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Murine B-1 B Cell Progenitors Initiate B-Acute Lymphoblastic Leukemia With Features of High Risk Disease[1]

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Abstract

B-1 and B-2 B cells derive from distinct progenitors that emerge in overlapping waves of development. The number of murine B-1 progenitors peaks during fetal development while B-2 B cell production predominates in adult bone marrow. Many genetic mutations that underlie B-acute lymphoblastic leukemia (B-ALL) occur in the fetus, at which time B-1 progenitor numbers are high. However, whether B-ALL can initiate in B-1 progenitors is unknown. We now report that *BCR-ABL* transformed murine B-1 progenitors can be B-ALL cells of origin and demonstrate that they initiate disease more rapidly than oncogene expressing B-2 progenitors. We further demonstrate that B-1 progenitors exhibit relative resistance to apoptosis and undergo significant growth following oncogene expression and propose that these properties underlie the accelerated kinetics with which they initiate leukemia. These results provide a developmental perspective on the origin of B-ALL and indicate B cell lineage as a factor influencing disease progression.

Introduction

B cell development initiates during embryogenesis (1, 2). However, rather than being a linear process in which all types of B cells are generated, at least two distinct waves of fetal B lymphopoiesis have been defined (3). During the first, B-1 progenitors are generated. These cells ultimately mature into innate-like B-1a and B-1b cells that provide defense against pathogens that include encapsulated bacteria (4, 5). Subsequently, B-2 B cell progenitors arise, and they are the predominant B cell progenitor population present in post-natal bone marrow (6). Most B lymphocytes in peripheral immune tissues are B-2 B cells, which are involved in adaptive immune responses (7).

In addition to differences in the kinetics with which they appear during embryogenesis, B-1 and B-2 progenitors can be distinguished by their cytokine responsiveness. For example, B-1 progenitors proliferate more vigorously to interleukin-7 (IL-7) than B-2 progenitors (8). In addition, B-1, but not B-2, progenitors can be distinguished based on their ability to respond to thymic stromal lymphopoietin (TSLP) (6, 9). It was initially proposed that TSLP

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had no effect on adult pro-B cells and its actions were limited to B cell progenitors in fetal tissues (10). However, we subsequently demonstrated that B cell lineage defined TSLP responsiveness. Thus, B-1 progenitors from fetal liver and young adult bone marrow responded to TSLP while B-2 progenitors, regardless of whether they were isolated from fetal or adult tissues, could not do so (6). Taken together with the differences in the kinetics with which they arise, these observations demonstrate that B-1 and B-2 progenitors exhibit distinct properties.

B-Acute Lymphoblastic Leukemia (B-ALL) is the most common pediatric malignancy (11, 12). While up to 80% of B-ALL patients respond well to treatment and survive long-term, the remaining 20% often have a poor prognosis (13). Various chromosomal translocations, such as *ETV6-RUNX1* (*TEL-AML1*), *MLL-AF4* and *BCR-ABL*, frequently underlie B-ALL and have been used to stratify patients into risk groups predictive of survival or therapeutic response (11, 14). For example, children with the *BCR-ABL* translocation, which encodes the Philadelphia (Ph) chromosome, are classified as high risk based on elevated leukocyte counts at diagnosis, central nervous system involvement, and poor response to treatment (15-18). Many of these chromosomal translocations occur in the human fetus (19, 20). Interestingly, this is the time that murine B-1 progenitor numbers peak (3). If human B-1 progenitor numbers are at their maximum at this time as well, then some infant and pediatric B-ALL cases could be B-1 malignancies. However, whether B-ALL can arise in B-1 progenitors has not been tested.

We now report that B-1 progenitors can be ALL cells of origin and demonstrate that *BCR-ABL* transduced B-1 progenitors initiate disease more rapidly than oncogene expressing B-2 progenitors. We further propose, based on *in vitro* modeling of ALL, that the high proliferative potential and relative resistance to oncogene-induced apoptosis of B-1 progenitors underlies the accelerated kinetics with which they initiate leukemia. Together, these results further define differences between the B-1 and B-2 lineages and provide a novel developmental perspective on the origins of pediatric ALL.

Materials and Methods

Mice

Swiss Webster (SW), CB17.SCID (SCID), and RAG-2/SJL (RAG, B6.SJL(129S6)-Ptprca/ BoCrTac-Rag2tm1) mice were obtained from Taconic Farms (Germantown, NY). TSLP receptor deficient (*CRLF2$^{-/-}$*) mice were a generous gift of Dr. James Ihle at St. Jude Children's Research Hospital (21). C57BL/6 (B6) and *IL-7R$\alpha^{-/-}$* (B6.129S7-*Il7r^{tm1Imx}*/J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). In initial experiments B cell progenitors were harvested from SW fetal liver at embryonic day 15. Otherwise, progenitors were purified from 4-6 week old SW, B6 and *CRLF2$^{-/-}$* mice and 1.5 to 3 week old *IL-7R$\alpha^{-/-}$* mice. All animals were housed and/or bred in the vivarium of the Division of Laboratory Animal Medicine, University of California at Los Angeles. Animal care and use were conducted according to the guidelines of the Institutional Animal Care and Use Committee.

Flow cytometry

Cell suspensions from spleen were prepared by crushing tissue between frosted slides in Ca^{2+} Mg^{2+}-free PBS. Bone marrow cell suspensions were prepared by flushing bones with Ca^{2+} Mg^{2+}-free PBS. When necessary, red blood cells were lysed with ACK buffer. All samples were incubated with anti-CD16/CD32 (FcγRII-III; clone 2.4G2) to block nonspecific labeling. Optimum working dilutions for the following antibodies were determined before use: CD19 (clone 1D3), CD45R (B220, clone RA3-6B2), CD93 (AA4.1, clone C1qRp), and CD43 (clone S7). Mature Lineage cells were detected with an antibody cocktail that included: goat anti-mouse IgM, anti CD3ϵ (clone 145-2C11), CD8α (clone 53-6.7), TCRβ (clone H57-597), TCR$\gamma\delta$ (clone UC7-13D5), NK1.1 (clone PK136), Ly-6C (clone AL-21), CD11b (clone M1/70), Ter-119 (clone Ter-119), and Gr-1 (clone RB6-8C5). Anti-IgM was obtained from Southern Biotechnology (Birmingham, AL) and other antibodies were purchased from e-Biosciences (San Diego, CA). Cells were stained for 30 min at 4°C, washed with Ca^2 Mg^{2+}-free PBS and analyzed on an LSRII (BD Biosciences). B-1 progenitors, defined as Lineage negative (Lin$^—$) CD19$^+$ CD45R(B220)$^{—/low}$ CD93$^+$ and B-2 progenitors, defined as Lin$^—$ CD19$^—$ CD45R(B220)$^+$ CD93$^+$ CD43$^+$, were isolated on an FACSAria (BD Biosciences, San Jose, CA). In some experiments, GFP expression was used to sort transduced cells following culture or B-ALL tumor cells from the spleen of diseased recipients. The frequency of GFP$^+$ AnnexinV^{+high} and Propidium Iodide (PI)$^+$ cells was determined by staining cells with a PE AnnexinV Apoptosis kit (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions and analyzing them on an LSRII.

