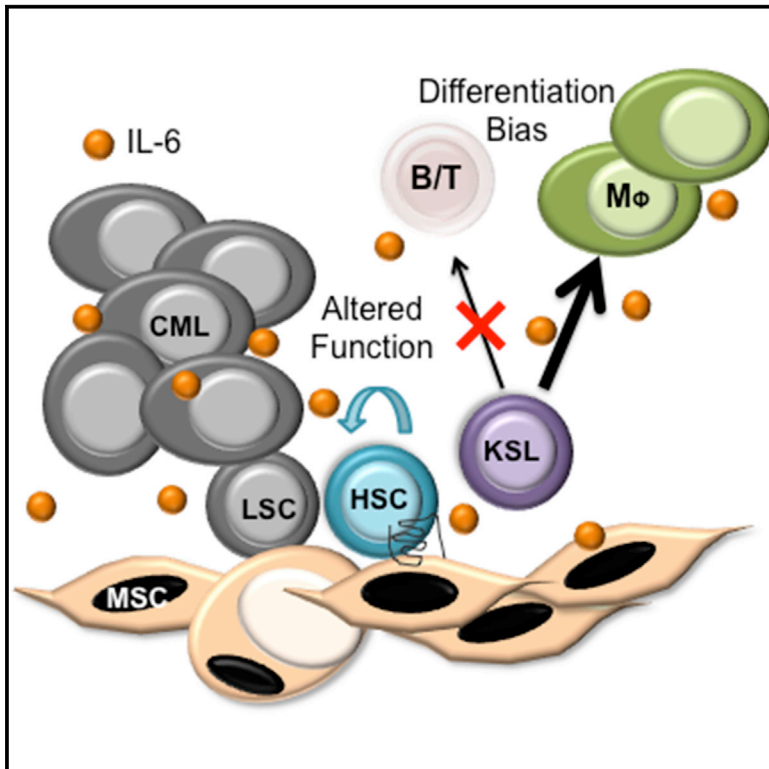


Cancer Cell

Treatment of Chronic Myelogenous Leukemia by Blocking Cytokine Alterations Found in Normal Stem and Progenitor Cells

Graphical Abstract



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In Brief

Welner et al. investigated whether chronic myelogenous leukemia (CML) cells alter normal hematopoietic stem/progenitor cells (HSPCs) in addition to outcompeting them. They show that CML cells promote proliferation, alter differentiation, and reduce self-renewal capacity of neighboring normal HSPCs via IL-6.

Highlights

- Leukemic cells alter neighboring non-transformed hematopoietic progenitor cells
- Imprinting of the leukemia on the normal progenitor cell counterparts mimics CML
- The pro-inflammatory cytokine IL-6 mediates these changes in normal hematopoiesis
- Treatment eliminates the leukemia upon targeted therapy against the IL-6 cytokine

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Treatment of Chronic Myelogenous Leukemia by Blocking Cytokine Alterations Found in Normal Stem and Progenitor Cells

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SUMMARY

Leukemic cells disrupt normal patterns of blood cell formation, but little is understood about the mechanism. We investigated whether leukemic cells alter functions of normal hematopoietic stem and progenitor cells. Exposure to chronic myelogenous leukemia (CML) caused normal mouse hematopoietic progenitor cells to divide more readily, altered their differentiation, and reduced their reconstitution and self-renewal potential. Interestingly, the normal bystander cells acquired gene expression patterns resembling their malignant counterparts. Therefore, much of the leukemia signature is mediated by extrinsic factors. Indeed, IL-6 was responsible for most of these changes. Compatible results were obtained when human CML were cultured with normal human hematopoietic progenitor cells. Furthermore, neutralization of IL-6 prevented these changes and treated the disease.

INTRODUCTION

Most hematopoietic stem cells (HSCs) reside in the bone marrow and self-renew as necessary to maintain their numbers (Mercier et al., 2012). Additionally, a fraction of HSCs develop into progenitor cells that become lineage restricted and that undergo extensive proliferation and differentiate to produce mature hematopoietic cells (Mayle et al., 2013; Venezia et al., 2004; Wilson et al., 2008). However, these normal processes are severely compromised with leukemia (Colmone et al., 2008; Hartwell et al., 2013; Hu et al., 2009; Krause et al., 2013; Schepers et al., 2013). While this could result from overcrowding by leukemic cells, it has been shown to occur with even low leukemic burden (Colmone et al., 2008). Considerable progress has been made in defining cells within marrow that support normal hematopoiesis

(Morrison and Scadden, 2014). Referred to as niches, these environments are thought to include multipotent stromal cells (MSCs), osteoblasts, and endothelial cells. Additionally, there is now evidence that leukemia alters their functions (Raaijmakers et al., 2010; Reynaud et al., 2011; Schepers et al., 2013; Zhang et al., 2012). However, consequences of those changes and the direct impact of the leukemic cells on stem and progenitor cells have not been adequately explored.

Myeloproliferative neoplasms are clonal disorders propagated by transformed HSCs. Chronic myelogenous leukemia (CML) is one such disorder, and it is characterized by a reciprocal translocation of the t(9;22)(q34;q11) loci. As a result, transformed cells express the BCR/ABL fusion protein (Ben-Neriah et al., 1986; Hooberman et al., 1989; Levine and Gilliland, 2008; Savona and Talpaz, 2008; Sawyers, 1999; Witte, 1988). This deregulated

Significance

It has generally been assumed that normal hematopoietic stem and progenitor cells (HSPCs) are simply out-competed for space by malignant cells. We have shown that the leukemic cells are able to modify the differentiation potential of non-transformed cells while promoting their own maturation. The leukemic environment imprints functional and transcriptional programs onto the non-transformed progenitors. However, by specifically blocking the IL-6 cytokine, we could inhibit the differentiation bias and eliminate the bulk of the disease. Additionally, these observations were also found in human CML cells, and leukemic-exposed human progenitors could be protected by anti-IL-6. These findings implicate a drug-targetable mechanism that could account for significant malignancy-related abnormalities in CML.

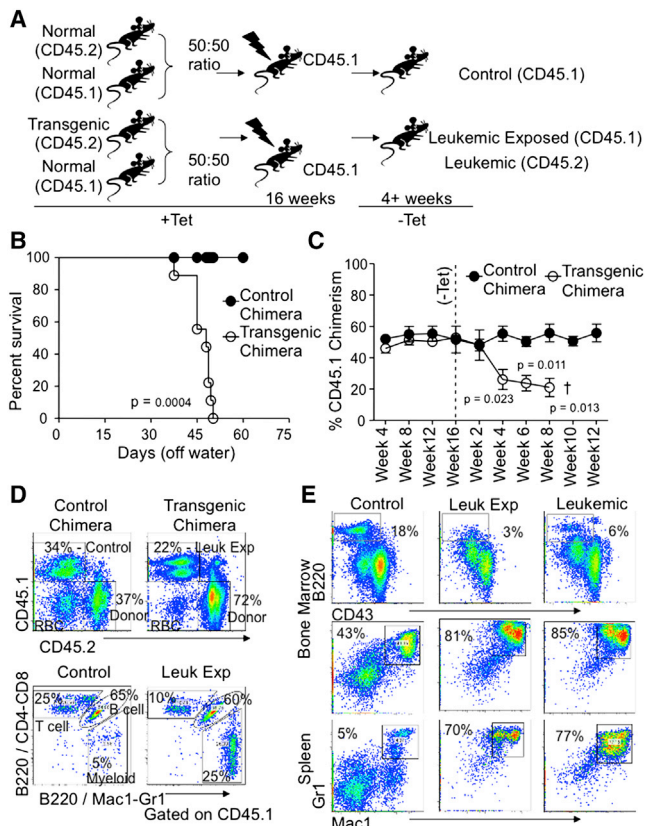


Figure 1. Experimental Design and Establishment of a Chimera Chronic Leukemia Model

(A) Overview of the experimental design for generation of CML chimeras, 2×10^6 whole bone marrow (WBM) cells from either C57BL/6 mice (CD45.2; Control) or Scl-tTA-BCR-ABL (CD45.2; Transgenic) mice mixed with 2×10^6 C57BL/6 cells congenic for the CD45 locus (CD45.1; Normal) and transplanted into mice on Tet. Chimerism was established for 16 weeks before Tet withdrawal and induction of BCR-ABL in those cells carrying the two transgenes. Tissue cells were obtained 4 weeks after induction, and CD45.1⁺ cells from control transplants, leukemic-exposed cells (CD45.1⁺ cells from leukemic mice), or leukemic cells (CD45.2⁺ transgenic cells from leukemic mice) were analyzed.

