Published in final edited form as:
Genesis. 2010 May ; 48(5): 309–316. doi:10.1002/dvg.20619.

p89c-Myb is not required for fetal or adult hematopoiesis

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Abstract

The *c-myb* gene encodes two proteins, termed p75 and p89. Of these, the larger isoform is transcribed from an alternatively spliced message that contains an additional exon, exon 9A. Disruption of the c-myb locus in mice results in embryonic lethality due to defective hematopoiesis and in the adult, tissue-specific inactivation of c-myb in hematopoietic tissues blocks differentiation along several lineages. The *c-myb* knock-out mouse models described thus far result in the disruption of both the p75 and p89 isoforms, making it impossible to assign a definitive role to $p89^{c\text{-}Myb}$ in development and hematopoiesis. We have therefore generated a null-mutant mouse where exon 9A has been systemically deleted that results in the absence of only the p89-myb transcript and protein. Unlike disruption of both forms of the *c-myb* gene, loss of only the p89-encoding isoform does not have any deleterious effects on mammalian hematopoiesis and development.

Keywords

c-Myb; hematopoiesis; p89; exon 9A; knock-out mice

Introduction

From embryogenesis to adulthood, hematopoiesis occurs via a series of proliferative and differentiative pathways, where pluripotent, multipotent and lineage-committed cells and intermediates develop into various types of mature and functional blood cells. Like all developmental processes, hematopoiesis is regulated, in part, by the actions of transcription factors. One such transcription factor, *c-myb*, has been shown to play a critical regulatory role in the development of the hematopoietic system. Systemic disruption of the *c-myb* locus in mice results in embryonic lethality at E15 due to defective hematopoiesis, erythropoiesis in particular, in the fetal liver (Mucenski et al., 1991). Cells belonging to other hematopoietic lineages are also reduced in number, with the exception of macrophages and mature megakaryocytes (reviewed in Emambokus et al., 2004). A role for *c-myb* in adult hematopoiesis has been shown more recently using several conditional knock-out and partial loss-of-function mutant c-Myb mice. In adult T-lymphocytes, disruption of the *c-myb* locus results in a block in thymocyte development at the DN3 stage, causing defects in proliferative responses of mature thymocytes, proliferation and survival of double-positive (DP) thymocytes and differentiation of the single-positive (SP) CD4 and CD8 populations (Bender et al., 2004; Lieu et al., 2004). B-lymphocytes isolated from CD19-cre/*c-myb* floxed mice are arrested at the transition from the pro-B to pre-B stage of development (Thomas et al., 2005) and more recently, use of the Mb1-cre strain that drives deletion in early progenitors has revealed that differentiation is blocked beyond the pre-pro-B stage (Fahl et

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al., 2009). In addition to T- and B-lymphocytes, other tissue-specific, conditional knock-out c-*myb* mouse models have revealed that c-myb expression is also required for proper erythropoiesis and myelopoiesis (Vegiopoulos et al., 2006; Garcia et al., 2009; Lieu & Reddy, 2009). The fact that c-*myb* is required for hematopoietic stem cell (HSC) development and maintenance (Garcia et al., 2009; Lieu & Reddy, 2009) underscores the importance of this gene in hematopoiesis.

The *c-myb* gene encodes two proteins, termed p75 and p89. Of these, the larger isoform is transcribed from an alternatively spliced message that contains an additional exon, exon 9A (Dasgupta & Reddy, 1989; Dudek & Reddy, 1989). $p75^{c\text{-}Myb}$ and $p89^{c\text{-}Myb}$ have been shown to differentially regulate c-Myb target genes. For example, we have previously shown that $p75^{c\text{-}Myb}$ negatively regulates the expression of GSTμ in murine myeloblastic cells and that over-expression of the p75 isoform accelerates their apoptotic death (Kumar et al., 2003). Interestingly, however, ectopic expression of $p89^{c\text{-}Myb}$ results in the up-regulation of GSTμ and delays the rate at which these cells apoptose in the absence of IL-3 (Kumar et al., 2003). Furthermore, studies with chicken (Woo et al., 1998) and human (O'Rourke et al.,2008) Myb genes show that $p89^{c\text{-}Myb}$ exhibits higher transactivational activities compared to its p75 counterpart. These results demonstrate that the two gene products, while structurally similar, do not necessarily have identical functions.

