Published in final edited form as:
Am J Hematol. 2011 January ; 86(1): 31–37. doi:10.1002/ajh.21915.

Characterization of chronic myeloid leukemia stem cells

Jonathan M. Gerber, M.D.[1], **Lu Qin, B.S.**[1], **Jeanne Kowalski, Ph.D.**[1], **B. Douglas Smith, M.D.**[1], **Constance A. Griffin, M.D.**[1], **Milada S. Vala, B.S.**[1], **Michael I. Collector, B.S.**[1], **Brandy Perkins, M.A.**[1], **Marianna Zahurak, M.S.**[1], **William Matsui, M.D.**[1], **Christopher D. Gocke, M.D.**[1], **Saul J. Sharkis, Ph.D.**[1], **Hyam I. Levitsky, M.D.**[1], and **Richard J. Jones, M.D.**[1]

[1]The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD.

Abstract

Though tyrosine kinase inhibitors have redefined the care of chronic myeloid leukemia (CML), these agents have not proved curative, likely due to resistance of the leukemia stem cells (LSC). While a number of potential therapeutic targets have emerged in CML, their expression in the LSC remains largely unknown. We therefore isolated subsets of $CD34^+$ stem/progenitor cells from normal donors and from patients with chronic phase or blast crisis CML. These cell subsets were then characterized based on ability to engraft immunodeficient mice and expression of candidate therapeutic targets. The $CD34^+CD38^-$ CML cell population with high aldehyde dehydrogenase (ALDH) activity was the most enriched for immunodeficient mouse engrafting capacity. The putative targets: *PROTEINASE 3*, *SURVIVIN*, and *hTERT* were expressed only at relatively low levels by the $CD34^+CD38^-ALDH^{high}$ CML cells, similar to the normal $CD34^+CD38^-ALDH^{high}$ cells and less than in the total CML $CD34^+$ cells. In fact, the highest expression of these antigens was in normal, unfractionated $CD34^+$ cells. In contrast, *PRAME* and *WT1* were more highly expressed by all CML $CD34^+$ subsets than their normal counterparts. Thus, ALDH activity appears to enrich for CML stem cells, which display an expression profile that is distinct from normal stem/progenitor cells and even the CML progenitors. Indeed, expression of a putative target by the total $CD34^+$ population in CML does not guarantee expression by the LSC. These expression patterns suggest that *PROTEINASE 3*, *SURVIVIN*, and *hTERT* are not optimal therapeutic targets in CML stem cells; whereas *PRAME* and *WT1* seem promising.

Keywords

chronic myeloid leukemia; CML; leukemia stem cell; WT1; PRAME

Introduction

The advent of tyrosine kinase inhibitors redefined the treatment of chronic myeloid leukemia (CML).[1] Complete cytogenetic response rates can now be seen in up to 90% of newly diagnosed patients in chronic phase (CP).[2] However, these “targeted” therapies have yet to prove curative. In fact, relapse is common after discontinuation of imatinib, even for patients in complete molecular remission at the time of cessation.[3,4] This appears to be due to resistance of CML stem cells to the pro-apoptotic effects of imatinib[5,6] and even newer tyrosine kinase inhibitors, such as dasatinib.[7] Indeed, primitive leukemic progenitors still can be readily detected in CML patients who have achieved complete cytogenetic remission on imatinib.[8] Blast crisis (BC) CML presents an even greater challenge, where in contrast to CP CML, tyrosine kinase inhibition rarely results in durable

Correspondence: Dr. Jonathan M. Gerber, MD, The Bunting-Blaustein Cancer Research Bldg, Room 205, 1650 Orleans St., Baltimore, MD 21231, Phone: 410-502-4423, Fax: 410-614-7279, jgerber2@jhmi.edu.

remission.[9] Hence, there has been a search for other therapeutic targets in CML, particularly on the leukemia stem cell (LSC), which in theory must be eradicated to achieve cure.[10–12]

A number of candidates have emerged as potential therapeutic targets in leukemia. Among the more promising are: the Wilm's tumor gene (*WT1*), *SURVIVIN*, the preferentially expressed antigen of melanoma (*PRAME*), *PROTEINASE 3* (*PR3*), and *hTERT* (the enzymatic component of telomerase). All of these are immunogenic, and each is over-expressed to varying degrees in many cancers, including CML.[13] Many of these candidate targets have been implicated in therapeutic resistance, including inhibition of apoptosis, and appear to correlate with prognosis.[13–15] There are ongoing vaccine trials targeting many of these antigens,[13,16] as well as early phase clinical trials of pharmacologic inhibitors of telomerase[17] and SURVIVIN.[18] Presumably, any such new therapies will have curative potential only if their targets are actually expressed by the LSC. However, the expression of these putative targets in CML stem cells is largely unknown.

Indeed, existing data are limited regarding the precise characterization of CML stem cells; and expression of a gene by the differentiated leukemic bulk does not necessarily guarantee expression by the LSC. In fact, in many respects LSC more closely resemble normal hematopoietic stem cells (HSC) than their own differentiated leukemic progeny.[19–21] Nonetheless, it is expected that qualitative or quantitative expression of some genes must distinguish LSC from their normal counterparts. Accordingly, an optimal therapeutic target would not only be highly expressed by the LSC (and ideally their progeny as well); but it would also be absent or only minimally expressed in normal HSC to avoid unacceptable toxicity.[13]

LSC research to date has been impeded by the relative rarity of these cells, as well as the lack of a consensus on their exact phenotype. LSC are often phenotypically defined as simply the $CD34^+$ leukemia cells or, occasionally, the more enriched $CD34^+CD38^-$ subset; but even the $CD34^+CD38^-$ cells are a heterogeneous population, of which the LSC constitute only a fraction.[19,22–24] The $CD34^+CD38^-$ population can be further refined for stem cells based on low side scatter and high aldehyde dehydrogenase (ALDH) activity.[23] ALDH, specifically the ALDH1A1 isoenzyme, mediates the biosynthesis of all-*trans*-retinoic acid, as well as the detoxification of a variety of compounds such as ethanol and active metabolites of cyclophosphamide;[25] and it is typically present at higher levels in adult stem cells, than in their differentiated progeny.[22–27] The fluorescently labeled ALDH1A1 substrate, Aldefluor, permits the isolation of viable normal and cancer stem cells.[22–24,26–28]

Here we report that CML stem cells are characterized by high ALDH expression, and that these cells have a unique expression profile of putative targets as compared to both the more differentiated CML progenitors and normal HSC.

