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CML stem cells and their interactions and adaptations to tyrosine kinase inhibitors

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ABSTRACT

Chronic myeloid leukemia (CML) is a hematopoietic stem cell malignancy, driven by the pathognomonic oncogenic fusion protein BCR::ABL1. Tyrosine kinase inhibitors (TKIs) targeting ABL1 have increased the life expectancy of patients with CML to near levels of age-matched healthy individuals. Intriguingly, the response to TKIs varies substantially and is related to observations that CML leukemic stem cells (LSCs) are less sensitive to TKIs. LSC-derived suboptimal response is suggested to explain failing treatment free remission (TFR) in approximately 60% of patients after stopping TKI treatment. Identification of novel and druggable targets on CML LSCs is a possible pathway for increasing TFR. Here we will focus on the role of CML LSCs in initial patient response to TKI therapy, and the possible interactions that LSC may experience in the bone marrow stroma. Adaptation of LSC and stroma is likely to play a central role in the heterogenous responses. Even if overall survival in CML is outstanding, deeper understanding of LSC biology may help more patients to avoid life-long therapy.

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Introduction

The concept of cancer stem cells was introduced by John Dick in the description of leukemic stem cells (LSCs) in acute myeloid leukemia [1]. This groundbreaking discovery led to the identification of various stem-like cells in most tumors. The definition of a LSC is that this cell may recapture the leukemic disease in vivo, typically in xenograft transplant experiments to immunodeficient mice. Both leukemias and solid tumors are hierarchically organized and likely sustained by a population of biologically distinct cancer stem cells [2]. The CML LSC and its progeny harbor the Philadelphia chromosome (Ph), the result of the reciprocal translocation, t(9;22)(q34; q11.2) forming the BCR::ABL1 gene [3]. The constitutively activated tyrosine kinase BCR::ABL1 drives cell survival and growth through phosphorylation of several intracellular signaling pathways [4-6]. Due to their capacity for selfrenewal and differentiation, CML LSCs both maintain the stem cell pool and expand the leukemic Ph⁺ clone, which matures into functional immune cells during the

chronic phase of CML (cp-CML) [7]. Early experiments in mice have provided functional proof of the fundamental role of the BCR::ABL1 gene and LSCs in the pathophysiology of CML [8].

Mature and differentiated leukemic cells from patients with cp-CML have been reported to be insensitive to tyrosine kinase inhibitor (TKI) treatment in vitro, despite ABL1 inhibition, and have a normal life cycle in patients [9]. The success of treatment depends on TKI-mediated inhibition of proliferation and clearance of most CML stem and progenitor cells (Lin⁻CD34⁺) [10-12]. However, primitive subpopulations of guiescent LSCs have been found to be insensitive to TKIs and persist through treatment [13-16]. Indeed, recent single-cell RNA sequencing (scRNAseq) analysis has confirmed that there is a gradient to TKI resistance among various stem cell populations, and that persisting populations also have a guiescent signature [17]. Quiescent LSCs are suggested to be a subset with particularly low metabolic activity, no DNA replication and with few cellular processes that can be targeted by

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therapy. The implications are that in a group of patients dosed with per oral TKI, a wide range of cells will be targeted. And these LSCs will be in very different milieu when the TKI molecule reaches the cell and inhibits BCR::ABL1.

Here, we will focus on what is known about interactions between LSCs and TKIs, as well as adaptations in the TKI-exposed LSCs in the context of cell fate: cell death, senescence and quiescence. Also, these TKI-induced LSC adaptations will be discussed in the context of the bone marrow (BM) microenvironment.

CML stem cell signatures

Distinguishing *BCR::ABL1*⁺ LSCs from *BCR::ABL1*⁻ HSCs and conducting high-dimensional differential analysis between these cell types, or across experimental conditions, has been a methodological challenge. Antibody based flow cytometry is widely used to capture multiparameter profiles of immune cells, but a reliable marker of BCR::ABL1 protein expression has been lacking, although innovative methods are being developed [18]. It is, however, generally accepted that CML LSCs and HSCs both are enriched among the Lin⁻CD34⁺CD38⁻ cells of the bone marrow. Researchers have been searching for a proxy marker, a surface antigen phenotype, that effectively distinguishes LSCs from normal HSCs by being exclusively or differentially expressed.

