that the reconstitution of Bid^{-/-} cells was significant lower than Bid^{+/+} cells within 24 weeks of transplantation, with significance observed as early as 4 weeks after transplantation (Fig. 1). The percentage of Bid^{+/+} cells was 2.66-fold higher than that of Bid^{-/-} cells (42.82 ± 8.04 for Bid^{+/+} and 16.11 ± 3.42 for Bid^{-/-}) at 24 weeks after transplantation. The ratio of CD45.2⁺ to CD45.1⁺CD45.2⁺, which indicated the ability of Bid^{+/+} or Bid^{-/-} cells to compete with the normal third-party bone marrow cells, was also significantly reduced in the Bid^{-/-} group. These results demonstrated a positive role of Bid in hematopoietic regeneration after transplantation.

Unaltered pool size and functions of HSCs and HPCs in Bid^{-/-} mice

HSCs and HPCs are mainly responsible for hematopoietic reconstitution in transplant recipients. To define the contribution of donor HSCs and HPCs to the defective hematopoietic reconstitution in the absence of Bid, we compared HSCs and HPCs pool sizes in the Bid^{-/-} and Bid^{+/+} bone marrow using multi-color flow cytometry. Interestingly, we found that the frequency of long term-HSC (LT-HSC) (IL-7R α ⁻Lin⁻Sca-1⁺c-kit⁺CD34⁻Flk2⁻), short term-HSC (ST-HSC) (IL-7R α ⁻Lin⁻Sca-1⁺c-kit⁺CD34⁺Flk2⁻),

multi-potent progenitors (MPPs) (IL-7R α ⁻Lin⁻Sca-1⁺c-kit⁺CD34⁺Flk2⁺), common lymphoid progenitors (CLP) (IL-7R α ⁺Lin⁻Sca-1⁺c-kit⁺), common myeloid progenitors (CMP) (IL-7R α ⁻Lin⁻Sca-1⁻c-kit⁺CD34⁺FcR⁺), granulocyte/monocyte progenitors (GMP) (IL-7R α ⁻Lin⁻Sca-1⁻c-kit⁺CD34⁺FcR⁺), and megakaryocyte/erythrocyte progenitors (MEP) (IL-7R α ⁻Lin⁻Sca-1⁻c-kit⁺CD34⁻FcR⁻) was not reduced in Bid^{-/-} mice (Fig. 2A). It suggests that the reduced hematopoietic reconstitution (Fig. 1) is not due to the pool size of HSCs and HPCs in Bid^{-/-} mice. Interestingly, the frequency of ST-HSC was even slightly higher in Bid^{-/-} mice. ST-HSC is much more efficient at rapidly reconstituting myelopoiesis and rescuing myeloablated recipients. The reconstitution seen within 5 weeks of transplantation is mainly from ST-HSC [22]. This could explain the pattern we saw at 4 weeks after transplantation in Figure 1. Those phenotypes of HSCs and HPCs were also compared in fully reconstituted mice with Bid^{-/-} and Bid^{+/+} cells. No significant difference of any populations was observed in Bid^{-/-} fully reconstituted mice (Supplemental Fig. 1).

To explore the potential functional alteration of HSCs due to Bid deficiency, we then performed functional assays on HSCs. Cell proliferation was tested by incorporation of Brdu and apoptosis was measured by staining with Annexin V. As seen in Figures 2B and 2C, the percent of Brdu⁺ and percent of Annexin V⁺DAPI⁻ cells in the LT-HSC population were not significantly different between Bid^{-/-} and Bid^{+/+} mice. We next performed *in vitro* HSC differentiation at the single HSC level. Single HSCs were sorted into wells containing cytokine cocktail medium to promote differentiation. After 10 days, colonies in individual wells were stained with May-Gruenwald-Giemsa solution. Neutrophils, macrophages, erythroblasts and megakaryocytes were identified by their morphology [26, 27]. The frequency of single HSC-generated in all lineages was calculated and no significant difference was seen between Bid^{-/-} and Bid^{+/+} (Fig. 2D). The proliferation of HSC in fully engrafted mice with Bid^{+/+} and Bid^{-/-} bone marrow cells was also compared at the single-cell level. We sorted single HSCs into individual wells containing the cytokine cocktail medium and counted the cells every 24h for 4 days to compare the percentage of divided cells, dead cells and the average cell number in each well. No significant difference was observed (Supplemental Fig. 2).