Methods

Patient and normal donor specimens

Bone Marrow and/or peripheral blood samples were obtained from a total of 14 patients with CML (10 CP and 4 myeloid BC). None of the BC CML patients had a duplicated Philadelphia chromosome. One of the BC CML patient samples was obtained at relapse from a patient who had been off of all treatment for several months, with imatinib-resistant disease at the time of relapse. All of the other patients were newly diagnosed and not yet treated at the time of sample collection. Demographic and clinical characteristics of the CML patients are displayed in Table I. A total of 15 normal samples (12 of which were used

in the gene expression analyses and 3 of which were used for quantification of protein expression) were obtained as excess material from allogeneic bone marrow harvests. Informed consent was obtained from all patients and healthy donors prior to sample collection in accordance with the Declaration of Helsinski, under a research protocol approved by the Johns Hopkins Institutional Review Board.

Mononuclear cells were isolated from fresh samples by Ficoll-Paque (GE Healthcare Life Sciences, Piscataway, NJ, USA) density (=1.077) centrifugation. $CD34^+$ cells were selected by Miltenyi Biotec (Auburn, CA, USA) microbeads (binding the class II CD34 epitope) and column per manufacturer's instructions. Next, ALDH1A1 activity was assessed in the $CD34^+$ cells by staining with Aldefluor (Aldagen, Durham, NC, USA) per manufacturer's guidelines; this was followed by labeling with monoclonal phycoerythrin (PE)-conjugated anti-CD34 (binding the class III CD34 epitope) and allophycocyanin (APC)-conjugated anti-CD38 (all antibodies purchased from BD Biosciences, San Jose, CA, USA). Cells were then sorted using a FACSAria (BD Biosciences) into 3 fractions: $CD34^+$ total (unfractionated), $CD34^+CD38^-ALDH^{low}$, and (low side scatter) $CD34^+CD38^-ALDH^{high}$. Representative examples of the staining profile and gating strategy for a normal sample, a CP CML sample, and a BC CML sample are depicted in Figure 1. Purity of the sorted cell fractions was >98% on reanalysis.

Fluorescence in situ hybridization (FISH)

Isolation of leukemic cells was confirmed by FISH for *BCR-ABL* on cytospins of 2×10^4 cells from each sorted cell fraction, fixed in 3:1 Methanol: Glacial Acetic acid (Sigma-Aldrich, St. Louis, MO, USA). FISH was performed by the Johns Hopkins Cytogenetics Core, using the Vysis LSI *BCR-ABL* Dual Color, Dual Fusion translocation probe (Abbot Molecular, Des Plaines, IL, USA) per manufacturer's instructions and a fluorescence microscope with a triple-band pass filter for DAPI, Spectrum Orange, and Spectrum Green.

NOD/SCID-IL2Rγ^{null} (NOG) mouse transplants

For a subset of CML patients (4 CP and 4 BC) from whom sufficient cellular yields of the isolated CD34 subsets were obtained, NOG mouse transplantation was used as a functional assay for stem cells. Following irradiation with 300cGy (via Cesium irradiator), 3 mice per cell fraction were injected (via tail vein) with 10^4 –10^5 cells; for any given sample, equal cell numbers from all fractions were transplanted. Mice were sacrificed >3 months later, and bone marrow was harvested at necropsy. The harvested mouse bone marrow was treated with RBC lysis buffer (Sigma-Aldrich) and then stained with an APC-conjugated monoclonal antibody against human CD45 (BD Biosciences). Cells were then analyzed on the FACSAria cell sorter and the human $CD45^+$ population (if present) collected and cytospun onto slides for analysis of *BCR-ABL* by FISH. A sample fraction was considered to have successfully engrafted if at least 1 transplanted mouse had ≥ 0.1% human $CD45^+$ cells detectable, with a cytospin of those cells positive for *BCR-ABL* by FISH. All mouse research was performed under a protocol approved by the Johns Hopkins Animal Care and Use Committee and complied with National Institutes of Health and American Veterinary Medical Association guidelines.

Reverse transcription polymerase chain reaction (RT-PCR)

Aliquots of 10^4 – 5×10^5 cells from the three $CD34^+$ subsets isolated from 10 CP CML patients, 4 BC CML patients, and 12 normal donors were pelleted, flash frozen, and stored at −80C until assay. Aliquots of the K562 cell line (American Type Culture Collection, Manassas, VA, USA) were similarly pelleted and frozen, for use as a control between assay runs. RNA was extracted with the RNeasy kit (Quiagen) per manufacturer's instructions. Expression of *BCR-ABL*, *SURVIVIN*, PR3, *PRAME*, *hTERT*, and *WT1* mRNA was

quantified by real-time, one-step RT-PCR using sequence-specific probes per the QuantiTect Probe RT-PCR kit (Qiagen, Valencia, CA, USA). Standard curves were generated using plasmids for each candidate and validated on fixed aliquots of K562 cells. Taqman primers were obtained from Applied Biosystems (Foster City, CA, USA; *GAPDH* 4326317, *hTERT* 162669, *SURVIVIN* 153353, *WT1* 240913, *PRAME* 196132, and PR3 1957752) and Ipsogen (Marseilles, France; *BCR-ABL* IPPF-70). mRNA expression of each putative target was also measured in 10^4 cell aliquots of the K562 cell line as a control. Candidate target expression was normalized to 10^4 copies of *GAPDH* mRNA. All RT-PCR reactions were performed in duplicate and run on the ABI 7500 machine (Applied Biosystems).

