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Adult Hematopoietic Stem and Progenitor Cells require either *Lyl1* or *Scl* for survival

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SUMMARY

Scl and *Lyl1* encode two related basic-helix-loop-helix transcription factors implicated in T-cell acute lymphoblastic leukemia. Previous studies have shown that *Scl* is essential for embryonic and adult erythropoiesis whilst *Lyl1* is important for B-cell development. Single knockout mice have not revealed an essential function for *Scl* or *Lyl1* in adult hematopoietic stem cells (HSCs). To determine if maintenance of HSCs in single-knockout mice is due to functional redundancy, we generated *Lyl1;Scl*-conditional double knockout mice. Here, we report a striking genetic interaction between the two genes, with a clear dose-dependence for the presence of *Scl* or *Lyl1* alleles for HSC function. Bone marrow repopulation assays and analyses demonstrated rapid loss of hematopoietic progenitors due to apoptosis. The function of HSCs could be rescued by a single allele of *Lyl1* but not *Scl*. These results show that expression of at least one of these factors is essential for maintenance of adult HSC function.

Keywords

Hematopoietic Stem Cells; *Scl*; *Lyl1*; Apoptosis; Knockout

INTRODUCTION

Bone marrow Hematopoietic Stem Cells (HSCs) can self-renew and differentiate to generate the diverse lineages of the blood. Critical in hematopoietic development and adult homeostasis are a number of transcription factors, many of which are dysregulated in human leukemias (Look, 1997; Murre, 2000; Rabbitts, 1994; Shivdasani and Orkin, 1996; Tenen, 2003).

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The Stem Cell Leukemia gene (*SCL*/TAL1), first identified at a translocation breakpoint in T-cell acute lymphoblastic leukemia (T-ALL), is a tissue-restricted member of the basic Helix-Loop-Helix (bHLH) class of transcription factors that has been termed a master regulator of hematopoiesis because gene-targeting studies have shown that it is essential for the formation of hematopoietic stem cells (Robb et al., 1995; Shivdasani et al., 1995). However, conditional deletion of *Scl* in adult HSCs has relatively little effect: *Scl*-null adult HSCs have impaired short-term repopulating ability, predominantly of the myeloid lineage, but no defects in long-term repopulation or self-renewal (Curtis et al., 2004). Thus, *Scl* is essential for the formation but not maintenance of adult HSCs (Mikkola et al., 2003).

A possible explanation for redundancy of *Scl* in adult HSCs is expression of the related bHLH transcription factor *Lyl1*. However, *Lyl1* cannot rescue early lethality of *Scl*-null mice despite similar patterns of embryonic expression (Giroux et al., 2007) and enforced expression of *Lyl1* in $Scl^{-/-}$ embryonic stem cells did not rescue their defective development (Porcher et al., 1999), arguing that *Lyl1* cannot compensate for *Scl in developmental hematopoiesis*.

In adult hematopoiesis, *Lyl1* is expressed more broadly than *Scl*, in myeloid and B-cell lineages as well as HSCs. In contrast to *Scl*, *Lyl1* is dispensable for hematopoietic development. Transplantation assays of *Lyl1*-null HSCs revealed defective lymphoid but normal myeloid repopulation, suggesting that *Lyl1* is also dispensable for adult HSCs (Capron et al., 2006). To examine the possibility that *Scl* and *Lyl1* can compensate for each other in adult HSCs, we generated *Lyl1;Scl-conditional* double knockout mice to study the potential genetic interaction between the two genes in adult hematopoiesis.

RESULTS

Strong selective pressure against loss of Scl in Lyl1-null myeloid progenitors

To examine possible functional redundancy between *Lyl1* and *Scl*, we generated Mx-Cre;$Lyl1^{-/-}$;$Scl^{fl/fl}$ mice. Blood counts including platelets in mice lacking one or both alleles of *Lyl1* were normal prior to deletion of the Scl^{fl} alleles (Figure S1A). Four weeks after deletion of the Scl^{fl} allele (Scl^{Δ}), Mx;$Lyl1^{-/-}$;$Scl^{\Delta/\Delta}$ mice had a higher platelet count compared with Mx;$Lyl1^{+/-}$;$Scl^{\Delta/\Delta}$ mice (Figure S1B). Other blood parameters including red cell and B-cells were normal in Mx;$Lyl1^{-/-}$;$Scl^{\Delta/\Delta}$ and control mice (data not shown). Unlike the original report (Capron et al. Blood 2007), our *Lyl1*-null colony had normal numbers of peripheral blood B-cells.

Eight weeks after poly(I:C) we observed reduced Meg-CFC and BFU-E and increased mast-CFC in control Mx;$Lyl1^{+/+}$;$Scl^{\Delta/\Delta}$ and Mx; $Lyl1^{+/-}$;$Scl^{\Delta/\Delta}$ mice as we previously described (Hall et al., 2003; Salmon et al., 2007). Remarkably, Mx;$Lyl1^{-/-}$; $Scl^{\Delta/\Delta}$ mice had normal numbers of BFU-E (Figure S1C) and Meg-CFC and no aberrant mast cell differentiation (Figure 1A). There were two possible explanations for improved platelet count and progenitor growth in Mx;$Lyl1^{-/-}$;$Scl^{\Delta/\Delta}$ mice: either selective outgrowth of non-deleted, *Scl*-expressing ($Scl^{fl/fl}$) progenitors, or the absence of *Lyl1* rescued $Scl^{\Delta/\Delta}$ growth defects. Southern blot of hematopoietic organs from Mx;$Lyl1^{-/-}$;$Scl^{\Delta/\Delta}$ mice showing almost no deleted Scl^{fl} allele suggested that selection of non-deleted $Scl^{fl/fl}$ cells was the most likely explanation (Figure S1D). To definitively demonstrate that myeloid and erythroid colonies generated from Mx;$Lyl1^{-/-}$;$Scl^{\Delta/\Delta}$ mice were derived from $Scl^{fl/fl}$ progenitors, single colonies were genotyped, revealing that all myeloid and BFU-E progenitors from Mx;$Lyl1^{-/-}$;$Scl^{\Delta/\Delta}$ mice had at least one Scl^{fl} allele (Figure 1B). In contrast, all colonies from control Mx;$Lyl1^{+/-}$;$Scl^{\Delta/\Delta}$ mice were *Scl*-deleted ($Scl^{\Delta/\Delta}$). These results indicate that $Scl^{\Delta/\Delta}$ myeloid progenitors have a significant growth disadvantage in the absence of *Lyl1*.