(B) Survival curves of control and transgenic chimeric mice after Tet withdrawal.

(C) The CD45.1 chimerism results are based on the staining of lymphoid (B220/CD3) and myeloid (Mac1/Gr1) cells in PB versus CD45.2 (control or leukemic) over time; $n = 4$ per time point. Error bars represent mean \pm SEM.

(D) Upper panel: representative FACS profiles from four experiments characterizing the PB of control or leukemic mice showing the chimerism of CD45.1 versus CD45.2 from control or transgenic chimeric mice 4 weeks after Tet removal. Lower panel: FACS profiles of CD45.1 cells in the PB of control or leukemic mice at 4 weeks after Tet removal. Cells were stained for B220/CD3 and B220/Mac1-Gr1. Cells that stain positive for both fluorochromes are B-lineage cells (B220⁺), while those that stain only for B220/CD4 and CD8 are T-lineage cells (CD4/8), and those cells that are positive for only B220/Mac1-Gr1 are of the myeloid lineage (Mac1-Gr1). Leuk Exp, leukemic exposed.

(E) Representative FACS profiles from the bone marrow gating for CD45.1⁺ B-lineage cells (B220⁺ CD43⁺) of either control or transgenic chimeric mice at 4 weeks after Tet withdrawal. Dot plots from leukemic cells (CD45.2) from the transgenic chimeric mice are shown for comparison (right). Similarly, bone marrow and spleens from control and transgenic chimeric mice were analyzed for the CD45.1⁺ myeloid-lineage cells (Mac1⁺ and Gr1⁺), and leukemic cells (CD45.2⁺) are shown for comparison, $n = 5$.

See also Figure S1.

tyrosine kinase promotes leukemic growth by disrupting signaling pathways involved in cell survival, proliferation, and differentiation. The chronic phase of CML presents with increased numbers of circulating progenitors, anemia, and splenomegaly (Petzer et al., 1996). At this time, leukemia-initiating cells (LICs) that can propagate disease are still present (Schemionek et al., 2010; Zhang et al., 2010) and retain the ability to make all blood cells, generating a vast expansion of malignant myeloid cells that displace normal hematopoiesis (Fialkow et al., 1977). In mice, these transformed progenitors are phenotypically similar to normal HSCs and are enriched within the Lin⁻ Sca1⁺ c-Kit^{Hi} fraction of bone marrow (KSL) (Holyoake et al., 1999; Hu et al., 2006; Maguer-Satta et al., 1996; Wang et al., 1998). Furthermore, processes that control normal HSC functions are also essential for LIC maintenance (Heidel et al., 2012; Lessard and Sauvageau, 2003; Reynaud et al., 2011; Warr et al., 2011; Zhao et al., 2007).

The chronic phase of CML cannot be efficiently modeled by transplantation of human cells into immunodeficient mice (Dazzi et al., 1998; Zhang et al., 2010). Therefore, our laboratory developed a BCR-ABL-inducible mouse model that results in expression of this oncogenic fusion under the control of a tetracycline (Tet)-regulated 3' enhancer of the murine stem cell leukemia (SCL) gene (Koschmieder et al., 2005; Schemionek et al., 2010). SCL-tTA \times BCR-ABL double transgenic mice develop a disease similar to the chronic-phase CML observed in patients. BCR-ABL expression following Tet withdrawal results in neutrophilic leukocytosis and splenomegaly. The chronic phase is characterized by progressive myeloid expansion, with accumulation of myeloid progenitors and mature granulocytes in the marrow and peripheral blood (PB) (Koschmieder et al., 2005; Schemionek et al., 2010). These CML cells are functionally heterogeneous and capable of maintaining a normal hierarchical differentiation process (Reynaud et al., 2011; Zhang et al., 2012).

This model made it possible to study normal hematopoietic cells while in close proximity to their leukemic counterparts by transplanting inducible leukemic transgenic marrow cells together with normal marrow cells and monitoring the effects of exposure of leukemic cells on normal HSPC function. It is important to note that the leukemic cells altered the properties of normal HSCs and progenitors, and preventing these environmental changes has important therapeutic implications for the disease.

RESULTS

Leukemic CML Cells Induce a Myeloproliferative Effect on Normal Hematopoietic Cells

Previous studies from our laboratory and others using this inducible model of CML identified leukemia-initiating activity in the stem/progenitor fraction of the marrow (Reynaud et al., 2011; Schemionek et al., 2010; Zhang et al., 2010). To explore the effects of leukemic cells on normal hematopoietic (leukemic-exposed) cells, C57BL/6-CD45.1 (normal) plus SCL-tTA \times BCR-ABL-CD45.2 (transgenic) whole marrow cells were transplanted into lethally irradiated C57BL/6-CD45.1 mice. As controls, 2×10^6 whole marrow cells from C57BL/6-CD45.1 (normal) plus the same number from C57BL/6-CD45.2 (normal) mice were transplanted (Figure 1A). Recipients were then kept on Tet water for 16 weeks to establish homeostasis within the chimeric mice. Upon removal of Tet, mice bearing transgenic

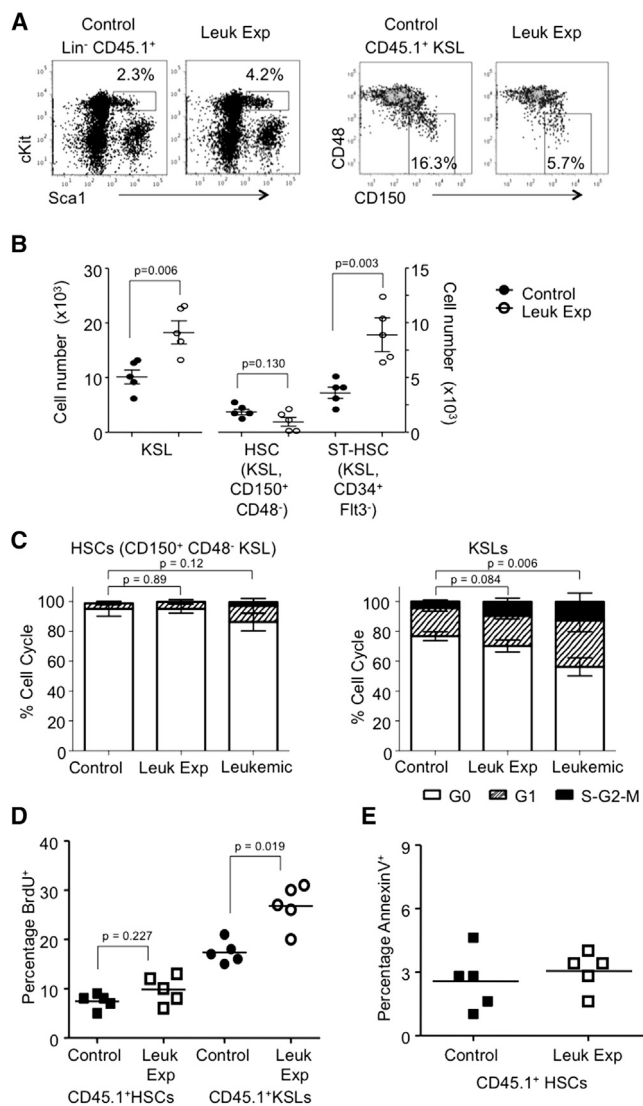


Figure 2. Normal Progenitor Profiles within the Leukemic Environment

(A) Flow cytometry profiles for control and leukemic-exposed (Leuk Exp) progenitors (CD45.1⁺) of Lin⁻ ckit^{hi} Sca1⁺ (KSL) and Lin⁻ ckit^{hi} Sca1⁺ CD150⁺ CD48⁻ (HSC), in either control or leukemic marrow at 4 weeks after Tet withdrawal, n = 5.