Protein quantification

Cells were fixed in 2% paraformalin, then permeabilized in 0.1% Triton-X-100 in PBS (Sigma-Aldrich), and then single-stained with unconjugated rabbit monoclonal antibody against PR3 or WT1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or unconjugated rabbit polyclonal antibody against PRAME (Abcam, Cambridge, MA, USA). Surface membrane expression of PRAME was also analyzed on fresh cells (neither fixed nor permeabilized).[29] Cells were then secondarily stained with PE-conjugated goat anti-rabbit antibodies (BD Biosciences) and analyzed on the FACSAria. Due to limited quantities of CML samples, these experiments were performed only on normal donor samples and the K562 cell line.

Statistical considerations

mRNA expression of each of the six genes (*PROTEINASE 3*, *SURVIVIN*, *hTERT*, *WT1*, *PRAME*, and *BCR-ABL*) was quantified (per 10^4 copies of *GAPDH*) from all 3 cell fractions ($CD34^+$ total, $CD34^+CD38^-ALDH^{low}$, and $CD34^+CD38^-ALDH^{high}$) and compared among normal, CP CML, and BC CML. To account for the correlation among the three cell fractions obtained from the same patient, generalized estimating equations (GEE) were used (assuming a compound symmetry correlation structure) for model estimation and hypothesis testing of mean differences based on a Chi-squared statistic. The vector of expression from the cell fractions among patients was modeled as a function of clinical phase of CML (normal, CP, BC), cell fraction, and the interaction of the two. A log (base 10) transformation was applied to all expression data. A similar statistical approach was used to examine *BCR-ABL* FISH data (expressed as percentage of cells with the *BCR-ABL* fusion gene), whereby GEE was used to model the FISH percentage of cell fractions for corresponding hypothesis tests of equal means among cell fractions and between clinical phases. All reported *p*-values are by Chi-squared test, with values <0.05 considered significant.

Correlation in expression between the $CD34^+$ total and $CD34^+CD38^-ALDH^{high}$ groups was explored using Spearman rank for *BCR-ABL*, *WT1*, and *PRAME* (reported *p*-values are also by Chi-squared test). *PR3*, *SURVIVIN*, and *hTERT* expression were further examined for mean differences between the $CD34^+$ total and $CD34^+CD38^-ALDH^{high}$ cell fractions within each of CP and BC CML by Chi-squared test.

Results

The $CD34^+CD38^-ALDH^{high}$ cell fraction is highly enriched for CML stem cells

$CD34^+$ cell fractions were isolated from 10 patients with CP CML and 4 patients with BC CML. CP CML staining patterns were quite reproducible and closely mirrored that of normal samples, whereas staining patterns of BC CML were more variable (Figure 1). The majority of the cells (on average >90%) from all fractions of all CML patients were positive

for *BCR-ABL* by FISH, with the exception of the $CD34^+CD38^-ALDH^{high}$ fraction of one CP CML patient (which was only 39.5% *BCR-ABL* FISH-positive) (Table II). The $CD34^+CD38^-ALDH^{high}$ cells constituted <5% of the total $CD34^+$ cells and about 20% of the total $CD34^+CD38^-$ population (and typically, <0.1% of the total mononuclear cells). There were no statistically significant differences in the mean percentage of *BCR-ABL* FISH-positive cells between CP and BC CML or between the $CD34^+$ cell subsets within each disease phase.

Stem cell function was defined by the ability to engraft immunodeficient mice.[30,31] The transplanted $CD34^+CD38^-ALDH^{high}$ cell fraction from all 8 CML patients (4 CP and 4 BC) produced leukemic (by FISH for BCR-ABL) engraftment in irradiated NOG mice (median 3%, range 0.1 – 31%, of total RBC-lysed marrow cells). A representative example of an engrafted CML sample is shown in Figure 2 (right panel). Equal numbers of the total $CD34^+$ cells also produced leukemic engraftment, but less efficiently; 7/8 patient samples engrafted, with a median population size of 0.1% (range 0.1–1.3% of total RBC-lysed marrow cells). The $CD34^+CD38^-ALDH^{low}$ fraction engrafted in only 1 case, a BC CML sample. Normal $CD34^+CD38^-ALDH^{high}$ cells similarly produced reliable engraftment.

Expression of PR3, SURVIVIN, and hTERT by CML stem/progenitor cells parallels that in their normal counterparts

mRNA expression of the potential therapeutic targets and *BCR-ABL* was assessed by real time RT-PCR in each of the three $CD34^+$ cell subsets isolated from the CML patients and normal donors (Figure 3). There was a trend toward higher *BCR-ABL* mRNA expression in the $CD34^+CD38^-ALDH^{high}$ fraction of both CP (p=0.0568) and BC CML (p=0.081). *PR3* and *SURVIVIN* mRNA showed similar patterns of expression in normal cells, with levels lowest in the $CD34^+CD38^-ALDH^{high}$ cells, intermediate in the $CD34^+CD38^-ALDH^{low}$ population, and highest in the total $CD34^+$ fraction. Expression of *PR3* and *SURVIVIN* mRNA in both CP and BC CML paralleled that detected in the normal samples; the expression of these antigens was actually highest of all in the normal $CD34^+$ total fraction and decreased with clinical progression from CP to BC CML. *hTERT* mRNA was expressed at only very low levels in any of the $CD34^+$ cell fractions, especially in the $CD34^+CD38^-ALDH^{high}$ cells of both the normal controls and CML patients. In CP CML, expression levels of *PR3* (p=0.002), *SURVIVIN* (p=0.005), and *hTERT* (p=0.008) mRNA were significantly lower in the $CD34^+CD38^-ALDH^{high}$ fraction than in the total $CD34^+$ fraction. These same trends were seen in BC CML, though only borderline significant for *SURVIVIN* (p=0.056) and not statistically significant for PR3 and *hTERT*.

