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*J Immunol* 2014; 192:5171-5178; Prepublished online 21 April 2014;
doi: 10.4049/jimmunol.1303170
http://www.jimmunol.org/content/192/11/5171

Supplementary Material

http://www.jimmunol.org/content/suppl/2014/04/21/jimmunol.1303170.DCSupplemental

References

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The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Murine B-1 B Cell Progenitors Initiate B-Acute Lymphoblastic Leukemia with Features of High-Risk Disease

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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 whereas 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. In the present study, we 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 do 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 we 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.

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). Although 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 chromosome, are classified as high risk based on elevated leukocyte counts at diagnosis, CNS 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 do 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 underlie the accelerated kinetics with which they initiate leukemia. Taken 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, and RAG-2/SJL (RAG, B6.SJL(129S6)-Ptprca/PtprcaBoCrTac-Rag2tm1Ikr/J) mice were purchased from Taconic Farms (Germantown, NY). TSLP receptor–deficient (CRLF2−/−) mice were a gift of Dr. James Ihle at St. Jude Children’s Research Hospital (21). C57BL/6 (B6) and IL-7Ra−/− (B6.129S7-Il7rtm1Imx/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- to 6-week-old SW, B6, and CRLF2−/− mice and 1.5- to 3-week-old IL-7Ra−/− 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 Ca²⁺/Mg²⁺-free PBS. Bone marrow cell suspensions were prepared by flushing bones with Ca²⁺/Mg²⁺-free PBS. When necessary, RBCs were lysed with ACK buffer. All samples were incubated with anti-CD16/CD32 (FcγRIII; clone 2.4G2) to block nonspecific labeling. Optimum working dilutions for the following Abs were determined before use: CD19 (clone ID3), CD45R (B220), clone RA3-6B2, CD93 (A41-1, clone C34μg), and CD33 (clone s7). Mature lineage (Lin) cells were detected with an Ab mixture that included goat anti-mouse IgM, anti-CD3ε (clone 145-2C11), CD8α (clone 53-6.7), TCRβ (clone H57-597), TCRγδ (clone U7-13D5), 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 SouthernBioTech (Birmingham, AL) and other Abs were purchased from eBioscience (San Diego, CA). Cells were stained for 30 min at 4°C, washed with Ca²⁺/Mg²⁺-free PBS, and analyzed on an LSR II (BD Biosciences). B-1 progenitors, defined as Lin⁻CD19⁻CD45R⁻ (B220)⁻/lowCD3⁻ and B-2 progenitors, defined as Lin⁻CD19⁻CD45R⁻ (B220)⁺/highCD3⁺, were isolated on a FACS Aria (BD Biosciences, San Jose, CA). In some experiments, GFP expression was used to sort transduced cells following culture or B-ALL tumors from the spleen of diseased recipients. The frequency of GFP⁺ cells from the spleen of diseased animals on a FACS Aria and tested for their levels of Ca²⁺, Mg²⁺-free PBS, and analyzed on an LSR II (BD Biosciences). Mature lineage (Lin) cells were detected with an Ab mixture that included goat anti-mouse IgM, anti-CD3ε (clone 145-2C11), CD8α (clone 53-6.7), TCRβ (clone H57-597), TCRγδ (clone U7-13D5), 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 SouthernBioTech (Birmingham, AL) and other Abs were purchased from eBioscience (San Diego, CA). Cells were stained for 30 min at 4°C, washed with Ca²⁺/Mg²⁺-free PBS, and analyzed on an LSR II (BD Biosciences). The frequency of GFP⁺ cells that expressed Stat5 was then determined on an LSR II.

Phospho-flow analysis
BCR-ABL–expressing (i.e., GFP⁺) B-ALL cells were isolated from the spleen of diseased animals on a FACS Aria and tested for their levels of activated Stat5α as follows. Cells were fixed with 0.5% methanol-free formaldehyde for 10 min at 4°C, washed with Ca²⁺/Mg²⁺-free PBS, and permeabilized with 70% ice-cold methanol in a dropwise manner and constant agitation. After washing, the cells were incubated for 30 min with anti-CD16/CD32 (FcγRIII, clone 2.4G2) as a blocking agent and Pacific Blue–labeled mouse anti-Stat5α (clone 45/Stat5pY699) or mouse IgG1 isotype control (clone MOPC-21) (all from BD Biosciences). The frequency of GFP⁺ cells that expressed Stat5α was then determined on an LSR II.

Ki-67 immunostaining
BCR-ABL–expressing (i.e., GFP⁺) B-ALL cells were isolated from the spleen of diseased animals on a FACS Aria, fixed and permeabilized with BD Cytofix/Cytopermeabilization solution, and washed with 1× BD Perm/Wash buffer as per the manufacturer’s instructions. The cells were then incubated for 30 min with anti-CD16/CD32 (FcγRIII, 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 min. The cells were then washed with 1× BD Perm/Wash buffer and analyzed on an LSR II (Becton Dickinson) located in the Broad Stem Cell Research Center at the University of California at Los Angeles.

