

## Conditional deletion of the *Bcl-x* gene from erythroid cells results in hemolytic anemia and profound splenomegaly

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### SUMMARY

**Bcl-x** is a member of the Bcl2 family and has been suggested to be important for the survival and maturation of various cell types including the erythroid lineage. To define the consequences of Bcl-x loss in erythroid cells and other adult tissues, we have generated mice conditionally deficient in the *Bcl-x* gene using the Cre-loxP recombination system. The temporal and spatial excision of the floxed *Bcl-x* locus was achieved by expressing the Cre recombinase gene under control of the MMTV-LTR. By the age of five weeks, Bcl-x conditional mutant mice exhibited hyperproliferation of megakaryocytes and a decline in the number of circulating platelets. Three-month-old animals suffered from severe hemolytic anemia, hyperplasia of immature erythroid cells and profound enlargement of the spleen. We demonstrate that Bcl-x is only required for the survival of erythroid cells at the end of maturation, which

includes enucleated reticulocytes in circulation. The extensive proliferation of immature erythroid cells in the spleen and bone marrow might be the result of a fast turnover of late red blood cell precursors and accelerated erythropoiesis in response to tissue hypoxia. The increase in cell death of late erythroid cells is independent from the proapoptotic factor Bax, as demonstrated in conditional double mutant mice for Bcl-x and Bax. Mice conditionally deficient in Bcl-x permitted us for the first time to study the effects of Bcl-x deficiency on cell proliferation, maturation and survival under physiological conditions in an adult animal.

Key words: Bcl-x, Bax, Bcl2 family, Erythropoiesis, Hyperplasia, MMTV-LTR, Cre recombinase

### INTRODUCTION

The production of mature erythroid cells from pluripotent hematopoietic stem cells requires the coordinated action of different cytokine signaling pathways to assure controlled cell proliferation, survival, differentiation and death. The homeostasis of erythroid cells is sustained by factors within these signaling cascades that affect the survival of progenitor cells and the turnover of mature red blood cells. Erythropoiesis occurs in distinct stages that are characterized by the site of erythroid cell origination, the expression of embryonic and adult globins, and the cytokines that trigger the developmental program. Primitive erythropoiesis begins around embryonic day 7 (E7) when nucleated red blood cells originate in the blood islands of the yolk sac. These cells express embryonic globins and are not dependent upon erythropoietin (EPO; Wu et al., 1995; Lin et al., 1996). EPO is the major cytokine for

definitive and adult erythropoiesis. Definitive erythropoiesis starts at approximately day E10 when the site of red blood cell production shifts to the fetal liver and the erythrocytes express predominantly adult globins. At birth, erythroblastic islands in the bone marrow and the red pulp of the spleen become dominant sites of erythrocyte production.

Several genes have been shown to promote proliferation, differentiation and survival of erythroid cells. The GATA family of transcription factors and their transcriptional co-activators have emerged as key players for erythropoiesis (Pevny et al., 1991; Orkin, 1998). GATA1 is required for very early stages of red blood cell development (Weiss et al., 1994; Fujiwara et al., 1996), and GATA consensus motif target sites are found in the regulatory elements of genes expressed in erythroid cells including the EPO receptor. In contrast to GATA1, the ligand EPO, the EPO receptor (Wu et al., 1995; Lin et al., 1996) and its associated kinase JAK2

(Parganas et al., 1998) are required only for definitive erythropoiesis.

The survival of definitive erythrocytes that originate in the fetal liver and the bone marrow of adults depend on both GATA and EPO signaling (Weiss et al., 1994; Wu et al., 1995). Both signaling cascades are known to induce the expression of the antiapoptotic factor Bcl-x<sub>L</sub> (Silva et al., 1996; Gregory et al., 1999). Therefore, Bcl-x<sub>L</sub> is suggested to be the critical cell survival factor at the intersection of the EPO and GATA signaling pathways. *Bcl-x* (*Bcl2l* – Mouse Genome Informatics) is a member of the *Bcl2* gene family (Boise et al., 1993) that shares homology within four conserved regions (BH1-4 domains), which control the ability of these proteins to dimerize and function as regulators of apoptosis. The Bcl2 family consists of factors that prevent apoptosis, such as Bcl-x<sub>L</sub>, Bcl2, Bcl-w (Bcl2l2 – Mouse Genome Informatics), and of members that promote programmed cell death, e.g. Bax, Bak and Bad (Adams and Cory, 1998; Chao and Korsmeyer, 1998). The *Bcl-x* gene is expressed predominantly in its long-form, *Bcl-x<sub>L</sub>* (Gonzalez-Garcia et al., 1994); however, other alternative splice variants such as *Bcl-x<sub>S</sub>* have been detected (Boise et al., 1993; Fang et al., 1994). In contrast to Bcl-x<sub>L</sub>, the short-form Bcl-x<sub>S</sub> can function as a proapoptotic factor in vitro, but its expression has not been detected in erythroid cells (Gregoli and Bondurant, 1997). Deficiency in Bcl-x leads to embryonic lethality, owing to massive apoptosis of immature erythroid cells in the fetal liver (Motoyama et al., 1995). During erythropoiesis, the expression of *Bcl-x* is strongly increased in terminally differentiated erythroblasts (Gregoli and Bondurant, 1997). Recently, it has been demonstrated in vitro that Bcl-x prevents apoptosis predominantly at the end of erythrocyte maturation (Motoyama et al., 1999).

The generation of chimeric mice by injection of Bcl-x-deficient ES cells into wild-type blastocysts (Motoyama et al., 1995, 1999) is a method with which to study the loss of this gene in adult animals and to bypass embryonic lethality. However, the ability of Bcl-x-deficient precursors to contribute to the generation of various organs (e.g. bone marrow and brain) is limited, since these cells are likely to be eliminated early in life and to be replaced by cells that are derived from the wild-type blastocyst. Secondly, the amount of Bcl-x-deficient cells that contribute to the formation of tissues may vary among chimeras, since the contribution of wild-type and mutant cells to form an organ is a stochastic process and can hardly be controlled technically. We have chosen a different approach that allows us to delete the *Bcl-x* gene conditionally using the Cre-loxP recombination system. We have used homologous recombination to generate mice that contain the *Bcl-x* gene flanked by loxP sites (Rucker et al., 2000). The temporal and spatial excision of *Bcl-x* was achieved by using MMTV-Cre transgenic mice that express the Cre recombinase in various secretory tissues and the hematopoietic system (Wagner et al., 1997). Here, we describe the phenotypic analysis of adult mice that are Bcl-x deficient in hematopoietic precursors. The Bcl-x conditional knockout model allows us for the first time to study the effects of Bcl-x deficiency on proliferation, differentiation and cell survival of immature and mature erythrocytes under physiological conditions in an adult animal.