WT1 and PRAME are over-expressed in CML

Mean *WT1* mRNA expression was significantly higher in CP (p=0.038) and BC (p=0.042) CML than normal, with 2 to 5-fold higher expression in each $CD34^+$ fraction versus the corresponding normal fraction (Figure 3). Mean expression of *WT1* mRNA did not differ significantly between CP and BC CML (p=0.498). *PRAME* mRNA was expressed at very low to undetectable levels in all of the normal samples, especially the $CD34^+CD38^-ALDH^{high}$ population (Figure 3). All three CP CML CD34 cell fractions expressed *PRAME* mRNA levels on average that were >30-fold higher than the normal $CD34^+CD38^-ALDH^{high}$ cells and >4-fold higher than the corresponding normal fraction (p=0.056 for mean *PRAME* expression in CP CML vs. normal). *PRAME* mRNA was significantly more highly expressed in all BC CML $CD34^+$ fractions; >50-fold on average higher than the corresponding CP CML fractions (p=0.039 for CP vs. BC CML) and >300-fold higher on average than any normal fraction (p=0.032 for BC CML vs. normal). Notably, there was a statistically significant correlation between mRNA expression in the

$CD34^+$ total fraction and that in the $CD34^+CD38^-ALDH^{high}$ fraction for both *WT1* (Spearman's rho=0.798; $p<0.001$) and *PRAME* (Spearman's rho=0.806; $p<0.001$).

PR3, WT1, and PRAME protein expression mirrored mRNA expression

PR3 protein levels by flow cytometry paralleled mRNA expression; unfractionated normal $CD34^+$ cells exhibited markedly higher pr3 expression than the normal $CD34^+CD38^-ALDH^{high}$ cell subset (Figure 4, left panel). *WT1* protein expression also correlated with mRNA expression, with higher levels in K562 cells than in normal unfractionated $CD34^+$ cells (Figure 4, middle panel). Likewise, *PRAME* protein expression correlated with mRNA levels. Intense PRAME surface membrane staining was seen in the K562 cells, substantially higher than that in normal unfractionated $CD34^+$ cells (Figure 4, right panel).

Discussion

The $CD34^+CD38^-ALDH^{high}$ cell population was highly enriched for CML stem cells, as determined by its ability to engraft immunodeficient mice. Equal numbers of unfractionated $CD34^+$ CML cells produced about 30-fold less chimerism, and little to no engraftment was generated by the $CD34^+CD38^-ALDH^{low}$ CML cells. Most importantly, the $CD34^+CD38^-ALDH^{high}$ CML stem cells exhibited a unique expression pattern, distinct from both their own leukemic progeny and the normal hematopoietic stem/progenitor cells.

Expression of *PR3*, *SURVIVIN*, and *hTERT* was highest in the more differentiated $CD34^+$ total cells of both CML and normal samples, with levels lowest in BC CML. This pattern is consistent with markers of differentiation. Indeed, *PR3*, the antigen for which this pattern was most prominent, has been proposed as a marker of myeloid differentiation.[32] Furthermore, *PR3*, *SURVIVIN*, and *hTERT* were all expressed at lower levels in each of the CML $CD34^+$ subsets analyzed (including the $CD34^+CD38^-ALDH^{high}$ fraction), than in the normal unfractionated $CD34^+$ cells. Protein levels of *PR3* reflected the mRNA expression levels, and the same has been reported previously for *SURVIVIN*[33] and *hTERT*.[34] These data suggest that efforts to target these antigens in CML stem cells may be compromised by substantial toxicity to normal hematopoietic cells. Additionally, these results illustrate that the $CD34^+$ total fraction in CML is not necessarily representative of the LSC and underscore the importance of assessing target expression in refined LSC populations.

Conversely, given their more selective expression, including at the level of the LSC, *WT1* and *PRAME* may be more suitable therapeutic targets in CML. WT1 appears to act downstream of BCR-ABL and has been implicated in inhibition of apoptosis;[14,35] it has also been proposed as a marker for minimal residual disease.[36,37] In contrast to the somewhat disappointing experience to date with vaccines against BCR-ABL, early promise has been seen with WT1 vaccines in myeloid leukemias.[38] Additionally, *WT1* siRNA has been demonstrated to decrease proliferation and increase apoptosis of leukemic cell lines, including K562, supplying further evidence of a critical role for this antigen.[35,39] It remains to be determined whether the degree of over-expression of *WT1* in both CP and BC CML provides a sufficient therapeutic window to be exploited.

PRAME may be a superior target, given its minimal to null expression in the normal $CD34^+$ cells, particularly the $CD34^+CD38^-ALDH^{high}$ subset. In fact, *PRAME* appears to have very limited expression in any normal adult tissue, other than testis.[13] Our results support existing data that *PRAME* expression increases from CP to advanced phase CML, suggesting a role in leukemic progression.[40] Furthermore, PRAME has been demonstrated to interfere with retinoic acid signaling[41] and may thus contribute to the inhibition of differentiation in advanced phase CML.[42] Though PRAME has been recommended as a

vaccine target in CML,[12,16] to our knowledge, there are yet no active clinical trials specifically targeting it.

Interestingly, investigators at our institution have developed a K562-based vaccine engineered to produce GM-CSF;[43] this vaccine has shown promise in early phase clinical trials against CML.[44] The K562 cell line, upon which the vaccine is based, expresses low levels of *PR3*, but high levels of both *WT1* and *PRAME*, similar to the expression pattern seen in CML stem cells (Figure 3 & Figure 4). Thus, this vaccine may be capable of selectively delivering CML stem cell antigens to the immune system. Since both *WT1* and *PRAME* mRNA expression were similar in all leukemic $CD34^+$ cell fractions, the $CD34^+$ total fraction could serve as a surrogate marker for the LSC with regard to these two targets, facilitating clinical screening of patients.

Yong *et al* previously reported on antigen expression by phenotypically defined populations of CML stem and progenitor cells.[12] They, too, found *WT1* mRNA to be more highly expressed in CML stem cells than in normal hematopoietic stem/progenitor cells and *PRAME* mRNA to be over-expressed in advanced phase CML. Likewise, they also found substantially higher levels of *PR3* mRNA expression in the differentiated CML and normal $CD34^+$ subsets than in their more primitive counterparts.[12] Importantly, our results confirm these findings in functionally defined CML stem/progenitor populations and also demonstrate that protein expression of these putative therapeutic targets correlates with mRNA levels.