Lyl1;Scl double knock-out hematopoietic stem cells cannot repopulate lethally irradiated recipients or contribute to steady state hematopoiesis

To determine whether the defect observed in myeloid progenitors extended to HSCs, we performed competitive repopulation assays using whole bone marrow obtained from Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ mice. Mice were injected three times with poly(I:C) on alternate days and bone marrow cells harvested for transplantation the day following the last injection to minimize potential outgrowth of non-deleted *Scl*$^{fl/fl}$ HSCs (Figure S2A). Peripheral blood analysis revealed that Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ bone marrow cells contributed to less than 1% of the recipients' peripheral blood production compared with WT bone marrow donor cells, which comprised approximately 40% of hematopoiesis by sixteen weeks (Figure S2B). Both *Lyl*$^{-/-}$ and Mx;*Scl*$^{\Delta/\Delta}$ single knockout bone marrow cells exhibited milder repopulating defects as previously reported (Capron et al., 2006; Curtis et al., 2004). Even with a 25-fold excess of transplanted donor cells, Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ gave rise to only 4% engraftment sixteen weeks post transplant (Figure S2C).

The inability of Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ HSCs to repopulate and expand in lethally irradiated recipients could be due to a homing defect. Therefore, we repeated the transplantation assay inducing deletion of the *Scl* alleles after, rather than before, transplantation (Figure 1C). Five to six weeks after transplant, recipient mice received three doses of poly(I:C) every other day to induce deletion of the *Scl*^{fl} allele and initial peripheral blood chimerism was determined two weeks later. Hematopoietic contribution by Mx;*Lyl1*$^{-/-}$;*Scl*$^{fl/fl}$ HSCs fell from 20% to less than 5% within two weeks after the start of poly(I:C) administration (Figure 1D), whereas there was no significant effect of poly(I:C) administration on contribution by WT or single knockout donor cells.

To determine the status of the *Scl* allele of the cells derived from Mx;*Lyl1*$^{-/-}$;*Scl*$^{fl/fl}$ HSCs after poly(I:C), we sorted donor CD45.2+ bone marrow cells into methylcellulose medium to generate colonies for PCR genotyping. All donor-derived cells in both transplant experiments contained at least one *Scl*^{fl} allele (Figure 1E and F). These non-deleted cells had escaped deletion but did not increase over time because the WT competitor cells had eliminated selective pressure for expansion. HSCs, therefore, require either *Lyl1* or *Scl* to contribute to steady-state adult hematopoiesis. To examine if one allele of *Lyl1* was sufficient to maintain HSC function in the absence of *Scl*, we tested donor bone marrow cells with the genotype Mx;*Lyl1*$^{+/-}$;*Scl*$^{fl/fl}$. Compared with Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ cells, Mx;*Lyl1*$^{+/-}$;*Scl*$^{\Delta/\Delta}$ cells provided stable hematopoiesis for at least sixteen weeks after deletion of the *Scl*^{fl} allele (Figure 1G). PCR genotyping of colonies derived from sorted donor Mx;*Lyl1*$^{+/-}$;*Scl*$^{\Delta/\Delta}$ cells confirmed the *Scl*-null (*Scl*$^{\Delta/\Delta}$) genotype (data not shown). These results demonstrate that a single allele of *Lyl1* in the absence of *Scl* is sufficient to maintain HSC activity.

Dosage effect of Lyl1 and Scl on HSC activity

The data above suggested that one WT *Lyl1* allele was sufficient to maintain adult HSC function. To examine if one WT *Scl* allele was sufficient, we crossed the *Lyl1*$^{-/-}$ mice to *Scl*$^{+/-}$ mice (Elefanty et al., 1998). By using *Scl*$^{+/-}$ mice rather than Mx;*Scl*$^{+/fl}$ mice, we avoid the complications of competition between HSCs bearing non-deleted *Scl*^{fl} and deleted *Scl*$^{\Delta}$ alleles. Since *Lyl1*$^{-/-}$;*Scl*$^{+/-}$ pups are not viable, as previously reported (Chan et al., 2007), we examined HSC activity using 14.5 dpc fetal liver, where HSCs reside during late development. *Lyl1*$^{+/-}$, *Lyl1*$^{-/-}$, *Lyl1*$^{+/-}$;*Scl*$^{+/-}$ and *Lyl1*$^{-/-}$;*Scl*$^{+/-}$ fetal liver cells were assayed by competitive transplantation against WT bone marrow (Figure 2A). *Lyl1*$^{-/-}$ fetal liver cells had a mild repopulation defect compared with *Lyl1*$^{+/-}$ or *Lyl1*$^{+/-}$;*Scl*$^{+/-}$ fetal liver cells (Figure 2B). Remarkably, *Lyl1*$^{-/-}$;*Scl*$^{+/-}$ fetal liver HSCs had a severe repopulation defect with almost complete absence of donor cells by eight weeks post-transplant. Thus, a single *Scl* allele in the absence of *Lyl1* was unable to maintain HSC activity.