Several cell surface markers have been identified to be either exclusively or differentially expressed on BCR::ABL1⁺ stem cells relative to BCR::ABL1⁻ stem cells (see box 1 in [19]). Notable examples include CD25, CD26 and IL1-RAP, all reported to be expressed on LSCs, but absent or at very low levels on HSCs [20-23]. Using mass cytometry, we have been able to reliably identify an increased expression of CD25 on a subset of Lin⁻CD34⁺CD38⁻ cells in peripheral blood (PB) collected from patients (n=8) with cp-CML (Figure 1a-c). These CD25⁺ stem cells were specifically depleted the first 7 days of treatment [24] and had a significant reduction in pSTAT5 Y694 already three hours after the first nilotinib tablet (Figure 1d and e). Follow up experiments have shown that this population of Lin-CD34⁺ CD38-CD25+ cells from PB also expresses CD26 (data not shown). In 2017, Warfvinge et al. found that the persistent CML LSC was Lin⁻CD34⁺ CD38^{-/low}CD45RA⁻cK-IT-CD26⁺ [17]. Later work by the same group further refined the distinction between LSCs and HSCs through analysis of combined single cell gene expression and cell-surface protein profiles. They were able to refine the signature of LSCs to be BCR::ABL1+CD26+CD35- and HSCs to be BCR::ABL1⁻CD26⁻CD35⁺. Interestingly, they further showed that the ratio of LSCs/HSCs was higher in patients with prospective treatment failure compared to optimal responders [25]. A recent comparison of gene expression profiles from different CML and



Figure 1. CD25 is expressed on CD34⁺CD38^{-/low} cells from peripheral blood collected from cp-CML. CD34⁺ cells from Gullaksen et al. [24] was clustered using PARC, and a heatmap showing the expression of phenotypic markers is shown in a). UMAP was used to generate a 2D representation of the various stem cell (SC) populations and is colored by their cluster affiliation in b). The single cell expression of CD25 (scaled) in the healthy bone marrow (HDBM, n=4) and peripheral blood (HDPB, n=4) is shown alongside the three cp-CML samples (collected: before, 3 h and 7 days after starting nilotinib treatment, n=8 each) in c). The single cell expression of pSTAT5 Y694 (scaled) is shown for the three cp-CML samples in d). Paired t-test of change of pSTAT5 Y694 (95th-percentile arcsinh transformed dual count) between samples collected before and 3 h after the first *per oral* dosing with the kinase inhibitor nilotinib in e).

normal stem cell subpopulations showed a six-fold increase of *CD93* in cp-CML LSCs compared to healthy HSCs. Flow cytometry sorting of Lin⁻CD34⁺CD93⁺ cells and xenografting into NOD-SCID IL2rg^{-/-} (NSG) mice showed that this population had the functional properties of CML LSCs. Furthermore, in both *ex vivo* and *in vitro* experiments this CD93⁺ LSC population persisted during TKI treatment [26].

The BCR::ABL1 gene expression signature

Recent advances in single cell RNA sequencing (scRNAseq) platforms have offered new opportunities of exploring CML LSCs, catalyzed by the identification of a BCR::ABL1 gene expression signature (GES). Using an enhanced BCR::ABL1-targeted single cell Smart-seq2 protocol able to both identify BCR::ABL1 transcripts as well as do whole-transcriptome sequencing, Giustacchini, et al. were able to establish a GES specific to stem cells expressing BCR::ABL1. They could also show that both BCR::ABL1+ and BCR::ABL1- stem cells from poor responders at diagnosis expressed more guiescenceassociated genes compared to patients achieving major molecular response (MMR). This indicates that BCR::ABL1 independent and cell-extrinsic or microenvironmental factors are involved in shaping response to therapy [27].