The systemic and conditional *c-myb* knock-out mouse models described thus far result in the disruption of both the p75 and p89 isoforms, making it impossible to assign a definitive role to $p89^{c\text{-}Myb}$ in development and hematopoiesis. To address this issue, we have generated a null-mutant mouse where exon 9A has been systemically deleted that results in the absence of only the p89-myb transcript and protein. The results of this study show that unlike systemic or conditional disruption of both forms of the *c-myb* gene, loss of only the p89-encoding isoform does not have any deleterious effects on mammalian hematopoiesis and development.

Results and Discussion

Generation of p89c-myb null mutant mice

To generate mice that specifically delete only the p89-encoding isoform of the *c-myb* gene and therefore, to determine the role of $p89^{c\text{-}myb}$ in cellular development, we have generated a targeting vector in which a portion of the *c-myb* locus has been sub-cloned into the pFlox gene-targeting vector and have flanked exon 9A with loxP sites (Fig. 1). Electroporation of this construct into RI mouse embryonic stem (ES) cells and subsequent selection in neomycin resulted in the isolation of three recombinant, clonal ES cell lines. Because the p89-specific exon (exon 9A) has been sub-cloned between the two loxP sites, these ES clones were subjected to a second round of transfection with a Cre recombinase-expressing plasmid, followed by selection in gancyclovir (Fig. 1). Clonal ES cell lines that contained the desired systemic, type I deletion were karyotyped and cells with a normal chromosomal make-up were used for blastocyst injections. Three male chimeric mice (greater than 50%) were then mated to wild-type C57BL/6 mice and these matings resulted in germline transmission of the disrupted p89*c-myb* allele (Fig. 1). The results from Northern and Western blot analyses of thymocyte-derived RNA and protein (Fig. 1) confirmed that p89 is not expressed in these animals and that they retain expression of the p75 isoform. More importantly, unlike previous *c-myb* null-mutant mouse models where both the p75 and p89 isoforms have been disrupted, p89-deficient mice are born in a Mendelian ratio and have a normal lifespan (data not shown), demonstrating that loss of p89c-Myb is compatible with development into adulthood.

Normal Thymocyte Development in p89c-Myb-deficient Mice

The expression level of *c-myb* is highest in hematopoietic cells and tissues, with the thymus and T-lymphocytes showing the greatest abundance of both mRNA and protein. We therefore sought to determine whether thymocyte development was affected in $p89^{-/-}$ mice. To address this issue, thymocytes were harvested from 6-8 week-old mice and subjected to flow-cytometric analysis using antibodies directed against CD4, CD8, CD25 and CD44. The results of this study, shown in figure 2a, reveal that there were no significant differences between the numbers of double-negative (DN), $CD4^+CD8^+$ double-positive (DP) or single positive (SP) $CD4^+$ and $CD8^+$ cells. We then examined whether there were any defects in the development of the DN population, as conditional disruption of the *c-myb* locus in thymocytes has been shown to arrest development at the DN3 stage (Bender et al., 2004; Lieu et al., 2004). Unlike our previous observations using Lck-cre-*c-myb* deficient thymocytes (Lieu et al., 2004), we did not observe any accumulation of $CD25^+$ cells within the DN population (Fig. 2b). Similar results were obtained using $p89^{+/-}$ heterozygous mice (data not shown).

Although these results suggested that the $p89^{c\text{-}Myb}$ isoform does not play a definitive role in thymocyte development, it was of interest to determine whether p89-deficient thymocytes were present in normal numbers within the periphery. We therefore isolated lymphocytes from spleens and whole blood from $p89^{+/+}$ and $p89^{-/-}$ mice and subjected them to flow cytometric analysis. The results of this experiment, shown in figure 2, panels C and D, revealed that there were comparable numbers of T-lineage ($CD3^+$) $CD4_+$ and $CD8_+$ cells in both the blood and spleens of the wild-type and null-mutant mice.