## MATERIALS AND METHODS

### Transgenic mice

Details of the MMTV-Cre transgenic lines and floxed *Bcl-x* mice have been published previously (Wagner et al., 1997; Rucker et al., 2000). All transgenes and targeted mutations were carried in a C57Bl6 and 129SvEv mixed background. DNA was extracted from tail biopsies of four-week-old mice to identify their genotypes. The floxed *Bcl-x* gene was discriminated from the wild-type locus using primers that amplify a sequence around the 5 prime loxP insertion site (5'-CGG TTG CCT AGC AAC GGG GC-3' and 5'-CTC CCA CAG TGG AGA CCT CG-3'). Expected PCR fragments were 300 bp for the floxed gene and 200 bp for the wild-type allele. This primer set was also used to determine the ratio of the two alleles as a marker for the recombination efficiency of Cre. Cre-mediated recombination of the *Bcl-x* gene was detected using a forward primer that binds upstream of the 5' loxP site (5'-CGG TTG CCT AGC AAC GGG GC-3' or 5'-AAT GGC CAG TAC TAG TGA ACC-3') and a reverse primer downstream of the second loxP site within the second intron (5'-TCA GAA GCC GCA ATA TCC CC-3'). The recombined allele showed a PCR fragment of approximately 150 bp in size. The presence of the *lacZ* reporter in the targeted Rosa26 locus was verified by using primers that amplify about 400 bp of the bacterial *lacZ* gene (5'-GAT CCG CGC TGG CTA CCG GC-3' and 5'-GGA TAC TGA CGA AAC GCC TGC C-3'). A PCR method to genotype the *Bax* wild-type allele and targeted locus has been described previously (Knudson et al., 1995; Rucker et al., 2000).

### Analysis of peripheral blood

Hematological examination of mice mutant for *Bcl-x* and/or *Bax*, and their wild-type controls was performed by phlebotomy from the retro-orbital plexus. Cell counts and measurements of total and direct bilirubin were executed at our NIH Clinical Pathology Department using standard assays. Erythrocytes on a blood smear were stained with May-Grünwald-Giemsa (Sigma) for morphological analysis. To discriminate reticulocytes from mature erythrocytes, EDTA-treated peripheral blood was mixed with a buffered Brilliant Cresyl Blue solution (Sigma), incubated for 20 minutes at 37°C and spread on glass slides. The reticulocyte percentile was determined by counting more than 1000 cells microscopically on more than two different areas of a slide.

### Staining techniques and histological examination of tissues

Tissues were fixed in 10% buffered formalin or in a solution containing 140 ml of ethanol, 10 ml of formaldehyde, 10 ml of glacial acetic acid and 40 ml of H<sub>2</sub>O. Paraffin sections were stained with Hematoxylin and Eosin or processed for immunohistochemistry as described earlier (Rucker et al., 2000). Antibodies used for staining of Bcl-x, Ter119 and B220 were purchased from PharMingen. Biotinylated secondary antibodies were detected with a Vectastain Elite ABC kit (Vector, Burlingame, CA). The slides were counterstained with Hematoxylin. TUNEL assay was performed according to the manufacturer's ApopTag kit protocol (Oncor, Gaithersburg, MD). Unlabeled nuclei were counterstained with Methyl Green. The X-Gal staining technique has been described previously (Wagner et al., 1997).

### Southern hybridization for efficiency of Cre-mediated recombination

DNA was extracted from spleen tissue of MMTV-Cre *Bcl-x*<sup>fl/fl</sup>, *Bcl-x*<sup>fl/fl</sup> and wild-type mice. DNA of two mice of each genotype was evaluated by Southern blot. 15 to 20 µg of DNA was digested with *EcoRI* and separated on a 0.7% agarose gel. The DNA was transferred to a GeneScreen Plus membrane (Dupont) and hybridized with a probe specific for *Bcl-x* and approximately 500 bp in size. The probe was generated by PCR using primers that bind downstream of the 3'

loxP site within the second intron of *Bcl-x* (5'-TAG TAG CTG TCG TGG TAG GCG-3' and 5'-GTT CAT TTA CAG ATT AGC TTT CAA G-3'). The Southern hybridization results in a 7.4 kb band for the wild-type or floxed locus and a 6.2 kb recombinant (knockout) allele.

#### Quantification of *Bcl-x* expression

Total RNA of *Bcl-x* mutant mice and their wild-type controls was isolated using standard guanidinium thiocyanate-phenol-chloroform extraction. SuperScript II kit (Gibco BRL) with oligo dT primers was used to perform the first strand synthesis. A 0.5  $\mu$ l aliquot was used for PCR using the ABI Prism 7700 Sequence Detection system and TaqMan PCR products (Perkin Elmer). Primers, probes and PCR conditions for *Bcl-x* and *Gapdh* were described previously (Rucker et al., 2000). The expression of various *Bcl2* family members was analyzed by RNase Protection Assay according to the mAPO-2 kit protocol (PharMingen).

#### Analysis of the relative transcriptional activity of erythroid differentiation markers

Erythroid cells from spleen tissues of Bcl-x-deficient mice and their wild-type controls were purified using the MACS separation system (Miltenyi Biotech, GmbH, Germany) and Ter119 labeled MicroBeads. Total RNA was extracted from the Ter119-positive cell fraction, and the cDNA first strand synthesis was performed as described above. PCR amplification of various differentiation factors was conducted using primers and PCR conditions that were published previously (Hodges et al., 1999; Weiss et al., 1994). Two aliquots were removed during the PCR after various cycles (determined empirically for each gene) to compare amplification products in the linear amplification range.

#### Flow cytometry and detection of apoptotic cells

One million cells of splenic single cell suspensions or overnight cultures from Bcl-x mutant and wild-type mice were incubated with different combinations of FITC-, PE-, PercP, and biotin-labeled antibodies. The following antibodies from PharMingen were used: anti-Ter119, anti-CD44 (clone IM7), anti-CD43 (clone S7), anti-CD11b (clone Mac-1), anti-Gr-1 (clone Ly-6G) and anti-B220 (clone RA3-6B2). Apoptotic cells were detected using merocyanine-540 (Sigma) at 1  $\mu$ g/ml or the standard annexin-V assay. Dead cells that were positive for 7-amino-actinomycin-D (PharMingen) were excluded from the apoptosis assay. Data were collected on a FACS Calibur and analyzed using CELLQuest software (Becton Dickinson).

## RESULTS

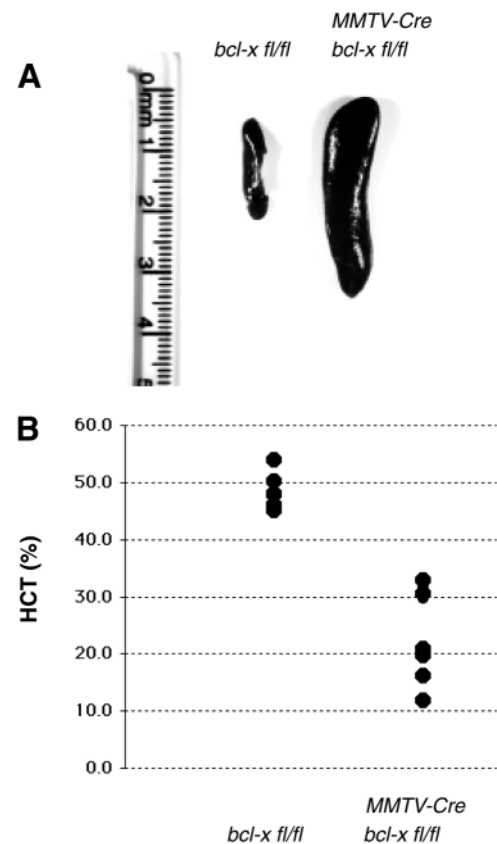
### Mice conditionally deficient in *Bcl-x* are anemic, and develop splenomegaly and severe thrombocytopenia

We have introduced loxP sites upstream of the promoter and in the second intron of the *Bcl-x* locus, and created mice that carry two conditional knockout alleles of the *Bcl-x* gene (Rucker et al., 2000). The introduction of the selectable marker gene in the upstream region of *Bcl-x* caused a dramatic loss of fetal germ cells in homozygous mutants. Therefore, we excised the selectable marker cassette by partial recombination in the germline of EIIa-Cre transgenic mice, and created a true floxed allele of *Bcl-x* with no additional sequences (Rucker et al., 2000). When bred to homozygosity, these mice exhibited no phenotypical abnormalities and they gave birth to normal litter sizes.

