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Eradicating chronic myeloid leukemia stem cells by IRAK1/4 inhibitors

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Abstract

Leukemia stem cells (LSCs) in chronic myeloid leukemia (CML) are quiescent, insensitive to BCR-ABL1 tyrosine kinase inhibitors (TKIs) and responsible for CML relapse. Therefore, eradicating quiescent CML LSCs is a major goal in CML therapy. Here, using a novel G₀ marker (G₀M), we narrowed down CML LSCs as G₀M- and CD27- double positive cells among the conventional CML LSCs. Whole transcriptome analysis revealed NF- κ B activation via inflammatory signals in imatinib-insensitive quiescent CML LSCs. Blocking NF- κ B signals by IRAK1/4 inhibitors together with imatinib eradicated mouse and human CML LSCs. Intriguingly, IRAK1/4 inhibitors attenuated PD-L1 expression on CML LSCs, and blocking PD-L1 together with imatinib also effectively eradicated CML LSCs in the presence of T cell immunity. Thus, IRAK1/4 inhibitors could eradicate CML LSCs through inhibiting NF- κ B activity and reducing PD-L1 expression. Collectively, the combination of TKIs and IRAK1/4 inhibitors is an attractive strategy to achieve a radical cure of CML.

(149 words)

Keywords

chronic myeloid leukemia, leukemia stem cells, imatinib, IRAK1/4, PD-L1

Introduction

Chronic myeloid leukemia (CML) results from the transformation of hematopoietic stem cells (HSCs) by BCR-ABL1 fusion protein^{1,2,3}. BCR-ABL1, which arises from the chromosomal translocation t(9;22), encodes a 210 kD chimeric protein (p210 BCR-ABL1) with constitutive tyrosine-kinase activity. BCR-ABL1 tyrosine kinase stimulates proliferation, activates pro-survival signaling pathways, inhibits apoptosis, and contributes to genomic instability to drive the disease^{4,5,6,7}. The development of the first BCR-ABL1 tyrosine kinase inhibitor (TKI) imatinib has dramatically improved CML therapy and the survival of CML patients^{8,9}. However, TKI treatment fails to eliminate CML leukemia stem cells (LSCs) even in patients reaching the stage of complete molecular responses, and the residual CML LSCs can be identified in a quiescent HSC fraction^{10,11,12,13,14}. Even the second generation TKIs nilotinib and dasatinib are not effective at eradicating quiescent CML LSCs^{15,16}. CML LSCs share many features with normal HSCs, such as the quiescent state, localization in the hypoxia niche, and self-renewal ability^{17,18,19,20,21}. Their quiescent state is one of the major reasons why CML LSCs are resistant to BCR-ABL1 TKIs^{22,23}. This is probably because quiescent CML LSCs are not absolutely dependent on BCR-ABL activity for their survival^{12,24}. In fact, the abrogation of the quiescent state of CML LSCs by Fbxw7 ablation increases their sensitivity to imatinib²⁵. Moreover, the combination of histone deacetylase (HDAC) inhibitors, which induce apoptosis in quiescent cancer cell lines, with imatinib induces apoptosis in quiescent CML LSCs^{26,27}. Thus, the ablation of the quiescence of CML LSCs is a key to enhancing their sensitivity to TKIs. On the other hand, imatinib partially

contributes to their quiescent state²⁸. Overall, how the quiescent state of CML LSCs is regulated in the presence of TKIs has yet to be elucidated.

NF- κ B activation by BCR-ABL1 has been observed in culture and in a mouse CML-like model^{29,30}. Inhibitors of NF- κ B activation attenuate the lymphoid and myeloid leukemogenesis by BCR-ABL1 and decrease the number of CML LSCs in the mouse model³¹. NF- κ B blockade by PS-1145, an I κ B kinase (IKK) inhibitor, is effective at suppressing the growth of imatinib-resistant CML cell lines and imatinib-resistant CML-chronic phase (CML-CP) patient bone marrow (BM) cells in vitro³². These reports indicate that NF- κ B activation is responsible for the TKI-resistance of CML LSCs.

Notably, CML LSCs express higher levels of IL-1 receptors (IL-1Rs) and are more sensitive to IL-1-induced NF- κ B signaling than normal HSCs. Accordingly, the combination of a recombinant IL-1R antagonist (IL-1RA) with a TKI resulted in significantly greater inhibition of CML LSCs compared with the TKI alone³³. Additionally, primitive CD34⁺CD38⁻ CML-CP cells proliferate and activate NF- κ B in response to IL-1 β stimulation³⁴. These reports indicate that the IL-1R-NF- κ B signal axis is important for the proliferation of CML LSCs.