Production of retroviral stocks
pMSCV40 retroviral vectors containing either a 5' LTR-driven p210 BCR-ABL internal ribosome entry site–enhanced GFP (EGFP; BCR-ABL/EGFP) or a 5' LTR-driven internal ribosome entry site EGFP (GFP only) were used to generate high-tier helper-free retrovirus supernatants following transfection of 293T cells. 293T cells were grown in 10-cm² tissue culture treated plates (Becton Dickinson) precoated with poly-l-lysine (Sigma-Aldrich) in IMDM (Mediatech, Manassas, VA) supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT), 1 mM l-glutamine, 100 U/ml streptomycin, and 100 μg/ml penicillin (complete IMDM; all from Life Technologies, St. Louis, MO). 293T transfections were performed by coprecipitating 15 μg retroviral vector with 15 μg eucaryotic packaging vector using the CalPhos mammalian transfection kit (BD Biosciences, San Jose, CA). Medium was replaced every 12 h for 3 d with complete IMDM. Viral stocks were prepared by pooling supernatants collected at 36 and 48 h after transfection. Viral titers were determined following infection of 3T3 cells with serial dilutions of the pooled virus supernatant and found to range between 2 × 10⁶ and 7 × 10⁷ virus particles/ml.

Quantitative PCR
Flash-frozen aliquots of purified cells were processed for gene expression by quantitative PCR. Total RNA was extracted with the RNAeasy Plus micro kit and used to synthesize cDNA with the RT² First Strand kit (both from Qiagen, Valencia, CA) as per the manufacturer’s instructions. Reactions were run in 25-μl volumes with SYBR Green quantitative PCR master mix (Bio-Rad) as recommended by the manufacturer. Amplification efficiencies were routinely found to be between 95 and 105%, and all reactions were run in duplicate. RT² primer sets for p19 Arf, whose sequences were 5’-GGTCCGTCGATTGTGCACATG-3’ (forward) and 5’-TCGAATTTGACCCGTGTAGTGAG-3’ (reverse), and Gapdh (catalog no. PPM02946A) were purchased from SABiosciences. Presence of single PCR products was confirmed by melt curve analysis. Data were analyzed with Bio-Rad iQ software using the Pfaffl method as a relative reference.

Gene expression in tumor cells harvested from mice was analyzed using a custom RT² PCR array designed by Qiagen (Germantown, MD; no. CAPM11051) as per the manufacturer’s instructions. Expression of the following genes was examined: JAK1, JAK2, STAT1, STAT3, STAT5a, STAT5b, AKT1, MCL1, and GRIK2. The 2⁻ΔΔCt 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 a reference. Quality control for the custom array quantitative PCR reactions and gene expression analyses were performed on the Qiagen PCR array data analysis Web portal (http://pcrdatasanalysis.sabiosciences.com/pcr/arrayanalysis.php?target= upload).

Statistical analysis
Data are expressed as means ± SEM. Statistical significance for the differences between groups was determined by a two-tailed, unpaired Student 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⁻/CD45Rlow⁻CD19⁻ (6) and Lin⁻CD45R⁻CD93⁻/CD19⁻ (7) phenotypes, respectively (Supplemental Fig. 1), and they 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 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 h in vitro with retroviral vectors containing BCR-ABL/GFP or GFP only. Cells were then harvested, washed, and transplanted i.v. 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 d later, and by 40 d 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 d (Fig. 1A).

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

The above results show that recipients of $3 \times 10^4$ B-1 progenitors developed disease more rapidly than did 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 h following transduction when cells were injected into animals. We thus cultured cell aliquots for 64 h and then measured GFP expression in the transduced B-1 and B-2

**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 (FL) or young adult bone marrow (BM) and B-2 progenitors from young adult bone marrow. Cells were harvested from SW mice (n indicates number of recipients). Numbers of transduced cells injected per mouse are indicated in parentheses. The solid line indicates that mice received 30,000 (3E+04) B-1 progenitors and the dashed line indicates that mice received 43,000 (4.3E+04) 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 BM and/or FL of SW mice. Means ± SEM and p values are shown. Each symbol represents an individual mouse. (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.
progenitors. As shown in Fig. 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 Fig. 2B, recipients of $5 \times 10^5$ BCR-ABL/GFP–transduced B-1 progenitors still developed disease more rapidly than did recipients of $1 \times 10^5$ 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-7R$\alpha^{-/-}$ 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-7R$\alpha^{-/-}$ 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-7R$\alpha^{-/-}$ mice, albeit in reduced numbers, indicating that IL-7 signaling is required for their emergence in normal numbers (Fig. 3B).