Phospho-flow analysis

BCR-ABL expressing (ie., GFP$^+$) B-ALL cells were isolated from the spleen of diseased animals on a FACSAria and tested for their levels of activated Stat5 as follows. Cells were fixed with 0.5% methanol free formaldehyde for 10 min at 4°C, washed with Ca^2 Mg^{2+}-free PBS and permeabilized with 70% ice cold methanol in a drop wise manner and constant agitation. After washing, the cells were incubated for 30 min with anti-CD16/CD32 (FcgRII-III; clone 2.4G2) as a blocking agent and Pacific Blue™ labeled mouse anti-Stat5 (clone 47/Stat5(pY609)) or mouse IgG1k Isotype control (clone MOPC-21); all from BD Biosciences). The frequency of GFP$^+$ cells that expressed Stat5 was then determined on an LSRII.

Ki-67 immunostaining—BCR-ABL expressing (ie., GFP$^+$) B-ALL cells were isolated from the spleen of diseased animals on a FACSAria, fixed and permeabilized with BD cytoperm™ fixation and permeabilization solution, and washed with 1× BD Perm/Wash™ buffer as per manufacturer instructions. The cells were then incubated for 30 min with anti-CD16/CD32 (FcγRII-III; clone 2.4G2, eBioscience) as a blocking agent before adding PE-mouse anti-Ki-67 or PE-mouse IgG1 isotype control (Becton-Dickinson) for 30 minutes. The cells were then washed with 1× BD Perm/Wash™ buffer and analyzed on an LSRII (Becton-Dickinson) located in the Broad Stem Cell Research Center at UCLA.

Production of retroviral stocks

pMSCV40 retroviral vectors containing either a 5′ LTR-driven p210 *BCR-ABL* internal ribosome entry site (IRES) enhanced *GFP* (EGFP; *BCR-ABL/GFP*) or a 5′ LTR-driven IRES *EGFP* (*GFP*-only) were used to generate high-titer helper-free retrovirus supernatants following transient co-transfection of 293T cells. 293T cells were grown in 10 cm^2 tissue culture treated plates (Becton Dickinson) pre-coated with Poly-L-Lysine (Sigma) in Iscove's modified Dulbecco's minimum essential medium (IMDM, Mediatech, Manassas, VA) supplemented with 10% heat inactivated fetal calf serum (FCS, Hyclone, Logan, UT), 1mM L-glutamine, 100 U/ml streptomycin, and 100 μg/ml penicillin (complete IMDM; all from Gibco, St. Louis, MO). 293T transfections were performed by co-precipitating 15 μg of retroviral vector with 15 μg of the ecotropic packaging vector using the CalPhos Mammalian Transfection Kit (BD Biosciences, San Jose, CA). Medium was replaced every 12 hours for 3 days with complete IMDM. Viral stocks were prepared by pooling supernatants collected at 36 and 48 hours post-transfection. Viral titers were determined following infection of 3T3 cells with serial dilutions of the pooled virus supernatant and found to range between 2×10^6 and 7×10^6 virus particles/ml.

Cell culture and transductions

B-1 and B-2 progenitors were plated in 3 ml of RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, 1 mM L-glutamine, 100 U/ml streptomycin, 100 μg/ml penicillin, 50 μM β-mercaptoethanol, 50 μg/ml gentamycin, Stem Cell Factor (20 ng/ml), IL-3 (20 ng/ml), Flt-3L (10 ng/ml), IL-7 (30 ng/ml; all from Gibco, St. Louis, MO) and 4 μg/ml polybrene. 0.4 μm transwell inserts pre-seeded with confluent layers of the S17 stromal cell line were inserted into each well *GFP*-only or *BCR-ABL/GFP* retrovirus supernatants were added to the bottom wells containing the purified progenitor populations at t = 0 hours, 4 hours, 16 hours, and 20 hours of culture. After a total of 24 hours in culture at 37°C, 5% CO_2/air humidified incubator, the cells were harvested and viable cell counts were determined by eosin dye exclusion.

Transplantation

Transduced SW cells were injected intravenously into SCID mice while RAG mice were used as recipients of transduced cells from B6, *CRLF2*$^{-/-}$ and *IL-7Rα*$^{-/-}$ donors. The number of transduced cells injected is indicated in the figure legends. SCID and RAG recipients were conditioned with 300R or 550R, respectively, from a 137^{Cs} irradiator (120 R/min; Mark I-68A; JL Shepherd and Associates, San Fernando, CA) 12 to 24 hours prior to injection of cells. Recipients were sacrificed when they became moribund. Their tissues were tested for the presence of donor derived B-ALL tumor cells through co-expression of GFP and B lineage cell surface antigens as described above.

qPCR

Flash frozen aliquots of purified cells were processed for gene expression by qPCR. Total RNA was extracted with the RNeasy Plus micro kit and used to synthetize cDNA with the RT^2 First Strand kit (both from Qiagen, Valencia, CA) as per manufacturer instructions. Reactions were run in 25μl volumes with SYBR green qPCR master mix (BioRad) as

recommended by the manufacturer. Amplification efficiencies were routinely found to be between 95-105% and all reactions were run in duplicate. RT^2 Primer sets for p19 *Arf*, whose sequences were: 5′— GCTCTGGCTTTCGTGAACATG—3′ (forward) and 5′— TCGAATCTGCACCGTAGTTGAG—3′ (reverse), and *Gapdh* (catalog number PPM02946A) were purchased from SABiosciences. Presence of single PCR products was confirmed by melt curve analysis. Data were analyzed with Biorad IQ5 software using the Pfaffl method with *Gapdh* as a reference gene.

