Clinical Cancer Research

Identification of CD25 as STAT5-Dependent Growth Regulator of Leukemic Stem Cells in Ph$^+$ CML

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Abstract

Purpose: In chronic myelogenous leukemia (CML), leukemic stem cells (LSC) represent a critical target of therapy. However, little is known about markers and targets expressed by LSCs. The aim of this project was to identify novel relevant markers of CML LSCs.

Experimental Design: CML LSCs were examined by flow cytometry, qPCR, and various bioassays. In addition, we examined the multipotent CD25$^+$ CML cell line KU812.

Results: In contrast to normal hematopoietic stem cells, CD34$^+$/CD38$^-$ CML LSCs expressed the IL-2 receptor alpha chain, IL-2RA (CD25). STAT5 was found to induce expression of CD25 in Lin$^-$/Sca-1$^+$/Kit$^+$ stem cells in C57Bl/6 mice. Correspondingly, shRNA-induced STAT5 depletion resulted in decreased CD25 expression in KU812 cells. Moreover, the BCR/ABL1 inhibitors nilotinib and ponatinib were found to decrease STAT5 activity and CD25 expression in KU812 cells and primary CML LSCs. A CD25-targeting shRNA was found to augment proliferation of KU812 cells *in vitro* and their engraftment *in vivo* in NOD/SCID-IL-2Rγ$^{-/-}$ mice. In drug-screening experiments, the PI3K/mTOR blocker BEZ235 promoted the expression of STAT5 and CD25 in CML cells. Finally, we found that BEZ235 produces synergistic antineoplastic effects on CML cells when applied in combination with nilotinib or ponatinib.

Conclusions: CD25 is a novel STAT5-dependent marker of CML LSCs and may be useful for LSC detection and LSC isolation in clinical practice and basic science. Moreover, CD25 serves as a growth regulator of CML LSCs, which may have biologic and clinical implications and may pave the way for the development of new more effective LSC-eradicating treatment strategies in CML. *Clin Cancer Res; 22(8); 2051–61. ©2015 AACR.*

Introduction

Chronic myelogenous leukemia (CML) is a hematopoietic stem cell (SC) disorder defined by the reciprocal translocation t(9;22) and the related oncoprotein, BCR/ABL1 (1–3). It is generally appreciated that BCR/ABL1 is a major driver responsible for initiation and evolution of CML (2–4). Correspondingly, the BCR/ABL1-targeting tyrosine kinase inhibitor (TKI) imatinib induces major cytogenetic and molecular responses in a majority of patients with chronic phase (CP) CML (4, 5). However, although long-term disease control can be achieved in many patients, imatinib is usually unable to eliminate CML. This phenomenon is best explained by intrinsic and acquired drug resistance in leukemic stem cells, LSC (6–12). The intrinsic form of resistance is common to all LSC fractions and is considered to be independent of BCR/ABL1. By contrast, the acquired form of TKI resistance is caused by newly acquired, subclone-specific defects, including *BCR/ABL1* mutations (7–13).

The 'LSC hypothesis' is based on the observation that only a subset of leukemic progenitors exhibits long-term disease-propagating capacity (14–16). This concept has major implications for the development of curative treatment approaches (7–19). LSC research is currently focusing on LSC-specific targets and drugs capable of attacking LSCs (17–19). In CML and other leukemias, the development of such LSC-targeting concepts is a major challenge (17–19). Notably, many different factors, including multiple signaling cascades and the so-called SC niche, regulate the development and expansion of LSCs in CML (9–11, 17–19).

One important regulator of survival and growth of CML LSCs appears to be the transcription factor STAT5 (20–23). A number of

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Translational Relevance

Although chronic myelogenous leukemia (CML) is a stem cell disease, little is known about the expression and function of specific markers and targets in CML LSCs. We here describe that CD25 serves as a novel robust marker of CML LSCs that can be used for detection, enumeration, and isolation of LSCs in these patients. Moreover, CD25 was found to serve as a drug-inducible suppressor of LSC expansion, which may be relevant clinically and may pave the way for the development of new treatment strategies. Based on our work, a straightforward approach might be to combine BCR/ABL1 tyrosine kinase inhibitors that downregulate CD25 expression with targeted drugs promoting CD25 expression, such as the PI3K/mTOR blocker BEZ235. Indeed, these drug combinations produced highly synergistic antiproliferative effects on KU812 cells in the present study.

previous and more recent studies have shown that BCR/ABL1 triggers STAT5 activity in CML cells (20–23). In addition, however, STAT5 expression and activation may be regulated independently of BCR/ABL1 in CML cells (11, 24). Especially in LSCs, STAT5 expression may be induced by BCR/ABL1-independent mechanisms. Recent data suggest that STAT5 triggers production of reactive oxygen species and clonal instability and thereby promotes the occurrence of *BCR/ABL1* mutations (24).

CML LSCs are considered to represent a small subset of $CD34^+/CD38^-$ cells in the leukemic clone (7–10, 25–27). However, since normal bone marrow stem cells (BM SCs) also display this phenotype, additional markers need to be applied to differentiate normal BM SCs from CML LSCs. Recent studies have shown that CML LSCs specifically express IL-1RAP and dipeptidyl-peptidase IV, DPPIV = CD26 (28–30). As assessed by gene array analyses, CML LSCs may express additional markers (30–32). One of these aberrant markers appears to be the low-affinity receptor for IL-2, CD25 (30–32). However, little is known about the functional role of CD25 in human CML LSCs and the mechanisms contributing to abnormal CD25 expression.

In this study, we show that expression of CD25 on CML LSCs is triggered by STAT5 and that CD25 acts as a negative regulator of LSC growth in CML. In addition, we show that BCR/ABL1 TKIs downregulate STAT5- and CD25 expression in LSCs, whereas the PI3K/mTOR blocker BEZ235 promotes CD25 expression.

Materials and Methods

Reagents

A detailed description of reagents used in this study is provided in the Supplement. Monoclonal antibodies (mAb) used in this study are described in Supplementary Table S1.

Cell lines

The multipotent human $BCR/ABL1^+$ cell line KU812 was kindly provided by Dr. K. Kishi (Niigata University, Niigata, Japan) in 1989; K562 cells and murine Ba/F3 cells expressing various BCR/ABL1 mutants (M244V, G250E, Q252H, Y253H, E255K, E255V, T315I, F317L, F317V, F359V, and H396P) or wild-type BCR/ABL1 were kindly provided by Dr. M. Deininger (Huntsman Cancer Institute, University of Utah, Salt Lake City, UT) in 2013; and imatinib-resistant K562 cells (K562-R) were kindly provided by Dr. J. D. Griffin (Dana-Farber Cancer Center, Harvard Medical School, Boston, MA) in 1999. KCL-22 cells were purchased from the German Collection of Microorganism and Cell Culture (DSMZ) in 2010. The identity of KU812, K562, and K562-R cells was confirmed by DSMZ using nonaplex-PCR in 2010. All experiments were performed from these stocks, and cells were thawed from these stocks (or secondary stocks) every 1 to 3 months. Cell lines were maintained in RPMI 1640 medium, 10% fetal calf serum (FCS), and antibiotics at 37°C. K562-R cells were cultured in the presence of 1 μmol/L imatinib. Mouse M2-10B4 feeder cells were purchased from the American Type Culture Collection. Ecotropic retroviral packaging cell lines GP^+/E86 encoding for STAT5A-IRES-GFP, STAT5B-IRES-GFP (33), or the empty vector, and GP^+/E86 cells encoding for $p210^{BCR-ABL1}$-IRES-dsRED (23) were maintained in complete medium supplemented with 10% FCS as described (23, 33).