Despite the remarkable success of tyrosine kinase inhibitors against CML, these drugs do not seem to cure the disease.[3,4] This appears to be due to the failure of these agents to reliably eliminate the CML stem cells.[5–7,10,45] The long term implications of the persistence of CML stem cells in patients with responsive disease remain unclear. Moreover, in a significant minority of patients with CML, including most with BC CML, the disease will not respond to tyrosine kinase inhibition or ultimately progresses in spite of it.[9,11,45] Thus, the search for novel therapies, especially those that target the rare CML stem cells, remains imperative. Although CML stem cells share many characteristics with their normal counterparts, *WT1* and *PRAME*, like *BCR-ABL*, are selectively expressed by the CML stem/progenitor cells. These candidates thus hold promise as potential therapeutic targets in CML. Further work is warranted for their functional validation.

Acknowledgments

The authors would like to thank James P. Barber and Laura Morsberger for their expert technical assistance.

This work was supported by grants from the National Institutes of Health (PO1 CA15396, PO1 CA070970, and 5 T32 HL0075225); an American Society of Hematology Research Training Award for Fellows; and a Maryland Stem Cell Research Fund Post-Doctoral Fellowship grant (RFA-MD-07-3)

References

1. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med. 344:1031–1037. [PubMed: 11287972]
2. Kantarjian H, Talpaz M, O'Brien S, et al. High-dose imatinib mesylate therapy in newly diagnosed Philadelphia chromosome-positive chronic phase chronic myeloid leukemia. Blood. 103:2873–2878. [PubMed: 15070658]
3. Cortes J, O'Brien S, Kantarjian H H. Discontinuation of imatinib therapy after achieving a molecular response. Blood. 104:2204–2205. [PubMed: 15377577]
4. Rousselot P, Huguet F, Rea D, et al. Imatinib mesylate discontinuation in patients with chronic myelogenous leukemia in complete molecular remission for more than 2 years. Blood. 109:58–60. [PubMed: 16973963]

5. Graham SM, Jorgensen HG, Allan E, et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood. 99:319–325. [PubMed: 11756187]
6. Angstreich GR, Matsui W, Huff CA, et al. Effects of imatinib and interferon on primitive chronic myeloid leukaemia progenitors. Br J Haematol. 130:373–381. [PubMed: 16042686]
7. Copland M, Hamilton A, Elrick LJ, et al. Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction. Blood. 107:4532–4539. [PubMed: 16469872]
8. Bhatia R, Holtz M, Niu N, et al. Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment. Blood. 101:4701–4707. [PubMed: 12576334]
9. Shah NP. Advanced CML: therapeutic options for patients in accelerated and blast phases. J Natl Compr Canc Netw. 6 Suppl 2:S31–S36. [PubMed: 18397679]
10. Savona M, Talpaz M. Getting to the stem of chronic myeloid leukaemia. Nat Rev Cancer. 8:341–350. [PubMed: 18385684]
11. Heaney NB, Holyoake TL. Therapeutic targets in chronic myeloid leukaemia. Hematol Oncol. 25:66–75. [PubMed: 17441215]
12. Yong AS, Keyvanfar K, Eniafe R, et al. Hematopoietic stem cells and progenitors of chronic myeloid leukemia express leukemia-associated antigens: implications for the graft-versus-leukemia effect and peptide vaccine-based immunotherapy. Leukemia. 22:1721–1727. [PubMed: 18548092]
13. Greiner J, Dohner H, Schmitt M. Cancer vaccines for patients with acute myeloid leukemia--definition of leukemia-associated antigens and current clinical protocols targeting these antigens. Haematologica. 91:1653–1661. [PubMed: 17145602]
14. Svensson E, Vidovic K, Lassen C, et al. Deregulation of the Wilms' tumour gene 1 protein (WT1) by BCR/ABL1 mediates resistance to imatinib in human leukaemia cells. Leukemia. 21:2485–2494. [PubMed: 17728783]
15. Badran A, Yoshida A, Wano Y, et al. Expression of the antiapoptotic gene survivin in chronic myeloid leukemia. Anticancer Res. 23:589–592. [PubMed: 12680151]
16. Greiner J, Schmitt M. Leukemia-associated antigens as target structures for a specific immunotherapy in chronic myeloid leukemia. Eur J Haematol. 80:461–468. [PubMed: 18284627]
17. Phatak P, Burger AM. Telomerase and its potential for therapeutic intervention. Br J Pharmacol.
18. Nakahara T, Takeuchi M, Kinoyama I, et al. YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts. Cancer Res. 67:8014–8021. [PubMed: 17804712]
19. Bedi A, Zehnbauer BA, Collector MI, et al. BCR-ABL gene rearrangement and expression of primitive hematopoietic progenitors in chronic myeloid leukemia. Blood. 81:2898–2902. [PubMed: 8499629]
20. Wang JC, Dick JE. Cancer stem cells: lessons from leukemia. Trends Cell Biol. 15:494–501. [PubMed: 16084092]
21. Gal H, Amariglio N, Trakhtenbrot L, et al. Gene expression profiles of AML derived stem cells; similarity to hematopoietic stem cells. Leukemia. 20:2147–2154. [PubMed: 17039238]
22. Bonnet D. Normal and leukaemic stem cells. Br J Haematol. 130:469–479. [PubMed: 16098060]
23. Christ O, Lucke K, Imren S, et al. Improved purification of hematopoietic stem cells based on their elevated aldehyde dehydrogenase activity. Haematologica. 92:1165–1172. [PubMed: 17666374]
24. Pierre-Louis O, Clay D, Brunet de la GP, et al. Dual SP/ALDH Functionalities Refine the Human Hematopoietic Lin(−)CD34(+)CD38(−) Stem/Progenitor Cell Compartment. Stem Cells. 27:2552–2562. [PubMed: 19650038]
25. Brodsky RA, Jones RJ. Aplastic anaemia. Lancet. 365:1647–1656. [PubMed: 15885298]
26. Matsui W, Wang Q, Barber JP, et al. Clonogenic multiple myeloma progenitors, stem cell properties, and drug resistance. Cancer Res. 68:190–197. [PubMed: 18172311]
27. Jones RJ, Gocke CD, Kasamon YL, et al. Circulating clonotypic B cells in classic Hodgkin lymphoma. Blood. 113:5920–5926. [PubMed: 19188663]