Gene expression in tumor cells harvested from mice was analyzed using a custom RT^2 PCR Array designed by Qiagen (#CAPM11051; Germantown, MD) as per manufacturer instructions. Expression of the following genes was examined: Jak1, Jak2, Stat1, Stat3, Stat5a, Stat5b, Akt1, Mcl1, and Grb2. 2 Ct values for the custom array were calculated using the Pfaffl method with the means of the Ct values for *Actb* and *Hsp90ab1* housekeeping genes as reference. Quality control for the custom array qPCR reactions and gene expression analyses were performed on the Qiagen PCR Array Data Analysis Web Portal (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php?target=upload).

Statistical analysis

Data are expressed as mean ± SEM. Statistical significance for the differences between groups was determined by a two-tailed, unpaired Student's t test (α = 0.05). The Log-rank (Mantel-Cox) test was used to assess differences in the rate of ALL development between different groups of mice.

Results

B-1 progenitors initiate a rapidly developing leukemia

B-1 and B-2 progenitors can be resolved based on their Lin$^-$ CD93$^+$ CD45R$^{low/negative}$ CD19$^+$ (6) and Lin$^-$ CD45R$^+$ CD93$^+$ CD19$^-$ CD43$^+$ phenotypes (7), respectively (Supplemental Fig. 1), and have been shown to differentiate into mature B-1 and B-2 cells following transplantation into immunodeficient recipients (6) (Supplemental Fig. 2). Because progenitors for these two B cell lineages arise in overlapping waves of development (3, 6), we were able to isolate both populations from the same bone marrow (BM) samples from SW mice, although in initial experiments we also purified B-1 progenitors from day 15 gestation fetal liver. Purified populations were transduced for 24 hours in vitro with retroviral vectors containing *BCR-ABL/GFP* or *GFP*-only. Cells were then harvested, washed, and transplanted intravenously into immunodeficient recipients. *BCR-ABL* was used in this study because we previously demonstrated its potential to transform B-2 pro-B cells (22), thus providing a reproducible model system to examine the leukemogenic potential of B lineage progenitors.

Several mice that received *BCR-ABL/GFP* transduced B-1 progenitors derived from fetal liver or young adult bone marrow became moribund as early as 18 days later, and by 40 days all recipients had to be sacrificed due to severe wasting symptoms. *BCR-ABL/GFP* transduced B-1 progenitors from fetal liver or young adult bone marrow initiated B-ALL with similar kinetics (Fig. 1A). In contrast, recipients of *BCR-ABL/GFP* transduced B-2

progenitors first showed signs of disease at around day 30 after transplantation, and many mice survived past 60 days (Fig. 1A).

The tumor burden in recipient mice, assessed by quantifying the number of GFP$^+$ cells, was nearly two-fold higher in the spleen of mice that received transformed B-1 compared to B-2 progenitors (Fig. 1B). Regardless of whether they were derived from B-1 or B-2 progenitors, over 95% of the tumor cells in the mice were CD19$^+$ CD45R(B220)$^+$ surface IgM negative (sIgM$^-$) pro/pre-B cells (Fig. 1C and Supplemental Fig. 3).

B-1 and B-2 progenitors are transduced with comparable efficiency

The above results show that recipients of 3×10^4 B-1 progenitors developed disease more rapidly than recipients of a comparable number of *BCR-ABL* transduced B-2 progenitors. However, it was critical to determine whether the mice received a similar number of transduced cells. Although the *BCR-ABL* containing retroviral construct contained a *GFP* reporter gene, its expression was inefficient at 24 hours following transduction when cells were injected into animals. We thus cultured cell aliquots for 64 hours and then measured GFP expression in the transduced B-1 and B-2 progenitors. As shown in Figure 2A, there was no significant difference in their level of GFP expression, making it unlikely that differences in the number of transduced cells injected accounted for the more rapid development of B-1 progenitor ALL. This was further confirmed by comparing the kinetics of B-ALL development in recipients following injection of twice as many *BCR-ABL/GFP* transduced B-2 than B-1 progenitors. As shown in Figure 2B, recipients of 5×10^3 *BCR-ABL/GFP* transduced B-1 progenitors still developed disease more rapidly than recipients of 1×10^4 *BCR-ABL/GFP* transduced B-2 cells. This result indicates that, within the range of cell doses tested, the latency in B-ALL emergence was linked to the lineage and not the number of transduced cells transplanted.

B-1 progenitors from CRLF2$^{-/-}$ and IL-7Rα$^{-/-}$ mice can initiate B-ALL

B-1 progenitors proliferate vigorously in response to IL-7 (Table I) and are distinguished from B-2 progenitors by their TSLP responsiveness (6). To determine whether TSLP and IL-7 responsiveness were necessary for the development of B-1 progenitor ALL, we tested whether B-1 progenitors isolated from *CRLF2$^{-/-}$* mice, which do not express the TSLP receptor, and *IL-7Rα$^{-/-}$* mice can initiate disease following transduction with *BCR-ABL*. B-1 progenitors were easily detected in *CRLF2$^{-/-}$* mice, indicating that signaling through the TSLP receptor is not required for their formation and/or maintenance (Fig. 3A). B-1 progenitors are also present in *IL-7Rα$^{-/-}$* mice, albeit in reduced numbers, indicating that IL-7 signaling is required for their emergence in normal numbers (Fig. 3B).

Because *CRLF2$^{-/-}$* and *IL-7Rα$^{-/-}$* mice are on a B6 background, we first established that B-ALL also developed in that strain. Recipients of 1×10^4 *BCR-ABL* transduced B6 B-1 progenitors efficiently initiated leukemia, and all mice had succumbed by day 45 thereafter (Fig. 3C). In contrast, the kinetics with which disease initiated in recipients of BCR-ABL transduced B6 B-2 progenitors was delayed. In fact, some mice had not developed leukemia by 100 days following receipt of transduced cells. This was the case even though the efficiency with which the two B6 progenitor populations were transduced was comparable

(Fig. 3D) and a higher number of transduced B-2 progenitors was injected into recipients (Fig. 3C).

We then assessed whether *BCR-ABL* transduced CRLF2$^{-/-}$ and *IL-7Rα*$^{-/-}$ B-1 progenitors could initiate disease and found that they could do so. In this case, the kinetics with which B6, *CRLF2*$^{-/-}$, and *IL-7Rα*$^{-/-}$ B-1 progenitors developed disease was similar (Fig. 3C). As with SW progenitors, recipients of the *BCR-ABL* transduced B6, *CRLF2*$^{-/-}$, and *IL-7Rα*$^{-/-}$ B-1 progenitors developed tumors in their bone marrow and spleen (data not shown) that were primarily CD45R(B220)$^+$ sIgM$^-$ (Fig. 3E).