B-Lymphocyte Development Occurs Normally in p89c-Myb-deficient Mice

B-lymphocytes and their progenitors also express *c-myb* mRNA. As disruption of the *c-myb* locus has been shown to block B-lymphocyte development during the transition from the pro- to pre-B cell stage, we analyzed the distribution of B-cell subsets using the B220, CD43 and IgM surface markers in bone marrow-derived lymphocytes and did not detect any remarkable differences in the percentages of pro- and pre-B, immature and mature B-lymphocytes (figure 3A and B). Further examination of splenic B-lymphocytes using antibodies directed against B220, IgM and IgD also did not reveal any differences in the number of IgM^+IgD_+ cells (figure 3C), suggesting that loss of $p89^{c\text{-}Myb}$ does not negatively affect B-cell development.

Disruption of the p89c-Myb Locus Does not Affect Myelopoiesis or Erythropoiesis

We (Lieu et al., 2009) and others (Garcia et al., 2009) have recently reported that conditional disruption of the *c-myb* locus in adult bone marrow results in impaired myelopoiesis. To determine whether loss of the p89 allele produces a similar phenotype, we harvested bone marrow from the femurs of 8-12 week-old mice and subjected the cells to flow cytometric analysis using antibodies directed against Gr-1 and CD11b. Figure 4A shows that the percentages of monocytes (upper-left quadrant) and neutrophils (upper-right quadrant) did not differ significantly between the wild-type and nullizygous animals. Finally, to ensure that erythropoiesis occurred normally in the absence of $p89^{c\text{-}Myb}$, we also measured the number of $TER119^+$ cells present in the bone marrow of p89+/+ and −/− mice using FACS analysis. The results of this study, shown in figure 4B, revealed no differences in the percentages of $TER119^+$ cells between the two genotypes.

Taken together, the results of this study show that systemic disruption of the p89c-*myb* locus does not adversely affect hematopoiesis. This is in stark contrast to the phenotype that is caused when the locus that encodes the p75 isoform is disrupted, both in the embryo and adult hematopoietic tissues. While it is clear that p89-deficient mice develop normally into

adulthood and do not appear to have any defects with respect to hematopoietic cell development, our results do not preclude a role for $p89^{c\text{-}Myb}$ in the function and survival of cells in the hematopoietic compartment, particularly those in the stem cell and early multipotent progenitor niches. Transforming variants of $p89^{c\text{-}Myb}$ transform progenitors that are at an earlier stage of development when compared to their respective $p75^{c\text{-}Myb}$-expressing counterparts, suggesting that $p89^{c\text{-}Myb}$ may regulate gene expression in immature hematopoietic cells as opposed to more mature or lineage committed populations (Woo et al., 1998). The fact that p89 mRNA levels are high in immature human CD34+ cells but drastically decline as the cells differentiate along the myeloid (granulocytic) lineage also indicates that this isoform may play a role in more immature populations (O'Rourke et al., 2008). Because we examined the distribution and overall percentages of differentiated cell types, subtle differences in HSC and progenitor numbers would be difficult to observe.

In addition to regulating hematopoiesis at an early stage, it is also possible that p89-deficient hematopoietic cells develop might exhibit defects in survival pathways in the absence of growth factors. For example, 32Dcl3 and FDCP1 myeloid-lineage cells that ectopically express $p89^{c\text{-}Myb}$ proliferate normally in the presence of IL-3 and have identical proliferative rates as cells that ectopically express $p75^{c\text{-}Myb}$ (Kumar et al., 2003). However, in the absence of IL-3, p89-expressing cells undergo apoptosis at a slower rate than the control and p75-expressing cells. It is therefore conceivable that cells that lack $p89^{c\text{-}Myb}$ may be more sensitive to apoptotic stimuli or to changes in cytokine levels.