Homozygous *Bcl-x* floxed mice (*Bcl-x*<sup>fl/fl</sup>) were crossed into MMTV-Cre transgenic mice (Wagner et al., 1997) to generate a conditional knockout of *Bcl-x* in various secretory tissues, the

skin and the hematopoietic system. These tissues are known to highly activate MMTV-driven transgenes in a steroid hormone-dependent manner (Ross and Solter, 1985). Initially, we crossed the *Bcl-x* floxed allele into two independent MMTV-Cre transgenic lines to exclude the integration site of the Cre transgene as a cause of the mutant phenotype. Both MMTV-Cre transgenic strains (lines A and D) showed a very similar expression pattern in somatic tissues. In contrast to the D-line, MMTV-Cre mice of the A-line exhibit recombination in oocytes (Wagner et al., 1997). The excision of the *Bcl-x* gene in both MMTV-Cre transgenic lines leads to the same defects in the hematopoietic system that are described below, thus confirming that the transgene integration site per se has no influence. However, in preliminary studies MMTV-Cre line A *Bcl-x*<sup>fl/fl</sup> females show a reduced fertility and a 50% reduction of primordial follicles, which demonstrates the importance of this gene for female germ cell development (K.-U. W., J. A. Flaws and L. H., unpublished). Since the MMTV-Cre line A *Bcl-x*<sup>fl/fl</sup> mutants could have fluctuating levels of circulating steroid hormones, hence non-uniform Cre expression in other somatic tissues, we utilized the MMTV-Cre line D to study the loss of function of *Bcl-x* in the hematopoietic system and various secretory organs.

At birth, mice that were homozygous for the *Bcl-x* floxed allele and carried the MMTV-Cre transgene (MMTV-Cre *Bcl-x*



**Fig. 1.** Splenomegaly (A) and severe anemia (B) are the common phenotypic abnormalities in all 3- to 5-month-old *Bcl-x* conditional knockout mice (MMTV-Cre *Bcl-x*<sup>fl/fl</sup>) but not in their control population (*Bcl-x*<sup>fl/fl</sup>). Note the significant increase in size of the mutant spleen and the reduction of the hematocrit (HCT, or packed cell volume of peripheral blood).

$x^{fl/fl}$ ) were indistinguishable from their nontransgenic littermates. They did not develop any visible abnormalities until puberty, but soon displayed severe splenomegaly. By 3 to 5 months, the spleens of all mutant animals had expanded drastically and compressed all other abdominal organs, and an increased mortality was observed. The spleens of moribund mice (Fig. 1A) were dark red and had a weight of 3 to 5 g. Littermate control animals (*Bcl-x<sup>fl/fl</sup>* or MMTV-Cre *Bcl-x<sup>fl/wt</sup>*) did not exhibit any phenotypic abnormalities during their lifetime.

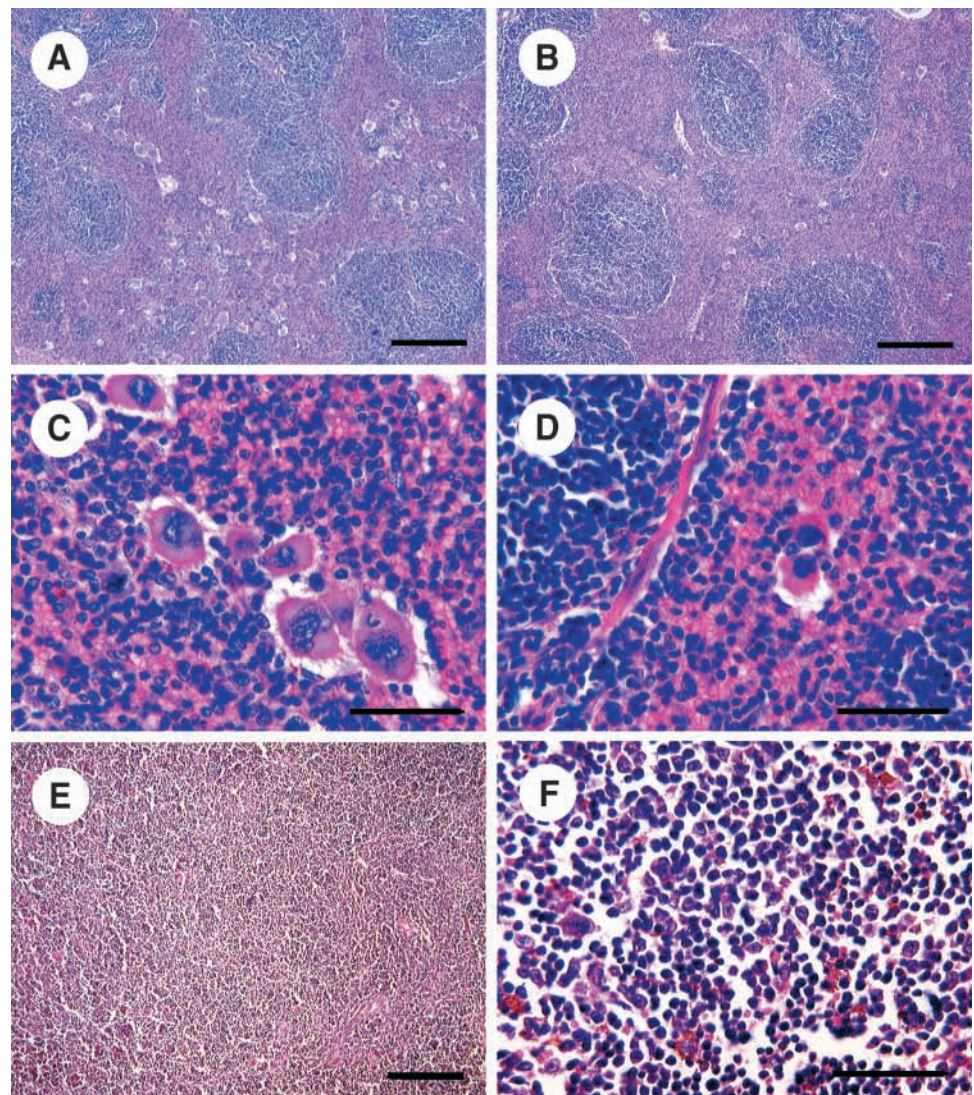
We analyzed peripheral blood taken from three-month-old animals, since splenomegaly could be a secondary effect of an imbalance of various cell types in circulation. *Bcl-x*-deficient mice exhibited profound anemia (Fig. 1B). The number of red blood cells (RBCs) was significantly reduced in the *Bcl-x* conditional knockouts ( $4.16 \pm 1.42 \times 10^6/\mu\text{l}$ ,  $n=8$ ) compared with their wild-type controls ( $10.15 \pm 0.64 \times 10^6/\mu\text{l}$ ,  $n=6$ ,  $P < 0.001$ ). Four out of eight mutant mice had erythrocyte counts below three million per  $\mu\text{l}$  blood (70% reduction), and the decline of RBCs in circulation was correlated with the extent of the enlargement of the spleen. No differences were observed between male and female *Bcl-x* mutant mice. Similar to the erythrocytes, the number of platelets was decreased in the *Bcl-x* mutants ( $14.3 \pm 13.4 \times 10^4/\mu\text{l}$ ,  $n=8$ ) compared with the controls ( $97.9 \pm 38.8 \times 10^4/\mu\text{l}$ ,  $n=6$ ,  $P < 0.001$ ). Five out of eight animals had less than  $10^5$  platelets per  $\mu\text{l}$  blood, i.e. less than 10% of the number of platelets found in normal mice. The clotting of the blood seemed to be delayed but not inhibited in these animals, and no deaths related to phlebotomy were recorded. There was no statistically significant difference in the amount of RBCs between the *Bcl-x* conditional knockout mice and their controls at four weeks of age ( $6.74$  and  $7.52 \times 10^6$  per  $\mu\text{l}$ ,  $n=6$ ). Likewise, the platelet count was very similar in these mutants compared with the wild-type littermates ( $51.9$  and  $63.3 \times 10^4$  per  $\mu\text{l}$ ,  $n=6$ ).