Taken together, these data indicate that the initiation and progression of B-1 progenitor B-ALL is not dependent on signaling through the TSLP or IL-7 receptor. Because deletion of *IL-7Ra* abrogates both IL-7 and TSLP signaling, these data further indicate that B-1 progenitors can initiate B-ALL even when both pathways are simultaneously disabled.

B-1 and B-2 progenitor tumors exhibit similar levels of proliferation

Even though the latency periods with which B-1 and B-2 progenitor ALL developed were distinct, once mice exhibited disease symptoms, their clinical course was indistinguishable and they rapidly succumbed within a week (data not shown). These observations suggested that the properties of established ALL tumor cells were similar, regardless of their B-1 or B-2 progenitor origin. We initially compared the expression of selected genes, which included Jak1, Jak2, Stat1, Stat3, Stat5a, Stat5b, Akt1, Mcl1, and *Grb2*, in the BCR-ABL signaling pathway in tumor cells. Mice received *BCR-ABL* transduced B-1 and/or B-2 progenitors from B6, *CRLF2*$^{-/-}$, and *IL-7Rα*$^{-/-}$ donors, and GFP$^+$ tumor cells were harvested from animals when they became moribund. These genes were generally expressed at similar levels in the tumors regardless of lineage or strain of origin (Fig. 4A).

Activation of JAK/STAT signaling is commonly observed in BCR-ABL transformed cells (23-25), so we next compared the activation of this pathway in B-1 and B-2 progenitor tumors. We isolated BCR-ABL expressing (ie., GFP$^+$) tumor cells from the spleen of mice that had received an injection of transduced B-1 and B-2 progenitors and examined Stat5 levels by phospho-flow analysis. As shown in Fig. 4B, levels were indistinguishable between the GFP-expressing tumor cells isolated from wild type, *CRLF2*$^{-/-}$ and *IL-7Rα*$^{-/-}$ mice. We also observed that the various tumor cells exhibited similar levels of proliferation as measured by Ki-67 (Fig. 4C).

The initial response of B-1 and B-2 progenitors to oncogene expression is distinct

The above results indicate that established B-1 and B-2 progenitor tumors are similar in terms of phenotype, gene expression, Stat5 activation, and proliferation. These observations suggest that events leading up to tumor formation, rather than the properties of ALL tumor cells, underlie the rapid development of B-1 progenitor ALL. In order to test this hypothesis, we assessed how the two progenitor populations responded to oncogene expression by transducing them with *BCR-ABL,* placing them in culture under B lymphopoietic conditions, and comparing their growth and survival sixty-four hours later.

The number of cells present in cultures seeded with *BCR-ABL/GFP* or *GFP*-only transduced B-1 progenitors was higher than in those initiated with similarly transduced B-2 progenitors (Fig. 5A). This result likely reflects the higher proliferative potential of B-1 progenitors compared to B-2 progenitors, irrespective of transduction (Table I). In addition, the distinct response of B-1 and B-2 progenitors to oncogene expression also affects cell recovery in the cultures. We used AnnexinV labeling to assess apoptosis in B-1 and B-2 progenitors. While BCR-expressing B lineage cells that bind AnnexinV at low/intermediate levels may not be apoptotic (26), some cells that bind high levels of AnnexinV (AnnexinV^{+high}) are undergoing cell death (27). As we show, very few B-1 and B-2 progenitors bind AnnexinV, and those that do are AnnexinV^{+high} (Supplemental Figure 4). Together, these observations indicate that AnnexinV^{+high} binding can be used as a marker for apoptosis in these B cell progenitors.

While transduction with the *GFP*-only vector induced cell death in both progenitor populations, the frequency and number of GFP$^+$ AnnexinV^{+high} and/or PI$^+$ apoptotic cells were highest in cultures established with *BCR-ABL* transduced B-2 progenitors (Figs. 5B and C). That cell death occurred following BCR-ABL expression is not unexpected, since this event is known to trigger an apoptotic response in B cell progenitors (28). However, the higher number of apoptotic cells in the cultures initiated with *BCR-ABL* transduced B-2 progenitors suggests that they are more sensitive to oncogene expression.

In order to define a genetic basis for the latter conclusion, we examined expression of the *p19^{Arf}* tumor suppressor gene in non-transduced, *GFP*-only transduced, and *BCR-ABL/GFP* transduced B-1 and B-2 progenitors. The expression of p19^{Arf} in B cell progenitors is known to trigger apoptosis through induction of p53 (28). *p19^{Arf}* was expressed in un-manipulated B-1 progenitors and its levels did not significantly change following transduction with *GFP* or *BCR-ABL*. In contrast, *p19^{Arf}* was not detected in un-manipulated B-2 progenitors, but expression was induced following BCR-ABL expression. This induction was not due to the transduction process alone, as *p19^{Arf}* levels were higher in the oncogene transduced compared to the GFP-only transduced cells (Fig. 5D). These differences in the response of B-1 and B-2 progenitors to BCR-ABL expression are particularly well illustrated when the relative level at which *p19^{Arf}* is induced is compared (Fig. 5E).

Discussion

The data in this manuscript demonstrate that B-1 progenitors can be B-ALL cells of origin and that, compared to B-2 progenitors, they initiate an aggressive form of the disease characterized by rapid onset and high tumor burden. These results provide a new conceptual framework for viewing the origins of infant and childhood B-ALL. While most focus has been placed on the specific chromosomal translocation and/or secondary mutations as key events in leukemia initiation and progression, our data indicate that B cell lineage may also be a significant factor. Specifically, our results indicate that intrinsic differences between B-1 and B-2 progenitors exist and influence their response to *BCR-ABL* expression. These results reinforce the concept that the cellular landscape in which oncogene expression occurs is an important determinant for ALL development. In this regard, we recently demonstrated that the potential of *BCR-ABL* to transform young and old pro-B cells was distinctly

different because of differences in patterns of gene expression between these progenitors (29).

A key finding of this study was that the kinetics with which B-1 and B-2 progenitor ALL developed was significantly different. This result was not related to the number of transduced cells introduced into the recipients, since B-1 and B-2 progenitors were transduced with similar efficiency. We considered the possibility that TSLP responsiveness was responsible for the aggressive nature of B-1 progenitor ALL, because B-1, but not B-2, progenitors are TSLP responsive (6, 9) and *CRLF2* expression has been associated with poor ALL outcome (17, 30, 31). Our data showing that *CRLF2* deficient B-1 progenitors still initiated rapid ALL suggest that TSLP responsiveness was not critical for the initiation of B-1 progenitor ALL. This was further confirmed by the observation that abrogation of *IL-7Rα* expression, which results in disruption of TSLP and IL-7 signaling, did not affect the initiation of B-1 progenitor ALL.

