Role of bcl-2 in the Development of Lymphoid Cells From the Hematopoietic Stem Cell

By Yumi Matsuzaki, Kei-ichi Nakayama, Keiko Nakayama, Takashi Tomita, Miu Isoda, Dennis Y. Loh, and Hiromitsu Nakauchi

To investigate the role of bcl-2 in lymphohematopoiesis, a long-term bone marrow reconstitution system was established. Transplantation of 1,000 c-Kit+ Sca-1+ and lineage markers negative cells from bcl-2−/− mouse bone marrow resulted in long-term reconstitution of nonlymphoid cells. However, T cells were totally absent and B-lymphocyte development was severely impaired at a very early stage of differentiation in the chimeric mouse. On the other hand, transplantation of day 14 fetal liver cells from bcl-2−/− mice resulted in generation of both T and B cells in the recipient, albeit transiently. These data suggest that bcl-2 plays a critical role in the development of lymphoid progenitor cells from the hematopoietic stem cell (HSC), but is not essential for the development of nonlymphoid cells and the self-renewal of HSC. In addition, lymphopoiesis from fetal liver HSC appears to be less dependent on bcl-2 than adult bone marrow HSC.

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MATERIALS AND METHODS

Mice. The bcl-2 targeted mice 13 were maintained by heterozygous matings in our animal facility. PCR screening was performed on the litters at 2 weeks of age by using two sets of primers in which the first primers were located in the neo gene and the second primers were located in the endogenous bcl-2 gene. First set, 5′-TGCTAAAGGCATGCTCAGACTG-3′ and 5′-ATTGCT-TCTTTTATACACAAAG-3′, second set, 5′-CGTCCCCCGCTTTCCACCTTTACGC-3′ and 5′-ATCTCCCTCCGATCCACCCATCCAC-3′. Genomic DNA (200 ng) was used as the template for the PCR (94°C for 30 seconds, 62°C for 30 seconds, 72°C for 1 minute and 30 seconds). C57BL/6-Ly5.1:Pep3b (B6-Ly5.1) mice were maintained in our animal facility and used for these studies at between 4 and 6 weeks of age.

Antibodies. For cell sorting, biotinylated rat monoclonal antibodies (MoAb), RA-6B2 (anti-B220), 18 M1/70 (anti-Mac-1), 19 RA3-8C5 (anti-Gr-1), 18 GK1.5 (anti-L3T4), 19 53-6.7 (anti-Ly-2), 19 and TER119 19 were used as lineage markers. Allophycocyanin (APC) conjugated ACK-2 (anti-c-kit) 25 and phycoerythrin conjugated Sca-
1 (anti-Ly 6A/E) were used as stem cell markers. B220, Mac-1, 100 ng/mL) were added. The cultures were prepared in 35-mm non-sense primers, and 5 U of Taq polymerase (Takara). After an initial

For analysis of reconstituted cells, mouse MoAbs A20.1 (anti-Ly 5.1) obtained from Dr Yamiko Saga (RIKEN, Tsukuba, Japan) and 104.2 (anti-Ly 5.2) obtained from Dr Hidetaka Yakura (Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan) were derivatized with Texas Red or biotin, respectively. B220 and Ly 2 were labeled with APC in our facility. PE-labeled Gr-1 and GK1.5, and FITC-labeled anti-Ly 5.1 (Texas Red), PE conjugated anti-Gr-1 and Mac-1, and APC conjugated anti-B220 and Thy-1. Four color FACS analysis was performed on the FACStarplus as described above.

**In situ colony assay.** A spleen colony assay was performed using a standard method. Ten to 14-week-old B6-Ly 5.2 female mice were lethally irradiated at a dose of 9.5 Gy total body irradiation. Sorted c-kit+ Sca-1+NK1.1+ bone marrow cells were injected into the irradiated mice intravenously via the retro-orbital plexus. After injection, the spleens were removed at day 12, fixed in Bouin’s solution, and macroscopically visible spleen colonies were counted.