Single cell RNA sequencing has been used by the Ong laboratory to investigate balanced fractions of sorted CD34⁺ stem cells and CD34⁻ immune cells from optimal responders, suboptimal responders and blast crisis (BC) patients (n=9, each) collected before starting treatment with TKIs. Gene set enrichment analysis of CD34⁺ stem cells from BC patients identified multiple hallmark features of BC relative to optimal responders. These signatures included a BC signature, stemness, and inflammatory signaling (IFNy, IFNa, IL2/STAT5 and IL6/JAK/STAT3) and relative quiescence. The authors then leveraged the BCR::ABL1 GES from Giustacchini, et al. to identify BCR::ABL1+ and BCR::ABL- stem cells, finding that most stem cells from the CML samples were indeed BCR::ABL1+. Analysis of LSCs from optimal responders showed increased functional erythroid gene expression programs. These patients also showed an increased expansion of erythroid progenitor (ERP) cells that displayed sensitivity to TKI in vitro. The sensitivity of ERPs to TKIs was observed in all patients, indicating that BCR::ABL1⁺ ERPs are intrinsically sensitive to TKIs. Increased activity of the MYC regulon, and regulons related to inflammatory responses was observed in LSCs of patients progressing to BC [28].

Huuhtanen et al. analyzed samples from untreated patients using scRNAseq using the GES to identify BM

BCR::ABL1⁺ and BCR::ABL1⁻ CD34⁺ stem cells in BM and investigated their interaction with immune cells from PB. Notably, they found that the most immature HSC (CD38-) expressed lower levels of BCR::ABL1 compared to more mature CD38⁺ populations, as previously observed [29]. By calculating significant ligand-receptor interactions they found that the PB immune system interacted more with the BCR::ABL1^{high}CD38⁺ populations compared to the BCR::ABL1^{low} HSC CD38⁻ populations. According to these results, LSCs with low BCR-ABL1 activity may evade immune cell recognition, aligning with clinical findings that show minimal BCR::ABL1 transcript levels persisting for years in patients [30].

TKIs and the CML stem cell

Kinase domain mutations

The mode of action for most TKIs involves targeting the ATP-binding pocket of the ABL1 kinase domain (KD) in its active (Type I TKIs) or inactive form (Type II TKIs) [31]. The first-generation TKI, imatinib, binds the inactive form [31,32]. The second-generation TKIs, such as nilotinib, dasatinib, and bosutinib, provide enhanced potency and binding characteristics. Nilotinib and bosutinib target the inactive form like imatinib, while dasatinib interacts with the active form, though bosutinib can also bind to an intermediate form [31,33-35]. While the two first generations of TKIs provided activities against many KD mutants, the T315I 'gatekeeper' mutation rendered them largely ineffective due to steric hindrance. To overcome this issue, the thirdgeneration type II TKI ponatinib applied a linear ethylene linker specifically to allow favorable van der Waals interactions with the gatekeeper residue [36]. Additionally, asciminib circumvents the effects of the T315I mutation by using an allosteric inhibition mechanism targeting a myristoyl-binding pocket, not the ATP-binding site (type IV TKI) thereby expanding the number of treatment options for clinically relevant mutations [37,38]. Compound mutations, where a single clone harbors two or more mutations, still pose significant challenges [39]. These BCR::ABL1 mutations, including the gatekeeper, have been identified in CD34⁺CD38^{-/+} cells, and whilst rare in the clinic, these necessitate personalized treatment strategies [40-42]. The diversity of KD mutations is largely attributed to cellular stress, inducing genomic instability in both LSCs and leukemic progenitors. This instability is associated with high levels of reactive oxygen species (ROS) and other oxidative DNA damage [42]. BCR::ABL1 has been demonstrated to function as a source of ROS, and to promote a dysfunctional cellular repair system that increases mutation rates and protects cells from apoptosis. However, even during TKI-mediated inhibition of BCR::ABL1, LSCs still contain considerable ROS levels, suggesting that BCR::ABL1 independent mechanisms, such as the Rac2-MRC-cIII pathway, also help to maintain a ROS *status quo* [43–46].

Signaling

In previous work, we have investigated the early effects of TKIs on BCR::ABL1 related signaling in PB immune cells of patients in clinical trials. Using mass cytometry, we were able to detect changes in signaling already within three hours of the patient receiving oral nilotinib, including increases is pSTAT3 Y705 in Lin⁻CD34⁺CD38⁺ cells, and reduction in pCRKL Y207 in basophils after 7 days of treatment. Analysis of signaling in leukemic cells after 7 days of treatment by principal component analysis revealed underlying differences in signaling status between responders and sub-optimal responders driven mainly by pCREB S133 and pSTAT3 Y705 [24]. Indeed, it has been shown by others that STAT3 plays an important role in intracellular signaling of BCR::ABL1⁺ cells, and it is activated by bone marrow derived factors that contribute in protecting BCR::ABL1⁺ cells [47,48]. The dual inhibition of JAK1 [49] or STAT3 [50] in addition to BCR::ABL1 reduces the survival of LSCs and may represent a novel treatment strategy for patients with inferior response to monotherapy.