Scl or Lyl1 is required for survival of adult progenitors and HSCs

The inability to identify Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ cells in Mx;*Lyl1*$^{-/-}$;*Scl*$^{fl/fl}$ mice after poly(I:C) or competitive transplantation (Figure 1) suggested that expression of either *Scl* or *Lyl1* is essential for the survival, growth or differentiation of adult HSCs. In order to determine the fate of Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ HSCs, we treated Mx;*Lyl1*$^{-/-}$;*Scl*$^{fl/fl}$ mice with poly(I:C) for six injections on alternate days and harvested bone marrow cells two hours after the last injection. Mx;*Lyl1*$^{+/-}$;*Scl*$^{fl/fl}$ mice were used as controls because they had normal repopulating activity (Figure 1G). Flow cytometric analysis of Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ bone marrow revealed a normal number of lineage negative cells (Lin^{neg}) but a variable, and on occasions dramatic, loss of Lin^{neg} c-Kit^{+} Sca-1^{-} cells (LK) and the more immature Lin^{neg} c-Kit^{+} Sca-1^{+} cells (LKS) (Figure 3A). Overall, there was a two-fold loss of LK cells and LKS cells immediately after *Scl* deletion (Figure 3B). Annexin V expression on LK cells revealed a three-fold increase in apoptotic cells (Figure 3C). PCR genotyping confirmed that most LKS cells were truly Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ but the LK fraction contained significant numbers of non-deleted *Scl*^{fl} cells (Figure 3D). Culture of Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ LKS generated ten-fold fewer colonies than control cultures (Figure 3E) and PCR genotyping demonstrated that all colonies from Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ LKS cells were derived from either *Scl*-heterozygous (*Scl*$^{\Delta/fl}$) or *Scl*-wild-type (*Scl*$^{fl/fl}$) cells (Figure 3F). Colonies from sorted Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ LK cells were mostly *Scl*-wild-type (Figure 3G), consistent with selection of non-deleted cells in the transition from LKS to LK cells (Figure 3D). Consistent with normal HSC activity of *Mx;Lyl1*$^{+/-}$;*Scl*$^{\Delta/\Delta}$ cells (Figure 1G), all colonies from *Mx;Lyl1*$^{+/-}$;*Scl*$^{fl/fl}$ LK cells were fully-deleted (Figure 3G).

Apoptosis of the Lyl1/Scl donor cells is cell intrinsic

The analyses of Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ mice immediately after poly(I:C) suggested that apoptosis was the explanation for the rapid loss of donor Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ cells in the transplanted mice (Figure 1B). To test this, recipients of bone marrow from Mx;*Lyl1*$^{-/-}$; *Scl*$^{fl/fl}$ mice were given one dose of poly(I:C) and were analyzed two days later. The contribution of Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ cells to bone marrow was three times lower in the poly(I:C)-treated mice than the PBS-treated controls as early as two days after treatment (Figure 4A). The percentage of apoptotic and dead cells, measured with Annexin V staining, was almost 50% of the total donor-derived cells, and three-fold greater than the controls. Analysis of the WT competitor cells in the same mice showed no difference (Figure 4B), suggesting that this phenotype is cell intrinsic and unique to the Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ cells.

In order to exclude the possibility of toxicity by poly(I:C) or an effect of the microenvironment, we induced deletion of the *Scl*^{fl} allele *in vitro*. Bone marrow progenitors (Sca-1^{+}) were isolated from *Lyl1*$^{-/-}$;*Scl*$^{fl/fl}$ mice, and transduced with an MSCV-based retroviral vector that carries the Cre recombinase gene coupled to eGFP, or a control GFP-only vector. Cells were cultured for 48 hours and the GFP-positive fraction was analyzed for apoptosis using Annexin-V (Figure 4C). Strikingly, 23% of the *Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ Sca1+ progenitor cells that were transduced with the Cre-expressing virus were apoptotic, four times higher than the control group (Figure 4D). This suggests that the increased rate of apoptosis of the Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ cells is cell intrinsic, independent of the environment and takes place rapidly after deletion of the *Scl* allele. These results demonstrate that HSCs and progenitors lacking both *Lyl1* and *Scl* cannot survive.

DISCUSSION

Scl has been extensively studied and found to be a crucial player in hematopoietic development; loss of *Scl* during embryonic development leads to absence of hematopoietic progenitors and vascular defects (Robb et al., 1995; Shivdasani et al., 1995). Surprisingly, conditional loss of *Scl* in the adult impacts short-term, but not long-term repopulation (Curtis et al., 2004), leading to the assertion that *Scl* is dispensable for maintenance of HSCs after development (Mikkola

et al., 2003). *Lyl1* is a related bHLH transcription factor with near identity in the bHLH region, and a similar expression pattern (Giroux et al., 2007). We thus hypothesized some redundancy between the two genes, despite the inability of *Lyl1* to rescue *Scl* during development (Chan et al., 2007; Porcher et al., 1999). By inducing *Scl* deletion on a $Lyl1^{-/-}$ background, we investigated a potential collaboration between these two genes for maintenance of adult hematopoiesis. Our results demonstrate that *Lyl1* is essential for maintaining normal HSC function in the absence of *Scl*. Specifically, *Scl*-null HSCs expressing a single allele of *Lyl1* ($Lyl1^{+/-}$;$Scl^{\Delta/\Delta}$) engraft normally in transplantation assays and can maintain long-term hematopoiesis whilst $Lyl1^{-/-}$;$Scl^{\Delta/\Delta}$ HSCs undergo rapid apoptosis. Therefore, adult *Scl*-null HSCs survive because of functional redundancy with *Lyl1*, indicating that bHLH factors are critical not only for the formation of HSCs but also for their maintenance in the adult.