Finally, as previously mentioned, $p89^{c\text{-}Myb}$ has been shown to exhibit increased transactivation activity when compared to $p75^{c\text{-}Myb}$ (Woo et al., 1998, O'Rourke et al., 2008). Although there are genes that are unique transcriptional targets of only one of the two isoforms, recent studies using the MCF7 breast tumor cell line have demonstrated that both $p75^{c\text{-}Myb}$ and $p89^{c\text{-}Myb}$ share some common target genes and transactivate them to similar degrees (O'Rourke et al., 2008). It is therefore possible that an overlap in target genes may account, in part, for the absence of a readily observable phenotype, as $p75^{c\text{-}Myb}$ and $p89^{c\text{-}Myb}$ could have some redundant functions in mature hematopoietic cells. Future studies with p89 null-mutant mice will allow us to determine the nature of the pathways that are influenced by $p89^{c\text{-}Myb}$ and the nature of the downstream genes that are affected by the absence of this c-Myb isoform.

Methods

Generation of p89 null-mutant mice

To construct the targeting vector, we isolated two *c-myb* genomic clones from a 129/SvJ mouse genomic library. One clone encoded exons 2-8, while the second encoded exons 9-14. A fragment of approximately 0.7 kb containing exon 9A was generated by PCR and sub-cloned into the BamHI site of the pFlox vector. The 5′ 3.0 kb (exons 7-9) and 3′ 4.0 kb (exons 10-12) flanking fragments were also generated by PCR and sub-cloned into the XbaI and XhoI sites, respectively. The targeting construct was linearized by NotI restriction digestion and introduced into R1 ES cells by electroporation. Transfected cells were grown in the presence of G418 for 10 days and individual neomycin-resistant clones were expanded, their genomic DNA digested with BamHI and the resulting DNA subjected to Southern blot analysis using a probe encompassing exons 13 and 14. Homologous recombinant clones were identifiable by the presence of two bands of 14.0 (wild-type, wt) kb and 12.5 kb (recombinant, $c\text{-}myb^{F[tkneo]}$) and three such clones were subsequently transiently transfected with the pPGK-Cre-bpA expression plasmid and selected in the presence of gancyclovir. Genomic DNA was isolated from gancyclovir-resistant sub-clones, digested with XbaI and analyzed by Southern blot analysis using the aforementioned probe. Recombination between the distal loxP sites generates the desired p89 *c-myb* null allele (c-

myb$^{\Delta}$; type I deletion), whereas recombination between the loxP sites that flank the *tk* and *neo* cistrons gives rise to a conditional p89 *c-myb*, floxed allele (c-myb^{F}; type II deletion). The wt and p89-*c-myb* null alleles are identified by bands of 6.1 kb and 5.4 kb, respectively. Chimeric mice were generated using standard microinjection techniques. Genomic DNA extracted from the tails was genotyped using Southern blotting as described above.

Northern and Western Blot Analyses

Northern blot analysis was performed as previously described by us (Baker & Reddy, 2000) using thymocytes isolated from 6-week-old p89$^{+/+}$, p89$^{+/-}$ and p89$^{-/-}$ mice. A full-length c-*myb* cDNA encoding p75 was used to detect the RNA that encodes the p75 isoform. An exon 9A fragment was used as a probe to detect the p89-encoding RNA. For Western blot analysis, thymocytes were harvested from 6-week old animals, and lysed in lysis buffer (20mM Tris-Cl, pH 7.5/ 150mM NaCl/ 1%/ NP-40/ 0.5 mM EDTA) supplemented with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and 0.5mM Na_3VO_4. 100μg of clarified total cell lysate was separated by SDS-PAGE (8%), transferred to a nitrocellulose membrane and probed using a commercially available c-Myb antibody (clone 1-1; Millipore, Billerica, Massachusetts) according to the instructions of the manufacturer. Proteins were visualized using Renaissance Chemiluminescence Plus Reagent (Perkin Elmer; Waltham, Massachusetts).

