Critical role for Gimap5 in the survival of mouse hematopoietic stem and progenitor cells

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Mice and rats lacking the guanosine nucleotide-binding protein Gimap5 exhibit peripheral T cell lymphopenia, and Gimap5 can bind to Bcl-2. We show that Gimap5-deficient mice showed progressive multilineage failure of bone marrow and hematopoiesis. Compared with wild-type counterparts, Gimap5-deficient mice contained more hematopoietic stem cells (HSCs) but fewer lineage-committed hematopoietic progenitors. The reduction of progenitors and differentiated cells in Gimap5-deficient mice resulted in a loss of HSC quiescence. Gimap5-deficient HSCs and progenitors underwent more apoptosis and exhibited defective long-term repopulation capacity. Absence of Gimap5 disrupted interaction between Mcl-1—which is essential for HSC survival—and HSC70, enhanced Mcl-1 degradation, and compromised mitochondrial integrity in progenitor cells. Thus, Gimap5 is an important stabilizer of mouse hematopoietic progenitor cell survival.

Self-renewing hematopoietic stem cells (HSCs) consist of phenotypically and functionally defined long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs), and multipotent progenitors (MPPs) and can progress into intermediate lineage-committed progenitors, including common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs; Weissman, 2000; Adolfsson et al., 2001; Christensen and Weissman, 2001). CLPs differentiate into early lymphoid progenitors that give rise to mature NK, T, and B cells. CMPs differentiate into granulocyte/monocyte progenitors (GMPs) that give rise to mature granulocytes and monocytes, and into megakaryocyte/erythroid progenitors (MEPs) that give rise to mature platelets and red blood cells (Weissman, 2000; Adolfsson et al., 2001; Christensen and Weissman, 2001). HSCs establish a balance between the self-renewal and hematopoietic differentiation, and the balance is strictly controlled by both extrinsic and intrinsic mechanisms (Weissman, 2000). Many cytokines, including stem cell factor (SCF), thrombopoietin, flt-2 and flt-3 ligands, IL-3, and IL-6, are involved in the regulation of HSC self-renewal and differentiation (Bodine et al., 1989; Broudy, 1997; Kimura et al., 1998; Metcalf, 1998). In addition, cell apoptosis plays an important role in regulating homeostasis of HSCs and their commitment and differentiation into distinct mature blood cells (Domen et al., 2000; Kondo et al., 2003). The Bcl-2 family of proteins is a key regulator of cell apoptosis. The BH3 domain–only proapoptotic Bcl-2 family members activate the multidomain death effector proteins Bax and Bak, which promote apoptosis by perturbing the permeability of the endoplasmic reticulum and mitochondrial outer membrane, whereas antiapoptotic Bcl-2 family members can sequester the BH3 domain–only members, preventing activation of Bax and Bak (Cheng et al., 2001; Wei et al., 2001; Scorrano et al., 2003). The antiapoptotic Bcl-2 family member Mcl-1 plays a specific role in the homeostasis of HSCs and other hematopoietic progenitors. Inducible deletion of Mcl-1 in mice results in the loss of HSCs and other hematopoietic progenitors (Opferman et al., 2005).
Gimap5 (GTPase of the immune-associated protein) genes, also known as IAN (immune-associated nucleotide-binding protein), constitute a family of seven (human and rat) or eight (mouse) guanosine nucleotide-binding proteins clustered in a small syntenic genomic region of human chromosome 7, rat chromosome 4, and mouse chromosome 6, respectively (Poirier et al., 1999; Dahéron et al., 2001; MacMurray et al., 2002; Stamm et al., 2002). A loss of Gimap5 function is the cause of T cell lymphopenia in rats as a result of a near complete loss of postthymic peripheral CD8 T cells and triggers a lethal autoimmune disease in the presence of the diabetogenic MHC locus Iddm1 (Plamondon et al., 1990; Groen et al., 1995; Iwakoshi et al., 1998; Hornum et al., 2002; MacMurray et al., 2002; Pandarpurkar et al., 2003; Michalkiewicz et al., 2004). Human Gimap5 has been shown to inhibit cell apoptosis induced by either okadaic acid or γ radiation (Sandal et al., 2003). No loss-of-function mutations have been described in human patients, but a polymorphism in the polyadenylation signal sequence of the human Gimap5 gene that reduces the abundance of properly terminated transcripts appears to be associated with the presence of islet autoantibodies in type 1 diabetes patients and with an increased risk of systemic lupus erythematosus (Hellquist et al., 2007; Shin et al., 2007). Gimap5 gene ablation in the mouse does not elicit spontaneous autoimmune disease but impairs the survival of peripheral T cells and of NKT cells, replicating the phenotype of the Gimap5-deficient rat (Schulteis et al., 2008). Moreover, a Gimap5-deficient mouse exhibits a marked reduction in the abundance of NK and B cells, mild thrombocytopenia, altered erythrocyte morphology, excessive extramedullary hematopoiesis in the liver, and a limited life-span of mutant mice of ~15 wk (Schulteis et al., 2008; Barnes et al., 2010). These observations strongly suggest that the biological function of Gimap5 in the mouse might extend beyond controlling the survival of peripheral CD8 T cells. In this paper, we show that Gimap5-deficient mice indeed exhibit a progressive loss of multilineage hematopoietic capacity that can be traced to defective function of HSCs and early progenitor populations in the BM, and that these defects are associated with a disruption of mitochondrial chaperon function of HSC70 for the apoptosis regulator Mcl-1.