28. Jones RJ, Barber JP, Vala MS, et al. Assessment of aldehyde dehydrogenase in viable cells. Blood. 85:2742–2746. [PubMed: 7742535]
29. Proto-Siqueira R, Figueiredo-Pontes LL, Panepucci RA, et al. PRAME is a membrane and cytoplasmic protein aberrantly expressed in chronic lymphocytic leukemia and mantle cell lymphoma. Leuk Res. 30:1333–1339. [PubMed: 16620968]
30. Sirard C, Lapidot T, Vormoor J, et al. Normal and leukemic SCID-repopulating cells (SRC) coexist in the bone marrow and peripheral blood from CML patients in chronic phase, whereas leukemic SRC are detected in blast crisis. Blood. 87:1539–1548. [PubMed: 8608245]
31. Lapidot T, Sirard C, Vormoor J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature. 367:645–648. [PubMed: 7509044]
32. Dengler R, Munstermann U, al-Batran S, et al. Immunocytochemical and flow cytometric detection of proteinase 3 (myeloblastin) in normal and leukaemic myeloid cells. Br J Haematol. 89:250–257. [PubMed: 7873374]
33. Badran A, Yoshida A, Wano Y, et al. Expression of the anti-apoptotic gene survivin in myelodysplastic syndrome. Int J Oncol. 22:59–64. [PubMed: 12469185]
34. Poole JC, Andrews LG, Tollefsbol TO. Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). Gene. 269:1–12. [PubMed: 11376932]
35. Elmaagacli AH, Koldehoff M, Peceny R, et al. WT1 and BCR-ABL specific small interfering RNA have additive effects in the induction of apoptosis in leukemic cells. Haematologica. 90:326–334. [PubMed: 15749664]
36. Tamaki H, Ogawa H. Monitoring of minimal residual disease using WT1 assay for patients with chronic myeloid leukemia who undergo allogeneic stem cell transplantation. Bone Marrow Transplant. 34:653–654. [PubMed: 15258561]
37. Cilloni D, Saglio G. WT1 as a universal marker for minimal residual disease detection and quantification in myeloid leukemias and in myelodysplastic syndrome. Acta Haematol. 112:79–84. [PubMed: 15179007]
38. Rusakiewicz S, Molldrem JJ. Immunotherapeutic peptide vaccination with leukemia-associated antigens. Curr Opin Immunol. 18:599–604. [PubMed: 16870418]
39. Glienke W, Maute L, Koehl U, Esser R, Milz E, Bergmann L. Effective treatment of leukemic cell lines with wt1 siRNA. Leukemia. 21:2164–2170. [PubMed: 17690705]
40. Radich JP, Dai H, Mao M, et al. Gene expression changes associated with progression and response in chronic myeloid leukemia. Proc Natl Acad Sci U S A. 103:2794–2799. [PubMed: 16477019]
41. Epping MT, Wang L, Edel MJ, Carlee L, Hernandez M, Bernards R. The human tumor antigen PRAME is a dominant repressor of retinoic acid receptor signaling. Cell. 122:835–847. [PubMed: 16179254]
42. Oehler VG, Guthrie KA, Cummings CL, et al. The preferentially expressed antigen in melanoma (PRAME) inhibits myeloid differentiation in normal hematopoietic and leukemic progenitor cells. Blood. 114:3299–3308. [PubMed: 19625708]
43. Borrello I, Sotomayor EM, Cooke S, Levitsky HI. A universal granulocyte-macrophage colony-stimulating factor-producing bystander cell line for use in the formulation of autologous tumor cell-based vaccines. Hum Gene Ther. 10:1983–1991. [PubMed: 10466632]
44. Smith BD, Kasamon YL, Kowalski J, et al. K562/GM-CSF immunotherapy reduces tumor burden in chronic myeloid leukemia patients with residual disease on imatinib mesylate. Clin Cancer Res. 16:338–347. [PubMed: 20048335]
45. Irvine DA, Heaney NB, Holyoake TL. Optimising chronic myeloid leukaemia therapy in the face of resistance to tyrosine kinase inhibitors--a synthesis of clinical and laboratory data. Blood Rev. 24:1–9. [PubMed: 20005615]