Patients and cell sampling

Sixty-three patients with $BCR/ABL1^+$ CML (32 females, 31 males) were examined for expression of CD25 on $CD34^+/CD38^-$ CML LSCs and $CD34^+/CD38^+$ CML progenitor cells. The median age was 54 years (range, 18–86 years). Most patients were examined at diagnosis (before treated with BCR/ABL1 TKI). The patients' characteristics are shown in Supplementary Table S2. Peripheral blood (PB) and/or BM cells (iliac crest or sternum) were collected at diagnosis and in the follow-up. Control samples included normal/reactive BM and other myeloid neoplasms (Supplementary Table S3). All donors gave written informed consent, and all studies were approved by the ethics committees of the Medical University of Vienna and the University of Veterinary Medicine Vienna.

Flow cytometry experiments

Phenotyping of $CD34^+/CD45^+/CD38^-$ (L)SCs and $CD34^+/CD45^+/CD38^+$ progenitor cells was performed on BM or PB cells (unseparated or Ficoll-isolated) by multicolor flow cytometry as described (34, 35) using fluorochrome-conjugated mAb shown in Supplementary Table S1. Flow cytometry was performed on a FACSCalibur (Becton Dickinson). A detailed description of the staining techniques is provided in the Supplement.

Retroviral infection of murine BM cells

Retroviral packaging GP^+/E86 cells encoding for STAT5A-IRES-GFP, STAT5B-IRES-GFP (33), or empty vector were mixed 1:1 with GP^+/E86 cells encoding $p210^{BCR/ABL1}$-IRES-dsRED (23) and seeded on gelatine (0.2%) precoated dishes. Single-cell suspensions from total wild-type BM cells of C57Bl/6N mice were prepared, and 3×10^6 cells/mL were cocultured with GP^+/E86 cells in DMEM supplemented with murine IL-3 (25 ng/mL), IL-6 (50 ng/mL), stem cell factor, SCF (50 ng/mL), and polybrene (7 μg/mL) for 72 hours. Thereafter, BM cells were stained with mAb PC61 directed against CD25 and analyzed by flow cytometry using a BD FACS-Canto II FACS device and BD FACS Diva software (Becton Dickinson). Six-to-eight–week-old male C57Bl/6N mice (20 g) were purchased from the Jackson Laboratory.

Gene array analyses

KU812 cells transduced with a random (RDM) shRNA or a CD25 shRNA were subjected to RNA isolation and gene array

analysis using Affymetrix technology. Details are provided in the Supplement. A complete set of mRNA data is available at Gene Expression Omnibus, accession number: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=orotekeollgdduf&acc=GSE60315.

Quantitative PCR and cytogenetics

RNA was isolated from CML cell lines, primary CML (stem) cells, and pooled colony-derived cells. CD25-, CD122-, CD132-, and BCR/ABL1 mRNA expression levels were measured by qPCR as reported (29, 35). Conventional cytogenetics and FISH were performed according to published protocols (36). A detailed description of the methods applied is provided in the Supplement.

Transfection studies

Knockdown studies with shRNAs were performed following published techniques (37, 38). In CD25 knockdown studies, 3 shRNAs against human CD25 (clone #1: 5′-AAATCTGTTGTTGTGACGAGG-3′; clone #2: 5′-TTTCTGTTCTTCAGGTTGAGG-3′; clone #5: 5′-TACTCTGTTGTAAATATGGAC-3′; Open Biosystems) expressed in pLKO.1 lentiviral vector containing the puromycine-resistance gene were applied. In select experiments ($CD34^+$ cells), the puromycine-resistance gene was replaced by mCherry. For knockdown of STAT5, an shRNA targeting STAT5A and STAT5B (5′-GCAGCAGACCATCATCCTG-3′; ref. 37) expressed in pLKO.1 containing the puromycine-resistance gene was used. The shRNA-mediated knockdown of CD25 was induced in KU812 cells and primary $CD34^+$ CML cells, and knockdown of STAT5A/STAT5B in KU812 cells. Transfected KU812 cells were selected by exposure to puromycin (2 μg/mL), and transduced primary $CD34^+$ CML cells by sorting for mCherry. Knockdown was confirmed by flow cytometry (CD25) or Western blotting (STAT5) according to published techniques (39). To evaluate the functional consequence of the CD25 knockdown, CD25 shRNA (clones #1, #2, and #5)-transduced KU812 cells and KU812 transduced with random (RDM) shRNA were mixed at 1:1 and cultured. Cell mixtures were periodically examined for CD25 expression by flow cytometry using phycoerythrin (PE)-conjugated mAb 2A3 against CD25. To study proliferation of KU812 cells and primary $CD34^+$ CML cells transduced with CD25 shRNA (clone #2) or RDM shRNA, ^{3}H-thymidine uptake experiments were performed. In a separate set of experiments, CD25 was lentivirally expressed in K562, K562-R, and KCL-22 cells. A precision LentiORF IL-2RA (CD25) gene construct (GFP tag) and a control construct was obtained from GE Dharmacon. Virus production and transduction of cells were performed as described above, and GFP^+ cells were selected by cell sorting. Purified cells were applied in mixing experiments (1:1 of empty vector control-transduced cells and CD25-transduced cells) as described above and analyzed 3 times/week using APC-conjugated CD25 mAb BC96.

Xenotransplantation assay

To investigate the functional role of CD25 in CML cells *in vivo*, KU812 cells transduced with shRNA against CD25 (clone #2) or with RDM shRNA were injected intravenously into nonirradiated nonobese diabetic NOD/SCID-IL-2R$\gamma^{-/-}$ (NSG) mice (5 per group). Ten-to-twelve-week-old, female NSG mice (20–30 g) were purchased from the Jackson Laboratory. Cells were resuspended in 0.15 mL RPMI with 10% FCS and antibiotics and injected into the tail vein of NSG mice (1×10^6 cells/mouse). After 6 weeks, blood was drawn to analyze engraftment. Then, mice were sacrificed and BM cells (tibia, femur, pelvis) and spleens were isolated and prepared for immunohistochemistry and flow cytometry. The size and weight of spleens were measured in each animal. Then, splenic samples were prepared as follows: each spleen was cut into small pieces with a scalpel and mashed through a small-sized filter to obtain single-cell suspensions. Long bones were flushed to recover BM cells for flow cytometry in order to document engraftment. Multicolor flow cytometry was performed using mAb 515 against CD44 for detection of KU812 cells. TO-PRO-3 (Invitrogen) was used to exclude nonviable cells.

Long-term culture-initiating cell assay

To investigate the long-term growth of sorted $CD34^+/CD38^-/CD25^+$ LSCs and $CD34^+/CD38^-/CD25^-$ (presumably normal) SCs obtained from 2 patients with CP CML, a long-term culture-initiating cell (LTC-IC) assay was performed as described (29). A detailed description of the method applied is provided in the Supplement.

Statistical evaluations

To determine the level of significance in differences in CD25 expression and pSTAT5 levels in LSCs in various donor-groups or before and after drug exposure, the Student *t* test was applied. Results were considered to be significantly different, when $P < 0.05$. Drug combination experiments were performed following published guidelines (40). Drug interactions were determined by calculating combination index (CI) values using Calcusyn software (Calcusyn; Biosoft) as described previously (40, 41). A CI of less than 1 indicates synergy, a CI of 1 an additive effect, and a CI of more than 1 an antagonistic effect.