**Long-term reconstitution assay.** Two thousand sorted c-kit+ Sca-1+NK1.1+ bone marrow cells from bcl-2 knockout or litter mate mice were injected into the irradiated B6-Ly 5.1 mice (9.5 Gy). After injection, peripheral blood cells were collected periodically and stained in four colors with donor-specific anti-Ly 5.2 (FITC) and recipient-specific anti-Ly 5.1 (Texas Red), PE conjugated anti-Gr-1 and Mac-1, and APC conjugated anti-B220 and Thy-1. Four color FACS analysis was performed on the FACStarplus as described above.

**FACS cell sorting.** Staining with antibodies. Total bone marrow cells from two or three bcl-2 knockout mice or their littermates were stained with a cocktail of biotinylated rat MoAbs specific for mouse differentiation antigens Gr-1, Mac-1, B220, TER119, CD4, and CD8 (see above for antibody designations) for 20 minutes at 4°C. After washing the cells three times with staining medium, cells were treated with streptavidin conjugated magnetic beads (BioMag; PerSeptive Diagnostics, Cambridge, MA), for 10 minutes at 4°C to remove lineage marker high positive cells. Unreacted cells were collected and stained with c-kit-APC, Sca-1-PE and SAV-Texas Red at 4°C for another 20 minutes. After a second wash, the cells were resuspended in staining medium at a final concentration of 1 × 10⁶ cells/mL supplemented with propidium iodide (PI, 1 μg/mL).

**RT-PCR studies on the expression of bcl-2.** Total RNA was extracted from 2 × 10⁶ sorted cells using an ISOGON total RNA isolating kit (Wako, Tokyo, Japan). After Ethanol Precipitation, the RNA pellet was dissolved in 12 μL of DEPC-treated water. After addition of 1 μL of 0.5 ng/mL oligo-dT primer, the reaction was incubated at 70°C for 10 minutes, and placed on ice for 2 minutes. The following reagents were then added: 4 μL of 5× PCR reaction buffer (500 mmol/L KCL, 200 mmol/L Tris-HCl, 25 mmol/L MgCl2); 2 μL of 0.1 mol/L DTT, 1 μL of a 10-nmol/L mixture of all four deoxynucleotide triphosphates and 1 μL of M-MLV reverse transcriptase at 200 U/mL (GIBCO-BRL). The reaction was incubated at 42°C for 60 minutes and then 180 μL of TE buffer was added. PCR reactions were performed in 50-μL volumes containing 10 μL of cDNA sample, 1× PCR buffer, 100 mmol/L of each of four deoxynucleotide triphosphates, 2 mmol/L of each sense and anti-sense primers, and 5 U of Taq polymerase (Takara). After an initial 5-minute incubation at 95°C, the 35 cycles of PCR reactions were carried out using the following conditions: denaturation at 95°C for
RESULTS

Lymphocyte development in bcl-2−/− mice. Analysis of lymphoid tissues from 4-week-old bcl-2−/− mice revealed a significant decrease in cell number both in the thymus and lymph nodes. In the thymus, cell number was decreased to 1/4, thymus weight to 1/50th, and the ratio of thymus weight/total body weight to 1/100th that of wild type mice (Fig 1A). Absolute number of cells in the c-Kit+/Pgp-1+ fraction that contains earliest T precursors is 12.0 ± 7.4 × 10^6 in litter mate (LM) and 0.7 ± 0.6 × 10^6 in bcl-2−/− thymus. As reported previously,13 bcl-2+/+ mice and +/− mice were not significantly different from each other. In spite of greatly diminished thymocyte cell numbers, FACS analysis did not show a loss of any thymocyte subpopulations (Fig 1B). Thus, the lack of bcl-2 did not result in the blockage of lymphocyte development at certain differentiation steps. The decrease in thymocyte cell number may be explained by either the death of thymocytes due to apoptosis at all developmental stages or a decrease in the supply of progenitor cells from BM to the thymus.

The severity of polycystic kidney (PCK) disease varied in each bcl-2−/− deficient mouse. Most mice died at 2 to 6 weeks of age due to uremia, but some survived longer. Interestingly, the long lived mice tended to have mild uremia and a less significant decrease in peripheral lymphocyte number including thymocyte cell number. Consequently, we speculated that the decrease in lymphocyte cell number was not intrinsic to stem cells, but secondary to other changes in the environment such as uremia caused by PCK disease. To rule out this possibility, we attempted a stem cell transplantation from bcl-2−/− mice into irradiated normal mice.

