Supporting Information

Henry et al. 10.1073/pnas.1005486107

SI Materials and Methods

Retroviral Transduction and BMT. The retroviral murine stem cell virus (MSCV)-ires-GFP (MiG) vector and constructs expressing either p190 or p210 Bcr-Abl in this vector were kindly provided by Zonghan Dai (Texas Tech University Health Sciences, Amarillo, TX) and Warren Pear (University of Pennsylvania, Philadelphia, PA). MSCV viruses were prepared by transient transfection of Pheonix-E cells, together with pCL-Eco or pCL-Ampho, and titered on REF52 cells using GFP as the marker so that similar multiplicities of infection for oncogenes and vector viruses were used. Harvested donor BM cells were enriched for c-Kit$^+$ cells by magnetic affinity cell sorting (Miltenyi Biotec) and then transduced in nonadhesive six-well plates using the spin-fection technique (centrifugation at 910 × *g* for 1.5 h in the presence 8 μg/mL polybrene). Cells were then washed once with PBS and transplanted into recipient mice. Young lethally irradiated (1,000-rad split dose) BALB/c female recipients were each injected with 2×10^6 unsorted BM cells from either 2-mo-old or 22- to 24-mo-old donors (prepopulation with young or aged competitors, respectively). Before lethal irradiation, mice were starved overnight to reduce small intestinal cytotoxicity. Four days after prepopulation, 1×10^5 MSCV-transduced cells were then transplanted i.v. into recipient mice. A subset of the transduced cells was further cultured for 2 d, and the percentage of GFP$^+$ cells was determined by flow cytometry as an estimate of the initial infection efficiency. For the experiments in Fig. 2*B* and Fig. S2, recipient mice were sublethally irradiated without prepopulation to achieve a higher fraction of cells expressing Bcr-Abl or vector. For the competitive BMTs shown in Fig. 1, whole BM was mixed at the indicated ratios (based on numbers of nucleated cells) and transplanted into lethally irradiated young BALB/c recipients (1.5–3 mo of age). Competitor GFP-transgenic BM cells were from young GFP-transgenic mice (1) backcrossed >10 times into the BALB/c background. The University of Colorado Institutional Animal Care and Use Committee approved all mouse experiments.

IL-7 Neutralization Experiments. M25 hybridoma cells were used to generate the anti–IL-7 neutralizing antibodies. Cells were passaged in BD/Falcon 100-mm Optilux nontissue culture-treated Petri dishes (Becton Dickinson) (10–12 mL per dish) every other day until they reached a density of $0.03–0.05 \times 10^6$ live cells/mL. Anti–IL-7 neutralizing antibody was purified from the supernatant: a HiTrap Protein G HP column was prepared (17-0404-03 or 17-0404-01; Amersham) and washed with 5 mL of filtered deionized water and then with 10 mL of filtered 1× PBS. Five liters of supernatant was collected and mixed slowly with ammonium sulfate at room temperature for 1 h. The ammonium sulfate suspension was centrifuged at 12,000 × *g* for 20 min. The supernatant was slowly decanted, and the pellet was resuspended in a total volume 10 times less than the original supernatant volume. Before putting supernatant on the column connected to an ATKA FPLC system (GE Healthcare), samples were centrifuged at 3,000 × *g* for 15 min at 4 °C. The supernatant was then loaded onto the column and pumped at a flow rate of 0.5 mL/min overnight continuously at 4 °C. On the next day, the flow-through was collected and the column was washed with 1× PBS until the OD reached baseline. Each fraction was saved for analysis. Elution buffer [10 mM glycine/HCL (pH 3.0) plus 100 mL of distilled water] was added to the column, and this fraction was collected. This sample was immediately neutralized by adding 2 μL/mL of neutralizing buffer (1.0 M Tris-OH + 0.01% sodium azide). After dialysis, samples were assayed for protein concentration and purity via Bradford assays and SDS/PAGE analyses.

Mice were irradiated and prepopulated with young BM cells. Four days later, mice were administered young c-Kit$^+$ cells that harbored p190 Bcr-Abl. At day +1, mice were injected i.p. with control IgG (5381-10MG; Sigma) or anti–IL-7 neutralizing antibodies at 0.5 mg per mouse every 4 d for 3 wk. This dose is half of that used previously to inhibit B lymphopoiesis (2), and although IL-7–mediated signaling was perturbed (Fig. 3), pro-B cells to mature B cells were still generated. Mice were killed, and experiments were performed as described in Fig. 3.

Flow Cytometric Analysis. Single-cell suspensions were plated in 96-well round-bottomed plates and washed in FACS buffer [3% FBS + 1× PBS + 2 mM EDTA (vol/vol)]. Following the wash, cells were stained in 50 μL of antibody solution (1:100 dilution of each antibody) for 30 min to 1 h on ice. Cells were washed once with 200 μL of FACS buffer and resuspended in 400 μL of FACS buffer for flow cytometric analysis. The following PharMingen antibodies against mouse were used: phycoerythrin (PE)-linked anti-B220, anti-CD43, anti-CD4, anti-CD8, anti-Ter119, anti-CD48, and anti–MAC-1; PE-Cy7–linked anti–MAC-1 and anti–IL-7Rα; allophycocyanin-linked anti-B220, anti-Thy1.2, anti-CD117, anti-Sca1, and anti-CD150; and PE-Cy7–linked antibiotin. PE-Cy7–conjugated anti-Mac1, anti-CD93, anti-CD19, anti–IL-7Rα, streptavidin-conjugated PeCy7, and biotin-conjugated anti-CD93 were purchased from Ebiosciences. Flow cytometry analysis was performed on a Cyan, Cytomics FC 500, or Cell Lab Quanta SC (Beckman Coulter) flow cytometer.