To further investigate whether the homing efficiency of HSCs/HPCs was decreased in the absence of Bid, we sorted CFSE-labeled HSCs (Flt3⁻LKS) from Bid^{-/-} and Bid^{+/+} bone marrow transplanted into the lethally irradiated mice. Bone marrow cells were harvested the day after transplantation and the frequency of CFSE⁺ cells in bone marrow CD45⁺ cells was measured by flow cytometry. The absolute number of CFSE⁺ cells per harvest was compared as the homing efficiency between Bid^{-/-} and Bid^{+/+} groups, and no significant difference was found (Fig. 2E).

The function of HPC was measured by CFU assay by plating bone marrow cells in methylcellulose medium containing cytokines. Colonies were compared after 7 days and no significant difference was observed between Bid^{-/-} and Bid^{+/+} mice (Fig. 2F).

Together, these data suggest that the reduced hematopoietic reconstitution in the absence of Bid was not directly attributable to HSCs or HPCs from the Bid^{-/-} mice.

Impaired development of T and B cells in Bid^{-/-} transplanted mice

Since HSCs and HPCs were not directly affected by the absence of Bid, we hypothesized that deficiencies in the development of specific cell lineages might contribute to the reduced hematopoietic reconstitution of Bid^{-/-} cells in the cBMT model. To find the specific lineage involved, we monitored the frequencies of T (CD3⁺), B (B220⁺) and myeloid (CD11b⁺) cells in peripheral blood in either Bid^{-/-} or Bid^{+/+} reconstituted leukocytes in the cBMT model at different time points after transplantation. We found that the percentage of T cells

in Bid^{-/-} reconstituted cells was consistently lower than in the Bid^{+/+} counterparts (Fig. 3). Bid^{-/-} T cells were almost completely unable to recover within 24 weeks of transplantation. The B cells also showed dramatically reduced frequency at weeks 4, 20 and 24 in Bid^{-/-} engrafted cells. Consequently, the proportion of myeloid cells was increased in Bid^{-/-} reconstituted cells (Fig. 3).

To define whether Bid is required for T and B cell development and differentiation in the setting of hematopoietic regeneration, we fully reconstituted hematopoiesis in the transplant mice by injecting 5×10^6 Bid^{-/-} or Bid^{+/+} bone marrow cells into lethally irradiated recipients. Notably, we found that the thymus and spleen were smaller in the Bid^{-/-} reconstituted mice. The number of cells (cellularities) in the thymus and spleen in Bid^{+/+} reconstituted mice were 3.4- and 1.8-fold higher, respectively, than in Bid^{-/-} reconstituted mice, while the white blood cell counts in peripheral blood and bone marrow were not significantly different between the two groups (Fig. 4A). We further compared the frequencies of T, B and myeloid cells in the reconstituted mice. T cells were significantly decreased in spleen and peripheral blood, B cells were significantly decreased in bone marrow and spleen, and myeloid cells were elevated in bone marrow and peripheral blood in Bid^{-/-} reconstituted mice compared to Bid^{+/+} reconstituted mice (Fig. 4B).

Given that thymus is the primary site for T cell development, the shrinkage observed in the thymus could represent a regenerating defect of T cells in the early developmental stage after transplantation. We measured T cell subsets in different developmental and transitional stages as (Fig. 5A): 1) triple negative stage (TN, CD3⁻CD4⁻CD8⁻) with four transitions from TN1 (CD3⁻CD4⁻CD8⁻CD25⁻CD44⁺) to TN2 (CD3⁻CD4⁻CD8⁻CD25⁺CD44⁺) to TN3 (CD3⁻CD4⁻CD8⁻CD25⁺CD44⁻) and to TN4 (CD3⁻CD4⁻CD8⁻CD25⁻CD44⁻); 2) immature single positive stage (ISP, CD3⁻CD4⁻CD8⁺); 3) double positive stage (DP, CD4⁺CD8⁺); and 4) single positive stage (SP, CD3⁺CD8⁺ or CD3⁺CD4⁺). The number of T cells was considerably decreased at ISP, DP, SP-CD4 and SP-CD8 stages in Bid^{-/-} reconstituted mice (Fig. 5B). These frequencies showed that cells accumulated at an early stage such as TN, especially at TN3, and were unable to transit smoothly to the mature phenotype (Fig. 5C, D). As a consequence, fewer SP-CD4 and SP-CD8 T cells exited from the thymus into the periphery, leading to a decrease in the total number of T cells (CD3⁺) in the peripheral blood and spleen in Bid^{-/-} reconstituted mice (Fig. 4B). In Bid^{-/-} reconstituted mice, both T cell subsets of CD3⁺CD4⁺ and CD3⁺CD8⁺ cells in the thymus and peripheral blood were reduced. However, the reduced ratio of CD4 to CD8 was only observed in the peripheral blood but not in the thymus (Fig. 6A). It suggests a distinct effect of Bid between CD4⁺ and CD8⁺ cells during the circulation rather than at the time of departure from the thymus.