RESULTS
Gimap5 deficiency causes progressive bone marrow failure
In contrast with younger animals, whole BM cellularity was dramatically reduced in 9–14-wk-old Gimap5-deficient mice as compared with age- and sex-matched littermates (Fig. 1 A). Diminished absolute cell numbers were observed in lymphoid (Thy1.2+ or B220+), myeloid (Mac-1+Gr-1+), and erythroid (Ter119+) BM subpopulations (Fig. 1 B). It is of note that the body weight of Gimap5-deficient mice was only slightly reduced compared with control wild-type mice (Fig. 1 C), which is unlikely to account for the dramatic reduction in the number of total BM cells and hematopoietic cells in the mutant mice. In addition, the mutant mice had more pronounced deficiency in lymphoid than myeloid lineages (Fig. 1 B). Within the B cell lineage, pro/pre–B progenitors (B220+IgM−) were markedly reduced, and immature (B220+IgM+) and mature (B220 hiIgM+) B cells were nearly absent in Gimap5-deficient mice (Fig. 1, D and E). Similarly, more mature myeloid (Mac-1+Gr-1+), and erythroid (Ter119+CD71 b) cells were more severely affected than their respective precursors, i.e., Mac-1+Gr-1 hi and Ter119+CD71 c cells (Fig. 1, F–I). These data show that Gimap5 deficiency leads to reduced production of multiple hematopoietic lineages, that the defect is more severe in lymphoid than myeloid differentiation, and that it is more severe in more mature stages of differentiation in either lineage.
**Gimap5 deficiency differentially affects HSCs and progenitors**

The effect of Gimap5 deficiency on multiple hematopoietic lineages indicated that the defect might arise from the stem cell and/or primitive progenitor compartment. FACS analysis of the BM from 9–14-wk-old animals demonstrated that the relative abundance of the Lin−IL7R−c-Kit+Scal+ (LSK) population in BM was increased approximately threefold, whereas their absolute numbers were comparable to those in wild-type mice (Fig. 2, A and B). In contrast, the relative abundance of the Lin−IL7R−c-Kit−Scal− (LK) population was comparable to that of wild-type littermates, the absolute number of LK cells was reduced between two- and threefold (Fig. 2, A and B). Among the LSK population comprising LT-HSC (CD34+Cd135−), ST-HSC (CD34+Cd135−), and MPP (CD34+Cd135+), both the relative and absolute abundance of LT-HSCs and ST-HSCs was increased, whereas the reverse was observed for MPP (Fig. 2, C and D). Hence, Gimap5−deficient BM contains approximately twice as many ST- and LT-HSC as wild-type marrow, which is balanced by a corresponding reduction in the absolute number of MPP to yield an apparent normal absolute number of the LSK compartment in the BM of Gimap5-deficient mice.

Analysis of the LK subpopulations revealed that the relative abundance of CMPs (CD34+FcyR−), GMPs (CD34+FcyR−), and MEPs (CD34+FcyR−) was similar, whereas the absolute numbers of these populations were markedly reduced in the BM of Gimap5-deficient relative to wild-type mice (Fig. 2, E and F). Similarly, although the relative representation of CLPs (Lin−IL7R−Scal−med−c-Kitmed) was normal, the absolute numbers of this population were reduced in the BM of Gimap5-deficient relative to wild-type mice (Fig. 2, G and H).

Consistent with this phenotypic analysis, Gimap5-deficient BM cells exhibited a substantially diminished capacity for cytokine-driven ex vivo colony formation in semisolid growth medium. In the presence of IL−3 plus Epo or IL−3 alone, the mixed CFUs (CFU−mix) were markedly reduced in BM cells from Gimap5-deficient relative to wild-type mice (Fig. 3 A). The numbers of mixed colonies formed in response to SCF and macrophage colonies in response to M-CSF were also markedly reduced in the mutant mice (Fig. 3 A). In addition, the numbers of colonies formed in response to the combination of IL−3, IL−6, and SCF were markedly reduced in the mutant mice (Fig. 3 A). It is of note that the total BM cell number and the absolute number of progenitors, including MPP and other more differentiated progenitors, were both markedly reduced in Gimap5-deficient relative to wild-type mice (Figs. 1 and 2). However, proportions of many types of hematopoietic progenitors (e.g., MEP) were not reduced in the BM of Gimap5-deficient relative to wild-type mice (Fig. 2).

The number of many types of hematopoietic progenitors should be similar within a same quantity of Gimap5-deficient and wild-type BM cells. Thus, marked reduction in the number of colonies formed by a same quantity of Gimap5-deficient relative to wild-type BM cells suggested that colony production per progenitor cell is likely impaired in the absence of Gimap5.

**Gimap5 is expressed in hematopoietic progenitors**

Measurement of Gimap5 mRNA by real-time quantitative PCR in BM subpopulations showed that Gimap5 mRNA was present not only in T and B cells, as reported previously (Nitta et al., 2006), but was also readily detectable in sorted LSK, LK, HSC, MPP, CLP, CMP, GMP, and MEP and distinct