Although IL-1Rs share their downstream signaling pathways with Toll-like receptors (TLRs) except for TLR3, TLR signaling in CML has not been well examined. IRAK1/4, two serine/threonine kinases, are mediators of IL-1R/TLR-NF- κ B signaling pathways. It was reported that the blockade of IRAK1/4 is effective at eradicating myelodysplastic syndrome (MDS) cells by inhibiting growth and inducing apoptosis through the deactivation of NF- κ B³⁵. The inhibition of IRAK1 is also effective at reducing the growth

of acute myeloid leukemia (AML) cell lines and primary AML patient cells³⁶. These studies indicate that IRAK1 is a key factor for the survival of myeloid leukemia cells. Therefore, the development of effective therapeutic approaches that target the IRAK1/4-NF- κ B pathway with TKIs is an attractive option for the eradication of CML LSCs.

The immune system plays an important role in the control of most malignancies including CML, and CML cells are susceptible to T cell immunity³⁷. Among hematologic neoplasms, CML is sensitive to graft-versus-leukemia immunity³⁸. The combination therapy of donor-derived CD8 T cells and imatinib results in prolonged leukemia-free survival of mice with BCR-ABL1-induced CML-like disease³⁹. In relation to this, CML cells express programmed cell death ligand 1 (PD-L1), and a high expression of PD-L1 on human CD34⁺ CML cells is a negative prognostic factor^{40,41}. Furthermore, PD-L1 is constitutively expressed on LSCs and leukemia progenitors in a mouse CML-like model. Finally, blocking the PD-1/PD-L1 interaction enhances the survival of CML mice in blast crisis⁴². These studies indicate that PD-L1 protects CML LSCs from T cell immunity. However, the efficacy of the combination of PD-1/PD-L1 blockade and TKIs on the eradication of CML LSCs has not been examined.

Previously we reported that G₀ marker (G₀M), a fusion protein between the fluorescent protein mVenus and p27K-, which is a p27 mutant lacking cyclin dependent kinase (Cdk) inhibitory activity, is a useful tool for visualizing the quiescent states of conventional long-term HSCs, and the stemness of the cells was correlated with the intensity of G₀M^{43,44}. In the present study, we applied G₀M to identify and visualize quiescent CML LSCs. Gene expression analysis revealed that the NF- κ B signal is important for their survival in

the presence of imatinib. The deactivation of NF- κ B by an IRAK1/4 inhibitor with imatinib was extremely effective at eradicating CML LSCs compared with imatinib alone in a mouse CML-like model and a xenograft model with primary CML-CP patient samples. Moreover, we found that the IRAK1/4 inhibitor attenuated PD-L1 expression on CML LSCs in addition to having proapoptotic function. These data indicate that the pharmacological inhibition of IRAK1/4 together with TKIs exerts a potent dual antileukemia effect partly through the assistance of antitumor immunity.

Results

Identification of CML LSCs by G₀ marker and CD27

We introduced G₀ marker (G₀M) to a mouse CML-like model to visualize quiescent CML LSCs. We retrovirally overexpressed BCR-ABL1 fusion gene in BM cells from 5FU-treated G₀M mice, in which Cre-inducible G₀M in a Rosa26 allele is regulated by Vav1-Cre⁴⁴, and then injected the cells into lethally irradiated wild-type recipient mice to develop mouse CML-like disease (Figure 1A). CML derived from BM cells carrying G₀M was developed in about 3 weeks, and imatinib treatment prolonged the survival of leukemic mice (Figure 1B), ensuring that G₀M did not affect CML development or imatinib treatment. To assess the expression of G₀M in CML LSCs, we subdivided the conventional CML LSC fraction (CML LSK; BCR-ABL⁺Lin⁻Sca1⁺cKit⁺) in the BM from

leukemic mice by G₀M and CD27, a marker for the CML stem/progenitor cell fraction ⁴⁵. The combination of CD27 and G₀M split the CML LSK fraction into four factions: G₀M⁺CD27⁺ (double positive: DP), G₀M⁻CD27⁺ (single positive: SP), G₀M⁺CD27⁻, and G₀M⁻CD27⁻ (double negative: DN) (Figure 1C). To evaluate the LSC potential, we performed colony-forming assays and secondary transplantation assays on these fractions. The colony-forming ability was highly enriched in the DP fraction (Figure S1A). Kinetic analysis of the chimerism of BCR-ABL1⁺ cells (GFP⁺ cells) in peripheral blood (PB) revealed that, although the levels of chimerism gradually increased in the PB of mice transplanted with DP cells, the engraftment of SP cells peaked at 25 days and then declined (Figure S1B). The secondary transplantation assay showed that mice engrafted with DP cells exclusively developed CML (Figure 1E). Hematoxylin and eosin (H&E) staining of these four fractions showed that both DP and SP cells were blast-like cells, CD27⁻G₀M⁺ cells were mast-like cells as previously reported ⁴⁶, and DN cells were differentiated cells (Figure S1C). Moreover, only DP cells could reconstitute the four fractions within CML LSK cells in the BM after secondary transplantation, whereas SP cells could reconstitute three fractions but not the DP fraction (Figures 1F and 1G). Imatinib did not affect the characteristics of the four fractions (Figures 1H-J and S1D). Importantly, imatinib augmented the proportion of DP cells in the CML LSK fraction and the absolute number of DP cells compared with vehicle (Figures 1C and 1D). These results demonstrated that CML LSCs were enriched in the DP fraction in the retroviral transduction model and were insensitive to imatinib.