For detection of intracellular phosphorylated proteins, BM cells were stained for surface markers as described above (cells were kept at 0–4 °C for the entire procedure). Sodium orthovanadate was added to the staining solution at 150 μM to inhibit tyrosine phosphatases. After washing with FACS buffer, the cells were resuspended in 50 μL of Reagent A (Fixation Media, catalog no. GAS001; Invitrogen) and incubated for 30 min on ice or overnight at 4 °C. One hundred microliters of FACS buffer was added per sample, and the cells were pelleted, resuspended in 100 μL of ice-cold 90% methanol (vol/vol), and incubated for 20 min on ice. The cells were then centrifuged, washed in 100 μL of FACS buffer, and resuspended in 40 μL of Regent B (catalog no. GAS002; Invitrogen) with 10 μL of PE-conjugated antibody specific for a phosphorylated (P-) protein or the appropriate isotype control. The following PE-linked antibodies were used: P-Akt (P-T308, catalog no. 558275), P-Akt (P-S473, catalog no. 560378), P-STAT3 (P-Y705, catalog no. 612569), P-STAT5 (P-Y694, catalog no. 612567), P-Erk (P-T202/P-Y204, catalog no. 612566), and control (rat IgG2b, catalog no. 556925). Following a 1-h incubation on ice, the cells were washed in 100 μL of FACS buffer, centrifuged, and resuspended in 400 μL of FACS buffer and analyzed on the Cyan cytometer. This protocol was modified from the one provided by Kent Teague (3).

In Vitro B-Progenitor Cultures. c-Kit–selected BM progenitors from old or young BALB/c mice were cultured on OP9 stromal cells supplemented with 1 ng/mL IL-7 and 10 ng/mL Flt3 ligand as previously described (4). Cell expansion was monitored by flow cytometry for GFP$^+$B220$^+$ cells in the cultures. The coefficient of selection was calculated by dividing the fold change in GFP percentages by the number of population doublings (PDs): $(GFP_1/GFP_0)/(PD_1 - PD_0)$. For the experiment shown in Fig. S6*C*, Bcr-Abl–expressing progenitors were diluted with untransduced pro-

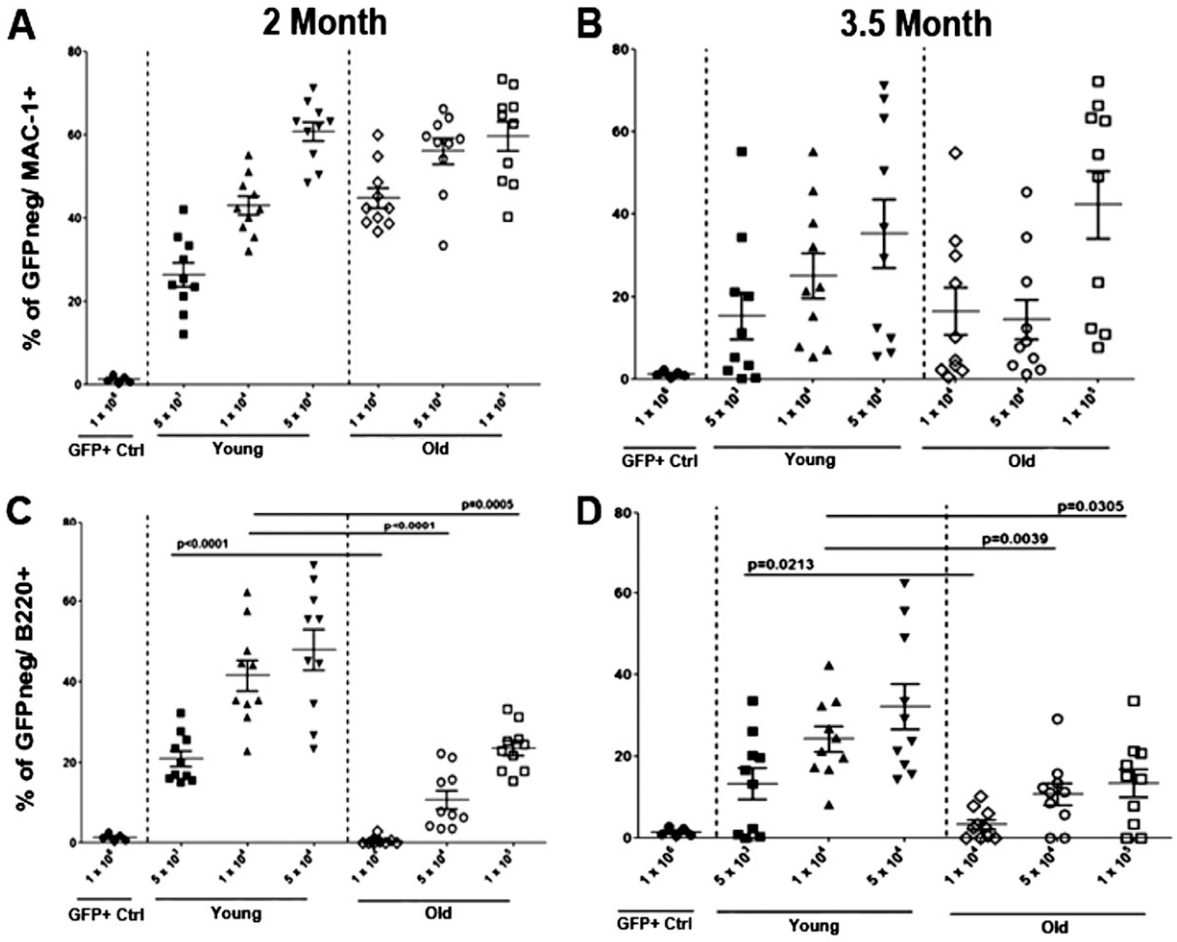
genitors/competitors of the indicated age to achieve a starting population with 5% Bcr-Abl$^+$B220$^+$GFP$^+$ cells.

Analysis of μ-Heavy Chain D-J Rearrangements by PCR. For assay design, primer sequences, and PCR conditions, we followed the method of Schlissel et al. (5) without modifications.

Pathology. Mice were monitored for disease development, as judged by increasing percentages of GFP$^+$ cells, white blood cell counts, and blast morphology in peripheral blood of transplanted animals as well as such symptoms as abnormal gait, frequent hind-limb paralysis, and labored breathing. Moribund animals were killed and examined for tumors as well as content of Bcr-Abl–expressing (GFP$^+$) cells in peripheral blood, spleen, and BM. Leukemia designation required >25% GFP$^+$ blasts in BM. For additional confirmation, touch preparations and tissue sections were obtained from spleen and liver and subjected to pathological examination. White blood cell and erythrocyte counts were determined using a Cell-Dyn 1700 System (Abbott Laboratories).