Examination of established B-1 and B-2 progenitor tumors did not provide insights into why B-1 progenitor ALL developed so rapidly. Tumor cells, regardless of whether they were derived from wild type, *CRLF2*$^{-/-}$, and/or *IL-7Rα*$^{-/-}$ mice, were homogeneous in terms of phenotype, gene expression, Stat5 activation, and proliferation. These results led us to consider that the initial response of B-1 and B-2 progenitors to oncogene expression might be distinct, and this was what was observed. The number of cells recovered from cultures initiated with *BCR-ABL* transduced B-1 progenitors was higher than in cultures initiated with *BCR-ABL* transduced B-2 progenitors. One reason for this is that the oncogene expressing B-1 progenitors exhibited lower levels of apoptosis compared to B-2 progenitors, which correlated with the differential response of these progenitors to $p19^{Arf}$ expression.

Un-manipulated B-1 progenitors appear to tolerate low levels of $p19^{Arf}$ and BCR-ABL expression did not induce it to higher levels. In contrast, un-manipulated B-2 progenitors did not express $p19^{Arf}$, but it was induced in these cells following oncogene expression. Based on these observations, we propose that the high levels of apoptosis in BCR-ABL expressing B-2 progenitors result from their relative sensitivity to $p19^{Arf}$. In addition, it is likely that the increased proliferative potential of B-1 progenitors, which distinguishes them from B-2 progenitors, further contributes to the increased number of cells harvested from the cultures initiated with the former cells. Together, these results highlight distinctions in how these two B cell progenitor populations respond to oncogene expression.

These observations allow us to propose a model for what occurs when *BCR-ABL* is expressed in B-1 progenitors *in vivo*. Because of their high proliferative potential and relative resistance to oncogene-induced death, a significant cohort of oncogene expressing B-1 progenitors poised to generate ALL would build in the individual. These cells may in turn be exposed to microenvironmental factors that further promote their survival and/or expansion. For example, even though IL-7 and TSLP responsiveness are not required for the development of B-1 progenitor ALL, the differential responsiveness of B-1 progenitors to these cytokines could still be a factor in the proliferation and/or survival of BCR-ABL expressing cells. The presence of a high number of BCR-ABL expressing B-1 progenitors may in turn increase the probability that these cells cross the threshold from *BCR-ABL*

expression to ALL initiation. In contrast, the lower resistance B-2 progenitors to oncogenic stress would result in a smaller pool of oncogene expressing cells in the individual. In some cases, all oncogene expressing B-2 progenitors may be eliminated, which would explain why some recipients of *BCR-ABL* transduced B-2 progenitors never developed leukemia. We cannot exclude the additional possibility that oncogene expressing B-1 progenitors are more prone to acquire key secondary mutations that further drive disease (17), but more extensive genetic profiling will be necessary to assess this possibility.

The results presented herein, which provide a developmental view of the origins of B-ALL, suggest that some aggressive forms of the disease may be malignancies of B-1 progenitors. This view allows clinical observations to be viewed from a new perspective. For example, one study stratified pediatric with Ph[+] B-ALL patients into three groups based on leukocyte counts at the time of diagnosis and response to chemotherapy. The estimates of five-year disease free survival for these patients ranged from 49% for those with the lowest leukocyte counts to only 20% for those with the highest number (15). It is tempting to speculate based on the present data that B-1 progenitors were transformed in the patients with the highest lymphocyte counts. B-1 B cells have been described in non-human primates (32) and humans (33). It will be interesting, once human B-1 progenitors are resolved, to determine whether their response to oncogene expression differs from that of B-2 progenitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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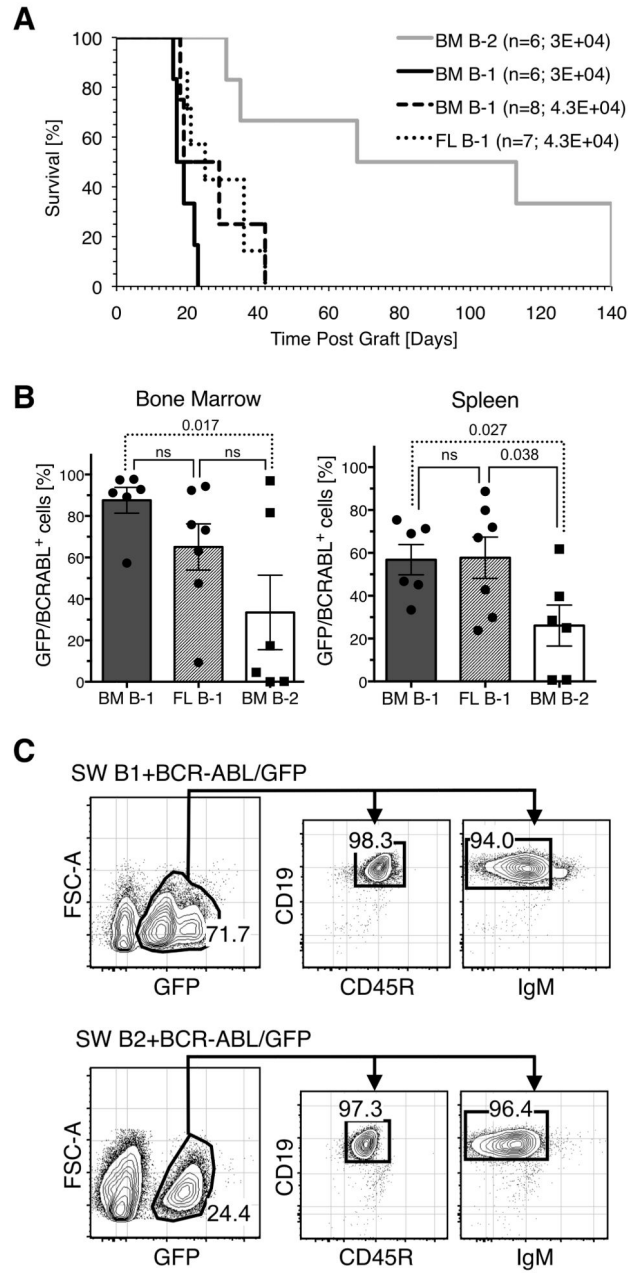
FIGURE 1.