Results

Human CML LSCs express IL-2RA (CD25)

As assessed by flow cytometry, $CD34^+/CD38^-$ LSCs expressed CD25 in 50 of 54 patients with CML (92.6%; Fig. 1A and B). CD25 was detected on LSCs in patients with CP (40/43), accelerated phase (AP, 2/3), and blast phase (BP, 8/8). As expected, highly enriched CML LSCs expressed CD25 mRNA in all samples tested (Fig. 1C). $CD34^+/CD38^+$ CML progenitor cells expressed only trace amounts or no detectable CD25 (Fig. 1C and D). During treatment with imatinib (14 patients) or other TKIs (3 patients), the numbers of $CD25^+$ LSCs decreased in the BM compared to pretreatment values (Fig. 1E). CML LSCs did not express IL-2RB (CD122) or IL-2RG (CD132) at the mRNA level (Fig. 1C) or protein level (Table 1). In the normal/reactive BM, $CD34^+/CD38^-$ SCs did not express CD25 (Fig. 1B). Moreover, CD25 was not detectable on $CD34^+/CD38^-$ SCs in various control groups, including 13 of 14 patients with idiopathic cytopenia of unknown significance (ICUS), other classical myeloproliferative neoplasms (MPN), or mastocytosis (Fig. 1B).

Aberrant expression of CD25 on CML cells is restricted to LSCs

As assessed by multicolor flow cytometry, $CD25^+$ CML LSCs coexpressed two other well-established LSC antigens, IL-1RAP and DPPIV (CD26). In addition, $CD25^+$ CML LSCs coexpressed Siglec-3/CD33, CD44, CD52, and KIT/CD117 (Table 1; Supplementary Fig. S1). Among more mature myeloid cells in the CML clone, only basophils were found to express CD25, with slightly higher expression levels observed in CML basophils compared

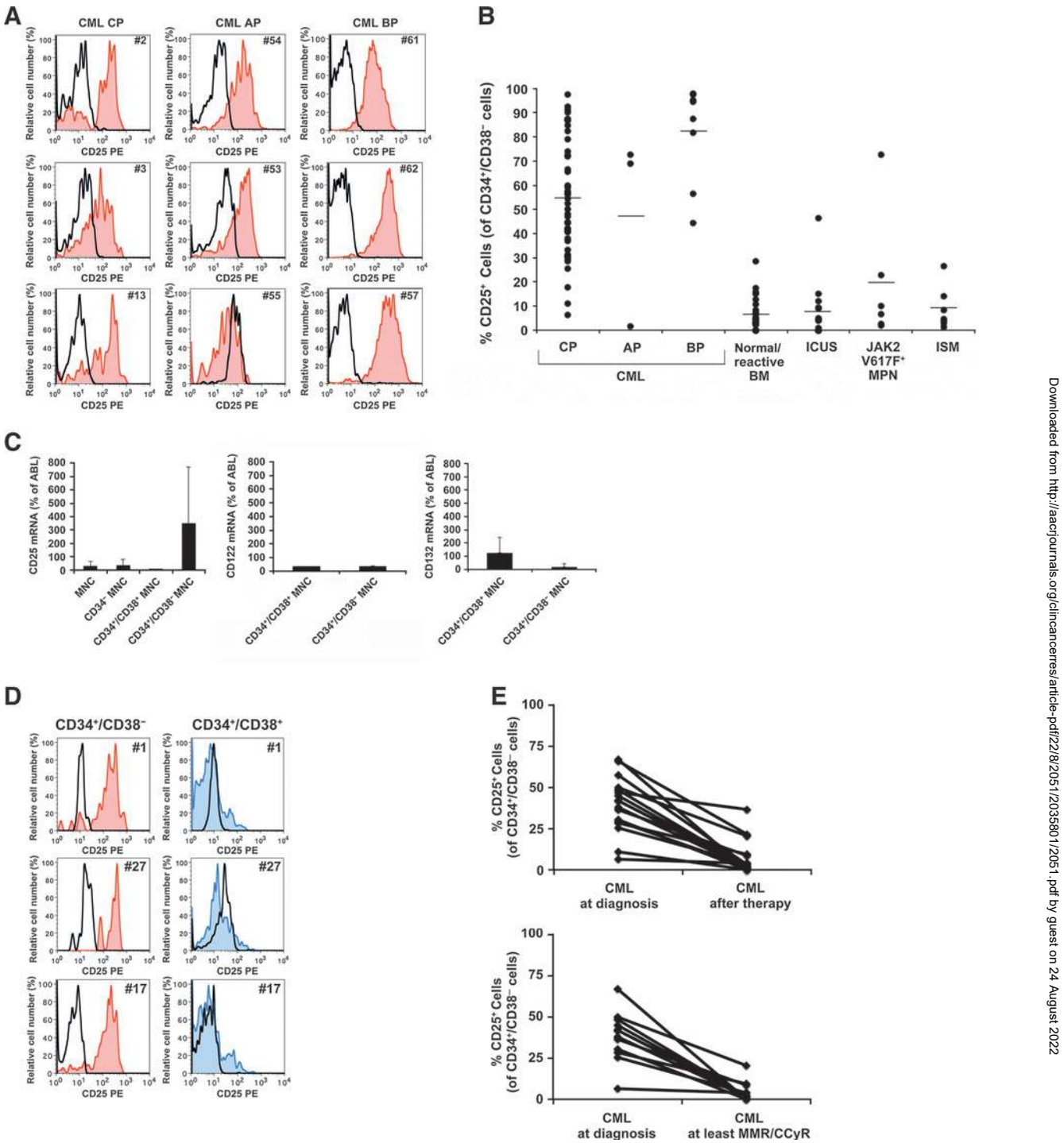

Figure 1.

$CD34^+/CD38^-$ stem cells in CML express CD25. A, CD25 expression on $CD34^+/CD38^-$ LSCs in 3 patients with CP CML, 3 in AP, and 3 in BP. Black histograms represent isotype-matched control antibodies, and red histograms represent CD25 expression. Patient numbers (#) refer to Supplementary Table S2. B, CD25 expression on $CD34^+/CD38^-$ LSCs in patients with CML (CP, $n = 43$; AP, $n = 3$; BP, $n = 8$), SCs in normal/reactive BM ($n = 24$), idiopathic cytopenia (ICUS, $n = 14$), JAK2 $V617F^+$ MPNs ($n = 6$), and indolent mastocytosis (ISM, $n = 7$). Results show the percentage of $CD25^+$ SCs. Horizontal bars indicate median values in each group. C, qPCR analysis of CD25– (left), CD122– (middle), and CD132– (right) mRNA expression in sorted $CD34^+/CD38^-$ LSCs, $CD34^+/CD38^+$ progenitors, $CD34^-$ cells, and total mononuclear cells (MNC) obtained from 3 patients with CML. mRNA levels are expressed as a percentage of ABL1 and represent the mean ± SD from three experiments. D, expression of CD25 on $CD34^+/CD38^-$ LSCs (left) and $CD34^+/CD38^+$ cells (right) in 3 patients with CML. Black open histograms represent isotype-matched control antibodies, red histograms CD25 expression on $CD34^+/CD38^-$ LSCs (left), and blue histograms CD25 expression on $CD34^+/CD38^+$ progenitors (right). E, percentage of $CD25^+$ SCs at diagnosis, after therapy for at least 9 months in all patients ($n = 17$, top), and at the time of major molecular response (MMR) and complete cytogenetic response (CCyR) ($n = 12$, bottom). Patients were treated with imatinib (400 mg/day, $n = 14$), dasatinib (100 mg/day, $n = 1$), or nilotinib (2 × 400 mg/day; $n = 2$).