Statistical Analysis. Statistical analyses were performed with using Prism 5 software from GraphPad. Differences in GFP intensity and mean fluorescent intensity were analyzed by the Student's exact *t* test or one-way ANOVA as indicated. Survival curves were analyzed by the log-rank (Mantel–Cox) test. All error shown is SEM.

1. Schaefer BC, Schaefer ML, Kappler JW, Marrack P, Kedl RM (2001) Observation of antigen-dependent CD8+ T-cell/ dendritic cell interactions in vivo. *Cell Immunol* 214: 110–122.
2. Guerrettaz LM, Johnson SA, Cambier JC (2008) Acquired hematopoietic stem cell defects determine B-cell repertoire changes associated with aging. *Proc Natl Acad Sci USA* 105:11898–11902.
3. Van De Wiele CJ, et al. (2004) Thymocytes between the beta-selection and positive selection checkpoints are nonresponsive to IL-7 as assessed by STAT-5 phosphorylation. *J Immunol* 172:4235–4244.
4. Kodama H, Nose M, Niida S, Nishikawa S, Nishikawa S (1994) Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells. *Exp Hematol* 22: 979–984.
5. Schlissel MS, Corcoran LM, Baltimore D (1991) Virus-transformed pre-B cells show ordered activation but not inactivation of immunoglobulin gene rearrangement and transcription. *J Exp Med* 173:711–720.



E

	Young			Old		
"test"cells	5,000	10,000	50,000	10,000	50,000	100,000
positive/total	6/10	10/10	10/10	6/10	8/10	10/10
HSC frequency	1 in 3,912			1 in 20,395		
95% confidence interval	1 in 2,212to 1 in 6,920			1 in 11,373 to 1 in 36,572		

Fig. S1. Limiting dilution assays reveal reduced numbers of functional HSCs and reduced contributions per HSC to B lymphopoiesis in BM from old mice. BM cells were harvested from young (2 mo of age) or old (24 mo of age) mice (the "test" cells). Different numbers of viable test cells (0.5×10^4 to 1×10^5) were mixed with 10^6 viable competitor GFP-transgenic BALB/c BM cells isolated from donors that were irradiated 8 wk prior with 5 Gy. Because irradiation can dramatically reduce the competitive ability of HSCs, we used competitor BM harvested from previously irradiated donors to ensure that contributions of "test" HSCs are not masked by nonirradiated GFP$^+$ competitors (1). The mixes were injected into lethally irradiated BALB/c recipients (10 mice per group). (*A* and *C*) At 2 mo posttransplantation, peripheral blood was stained with anti-Mac1 plus anti-B220 antibodies and the percentages of GFP$^+$ cells in the Mac1$^+$ myeloid and B220$^+$ B-cell lineages were determined. (*B* and *D*) At 3.5 mo posttransplantation (when contributions to the myeloid lineage should be HSC-dependent), BM was similarly stained and the percentages of GFP$^+$ cells in the MAC1$^+$ and B220$^+$ lineages were determined. Similar differences were observed in the common lymphoid progenitors (CLP), pre-B cell, and pro-B cell pools. (*E*) Numbers of functional HSCs were determined based on the ability of different doses of BM to contribute to hematopoiesis in the myeloid lineage (with a ≥4.0% contribution required to be scored as positive, based on the background levels of GFP^{neg} hematopoiesis detected in recipients of 100% GFP-transgenic BM). The frequencies of functional HSCs were determined with L-Calc software from Stem Cell Technologies. Results are representative of two experiments. The comparison of HSC frequencies in young and old mice is significant at $P < 0.001$ (two-tailed ratio of proportions test). HSC, hematopoietic stem cells; BM, bone marrow.

1. Marusyk A, Porter CC, Zaberezhnyy V, DeGregori J (2010) Irradiation selects for p53-deficient hematopoietic progenitors. *PLoS Biol* 8:e1000324.