Flow Cytometric Analysis

Single cell suspensions were prepared in phenol redfree RPMI supplemented with 5% heat-inactivated FBS. Cells were pre-incubated in Fc block (CD16/32) and then stained with the indicated fluorochrome-conjugated antibodies (BD Pharmingen, San Diego, CA; eBioscience, San Diego, CA). Samples were analyzed using either a FACSScan, FACSCalibur or a FACSAria (BD Immunocytometry Systems, San Jose, CA). Data were analyzed using either WinMDI or FlowJo (Treestar, Ashland, OR) software. The antibodies used are: CD4-PE or APC (GK1.5), CD8-FITC (53-6.7), CD3-PerCp-Cy5.5 (17A2), CD44-PerCP-Cy5.5 (IM7), CD25-PE (PC61), B220-PerCP (RA3-6B2), CD43-PE (S7), IgM-APC (R6-60.2), IgD-FITC (11-26), TER119-FITC (TER119), CD11b-PE-Cy7 (MI/70) and Gr-1-PE (RB6-8C5).

Acknowledgments

This work was supported by a grant from the National Institutes of Health National Heart, Lung and Blood Institute to EPR (5RO1HL085279).

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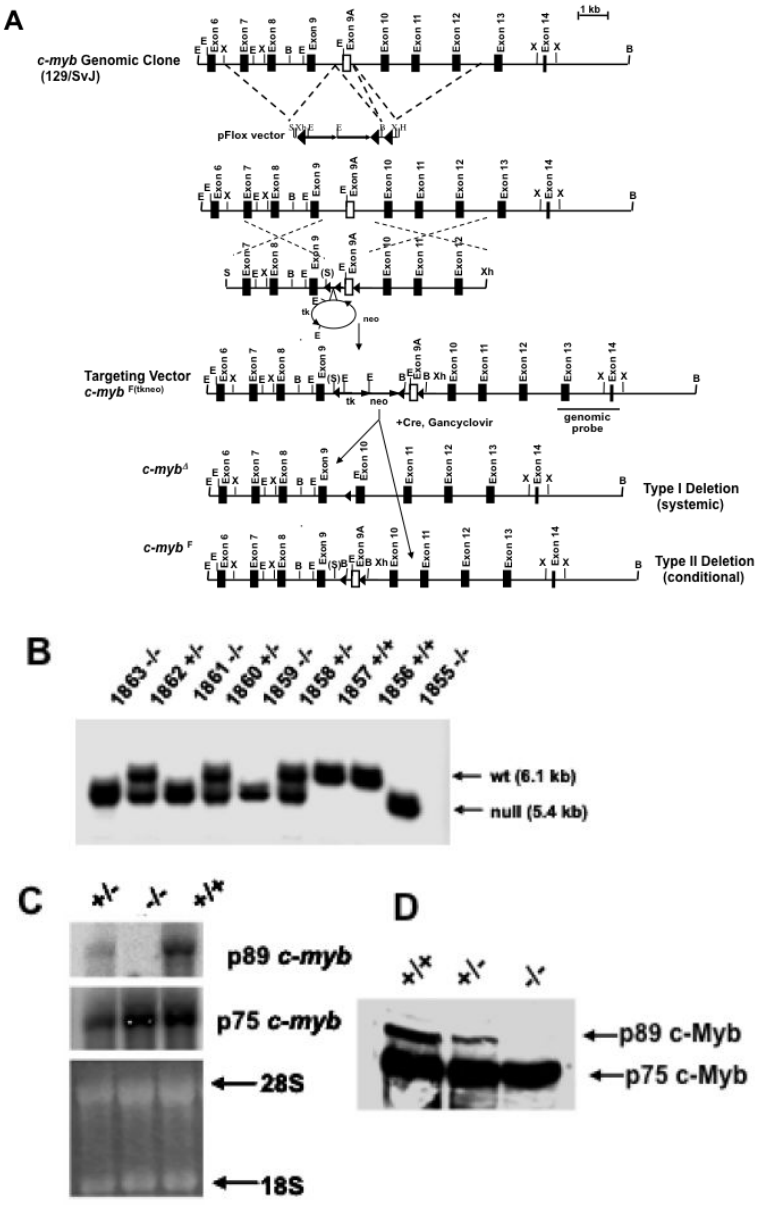

Figure 1.