A small proportion of donor T cells was also reported to be regenerated through the pathway of homeostatic peripheral expansion (HPE) in lethally irradiated recipients and those T cells should predominantly have a memory phenotype [28]. We thus examined the expression levels of memory cells in CD4 and CD8 T cells. Interestingly, the frequency of CD44 expression was significantly higher in both CD4 and CD8 peripheral blood cells in Bid^{-/-} reconstituted mice (2.5-fold for CD4 and 2.6-fold for CD8, n = 12) (Fig. 6B). Significant increases in absolute numbers of CD8⁺CD44⁺ memory cells were also seen in the Bid^{-/-} reconstituted mice. However, the absolute number of CD4⁺ memory cells was not different because of a significant reduction in CD4⁺ T cell counts (Fig. 6A). This result suggested that Bid contributes to the maintenance of memory T cells.

We also investigated the impact of Bid deletion on B cell development from CLP cells to pro-B cells (B220⁺CD43⁺) to pre-B (B220⁺CD43⁻IgM⁻) cells to immature B (IMB, B220⁺CD43⁻IgM⁺) cells, and to mature B (MB, B220^{high}CD43⁻IgM⁺) cells in bone

marrow (Fig. 7A). As stated earlier, CLP was similar in Bid^{-/-} and Bid^{+/+} reconstituted mice. However, we found that in Bid^{-/-} reconstituted mice, pro-B cells accumulated and mature B cells were reduced (Fig. 7B). Thus, the transition from pro-B to the late stages of B cells was clearly compromised.

Taken together, our results demonstrate that the lack of Bid reduced the hematopoietic reconstitution by impairing the regeneration and maturation of both T and B cell populations, but not the pool size and functions of HSCs and HPCs.

Discussion

Our current study demonstrates a positive effect of Bid on donor hematopoietic cell regeneration in irradiated recipients, as opposed to the conventional view on Bid as a pro-apoptotic protein. Under homeostasis conditions, Bid^{-/-} mice have been reported to show normal organ development with normal lymphoid tissues, such as spleen and thymus [8, 29]. In the BMT model, however, Bid appeared to act as an important regulator for the regenerative potential of donor hematopoietic cells after BMT, due to its primary effect on lymphoid cell maturation, but not on HSCs and HPCs in the transplant recipients.

In contrast, in a similar transplant model, our recent work showed that deletion of Puma, another BH3-only Bcl-2 family protein, did not impair transplantation efficiency and lineage maturation[3]. In fact, Puma mediated the apoptosis of HSCs and HPCs when exposed with γ -radiation[3]. Interestingly, challenging HSCs and HPCs from Bid^{-/-} mice with γ -irradiation did not show the resistance as seen in cells from PUMA^{-/-} mice (data not shown). Although Bid and Puma belong to the BH3-only protein group of the Bcl-2 family and both have been characterized as proapoptotic proteins, they exert very different functions in hematopoietic regeneration, implying that each BH3-only protein may target a unique signaling pathway and affect specific cell types. Thus, the biological effects of these molecules should be vigorously evaluated at both cellular and organism levels in order to accurately assess the significance of their effects under physiological as well as pathophysiological conditions.