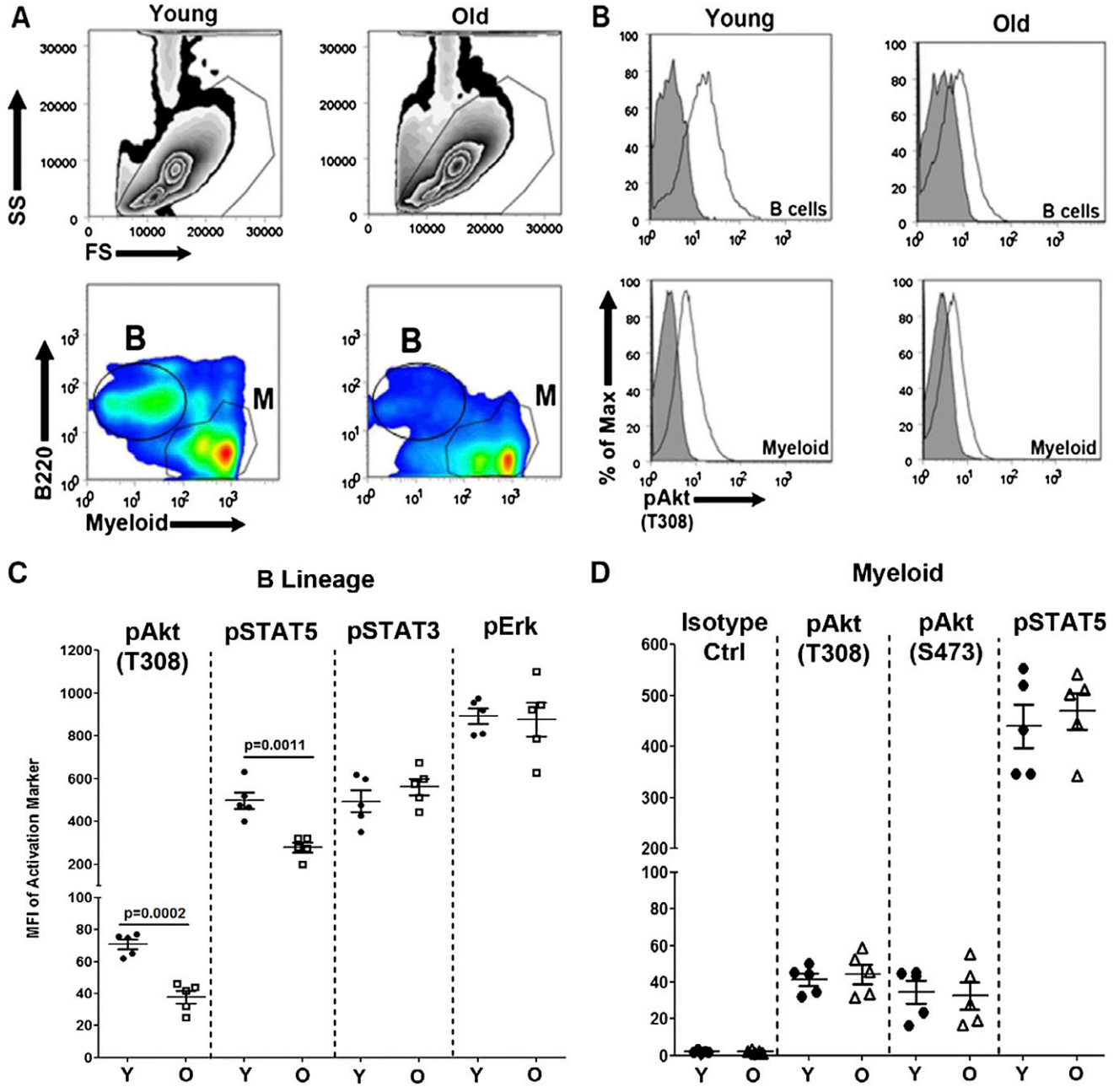


Fig. S2. Activation of Akt and STAT5 is reduced in old BM. BM was isolated from young (Y) and old (O) mice; stained with antibodies against B220 and Mac1; and processed for detection of intracellular P-Akt (T308 or S473), P-STAT5, and P-Erk by flow cytometry. (*A* and *B*) Gating strategy used for the quantitation of mean fluorescence intensity (MFI) shown. The "B" and "M" gates in *A* were used for P-Akt detection in the B-cell and myeloid lineages, respectively, as shown in *B*. (*C*) Detection of P-Akt, P-STAT5, P-STAT3, and P-Erk in cells gated on the $B220^{+}$ B-lineage population negative for other lineage markers (Mac1, Ter119, and CD3) is shown. (*D*) Detection of P-Akt and P-STAT5 in cells gated on the $B220^{neg}$ population positive for other lineage markers (Mac1, Ter119, and CD3) is shown. Greater than 90% of these gated cells will be myeloid (erythroid and other myeloid lineages).

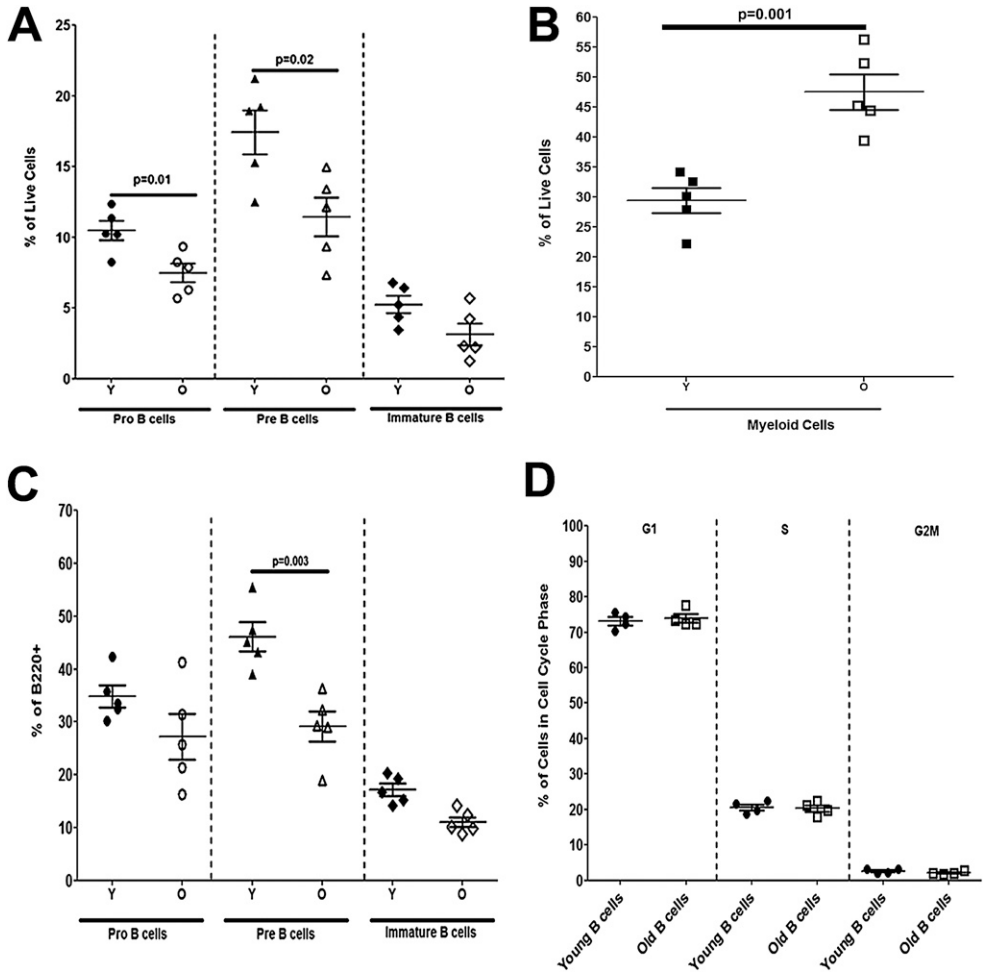


Fig. S3. Reduced pro-B cell and pre-B cell frequencies in old BM. The percentage of pro-B and pre-B cells is reduced in the BM of old mice. BM was stained with antibodies against B220, CD93, Mac1, and CD43, and the percentages of nucleated myeloid cells ($Mac1^{pos}B220^{neg}CD93^{neg}CD43^{neg}$), pro-B cells ($B220^{med}CD93^{+}CD43^{+}Mac1^{neg}$), pre-B/immature B cells ($B220^{med}CD93^{+}CD43^{neg}Mac1^{neg}$), or mature B cells ($B220^{high}CD93^{neg}CD43^{neg}Mac1^{neg}$) within total live BM (*A* and *B*) or $B220^{+}$ pools (*C*) are graphed. (*D*) B-cell progenitors in young (Y) and old (O) BM do not exhibit differences in cell cycle profiles. BM from young and old mice was stained with anti–B220-FITC, washed, permeabilized, and stained with propidium iodide to determine DNA content per cell. The fraction of cells in the indicated cell cycle stages is graphed. BM, bone marrow.