Table 1. Expression of surface antigens on $CD34^+/CD38^-$ and $CD34^+/CD38^+$ cells in patients with CML and in normal/reactive BM

		CP CML		AP CML		BP CML		Normal/reactive BM	
CD	Antigen	$CD34^+/CD38^-$	$CD34^+/CD38^+$	$CD34^+/CD38^-$	$CD34^+/CD38^+$	$CD34^+/CD38^-$	$CD34^+/CD38^+$	$CD34^+/CD38^-$	$CD34^+/CD38^+$
CD25	IL-2RA	++	+/−	++	+	++	++	−	+/−
CD26	DPPIV	++	+/−	++	+/−	+	+/−	−	−
CD33	Siglec-3	++	++	++	++	+	+	+	++
CD44	Pgp1	++	++	++	++	++	++	++	++
CD52	Campath-1	+	+/−	n.t.	n.t.	+	+	+	+
CD122	IL-2RB	−	−	n.t.	n.t.	−	−	−	−
CD132	IL-2RG	−	−	n.t.	n.t.	n.t.	n.t.	−	−
CD117	KIT/SCFR	++	++	++	++	n.t.	n.t.	++	++
n.c.	IL-1RAP	+	+	n.t.	n.t.	++	++	−	+/−

Abbreviations: IL-1RAP, IL1 receptor accessory protein; IL-2RB, IL-2 receptor beta; IL-2RG, IL-2 receptor gamma; n.c., not yet clustered; n.t., not tested; SCFR, stem cell factor receptor; ++, strongly expressed in a majority of cases; +, clear expression in majority of cases; +/−, expression in minority of cases or weak expression in majority of cases; −, no expression in a vast majority of cases.

with normal basophils (Table 2). In all other myeloid lineages examined, including monocytes and mast cells, CD25 was not detectable in our CML patients. These data suggest that aberrant expression of CD25 in the CML clone is restricted to LSCs. In a subset of patients, we separated the $CD25^+$ and the $CD25^-$ SC fractions from the same samples to near homogeneity (>95% purity) by cell sorting. In these experiments, all $CD25^+$ LSCs (100%) contained BCR/ABL1 by FISH, whereas only 45% of the $CD25^-$ SCs expressed BCR/ABL1 (Supplementary Fig. S2; Supplementary Table S4). The long-term proliferative (LTC-IC) capabilities of $CD25^+$ CML LSCs and normal $CD25^-$ SCs obtained from the same patients are shown in Supplementary Fig. S3.

STAT5 activity contributes to the expression of CD25 in LSCs

Because CD25 is a STAT5 target gene, we applied shRNA against STAT5, which led to a substantial knockdown of STAT5 expression in KU812 cells (Supplementary Fig. S4). The STAT5 knockdown resulted in a decreased expression of CD25 at the mRNA and protein level (Fig. 2A). A STAT5-targeting drug, pimozide, was also found to downregulate CD25 expression in KU812 cells (Fig. 2B). A similar effect was seen with the MEK inhibitors PD0325901 and RDEA119 (Fig. 2C), whereas the PI3K/mTOR blocker BEZ235 did not inhibit CD25 expression. Rather, BEZ235, the mTOR-targeting drug RAD001, and the PI3K inhibitor LY294002, were found to upregulate expression of CD25 in KU812 cells (Fig. 2D) as well as in the other CML cell lines examined (Supplementary Fig. S5A). Similar results were obtained with primary CML LSCs. Again, the STAT5 blocker pimozide decreased CD25 expression (Fig. 3A), and BEZ235 and RAD001 were found to augment CD25 expression on $CD34^+/CD38^-$ CML LSCs (Fig. 3B). Finally, BEZ235 was found to upregulate CD25 expression on Ba/F3 cells containing various BCR/ABL1 mutants, including E255K, F317L, and T315I (Supplementary Fig. S5B). As expected, BEZ235 was also found to inhibit proliferation in these cells (Supplementary Fig. S5C). Together, these data suggest that expression of CD25 on CML LSCs is regulated by a signaling cascade involving STAT5, PI3K, and mTOR.

Table 2. Expression of CD25, CD122, and CD132 on various cell types in CML and comparison with cells obtained from healthy donors[a]

	Expression of		
Types of cell	CD25	CD122	CD132
Control			
$CD3^+$ T cells	+/−	+/−	+
$CD19^+$ B cells	+/−	−	+/−
$CD14^+$ Monocytes	−	−	+/−
$CD123^+$ Basophils	+	−	+/−
CML			
$CD3^+$ T cells	+/−	+/−	+/−
$CD19^+$ B cells	+/−	−	+/−
$CD14^+$ Monocytes	−	−	−
$CD123^+$ Basophils	++	−	+/−

Abbreviation: CML, chronic myeloid leukemia.

[a]Expression of CD25, CD122, and CD132 was examined on PB or BM cell subsets by multicolor flow cytometry using monoclonal antibodies shown in Supplementary Table S1. Cells were analyzed in freshly obtained PB or BM samples (CML, $n = 5$; healthy controls, $n = 5$). Expression of CD25, CD122, and CD132 was determined as median fluorescence intensity (MFI) and is expressed as staining-index (SI), according to the formula: SI = MFI (test mAb)/MFI (isotype-control mAb). Results were scored as follows:
SI 0–1.3, −
SI 1.3–3, +/−
SI 3–10, +
SI >10, ++.

Effects of BCR/ABL1-targeting TKIs on expression of pSTAT5 and CD25 in CML cells

All three BCR/ABL1 TKIs tested (imatinib, nilotinib, and ponatinib) were found to inhibit the expression of pSTAT5 and of CD25 in KU812 cells (Fig. 3C). Nilotinib and ponatinib were also found to block CD25 expression in primary CML LSCs (Fig. 3D). However, the weaker BCR/ABL1 blocker imatinib showed no significant effect on expression of CD25 on primary CML LSCs (Fig. 3D). All three TKIs were found to downregulate expression of pSTAT5 in primary CML LSCs (Fig. 3E). Finally, we found that imatinib, nilotinib, and ponatinib downregulate the expression of CD25 in Ba/F3 cells expressing various mutant forms of BCR/ABL1, including E255K, G250E, F317V, and F317L (Supplementary Fig. S6).

Retroviral infection of murine SCs with STAT5 is followed by upregulation of CD25

Infection of BM stem cells of C57Bl/6 mice with a retroviral vector encoding either STAT5A or STAT5B, or coinfection of BCR/ABL1-p210 and STAT5, resulted in an enhanced expression of CD25 on $Lin^-/Sca\text{-}1^+/Kit^+$ (LSK) cells (Fig. 4). There was no difference in expression of CD25 on LSK cells when comparing cells infected with STAT5 alone with cells infected with both, STAT5 and BCR/ABL1 (Fig. 4). Interestingly, infection of BM cells with BCR/ABL1 alone was not followed by a major increase in expression of CD25 on LSK cells (Fig. 4). In line with this observation, infection with BCR/ABL1 alone also failed to substantially increase the expression of STAT5 in LSK cells (not shown). These data suggest that expression of CD25 in LSK cells