BCR-ABL transduced B-1 progenitors induce rapid and severe B-ALL. (**A**) Survival curves for recipients of *BCR-ABL* transduced B-1 progenitors from fetal liver or young adult bone marrow and B-2 progenitors from young adult bone marrow. Cells were harvested from SW mice (n = number of recipients). Numbers of transduced cells injected per mouse are indicated in parentheses. The solid line indicates that mice received 30,000 B-1 progenitors and the dashed line indicates that mice received 43,000 progenitors. Log-rank (Mantel-Cox) test results for differences in survival between groups were as follows: bone marrow B-2 (3E+04) versus bone marrow B-1 (3E+04), p = 0.002; bone marrow B-2 (3E+04) versus bone marrow B-1 (4.3E+04), p = 0.022; bone marrow B-2 (3E+04) versus fetal liver B-1

(3E+04), p = 0.07. Recipients of B-1 and B-2 progenitors transduced with *GFP*-only containing retroviruses never developed disease (data not shown). (**B**) Tumor burden in the bone marrow and spleen of recipients of *BCR-ABL* transduced B-1 and B-2 progenitors purified from the bone marrow (BM) and/or fetal liver (FL) of SW mice. Means ± SEM and p values are shown. Each symbol represents an individual mouse. ns = not significant. (**C**) Representative FACS plots showing the phenotype of tumor cells derived from *BCR-ABL* transduced B-1 and B-2 progenitors. BCR-ABL expressing cells, identified by their expression of GFP, were examined by FACS for expression of CD19, CD45R(B220), and sIgM.
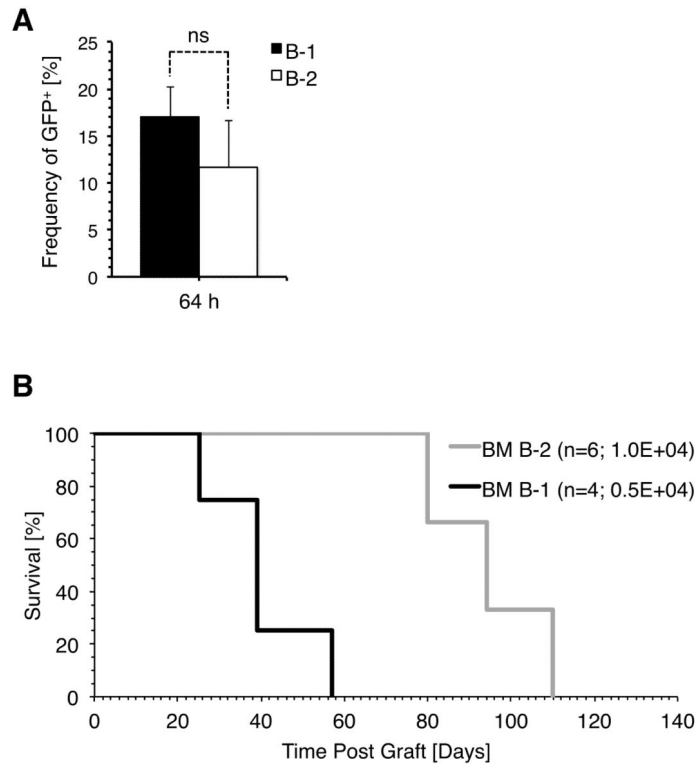
FIGURE 2.
B-1 and B-2 progenitors are transduced with comparable efficiency. (**A**) Frequency of GFP[+]
cells in aliquots of the *BCR-ABL* transduced cells used for the *in vivo* injections following 64
hours in culture. ns = not significant. (**B**) Survival curves for recipients of 5,000 *BCR-ABL*
transduced B-1 progenitors and 10,000 BCR-ABL transduced B-2 progenitors (n = number
of recipients). Log-rank (Mantel-Cox) test results for differences in survival between groups
for bone marrow B-2 versus bone marrow B-1, p = 0.0005.
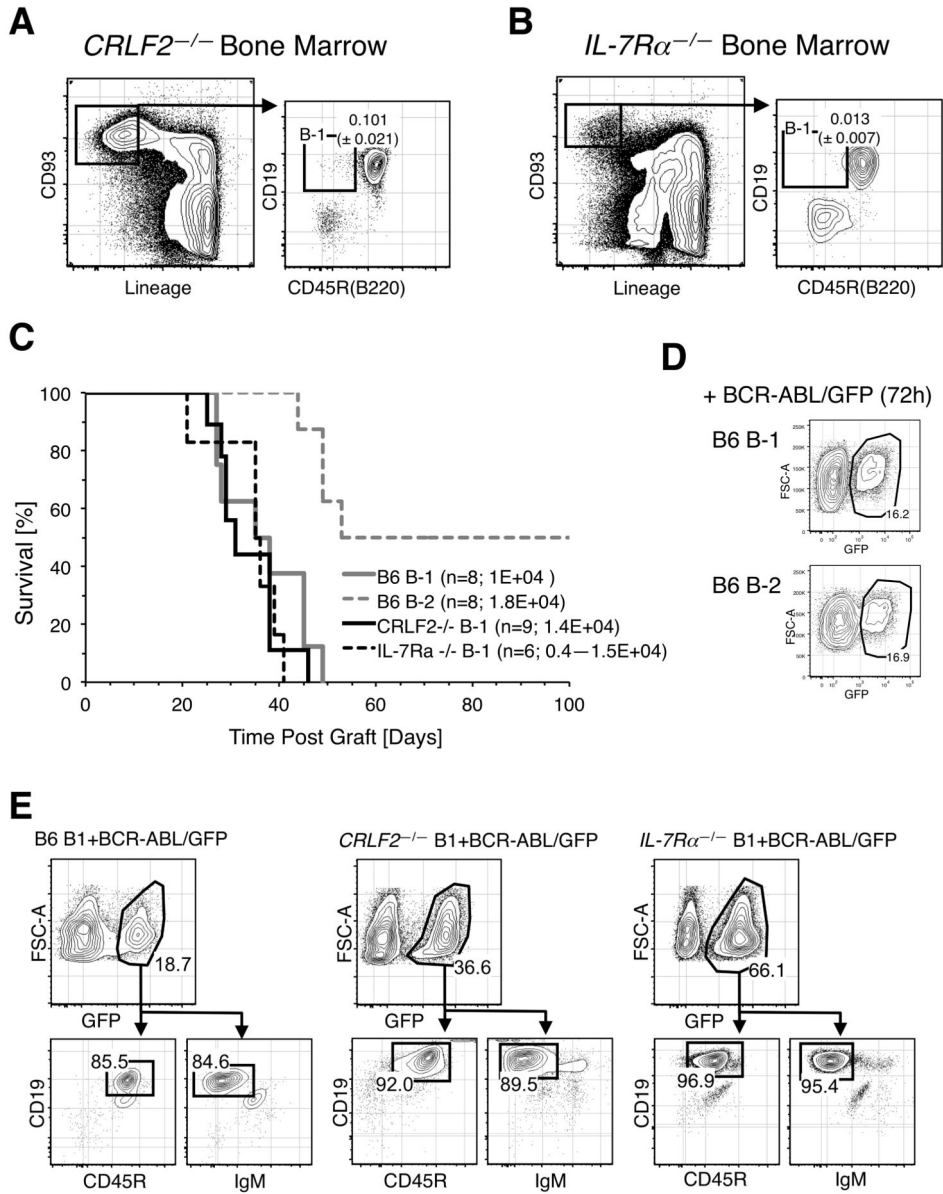
FIGURE 3.
Deletion of *CRLF2*$^{-/-}$ and *IL-7Rα*$^{-/-}$ does not affect the development of B-ALL from *BCR-ABL* transduced B-1 progenitors. (**A**) Purification strategy used to isolate B-1 and B-2 progenitors from the bone marrow of *CRLF2*$^{-/-}$ mice. The mean ± standard deviation frequency (sd) of B-1 progenitors is indicated. (**B**) Purification strategy used to isolate B-1 progenitors from the bone marrow of *IL-7Rα*$^{-/-}$ mice. The mean ± sd frequency of B-1 progenitors is indicated. (**C**) Survival curves for recipients of *BCR-ABL* transduced B6, *CRLF2*$^{-/-}$ and *IL-7Rα*$^{-/-}$ B-1 and B-2 progenitors (n = number of recipients). The number of transduced cells injected per mouse is indicated in parentheses. Due to the difficulty in isolating enough *CRLF2*$^{-/-}$ and *IL-7Rα*$^{-/-}$ B-2 progenitors, these populations were not tested in these experiments. (**D**) FACS plots showing the frequency of GFP$^+$ cells in aliquots of the *BCR-ABL* transduced B-1 and B-2 progenitors following 72 hours in