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*Figure 2. Increased numbers of HSCs and markedly reduced numbers of primitive hematopoietic progenitors in Gimap5-deficient mice. (A) LSK and LK populations in Gimap5-deficient mice. BM cells from wild-type or Gimap5-deficient mice were stained with anti-lineage cocktail (Mac-1, Gr-1, B220, CD4, CD8, and Ter-119), IL-7R, c-Kit, and Sca-1 antibodies. The percentages indicate LSK and LK cells in a gated DAPI+IL7R−c-Kit−Sca1− population. (B) Bar graphs show the numbers of LSK and LK in BM of wild-type and Gimap5-deficient mice. (C) LT-HSC, ST-HSC, and MPP populations in Gimap5-deficient mice. BM cells from wild-type or Gimap5-deficient mice were stained with anti-lineage cocktail (Mac-1, Gr-1, B220, CD4, CD8, and Ter-119), IL7R+, c-Kit+, and Sca-1 antibodies. The percentages indicate LT-HSC (CD34+Cd135−), ST-HSC (CD34+Cd135−), and MPP (CD34+Cd135+) in the gated LSK population. (D) Bar graphs show the numbers of LT-HSC, ST-HSC, and MPP in the BM of wild-type and Gimap5-deficient mice. (E) CMP, GMP, and MEP populations in Gimap5-deficient mice. BM cells from wild-type or Gimap5-deficient mice were stained with anti-lineage cocktail (Mac-1, Gr-1, B220, CD4, CD8, and Ter-119), IL7R−c-Kit−Sca1−, CD34, and CD135 antibodies. The percentages indicate LT-HSC (CD34+Cd135−), ST-HSC (CD34+Cd135−), and MPP (CD34+Cd135+) in the gated LK population. (F) Bar graphs show the numbers of LT-HSC, ST-HSC, and MPP in the BM of wild-type and Gimap5-deficient mice. (G) CLP population in Gimap5-deficient mice. BM cells from wild-type or Gimap5-deficient mice were stained with anti-lineage cocktail (Mac-1, Gr-1, B220, CD4, CD8, and Ter-119), IL7R−c-Kit−Sca1−, CD34, and CD4FcyR− antibodies. The percentages indicate CMP, GMP, and MEP in the gated LK population. (H) Bar graphs show the numbers of CMP, GMP, and MEP cells in BM of wild-type and Gimap5-deficient mice. (I) CLP population in Gimap5-deficient mice. BM cells from wild-type or Gimap5-deficient mice were stained with anti-lineage cocktail (Mac-1, Gr-1, B220, CD4, CD8, and Ter-119), IL7R−c-Kit−Sca1−, CD34, and CD4FcyR− antibodies. The percentages indicate CMP, GMP, and MEP in the gated LK population. (J) Number of wild-type and Gimap5-deficient mice. (K) Bar graphs show the numbers of CLP in BM of wild-type and Gimap5-deficient mice. Data shown are obtained from or representative of nine (A, B, G, and H) or seven (C−F) mice of each genotype. Error bars show ± SD. *P < 0.001; **P < 0.05.*
lineages of hematopoietic BM cells, including NK (NK1.1+), B (B220+), T (Thy1.2+), myeloid (Mac-1+), and erythroid (Ter119+) lineages (Fig. 3 B). Overall, the amounts of Gimap5 mRNA were slightly higher in lymphoid lineages than in myeloid lineages (Fig. 3 B). Thus, the cellular distribution of Gimap5 mRNA in the BM correlated with the range of cell subpopulations affected by Gimap5 deficiency.

**Gimap5 deficiency impairs hematopoietic progenitor repopulation ability**

To determine whether there is an intrinsic defect in the long-term repopulation capacity of Gimap5-deficient HSC, competitive repopulation experiments were performed. As determined 8 wk after transfer of an equal number of Gimap5-deficient (CD45.2) and wild-type competitor BM cells (CD45.1) into lethally irradiated wild-type BM experi-ments, markedly <50% of the total BM cells were derived from Gimap5-deficient donor cells (Fig. 3 C). This deficit was not limited to LK and CLP progenitors, myeloid cells (Mac-1+ or Gr-1+), lymphoid cells (B220+, Thy1.2+, or NK1.1+), and erythroid cells (Ter119+) but was also evident in the LSK compartment, including HSCs and MPPs. A corresponding underrepresentation of Gimap5-deficient cells was observed in the spleen of competitively repopulated recipients, including myeloid (Mac-1+ or Gr-1+), lymphoid (B220+, Thy1.2+, or NK1.1+), and erythroid (Ter119+) cells (Fig. 3 C). In contrast, in recipients that received equal numbers of CD45.2 wild-type and CD45.1 wild-type competitor BM cells, ~50% of total BM cells, including LSK/LK, HSC, MPP, and CLP progenitors, myeloid, lymphoid, and erythroid cells, and total splenic cells, including myeloid, lymphoid, and erythroid cells, displayed CD45.2 origin (Fig. 3 C). The impaired engrafting capacity of Gimap5-deficient hematopoietic progenitors was further confirmed in the competitive repopulation experiments 16 wk after BM transplantation (Fig. S1). It is of note that no significant differences in BM homing between Gimap5-deficient and wild-type hematopoietic progenitors were observed (Fig. 3 D).

In the Gimap5-deficient rat, occurrence of autoimmunity is strictly dependent on coinheritance of the appropriate (diabetogenic) MHC locus (Ono et al., 1988; Hanenberg et al., 1989; Huang et al., 1994). In the F344 rat lacking this particular MHC, no autoimmunity occurs (Fuller et al., 2006). In addition, Gimap5-null mice with a C57 or BALB/c background do not develop spontaneous autoimmunity (Schulteis et al., 2008). Gimap5 deficiency–caused autoimmunity is associated with reduction of regulatory T cells (T reg cells; Poussier et al., 2005; Hillebrands et al., 2006). To further rule out that the impairment of repopulation ability of Gimap5-deficient BM is a result of autoimmune attack, we examined T reg cell population and lymphocyte activation status in competitively reconstituted recipients. Recipients that received an equal number of Gimap5-deficient (CD45.2) and wild-type competitor BM cells (CD45.1) possessed a normal population of T reg cells, although these T reg cells were derived from wild-type but not Gimap5-deficient BM (Fig. S2 A). Control recipients that received equal numbers of CD45.2 and CD45.1 wild-type BM cells also had a normal population of T reg cells derived from both types of wild-type BM cells (Fig. S2 A). Consistent with the observation of normal T reg cell populations in both types of recipients, T and B cells from these recipients barely showed expression of activation
markers, such as CD69, CD80, or CD86 (Fig. S2, B and C). Thus, in the absence of immune activation, Gimap5-deficient BM cells display a reduced ability to competitively repopulate. These outcomes document that Gimap5 deficiency in a cell-autonomous manner impairs the ability of BM resident cells to competitively repopulate virtually all hematopoietic lineages, consistent with a defect in HSC and/or early progenitors.