Functional redundancy is a feature of other bHLH proteins in other cell types. For example, during development of skeletal muscle, MyoD and Myf5 exhibit functional redundancy early in the myogenic lineage in establishing myoblast identity (Rawls et al., 1998). In a similar way, a threshold may be required in adult HSCs in order to maintain optimal function. Increasing loss of additional alleles of *Lyl1* and *Scl* leads to proportional loss of HSC function, as illustrated most clearly in Figure 2.

Analyses immediately following poly(I:C) demonstrated a relatively pure population of Mx;$Lyl1^{-/-}$;$Scl^{\Delta/\Delta}$ cells within the LKS cell fraction, however these cells were unable to grow in culture (Figure 3). This failure to grow was reflected by the presence of cells with the non-deleted Scl^{fl} allele within the more mature LK progenitor cell fraction (Figure 3D). The increased expression of Annexin V on LKS and LK cells suggests that survival of primitive progenitors including HSCs is dependent on the presence of at least one of these bHLH factors. Because hematopoiesis is a dynamic process with many stem cells and progenitors generating progeny at any given time, a growth advantage of cells with one wild-type *Scl* allele would rapidly result in their predominance. Therefore, we cannot exclude the possibility that some $Lyl1^{-/-}$;$Scl^{\Delta/\Delta}$ cells remain alive but quiescent and that *Lyl1* or *Scl* are required for expansion of primitive progenitors *in vivo*.

The functional redundancy between *Lyl1* and *Scl* in adult HSCs is surprising given the inability of *Lyl1* to rescue *Scl*-null hematopoiesis (Chan et al., 2007; Porcher et al., 1999). However, the two genes have almost identical bHLH domains, and therefore similar DNA and protein binding properties to E-proteins (Miyamoto et al., 1996) and Lmo2 (Wadman et al., 1994), genes essential for hematopoietic development and lineage commitment. The rapid death of $Lyl1^{-/-}$;$Scl^{\Delta/\Delta}$ cells makes it challenging to determine the precise down-stream mediators of this effect. However, given the broad range of interactions of bHLH proteins and the wide range of transcriptional target genes, the phenotype that we observe in the double knockouts most likely arises due to disruption of multiple transcription networks. Transcriptional targets that could contribute to the phenotype include *c-kit*, *Gata2* and *Runx1*, which are targets of *Scl*, possibly also of *Lyl1*, and have been implicated in the regulation of HSC survival and proliferation (Krosl et al., 1998; Landry et al., 2008; Pimanda et al., 2007).

The clear dose-sensitivity for optimal HSC engraftment that is evident with decreasing allele number (Figure 2) highlights the relatively narrow tolerance for perturbations in the amounts of bHLH transcription factors for optimal hematopoiesis. Likewise, studies with knockout or over-expression of related bHLH factors, such as E2a and Id2 support this view (Cochrane et al., 2008;Engel and Murre, 2001;Ji et al., 2008). This redundancy, coupled with dose sensitivity, has important implications for the potential mechanisms that ensure fine-tuning of the dynamic production of various blood cell components under different types of demand.

Even though *Lyl1* and *Scl* are functionally redundant in adult HSCs, their relative roles during development and adult hematopoiesis appear to differ. Expression of Scl:Lyl1 chimeric proteins (Porcher et al., 1999) suggests that protein-protein interactions outside the bHLH mediate their distinct roles in development. Furthermore, the observation that *Scl* null HSCs with one intact allele of *Lyl1* function relatively normally (Figure 1G) while *Lyl1*-null HSCs with one allele of *Scl* engraft very poorly (Figure 2B) suggests that *Lyl1* is more important for adult HSCs. Their functional redundancy in adult stem cells may also account for their similar contribution to human T cell leukemia when aberrantly expressed. Interestingly, LYL1-positive T-ALLs display more primitive stem cell-like characteristics than SCL-positive samples (Ferrando et al., 2002), perhaps consistent with a more important role for LYL1 in human HSCs, as our data suggests for murine HSC. Further studies defining the common and distinct functions of these key bHLH proteins will provide valuable insights in both embryonic and adult hematopoiesis, as well as their leukemogenic properties.

EXPERIMENTAL PROCEDURES

Mice

Mice with a floxed *Scl* allele ($Scl^{fl/fl}$) and $Lyl1^{-/-}$ mice were generated as described before (Capron et al., 2006; Hall et al., 2003). $Scl^{fl/fl}$ were crossed to the Mx-Cre transgenic strain (Kuhn et al., 1995) and then to $Lyl1^{-/-}$ mice; their progeny was intercrossed to generate double knockouts, $Lyl1^{-/-}$; $Scl^{fl/fl}$; Mx-Cre. All mice are on a C57Bl/6 background and were housed in a specific-pathogen-free animal facility, AALAC-accredited, at Baylor College of Medicine (Houston, TX) or at Bio21 Institute (Melbourne, VIC).

Genotyping

Genomic DNA was isolated from mouse tails and digested in DNA lysis buffer (Gerard Biotech). Primer sequences used for PCR amplification of the *Lyl1* WT allele were: 5′-AAGCTGAGCAAGAACGAGGTGC-3′ and 5′-TCTGCTCCAACTTGATGGGTCTC-3′, and for the lacZ knockin allele: 5′-AGTGGGCGCCCTCTGTCCTCTA-3′ and 5′-TAATTCGCGTCTGGCCTTCCTGTA-3′. Primer sequences used for PCR amplification of floxed and deleted *Scl* alleles were 5′-TCCCAAGCCCAAAGATTTCCCCAATG-3′, 5′-GCAAGCTGGATGGATCAACATGGACCT-3′, and 5′-ATGCTTGGATGCTTGGTTCAGAG-3′. Primer sequences used for PCR amplification of the *Scl* lacZ knockin allele were: 5′-GGATGGCGGGGCACACGAGGTAA-3′ and 5′-TGCCAGTTTGAGGGGACGACGACA-3′

Deletion of the conditional *Scl* allele

The Scl^{fl} allele was deleted by inducing expression of the Mx-Cre by injecting mice intraperitoneally between 1 and 6 times with 300μg of polyinosinic-polycytidylic acid (poly (I:C); Sigma (St Louis, MO) in PBS. The extent of deletion was determined by performing either PCR or Southern blotting using a probe that distinguishes floxed, deleted and WT *Scl* alleles (Hall et al., 2003)

Peripheral Blood Analysis

For platelet counts, peripheral blood was collected from the retro-orbital plexus into tubes containing potassium EDTA (Sarstedt, Nümbrecht, Germany) and analyzed with an Advia 120 automated hematological analyzer (Bayer, Tarrytown, NY).