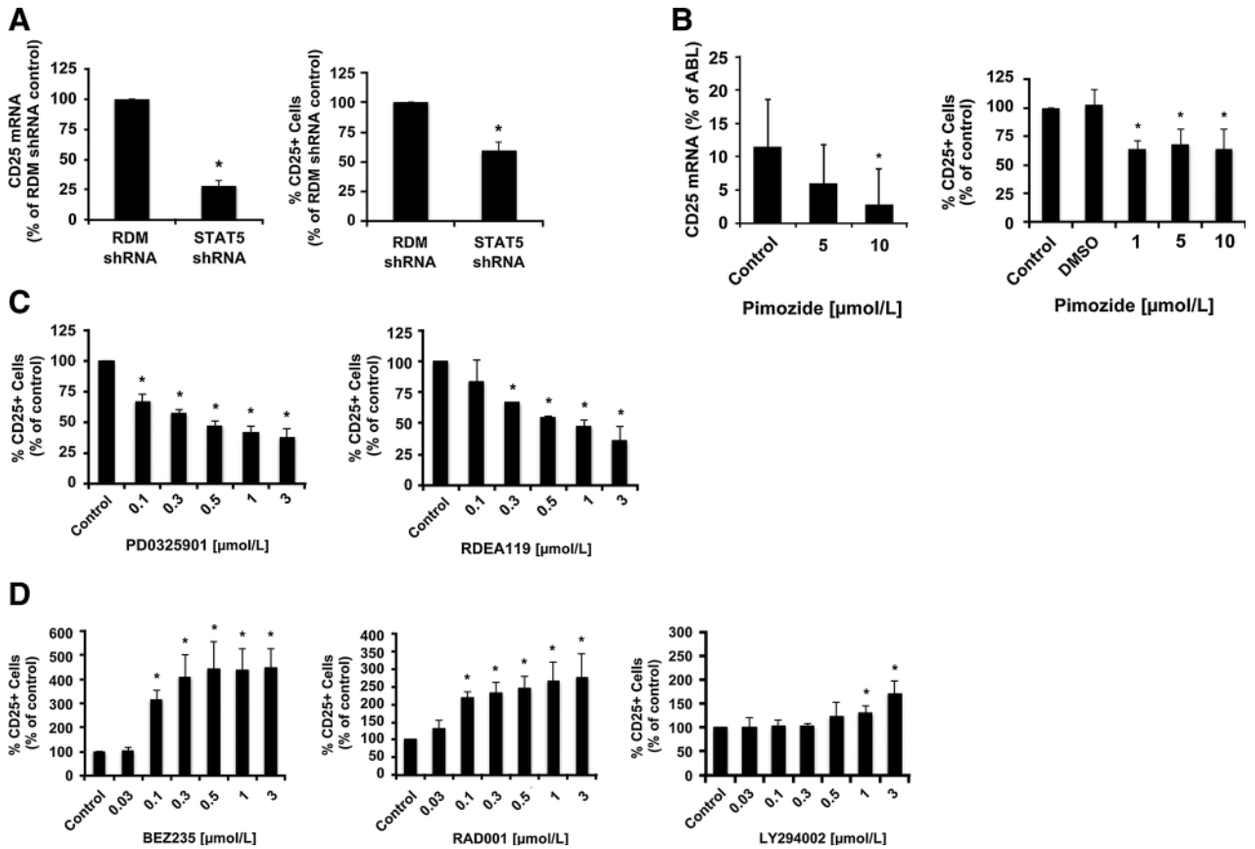

Figure 2.

Expression of CD25 on CML cells is dependent on STAT5 activity. A, KU812 cells transduced with shRNA against STAT5 or a random control (RDM) shRNA were analyzed for CD25 expression by qPCR (left) and flow cytometry (right). Results are expressed as a percentage of RDM control and represent mean ± SD from three (qPCR) or five (flow cytometry) experiments. Asterisk (*): $P < 0.05$ compared with RDM control. B, expression of CD25 mRNA levels (left) and CD25 surface levels (right) in KU812 cells after incubation in medium (control) or pimozide (1, 5, and 10 μmol/L) at 37°C for 24 hours. mRNA levels were quantified by qPCR, and surface expression by flow cytometry. Results are expressed as a percentage of ABL (qPCR) or percentage of medium control (flow cytometry), and represent the mean ± SD from five (qPCR) and four (flow cytometry) experiments. Asterisk (*): $P < 0.05$ compared with untreated cells (control). C, flow cytometric evaluation of CD25 expression on KU812 cells after incubation in control medium or with the MEK inhibitors PD0325901 or RDEA119 (each 0.1–3 μmol/L) at 37°C for 24 hours. Results are expressed as a percentage of control and represent the mean ± SD from 3 experiments. Asterisk (*): $P < 0.05$ compared with untreated cells (control). D, CD25 expression on KU812 cells after incubation in control medium, BEZ235, RAD001, or LY294002 (each 0.03–3 μmol/L) at 37°C for 24 hours. Results represent the mean ± SD from three independent experiments. Asterisk (*): $P < 0.05$ compared with untreated cells (control).

is triggered by STAT5 activity in the absence and presence of BCR/ABL1 in C57Bl/6 mice.

Identification of CD25 as a regulator of growth of CML cells

We next examined the potential biologic function of CD25 on CML LSCs. In initial experiments, we were unable to demonstrate any effects of IL-2 on growth of KU812 cells, K562 cells, or primary CML cells (Supplementary Fig. S7). In addition, preincubation with IL-2 did not alter responses of primary CML cells to the PI3K/mTOR inhibitor BEZ235 (Supplementary Fig. S7). Finally, we applied a random (RDM) shRNA and 3 different CD25-specific shRNAs (clone #1, #2, and #5) on KU812 cells. In these experiments, the knockdown of CD25 resulted in an increased proliferative capacity compared with cells transduced with an RDM shRNA (Fig. 5A). We also confirmed the growth advantage of CD25-negative KU812 cells *in vivo* in an NSG xenotransplantation model. NSG mice injected with CD25 shRNA–transfected (clone #2) KU812 cells showed significantly higher engraftment levels in the BM, PB, and spleen when compared with mice injected with control cells (Fig. 5B). In a next step, we transduced $CD34^+$ SC from 2 patients with CML CP (23%–30% of the $CD34^+$ cells expressed CD25; and >80% of the $CD34^+/CD38^-$ cells expressed CD25) with a specific CD25 shRNA (clone #2) or an RDM control shRNA. The knockdown of CD25 resulted in an enhanced proliferation of these $CD34^+$ CML cells (Fig. 5C). Finally, we expressed CD25 in the CD25-negative (or weakly CD25-positive) CML cell lines K562, K562-R, and KCL-22 by lentivirus-mediated transduction. In all three cell lines tested, transduction with CD25 resulted in decreased proliferation (Supplementary Fig. S8). Together, these data strongly suggest that CD25 acts as a "leukemia-suppressing" molecule in CML (stem) cells.

Identification of CD25-regulated target genes in KU812 cells

In an attempt to define the mechanism of CD25-induced growth inhibition in CML cells, we performed gene array analyses using KU812 cells transfected with RDM shRNA or CD25 shRNA (clone #2). In these analyses, a number of up- and down-regulated mRNA species were identified in CD25-depleted cells