Targeting of the mouse p89 *c-myb* locus. (A) Strategy for flanking exon 9A of the murine *c-myb* locus with loxP sites. The partial structure of the *myb* locus containing exons 6–14 is depicted as well as the targeting vector, which contains two selectable markers (arrows): thymidine kinase (tk) and neomycin resistant gene (neo). LoxP sites are shown as arrowheads. MybF[tkneo] is the product of homologous recombination that is used as a substrate for subsequent recombination by Cre recombinase. The position of the probe used to determine homologous recombination and for additional genomic Southern blotting studies of the targeted p89 *c-myb* allele structure is shown. Deletion of the tk-neo markers generates the conditional c-mybF allele in ES cells, whereas deletion of the loxP-flanked exon 9A (open box) generates the desired systemic, p89c-myb null mutant allele (c-myb$^{\Delta}$). B, *Bam*HI; E, *Eco*RI; S, *Sa*/I; X, *Xho*I. (B) Southern blot analysis of genomic DNA isolated from tails of p89+/+, +/− and −/− mice. Genomic DNA was isolated from the tails of mice

and subjected to Southern blot analysis as described in the Methods sections. (C) Northern blot analysis showing p89 *c-myb* RNA expression in thymocytes isolated from 6 week-old p89+/+, +/− and −/− mice. p89 expression was analyzed using an exon 9A probe. p75 expression was analyzed using the p75 cDNA as a probe. (D) p89 c-Myb protein expression in thymocytes isolated from 6 week-old p89+/+, +/− and −/− mice. Total cell lysates were subjected to Western blot analysis using a c-Myb antibody. p75 protein levels are unaffected and are also indicated.