Semi-solid culture of bone marrow progenitors

Bone marrow cells were cultured in 0.3% agar for 7 days, stained for acetylcholinesterase, followed by Luxol Fast Blue and hematoxylin/eosin and analyzed as described previously

(Metcalf, 1984). Recombinant cytokines were used at the following concentrations: 10ng/mL mIL-3 (R&D Systems, Minneapolis, MN), 50ng/mL SCF (Amgen Inc., Thousand Oaks, CA), and 10ng/mL human megakaryocyte growth and differentiation factor (MGDF; Amgen Inc.). For BFU-E, bone marrow cells were plated in 0.9% methylcellulose for seven days with 10ng/mL mIL-3, 50ng/mL SCF, and 4U/mL Erythropoietin (EPO; Amgen Inc.) and enumerated *in situ* after staining for hemoglobinized erythroid colonies using 2,7-diaminofluorene.

Competitive bone marrow transplantation assays

Competitive bone marrow transplantation assays were performed by intravenous injection of admixed CD45.2 donor whole bone marrow cells with CD45.1 competitor bone marrow. *Lyl1* and *Scl* single and double knockout mice carried the CD45.2 allele. Recipient C57Bl/6 mice had been lethally irradiated with a split dose of 10.5Gy, 3 hours apart. Sex- and age-matched C57Bl/6 mice were used as competitors for every experiment. The competitor cell dose was kept constant at 250,000 cells in all transplants except those shown in figure 1G, where the competitor dose was 500,000 cells.

Timed matings and E14.5 Fetal Liver transplants

Lyl1$^{-/-}$ females in estrous were put together with *Lyl1*$^{-/+}$;*Scl*$^{lacZ/+}$ males and were checked for vaginal plugs the next morning, designated as 0.5 days post-coitum (dpc). At 14.5dpc, fetal liver was dissected from the fetuses, single-cell suspensions were prepared in Hank's balanced salt solution (Gibco, Carlsbad, CA). Fetal liver cells were used in a 2:1 ratio with WT competitor bone marrow in a transplantation assay as described above. The genotypes were determined using a small amount of tissue from the fetuses prior to the transplant, prepared and genotyped as described above.

Flow cytometry and lineage analysis

For peripheral blood analysis, mice were bled at 4, 8,12 and 16 weeks post transplantation. Red blood cells were lysed and samples were stained with CD45.1-APC, CD45.2-FITC, CD4-pacific blue, CD8-pacific blue, B220-pacific blue, B220-PE-cy7, Mac1-PE-cy7 and Gr-1-PE-cy-7 antibodies (BD Pharmingen, eBiosciences). FacsARIA, FacsCalibur, LSRII and FACS-Scan flow cytometers were used for analysis and sorting.

Methylcellulose assay colony PCR

Whole bone marrow from recipient animals was harvested, stained with CD45.2-FITC antibody (eBiosciences) and sorted using a FACS-Aria. CD45.2 positive cells were plated in Methocult GF M3434 (StemCell Technologies Inc.) or 0.3% agar as described and incubated at 37°C, 5% CO2. Colonies formed at around day 10 were picked, DNA was isolated (Gerard Biotech DNA isolation kit) and genotyped using the *Scl*-WT/floxed primers used in mouse genotyping.

Annexin-V and cell death assays

Annexin V and PI or Fluoro-Gold staining was used to assess cell death and apoptosis. Briefly, cells were washed twice with cold PBS and incubated at room temperature in 1X binding buffer (10mM HEPES, 140mM NaCl, 2.5mM CaCl2) containing either Annexin V-APC (BD-Pharmingen) and PI or Annexin V-FITC; Sca1-PE; c kit-APC; lineage markers (B220, Ter119, Gr1, Mac1, CD3)-PerCP/Cy5.5 and Fluoro-Gold. Cells were analyzed by flow cytometry within one hour of staining.