(B) Numbers of control and leukemic-exposed (CD45.1⁺) progenitors were calculated per femur for Lin⁻ ckit^{hi} Sca1⁺ (KSL), Lin⁻ ckit^{hi} Sca1⁺ CD150⁺ CD48⁻ (HSC), or Lin⁻ ckit^{hi} Sca1⁺ CD34⁺ Flt3⁻ (ST-HSC) in either control or leukemic marrow, n = 5. Error bars represent mean ± SEM.

(C) CD45.1⁺ HSCs (Lin⁻ ckit^{hi} Sca1⁺ CD150⁺ CD48⁻; HSC) or CD45.1⁺ KSLs (Lin⁻ ckit^{hi} Sca1⁺; progenitors) were sorted from either control or leukemic environments, and then cell cycle status was determined using Pylonin Y and Hoechst, n = 5. Error bars represent mean ± SD.

(D) BrdU incorporation of CD45.1⁺ HSCs (KSL CD150⁺ CD48⁻) from control or leukemic mice was quantified, and lines indicate the mean; n = 5.

(E) Annexin V staining of control or leukemic-exposed HSCs was used to determine the apoptosis within this subset of progenitors, and lines indicate the mean, n = 5.

See also Figure S2.

cells developed a disease similar to chronic-phase CML with severe myeloid cell expansion, coincident with reduction of lymphoid-lineage cells in the marrow, spleen, and PB. The disease progressed so that ~4 weeks after induction, elevated PB leukocyte counts were observed (Figure S1A), with increased cellularity in the spleen and decreased cellularity in the marrow. Chimeric mice with transgenic cells succumbed to the disease with the median survival time of 46 days after Tet withdrawal (Figure 1B). All mice transplanted with leukemic cells developed splenomegaly, compared to control transplanted mice (Figure S1B). Leukemic cells altered the chimerism of normal cells (CD45.1⁺, leukemic exposed) within these same mice from the time of induction as compared to the control transplants. Chimerism is shown as the percentage of CD45.1 cells out of the total CD45-positive cells (CD45.1⁺ and CD45.2⁺) (Figure 1C).

Flow cytometry analysis of the PB from chimeric mice showed increased numbers of donor (CD45.2⁺) cells in leukemic transplants, compared to those observed in the control mice. Similar to those of the leukemic population, the leukemic-exposed (CD45.1⁺ normal from the leukemic mice) cells also have increased myeloid lineages with losses in both B and T lineages in the PB (Figures 1D and S1C). To demonstrate that CD45.1 marked only normal cells lacking the two transgenes, populations of CD45.1⁺ and CD45.2⁺ cells were sorted from leukemic mice, and only the CD45.2⁺-sorted cells contained both transgenes by PCR analysis: SCL-tTA and BCR-ABL (Figure S1D). Increased percentage and absolute numbers of myeloid cells could also be observed in the normal cells of the bone marrow and spleens of the leukemic mice as compared to control mice, with a phenotypic representation similar to that of the leukemic cells (CD45.2⁺) (Figure 1E). Therefore, our chimeric mouse model replicates the disease found in primary transgenic mice. It is important that accumulation of myeloid-lineage cells at the expense of lymphoid cells was observed in the non-transformed CD45.1⁺ leukemic-exposed cells, similar to that of the leukemic cells expressing the BCR-ABL transgene.

Alterations in Normal Stem and Progenitor Cells during Leukemia

As an initial approach to understand the alterations described earlier in mature lineages, we performed detailed analyses of stem and progenitor cells in chimeric marrow. Mice were analyzed at 4 weeks after leukemic induction, and subsets of early progenitor populations were evaluated: Lin⁻ ckit^{hi} Sca1⁺ (KSLs, referred to as progenitors), long-term HSCs (HSCs: CD150⁺ CD48⁻ KSLs, or SLAMF⁺ KSLs, referred to as stem cells), and short-term HSCs (ST-HSCs: CD34⁺ Flt3⁻ KSLs; Wilson et al., 2008) (Figure 2A; Figure S2A). Absolute numbers of normal leukemic-exposed HSCs were not significantly altered in the bone marrow of mice with CML. Meanwhile, there were significant increases in the leukemic-exposed total KSL and granulocyte-monocyte progenitor (GMP), including specific increases in ST-HSCs and multipotent progenitors (MPP), as compared to controls (Figure 2B; Figure S2A). Therefore, the CML leukemic environment had an altered capacity to support normal progenitor cells, changing the distribution of these populations within 4 weeks of disease.

We then assessed the proliferation status and cell cycle of these leukemic-exposed stem and progenitor cells. However,

there were no observed changes in the HSCs in cycle. Meanwhile, CD45.1⁺ progenitors from the leukemic environment were induced to proliferate (Figure 2C; Figure S2B). It was previously demonstrated that leukemic BCR-ABL⁺ HSCs have a hyperproliferative phenotype (Schemionek et al., 2010; Zhang et al., 2010). However, the leukemic-exposed HSCs showed little evidence of proliferation as assessed by bromodeoxyuridine (BrdU) incorporation. Conversely, many leukemic-exposed KSLs were dividing (Figure 2D; Figure S2C). The increase of BrdU incorporation could be from DNA damage by the leukemic environment; however, there was no evidence of increased apoptosis in HSCs from the leukemic environment (Figure 2E; Figure S2D). Thus, the leukemic environment promoted proliferation of normal progenitors but not the HSCs from which they derive.

Leukemic Cells Compromise Functions of Normal Stem and Progenitor Cells

We wondered if co-habitation with leukemic cells caused intrinsic changes in otherwise normal stem and progenitor cells. Normal CD45.1⁺ KSLs were sorted from either control or leukemic mice and co-cultured on OP9 stromal cells to allow differentiation to the B lineage. It has been previously shown that, in addition to B cells, myeloid cells can also be produced inefficiently under these conditions (Mohtashami et al., 2010; Wang et al., 2005; Welner et al., 2008). The yield of B-lineage cells produced from normal KSLs was severely decreased by leukemic exposure with only modest increases in myeloid lineage cells in this assay, similar to that observed when using leukemic cells (Figures 3A and 3B).

Many methods have been used for HSC purification (Challen et al., 2009), but they are functionally defined through transplantation (Kiel et al., 2005; Spangrude et al., 1995; Szilvassy et al., 1990). To determine how the leukemic cells and their altered environment impact normal HSCs or KSLs (progenitors), we transplanted control or leukemic-exposed HSCs into irradiated CD45.2 congenic recipients (Figure 3C). At 12 hr after transplantation, no significant defects in homing were observed from the HSC-transplanted mice, similar to results obtained with homing of leukemic cells (Figure 3D). To test whether the observed increase in leukemic-exposed progenitors reflects differences in function, we performed competitive repopulation transplants with limiting numbers of control or leukemic-exposed HSCs. It is interesting that an approximate 4-fold reduction in stem cell activity results from exposure to leukemic cells (Figure 3E). Therefore, a more rigorous test of HSC function was then performed with serial transplantation assays. Since there were modest differences in HSC numbers between control and leukemic-exposed populations, we adjusted for these in the serial transplantations. Residence in a leukemic environment diminished repopulation capability in one round of transplantation, and this was magnified when the cells were re-transplanted to additional recipients (Figure 3F). From similarly transplanted mice, lymphoid (B/T) and myeloid reconstitution in the PB was determined 16⁺ weeks post-transplant. Leukemic-exposed HSCs showed no skewing to either myeloid or lymphoid lineages (Figure 3G, top). However, when recipient mice were transplanted with KSLs/progenitors and analyzed at 8–10 weeks post-transplant, residence in the leukemic environ-

ment increased their myeloid cell production at the expense of lymphoid lineages (Figure 3G, bottom). Therefore, leukemic exposure caused discrete alterations in hematopoietic progenitors. KSLs/progenitors had reduced competency for generation of lymphopoiesis, whereas numbers of more rigorously defined HSCs were mostly unaffected, and they could produce all blood lineage cells. However, HSCs were not unscathed, because their self-renewal and re-populating ability on transplant were compromised.