culture. The frequencies of GFP$^+$ cells are indicated. (**E**) Representative FACS plots showing the phenotype of tumor cells derived from *BCR-ABL* transduced *CRLF2*$^{-/-}$ and *IL-7Rα*$^{-/-}$ B-1 progenitors. GFP$^+$ cells were examined by FACS for expression of CD19, CD45R(B220), and sIgM.
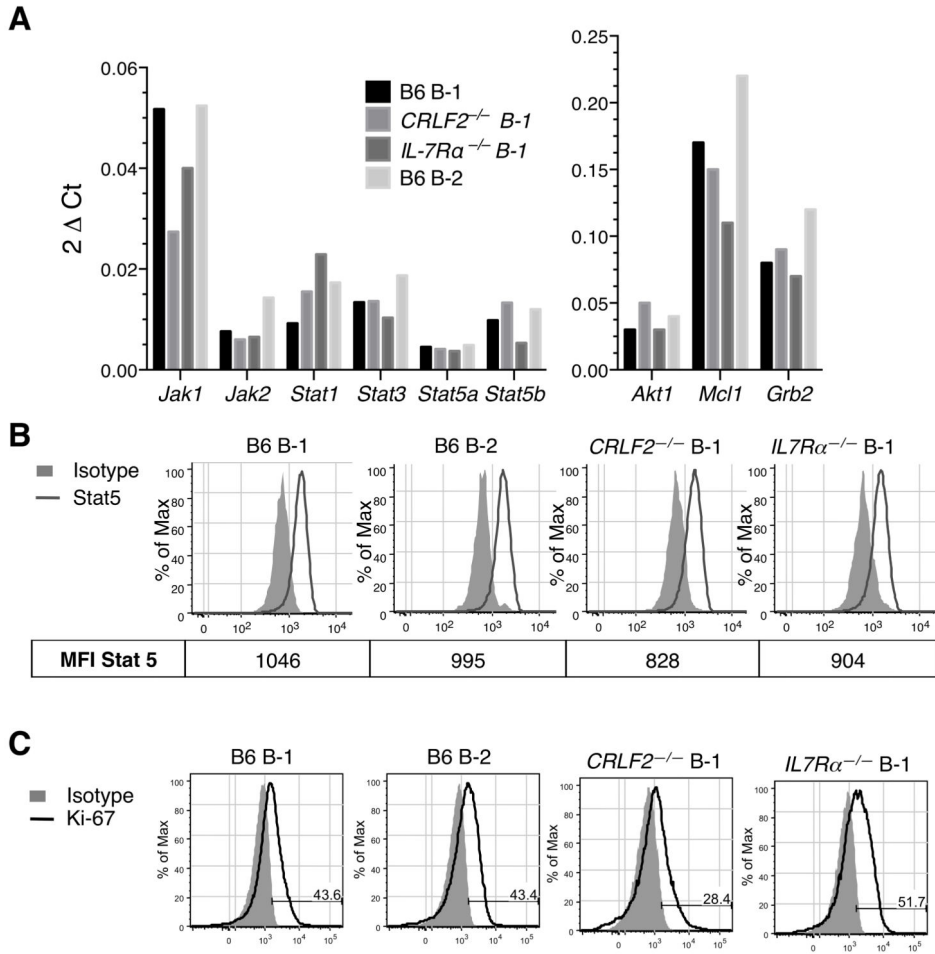
FIGURE 4.
B-1 and B-2 progenitor tumors exhibit similar properties. (**A**) Relative expression of genes involved in BCR-ABL signaling in tumor cells isolated from the spleen of mice that received *BCR-ABL* transduced progenitors from B6, *IL-7Rα*$^{-/-}$, and *CRLF2*$^{-/-}$ mice. 2 Ct values were calculated using the Pfaffl method with the Ct means of *Actb* and *Hsp90ab1* housekeeping genes as reference for the indicated genes. All reactions were run in duplicate. (**B**) Stat5 activation status in B6, *CRFL2*$^{-/-}$ and *IL-7Rα*$^{-/-}$ B-1 and B-2 tumor cells as determined by phospho-flow analysis. (**C**) Frequency of Ki-67$^+$ expression in the B6, *CRFL2*$^{-/-}$ and *IL-7Rα*$^{-/-}$ B-1 and B6 B-2 derived ALL tumor cells described in (A).
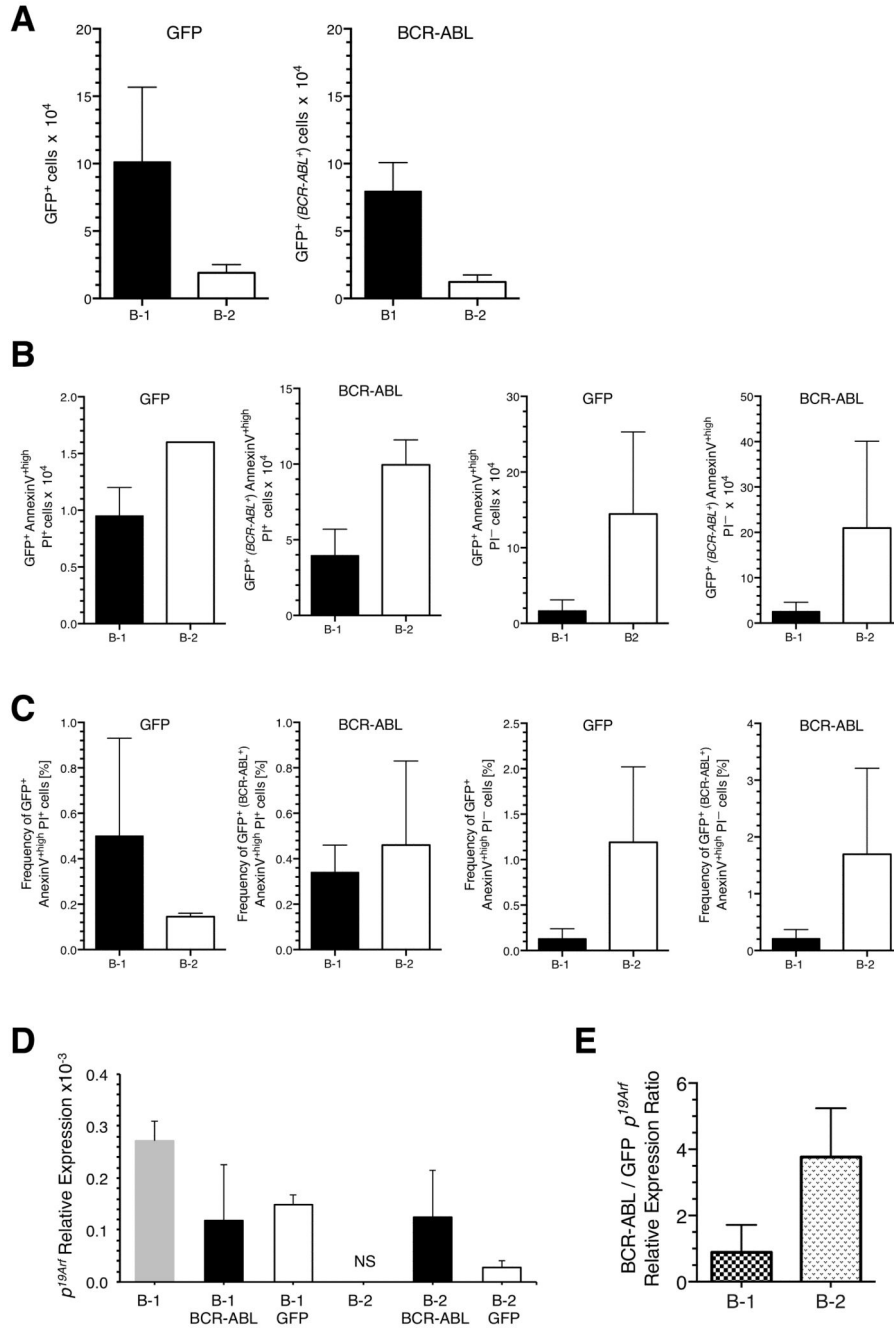
FIGURE 5.