Long-term reconstitution ability of bcl-2 deficient hematopoietic stem cells. One thousand cells positive for c-kit and Sca-1 but negative for lineage markers (Lin−) obtained from bcl-2−/− or bcl-2+/+ LM mouse BM were injected into irradiated C57BL/6-Ly5.1 recipient mice. After transplantation, peripheral blood was collected periodically and analyzed for donor-derived myeloid and lymphoid cells using antibodies specific for Ly5.1, Ly5.2, and other MoAbs. In the transplantation of LM-derived stem cells, successful lymphohematopoietic reconstitution was seen in all cases, whereas for the bcl-2−/− derived stem cells only 8 of 31 cases succeeded. However, when C57BL6 × 129F1 mouse was used as a recipient, in 6 of 6 cases, the graft was accepted (data not shown). Once engrafted, HSCs from bcl-2−/− mice maintained hematopoiesis for more than 32 weeks in all cases (Fig 2A). An in vitro colony formation assay did not reveal any significant defect in HSCs from the bcl-2−/− mice. On the other hand, CFU-S assay revealed that those from bcl-2−/− had significantly reduced CFU-S activity (Table 1). This may account for delayed and decreased chance of engraftment by the HSC derived from bcl-2−/− mice.

In the recipients of LM HSC, donor-derived lymphoid and myeloid cells were present in an approximately equal ratio. On the other hand, in recipients of bcl-2−/− mouse-derived HSCs, myeloid cells predominated over lymphocytes (Fig 2A). The small number of lymphoid cells that were observed in the early posttransplantation period were B220 positive B lineage cells, but T cells were not seen (data not shown).

To compare the adult BM and fetal liver (FL)-derived
HSC, we performed transplantation of day 14 FL cells. As shown in Fig 2B, FL-derived HSCs reconstituted the BM of irradiated normal mice just as LM-derived HSC. They differed from adult BM-derived HSCs, however, in that (1) a large number of lymphoid cells appeared in the first 10 weeks, which was then replaced by myeloid cells (Fig 2B); and (2) both T and B cells were present in the lymphoid fraction (data not shown).

Thus, HSCs from bcl-2−/− mice, either adult BM or FL-derived, supported the generation of nonlymphoid cells for up to 8 months. The generation of donor-derived T and B lymphocytes, however, was significantly impaired in the recipients. The lymphoid specific decrease in cell number in normal irradiated hosts clearly indicates that this defect is intrinsic to the hematopoietic cells of the bcl-2−/− mice and that expression of bcl-2 is required for the continuous supply of lymphoid cells.

Bcl-2 is required for early B-cell differentiation. Eight months after transplantation, recipient mice were killed and B-cell development in the bone marrow was analyzed by FACS. In the recipients of bcl-2−/− BM HSC, Gr-1 positive myeloid cells predominated with B220 positive cells being significantly reduced. The recipients of LM HSC, on the other hand, showed equal numbers of myeloid and B-lineage cells (Fig 3A). Within the B220 positive population, we found small numbers of both IgM+ and IgM− cells (Fig 3A, left). Similar results were obtained from the recipients of day 14 FL cells (Fig 3A, right).

Interestingly, although B220 positive cells were barely detectable in BM at 8 month posttransplantation, B-lineage cells were present in peripheral lymph nodes (Fig 3B). In contrast to B220+ cells derived from LM HSC, many of those derived from bcl-2−/− HSC were B220 positive but IgM negative. These B220+ IgM-cells in the peripheral lymph node may represent immature B cells, NK cells, or surviving plasma cells.

A significant decrease in B-lymphoid cells in the BM and the presence of B lymphocytes in the peripheral lymphoid organs may suggest that bcl-2 expression is more important for early B-cell development than for the survival of peripheral B lymphocytes.