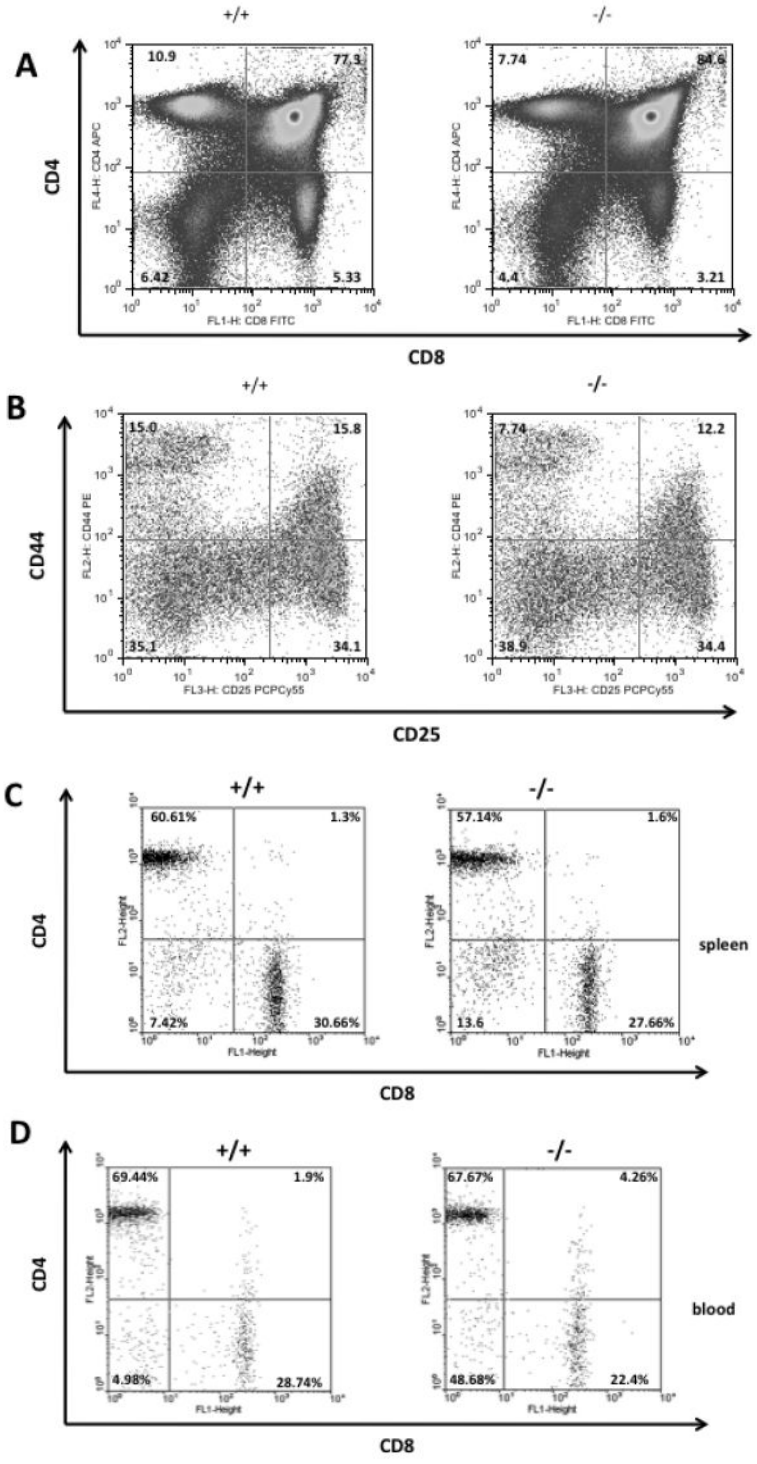

Figure 2.

Thymocyte Development in p89 null-mutant mice. Flow cytometric analysis of (A) DN, DP and SP CD4+ and CD8+ subsets, and of (B) CD44 and CD25 distribution in the DN thymocytes in p89+/+ and −/− mice. Percentages of CD4+ and CD8+ T-lineage

lymphocytes (CD3+) in (C) spleen and (D) whole blood of p89+/+ and −/− mice. n≥5 mice for each genotype.