The developmental stages of T cells in the thymus and B cells in the bone marrow have been well defined (Fig. 5A and Fig. 7A), thereby offering a quantitative measure to determine how Bid may potentially disrupt the normal differentiation process in the transplant model. We observed that the arrest of TN stage during early development of T cells in the thymus and the compromised transition of pro-B to pre-B cell development in the bone marrow. As known, TCR- β gene rearrangement, pre-TCR/CD3 assembly, or the activation of their downstream signaling molecules are essential for thymocyte development from the TN3 to TN4 stage [30–33]. And the transition from pro-B to pre-B cells requires the surface expression of pre-B cell receptor (pre-BCR) and its signaling, which is dependent upon the presence of a rearranged functional IgH gene by the V(D)J recombination step [34]. It has been reported that the up-regulation of Bcl-2 is associated with the arrest of the TN3 stage of T cell development[35]. It has been reported that ongoing TCR rearrangement and induced DNA damage responses are associated with Bid expression and p53 activity. *In vivo* deletion of Bid suppresses pre-T cell death [36]. It is similar to our data which suggests that Bid is important for the β -selection. Dramatic reduced regeneration of SP T cells in the thymus might also indicate that Bid is critical for positive selection although the molecular partners of Bid during the process need to be further defined. It is not clear how Bid might affect these steps based on its known molecular functions in apoptosis and cell proliferation. Nevertheless, we could speculate that Bid might have other unknown functions in regulating cellular differentiation.

Interestingly, despite the fact that overall T cell regeneration was compromised in the absence of Bid, memory T cells, especially CD8⁺CD44⁺ memory T cells, were well maintained. Although CD44 expression in the BMT model is independent of MHC disparity, the expression of the CD44 marker in peripheral T cells is associated with a susceptibility to apoptosis. Increased apoptosis in these T cells has been associated with decreased expression of anti-apoptotic Bcl-2 and Bcl-X_L [37]. Conversely, deletion of a pro-apoptosis molecule, such as Bid, might reduce apoptosis. The relative increase of CD44⁺ T cells in Bid^{-/-} reconstituted mice may suggest that Bid is able to induce apoptosis of this type of cell. Previous studies have indicated that Bid is particularly critical in apoptosis of the so-called type II cells triggered by the death receptor pathway [8]. Both type I and type II lymphoid cells have been described [38] and it may be speculated that the CD44⁺ T cells are type II cells from this aspect. As known, the peripheral T cell pool is maintained by the exporting of naïve T cells from the thymus and by post thymus expansion of activated/memory T cells. In Bid^{-/-} reconstituted mice, we observed that both naïve CD4 and CD8 T cells were reduced when they were exported from the thymus into the peripheral circulation, however, the ratio of CD4 to CD8 in the thymus was similar to that in Bid^{+/+} reconstituted mice. Thus, the maintained CD8⁺CD44⁺ memory T cells in the peripheral blood in Bid^{-/-} reconstituted mice could explain the reduced ratio of CD4 to CD8 in peripheral blood (Fig. 6)

We did observe an increase of myeloid cells after transplantation (Fig. 3 and Fig. 4) as reported [21]. However, the relative increase of myeloid in Bid^{-/-} transplanted cells could not compensate the reduced reconstitution due to reduced regeneration of lymphoid cells (Fig. 1 and Fig. 4). Therefore, in the BMT model, the effect of Bid seems more dramatic on lymphoid cells rather than on myeloid cells. It was also previously reported that the incidence of chronic myelomonocytic leukemia (CMML) and the related mortality increased significantly in aged Bid^{-/-} mice [21]. Monocytes and granulocytes are predominant in the bone marrow [21]. In our current study, we found no cases of CMML or other hematopoietic malignancies during 6 months of transplantation (n = 24). However, we cannot rule out that myeloid leukemia might develop in a prolonged period after transplantation or in a serial transplantation of Bid^{-/-} cells. Nevertheless, hematological malignancies, if any, due to the relative expansion of myeloid cells in the Bid^{-/-} transplant mice, might only occur in rare precursor cells after a highly selective process involving additional molecular abnormalities. On the other hand, Bid seems to serve as an important regulator for balancing the regeneration between lymphoid and myeloid cells after transplantation. Therefore, this study may help develop new strategies to further improve the outcome of HSCT for patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Reduced repopulation of Bid^{-/-} hematopoietic cells in a competitive BMT model

Donor bone marrow cells (2×10^6) from Bid^{-/-} or Bid^{+/+} mice (CD45.2⁺) were mixed with an equal number of competitor bone marrow cells from the F1 mice of C57BL/6 by B6.SJL mice (CD45.1⁺CD45.2⁺) and co-transplanted into pre-lethally irradiated (10 Gy) recipients (CD45.1⁺). The reconstitution of donor hematopoietic cells was monitored every 4 weeks by calculating the frequency of donor cells as a percent of CD45.2⁺ (A), or the ratio of donor cells to competitors (B). Plots show the data from one of two experiments. Mean $\pm$ standard deviation (SD) (n = 4–5 mice/group) is shown. * p < 0.05. **p < 0.01.