#### Megakaryocyte and erythroblast amplification in the spleens of *Bcl-x* conditional knockout mice

A histological analysis was performed on spleens of five-week and three- to five-month-old *Bcl-x* conditional mutants and their controls. The architecture and the amount of the white and red pulp differed between wild-type and

knockout mice and was age dependent. In five-week-old animals, the mutant spleen was normal or only slightly enlarged, and exhibited approximately the same ratio of white and red pulp as wild-type controls (Fig. 2A to 2D). However, there was a significant increase in the number of megakaryocytes within the red pulp of the mutant spleen (Fig. 2A,C). The architecture of the spleen changed by 3 to 5 months of age when the mutant mice exhibited severe splenomegaly (Fig. 2E,F). The spleen was filled with small nucleated cells and had lost the classical composition of distinct areas of white and red pulp. Megakaryocytes were still found, but their relative amount compared with the amplified small nucleated cells was markedly decreased.

Initial studies using flow cytometry revealed that the hyperproliferated cells in the spleen and bone marrow were neither B nor T cells (not shown), but in fact, the vast majority was positive for Ter119, a marker of the erythroid lineage from



**Fig. 2.** Hematoxylin and Eosin staining of spleen tissue from *Bcl-x* conditional knockout mice (A,C,E, F) and their wild-type controls (B,D). (A-D) 5-week-old animals. (E,F) Drastic amplification of erythroid cells in the spleen of *Bcl-x* conditional knockouts. Note the significant amount of megakaryocytes present in the 5-week old mutant spleen (A,C). Scale bars: 0.2 mm in A,B, E); 0.05 mm in C,D,F).

early erythroblast stages throughout mature erythrocytes. Using immunohistochemistry with an anti-mouse Ter119 antibody and a marker that reacts specifically with B cells (anti-CD45R/B220 antibody), we demonstrated a sharp increase in the erythroblast cell population and a dramatic decline in the number of B lymphocytes in the spleens of the *Bcl-x* conditional mutant mice (Fig. 3). By five month of age, erythroblast cells had infiltrated every part of the massively enlarged spleen, and residing B lymphocytes were only found in a few restricted areas (Fig. 3F) that covered less than 2% of the whole cross section. A similar decline in the relative number of T cells was confirmed with an antiserum against CD4 and CD8 (not shown).

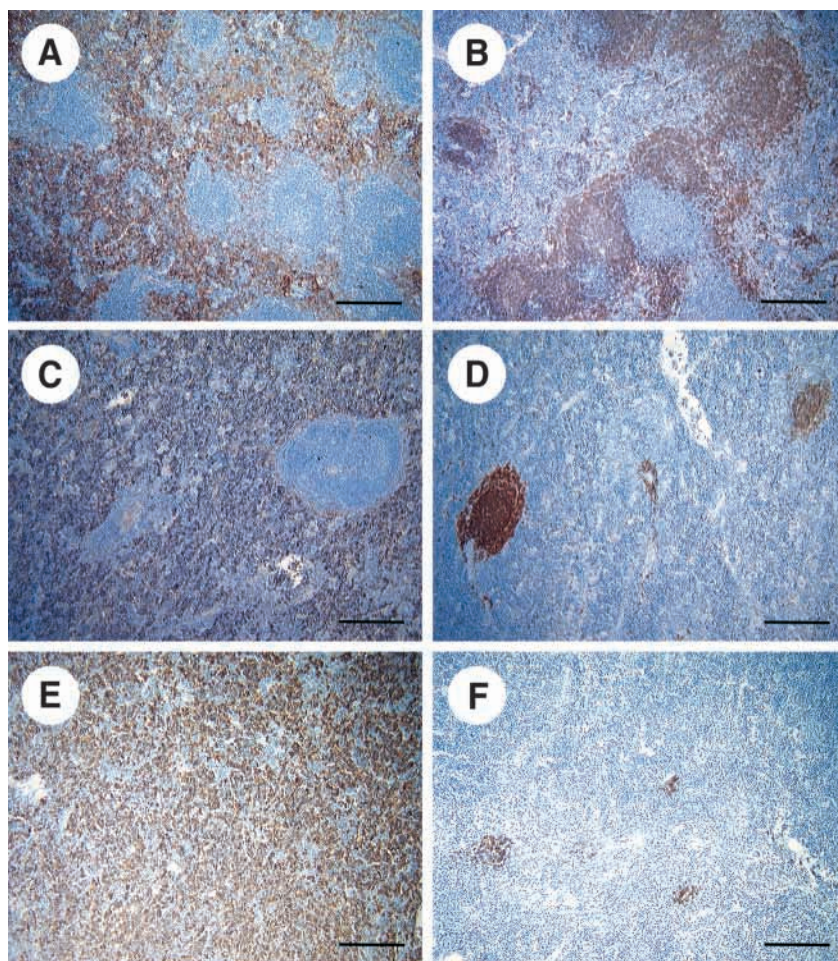
### Hyperproliferated erythroblast cells and megakaryocytes lack *Bcl-x*

We analyzed the timing and efficiency of the Cre-mediated recombination in erythroblasts and megakaryocytes of the spleen by Southern blot (Fig. 4A). Genomic DNA was isolated from spleens of two *Bcl-x* conditional mutants with severe splenomegaly: two *Bcl-x<sup>fl/fl</sup>* and two *Bcl-x<sup>wt/wt</sup>* controls. The floxed and the wild-type allele for *Bcl-x* had the same size of the *EcoRI* restriction fragment (7.4 kb), and the recombined (null) allele was 6.2 kb in size. The Southern blot (Fig. 4B) revealed that almost all of the erythroblasts in the conditional mutants had undergone Cre-mediated recombination of both floxed *Bcl-x* alleles. The results were reconfirmed with *XbaI* restriction digest that exhibited a 5 kb fragment for the wild-type allele and an 11 kb fragment for the recombined allele (not shown). Furthermore, we confirmed the extensive recombination by X-Gal staining (Fig. 4C) in mice that carry the MMTV-Cre, both floxed alleles for *Bcl-x*, and the *Rosa26* reporter locus (MMTV-Cre *Bcl-x<sup>fl/fl</sup> Rosa26<sup>lox-Stop-lox-LacZ<sup>wt</sup></sup>*). In addition, we used the *Rosa26-lox-Stop-lox-LacZ* reporter gene to analyze the recombination efficiency in the *Bcl-x* conditional mutants at five weeks of age. We investigated whether the megakaryocytes had expressed the MMTV-Cre transgene, and therefore, whether the deletion of the *Bcl-x* gene had contributed to their rapid amplification. The results shown in Fig. 4D demonstrated clearly that the vast majority of the megakaryocytes in the *Bcl-x* conditional knockout mice had expressed Cre. Also, a significant number of erythroblasts had active  $\beta$ -galactosidase in their cytoplasm, suggesting an activation of the Cre transgene in these cells at five weeks of age. Since many of these erythroblasts have very little cytoplasm, the blue X-Gal staining is mostly occluded by the red nuclear counter stain, and the extent of recombination is underestimated. No X-Gal-positive cells were detected in the absence of the *Rosa* reporter locus in the *Bcl-x* conditional mutants (Fig. 4E).