Normal Progenitors Resemble Transformed CML with Respect to Molecular Features

Bystander effects of residence within the leukemic environment were then explored with genome-wide gene profiling of purified control or leukemic-exposed HSCs and KSLs. The data were interrogated with several unbiased approaches, focusing on lineage-specific transcription factors that could account for changes in function and differentiation toward the myeloid lineage (Figure 4A). Gene set enrichment analysis (GSEA) was used to seek similarities between these normal progenitors and ones with a CML signature. Surprisingly, leukemic-exposed KSLs acquired a BCR-ABL⁺ signature of the leukemic cells with which they reside. In contrast, transcription patterns in leukemic-exposed HSCs were altered but not to the degree in which they resembled the leukemic signature (Figure 4B).

We wanted to further investigate the HSC microarray data to uncover why we see differences in self-renewal and engraftment upon transplantation. There were 1,468 differentially expressed genes in the leukemic-exposed HSCs versus control HSCs (Figure S3A). GSEA was used to determine the correlation of each dataset with known “stem-ness”-related genes. We observed bias in control HSCs relative to leukemic-exposed HSCs, further supporting their ability to repopulate mice upon transplantation (Figure S3B). Significant to this study were changes observed in signaling pathways potentially altered by the leukemic environment which might account for the changes in lineage specification specific to the KSLs/progenitors. Several pathways were shown to be dysregulated in the leukemic-exposed KSLs (Figure S3C) and might account for the changes we see in engraftment; these will be a focus of our future work. Unlike our findings with KSLs/progenitors, the leukemic-exposed HSCs showed no consistent lineage bias in myeloid or lymphoid lineage specific transcription factors (Figure S3D).

From our analysis of leukemic-exposed HSCs and KSLs/progenitors, we thought that the inflammatory environment might mediate many of the observed changes. From our model, we measured serum cytokine levels (Figure 4C). Consistent with data from others (Reynaud et al., 2011; Zhang et al., 2012), several inflammatory cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6, and IL-1 α , were significantly increased during leukemogenesis. These inflammatory cytokines provide one way that the leukemic environment might impose malignant cell properties on untransformed cells that reside there, as seen with the BCR-ABL GSEA earlier (Figure 4B). Additionally, GSEA showed that there was an inflammatory signature in the leukemic cells that was absent in the leukemic-exposed cells, suggesting that the leukemic cells are the source of these cytokines (Figure S3E) (Kleppe et al., 2015). From the current literature and known functions of each

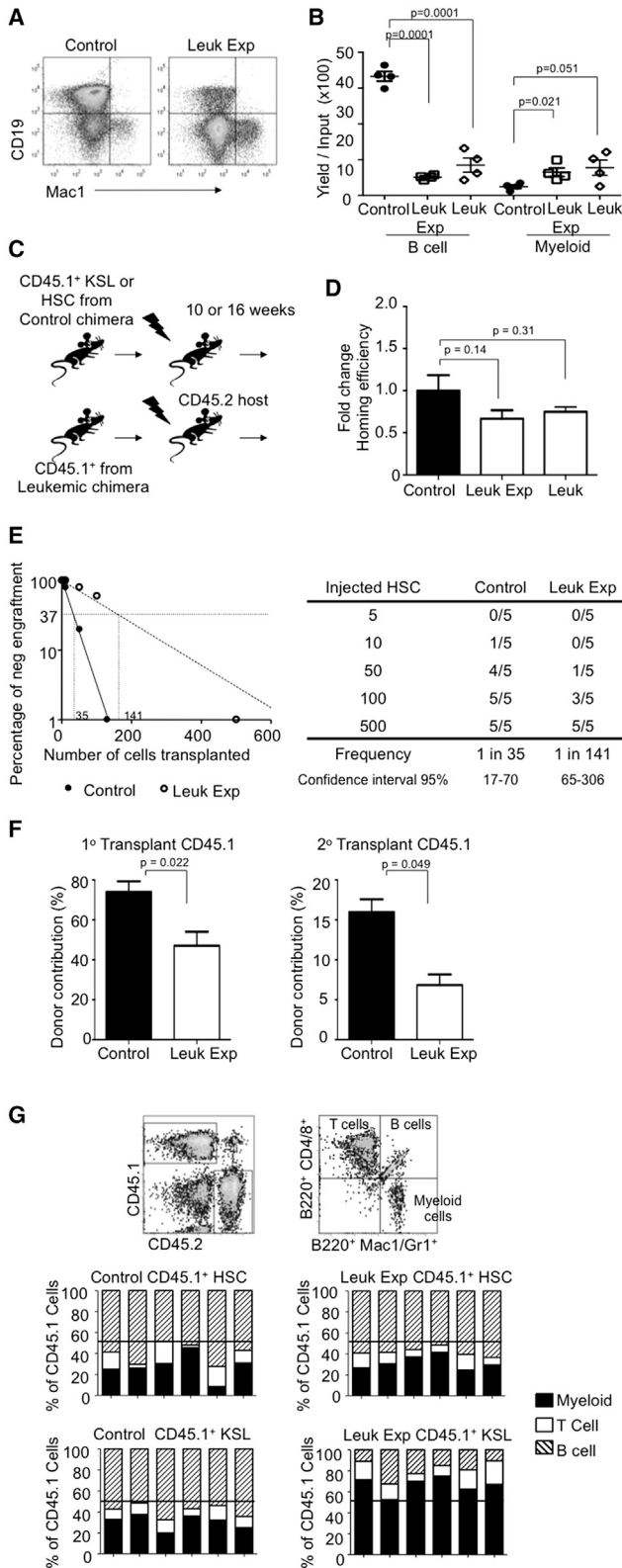


Figure 3. Stem and Progenitor Cell Memory of Leukemic Exposure
(A) CD45.1⁺ KSLs from either control or leukemic mice were sorted and placed in OP9 co-culture assay. Flow cytometry analysis of CD19 and Mac1 in these cultures after 14 days is shown; n = 4. Leuk Exp, leukemic exposed.

of these cytokines, IL-6 was further investigated for this cytokine's role in altering the normal KSL functions within the CML environment. Again, we used GSEA to ask whether normal KSLs from the leukemic mice were aligned with an "IL-6 signature," as this receptor has little to no expression in HSCs (Figure S3F) (Maeda et al., 2005). Indeed, this was the case for the leukemic-exposed progenitors but not leukemic-exposed HSCs (Figure 4D). Our own observations were consistent with recent findings that IL-6 was elevated in the leukemic cells of this mouse model (Figure S3G) (Reynaud et al., 2011).

Furthermore, we wondered whether current therapy was able to reverse the impact of normal hematopoiesis within CML specifically with regard to IL-6 production. Currently, imatinib (IM) is the first-line treatment for CML. Therefore, IM was started 2 weeks after Tet withdrawal, a time point when disease was consistently initiated as determined by flow cytometry. Mice were treated by gavage every other day for 4 weeks. After this treatment, B lymphopoiesis and myeloid differentiation were restored to their normal ratios (Figure S3H). Additionally, we sorted CD45.1⁺ KSLs from the control, IM-treated leukemic, or leukemic mice and determined their ability to make B-lineage cells in vitro. Indeed, treated mice had restored B-lineage potential (Figure S3I). Moreover, the levels of IL-6 in the serum were reduced to near-normal levels, even in the presence of minimal residual disease (2%–15% CD45.2⁺ cells) (Figure S3J). The phenomenon of altered inflammatory environment by IM has been recently observed by others as well (Zhang et al., 2010). Taken

(B) Quantification of B cells (CD19⁺) and myeloid-lineage cells (Mac1⁺) from OP9 co-cultures were plotted from CD45.1⁺ KSLs from either control or leukemic-exposed and CD45.2⁺ leukemic KSLs 14 days after initiation; n = 4. Error bars represent mean ± SEM.