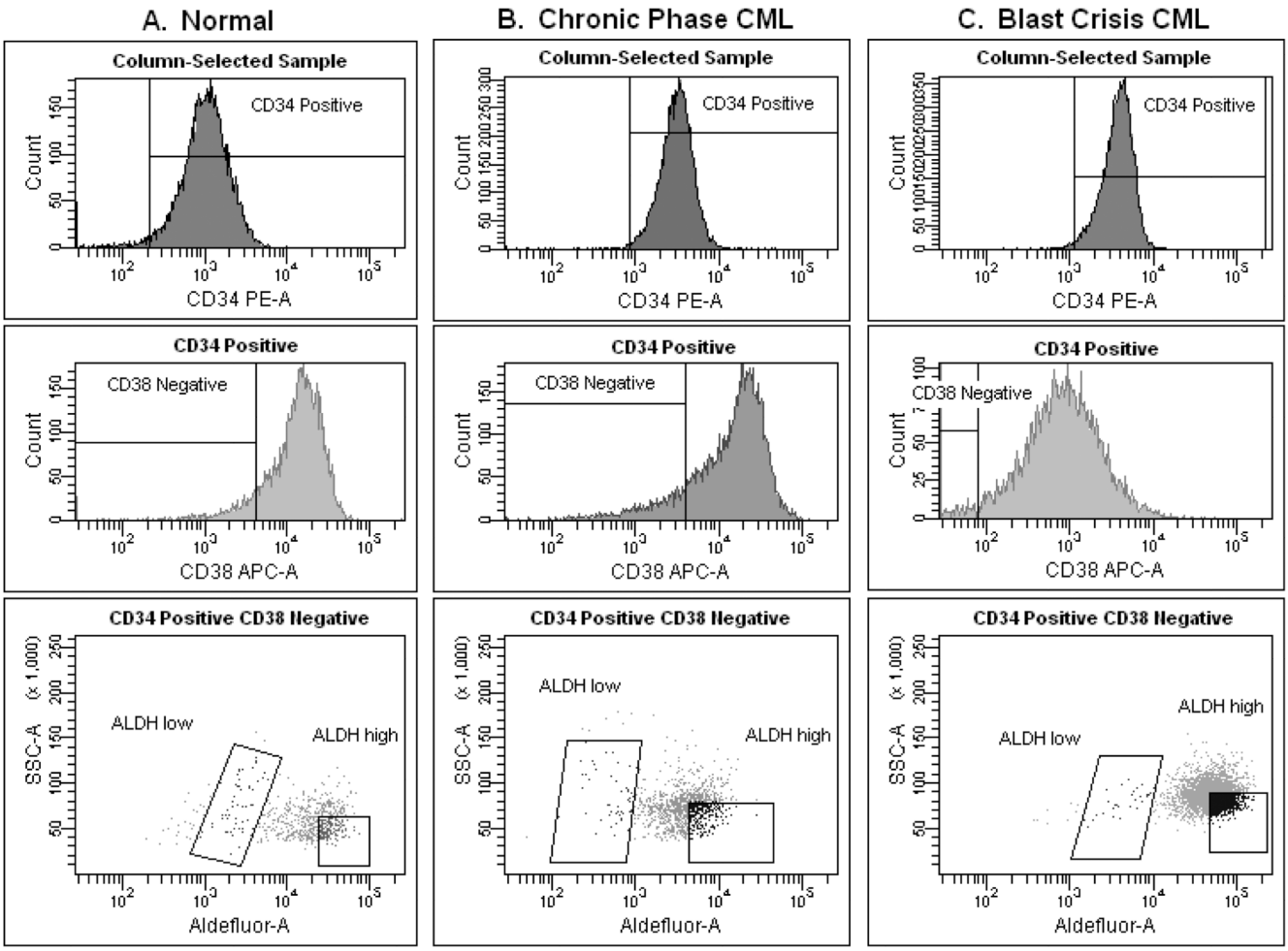

Figure 1. Staining profiles and gating strategies

Representative staining patterns and gating strategies are depicted for a normal sample (A), a chronic phase CML sample (B), and a blast crisis CML sample (C). Following Ficoll-Paque density centrifugation and $CD34^+$-selection via magnetic bead and column, each sample was stained (for CD34, CD38, and ALDH) and then sorted. The $CD34^+$ total, $CD34^+CD38^-ALDH^{low}$, and (low side scatter) $CD34^+CD38^-ALDH^{high}$ populations were collected and analyzed from each sample. Given the variation across samples, attempts were made to isolate equivalent populations based on relative size and staining.

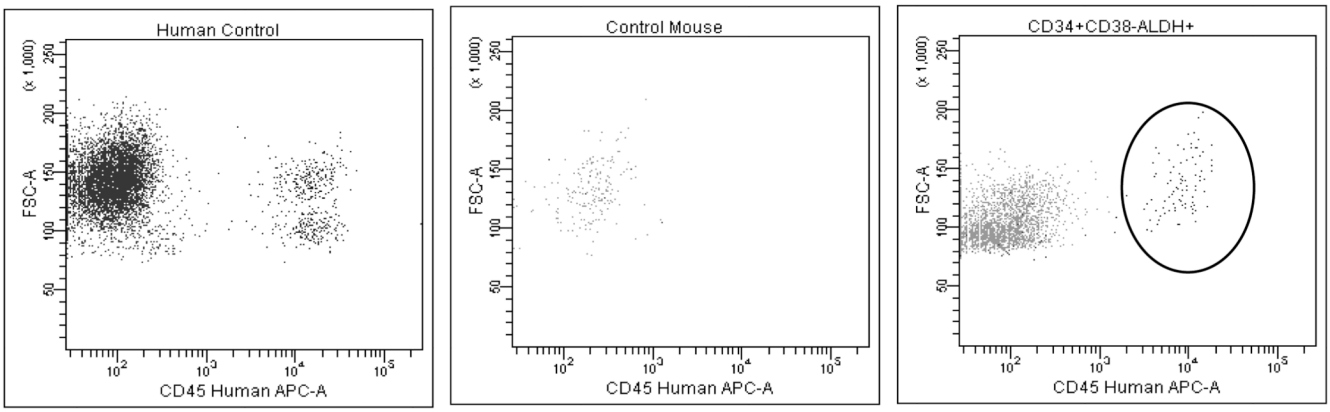

Figure 2. The CD34^{+}CD38^{-}ALDH^{high} fraction was highly enriched for NOG mouse engrafting capacity

Left panel: a human control, depicting a CD45^{+} cell population. Middle panel: an untransplanted NOG mouse control, lacking a detectable human CD45+ population. Right panel: a NOG mouse which engrafted with a detectable human CD45+ population after injection with the CD34^{+}CD38^{-}ALDH^{high} fraction of a CP CML sample several months prior; 98% of the sorted human CD45+ cells from this sample were positive for *BCR-ABL* by FISH.

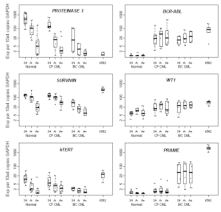

Figure 3. mRNA expression of *PROTEINASE 3, SURVIVIN, hTERT*, BCR-ABL, *WT1*, and *PRAME* in CD34$^+$ cell subsets of normal controls and CML patients

Boxplots of log (base 10) mRNA expression (per 10^4 copies of GAPDH) of each candidate target by clinical phase (Normal, Chronic Phase (CP) CML, or Blast Crisis (BC) CML) and within phase by cell phenotype: CD34$^+$ total (34), CD34$^+$CD38$^-$ALDH^{low} (A−), and CD34$^+$CD38$^-$ALDH^{high} (A+). The K562 cell line is included as a control. Each dot refers to an individual sample. Horizontal lines indicate median expression.