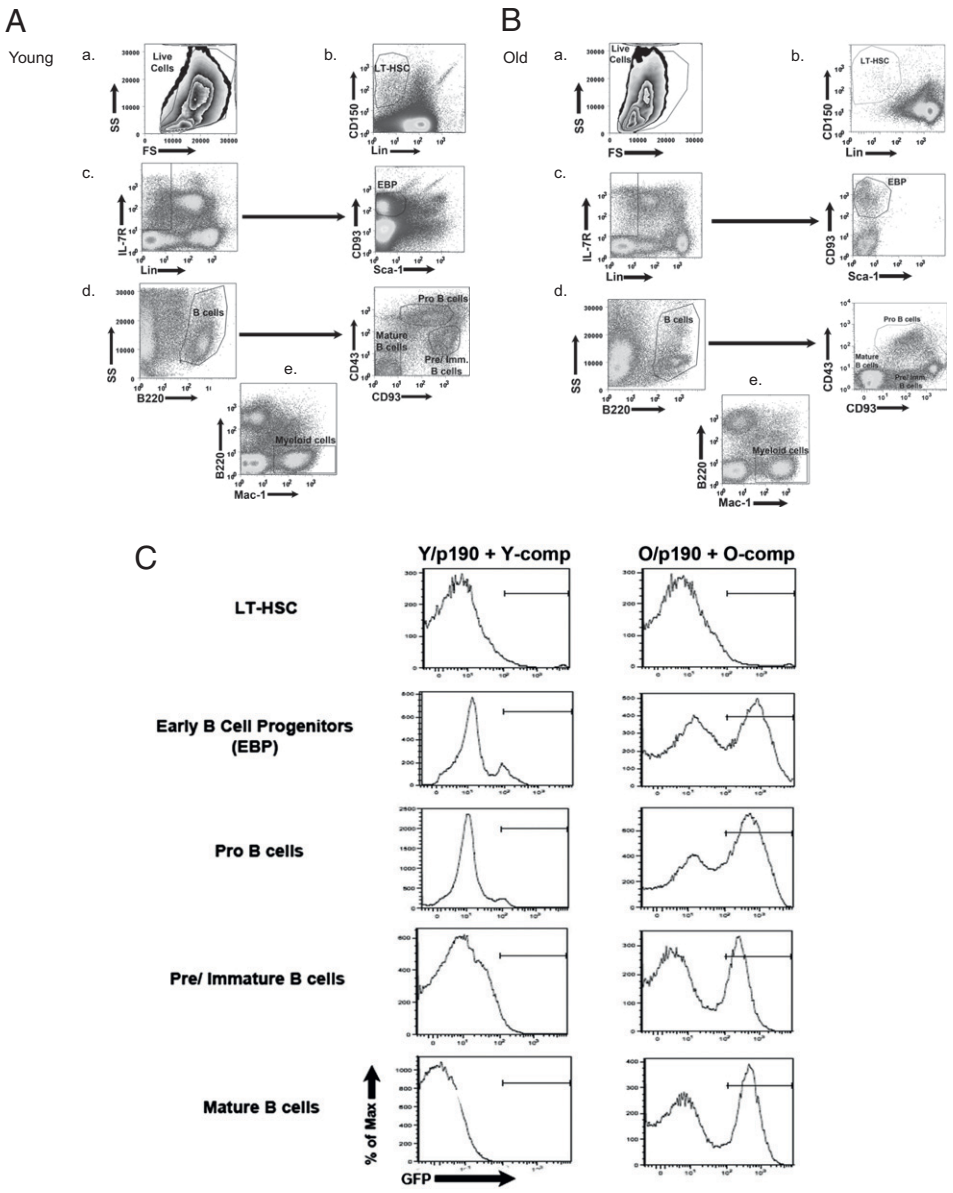


Fig. S4. Representative flow cytometric plots and the gating strategy used for the quantitation shown in Fig. 2*A* and Fig. S5 of Bcr-Abl and vector (respectively) contributions to different hematopoietic populations. The gating strategy for detection of LT-HSC, EBPs, pro-B cells, pre-B/immature cells, mature B cells, and myeloid cells is shown for a representative young mouse (*A*) and a representative old mouse (*B*). (*C*) Examples of percentage of cells expressing GFP as determined in these gated populations. Further details are provided in Fig. 2*A* and Fig. S5.