(C) Cartoon showing that control or leukemic-exposed CD45.1⁺ KSLs or HSCs (Lin⁻ ckit^{hi} Sca1⁺ CD150⁺ CD48⁻) were sorted from the primary chimeras and transplanted into sublethally irradiated C57BL/6 (CD45.2) hosts. KSL-transplanted mice were analyzed at 10 weeks post-transplant, while HSC-transplanted mice were analyzed after ≥ 16 weeks post-transplant.

(D) CD45.2⁺ leukemic HSCs and CD45.1⁺ HSCs were sorted from control and/or leukemic mice and then labeled with CFSE (5(6)-carboxyfluorescein N-hydroxysuccinimidyl ester). These cells were then transplanted into lethally irradiated mice for 12 hr, at which time the femurs were analyzed for the presence of CFSE and CD45.1⁺ cells. Plots show homing efficiency relative to the control HSCs; n = 5. Error bars represent mean ± SD.

(E) Limiting dilution CRU (competitive repopulation unit) assay is shown. The indicated numbers of control or leukemic-exposed HSCs were transplanted along with 2 × 10⁵ bone marrow cells of a competitor (CD45.2⁺) into lethally irradiated hosts (CD45.2⁻). Reconstitution was evaluated in the blood at 16 weeks post-transplantation (p = 0.0001). Mice with CD45.1⁺ chimerism <0.3% were considered as non-responders.

(F) For serial transplantation, 2 × 10⁶ sorted CD45.1⁺ bone marrow cells from either control or leukemic mice and appropriately adjusted per HSC numbers of CD45.1⁺ cells from leukemic mice were transplanted into lethally irradiated CD45.2 hosts. Rounds of transplantations were performed at 16-week intervals. The proportion of PB generated from the CD45.1⁺ cells in recipient mice was determined for each round of transplantation. Bar graphs indicate chimerism after each round of transplantation; n = 4. Error bars represent mean ± SD.

(G) PB samples from either 10 weeks post-transplant for CD45.1⁺ KSLs or 16 weeks post-transplant for CD45.1⁺ HSCs transplants, respectively, were stained with CD45.1, CD45.2, and DAPI for viability. The bars show percentages of the donor-type cells (CD45.1) that expressed myeloid (Gr-1 and CD11b, filled), B-lineage (B220, striped), or T-lineage (CD4/8, open) markers in the blood of individual recipients.

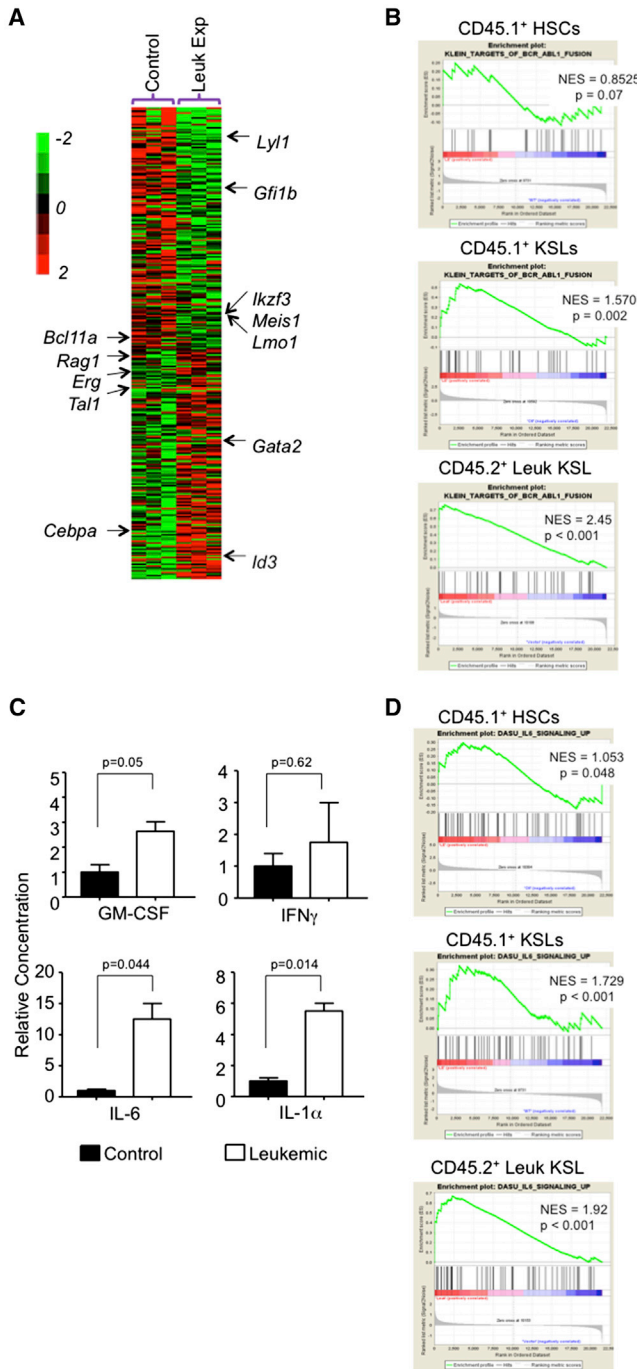


Figure 4. Microarray Data Points to Cytokine Changes in the Progenitor Cells from the Leukemic Exposure

(A) Hierarchical clustering with average linkage was used to generate the heatmap of lineage-specific transcription factors from the control and leukemic-exposed (Leuk Exp) progenitors.

(B) Enrichment plots of GSEA using control, leukemic-exposed, and leukemic HSC and KSL expression data against a list of BCR-ABL-related gene changes.

(C) We used an antibody-based protein array to measure cytokine changes in the sera from our chimera mice, both control and transgenic. Results are shown based on the normalization of the control sera. Error bars represent mean \pm SD.

together, the leukemic bystander effects are dependent on tyrosine kinase activity, can drive IL-6 production, and are likely reversible.

The Leukemic Environment Changes Normal Progenitors through IL-6

The aforementioned findings raised questions related to reversibility or prevention of disease through protection of normal hematopoiesis by manipulation of the CML inflammatory environment. We designed experiments to determine whether the observed IL-6 dysregulation contributed to the changes described earlier in the leukemic-exposed stem and progenitor cells. Therefore, we analyzed chimeric mice lacking essential components of the IL-6 signaling pathway, specifically IL-6R α . Consequently, MxCre⁻ IL-6R α ^{fllox/fllox} and MxCre⁺ IL-6R α ^{fllox/fllox} mice were treated with poly:I:C every other day for three total injections to excise IL-6R α . At this point, the mice had no obvious abnormalities in hematopoiesis. Since these mice were on a CD45.2⁺ background similar to our BCR-ABL leukemic model, we used lineage-depleted marrow cells from the MxCre⁻ IL-6R α ^{fllox/fllox} and MxCre⁺ IL-6R α ^{Δ/Δ} mice and labeled these two groups of cells with a lentiviral GFP. GFP⁺ cells were sorted and co-transplanted with control or transgenic (BCR-ABL) bone marrow into lethally irradiated congenic recipients and kept on Tet treatment to prevent disease (Figure 5A). At 16 weeks post-transplantation, we induced the expression of BCR-ABL (Tet removal), and the GFP⁺ cells were then analyzed for their ability to make either lymphoid- or myeloid-lineage cells (Figure 5B). MxCre⁺ GFP⁺ cells maintained their B lymphopoiesis, even though they were in a leukemic environment, implicating IL-6 signaling in lymphoid-lineage suppression and myeloid expansion. Furthermore, GFP⁺ MxCre⁺ IL-6R α ^{Δ/Δ} KSLs from the leukemic mice had no intrinsic changes as assayed by our B-lineage differentiation in vitro assay (Figure 5C). Even though normal differentiation from the leukemic-exposed cells was restored in the IL-6R α ^{Δ/Δ} -transplanted cells, this did not hinder the progression of CML disease in the CD45.2⁺ cells.