Integrin-linked kinase (ILK) is found to be overexpressed in CD34⁺ CML cells, with higher levels in TKI non-responders compared to responders or normal HSCs. This overexpression is associated with the phosphorylation of STAT3 Y705. ILK, along with PINCH1 and PARVB, is highly elevated in the CD34+CD38- CML cells, compared to more mature CD38+ subsets or terminally differentiated CD34⁻ cells, with the highest levels observed in TKI non-responders. ILK expression increased significantly when CD34⁺ CML cells were in direct contact with stromal cells or exposed to imatinib, but not when separated by a trans-well insert, highlighting the importance of physical niche interactions for ILK upregulation. Therapeutic inhibition of ILK reduces STAT3 phosphorylation and suppresses STAT3-dependent genes, such as CD44 and PTPN2, which mediate resistance and niche protection. ILK inhibition also disrupts mitochondrial OXPHOS, decreases ROS levels, and impairs mitochondrial function. In TKI non-responders, ILK inhibition synergizes with TKIs to target dormant LSCs by disrupting mitochondrial metabolism, adipogenesis, and the Wnt/ β -catenin pathway, as shown by reduced GSK3 β phosphorylation and Wnt-related gene expression. These effects were validated in patient-derived xenograft models, where ILK inhibition significantly impaired TKI-resistant LSC engraftment and self-renewal without affecting normal HSCs [51].

TKI treatment may increase LSC quiescence through altering tyrosine kinase dependent signaling pathways. For example, the inhibition of BCR::ABL1 by TKIs can lead to FOXO3a mediated inhibition of p53 repressor BCL6, promoting cell survival [52]. Differences in cell cycle regulation have also been observed in primary stem cells from the bone marrow of CML patients. In cells treated with TKIs (dasatinib, nilotinib, imatinib) they found a nuclear location and decrease in p18 and p57 protein levels, both of which are known to negatively regulate the transition from G_1 to S phase, which could correlate to LSC quiescence [53].

Adaptations to TKI in the microenvironment

Interactions with mesenchymal stem cells

One way in which the LSC adapts to TKI treatment is by leveraging the supportive milieu in the bone marrow microenvironment. Through interactions with stromal cells, which release cytokines and growth factors, and their ability to thrive under hypoxic conditions in the microenvironment, the LSC is supported and can persist even in the presence of TKI therapy [54,55]. The bone marrow compartment is divided into sinusoids and specific niches. Each of these has unique cell populations and cell signaling providing HSC maintenance, direction of differentiation and release of mature blood cells [56]. These niches are controlled by secretion of cytokines and growth factors from the mesenchymal stem cells (MSCs). Even though the MSCs in CML patients do not express the BCR::ABL1 protein, there may be a genetic signature distinguishing these MSCs from a leukemic microenvironment from normal MSCs. A CML-associated gene signature, characterized by the upregulation of BMP1, MET, MITF, NANOG, and PDPN mRNA, persisted in MSCs from patients in deep molecular response. This suggests that the gene signature is likely established during the early leukemogenic phase and continues to support LSCs even after deep molecular response is achieved [57]. The adaptations of LSCs to the hypoxic environment of the stem cell niche are different from HSCs. In contrast to the HSCs, the TKI-insensitive LSC population adapts to treatment by rewiring their metabolic profile to maintain a quiescent state with restoration of OXPHOS levels thus less sensitive

to HIF1 α degradation. Interestingly, co-suppressing BCR:: ABL1 and HIF-1 α could be a way to eradicate the CML LSC [46]. Interactions between MSCs and CML leukemic stem cells through the WNT/ β -catenin pathway significantly enhance LSC proliferation. This pathway, crucial for both the survival and increased self-renewal of CML progenitors during blast crisis, facilitates the resistance of LSCs to TKIs. Increased β -catenin expression, mediated by these stromal cell interactions, serves as a protective mechanism against TKI therapy, highlighting the critical role of the microenvironment in supporting CML resistance [55,58–60].