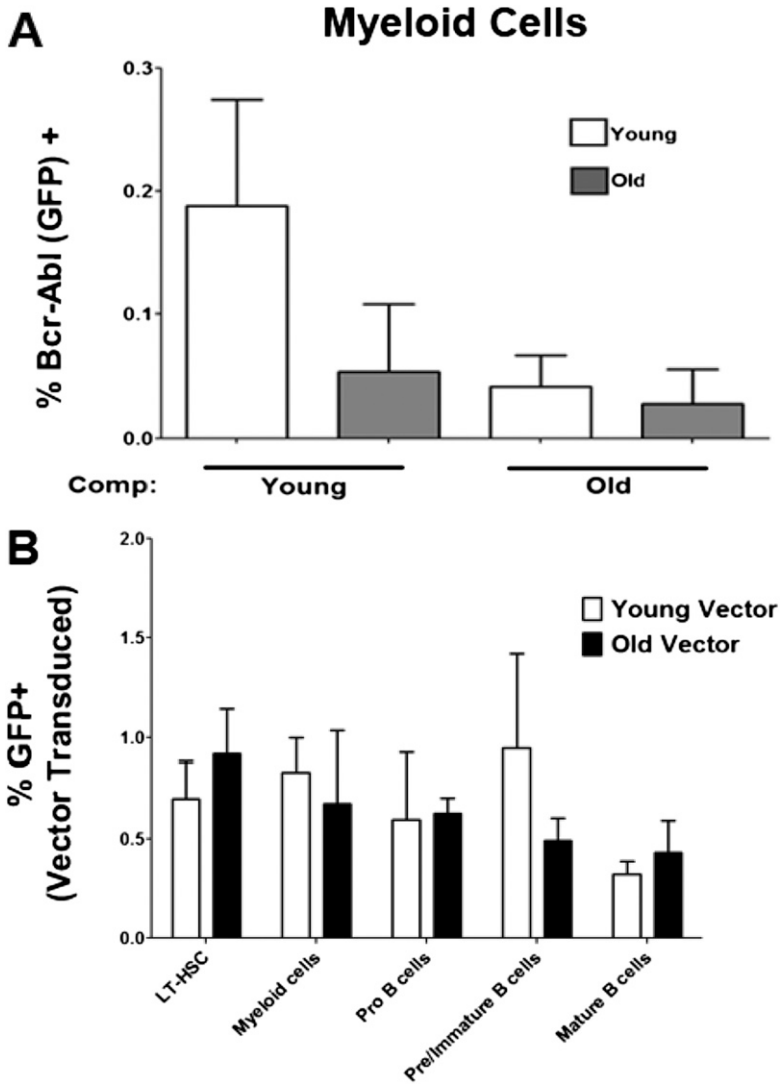


Fig. S5. Contributions of p190 Bcr-Abl to the myeloid lineage is not age-dependent, and donor age does not influence vector contributions to hematopoiesis. (*A*) Mice were transplanted and BM was analyzed as described in Fig. 2*A*, and contributions of Bcr-Abl/GFP$^+$ cells to the myeloid lineage (Mac1$^+$B220^{neg}) are graphed. Differences between young and old groups were not significant. (*B*) Mice were transplanted and BM was analyzed as described in Fig. 2, except that prepopulated mice received ~1 × 10^5 MiG-vector–transduced Kit-selected BM cells from young or old donors. The percentages of GFP$^+$ cells among HSCs (CD150$^+$CD48^{neg}Lin^{neg}), myeloid cells (Mac1$^+$B220^{neg}), pro-B cells (B220^{med}CD93$^+$CD43$^+$Mac1^{neg}), pre-B/immature B cells (B220^{med}CD93$^+$CD43^{neg}Mac1^{neg}), and mature B cells (B220^{high}CD93^{neg}CD43^{neg}Mac1^{neg}) in BM of mice transplanted with Bcr-Abl–transduced cells were determined by flow cytometry at 3 wk posttransplantation. Differences between young and old groups were not significant.

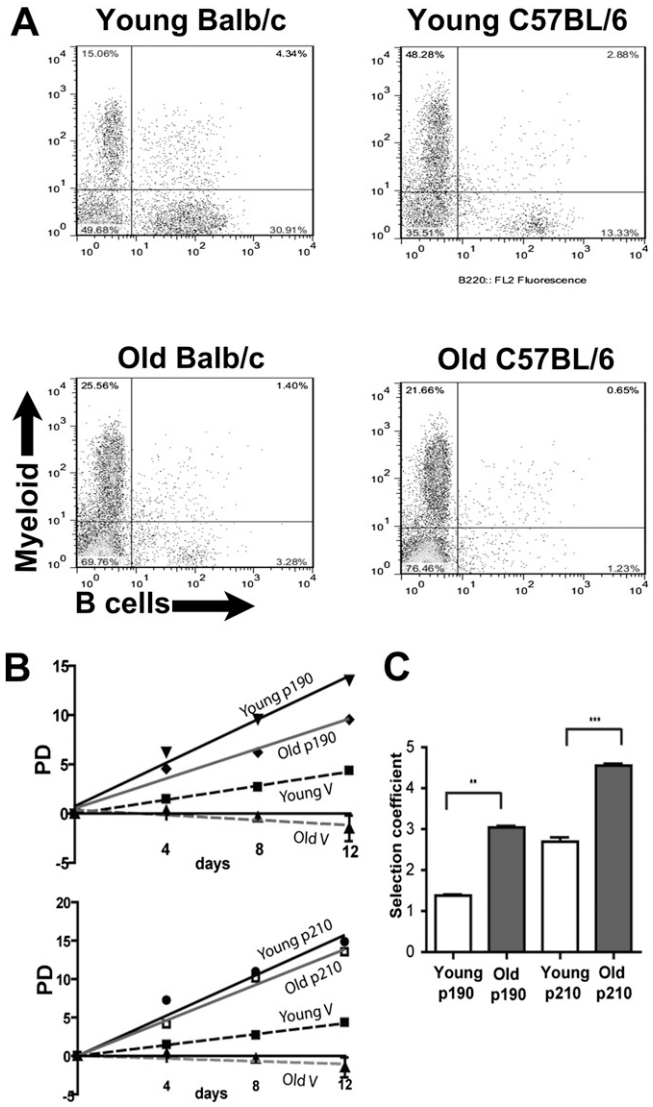


Fig. S6. Age-associated decreased B-progenitor maturation and expansion, with rescue of impaired expansion by Bcr-Abl expression. (*A*) We capitalized on the ability of B progenitors to expand on OP9 BM stromal cultures (1). c-Kit–selected BM progenitors from young or old C57BL/6 or BALB/c mice were cultured on OP9 stromal cells supplemented with IL-7 and Flt3 ligand for 4 d and then analyzed by flow cytometry for the expression of Mac1 (myeloid lineage) and B220 (B-cell lineage). Representative flow cytometric plots are shown. We consistently observed reduced differentiation of old c-Kit–selected progenitors into the B-cell lineage in both strain backgrounds at day 4, although old progenitors would eventually commit to the B-cell lineage (with >50% $B220^+$ by days 7–10). B progenitors in these cultures displayed a pro-B– to early pre-B–like phenotype ($B220^+CD43^+$). (*B*) Once more than 50% of progenitors in both young and old cultures were $B220^+$, cultures were transduced with MiG-p190 Bcr-Abl, MiG-p210 Bcr-Abl, or MiG vector (V). Transduced cells were returned to culture on OP9 stromal cells with IL-7 and Flt3 ligand. Cell expansion was monitored by flow cytometry for GFP^+B220^+ cells in the cultures. Young vector-expressing B progenitors were capable of substantial expansion. In contrast, old vector-expressing B progenitors largely failed to expand. The expression of p190 or p210 Bcr-Abl endowed young B progenitors with a selective advantage, leading to even greater expansion. The greater advantage provided by Bcr-Abl to young B progenitors in vitro relative to in vivo may represent adaptation by Bcr-Abl to the culture shock inherent in vitro. Strikingly, the expression of Bcr-Abl rescued the proliferative defect of the old B progenitors, leading to substantial expansion. (*C*) To determine the selective advantage provided by expression of Bcr-Abl, we calculated coefficients of selection as a fold change in GFP percentages relative to the number of PDs. The coefficient of selection was calculated by dividing the fold change in GFP percentage over 4 d by the number of PDs: $(GFP_1/GFP_0)/(PD)$, where GFP_0 is the initial GFP percentage and GFP_1 is the GFP percentage after 4 d. PD is calculated by the $\log_2$ of the fold change in cell numbers over the 4 d (e.g., a twofold increase represents 1 PD). The coefficient of selection was much greater for Bcr-Abl expression in old progenitors relative to young. Thus, given that old B progenitors do not substantially expand ex vivo without Bcr-Abl expression, we observed a significantly greater increase in the fraction of cells expressing Bcr-Abl over each PD. In all, the OP9 model allowed us to study the effects of Bcr-Abl in aged B lymphopoiesis in a more controlled setting. These experiments demonstrate that the inherent defects in cell expansion for aged B progenitors can be reversed by Bcr-Abl expression in vitro, leading to strong selection for Bcr-Abl. $^{**}P$ values between 0.01 and 0.001; $^{***}P < 0.001$.