**Gimap5 sustains HSC quiescence and survival**

To characterize the role of Gimap5 for HSC function, the effect of Gimap5 deficiency on in vivo proliferation and survival of HSC was examined by measuring in vivo BrdU incorporation and Annexin V/DAPI staining, respectively. The fraction of BrdU+ HSC-enriched LSK cells in Gimap5-deficient mice was more than twofold higher than that in wild-type counterparts (Fig. 4, A and B). Within the LSK compartment, ~20% of Gimap5-deficient HSC had incorporated BrdU, compared with ~2% of wild-type HSC. The increased BrdU incorporation in Gimap5-deficient MPP was slightly less pronounced (Fig. 4, A and B). Conversely, the fraction of more differentiated BrdU+ Gimap5–deficient LK cells was only slightly elevated when compared with that of corresponding wild-type cells (Fig. 4, A and B). RNA and DNA content analysis by Pyronin Y and DAPI staining further revealed that the proportions of Gimap5-deficient CD135– LSK cells (LT- and ST-HSC) and CD150+ LSK cells (LT-HSC) in the S + G2/M phases were markedly increased when compared with those of wild-type controls (Fig. 4 C). In addition, Annexin V/DAPI staining revealed that the relative abundance of apoptotic LSK cells was markedly higher in Gimap5–deficient relative to wild-type mice (Fig. 4 D). Consistent with earlier findings in T cell line (Nitta et al., 2006), increased apoptosis was noted in more differentiated or committed hematopoietic cells, including B cells at different developmental stages (unpublished data). Furthermore, a marked increase of apoptosis was also observed in the Gimap5-deficient CD135– LSK population, comprising LT- and ST-HSC (Fig. 4 E). These data demonstrate that the loss of Gimap5 function not only causes apoptosis of hematopoietic stem and progenitor cells but also triggers a marked loss of HSC quiescence. The overall preponderance of the proliferative effect of Gimap5 deficiency in stem cells is consistent with, and explains, the increased absolute number of these cells (Fig. 2, C and D). The loss of HSC quiescence in Gimap5–deficient mice could be caused by a reduction in numbers of hematopoietic progenitors and differentiated cells in these mutant mice. To test this possibility, cell proliferation of Gimap5–deficient HSC was examined in competitively reconstituted recipients that had normal numbers of all hematopoietic lineages derived from wild-type CD45.1 BM (Fig. 3 C). It is of note that CD45.2+ Gimap5–deficient HSC in competitively reconstituted recipients displayed comparable BrdU incorporation relative to CD45.2+ wild-type HSC in control recipients (Fig. 4 F). Thus, the loss of quiescence in HSCs in Gimap5–deficient mice is the result of an apoptosis-caused reduction in numbers of hematopoietic progenitors and differentiated cells.

**Gimap5 stabilizes Mcl-1 by strengthening the association of Mcl-1 with HSC70**

To understand the molecular mechanism underlying the regulation of hematopoietic progenitor apoptosis by Gimap5, we sought to identify molecules capable of interacting with Gimap5. Bcl-2 family members are important regulators of cell survival/apoptosis and each member plays a specific role
of proteins. The interaction of Gimap5 with endogenous HSC70 was confirmed by coimmunoprecipitation/Western blot analysis with the indicated antibodies. (B) Cell lysates from parental or Gimap5nFL 70Z/3 cells were incubated with Sepharose beads conjugated with anti-Flag. The immune complex was subjected to Western blot analysis with the indicated antibodies. (C) Cell lysates from parental or Gimap5nFL 70Z/3 cells were immunoprecipitated with anti–Mcl-1 or anti–Bcl-xL or control IgG. The immune complex was subjected to Western blot analysis with the indicated antibodies. (D) Cell lysates from parental or Gimap5nFL 70Z/3 cells were immunoprecipitated with anti-HSC70. The immune complex was subjected to Western blot analysis with the indicated antibodies. (E) The cytosol and mitochondria fractions were extracted from parental or Gimap5nFL 70Z/3 cells and subjected to Western blot analysis with the indicated antibodies. Tom20 was used as a marker of mitochondria fraction. (F) Gimap5nFL 70Z/3 cells were applied to glass slides and subjected to immunofluorescence analyses with anti-Flag (green) with a combination of anti–Mcl-1 or anti–HSC70 (red). Data shown are representative of three (A and E) or two (C, D, and F) independent experiments.
that Gimap5, HSC70, and Mcl-1 form a ternary protein complex on mitochondria.

Deletion of the Gimap5 gene abolished Gimap5 protein expression (Fig. 6 A), and we examined the effect of Gimap5 deficiency on the interaction between HSC70 and Mcl-1/ Bcl-xL. Immunoprecipitation of endogenous Mcl-1 or Bcl-xL resulted in coimmunoprecipitation of endogenous HSC70 in wild-type BM-derived B cell progenitors (Fig. 6 B). However, in the absence of Gimap5, the amount of endogenous HSC70 that was coimmunoprecipitated with endogenous Mcl-1 or Bcl-xL was dramatically reduced (Fig. 6 B). In addition,
Gimap5 deficiency markedly reduced the amount of endogenous HSC70 that was communoprecipitated with endogenous Bcl-2 (unpublished data). It is of note that both wild-type and mutant cells expressed the same amount of HSC70 proteins (Fig. 6 B). Thus, Gimap5 is required for the firm interaction between Mcl-1 or Bcl-xL and HSC-70.

We next examined the effect of Gimap5 deficiency on the cellular localization of Mcl-1 or Bcl-xL and HSC-70. Subcellular fractionation analysis demonstrated that endogenous Gimap5 exclusively localized in mitochondria-rich fraction in wild-type but was absent in Gimap5-deficient BM-derived primary B cell progenitors (Fig. 6 C). It is of note that the cellular localization of Mcl-1 and Bcl-xL was the same in wild-type and Gimap5-deficient primary B cell progenitors (Fig. 6 C). Similarly, the cellular localization of HSC70 was also the same in both wild-type and Gimap5-deficient primary B cell progenitors (Fig. 6 C). Furthermore, exclusive mitochondria localization of endogenous Gimap5 was observed in wild-type BM-derived hematopoietic progenitors (Fig. 6 D). Again, the cellular localization of Mcl-1, Bcl-xL, Bcl-2, and HSC70 was the same in wild-type and Gimap5-deficient hematopoietic progenitors (Fig. 6 D). Thus, Gimap5 deficiency does not affect cellular localization, including mitochondrial localization, of Mcl-1, Bcl-xL, Bcl-2, and HSC70.