Direct cell-cell contact between LSCs and stromal cells has also been shown to provide protection against TKI treatment [61]. Pro-survival stimulation and activation of alternative survival pathways through direct cell-cell contact can be related to the expression of IL-1RAP, CD26 and CD25 on LSCs [21,62-64]. The chemokine CXCL12 is suggested to be important in stem cell niches. The CXCL12-abundant reticular (CAR) cells release CXCL12 to stimulate proliferation of HSCs, and a short-time ablation of CAR cells in a mouse model in vivo resulted in quiescent HSCs [65]. Interestingly, an increased expression of CD26 disrupts the CXCL12/CXCR4 axis and is suggested to play a part in the extramedullary spread of LSCs [21]. It has been demonstrated that loss of CXCL12 secretion from MSCs results in the expansion of LSCs and sensitizing them to TKI-treatment, suggesting that CXCL12 is critical for maintaining quiescence [62]. Furthermore, CD26 expression on LCS is suggested as a route of LSC escape by inhibiting the CXCL12/CXCR4 axis and releasing the LSCs from the niche [20,21]. Imatinib resistance may be linked to this mechanism by upregulation of CXCR4, thus forcing the CML LSC to home back to the protective bone marrow stroma [66]. The effect of expressing IL-1RAP on CML LSC sensitizes them to IL-1 signaling, promoting a pro-leukemogenic environment [63]. Inhibition of IL-1 signals has been suggested to aid in modulating the sensitivity to TKI treatment [67]. Also, increased TNFa-mediated alteration in the CML BM stromal niche has been suggested to enhance LSC maintenance and growth through the CXCL1-CXCR2-axis [68]. The CD25 expression on LSC likely replaces the BCR::ABL1 oncogene addiction pushing the LSC to a STAT5-dependent proliferation pathway. By stimulating the microenvironment to release factors increasing STAT5 signaling, the LSCs maintains their proliferation asset in the microenvironment [69].

The TGF- β family members Bone morphogenetic protein 2 (BMP2) and BMP4 levels are significantly elevated in the BM plasma of CP-CML patients at

diagnosis compared to healthy donors, with these BMPs influencing the maintenance and expansion of leukemic CD34⁺ cells [70]. Indeed, LSCs rely on BMP4 secreted by stromal cells, which binds to BMPR1B receptors on LSCs, activating the Smad1/5/8 and JAK2-STAT3 pathways to maintain their quiescent state after TKI treatment. This was demonstrated using CD34⁺CD38⁻ cells from CML patients in remission and MSCs from both CML patients and healthy donors. Direct adhesion to BMP4-producing stromal cells reinforces this quiescence, creating a localized niche that supports resistance to ongoing TKI therapy [71].

Cell-cell communication through exosomes and tunneling nanotubes (TNT) is shown to promote cell CML LSC survival [72,73]. Exosomes made by MSCs can be released with content like proteins, mRNA and microRNA [74]. Exosome release from human bone marrow microenvironment-derived mesenchymal stem cells (hBMMMSC) can inhibit the proliferation of CML cells through miR-15a and arrest the cell cycle in the G0/G1. This could likely be affecting the CML LSCs as well.

Tunneling nanotubes

Tunneling nanotubes (TNT) are dynamic actin-based cytoplasmic extensions, which facilitate intercellular communication and the transport of various cellular components, including mitochondria and oncoproteins [75–77]. For example, the transfer of mitochondria between MSCs and tumor cells has been shown to promote metastasis and increase tumor cell tolerance of oxidative stress [78]. TNT-mediated transfer between stromal cells and imatinib-treated CML cells is suggested to facilitate protection against the effects of imatinib. In addition, they demonstrate how cellular stress may increase the bi-directional transfer of cytoplasmic and membrane compartments through TNTs, promoting mutual survival and spreading resistance throughout the leukemic population [73].