Retroviral transduction of *Lyl1*$^{-/-}$;*Scl*$^{fl/fl}$ cells

MSCV-Cre-IRES-GFP and MSCV-IRES-GFP retroviruses were generated as previously described. (Chambers et al., 2007). Transdused Sca1+ cells were grown in culture for 48 hours and Annexin V apoptosis analysis was performed by flow cytometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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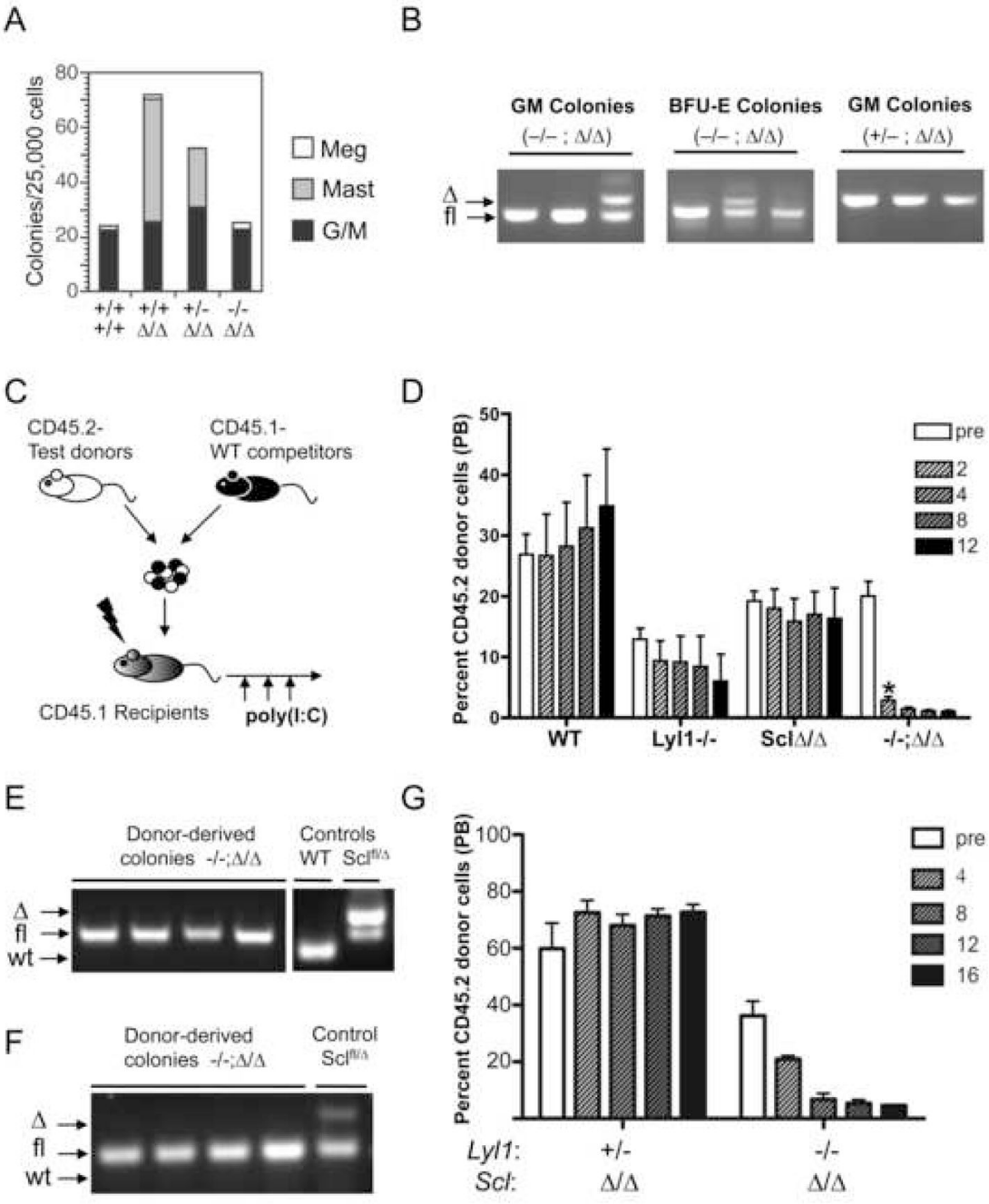

Figure 1.

Lyl1$^{-/-}$;*Scl*$^{\Delta/\Delta}$ HSCs cannot contribute to steady-state hematopoiesis.

(A) Myeloid colony numbers and types generated from agar cultures of 25,000 bone marrow cells four weeks after poly(I:C) (mean, n=3 mice). (B) PCR genotyping of the *Scl*^{fl} allele in single granulocyte/macrophage (GM) or BFU-E colonies grown from bone marrow of Mx;*Lyl1*$^{+/-}$;*Scl*$^{\Delta/\Delta}$ and Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ mice. (C) Schematic of experimental design. Whole bone marrow from CD45.2 WT, *Lyl1*$^{-/-}$, Mx;*Scl*$^{fl/fl}$, and Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ mice was isolated and transplanted into lethally irradiated recipients (n=8). Engraftment was assessed prior to poly(I:C) four weeks after transplant. Recipient mice then received three doses of poly(I:C) on alternate days, and analyzed initially two weeks after first treatment. (D) Contribution of donor

cells with the indicated genotypes to peripheral blood, as analyzed by flow cytometry using the CD45.2 marker. (E, F) PCR genotyping for the Scl^{fl} allele of donor-derived cells in recipient mice ("E"-colonies from pIpC treatment before transplant, and "F" after, the transplant). CD45.2+ cells were sorted and cultured in methylcellulose medium and colonies genotyped for excision of the floxed *Scl* allele. Representative colony PCR shows that all of the engrafted donor-derived cells are non-deleted for the *Scl* allele. (G) Competitive transplant assay as in (C) to assess the function of Mx;$Lyl1^{+/-}$;$Scl^{\Delta/\Delta}$ HSCs. A three-fold excess of donor cells was used and peripheral blood contribution was assessed at four-week intervals. All experiments were repeated at least twice with similar results each time.