1. Kodama H, Nose M, Niida S, Nishikawa S, Nishikawa S (1994) Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells. *Exp Hematol* 22:979–984.

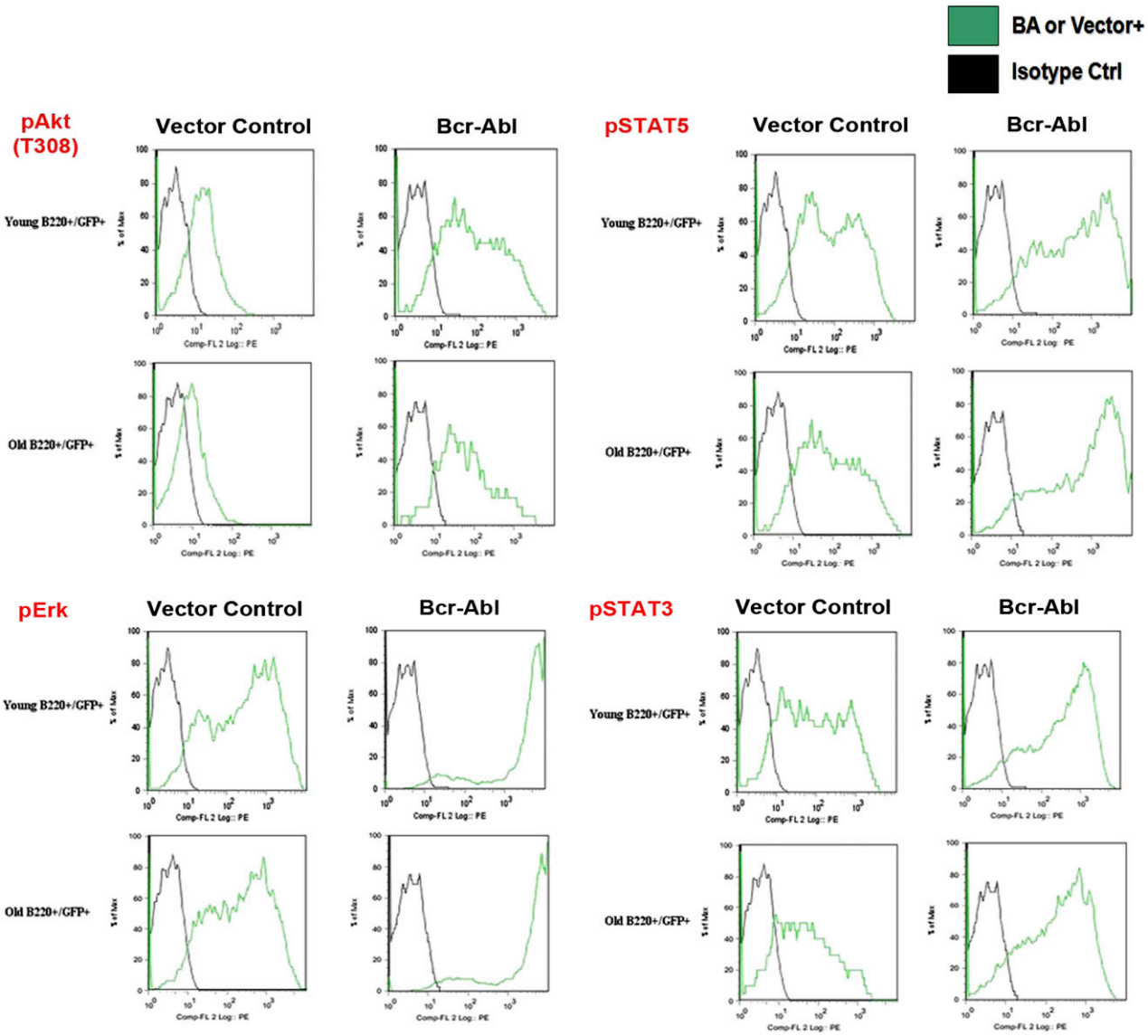


Fig. S7. Representative flow cytometric plots used for the quantitation shown in Fig. 2*B*. BM cells described in Fig. 2*B* were analyzed for P-Akt, P-STAT3, P-STAT5, and P-Erk, together with surface staining for B220, Mac1, CD3, and Ter119 by flow cytometry. The detection of the indicated phosphorylated effector protein within B-lineage cells ($B220^{+}Mac^{neg}CD3^{neg}Ter119^{neg}$) is graphed for both MiG vector- and MiG-p190–expressing cells (green trace) as well as for isotype control (Ctrl) cells (black trace). BA. BA, Bcr-Abl.