For therapeutic applications where targeting the inflammatory environment might rescue normal differentiation as well as perturb the leukemic cells, we attempted to block IL-6 using an antibody against the cytokine. Therefore, chimeras were prepared, and 16 weeks later, leukemia was induced by withdrawal of Tet. After 2 weeks off Tet when increased CD45.2⁺ cells are observed in the PB, groups of mice received either immunoglobulin G (IgG) control antibody or an anti-IL-6 every other day (0.2 mg/kg) for 6 weeks. Remarkably, the anti-IL-6-treated mice showed no signs of disease such as splenomegaly or myeloid expansion in hematopoietic tissues (Figures 5D and 5E). Only residual disease could be detected in the bone marrow of these mice (Figure S4A). It should be noted that this treatment did not eliminate the leukemic-initiating cells, as mice taken off anti-IL-6 therapy eventually developed CML-like disease (Figure S4B). These treatments were unique to IM and anti-IL-6, as treatment with anti-GM-CSF or anti-IL-1 α did not lead to significant cell death of leukemic bone marrow cells in culture

(D) Enrichment plots of GSEA using control, leukemic-exposed, and leukemic HSC and KSL expression data against a list of IL-6-related gene changes. See also Figure S3.

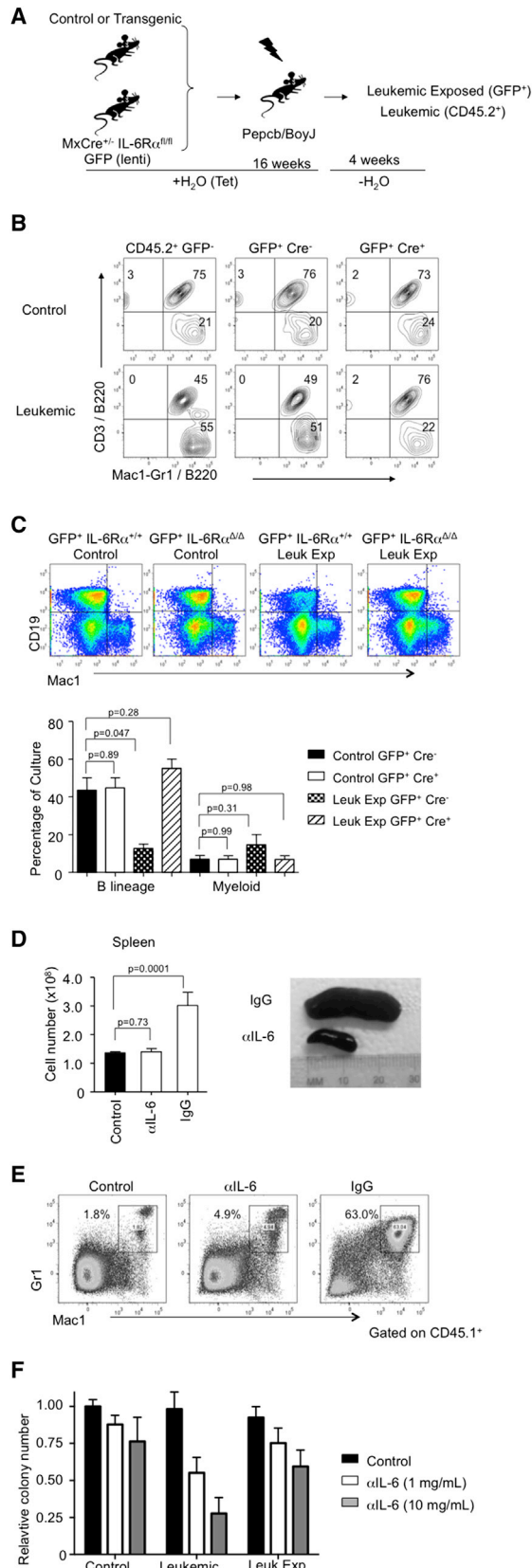


Figure 5. Inhibition of IL-6 Signaling Protects Normal Progenitor Cells

(A) Cartoon of the method used to determine leukemic-exposed KSLs skewing to the myeloid lineage was dependent on IL-6R α signaling. CD45.2⁺ BCR-ABL leukemic cells were transplanted with CD45.2⁺ GFP⁺ MxCre⁺ IL-6R α ^{Δ/Δ} cells or CD45.2⁺ GFP⁺ MxCre⁻ IL-6R α ^{fl/fl} cells and kept on Tet for 16 weeks before the removal of Tet. Pepcb/BoyJ is the mouse name for CD45.1 B6 mice. lenti, lentivirus.

(B) Contour plots from staining of PB gating on GFP⁺ and GFP⁻ cells for B220/CD3 and B220/Mac1-Gr1. Top panels show the control chimera, while the bottom panels show the leukemic. GFP⁺ MxCre⁻ cells (center panels) contain IL-6R α ^{fl/fl} cells. GFP⁺ MxCre⁺ cells are excised for IL-6R α (right panels); n = 4.

(C) GFP⁺ KSL cells from control or leukemic mice were sorted and co-cultured on OP9 stromal cells for B-lineage differentiation. Flow cytometry plots show CD19 versus Mac1 staining from control and leukemic mice containing either IL-6R α ^{fl/fl} or MxCre⁺ IL-6R α ^{Δ/Δ} cells. Quantification of the percentages of B-lineage cells and myeloid cells derived from these cultures are plotted; n = 4. Error bars represent mean \pm SD.

(D) Splenic numbers and morphology from IgG- and anti-IL-6-treated leukemic mice are shown. Mice were injected intravenously with anti-IL-6 diluted in PBS (0.2 mg/kg). Antibody-injected controls were similarly injected with IgG in PBS. Two weeks after removal from Tet, mice were injected every other day for 6 weeks. Mice were then euthanized, and total cell numbers from the spleens of control, leukemic IgG-, and anti-IL-6-leukemic-treated mice were obtained; n = 5. Error bars represent mean \pm SD.

(E) Flow cytometry plots of CD45.1⁺ myeloid cells (Mac1⁺ Gr1⁺) from the spleens of control or leukemic IgG- and anti-IL-6-treated mice. Spleens were stained for viable CD45.1, CD45.2, Mac1, and Gr1; n = 4.

(F) Sorted control, leukemic-exposed, and leukemic KSLs were cultured in colony assays in the presence or absence of anti-IL-6 at two doses. Colonies were counted, and relative colony numbers were determined compared to untreated cells for each population; n = 3. Error bars represent mean \pm SD. See also Figure S4.

(Figure S4C). Moreover, treatment with anti-IL-6 in colony assays with control, leukemic-exposed, or leukemic KSL showed that the leukemic cells were specifically sensitive to this treatment with respect to colony formation (Figure 5F). These data suggest that the BCR-ABL leukemic environment promotes the disease and impacts the neighboring non-transformed cells through IL-6. Furthermore, this environment can be altered through blocking the inflammatory signature.