We have demonstrated low numbers or absence of TNTs between BCR::ABL1⁺ cell lines (Kcl-22 and K562) and between primary cells from CML patients, likely due to the decreased adherent functions of CML cells. Interestingly, treatment with imatinib, nilotinib or interferon- α (IFN α) facilitated TNT formation in these cells [79]. Thus, certain treatments might enhance resistance to drug-induced apoptosis by facilitating increased adherence, subsequent formation of TNTs and ultimately the sharing of survival-promoting organelles (such as mitochondria) and resistance factors. This enhanced intercellular connectivity *via* TNTs as a response to TKIs could represent a survival

strategy for leukemic cells, allowing them to evade the cytotoxic effects of targeted therapies. Altogether, these findings underscore the need for further research on the potential role of TNTs in LSC persistence in CML.

CML LSC and its role in relapse after TKI cessation

In recent years, both clinical trials and practice have shown that treatment discontinuation is the emerging therapeutic goal for patients with CML [80,81]. However, more than 50% of patients in treatment free remission (TFR) studies had a molecular relapse reaching BCR::ABL transcript levels over 0.1% and subsequently restarted TKI treatment [82,83]. Inhibiting BCR::ABL1 with imatinib or other TKIs reduces the overall proliferation but does not efficiently eliminate the guiescent fraction of LSCs (Figure 2). A subset of these primitive and guiescent LSCs likely retain a leukemia-initiation capacity and are responsible for molecular relapse after stopping TKI treatment, even in patients without detectable BCR::ABL1 [84,85]. Conversely, patients may be off TKI treatment for years with low levels of measurable BCR::ABL1. The clinical diagnostic application of using CD26 to identify persisting LSCs during discontinuation of TKI is being evaluated in clinical trials [86-88]. A recent study evaluated the linages of residual BCR::ABL1 DNA positive cells in blood of patients in long term TFR. Among these patients, no BCR::ABL1 DNA was detected in granulocytes but in B and T cells. This suggests that detection of BCR::ABL1 in patients off TKI treatment is not necessarily evidence for persisting multipotent CML cells [89].

The immune system plays an important role in obtaining disease control also in CML when using

targeted therapy, as certain immune profiles have been proposed to facilitate successful TFR [90–92]. The interplay between the immunological host and *BCR::ABL1*⁺ cells may be a key in the gradual eradication of LSCs and to protect against overt relapse. Maybe prolonged TKI treatment facilitate education of anti-CML immunological mechanism, illustrated by the lower 10% chance of molecular relapse in patients treated for five years or longer [93].

Targeting CML LSCs is challenging due to the many features in common with normal HSCs. A possible strategy is to utilize small molecule CBP/β-catenin antagonists to block the interaction between CBP and β-catenin, modulating the WNT/catenin signaling pathway and thereby affecting cell division of cancerous stem cells (CSCs) [94]. Others have proposed a Bcl-2-inhibitor-loaded immunoliposome for targeting CD26+ LSCs without harming CD26- HSCs, proposed to be used after TKI cessation to prevent relapse [95]. Future research will need to focus on eradicating these LSCs to promote TFR in CML patients. This may be achieved through combining TKIs with LSCs-targeted inhibitors. Perhaps a combination of deep immuno-profiling and mapping of the stromal interaction with the CML stem cells is needed to understand the difference between the successful deep responder and the suboptimal treated CML patient. Understanding the context of CML stem cell adaptations to TKIs may introduce new therapy principles to secure a cure in more patients.

Concluding remarks

CML is a prototypic malignant stem cell disease where the response to TKIs may be explained by the number



Figure 2. Leukemic stem cells (LSC) and relapse after TKI cessation. Tyrosine kinase inhibitor (TKI), tunneling nanotube (TNT), kinase domain mutation (KD mut), treatment free remission (TFR), leukemic stem cells (LSC).

LSC present at diagnosis. Potential ABL1-mutations that interfere with TKI action will also directly define the response. So far it is not known if co-occurrence of other mutations related to myeloid neoplasia influences the TKI sensitivity. Epigenetic and other adaptive mechanisms in the LSCs after start of TKI therapy are poorly understood but based on observations in other TKI-treated malignancies these mechanisms may provide significant TKI resistance. Interplay between LSCs and stromal cells, like MSCs, is suggested to assist TKI-stressed LSCs. Maybe the most important therapeutic control of LSCs under TKI treatment is mediated by the cellular immune system. Together, the various resistance mechanisms provided by TKI-treated LSCs suggest new consolidation or maintenance therapies that should be developed to offer more CML patients a cure.

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