Mcl-1 is eliminated by proteasome-dependent protein degradation and withdrawal of cytokines can trigger Mcl-1 elimination (Zhong et al., 2005; Maurer et al., 2006). Previous studies have shown that Mcl-1 and Bcl-xL associate with HSP70, which stabilizes Mcl-1 and Bcl-xL (Jacobs and Marnett, 2007, 2009; Stankiewicz et al., 2009). To this end, we examined the effect of Gimap5 deficiency on the stabilities of Mcl-1 and Bcl-xL. As expected, withdrawal of IL-7 prompted Mcl-1 degradation in wild-type primary B cell progenitors (Fig. 6 E). Importantly, the levels of Mcl-1 protein subsequent to IL-7 withdrawal were markedly lower in Gimap5-deficient relative to wild-type primary B cell progenitors (Fig. 6 E). In contrast, the levels of Bcl-xL and Bax proteins were largely stable after IL-7 withdrawal in both wild-type and Gimap5-deficient progenitors (Fig. 6 E). It is of note that IL-7 withdrawal also accelerated Bcl-2 degradation in Gimap5-deficient relative to wild-type primary B cell progenitors (Fig. 6 E). Therefore, Gimap5 deficiency reduced the stabilities of Mcl-1 and Bcl-2, but not Bcl-xL or Bax, in B cell progenitors. Furthermore, we examined the effect of Gimap5 deficiency on the stabilities of Bcl-2 family members in BM-derived hematopoietic progenitors. Withdrawal of cytokines (IL-3, IL-6, and SCF) accelerated Mcl-1 degradation in Gimap5-deficient relative to wild-type hematopoietic progenitors (Fig. 6 F). Cytokine withdrawal induced Bcl-xL degradation in wild-type hematopoietic progenitors, but lack of Gimap5 did not influence this process (Fig. 6 F). It is of note that the levels of Bcl-2 proteins were stable after cytokine withdrawal in both wild-type and Gimap5-deficient progenitors (Fig. 6 F). Therefore, Gimap5 deficiency reduced the stabilities of Mcl-1, but not Bcl-xL or Bcl-2, in hematopoietic progenitors.

The Bcl-2 family, including Mcl-1, is essential for maintaining mitochondria integrity (Gross et al., 1999; Kuwana et al., 2005). Thus, we examined the effect of Gimap5 deficiency on the integrity of mitochondria. The mitochondrial potential ($\Delta\Psi_m$) is an indicator for mitochondria integrity and can be assessed by the potential sensing dye JC-1 (Smiley et al., 1991; Harris and Thompson, 2000). JC-1 accumulates within the intact mitochondria as bright red J-aggregates but remains in the cytoplasm as a green fluorescent monomer when mitochondria lose $\Delta\Psi_m$ (Smiley et al., 1991). A greater number of Gimap5-deficient BM-derived primary B cell progenitors displayed a decrease of mitochondrial $\Delta\Psi_m$ compared with corresponding wild-type B progenitors (unpublished data). Similarly, a larger proportion of Gimap5-deficient BM-derived hematopoietic progenitors displayed a decrease of mitochondrial $\Delta\Psi_m$ compared with corresponding wild-type progenitors after cytokine withdrawal (Fig. 6 G). Thus, Gimap5 deficiency results in a decrease of $\Delta\Psi_m$, indicating impaired integrity of mitochondria.

In addition, the in vitro BrdU labeling assay demonstrated that cytokine-mediated cell proliferation was comparable between Gimap5-deficient and wild-type B cell and hematopoietic progenitors (Fig. 6, H and I). Consistent with the findings that Gimap5 deficiency reduced Mcl-1 stability, Annexin V and 7-AAD staining assay detected that cytokine withdrawal–induced cell apoptosis was markedly enhanced in Gimap5-deficient relative to wild-type primary B cell or hematopoietic progenitors (Fig. 6, J and K). Therefore, Gimap5 deficiency increases cytokine withdrawal–induced cell apoptosis of B cell and hematopoietic progenitors. It is of note that expression of Gimap5, but not GFP alone, by retrovirus-mediated gene transfer restored the survival of BM-derived Gimap5-deficient hematopoietic progenitors to the same level as was observed in wild-type hematopoietic progenitors transduced with GFP alone (Fig. S3). Expression of Flag-tagged Gimap5 restored the survival of BM-derived Gimap5-deficient hematopoietic progenitors to the same extent as wild-type Gimap5 (Fig. S3). Collectively, these data demonstrate that Gimap5 deficiency impairs the interaction between Mcl-1 and HSC70, decreases the stability of Mcl-1, and reduces the integrity of mitochondria, resulting in an increase of cell apoptosis.

**DISCUSSION**

Previous studies have demonstrated that Gimap5 is essential for the survival and development of T lymphocytes, including T reg cells, and its mutation leads to T lymphopenia in rat and mouse (Plamondon et al., 1990; Groen et al., 1995; Iwakoshi et al., 1998; Hernández-Hoyos et al., 1999; Whalen et al., 1999; Hornum et al., 2002; MacMurray et al., 2002; Pandarpurkar et al., 2003; Michalkiewicz et al., 2004; Pousset et al., 2005; Nitta et al., 2006; Schulteis et al., 2008). However, the consequences of Gimap5 deficiency in the mouse are not limited to T cell defects but extend to defective development of B cells and NK cells (Schulteis et al., 2008). All these abnormalities in the Gimap5-deficient mouse are confirmed in the sphinx mouse strain, which exhibits a loss of Gimap5 protein.
expression secondary to ENU-induced mutagenesis (Barnes et al., 2010). In addition, excessive hematopoiesis in the liver, and to a lesser extent in the spleen, is a phenotypic hallmark of mice lacking Gimap5 (Schulteis et al., 2008; Barnes et al., 2010). A markedly increased number of hepatic hematopoietic foci can already be detected in 3-wk-old Gimap5-deficient mice and becomes progressively dominant in older mice (12–15 wk; Schulteis et al., 2008). Although extramedullary hematopoiesis is usually considered a compensatory response to reduced BM output or outright failure, no such defects in marrow cellularity or cellular composition were detected in relative young Gimap5-deficient mice with mixed genetic background (C57BL/6 and 129; Schulteis et al., 2008; Barnes et al., 2010). In this paper, we demonstrate that Gimap5 is expressed in HSCs, hematopoietic progenitors, and differentiated blood cells and is critical for their survival. Gimap5 deficiency results in reduction in primitive hematopoietic progenitors and multiple hematopoietic lineages. The reduction of committed progenitors and differentiated cells leads to a breakdown of quiescence of Gimap5-deficient HSCs. In addition, Gimap5-deficient HSCs have impaired engrafting ability. Gimap5-deficient HSCs, hematopoietic progenitors, and differentiated blood cells all have increased apoptosis.