PROTEINASE 3, *SURVIVIN*, and *hTERT* expression in CP and BC CML paralleled that in normal cells. Expression of each of these genes was highest in the normal CD34$^+$ total cells and low in the CP and BC CML CD34$^+$CD38$^-$ALDH^{high} cells. In contrast, BCR-ABL, *WT1* (p=0.038 for CP CML and p=0.042 for BC CML vs. normal), and *PRAME* (p=0.056 for CP CML and p=0.032 for BC CML vs. normal) exhibited significantly higher expression in CML than in normal. *BCR-ABL* and *WT1* did not differ significantly between cell fractions or between CP and BC. *PRAME* expression also did not differ significantly by cell fraction, but it did significantly increase from CP to BC (p=0.039).

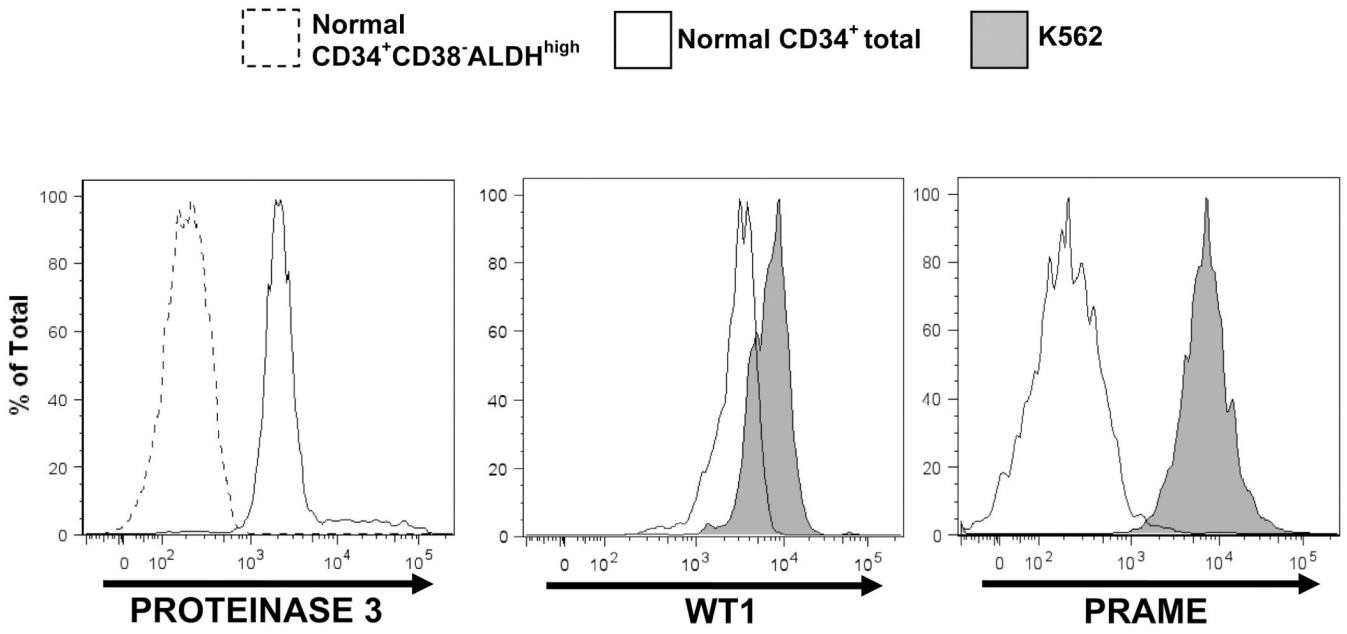

Figure 4. Protein staining by flow cytometry mirrors mRNA expression for *PROTEINASE 3, WT1*, and *PRAME*

Left panel: PROTEINASE 3 staining was markedly higher in normal $CD34^+$ total cells (solid curve) than in normal $CD34^+CD38^-ALDH^{high}$ cells (dotted curve). Middle panel: WT1 staining was higher in the K562 cell line (shaded) than in normal $CD34^+$ total cells (solid curve). Right panel: PRAME staining was substantially higher in the K562 cell line (shaded) than in normal $CD34^+$ total cells (solid curve).

Table I

Patient Characteristics

Sample #	Disease Phase	Age	Sex	Race	*BCR-ABL* FISH	*BCR-ABL* PCR*	Other chromosomal abnormalities
1	chronic	39	Male	Hispanic	96.5%	144	None
2	chronic	53	Male	African-American	95.5%	533	None
3	chronic	43	Female	White	81.5%	645	None
4	chronic	54	Male	African-American	73.5%	902	None
5	chronic	48	Male	Hispanic	93.0%	531	None
6	chronic	59	Male	White	94.5%	379	Inv 2
7	chronic	50	Female	White	83.5%	857	None
8	chronic	62	Female	White	96.5%	562	None
9	chronic	25	Male	White	96.0%	1495	t(9;16)
10	chronic	68	Male	White	96.5%	345	None
11	blast	52	Male	White	70.5%	615	Inv 3 & t(1;10)
12	blast	62	Female	White	82.0%	433	+8
13	blast	64	Female	African-American	93.5%	808	del 12p†
14	blast	37	Female	African-American	87.5%	195	t(2;13)

*copies of *BCR-ABL* per 1,000 copies *ABL*

†subclone present in 5/20 metaphases with additional abnormalities of +7, +8, and +21

Table II

Percentages of each sorted cell fraction positive for *BCR-ABL* by FISH in chronic phase (CP) and blast crisis (BC) CML.

	$CD34^+$ total	$CD34^+CD38^-ALDH^{low}$	$CD34^+CD38^-ALDH^{high}$
CP CML mean	94%	92.8%	85.2%
CP CML median	97.5%	95.5%	94%
CP CML range	70–99.5%	79–99%	39.5–100%
BC CML mean	98.4%	93.3%	98.6%
BC CML median	98.5%	92.8%	98.5%
BC CML range	97–99.5%	88–99.5%	98.3–99%