IL-6 Disrupts Normal Stem and Progenitor Cell Function in Human CML

These experimental animal findings may lead one to question their relevance to human disease, but chronic-phase CML has not been satisfactorily reproduced by transplantation into immunodeficient mice (Dazzi et al., 1998) (as well as the observations of R.S.W. and G.A.). Therefore, we exploited culture approaches similar to those described to evaluate bystander changes in our mouse model. We cultured lentiviral GFP-labeled, sorted CD34⁺ adult bone marrow cells that were mixed with bone marrow cells from either control (healthy) or chronic-phase CML patients. As seen with our mouse model of CML, human cultures had increased IL-6 as assessed by real-time PCR (Figure 6A). Furthermore, CML bone marrow exposure caused GFP⁺ CD34⁺ progenitors to expand more than twice that observed from co-culture with normal patient bone marrow (Figure 6B). This increase in GFP⁺ cells was inhibited by addition of an anti-IL-6 antibody. Additionally, CD34⁺ progenitors co-cultured with CML bone marrow had reduced B-lineage potential as assayed during

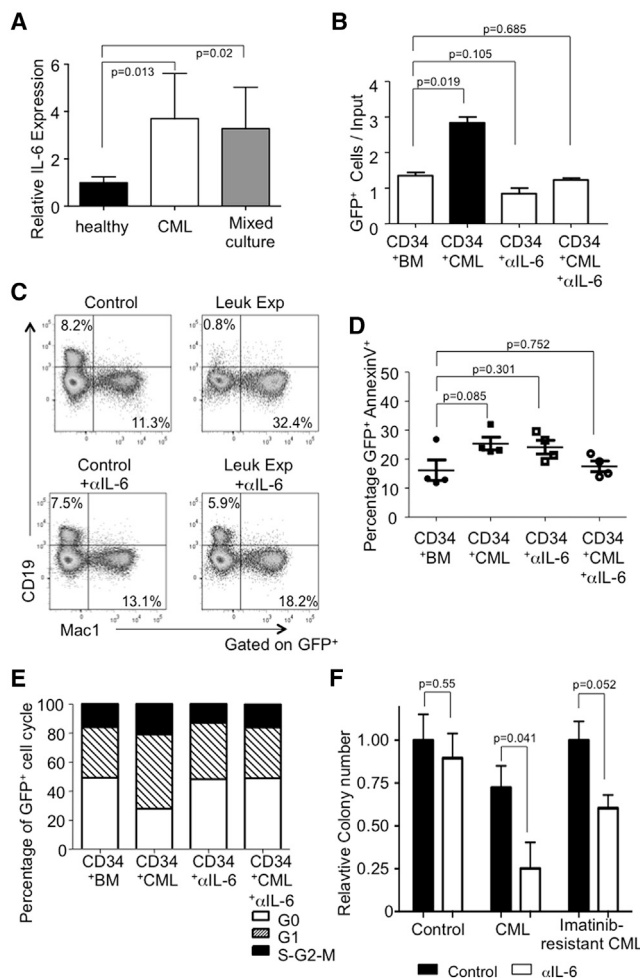


Figure 6. Increased Proliferation and Skewed Differentiation of Normal Human CD34⁺ Progenitors when Mixed with Human CML Cells

(A) Cytokine IL-6 mRNA levels from cultured patient bone marrow (healthy, chronic-phase CML, or mixed) were measured against GADPH and normalized to healthy controls; $n = 5$. Error bars represent mean \pm SD.

(B) Bar graph showing the yield per input of GFP⁺ cells when cultured with control or CML bone marrow with or without anti-IL-6. Bead-enriched CD34⁺ cells from human adult bone marrow were labeled with a lentiviral GFP reporter. These cells were then cultured with whole adult bone marrow from healthy patients (control) or those with chronic-phase CML (leukemic exposed) on stromal cells (MS5). Additionally, wells contained either IgG or anti-IL-6 as indicated (5 μ g/ml). These cells were cultured for 96 hr with Flt3 ligand (Flt3L), thrombopoietin (TPO), and IL-3 and then counted and analyzed by flow cytometry for total GFP⁺ cells; $n = 3$. Error bars represent mean \pm SD.

(C) CD34⁺ GFP-marked cells were co-cultured with healthy bone marrow (Control) or chronic-phase CML (leukemic exposed; Leuk Exp) on stromal cells. These cultures were maintained with IL-7, stem cell factor (SCF), and Flt3L for 1 week and then an additional 3 weeks with only SCF and Flt3L. As indicated, some wells contained anti-IL-6. After the 4-week culture, GFP⁺ human cells were analyzed by flow cytometry for expression of CD19 and Mac1; $n = 3$.

(D) GFP⁺ CD34⁺ cells from either control or leukemic-exposed cultures were stained for annexin V to determine the relative apoptosis in these conditions, and lines indicate the mean; $n = 3$. Error bars represent mean \pm SEM.

(E) Sorted GFP⁺ CD34⁺ cells were analyzed from control and CML cultures (with or without anti-IL-6) after staining with Pylonin Y and Hoescht to determine cell cycle status; $n = 3$.

stromal cell co-cultures. Similar to the mouse studies, treatment with anti-IL-6 was able to restore B-lineage potential (Figure 6C). From these culture conditions, we were able to determine that CML cells did not significantly increase apoptosis of the GFP-marked cells (Figure 6D). Moreover, CML cells caused not only the expansion of the GFP⁺ CD34⁺ progenitors but also their division, a phenomenon that was also IL-6 dependent (Figure 6E). As seen with our murine studies, anti-IL-6 was able to inhibit colony formation of both chronic-phase and IM-resistant CML bone marrow patient samples (Figure 6F). We conclude that changes in cytokine levels, specifically those of IL-6, in the CML environment are responsible for altered differentiation and function of normal stem and progenitor cells. Our models underscore the significant effect of the leukemic cells and environment on conditioning both leukemic and normal stem and progenitor cells. We conclude that changes in cytokine levels, specifically those of IL-6, in the CML are mostly responsible for the altered differentiation and function of normal stem and progenitor cells while promoting the disease.

DISCUSSION

Our models underscore the significance of the leukemic cells on conditioning both leukemic and normal stem and progenitor cells. First, and confirming the recent work of others (Begley and Ellis, 2012; Krause et al., 2013; Reynaud et al., 2011; Schepers et al., 2013), leukemic cells alter neighboring non-transformed hematopoietic cells; however, the direct impact on stem and progenitor cell function was not previously addressed. Second, these leukemic-exposed normal cell counterparts acquire characteristics of the transformed cells, with changes dependent on their stage of differentiation. That is, HSCs are altered differently from the changes observed in KSLs/progenitors. Characteristics imprinted on leukemic-exposed HSCs included reconstitution defects but not homing or differentiation potential. Alterations in KSLs were much more striking. Exposure of these progenitors to leukemic cells induced loss of lymphopoietic potential and enhanced myeloid potency. In addition, the normal progenitors entered cell cycle and mimicked leukemic cells with respect to gene expression. Third, an IL-6 signature was prominent in the transcriptome profile, leading us to functional studies involving this cytokine. Furthermore, IL-6 signaling was necessary for acquisition of the characterized bystander properties by normal progenitors. Finally, human leukemic cells perturbed neighboring progenitors through the production of IL-6 similar to that demonstrated by our mouse model. We believe that these observations will focus attention on how changes in normal hematopoietic cells that reside near transformed ones in bone marrow can lead to therapeutics targeting the leukemic cells by altering the inflammatory profile from the disease.

Our model was previously shown to closely resemble the chronic phase of human CML (Koschmieder et al., 2005). Moreover, we are encouraged that two recent reports are closely relevant to our study and show the reproducibility of the science

(F) Bone marrow from healthy, chronic-phase CML, or IM-resistant CML was cultured in Methocult4435 for colony formation with or without human anti-IL-6. Colony numbers are presented as relative to the healthy untreated control; $n = 2$. Error bars represent mean \pm SD.

(Begley and Ellis, 2012; Schepers et al., 2013; Zhang et al., 2012). In these studies, they lethally irradiated our inducible BCR-ABL transgenic or wild-type controls. The animals were then used as recipients of HSPCs from leukemic or normal marrow from non-irradiated mice. The leukemic host, mainly the stromal niche, was a poor host for normal hematopoiesis. It is important to note, however, that one group did not record IL-6 overexpression from the environment but rather the changes in the cellular environment (Zhang et al., 2012).

In our work, we closely monitored the normal hematopoietic cells over time following BCR-ABL activation and observed rapid changes in progenitors. After 1 month of leukemic exposure, KSL numbers were greatly expanded, and they had almost completely lost their ability to generate lymphocytes. This might be explained in terms of selective growth of myeloid precursors and/or re-direction away from lymphopoiesis. HSC changes were more subtle and obvious only in transplantation assays, a phenotype that remained even after treating the mice with anti-IL-6. Although we saw little changes in proliferation and differentiation of HSCs, these changes may occur at earlier stages of the disease, particularly at onset from perturbation of leukemic stem cells within a similar niche (Zhang et al., 2012). Our data and others suggest that HSC express little to no IL-6R α and may, therefore, be less responsive to this specific inflammatory cytokine (Maeda et al., 2005). We are interested to determine other cytokines or environmental changes specifically mediating changes in HSCs. However, we can conclude that residence near leukemic cells provides several disadvantages to cells lacking the BCR-ABL fusion protein. Their inability to self-renew and sustain balanced hematopoiesis would allow them to be out-competed by transformed cells over time.