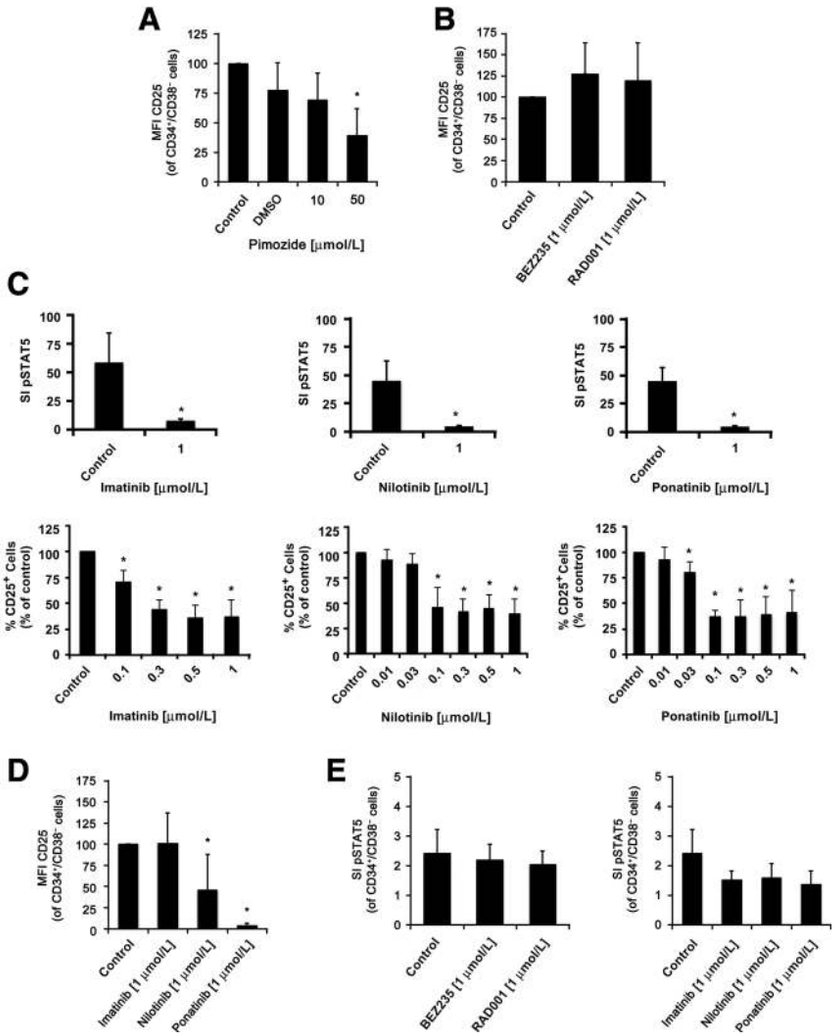

Figure 3.
Expression of CD25 on LSCs is dependent on STAT5 activity. A, CD25 expression on $CD34^+/CD38^-$ LSCs was analyzed by flow cytometry after incubation in control medium, pimozide (10 or 50 μmol/L), or DMSO at 37°C for 24 hours. Results show CD25 expression (median fluorescence intensity) on $CD34^+/CD38^-$ LSCs as a percentage of control and represent the mean ± SD from three independent experiments. Asterisk (*): $P < 0.05$ compared with untreated cells (control). B, CD25 expression on $CD34^+/CD38^-$ LSCs after incubation in control medium, BEZ235, or RAD001 (each 1 μmol/L) at 37°C for 24 hours. Results show CD25 expression on $CD34^+/CD38^-$ LSCs as a percentage of control and represent the mean ± SD from five independent experiments.
C, expression of pSTAT5 (top) and CD25 (bottom) in KU812 cells after incubation in control medium or TKIs (imatinib, nilotinib, ponatinib, each 0.01–1 μmol/L) at 37°C for 4 hours (pSTAT5) or 24 hours (CD25). Results are expressed as staining index (SI; pSTAT5) or as a percentage of control (CD25), and represent the mean ± SD from at least three independent experiments. Asterisk (*): $P < 0.05$ compared with untreated cells (control). D, CD25 expression on $CD34^+/CD38^-$ LSCs after incubation in control medium or TKIs (imatinib, nilotinib, or ponatinib, each 1 μmol/L) at 37°C for 24 hours. Results show CD25 expression on $CD34^+/CD38^-$ LSCs as a percentage of medium control and represent the mean ± SD of five independent experiments. Asterisk (*): $P < 0.05$ compared with untreated cells. E, expression of pSTAT5 in $CD34^+/CD38^-$ LSCs after incubation in control medium, BEZ235, RAD001 (left), or TKIs (imatinib, nilotinib, ponatinib, 1 μmol/L each; right) at 37°C for 4 hours. Results represent the mean ± SD from five independent experiments.

(Supplementary Table S5). Among the top upregulated gene products, a number of mediators of cell growth, proliferation, or survival were identified, including several members of the DAZ-family, RAB27B, IGFBP7, SSX2, RASGEF1A, or IGF-1 (Supplementary Table S5A). Downregulated genes included several "tumor-suppressor" genes, genes involved in lymphocyte activation, inflammatory response, and cell signalling (Supplementary Table S5B). Together, these data suggest that multiple target genes may contribute to CD25-induced growth inhibition in CML cells. In a next step, we performed pathway analyses in order to detect additional genes or gene patterns relevant to CD25-induced growth inhibition. However, no additional genes or pathways potentially involved in CD25-induced suppression of SC growth could be identified (Supplementary Fig. S9). We also compared cell-cycle progression, mitosis, and apoptosis in shRNA-transduced versus RDM control shRNA-transduced KU812 cells. In these experiments, we were able to show that the shRNA-induced knockdown of CD25 resulted in a slightly increased number of mitotic cells compared with RDM-transduced cells (Supplementary Fig. S10). By contrast, we were unable to detect any changes in the percentage of apoptotic cells after CD25 shRNA transduction (Supplementary Fig. S10). Finally, shRNA-induced knockdown of CD25 did not alter cell-cycle progression in KU812 cells (Supplementary Fig. S10).

Identification of CD25 as a BEZ235-induced growth inhibitor: a rationale for the design of drug combinations with synergistic effects

In a final step, we asked whether CD25 can serve as a secondary "drug effector" blocking the growth of CML cells. The shRNA-induced knockdown of CD25 in KU812 cells was not followed by any changes in their responses to imatinib, nilotinib, or ponatinib (comparable IC_{50} values; Supplementary Fig. S11A). However, the shRNA-induced knockdown of CD25 resulted in a decreased response of KU812 cells to BEZ235 (Supplementary Fig. S11A). Since BEZ235 upregulates CD25 in KU812 cells, these data suggest that CD25 may serve as a secondary target mediating BEZ235-induced growth inhibition. Because we also noted that BCR/ABL1 TKIs decrease CD25 expression, we asked whether combined application of BEZ235 and BCR/ABL1 TKIs would result in cooperative growth-inhibitory effects. Indeed, combinations of BEZ235 and nilotinib and BEZ235 and ponatinib resulted in strong synergistic growth-inhibitory effects in KU812 cells (Supplementary Fig. S11B).