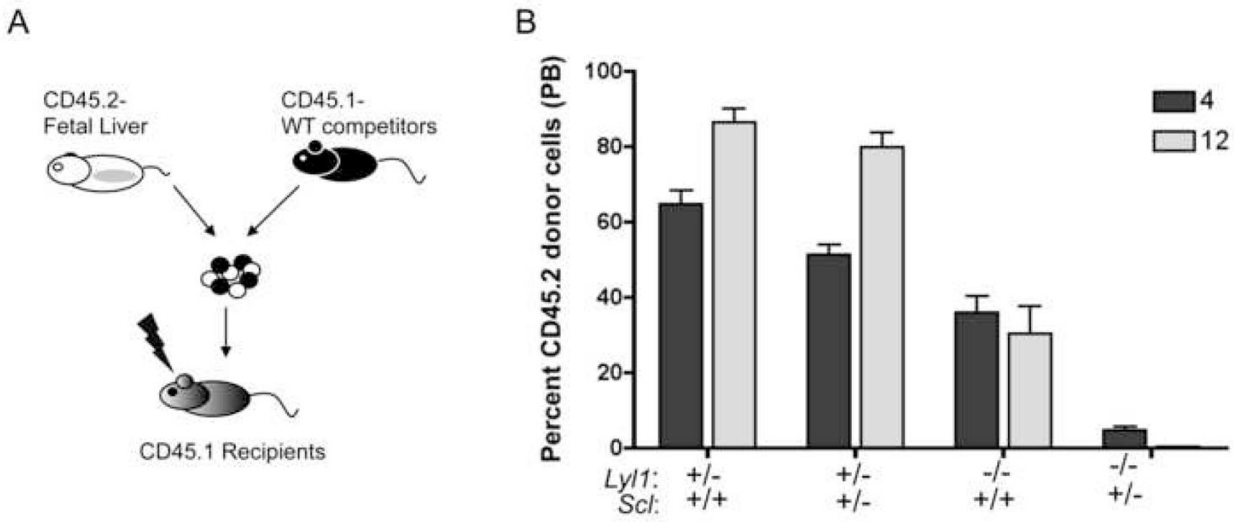

Figure 2. Dose dependence of *Lyl1* and *Scl* alleles on hematopoietic stem cell engraftment potential

(A) Schematic of the transplantation scheme. Fetal liver (FL) cells from $Lyl1^{+/-}$, $Lyl1^{-/-}$, $Lyl1^{+/-}$;$Scl^{+/-}$ and $Lyl1^{-/-}$;$Scl^{+/-}$ embryos (CD45.2) at 14.5 dpc were isolated, mixed at a 2:1 ratio with WT competitor bone marrow (CD45.1) (500,000 FL vs 250,000 BM cells) and transplanted into lethally irradiated recipients. (B) Peripheral blood (PB) analysis showing the proportion of nucleated cells derived from the test population at four and twelve weeks after transplantation (mean +/− SEM, n=7). Two replicates of the experiment showed the same results.

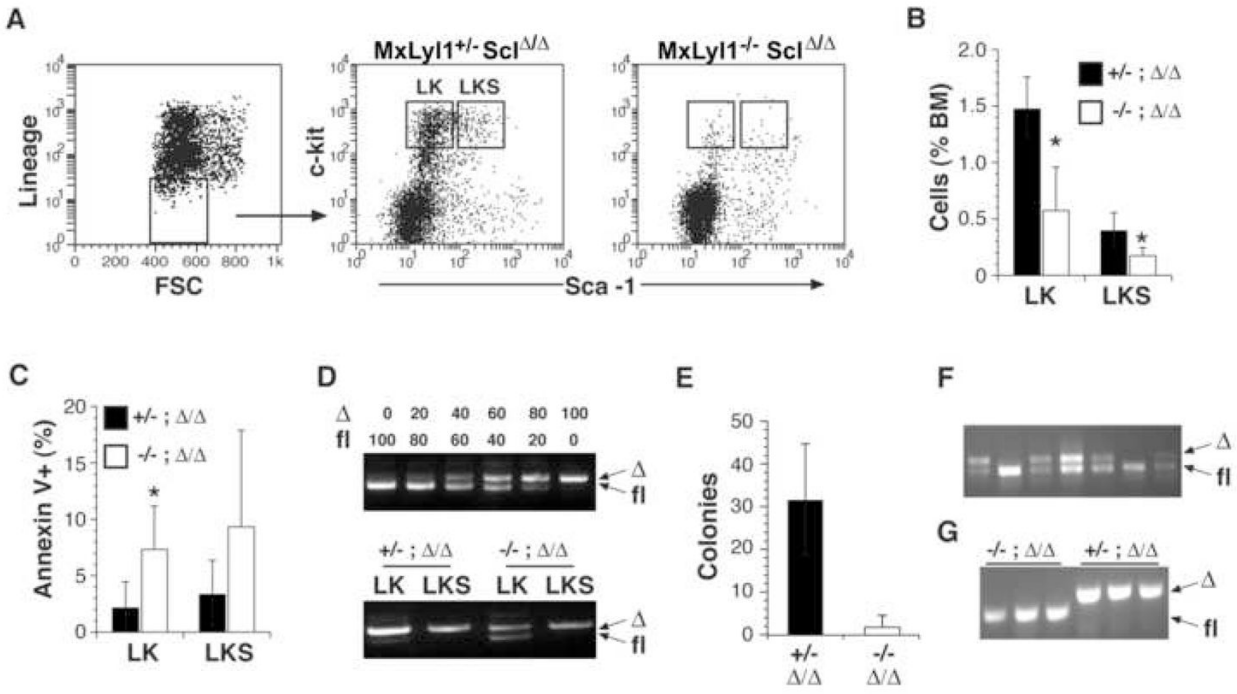

Figure 3. Analysis of hematopoiesis in Mx;Lyl1$^{-/-}$;Scl$^{\Delta/\Delta}$ mice immediately after poly(I:C)