BCR-ABL transduced B-1 progenitors exhibit enhanced survival compared to B-2 progenitors. (**A**) Number of GFP+ cells recovered from cultures established with *GFP*-only (GFP) and *BCR-ABL/GFP* (BCR-ABL) transduced B-1 and B-2 progenitors. Cells were isolated from SW bone marrow, transduced with *BCR-ABL-GFP* or *GFP*-only vectors and analyzed following 64 hours in culture. Results show the mean ± SEM of 4 independent experiments. (**B**) Total number and (**C**) frequency of GFP+ AnnexinV+high propidium iodide (PI)+ and GFP+ AnnexinV+high PI— cells in cultures established with *GFP*-only (GFP) and

BCR-ABL/GFP (BCR-ABL) transduced B-1 and B-2 progenitors isolated from SW bone marrow following 64 hours in culture. Results show the mean ± SEM of 2 independent experiments. (**D**) Relative expression of *p19^{Arf}* in non-transduced, *BCR-ABL/GFP*-transduced, and *GFP*-only transduced B-1 and B-2 progenitors. (**E**) Relative levels of *p19^{Arf}* expression in B-1 and B-2 progenitors 72 hours following transduction. Data represent the ratio of $2^{-\ Ct}$ *p19^{Arf}* values in *BCR-ABL* transduced cells divided by the $2^{-\ Ct}$ *p19^{Arf}* values in *GFP*-only transduced cells for each progenitor population. $2^{-\ Ct}$ were calculated using the Pfafl method using *Gapdh* as a reference gene. The data are based on two independent experiments.

Table I

B-1 cells from fetal liver and bone marrow proliferate extensively in the presence of IL-7[a]

Origin	Cells	Cells seeded $\times 10^5$	IL-7	Cell Recovery $\times 10^5$	Fold Expansion
E15 FL	B-1	0.34	+	70.00	205.0
			−	0.48	1.4
	B-2	0.34	+	7.6	2 2.
			−	0.3	0.9
Adult BM	B-1	0.06	+	10.0	166.6
			−	0.0	0.0
	B-2	0.06	+	0.4	6.7
			−	0.0	0.0

[a]Cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, 1 mM L-glutamine, 100 U/ml streptomycin, 100 μg/ml penicillin, 50 μM β-mercaptoethanol, 50 μg/ml gentamycin, Stem Cell Factor (20 ng/ml), IL-3 (20 ng/ml), Flt-3L (10 ng/ml) with or without IL-7 (30 ng/ml) for 7 days at 37°C in a 5% CO_2/air humidified incubator, at which time the number of live cells were counted by eosin dye exclusion.