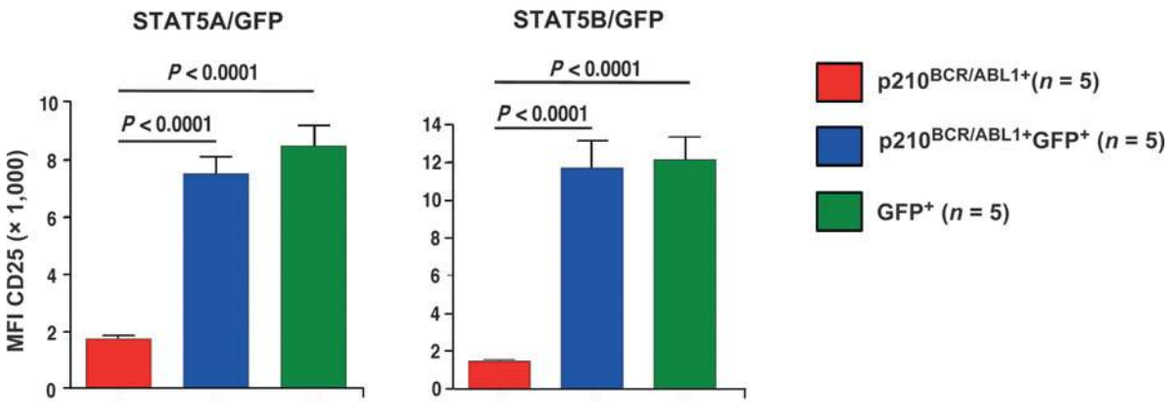

Figure 4.
Infection of STAT5A/B is followed by upregulation of CD25 in mouse LSK cells. CD25 expression in $Lin^-/Sca\text{-}1^+/Kit^+$ LSK cells after infection of murine C57Bl/6 BM cells with retroviral constructs encoding $p210^{BCR/ABL1}$ (red bars), STAT5A/GFP (left, green bar) or STAT5B/GFP (right, green bar), or combinations of $p210^{BCR/ABL1}$ and STAT5/GFP isoforms (blue bars). BM cells were infected as described in the text. Expression of CD25 on LSK cells was analyzed by multicolor flow cytometry using an antibody against CD25. Results are expressed as median fluorescence intensity (CD25 expression) and represent the mean ± SD from five independent experiments. Asterisk (*) indicates $P < 0.05$ compared with $p210^{BCR/ABL1}$-infected cells.

Discussion

Although LSCs are an emerging new target of therapy, little is known about disease-specific markers and targets expressed in CML LSCs. We here show that CML LSCs aberrantly express CD25 and that STAT5 triggers the expression of CD25 on LSCs. Moreover, we show that shRNA-induced CD25-depletion in CML cells is associated with enhanced proliferation *in vitro* and enhanced engraftment of KU812 cells in NSG mice. These data suggest that CD25 is a novel biomarker of CML LSCs and that it acts as a 'leukemia-suppressing' molecule, which may have clinical and diagnostic implications.

Recently, gene array studies have shown that CML LSCs express CD25 mRNA in excess over normal SCs (30–32). In the current study, we confirmed these results at the mRNA and protein level. In particular, CD25 was found to be expressed on CML LSCs in almost all patients tested, independent of the phase of disease. The levels of CD25 on LSCs varied from donor to donor, but CD25 was detectable in >90% of all patients with CML. By contrast, in most control samples tested, normal $CD34^+/CD38^-$ BM SCs did not express CD25. After successful treatment with imatinib, the numbers of $CD25^+$ SCs in the BM decreased substantially in our CML patients. However, in a few patients entering a major molecular response (BCR/ABL1 <0.1%), $CD34^+/CD38^-$ SCs still expressed CD25, even when CD26 and IL-1RAP were no longer detectable. This phenomenon may have several explanations. First, these cells may represent an early (BCR/ABL1-negative) phase of LSC evolution (42, 43). An alternative explanation would be that normal SCs in these patients were in an activated state and therefore expressed CD25.

Recent data suggest that LSCs in patients with acute myeloid leukemia express CD25 (44). In the present study, we asked whether CD25 is specifically expressed on CML LSCs among MPNs. However, we were neither able to detect CD25 on SCs in patients with *JAK2*-mutated MPNs nor in patients with systemic mastocytosis, which was an unexpected result, because STAT5 activation occurs downstream of *JAK2* V617F and *KIT* D816V. One explanation may be that additional (disease- or cell-specific) factors, apart from STAT5, are required for induction of CD25 expression in LSCs. We therefore asked whether aberrant expression of CD25 is detectable in various lineages in the CML clone. However, we were unable to detect substantial amounts of CD25 in other cell types in our CML patients, including $CD34^+/CD38^+$ progenitor cells and more mature myeloid cells in the CML clone. These data suggest that aberrant expression of CD25 is confined to the LSC fraction in CML.

A number of previous studies have shown that CD25 is a STAT5 target gene (45, 46). Other studies have shown that BCR/ABL1 directly triggers STAT5 activation (20–23). In the present study, we were able to show that expression of CD25 on CML LSCs is dependent on STAT5 activity. First, the shRNA-induced knockdown of STAT5 resulted in a decreased expression of CD25. Furthermore, the STAT5-targeting drug pimozide was found to inhibit expression of CD25 in KU812 cells and primary CML LSCs. Finally, infection of murine BM SCs with STAT5A or STAT5B resulted in an enhanced expression of CD25. An interesting observation was that CD25 expression on murine CML LSCs (LSK cells) was induced by STAT5 rather than by BCR/ABL1, contrasting recently published results (32). One explanation for these discrepant data would be differences in the transfection assay or transfection efficacy. Another explanation may be that BCR/ABL1 triggers STAT5 activation rather than STAT5 production in LSK cells.

Imatinib and other BCR/ABL1 TKIs remain the standard of treatment in patients with Ph^+ CML (4, 5, 47). However, the potency of nilotinib and ponatinib against BCR/ABL1 clearly exceeds the potency of imatinib (48–50). We asked whether exposure to TKIs would result in a decreased expression of activated STAT5 and CD25. In KU812 cells, all three TKIs were found to inhibit the expression of pSTAT5 and expression of CD25, without major differences in IC_{50} values. In primary patient-derived LSCs, however, ponatinib was the most potent compound, followed by nilotinib, whereas imatinib showed only little if any effect. These data may be explained by the fact that ponatinib and nilotinib are more potent inhibitors of BCR/ABL1. Alternatively, CD25 expression in LSCs is dependent not only on BCR/ABL1 and STAT5 but also on other targets recognized by ponatinib and nilotinib, but not imatinib.