It has previously been suggested that cytokines create a pro-inflammatory environment during CML development and that this provides a proliferative advantage to leukemic cells (Holyoake et al., 1999; Zhang et al., 2012; Zhang and Ren, 1998). We observed a conspicuous IL-6 signature in leukemic-exposed progenitors, and there are precedents for the importance of this factor in CML. Two groups observed that serum IL-6 was elevated in sera of leukemic animals (Reynaud et al., 2011; Zhang et al., 2012). Additionally, IL-6 secretion increases with CML development in patients (Anand et al., 1998; Reynaud et al., 2011; Schmidt et al., 2011; Zhang et al., 2012). Furthermore, Reynaud et al. (2011) found that disease progression was inhibited when BCR-ABL was induced in hematopoietic cells of IL-6-deficient mice. IL-6 is also capable of blocking a discrete early stage of lymphopoiesis (Maeda et al., 2005). We are not the first to find this inflammatory signature in CML; however, we were able to use antibody therapy to target the inflammatory environment in this model of leukemia.

We found that IL-6 drives normal progenitor changes in our chimeric animal model of CML. Indeed, treatment of the mice with neutralizing anti-IL-6 allowed progenitor composition and differentiation to proceed normally for at least 6 weeks after BCR-ABL activation. Tumor-initiating cells must have been spared because the disease developed when antibody therapy was discontinued. HSC can propagate CML disease but are unlikely to be direct targets of IL-6 because they lack critical receptor components (Raaijmakers et al., 2010; Reynaud et al., 2011; Schemionek et al., 2010). Thus, factors other than IL-6 might account for

our finding that untransformed HSCs had reduced self-renewal ability after residence in leukemic marrow and during therapy. Moreover, Schepers et al., (2013) provide evidence that this loss of HSC function could be accomplished by reprogramming stromal cells as a result of the leukemic cells or environment. In that context, it is interesting that, in human CML, chemokines can inhibit the proliferation of normal hematopoietic cells while sparing transformed ones, and similar interactions may be occurring within the niche (Cashman et al., 1998; Eaves et al., 1993a, 1993b). The source of IL-6 produced in CML is still in question and might involve multiple types of BCR-ABL-transformed cells. The study by Zhang et al. (2012), noted earlier, suggested that radio-resistant stromal cells were not an important source of IL-6 in a transplantation model similar to ours. On the other hand, inflammation driven by TLR ligands and T-cell-produced interferons can elicit IL-6 production in stromal elements (Zhao et al., 2014).

In addition to the results we observed in mice, neutralization of IL-6 protected human CD34⁺ cells when exposed to CML cells. Given the contribution of IL-6 to a wide range of human inflammatory diseases, clinically effective strategies have already been developed for its neutralization (Tanaka et al., 2011). Our results suggest that this approach might be an effective therapy in drug-resistant CML or other leukemias with pro-inflammatory signatures, specifically, those in which the leukemic cells are also found to have an adverse impact on untransformed bystander hematopoietic cells. From studying the non-transformed cells in other models of leukemia, we hope to find and target additional cell-autonomous environmental changes that are necessary for propagation of the leukemic clones.

EXPERIMENTAL PROCEDURES

Mice/Human Samples

Inducible, transgenic SCL-tTa/BCR-ABL mice (Koschmieder et al., 2005) were backcrossed into the B6 background and were maintained with administration of Tet in the drinking water (0.5 g/l). BCR-ABL expression was induced by Tet withdrawal for a minimum of 3 weeks. Mice were housed in a sterile barrier facility approved by the Institutional Animal Care and Use Committee (IACUC) at the Beth Israel Deaconess Medical Center. Human samples were collected with informed consent and approved by the institutional review board at the Beth Israel Deaconess Medical Center.

Flow Cytometry

Single-cell suspensions from various organs were analyzed by flow cytometry using the following monoclonal antibodies conjugated with phycoerythrin (PE), PE-CY7, fluorescein isothiocyanate, allophycocyanin (APC), APC-Cy7, or eFluor 450 obtained from BD PharMingen (BD), BioLegend, or eBioscience: Mac-1/CD11b (M1/70), Gr-1 (8C5), CD3 (KT31.1), CD4 (GK1.5), CD8 (53-6.7), B220 (RA3-6B2), CD19 (1D3), TER119 (TER-119), Sca1 (E13-161-7), c-Kit (2B8), CD16/32 (2.4G3), Thy-1.2 (53-2.1), CD135 (AF2 10.1), CD48 (HM48-1), CD45.1 (A20), CD45.2 (104), and CD150 (TC15-12F12.2). Stained cells were analyzed with an LSRII flow cytometer and sorted using a FACSAria II (BD Biosciences). Viable cells were identified by DAPI exclusion. Human stem cells/primitive progenitors were isolated by sorting CD34⁺CD38⁻ cells. Diva software (BD) and FlowJo (Tree Star) were used for data acquisition and analysis, respectively.

Proliferation and Cell Cycle Analysis

For proliferation analysis, BrdU was administered intraperitoneally (1 mg per mouse), bone marrow cells were obtained 16 hr post-injection, and BrdU incorporation into stem and progenitor populations was analyzed (Cappella et al., 2008). Cell cycle was also analyzed using Pylonin Y and Hoescht labeling as previously described (Staber et al., 2013).

Limiting Dilution Assay

HSCs or total bone marrow cells were collected as CD45.1⁺ from leukemic or control mice 4 weeks following removal of Tet water; then, the number of cells indicated to be injected per mouse was sorted into individual wells of a 96-well plate containing 10⁴ wild-type CD45.2⁺ bone marrow (rescue cells) in PBS. The contents of individual wells were injected into the retro-orbital venous sinus of lethally irradiated CD45.2⁺ recipients (600 rads twice with a 3-hr interval). PB was obtained from each mouse each month after transplantation for at least 4 months and analyzed by fluorescence-activated cell sorting (FACS). Cells were stained with anti-CD45.1 and CD45.2 antibodies to distinguish donor-derived cells from the host cells, as well as lineage-specific antibodies Mac1, Gr1, B220, and CD3, to identify myeloid, B, and T lineages. A recipient mouse was considered positive if CD45.1⁺CD45.2⁻ cells were present in myeloid and B and/or T cells and also constituted more than 0.3% of the cells in the PB.

Bone Marrow Transplantation

For the initial experimental setup, 2 × 10⁶ CD45.1⁺ bone marrow cells and 2 × 10⁶ CD45.2 bone marrow cells from SCLTα/BCR-ABL mice were transplanted through retro-orbital injection into lethally irradiated CD45.1 mice. Mice were maintained on Tet before (2 weeks) and after transplantation for 16 weeks to establish chimerism before induction of the transgenes.

Microarray Analysis

Robust multichip average (RMA) was run for all CEL files generated in this study to obtain the probe-set expression data. The probe-set expression data were then normalized using the cross-correlation method. GSEA was carried out with the normalized data by comparing the leukemic-exposed HSCs or KSLs to their corresponding controls, respectively. The gene set database used in GSEA was the msigdb v3. For heatmap presentation of the significantly changed transcription factors in the leukemic-exposed versus the control setting, the normalized data were further log₂ transformed, and the subtraction of the mean of the means was carried out for each gene. Also, hierarchical clustering with average linkage was used to generate the heatmap.

Statistical Analysis

The statistical significances were assessed by Student's unpaired t test using the GraphPad software.

ACCESSION NUMBERS

The Gene Expression Omnibus accession number for microarray data reported in this paper is GSE59337 for microarray data.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.ccell.2015.04.004>.

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