T-cell differentiation in the thymus. Although transplantation of BM HSC from bcl-2−/− mice generated a small number of B cells throughout the experimental period, T cells were barely detectable. We therefore analyzed carefully T-cell differentiation in the thymus. As shown in Fig 3C, transplantation of HSC from adult bcl-2−/− mouse BM resulted in the generation of CD4CD8 double positive cells but not of single positive cells at 2 months after transplantation. These double positive cells do not express detectable amounts of TCR up to 3 weeks after transplantation of either bcl-2−/− or bcl-2+/− HSC. Normal T-cell development was then observed in the recipient of bcl-2+/− HSC but not in those of bcl-2−/− HSC (Fig 3E). Indeed, PCR analysis revealed a total absence of TCR β-chain gene rearrangement (Fig 3F). The emergence of such double positive cells after irradiation has been reported elsewhere. In contrast, injection of day 14 FL cells resulted in normal thymocyte differentiation at 2 months after transplantation (Fig 3C, right). In the peripheral lymph nodes, mature T cells were found in the recipients of bcl-2−/−/FL cells but not of adult BM HSC.

At 8 months, the total thymocyte cell number in the recipient of KO HSC was reduced to \( \frac{1}{100} \) (LM, 2.1 ± 0.7 \( \times \) 10⁶; Table 1). 

Table 1. In Vivo and In Vitro Colony Forming Activity of bcl-2

<table>
<thead>
<tr>
<th>Knockout Hematopoietic Stem Cells</th>
<th>HPP-CFU* /100 Cells</th>
<th>CFU-C1 /100 Cells</th>
<th>CFU-S± /1,000 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>bcl-2−/−</td>
<td>12.5 ± 1.5</td>
<td>16.2 ± 7.6</td>
<td>4.6 ± 1.3†</td>
</tr>
<tr>
<td>bcl-2+/−</td>
<td>14.5 ± 2.5</td>
<td>19.0 ± 1.1</td>
<td>7.7 ± 1.7</td>
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* Numbers of colonies in the presence of IL-1, IL-3, M-CSF, GM-CSF, and SCF/KL at day 14.
† Number of colonies in the presence of IL-3 and Epo at day 14.
‡ Cells were injected into irradiated B6 mice intravenously and the number of spleen colonies were counted at day 12.
§ \( P < .01 \)
KO, 1.5 ± 0.3 × 10^6) leaving predominantly double negative cells in both adult and fetal HSCs transplanted animals (Fig 3C). These double negative cells were Mac-1^+ non T lineage cells (data not shown). Thus, adult bcl-2^−/− BM HSC are not capable of supporting T lymphopoiesis, but, those from fetal liver can for at least the first 2 to 3 months. In the recipients of FL cells, some CD8 positive T cells were detected 2 months after transplantation in the lymph node. However, by 8 months, CD8^+ cells were no longer detectable. These data support the notion that CD8 positive T cells are more dependent on bcl-2 expression for their survival in the periphery. FACS analysis of the CD4^+ T cells remaining in lymph nodes revealed Pgp-1^hi Mel-14^+, so-called memory phenotype.

Intrathymic injection of bcl-2 deficient hematopoietic stem cells. In contrast to the high level of chimerism (>90%) in bone marrow and peripheral blood, less than 20% of cells in lymph nodes and thymus were donor bcl-2^−/− HSC derived (Fig 4). Because myeloid cells in BM and peripheral blood were supplied normally over an 8-month period, transplanted HSCs were present and functioning. However, T-cell differentiation was not detectable in the recipient’s thymus. Therefore, it was likely the supply of T progenitor cells from BM was defective in bcl-2^−/− HSC recipients. In order to test this hypothesis, we attempted to bypass this step in the pathway by directly injecting HSCs into the recipient’s thymic lobe.

Four weeks after injection of 2 × 10^6 of c-Kit^+ Sca-1^+ Lin^- cells into the thymic lobes of a sublethally irradiated Ly5.1 congenic mouse, 4-color FACS analysis was performed. As shown in Fig 5, the thymus repopulating ability of the bcl-2^−/− HSC was significantly lower than that of BM HSC. Although the number of donor-derived thymocytes was small (LM, 8.4 ± 5.1 × 10^5; KO, 0.6 ± 0.5 × 10^5), they showed a normal pattern of thymocyte differentiation as evidenced by CD4, CD8, and TCR expression. Thus, the major reason for the absence of thymocyte differentiation in bcl-2^−/− HSC recipients appears to be in the migration process.