Because CRLF2$^{-/-}$ and IL-7R$\alpha^{-/-}$ mice are on a B6 background, we first established that B-ALL also developed in that strain. Recipients of $1 \times 10^5$ 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 were delayed. In fact, some mice had not developed leukemia by 100 d 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-7R$\alpha^{-/-}$ B-1 progenitors could initiate disease and found that they could do so. In this case, the kinetics with which B6, CRLF2$^{-/-}$, and IL-7R$\alpha^{-/-}$ B-1 progenitors developed disease were similar (Fig. 3C). As with SW progenitors, recipients of the BCR-ABL–transduced B6, CRLF2$^{-/-}$, and IL-7R$\alpha^{-/-}$ 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-7R$\alpha$ 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-7R$\alpha^{-/-}$ 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 (i.e., 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-7R$\alpha^{-/-}$ 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. 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 64 h 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 with B-2 progenitors, irrespective of transduction (Table I). Additionally, the distinct
response of B-1 and B-2 progenitors to oncogene expression also affects cell recovery in the cultures. We used annexin V labeling to assess apoptosis in B-1 and B-2 progenitors. Whereas BCR-expressing B lineage cells that bind annexin V at low/intermediate levels may not be apoptotic (26), some cells that bind high levels of annexin V (annexin V[^high]) are undergoing cell death (27). We found that very few B-1 and B-2 progenitors bind annexin V, and those that do are annexin V[^high] (Supplemental Fig. 4). Taken together, these observations indicate that annexin V[^high] binding can be used as a marker for apoptosis in these B cell progenitors.

Whereas transduction with the GFP-only vector induced cell death in both progenitor populations, the frequency and number of GFP[^+], annexin V[^+[^high] and/or PI[^+] apoptotic cells were highest in cultures established with BCR-ABL–transduced B-2 progenitors (Fig. 5B, 5C). That cell death occurred following BCR-ABL expression is not unexpected, because 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.

**Table I.** B-1 cells from fetal liver and bone marrow proliferate extensively in the presence of IL-7

<table>
<thead>
<tr>
<th>Origin</th>
<th>Cells Seeded × 10^5</th>
<th>IL-7</th>
<th>Cell Recovery × 10^3</th>
<th>Fold Expansion</th>
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<tbody>
<tr>
<td>Embryonic day 15 fetal liver</td>
<td>B-1 0.34</td>
<td>+</td>
<td>70.00</td>
<td>205.0</td>
</tr>
<tr>
<td></td>
<td>B-2 0.34</td>
<td>+</td>
<td>7.6</td>
<td>2.2</td>
</tr>
<tr>
<td>Adult bone marrow</td>
<td>B-1 0.06</td>
<td>+</td>
<td>10.0</td>
<td>166.6</td>
</tr>
<tr>
<td></td>
<td>B-2 0.06</td>
<td>+</td>
<td>0.4</td>
<td>6.7</td>
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Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 1 mM L-glutamine, 100 U/ml streptomycin, 100 μg/ml penicillin, 50 μM 2-ME, 50 μg/ml gentamicin, stem cell factor (20 ng/ml), IL-3 (20 ng/ml), and Flt-3L (10 ng/ml) with or without IL-7 (30 ng/ml) for 7 d at 37˚C in a 5% CO2/air-humidified incubator, at which time the numbers of live cells were counted by eosin dye exclusion.

**FIGURE 3.** Deletion of CRLF2[^+/^−] and IL-7R[^α]/[^−/^−] 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 means ± SD frequency of B-1 progenitors is indicated. (B) Purification strategy used to isolate B-1 progenitors from the bone marrow of IL-7R[^α]/[^−/^−] mice. The means ± SD frequency of B-1 progenitors is indicated. (C) Survival curves for recipients of BCR-ABL–transduced B6, CRLF2[^+/^−], and IL-7R[^α]/[^−/^−] B-1 and B-2 progenitors (n indicates number of recipients). The number of transduced cells injected per mouse is indicated in parentheses. Owing to the difficulty in isolating enough CRLF2[^+/^−] and IL-7R[^α]/[^−/^−] 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 h 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-7R[^α]/[^−/^−] B-1 progenitors. GFP[^+] cells were examined by FACS for expression of CD19, CD45R (B220), and sIgM.
In contrast, significantly change following transduction with expressed in unmanipulated B-1 progenitors and its levels did not only–transduced, and GFP progenitors. The expression of p19Arf in B cell progenitors is known to trigger apoptosis through induction of p53 (28).