Figure 2. Quantitation of HSCs and HPCs in Bid^{-/-} or Bid^{+/+} bone marrow and functional analyses

(A) Bone marrow cells were harvested and stained with antibodies associated with HSCs and HPCs. Flow cytometry identified LT-HSC, ST-HSC, MPP, CLP, CMP, GMP, and MEP cells. Shown is the average frequency of each population from four mice at homeostasis. Error bars represent SDs. (B) Proliferation of HSCs was measured by incorporation of BrdU. Shown is the frequency of BrdU⁺ cells in the LT-HSC population (n = 3). (C) Flow cytometry for apoptosis analysis. The percentage of early apoptotic (Annexin V⁺DAPI⁻) LT-HSCs was compared between Bid^{-/-} and Bid^{+/+} mice (n = 7). (D) *In vitro* single cell differentiation potential was measured as described in the Methods. The frequency of single HSC-generated in all lineages of neutrophils, macrophages, erythroblasts and megakaryocytes (nmEM) is plotted (n = 180). (E) HSCs were sorted from pooled mice (n = 8–10) and labeled with CFSE before injecting into recipients (n = 3) for the homing assay. The total number of homed HSCs in the bone marrow/harvest one day after injection was compared between Bid^{-/-} and Bid^{+/+}. (F) CFU assay performed by plating 1–2 × 10⁴ bone marrow cells in triplicate into methylcellulose medium with cytokines as described in the Methods. Average frequency of total CFU, CFU-GM, CFU-GEMM and BFU-E in the plot was from one of three experiments.

Figure 3. Multi-lineage generation in the cBMT model by flow cytometry

(A) Donor (R1 region), competitor (R2 region) and recipient (R3 region) were distinguished with anti-mouse CD45.1 and CD45.2 antibodies. T, B and myeloid cell lineages in donor cells were measured using anti-mouse CD3, B220, and Mac-1, and gated for the R1 region. Flow Cytometry plots shown from one mouse 24 weeks after cBMT with Bid^{+/+} as a donor. (B) Every 4 weeks after transplantation, the frequency of T, B and myeloid cells from donor (R1) and competitor (R2) cells was plotted, and the group of Bid^{-/-} as donors and the group of Bid^{+/+} as donors were compared. The mean $\pm$ SD (n = 5 mice/group) is shown. * p < 0.05. **p < 0.01.

Figure 4. Comparison of total cell number in hematopoietic organs and multiple lineages in fully reconstituted $Bid^{-/-}$ or $Bid^{+/+}$ mice

(A) Total cell numbers in the thymus, spleen, bone marrow and total white blood count in the blood of fully reconstituted mice are shown. (B) Total T, B and myeloid cells in the spleen and bone marrow, and the concentration of T, B and myeloid cells in the blood are shown. Mean and SD (n = 8–13 mice/group) are indicated. * p < 0.05. **p < 0.01.

Figure 5. T cell development in thymus

(A) Phenotypic diagram from flow cytometric analysis and the sequence of early T cell development in the thymus associated with its phenotypes are illustrated. (B) The total cell number and the frequency of each T cell subset at different developmental stages were calculated and plotted. Mean and SD (n = 5–7 mice/group) are indicated. * p < 0.05. **p < 0.01.

Figure 6. T cell subsets in reconstituted mice

(A) Numbers of CD4⁺ and CD8⁺ T cells in blood and the ratio of CD4⁺ to CD8⁺ were calculated after flow cytometric analysis (n = 12 mice/group). In the thymus, the number of SP-CD4 and SP-CD8 and the ratio of SP-CD4 to SP-CD8 were plotted (n=5–7 mice/group). (B) Representative plots showed differences in CD44 expression in CD4⁺ and CD8⁺ T cells between Bid^{-/-} and Bid^{+/+} reconstituted mice. The absolute cell numbers of CD4⁺CD44⁺ and CD8⁺CD44⁺ memory cells in peripheral blood are shown (n = 12 mice/group). **p < 0.01.

Figure 7. B cell development in the bone marrow of reconstituted mice

(A) Phenotypic diagram from flow cytometric analysis and the sequence of early B cell development in bone marrow associated with its phenotypes are illustrated. (B) The frequency of each B cell subset at different developmental stages was calculated and plotted. Mean and SD (n = 6 mice/group) are indicated. * p < 0.05. **p < 0.01.