The loss of the *Bcl-x* gene was validated by using a quantitative RT-PCR assay (Rucker et

al., 2000). The *Bcl-x* mRNA levels in the three-month-old knockout mice were 0.5% compared with wild-type controls (data not shown). We also used an RNase protection assay (RPA) to compare the expression levels of the most prominent members of the Bcl2 family (Fig. 5). *Bcl-x*, *Bak*, *Bad* and *Bfl1* (Bcl2a1a – Mouse Genome Informatics) were highly expressed, whereas mRNAs of *Bcl2* and *Bcl-w* were barely detectable in wild-type mice (Fig. 5A,B, lane 1). The results of the RPA demonstrated a gradual downregulation of *Bcl-x* at five weeks of age (Fig. 5A, lane 2), and undetectable message in mice with severe splenomegaly (Fig. 5B, lane 2). *Bax*, *Bak* and *Bad* mRNA levels remained unchanged compared with the *L32* and *Gapdh* loading controls. However, *Bfl-1*, another antiapoptotic factor, exhibited a marked downregulation, possibly due to the relative (and possibly absolute) decrease in the number of B and T lymphocytes in the mutant spleen. This is supported by the fact that *Bfl-1* expression has been localized to the white pulp of a normal spleen (Jung-Ha et al., 1998).

The loss of *Bcl-x* in the megakaryocytes and erythroblast cells was again verified using immunocytochemistry. Formaldehyde-fixed paraffin sections of *Bcl-x* conditional



**Fig. 3.** Immunohistochemistry for Ter119 (A,C,E) and B220 (B,D,F) on spleen tissue from *Bcl-x* conditional knockout mice (C-F) and their wild-type controls (A,B). (A-D) 5-week-old animals. Note the drastic amplification of Ter119 positive erythroblast cells in the spleen of *Bcl-x* conditional knockouts (E) and the significant decline in the number of B lymphocytes (F) in 3-month-old mutants with marked splenomegaly. Scale bars: 0.2 mm.

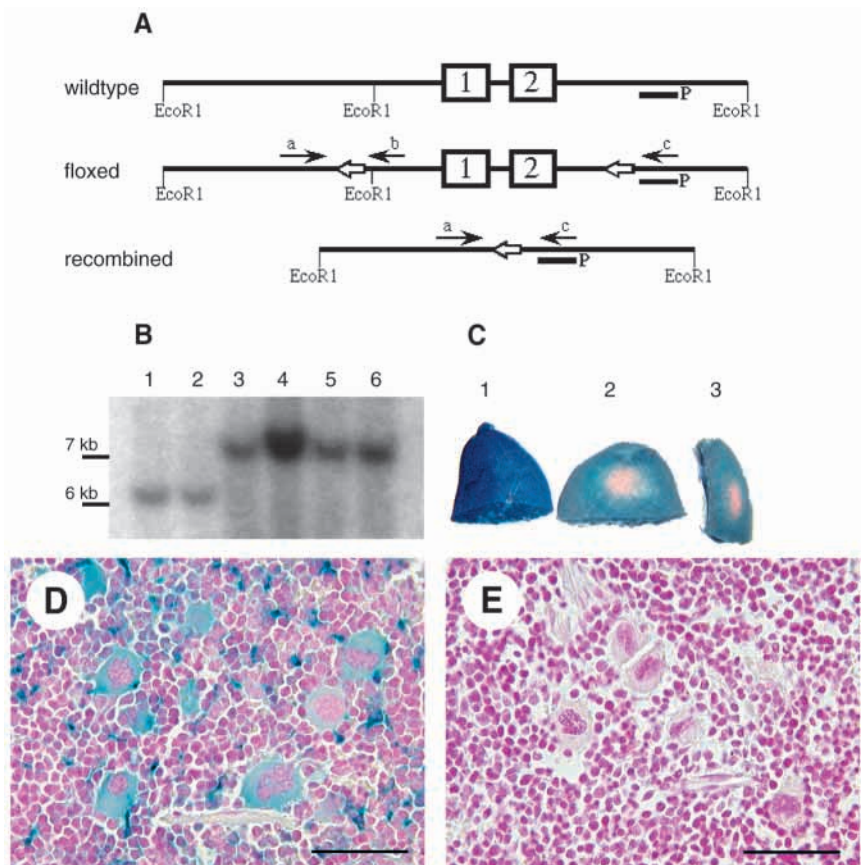
knockout mice and their wild-type controls were stained with a Bcl-x antibody. Histological figures from this experiment were deposited in HistoBank (<http://histology.nih.gov>), a new tool for Internet-based direct submission of high-resolution images (Evans et al., 2000). The Bcl-x protein was highly abundant in megakaryocytes and erythroblasts of a normal spleen (HistoBank # 1435 and 1444); however, it was significantly decreased in the majority of the accumulated megakaryocytes in the spleen of five-week-old mutants (HistoBank # 1440 and 1446). At three to five months of age, the hyperplastic erythroid cells showed considerably less Bcl-x staining (HistoBank # 1442 and 1448). The latter staining was comparable with background staining (HistoBank # 1439, 1441, 1443, 1445, 1447 and 1449).

### Differentiation status and viability/apoptosis of the hyperproliferative erythroid cells

It has been suggested from in vitro studies that Bcl-x is essential at a late stage of definitive erythroid maturation (Gregory et al., 1999; Motoyama et al., 1999). Bone marrow and spleens of our conditional knockout model massively accumulate Bcl-x-deficient erythroblasts. However, the numbers of mature erythrocytes in the peripheral blood were decreased. Both observations could suggest a block of differentiation caused by the absence of Bcl-x. To detect such a block, we analyzed the expression of several genes that are known to be differentially regulated during various stages of erythroid maturation. The analysis was performed on purified Ter119 cells from two conditional knockouts and two age-matched wild-type controls. We used an RT-PCR assay with different numbers of cycles (Weiss et al., 1994) to determine empirically the relative amounts of mRNA transcripts in the logarithmic amplification range (Fig. 6). The results indicated that neither GATA1, nor the GATA target genes such as  $\alpha$ - and  $\beta$ -globin, the EPO receptor, and transcription factors SCL and EKLF are deregulated. However, erythroid cells of both mutant animals had considerably less amounts of *Myb* mRNA compared to their wild-type controls. A decreased expression of *Myb* is known to be important for the initiation of erythroid cell differentiation (Clarke et al., 1988).

Differentiation of erythroid cells was not entirely blocked upon deletion of the *Bcl-x* gene since increased amounts of reticulocytes were produced and released into circulation (see next paragraph). However, the mutant mice were severely anemic, and we hypothesize that significant numbers of erythroid cells die prematurely in the bone marrow and spleen or as mature erythrocytes in circulation. Therefore, we determined the

steady-state level of erythroblasts that exhibited early markers for apoptosis in the spleen and bone marrow using flow cytometry with anti-Ter119, merocyanine-540 or annexin-V and 7-amino-actinomycin-D to exclude dead cells. We were unable to detect a significant difference in the number of dying erythroid cells between the wild-type and *Bcl-x* mutant tissues ( $0.64 \pm 0.35\%$ ,  $n=6$ ;  $0.61 \pm 0.02\%$ ,  $n=4$ , respectively). We also used splenocytes that were cultured for 16 hours in the presence of 2 U/ml EPO and other growth factors (Motoyama et al., 1999) to verify the feasibility of our apoptosis assay.