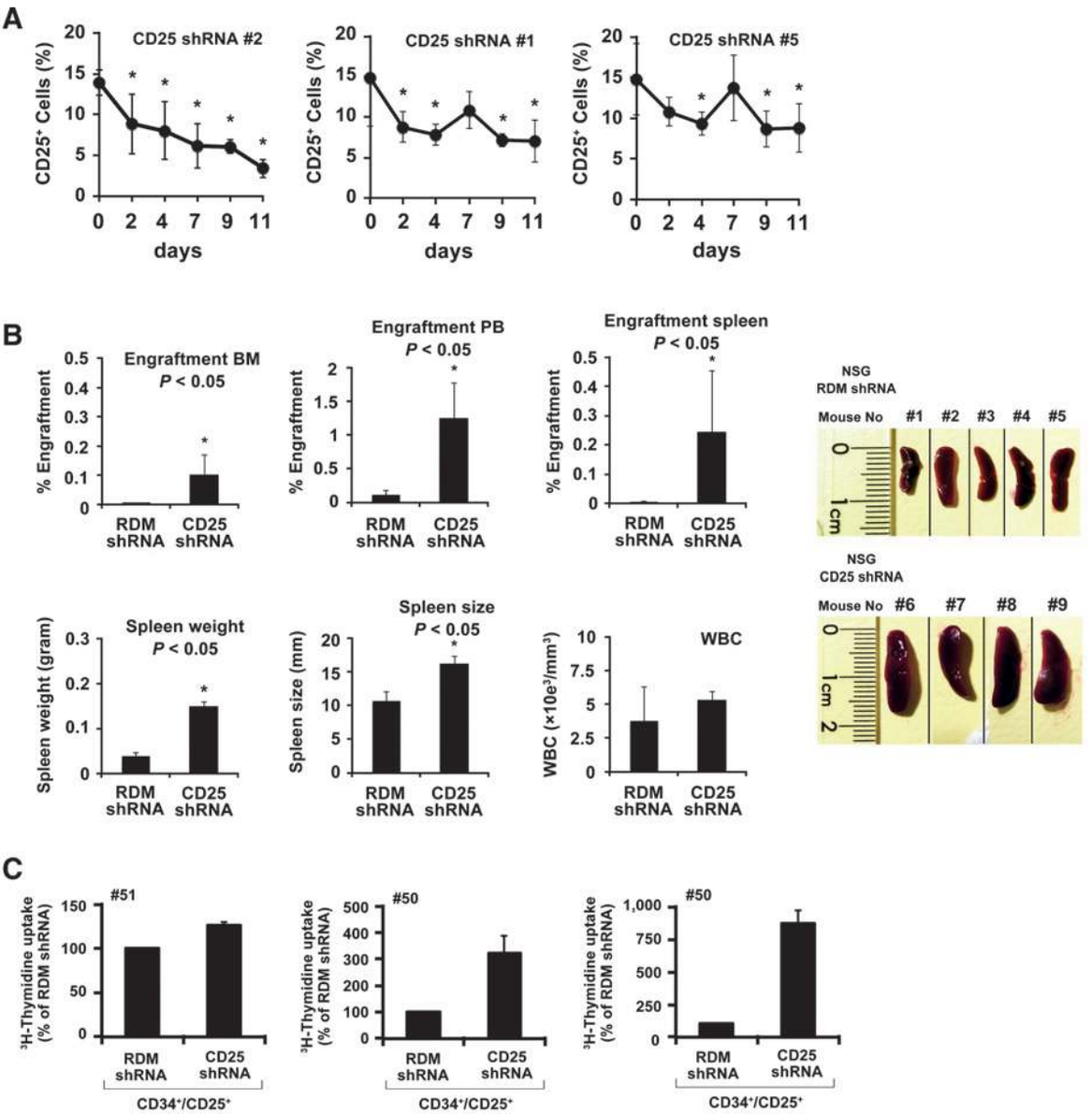

Figure 5.

Evaluation of CD25 as a functional target on CML cells. A, CD25 shRNA–transduced cells (shRNA clones #2, #1, #5 as indicated) were mixed 1:1 with KU812 cells transduced with an RDM shRNA and cultured at 37°C. Expression of $CD25^+$ cells was analyzed 3 times a week by flow cytometry using the PE-conjugated mAB 2A3 against CD25. Results are expressed as a percentage of $CD25^+$ cells and represent the mean ± SD from at least 4 independent experiments. Asterisk (*) indicates $P < 0.05$ compared with CD25 expression on day 0. B, NSG mice were injected with CD25 shRNA (clone #2) or RDM shRNA–transduced KU812 cells (5 mice/group). After 6 weeks, NSG mice were analyzed for engraftment of $CD44^+$ cells (expressed as %) in the BM, PB, and spleen by flow cytometry using an antibody against CD44 (top). The spleen weight, spleen size, and white blood count (WBC) are shown in the bottom plots. Results represent the mean ± SD from five mice per group. Asterisk (*): $P < 0.05$ compared with control mice. The images in the very right plots show the spleen size of NSG mice (#1–5: NSG mice injected with KU812 cells transduced with RDM shRNA; #6–9: NSG mice injected with KU812 cells transduced with CD25 shRNA). C, primary $CD34^+$ stem/progenitor cells from 2 patients with CML CP were transduced with a CD25 shRNA (clone #2) or an RDM control shRNA. After 24 hours (recovery time), ^{3}H-thymidine uptake was measured in highly enriched (sorted for mCherry) transduced cells. Results are expressed as a percentage of control (^{3}H-thymidine uptake in RDM shRNA–transduced cells = 100%) in three independent experiments (one experiment with cells from patient #51 and two experiments with cells from patient #50).

Next, we explored the functional role of CD25 in CML LSCs. In a proliferation assay, IL-2 did not modulate growth of CML cells even when applied at high concentrations. In line with this observation, CML LSCs and KU812 cells stained negative for IL-2RB (CD122) and IL-2RG (CD132). Recent data suggest that CD25 expression on LSK cells correlates with their proliferative capacity *in vivo* (32). By contrast, we observed a growth-promoting effect of various CD25 shRNAs in KU812 cells. In fact, the shRNA-induced knockdown of CD25 was found to be associated with an enhanced proliferation *in vitro* and with a significantly increased

engraftment of KU812 cells in NSG mice. These data suggest that CD25 is involved in the regulation of growth of KU812 cells. Moreover, we were able to show that lentivirus-mediated expression of CD25 in the CD25-negative cell lines K562, K562-R, and KCL-22 leads to reduced proliferation. Finally, we were able to show that a knockdown of CD25 in primary $CD34^+$ CML stem- and progenitor cells is associated with enhanced growth. All in all, these data suggest that CD25 is a negative regulator of growth of CML stem cells.

We next attempted to explore mechanisms underlying the CD25-mediated growth inhibition. In these analyses, slightly different numbers in mitotic KU812 cells were found, whereas we were unable to detect any major differences in cell-cycle distribution when comparing CD25 shRNA-transduced cells with RDM shRNA–transduced cells. Alternatively, CD25 may serve as a receptor for an as yet unknown negative regulator of stem cell survival or proliferation. In order to address these questions, we performed gene array studies on shRNA-transduced KU812 cells. In these studies, we were able to identify a number of regulated genes that might be responsible for the different growth kinetics (mitotic rate) in KU812 cells. These genes include several members of the DAZ family, RAB27B, IGFBP7, SSX2, RASGEF1A, and IGF-1. We hypothesize that these genes act together to promote growth in CD25-depleted CML cells. In fact, no other critical genes or pathways that could explain the CD25-mediated growth inhibition in KU812 cells were identified in this study.

Because CD25 expression in CML cells may be associated with reduced growth, we screened for drugs that would upregulate CD25 expression in CML cells. Whereas most drugs were found to downregulate the expression of STAT5 and CD25 in CML cells, the PI3K/mTOR blocker BEZ235, the mTOR-targeting drug RAD001 (everolimus), and the PI3K inhibitor LY924002 were found to upregulate CD25 expression. We were therefore interested to learn whether drug-induced upregulation of CD25 contributes to the antineoplastic effects of these two agents. Indeed, we found that shRNAs against CD25 reduce the sensitivity of KU812 cells against BEZ235, suggesting that CD25 may act as secondary drug effector of BEZ235. Based on these observations, we were interested to know whether BEZ235-induced upregulation of CD25 may enhance the effects of BCR/ABL1 TKIs that were found to downregulate CD25 expression. To address this question, we performed drug combination experiments and found that BCR/ABL1 TKIs synergize with BEZ235 in producing growth inhibition in CML cells. Although it remains unknown whether CD25 is indeed a critical target mediating drug synergy, our data suggest that these two types of drugs utilize different mechanisms of action, which may explain synergistic effects. A related hypothesis would be that TKI-induced CD25 downregulation in CML LSCs is part of an escape mechanism, and that this escape is disrupted by addition of PI3K/mTOR blockers.

Together, our data show that CML LSCs aberrantly express CD25 and that STAT5 triggers expression of CD25 in CML LSCs. Moreover, our data show that CD25 expression in LSCs is functionally relevant and associated with decreased proliferative capacity. These data may have implications for the biology of CML and should facilitate LSC detection and isolation. Moreover, CD25 may serve as a potential indirect drug target of drug therapy.

Disclosure of Potential Conflicts of Interest

T.L. Holyoake reports receiving speakers bureau honoraria from Ariad and Novartis, and commercial research grants from Bristol-Myers Squibb, Constellation Pharmaceuticals, Novartis, and Roche. P. Valent is a consultant/advisory board member for Ariad, Bristol-Myers Squibb, Novartis and Pfizer, and reports receiving commercial research support from Ariad and Novartis. No potential conflicts of interest were disclosed by the other authors.

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