Expression of bcl-2 in normal lympho-hemopoietic cells. The above data show that HSCs from bcl-2^−/− mice can reconstitute BM with the exception of the lymphoid compartments. Both T and B lymphopoiesis were severely disturbed at a very early progenitor cell level. We therefore analyzed bcl-2 expression at various stages of differentiation from HSCs to T lymphocytes by RT-PCR (Fig 6). Sca-1^+ c-Kit^+ Lin^- BM HSCs expressed only a trace amount of bcl-2, whereas c-Kit^- Pgp-1^- CD25^- Lin^-, the common lymphoid precursor cells, were found to express a large amount of bcl-2. As reported previously, expression of bcl-2 is decreased at the double positive stage but upregulated in single positive thymocytes. Interestingly, bcl-2 was highly expressed in Sca-1^- c-Kit^- Lin^- cells in BM. The cells in this fraction formed neither myeloid nor erythroid colonies. We are currently in the process of characterizing the cells in this fraction.

DISCUSSION

Adult and fetal HSC are different in bcl-2 dependency. We have previously reported that lymphoid cells develop normally for 1 to 2 weeks after birth in bcl-2^−/− mice. In this report, however, we describe the absence of T lymphopoiesis and significantly retarded B lymphopoiesis after transplantation of bcl-2^−/− BM HSC into normal mice. This discrepancy may be explained by a difference in the requirement of bcl-2 between embryonal and adult HSC for development of lymphoid cells. An alternative explanation is the difference in the embryonal and adult microenvironment. It is known that the serum steroid level is low in the embryo, but increases rapidly after birth. Lymphoid progenitor cells may die by apoptosis in the presence of a high concentration of steroid in the absence of bcl-2. To clarify this issue, we transplanted bcl-2^−/− FL cells into lethally irradiated adult mice. Donor-derived lymphocytes developed normally for at least the first 2 months, suggesting that lymphopoiesis from FL HSC is less dependent on bcl-2 than that from adult BM HSCs.

There are several possibilities to account for age associated change in bcl-2 dependency. First, fetal lymphoid progenitor cells may use a molecule(s) other than bcl-2 for their survival. Bcl-xL, for example, is one possible candidate.

Role of bcl-2 in early lymphoid progenitors. We, and others, have previously shown that the most immature cells in the thymus are capable of differentiating into T, B, NK, and dendritic cells but not myeloid or erythroid cells. It is of note that although development of both T and B cells was affected by the lack of bcl-2, its effect was more profound on T cells than B lineage cells. Furthermore, the results of the intrathymic injection experiments indicate that the development of T cells is disturbed at a pre-thymus level. These data suggest that lack of bcl-2 impedes lymphopoiesis at a very early stage common to both T and B lineages. This notion is further supported by RT-PCR studies that show higher expression of bcl-2 in committed progenitors than in primitive BM HSCs.

As a small number of thymocytes developed after direct injection of HSCs in the thymic lobes, these HSCs do have the potential to differentiate into T cells albeit at low efficiency. It is therefore assumed that the absolute number of common lymphoid progenitor cells is decreased in the thymus of these mice. These common lymphoid progenitor cells may die of apoptosis before they reach the thymus, while B progenitors stay in the BM and some of them differentiate into mature B cells. This difference in the requirement for migration may have resulted in the observed difference in T and B lymphopoiesis.