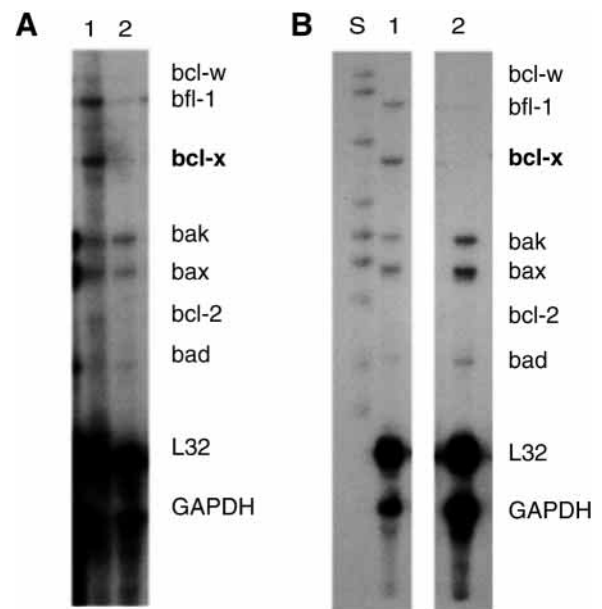
**Fig. 4.** Efficiency of the Cre-mediated recombination in erythroblasts and megakaryocytes of the spleen from MMTV-Cre *Bcl-x*<sup>fl/fl</sup> mice. (A) Southern blot strategy to quantify the amount of recombination. *EcoR1* was used for restriction digest of genomic DNA, and after Southern transfer to a membrane, the DNA was hybridized with an internal probe (P), resulting in a 7.4 kb band for the wild-type or floxed allele and a 6.2 kb band for the recombined locus. Alternatively, PCR can be used to detect the recombination event using primer pairs a and b (floxed versus wild type) or a and c for the knockout allele. (B) Nearly 100% of the hyperplastic erythroid cells in the MMTV-Cre *Bcl-x*<sup>fl/fl</sup> mice have lost both floxed alleles (lanes 1 and 2), whereas the floxed allele remains unaltered in *Bcl-x*<sup>fl/fl</sup> mice without the MMTV-Cre transgene (lanes 3 and 4). Lanes 5 and 6 show DNA from wild-type controls. (C) X-Gal staining of whole-mount spleen tissue from *Bcl-x* conditional knockout mice (1 and 2) and a wild-type control (3). The mutant spleen #1 also contains a reporter transgene (MMTV-Cre *Bcl-x*<sup>fl/fl</sup> *Rosa26*<sup>lox-Stop-lox-LacZ</sup><sup>wt</sup>) that results in the expression of  $\beta$ -galactosidase (*lacZ*) and blue staining as the result of Cre activation, indicating that all hyperproliferative cells express or have expressed Cre recombinase. (D) X-Gal staining on histological sections of a 5-week-old *Bcl-x* mutant mouse that also carries the *lacZ* reporter transgene (MMTV-Cre *Bcl-x*<sup>fl/fl</sup> *Rosa26*<sup>lox-Stop-lox-LacZ</sup><sup>wt</sup>). Note the blue staining in the megakaryocytes, indicating that the loss of *Bcl-x* leads to their significant amplification. The counterstain was Nuclear Fast Red. No blue staining was detected in mutant animals (MMTV-Cre *Bcl-x*<sup>fl/fl</sup>) without the reporter transgene (E), suggesting no endogenous  $\beta$ -galactosidase in megakaryocytes. Scale bars: 0.05 mm.

Cultured erythroid cells of a wild-type spleen exhibited an apoptotic rate of approximately 2.5% that was elevated to 4% when these cells were grown in the presence of anti-Fas (clone Jo2, PharMingen) to induce apoptosis. Cultured *Bcl-x* mutant erythroblasts exhibited a sharp increase in apoptosis of 12%, and yet anti-Fas was less effective (15% apoptotic cells). Thymocytes from a wild-type animal were used as another positive control to verify the Fas activation, which resulted in a threefold increase in apoptotic T cells. All assays were performed in duplicate.

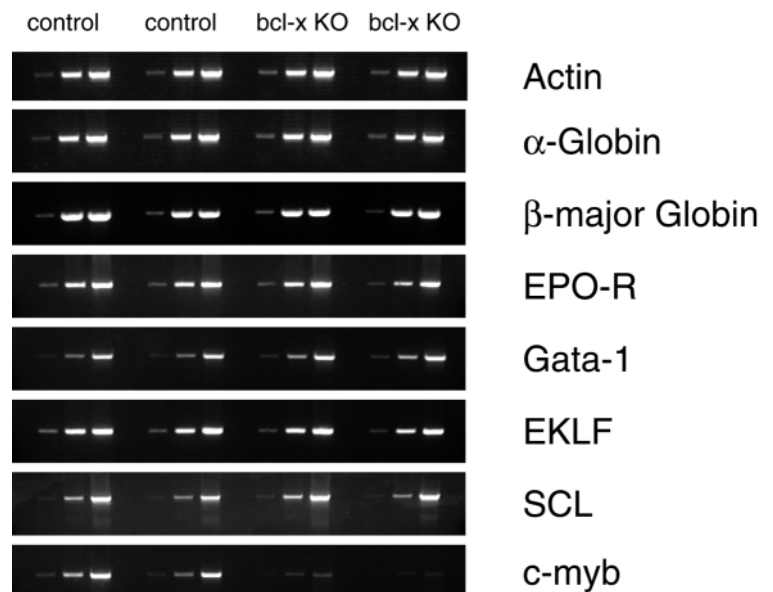
Our data showed a discrepancy between the relative amounts of apoptotic cells in vitro and in vivo. Phagocytosis of dying cells could be one explanation for the low percentile of apoptotic cells in vivo. Additionally, the relative amount of dead cells could have been decreased by a high steady-state level of erythroid cell multiplication in the mutant spleen that resulted in advancing splenomegaly. However, we hypothesize that the *Bcl-x* mutant cells die faster in vivo without exhibiting all markers for early apoptosis. To further examine this hypothesis, we performed a TUNEL assay (terminal transferase deoxytidyl uridine end labeling) on paraffin sections of normal and *Bcl-x*-deficient spleen tissue to examine the nuclear degradation as a late event in apoptosis. Erythroid cells in the red pulp of a wild-type spleen exhibited a moderate amount of apoptotic cells with a distinct nuclear labeling (HistoBank # 1451). In contrast, TUNEL-labeled DNA in the mutant spleen was not always confined to the nuclei, and excessive amounts of TUNEL-positive material was observed in the intercellular space (HistoBank # 1450) suggesting a rapid release of degraded DNA from lysed or fast dying cells that were not absorbed by macrophages. An increase in cell lysis could also explain the noticeable presence of eosinophilic material in the intercellular space between erythroblasts in the mutant spleen (Fig. 2F) and higher levels of unconjugated bilirubin in the serum (see next section).