Members of the Bcl-2 family play important and unique roles during hematopoiesis. Mcl-1 is essential for the survival of HSCs and other early hematopoietic progenitors (Opferman et al., 2005). Inducible deletion of Mcl-1 in mice results in the loss of HSCs and other hematopoietic progenitors (Opferman et al., 2005). Mcl-1 also plays an important role in the maintenance of the mature lymphocytes (Opferman et al., 2003). Bcl-2 is critical for the survival of mature lymphocytes but not for early hematopoiesis (Veis et al., 1993). Loss of Bcl-2 leads to massive apoptosis of T and B lymphocytes (Veis et al., 1993). Bcl-xL is important for survival of erythrocytes and T cells, and Bcl-xl-deficient mice display a massive loss of immature erythrocytes and double-positive early T cells (Ma et al., 1995; Motoyama et al., 1995, 1999). Proapoptotic Bim is critical for the survival of hematopoietic progenitors, and its deficiency results in an increase in myeloid and lymphoid but not erythroid cells (Bouillet et al., 1999). In addition, the proapoptotic family members Puma and Nix are implicated in regulating the survival of lymphocytes and erythrocytes, respectively (Erlicher et al., 2006; Diwan et al., 2007). Importantly, Gimap5 plays a critical role in stabilizing Mcl-1 in BM-derived hematopoietic progenitors. Thus, Gimap5 regulates the survival of HSCs and other early hematopoietic progenitors at least partially through stabilizing Mcl-1. In addition to Mcl-1, Gimap5 can stabilize Bcl-2 in BM-derived B cell progenitors. Accordingly, destabilization of both Mcl-1 and Bcl-2 proteins by Gimap5 deficiency may explain the more severe reduction and obvious loss of viability in mature lymphoid lineages relative to early hematopoietic progenitors and myeloid lineages in Gimap5-deficient mice.

Mcl-1 expression is up-regulated by SCF stimulation in HSCs and by IL-7 in lymphoid progenitors (Opferman et al., 2003, 2005). Withdrawal of cytokines triggers Mcl-1 elimination by proteasome-dependent protein degradation (Zhong et al., 2005; Maurer et al., 2006). With a loss of Gimap5, cytokine withdrawal–induced degradation of Mcl-1 is markedly enhanced. Gimap5-deficient hematopoietic cells are more prone to apoptosis than wild-type corresponding cells, especially when cytokines are limiting. Consequently, Gimap5-deficient hematopoietic progenitors, including HSCs, fail to compete with wild-type counterparts in competitive engraftment. The Gimap5 deficiency–induced loss of the cellularity of all hematopoietic lineages becomes more pronounced with age. A nearly complete loss of B cell populations and an associated increase in apoptotic cells in the BM of aged Gimap5-deficient mice (unpublished data) is consistent with progressive depletion of HSCs in these mutant mice. In contrast, overexpression of Gimap5 might confer hematopoietic cells a survival advantage and resistance to cytokine insufficiency–induced cell death. In fact, Gimap5 is up-regulated in human B cell lymphomas, indicating that a gain of Gimap5 function might contribute to the development of B cell malignancy by inhibiting cell apoptosis (Zenz et al., 2004).

Our current studies and several of the previous studies have demonstrated that Gimap5 predominantly localizes on mitochondria (Dahéron et al., 2001; Hornnum et al., 2002; Pandarpurkar et al., 2003; Zenz et al., 2004; Keita et al., 2007), although some other studies have indicated that Gimap5 is also expressed on ER membrane (Stamm et al., 2002). Lack of Gimap5 in rat T cells alters the composition of mitochondrial membrane protein complexes, resulting in loss of mitochondrial integrity and increased spontaneous apoptosis (Pandarpurkar et al., 2003). A hairpin RNA–mediated Gimap5 knockdown induces the mitochondria-mediated apoptosis in a T cell line (Nitta et al., 2006). A previous study has detected the interaction between Gimap5 and Bcl-2 when these molecules are overexpressed in T cell line (Nitta et al., 2006). The same study has also found the association between Gimap5 and Bcl-xL when they are overexpressed in T cell line (Nitta et al., 2006). In addition, Bax, a proapoptotic member of Bcl-2 family, has been shown to associate with Gimap5 after its apoptosis-induced translocation onto mitochondrial membrane (Nitta et al., 2006). Thus, the association between the Gimap family proteins and the Bcl-2 family proteins might be the key to maintaining the integrity of mitochondrial membrane. However, the mechanism by which Gimap5 maintains mitochondrial integrity through the association with the Bcl-2 family proteins has been unclear. In this study, we have discovered that Gimap5 physically interacts not only with Bcl-xL and Bcl-2 but also with Mcl-1 and HSC70, a member of the Hsp70 family. Thus, Gimap5 appears to strengthen the association between Mcl-1/Bcl-xl/Bcl-2 and HSC70 on mitochondria. It is of note that removal of Mcl-1 is regulated by ubiquitination and subsequent degradation (Stankiewicz et al., 2009). Importantly, our findings have demonstrated that HSC70 may...
not directly associate with Mcl-1, and Gimap5 is required to connect HSC70 and Mcl-1 together to form the HSC70–Gimap5–Mcl-1 ternary complex. Lack of Gimap5 impairs the association of HSC70 with Mcl-1, resulting in a rapid degradation of Mcl-1 and subsequent cell apoptosis. It is of note that Gimap5 deficiency has no effect on Mcl-1 mitochondrial localization. In addition, HSC70 associates with Bcl-xL and Bcl-2 and prevents them from degradation (Jacobs and Marnett, 2007, 2009). We have found that Gimap5 markedly strengthens the association of HSC70 with Bcl-xL and Bcl-2. However, lack of Gimap5 impairs the association of HSC70 with Bcl-xL and Bcl-2 but only results in a rapid degradation of Bcl-2 in B cells. Again, Gimap5 deficiency has no effect on the cellular localization of Bcl-xL and Bcl-2. Gimap5 specifically stabilizes Mcl-1 and Bcl-2 but not Bcl-xL, indicating that an additional mechanism is involved in regulating Bcl-xL stabilization. Bcl-2 family members play a critical role in maintaining mitochondrial outer membrane integrity (Newmeyer and Ferguson-Miller, 2003; Green, 2005). Thus, Gimap5 maintains mitochondrial integrity and subsequent cell viability through stabilization but not mitochondrial localization of Mcl-1 and Bcl-2. A limitation of the current analysis is that all of the biochemical studies were performed in cell lines and in vitro BM culture–derived hematopoietic or B cell progenitors. Regulation of survival of these cells in vitro may not be exactly same as that of HSCs or hematopoietic B cell progenitors. Levels of Gimap5 transcription were normalized to β-actin abundance. The primers used for β-actin were: 5’ primer, 5’-CCACAGCTGAGGGAATACT-3’; and 3’ primer, 5’-CTTCTCCAGGGAGGAAGAGG-3’. Real-time PCR was performed in triplicate at 95°C for 2 min and then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min using the 7500 Real-Time system (Applied Biosystems) in 25-µl reaction volumes containing cDNA, primers, and iQ SYBR Green Supermix (Bio-Rad Laboratories). Data were analyzed using the 7500 system analyzing software and relative transcription was calculated using the ΔCT method.