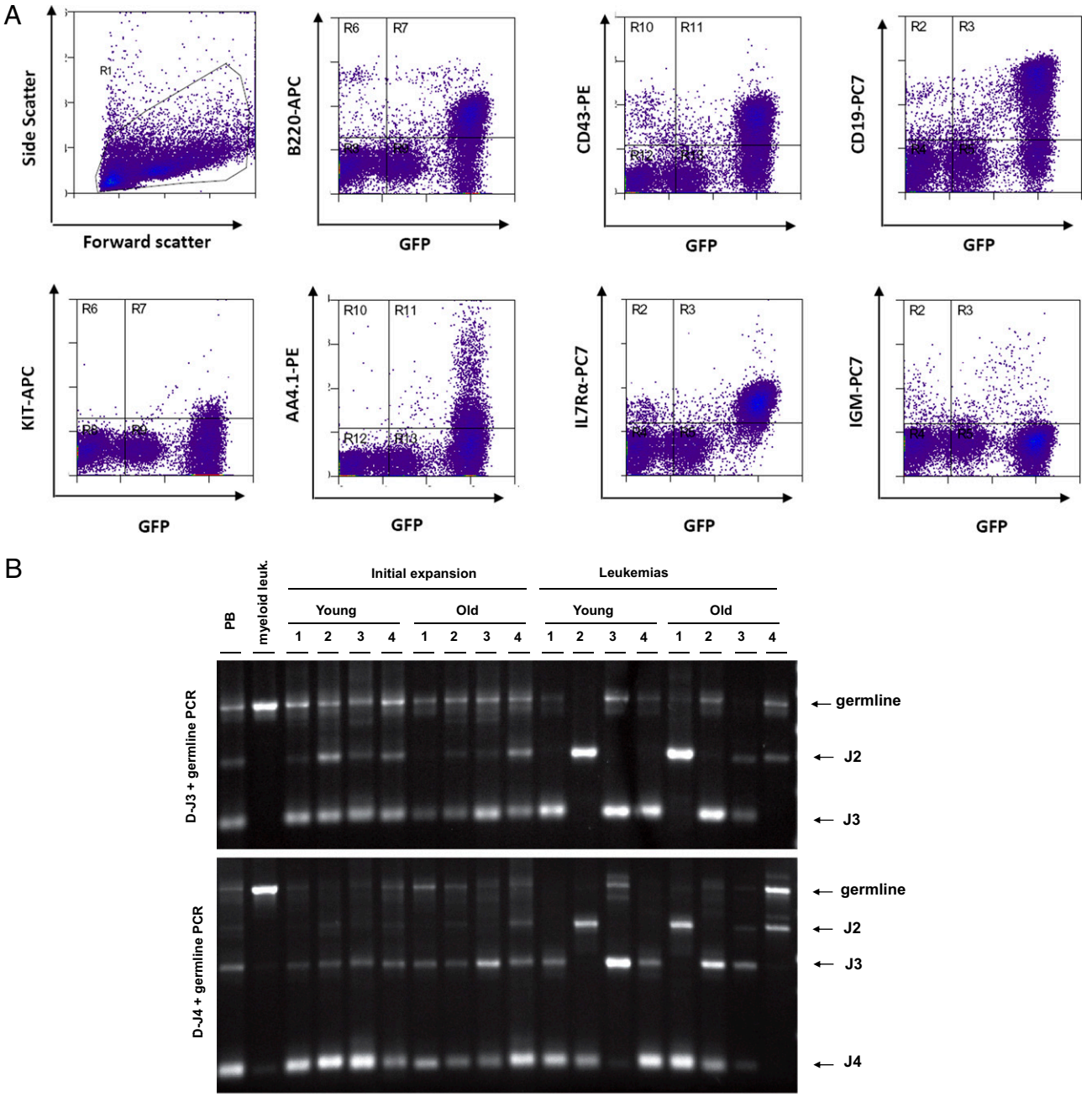


Fig. S8. Although the initial expansion of Bcr-Abl–expressing cells is polyclonal, most leukemias that develop in Bcr-Abl–expressing young or old progenitors are pro-B cell–like and monoclonal. (*A*) Most leukemias that developed in recipients of p190 or p210 Bcr-Abl–transduced BM cells (with the exception of p210-transduced cells in recipients with *Rag2*$^{-/-}$ competitors) exhibited a pro-B–like phenotype. An example of flow cytometric analysis of leukemia from a recipient of p190-transduced old cells with old competitor BM is shown, but similar phenotypes were observed for other B-lineage leukemias. The antibodies used to detect the indicated cell surface markers are shown on the *y* axis, and GFP expression is depicted on the *x* axis. A summary of the lineage types for leukemias that developed in all groups shown in Fig. 4. is presented in Table S1. (*B*) D-J rearrangements for the μ-heavy chain were analyzed by PCR using D and J3 primers or D and J4 primers for either Bcr-Abl^{+}B220^{+} cells isolated from mice during early expansions (~3 wk) or from leukemias that developed in transplanted mice (from young or old progenitors). Peripheral blood (PB) is used as a control, because all possible D-J rearrangements are present. As another control, Bcr-Abl–initiated myeloid leukemias only exhibit germline configurations. Each of the PCR reactions has two forward primers and one reverse primer. Forward primers are a degenerate primer for D minigene that is specific to 9 of 10 murine D minigenes (DH primer) and a germline (Mu0) primer. The reverse primer is specific for J3 (*Upper*) or J4 (*Lower*). DJ rearrangement results in a loss of amplification product with Mu0 and generation of a product with DH primer.

Table S1. Leukemia typing

Bcr-Abl p190	B-cell lymphoid	Myeloid	T-cell lymphoid	Mixed B/M
Young competition	3	1	—	—
Old competition	9	1	—	1
RμKI competition	3	—	—	—
Rag competition	4	—	—	—
Young competition	6	1	—	1
Old competition	9	4	1	1
RμKI competition	3	—	—	1
Rag competition	2	8	1	1*

Leukemia typing was performed as described in Fig. S8*A*, except that antibodies recognizing the myeloid markers GR-1 and Mac-1 were also included. The leukemias were typed as "B-cell" or "myeloid" if over half of GFP$^+$ cells were B220$^+$ or Mac-1/GR-1$^+$. Mixed B-cell/myeloid (B/M) leukemias contained at least 25% of both B-cell and myeloid markers.

*B-cell and myeloid markers were expressed on the same leukemia cells for this mixed B/M leukemia (whereas for other mixed leukemias, these markers were expressed on different leukemia cells).