### Bcl-x deficiency results in an increase of reticulocytes into the peripheral blood and in hemolytic anemia

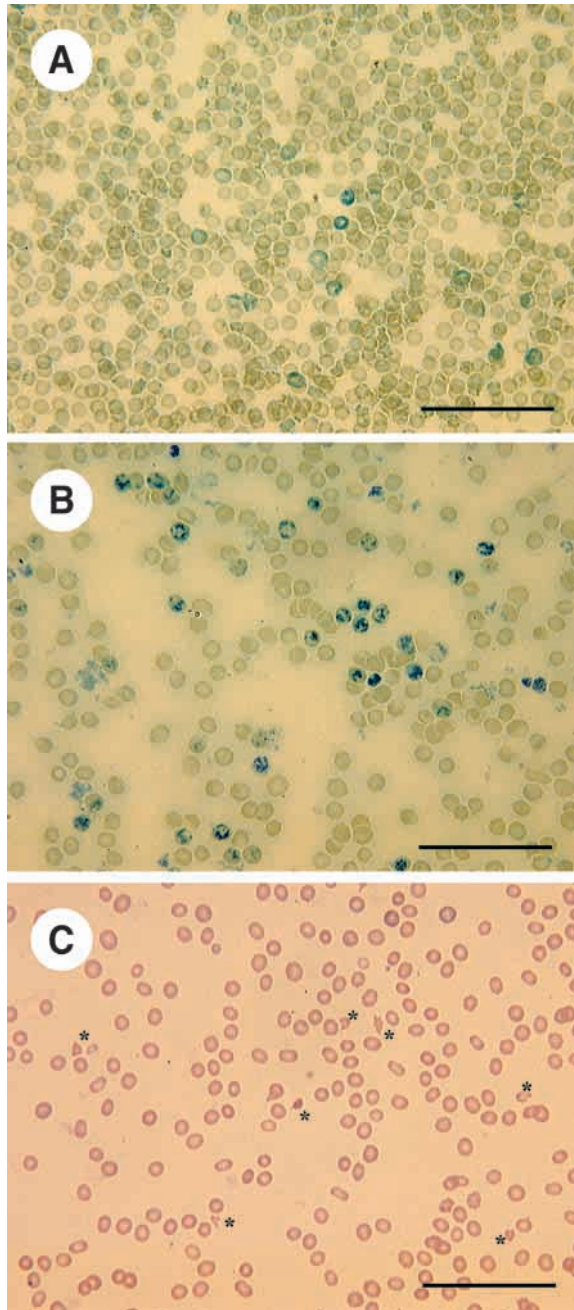
A complete block of erythropoiesis would result in lethality within a few months based on the suggested half-life of erythrocytes of about 60 days in mice. However, the majority of *Bcl-x* mutant mice survive the first five months of their life despite severe splenomegaly and anemia, suggesting no inhibition or only an incomplete block of erythropoiesis. The mutant phenotype also indicates that not all *Bcl-x*-deficient cells die because of programmed cell death. Therefore, it has to be assumed that increasing amounts of *Bcl-x*-deficient RBCs in the form of reticulocytes have to be constantly released into circulation. We have stained erythrocytes from the peripheral blood of *Bcl-x* conditional mutants and controls with a supravital dye (brilliant cresyl blue) and estimated the amount of cells that exhibit a blue granular network, which is typical for reticulocytes (Fig. 7A,B). The number of reticulocytes in circulation was significantly increased in the *Bcl-x* mutants. In these animals 15% of the circulating RBCs were reticulocytes, whereas the wild-type mice had only 0.9% polychromatophilic erythrocytes. When corrected to the



**Fig. 5.** RNase protection assay of various *Bcl2* family members including *Bcl-x* and two housekeeping genes (*L32* and *Gapdh*) on spleen tissue of 5-week- (A) and 3-month- (B) old animals. Lane 2 (A,B) shows RNA from *Bcl-x* conditional mutants compared with their wild-type controls (lane 1, A,B). Lane S represents the unprotected standard that runs higher than the protected fragments. Note the significant downregulation of *Bcl-x* mRNA as the result of Cre-mediated recombination and the correlated decrease of *Bfl1* message in response to a relative depletion of lymphocytes in the conditional mutants.



**Fig. 6.** RT-PCR assay of various differentiation factors. The isolation of total RNA from purified erythroid cells and the first strand synthesis was performed as described in Material and Methods. RNA from two mutant spleens and two corresponding wild-type controls were used in this experiment. The PCRs have been performed in triplicate with a variation in the numbers of cycles to keep the amplification of reverse transcribed cDNA in the logarithmic phase for better comparison of the relative transcriptional activity between animals. Note the significant lower levels of *Myb* mRNA in the *Bcl-x* conditional knockouts compared with the controls.



**Fig. 7.** Brilliant Cresyl Blue (A,B) and May-Grünwald-Giemsa (C) staining of peripheral blood from *Bcl-x* mutants (B,C) and a wild-type control (A). Note the significant increase of reticulocytes (B) and the occurrence of schistocytes (C, asterisks) in the blood of anemic MMTV-Cre *Bcl-x*<sup>fl/fl</sup> mice. Scale bars: 0.05 mm.

decreased total number of circulating RBCs (less than 30% compared with the wild-type controls) there was still more than a threefold increase in the release of reticulocytes in the mutant animals. An excess of larger reticulocytes in the blood leads frequently to an elevated mean corpuscular volume (MCV) of the RBCs. Measurements on RBCs of eight mutant mice and six wild-type mice revealed a 27% increase in the MCV (60.9±6.0 fl and 47.7±0.9 fl, respectively,  $P<0.001$ ) and a 34% increase in the mean corpuscular hemoglobin content

(21.0±3.2 pg and 15.5±0.4 pg, respectively,  $P<0.001$ ). In addition, the mean corpuscular hemoglobin concentration was elevated in the mutants versus the controls (34.9±1.9 g/dl and 32.5±0.6,  $P<0.001$ ).

Despite an increased release of reticulocytes into circulation, the red blood cell count was greatly reduced in the *Bcl-x* conditional knockout. Consequently, the loss of *Bcl-x* could have an influence on the half-life of circulating erythrocytes through an unknown mechanism. We next analyzed the bilirubin levels in the serum of four mutant and four wild-type mice to determine a possible effect of *Bcl-x* on the endurance of the erythrocytes. Indeed, the unconjugated bilirubin was elevated in three-month-old mutant mice (2.06±1.15 mg/dl) compared with the age-matched wild-type controls (0.39±0.13 mg/dl,  $P<0.01$ ). Initially, we hypothesized that the elevation of the bilirubin in the blood plasma was caused solely by the lysis of erythroid cells in the spleen (extravascular hemolysis). However, we have also observed schistocytes in a Giemsa-stained blood smear (Fig. 7C), suggesting that there is a certain degree of intravascular hemolysis present in our *Bcl-x*-deficient mouse model.