Flow cytometry. Single-cell suspensions of BM cells were treated with Gey’s solution to remove red blood cells and resuspended in PBS supplemented with 2% FBS. The cells were then stained with a combination of fluorescence-conjugated antibodies. PE-conjugated anti–Mac-1, anti–B220, anti–Gr-1, anti–Ter-119, anti–c–Kit, anti–FceγR, and anti–FoxP3; FITC-conjugated anti-CD34, anti-CD71, Sca-1, and anti-CD150; Biotin-conjugated anti-CD4, anti-CD8, anti-Ter119, and anti-CD13; APC-conjugated anti-B220, anti–c–Kit, anti–Mac-1, and anti–IgM; PE-Cy5–conjugated anti–CD80; PE-Cy7–conjugated anti-CD69 and anti-B220; and PE-Cy5.5–conjugated anti-streptavidin were purchased from eBioscience. PE-conjugated anti–Thy1.2, anti–CD4, anti–CD8, and anti–CD86; Biotin-conjugated anti–Gr-1, anti–Mac-1, anti–B220, and anti–IL-7R; and FITC-conjugated anti-CD45.2, anti–BrdU, and anti–Annexin V were purchased from BD. DAPI was added after staining to exclude the dead cells. Samples were applied to a flow cytometer (LSRII, BD). Data were collected and analyzed using CellQuest or FACSDiva software (BD).

In vivo and in vitro BrdU incorporation assay. Mice were intraperitoneally injected with 1 mg BrdU (Sigma-Aldrich) in 0.2 ml PBS 2 h before analyses. The BM cells from BrdU-injected mice were incubated with Bio-conjugated CD135, followed by staining with the combination of PE-conjugated lineage cocktail (anti–Mac-1, Gr-1, B220, CD4, CD8, Ter-119, and IL-7R), PE-cy7–conjugated anti–Sca-1, APC-conjugated anti–c–Kit, and PE-Cy5–conjugated anti-streptavidin. Finally, the cells were fixed and then stained with FITC-conjugated anti–BrdU and DAPI. For in vitro BrdU incorporation, IL-7 BM culture–derived B cell or IL-3 + IL-6 + SCF BM culture–derived hematopoietic progenitors were cultured in the presence of 10 µM BrdU for 1 h. Then, B cell progenitors were stained with APC-conjugated anti–B220, FITC-conjugated anti–BrdU, and 7–AAD, whereas hematopoietic progenitors were stained with lineage cocktail, anti–BrdU, and DAPI. The degree of BrdU positivity was analyzed by FACS.

Cell apoptosis assay. The BM cells from wild-type and Gimap5-deficient mice were stained with the combination of PE-conjugated lineage cocktail (Mac-1, Gr-1, B220, CD4, CD8, Ter-119, and IL-7R), PE-Cy7–conjugated Sca-1, and APC–conjugated anti–c–Kit, followed by staining with FITC-conjugated Annexin V and DAPI. The degree of Annexin V+ and 7–AAD was analyzed by FACS. For the detection of cytokine withdrawal–induced cell apoptosis, IL-7 BM culture–derived B cell or IL-3 + IL-6 + SCF BM culture–derived hematopoietic progenitors were cultured in the absence of cytokines for the indicated times, followed by the staining with APC-conjugated Annexin V and 7–AAD. The degree of Annexin V+ cells was analyzed by FACS.

MATERIALS AND METHODS

Mice. Gimap5-deficient mice were generated as previously reported (Schulteis et al., 2008) and were on the C57BL/6 background. Experimental and control mice were 9–14 wk old. Mice were maintained in the Biological Resource Center at the Medical College of Wisconsin (MCW). All animal protocols were approved by the MCW Institutional Animal Care and Use Committee.