(A) Flow cytometric analysis of bone marrow cells from Mx;*Lyl1*$^{+/-}$;*Scl*$^{fl/fl}$ and Mx;*Lyl1*$^{-/-}$;*Scl*$^{fl/fl}$ mice two hours after the final poly(I:C) injection. Viable cells were stained for expression of lineage markers (Mac-1, B220, CD3, TER119), c-kit and Sca-1. The gates used for lineage negative (Lin^{neg}), Lin^{neg} c-Kit^{+} Sca-1^{-} (LK) and Lin^{neg} c-Kit^{+} Sca-1^{+} (LKS) cells are shown. (B) Percentage of LK and LKS cells of bone marrow cells from Mx;*Lyl1*$^{+/-}$;*Scl*$^{fl/fl}$ and Mx;*Lyl1*$^{-/-}$;*Scl*$^{fl/fl}$ mice. Mean and SD of at least 7 mice. * P<0.05 Student's *t*-test. (C) Percentage of viable LK and LKS cells expressing Annexin V (n>4 mice). * P < 0.05. (D) Dose titration curve for PCR genotyping (top gel). PCR genotyping of sorted cells (bottom gel). (E) Myeloid colony numbers generated from agar cultures of 250 LKS cells (mean +/− SD, n=3 mice). (F) PCR genotyping for deletion of the *Scl*^{fl} alleles in single granulocyte/macrophage colonies generated from culture of *Mx;Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ LKS cells. (G) PCR genotyping for deletion of the *Scl*^{fl} alleles in colonies generated from culture of Mx;*Lyl1*$^{-/-}$;*Scl*$^{\Delta/\Delta}$ and Mx;*Lyl1*$^{+/-}$;*Scl*$^{\Delta/\Delta}$ LK cells. Experiments were repeated multiple times with consistent results.

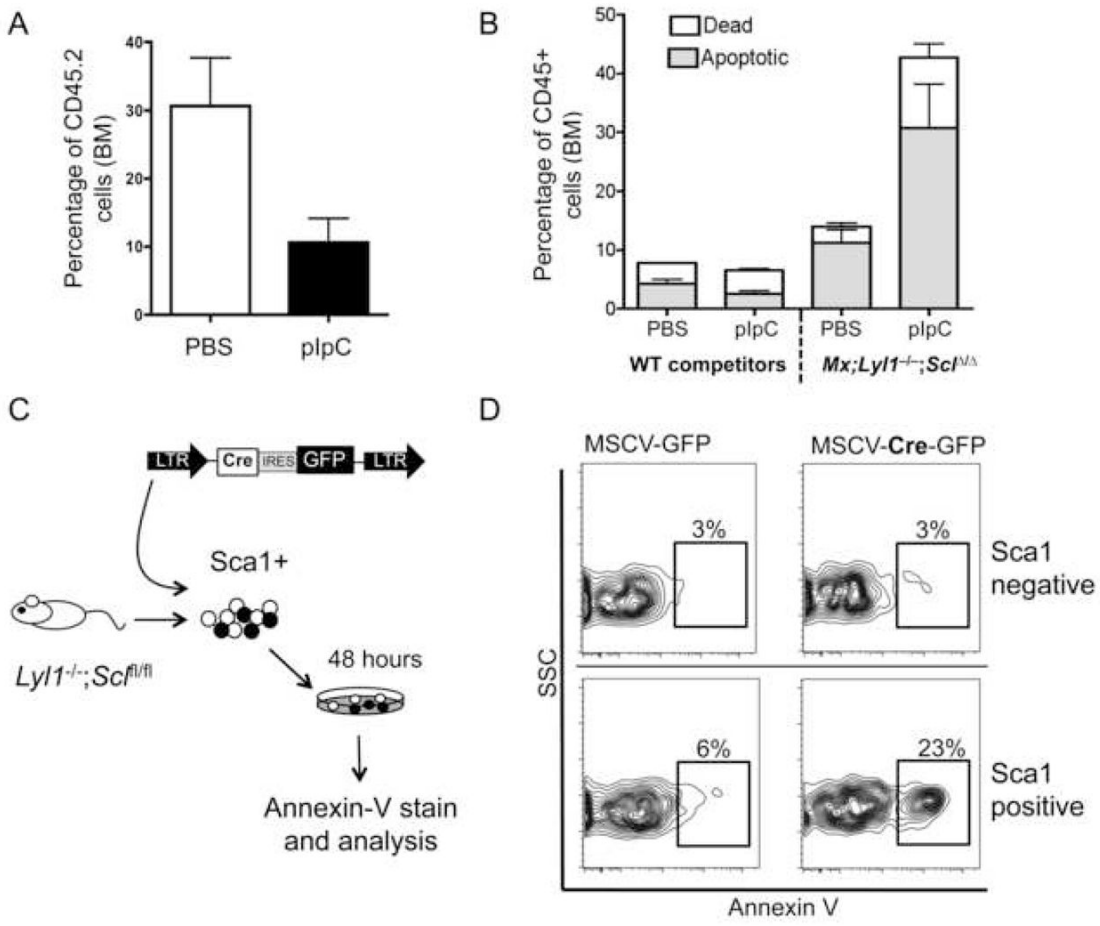

Figure 4. Apoptosis of the Mx;Lyl1$^{-/-}$;Scl$^{\Delta/\Delta}$ cells is cell intrinsic

(A) Bone marrow engraftment of Mx;*Lyl1*$^{-/-}$;*Scl*$^{fl/fl}$ cells treated with either PBS or poly(I:C). Peripheral blood (PB) chimerism was analyzed two days after treatment. Mice treated with poly(I:C) had a three-fold lower engraftment than mice treated with PBS (mean +/− SEM, n=4). (B) Annexin V staining on the remaining donor-derived population in recipients shows that Mx;*Lyl1*$^{-/-}$;*Sc*$^{\Delta/\Delta}$ donor-derived cells undergo apoptosis at three times higher rates than controls. The experiment was carried out four times with consistent results. (C) Experimental schematic. Sca1^{+} cells were cultured for 48 hours after transduction with a Cre-expressing virus and analyzed by Annexin V staining. (D) Representative flow cytometry plots of *in vitro* deletion of the *Scl*^{fl} alleles. The plots shown have been gated from the GFP-positive populations and show specific apoptosis of *Lyl1*$^{-/-}$;*Scl*$^{fl/fl}$ cells transduced with the Cre-expressing retrovirus. Transduction experiments were carried out twice with similar results.