#### Erythroid hyperplasia and hemolytic anemia are not dependent upon Bax

Bax is a proapoptotic factor of the *Bcl2* gene family. Although Bax homodimers are involved in the induction of apoptosis (Gross et al., 1998), it is suggested that this protein antagonizes the antiapoptotic function of *Bcl-x* and other family members by heterodimerization (Oltvai et al., 1993; Sedlak et al., 1995). *Bax* mRNA is highly expressed in normal and *Bcl-x*-deficient erythroid cells (Fig. 5). To evaluate whether Bax contributes to the death of *Bcl-x*-deficient erythroid cells, we created a conditional double knockout of Bax and *Bcl-x* by crossing two *Bax* null alleles (Knudson et al., 1995) into the *Bcl-x* conditional gene deletion mice (MMTV-Cre *Bcl-x*<sup>fl/fl</sup> *Bax*<sup>null/null</sup>). Double mutant mice developed normally until shortly after puberty when they revealed the same abnormalities in erythroid cells as the *Bcl-x* conditional mutants. They exhibited erythroid hyperplasia, severe anemia and thrombocytopenia, and increased morbidity was observed in pregnant females. We have analyzed peripheral blood from two-and-a-half-month-old male mice of all relevant genotypes: conditional double mutants, *Bax*-null mice, *Bcl-x* conditional null mice and wild-type controls. Since both genders generally have a different onset of puberty and therefore activation of the Cre transgene, we have used only male mice in this experiment. The number of erythrocytes was essentially the same in wild-type and *Bax*-null mice (10.04±0.85×10<sup>6</sup>/μl,  $n=6$ ; 9.35±0.67×10<sup>6</sup>/μl,  $n=6$ ). The *Bcl-x* conditional mutants and the *Bax*/*Bcl-x* double mutants exhibited a drastic reduction of the numbers of RBCs (5.76±1.24×10<sup>6</sup>/μl,  $n=5$ ; 4.54±1.56×10<sup>6</sup>/μl,  $n=4$ ) as compared with wild-type controls and *Bax*-null mice ( $P<0.001$ ). The subtle difference between the *Bcl-x* conditional mutants and the double mutants was statistically insignificant. Furthermore, the numbers of platelets were decreased in the conditional *Bcl-x* mutants and *Bcl-x*/*Bax* double mutants by more than 85% (data not shown). The results of the peripheral blood counts indicate clearly that Bax does not compensate for the loss of *Bcl-x* during erythropoiesis. Bax does not play a major role in the development of erythroid hyperplasias, increased cell death of erythroblasts or hemolytic anemia in



our Bcl-x-deficient mouse model. In contrast, the balance of Bcl-x and Bax controls cell survival or apoptosis in other cell types, such as developing neurons (Shindler et al., 1997) as well as male and female gonads (Rucker et al., 2000).

## DISCUSSION

### The MMTV-LTR targets Cre expression to definitive erythrocytes in the bone marrow and spleen but not the fetal liver

One requirement for the development of an adult mouse model for Bcl-x deficiency in the erythroid cell lineage was the temporal and spatial activation of the MMTV-Cre transgene. An extensive activation of Cre at any stage of definitive erythropoiesis should lead to embryonic lethality, as shown in the conventional knockout model (Motoyama et al., 1995). Therefore, initial studies were performed on double transgenic mice that carry the MMTV-Cre line D transgene and the Rosa26-lox-Stop-lox-lacZ targeted reporter construct (Soriano, 1999) to determine the temporal activity of the MMTV-Cre gene in embryonic and adult hematopoietic tissues. X-Gal staining was used to monitor the Cre-mediated recombination on the level of a single cell in these animals. Several organs known to express the MMTV-Cre transgene (Wagner et al., 1997) were analyzed by X-Gal staining during embryogenesis and in postnatal and adult animals (K.-U. W. and L. H., unpublished). Expression of bacterial  $\beta$ -galactosidase was detected in less than 10% of fetal liver cells at embryonic days 13 and 16. This explains the absence of embryonic lethality in the MMTV-Cre line D *Bcl-x<sup>fl/fl</sup>* population. While less than 10% cells in the spleen were recombined at day 6 post-partum, more than 70% of the cells of the red pulp were X-Gal-positive in five-week-old mice. The strong activation of the MMTV-Cre transgene around puberty was expected since the MMTV-LTR is greatly induced by steroid hormones. We also suggest that the activation of MMTV-LTR in the hematopoietic system of this particular strain is under tight control by sex hormones, specifically estrogen, since lethality from Bcl-x deficiency was fivefold higher in females that had given birth to at least one litter.

### Differentiation and cell death in Bcl-x-deficient erythroid cells

Many transgenes that are randomly inserted into the genome are expressed mosaically in a particular cell type. This phenomenon is particularly valid for constructs that are regulated by the MMTV-LTR (Hennighausen et al., 1995). Therefore, we did not expect that the MMTV-Cre transgene would excise all copies of the floxed *Bcl-x* gene in all hematopoietic cells after its activation by steroid hormones. However, erythroblasts in the Bcl-x conditional mutant mice with marked splenomegaly exhibited a near complete deletion of the *Bcl-x* gene. Since we observed a selective amplification of Bcl-x-deficient cells, it cannot be excluded that Bcl-x-deficient erythroblasts have a slight delay in maturation at a later stage of development as proposed by Gregory et al. (1999). If there is a delay in differentiation it has to be at the very end of maturation and cannot be monitored with differentiation markers that have been used previously (Weiss et al., 1994) and applied to our studies. It can be excluded that Bcl-x-deficient cells have a block in differentiation like GATA1

null cells, since all differentiation factors are expressed at similar levels. Second, increasing numbers of reticulocytes are released into circulation, and third, large amounts of erythroblasts undergo apoptosis or hemolysis, and therefore never reach the final differentiation stage. The phenomenon of a high steady-state level of immature erythroid cells in the spleen and bone marrow can therefore be caused by a fast turnover of mature cells and a more extensive proliferation of immature cells, owing to positive compensating mechanisms promoted by EPO in response to tissue hypoxia (Krantz, 1991) or to missing negative feedback loops (De Maria et al., 1999).

Although it is possible that the mutant phenotype could have been caused by the loss of Bcl-x function in cells supporting hematopoiesis and not erythroid cells, our animal model clearly supports earlier findings of in vitro differentiation assays that Bcl-x is essential for the last phase of erythropoiesis (Motoyama et al., 1999). We suggest that the function of Bcl-x as a cell survival factor might not only be restricted to nucleated cells where classical markers of apoptotic cell death can be analyzed. Increased numbers of reticulocytes in the *Bcl-x* mutants indicate that the bone marrow and spleen are responding to the anemic situation with accelerated erythropoiesis and increased release of enucleated erythrocytes. However, Bcl-x-deficient mice still suffer from severe anemia, which could suggest that these maturing erythrocytes have a shorter half-life and hemolyze prematurely. Similarly, erythroid hyperplasia is more pronounced in individuals with hemolytic anemia than non-hemolytic anemia. The expansion of Bcl-x function as a survival factor for cells without a nucleus would demand a new definition for apoptosis, which is generally believed to be a phenomenon for nucleated cells. Our hypothesis that Bcl-x is important for the survival of reticulocytes is supported by earlier findings on in vitro differentiated mouse and human erythroid cells (Gregoli and Bondurant, 1997). The translation of the Bcl-x protein is sharply increased at the time of maximal hemoglobin synthesis and remains to accumulate when the majority of erythroblasts has undergone enucleation to form reticulocytes.

It is known from recent studies that Bcl-x regulates cell survival by at least two distinct mechanisms: heterodimerization with other Bcl2 family members and sustained ion-channel formation (Minn et al., 1999). The configuration of ion channels might be the more potent function in erythroid cells since the Bcl-x/Bax counteractive mechanism does not appear to regulate cell survival. These ion channels might control processes such as mitochondrial ATP/ADP exchange or cytochrome C release (Minn et al., 1997; Shimizu et al., 1999; Vander et al., 1999). It can therefore be assumed, that Bcl-x is important until the reticulocyte stage when mitochondria are still present. Mitochondria are progressively eliminated from mature erythrocytes as they meet their energy needs by anaerobic glycolysis instead of the Krebs cycle. If Bcl-x function is largely restricted to mitochondria in RBCs it can be predicted that its role as a survival factor, pore-channel-forming unit or countermeasure against free radicals has to diminish at the very end of erythrocyte maturation.

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