Real-time quantitative RT-PCR analysis. Subsets of hematopoietic progenitors and differentiated cells were sorted from BM cells of 2-mo-old wild-type mice by flow cytometry. Total RNA was isolated from the indicated cells. First-strand cDNA was synthesized from the total RNA with the SensiScript RT kit (QIAGEN) using random primers (Invitrogen) according to the manufacturer’s protocol. Real-time PCR was performed to quantitate Gimap5 using the following primers: 5’ primer, 5’-GCTGACAGCAGCCGTTGGG–3’; and 3’ primer, 5’-TGGGCCTCTGTCTTTGAAC–3’. Levels of Gimap5 transcription were normalized to β-actin abundance. The primers used for β-actin were: 5’ primer, 5’-CCACAGCTGAGGGAATACT-3’; and 3’ primer, 5’-CTTCTCCAGGGAGGAAGAGG-3’. Real-time PCR was performed in triplicate at 95°C for 2 min and then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min using the 7500 Real-Time system (Applied Biosystems) in 25-µl reaction volumes containing cDNA, primers, and iQ SYBR Green Supermix (Bio-Rad Laboratories). Data were analyzed using the 7500 system analyzing software and relative transcription was calculated using the ΔCT method.
Measurement of mitochondrial membrane \( \Delta \Psi_{\text{m}} \), IL-3 + IL-6 + SCF

BM culture–derived CD34+ hematopoietic progenitors were cultured in the presence or absence of cytokines for the indicated times, and then incubated with 2.5 µg/ml \( \Delta \Psi_{\text{m}} \) indicator JC-1 in complete medium at 37°C for 30 min. After washing with PBS, the detection of JC-1 staining was performed by FACS analysis.

Mass spectrometry analysis. 5 × 10^4 70Z/3 or 70Z/3 cells that stably express Flag-tagged Gimap5 were lysed in lysis buffer (10 mM Tris, pH 7.6, 50 mM NaCl, 100 mM NaCo3, 5 mM DTT, 2% Triton X-100, 3 µg/ml aprotinin, 2 µg/ml pepstatin A, and 1 µg/ml leupeptin) at 4°C. Cell lysates were precleared with Sepharose beads and subsequently incubated with the anti-flag–conjugated Sepharose beads at 4°C for overnight. After washing five times with cold lysis buffer, bound proteins were eluted in 2× Laemmli buffer at 100°C for 5 min and subjected to 10% SDS-PAGE and Coomassie blue staining. The proteins that appeared in cell lysates derived from 70Z/3 cells expressing Flag-tagged Gimap5, but not parental 70Z/3 cells, were subjected to in-gel tryptic digestion and a MALDI-TOF mass spectrometry analysis. The mass spectrometry data were analyzed with the ProteinProspector programs (University of California, San Francisco).

Mitochondria preparation. The cells were incubated in the homogenization buffer (250 mM sucrose, 20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 3 µg/ml aprotinin, 2 µg/ml pepstatin, and 1 µg/ml leupeptin) on ice for 10 min and then lysed with 100 strokes of a dounce homogenizer. The cell lysates were centrifuged at 4°C at 7,000 g for 10 min. The supernatant was centrifuged at 4°C at 7,000 g for 10 min and the pellets were collected as the mitochondria. The supernatant was further centrifuged at 4°C at 15,000 g for 10 min and the supernatant was collected as the cell cytosol fraction.

CFU assays. BM cells were plated in duplicate in methylcellulose with recombinant cytokines specific for each of the assays as previously described (Parganas et al., 1998). For the Epo + IL-3 assay, 2 × 10^5 cells/dish were cultured in 10 ng/ml of recombinant murine M-CSF (R&D Systems), and colonies were scored at day 12. For the CFU-Mix assay, 1.5 × 10^5 cells/dish were cultured in 10 ng/ml of recombinant murine IL-3, 50 ng/ml of recombinant murine SCF (R&D Systems), and 10 ng/ml of recombinant murine IL-6 (R&D Systems) or 10 ng/ml of recombinant murine IL-3 alone, or 2 × 10^5 cells/dish were cultured in 50 ng/ml of recombinant murine SCF alone. The colonies were scored at day 6 or 12. For the CFU-M assay, 1.5 × 10^6 cells/dish were cultured in 10 ng/ml of recombinant murine M-CSF (R&D Systems), and the colonies were scored at day 12.

Competitive repopulation assay. 10^7 BM cells (CD45.2+) from wild-type or Gimap5-deficient mice were mixed 1:1 with the competitor CD45.1 BM cells from wild-type B6 SJL mice and transplanted into lethally irradiated (1,100 cGy) wild-type B6 SJL mice by retroorbital injection. The recipient mice were analyzed 8 or 16 wk after BM transplantation.

Immunofluorescence microscopy. 70Z/3 cells that stably express Flag-tagged Gimap5 were washed twice in PBS, resuspended in PBS (5 × 10^6 cells/ml), and applied to glass slides in 10-µl aliquots. After cells completely dried, the slides were fixed for 30 min in 4% buffered formalin (Thermo Fisher Scientific), permeabilized by incubation in 0.2% Triton X-100 in PBS for 15 min, and blocked for 1 h at room temperature with 10% bovine serum albumin in PBS. After blocking, the slides were incubated for 2 h at room temperature with mouse anti-Flag with a combination of rabbit anti–Mcl-1 (Rockland Immunocchemicals), or rat anti–HSC70 (Santa Cruz Biotechnology, Inc.), respectively. Slides were washed three times in PBS and incubated for additional 1 h at room temperature with PE-conjugated goat anti-rabbit IgG (SouthernBiotech), APC-conjugated goat anti–mouse IgG1 (BD), or donkey anti-rat IgG (eBioscience), respectively. Slides were then washed three times with PBS, mounted with Vectorshield (Vector Laboratories), and examined for fluorescence using a confocal microscope (FlowView multiphoton LSM; Olympus). Images were obtained and processed using FlowView software.

BM homing assay. BM homing assay was performed as previously described (Cheng et al., 2000; Maillard et al., 2009). In brief, 2 × 10^7 BM cells from wild-type or Gimap5-deficient mice were intravenously injected into lethally irradiated (1,100 rad) CD45.1-B6-SJL recipients. 6 h later, the percentage of CD45.2+ donor hematopoietic progenitors in the recipient BM was determined by FACS.

Online supplemental material. Fig. S1 shows the engrafting capacity of Gimap5-deficient hematopoietic progenitors. Fig. S2 shows the T reg cell population and lymphocyte activation status in competitively reconstituted recipients. Fig. S3 describes the rescue of Gimap5-deficient hematopoietic progenitors from cytokine withdrawal–induced apoptosis by the expression of wild-type or Flag-tagged Gimap5. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101192/DC1.

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