Recently, it was reported that expression of bcl-2 not only suppresses apoptosis, but induces cell proliferation. As reported for erythroid cells, the supply of lymphocytes may...
Fig 3. Four-color flow cytometry analysis of T and B lymphocyte subpopulations in the thymus, BM, and lymph node of chimeric mice. The mice were killed at 2 or 8 months after injection of HSCs. Gate used to define donor-derived cells were set according to the expression of Ly5.2 and Ly5.1. Results shown are representative of eight experiments. B cell subpopulations in BM (A) and lymph node (B) of chimeric mice. Single cell suspensions of BM cells were stained with donor-specific MoAb (anti-Ly5.2), B220, Gr-1, and IgM. The two-color contour plots of the expression of B220 and Gr-1 are shown (A, upper) and expression of B220 and IgM is shown (A, lower). Two color contour plots of B220 and IgM expression analyzed at 2 months (upper) and 8 months (lower) are shown in B. CD4 and CD8 expression on donor-derived cells in thymus (C) or lymph node (D). Two-color contour plots from analysis at 2 months after injection (upper) and 8 months after injection (lower) are shown.
be regulated, at least in part, at the common lymphoid progenitor level by way of cytokine dependent apoptosis. An increase of lymphoid progenitor cells in BM should increase the chance of those cells reaching the thymus for maintenance of T lymphopoiesis. Based on these data, we believe that the supply of progenitor cells to the thymus is mediated by lymphoid committed progenitor cells but not by the HSCs themselves.

**Generation of aberrant CD4<sup>+</sup>CD8<sup>-</sup> TCR<sup>+</sup> T cells.** We observed appearance of CD4<sup>+</sup>CD8<sup>-</sup> TCR<sup>+</sup> T cells in the recipients of both bcl-2<sup>−/−</sup> and <sup>−/−</sup> HSC shortly after transplantation. The difference is that in normal mice, these cells
production in the thymic or extrathymic environment that may in turn render T progenitors express CD4CD8 by as yet unknown mechanisms.

Are memory T cells bcl-2 independent? In the recipients of FL cells, we found CD4⁺ T cells in the lymph node even 8 months after transplantation. Thus, these T cells may have survived because they were stimulated through their T-cell receptors, or they may be memory T cells. There have been reports showing that antigen stimulation of peripheral T cells generates two subgroups. In one group, there is an increase of bcl-2 and the cells survive and turn into memory cells, while cells in the other group fail to increase bcl-2 expression and consequently die of apoptosis.39,40 On the other hand, it is known that CD4⁺ cells are relatively resistant to apoptosis even without bcl-2. Moreover, it was shown that antigen stimulation further rendered the cells from bcl-2⁻/⁻ mice even more resistant to cell death.11 Thus, increased bcl-2 expression may not necessarily indicate differentiation into memory T cells.

Susceptibility of bcl-2⁻/⁻ HSC to allograft rejection. During the transplantation experiments, we noticed that HSCs from bcl-2⁻/⁻ mice had a significantly lower probability of successful engraftment (25.8% in bcl-2⁻/⁻ and 100% in LM HSC). Due to the difficulty in obtaining large numbers of bcl-2⁻/⁻ mice, we prepared HSCs from a donor mouse singly. Two interesting features were found. First, the engraftment was either as good as that of LM controls or aare eventually replaced by normally developing DP TCRblind thymocytes, whereas in SCID or bcl-2⁻/⁻ mice, they remain in the thymus for several months. Appearance of such abberant DP cells have been reported in SCID or RAG KO studies.30,31 It is not yet clear how CD4⁺CD8⁻ TCR negative cells are generated after irradiation. Radiation to T cell progenitors may somehow stimulate them to express CD4 and CD8 independent of TCR-mediated signals. However, this is clearly not the case, since nonirradiated T-cell progenitors can become double positive in the irradiated host as shown in this study. Irradiation cause changes in cytokine/hormone
Fig 6. Expression of bcl-2, EF1α was examined by RT-PCR in total cellular RNA isolated from various cell fractions in thymus and BM of C57BL/6 mice. Four-color combinations of reagents specific for c-Kit, CD44, CD25, and CD4+CD8 mixture were used to discriminate three fractions in CD4/CD8 double negative thymocytes. In BM, three-color combinations with c-Kit, Sca-1, and a lineage mixture were used to discriminate two fractions associated with Kit and Sca-1 expression in Lin-cells. Similar results were obtained in three other experiments.

F1 recipients, successful engraftment was observed in all cases. These data may indicate that bcl-2 expressed in BM HSCs has a role in resisting attacks by host cytotoxic T cells or NK cells.

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