To define a genetic basis for the latter hypothesis, we examined expression of the \( p19^{Arf} \) tumor suppressor gene in nontransduced, GFP only–transduced, and B-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 unmanipulated 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 unmanipulated 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 with 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 study demonstrate that B-1 progenitors can be B-ALL cells of origin and that, compared with 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. Whereas 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 were significantly different. This result was not related to the number of transduced cells introduced into the recipients, because 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-7Ra 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-7Ra–/– 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 with B-2 progenitors, which correlated with the differential response of these progenitors to \( p19^{Arf} \) expression.
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– and BCR-ABL/GFP–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 h in culture. Results show the means ± SEM of four independent experiments. (B) Total number and (C) frequency of GFP+annexin V+PI− and GFP+annexin V+PI− cells in cultures established with GFP only– and BCR-ABL/GFP–transduced B-1 and B-2 progenitors isolated from SW bone marrow following 64 h in culture. Results show the means ± SEM of two independent experiments. (D) Relative expression of p19Arf in nontransduced, BCR-ABL/GFP–transduced, and GFP only–transduced B-1 and B-2 progenitors. (E) Relative levels of p19Arf expression in B-1 and B-2 progenitors 72 h following transduction. Data represent the ratio of $2^{-\Delta\Delta C_t} p19Arf$ values in BCR-ABL/GFP–transduced cells divided by the $2^{-\Delta\Delta C_t} p19Arf$ values in GFP only–transduced cells for each progenitor population. The $2^{-\Delta\Delta C_t}$ values were calculated using the Pfaffl method using Gapdh as a reference gene. The data are based on two independent experiments.
Unmanipulated B-1 progenitors appear to tolerate low levels of p19Arf, and B-2 progenitors did not induce it to higher levels. In contrast, unmanipulated B-2 progenitors did not express p19Arf, 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 p19Arf. Additionally, 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. Taken 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 in this study, 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 Philadelphia chromosome B-ALL patients into three groups based on leukocyte counts at the time of diagnosis and response to chemotherapy. The estimates of 5-y 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 leukocyte counts. B-1 B cells have been described in nonhuman primates (32) and humans (33). It will be interesting, once nonhuman primates (32) and humans (33). It will be interesting, once

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References


Disclosures

The authors have no financial conflicts of interest.
Figure S1: Enrichment of B-1 and B-2 progenitors by Flow Cytometry. 
(A) Representative FACS plots showing the staining and gating strategy used to isolate B-1 and B-2 progenitors from 5 weeks old Swiss Webster Bone Marrow. Plots B and C are representative of the level of purity for B-1, defined as Lineage$^-\text{CD93}^+\text{CD19}^+\text{CD45R}^-$ (B), and B-2, defined as Lineage$^-\text{CD93}^+\text{CD19}^+\text{CD45R}^+\text{CD43}^+$ (C), progenitors following sorting on the FACSARia (Becton Dickinson) located in the Jonsson Cancer Center Flow Cytometry core at UCLA. Enrichment levels of 98% were routinely achieved. The lineage cocktail includes: anti-mouse IgM, anti-CD3ε, CD8α, TCRβ, TCRγδ, NK1.1, Ly-6C, CD11b, Ter-119 and Gr-1.
Figure S2: Developmental potential of GFP-only transduced B-1 and B-2 progenitors. (A) Representative FACS plots showing the frequency of GFP+ cells in the Bone Marrow (BM), Spleen (SPL) and Peritoneal Wash (PW) of RAG/SJL mice transplanted with 3x10⁴ GFP-only transduced SW B-1 progenitors 22 days post-transplant. As expected transduced B-1 cells reconstituted mostly B-1 cells in the PW while no B lineage cells were present in the BM and limited B cells were found in the SPL. One of 3 mice is shown. (B) Representative FACS plots showing the frequency of GFP+ cells in the BM, SPL and PW of RAG/SJL mice transplanted with 3x10⁴ GFP-only transduced SW B-2 progenitors 75 days post-transplant. As expected no GFP+ cells were present in any of the transplanted animals due to the fact that B-2 progenitors usually reconstitute a transient wave of B cell development undetectable at the time tested. One of 3 mice is shown. For comparison purpose, mice were processed at the earliest time their counterparts transplanted with BCR-ABL/GFP transduced B-1 or B-2 progenitors showed signs of B-ALL.
Figure S3: Representative FACS plots showing the gating criteria for sIgM and CD19 expression on BM cells from normal B6 mice.
Figure S4. AnnexinV Does Not Label Immature B Cell Progenitors. B-1 and B-2 progenitors were resolved in the bone marrow of 4 week old B6 mice (n=3) as indicated in combination with 7-AAD and AnnexinV (PE AnnexinV Apoptosis kit, BD Pharmingen, San Diego, CA) according to the manufacturer’s instructions. Values on graph represent the mean ± (standard deviation) frequency of cells within the indicated gates. Because the levels of AnnexinV+high B-1 and B-2 progenitors were low, we used this technique to determine the frequency of apoptotic cells following transduction with BCR-ABL/GFP and GFP-only vectors in our study.