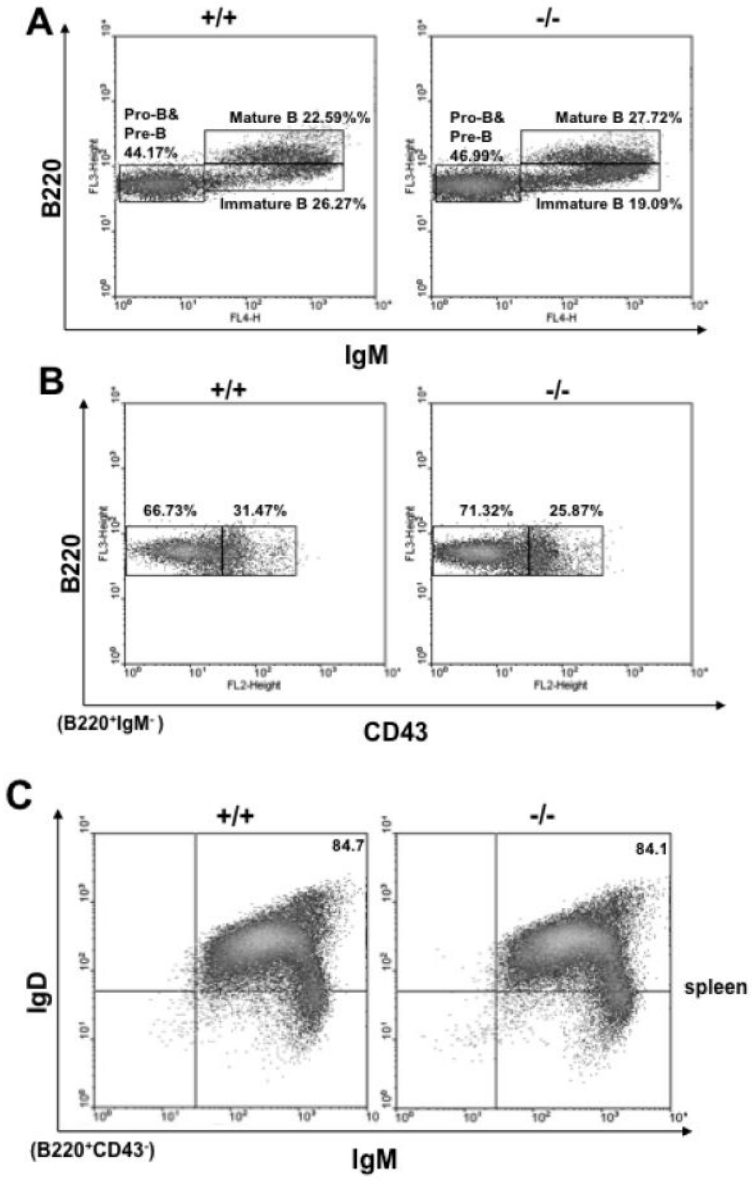

Figure 3.

B-lymphocyte Development in p89 null-mutant mice. Flow cytometric analysis of bone marrow-derived cells stained with (A) anti-B220 and anti-IgM or (B) anti-B220 and anti-CD43. The percentages of cells in each population or gate are indicated. (C) Splenocytes were stained with antibodies directed against B220, CD43, IgM and IgD. The number of IgM^+IgD^+ double-positive cells in the indicated gate are shown. n≥5 mice for each genotype.

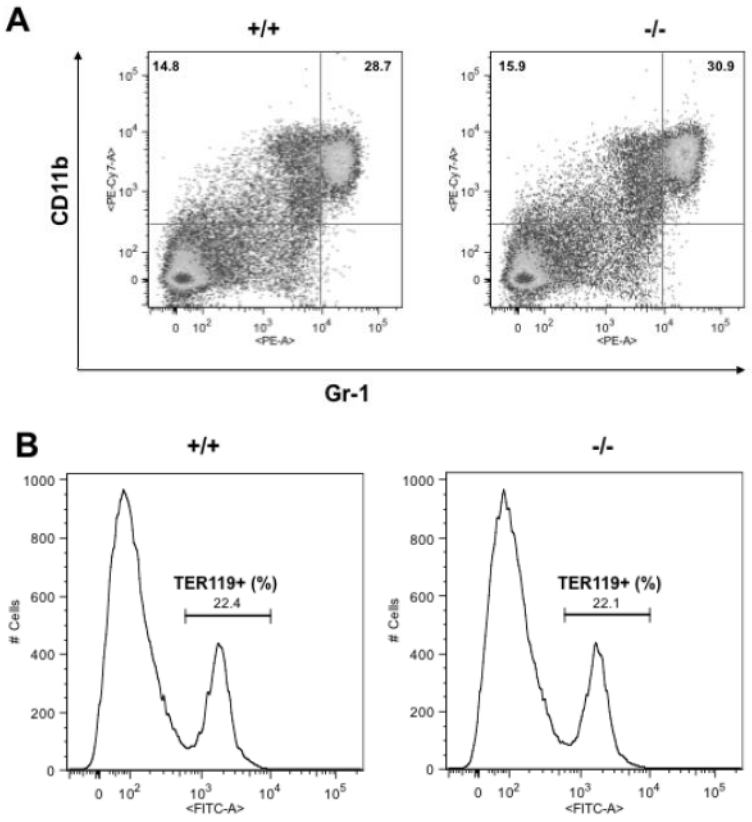

Figure 4.

Myeloid and Erythroid Development in p89 null-mutant mice. (A) Bone marrow-derived cells were subjected to flow cytometric analysis using antibodies directed against Gr1 and CD11b. The numbers of neutrophils and monocytes are indicated in the upper-right and upper-left quadrants, respectively, are indicated. (B) Bone marrow-derived cells were subjected to flow cytometric analysis using anti-TER119. The percentage of $TER119^{+}$ cells is shown. n≥5 mice for each genotype.