Upregulation of NF- κ B signaling pathways in imatinib-resistant CML LSCs

To clarify the molecular basis of the LSC potential of DP cells, we performed whole-transcriptome (RNA-seq) analysis of SP and DP cells from vehicle- and imatinib-treated CML mice. Principal-component analysis (PCA) and subsequent Enrichr analysis⁴⁷ along with a comparison to normal HSCs⁴⁴ revealed that DP cells from vehicle-treated mice and DP cells from imatinib-treated mice (IMDP cells) had more HSC features than SP cells from vehicle-treated mice or SP cells from imatinib-treated mice (IMSP cells) (Figures 2A and 2B). Representative features for DP and IMDP cells comprised the NF- κ B signaling pathway, inflammatory response, IL-2/STAT5 signaling, interferon gamma response and hypoxia (Figure 2C). These features are equivalent to CML LSC features, confirming that DP cells represent CML LSCs. Next, we assessed the impact of imatinib on gene expression changes in CML LSCs. PCA clearly separated the four CML populations (Figure 2D). The subsequent Enrichr analysis showed that negatively correlated features of PC2, which are equivalent to imatinib-resistant features, comprised TLR, TRAF6-mediated and IL-1 β processing pathways, whereas the negatively correlated features of PC1, which are equivalent to CML LSC features, comprised inflammatory response and type II interferon signaling pathways (Figures 2E and 2F). These data indicated that signaling pathways related to NF- κ B activation via inflammatory signals were crucial for the maintenance of CML LSCs in the presence of imatinib.

Combination of IRAK1/4 inhibitor and imatinib is effective at eradicating CML

LSCs

We next examined whether inflammatory signals in CML LSCs are crucial for the cell survival in the presence of imatinib. TLR1, TLR2, TLR4, and TLR6 were expressed on CML stem/progenitor cells, and TLR1 and TLR6 were highly expressed on IMDPs. IL-1R1 was highly expressed on IMSP and IMDP cells (Figure S2). These data suggested that the inflammatory signals on CML LSCs in the presence of imatinib were transmitted through TLRs/IL1Rs. TLRs except for TLR3 and IL1Rs transduced their signals through Myd88, IRAK1/4 and TRAF6 to NF- κ B to activate an inflammatory signaling cascade, and IRAK1/4 inhibitor could block the signals (Figure 3A). To block TLR/IL1R-NF- κ B inflammatory signaling pathways in CML LSCs in the presence of imatinib, we assessed the efficacy of IRAK1/4 inhibitor. We treated CML mice with imatinib, IRAK1/4 inhibitor or the combination of these. The combination greatly prolonged the survival of CML mice and reduced the splenomegaly of CML mice, whereas IRAK1/4 inhibitor alone did not (Figures 3B and 3C). In addition, the imatinib-induced increase of the absolute number of DP cells in the BM from CML mice was suppressed by the combination (Figures 3D and 3E). Moreover, the combination deactivated NF- κ B in DP cells, whereas neither imatinib nor IRAK1/4 inhibitor alone did (Figure 3F). These results demonstrated that NF- κ B activation via inflammatory signals was crucial for the survival of CML LSCs in the presence of imatinib, and NF- κ B activation via either BCR-ABL1 or inflammatory signals was enough to maintain CML LSCs. Collectively, the combination of IRAK1/4 inhibitor and imatinib is an attractive therapeutic strategy for the eradication of imatinib-insensitive CML LSCs.

Combination of IRAK1/4 inhibitor and imatinib is effective at reducing human CML LSCs

We extended the examination of the efficacy of IRAK1/4 inhibitor on CML LSCs to newly diagnosed CML-CP patient samples. CML CD34⁺ cells were isolated from the BM mononuclear cells of CML-CP patients (Table S1) by immunomagnetic column separation. The CML CD34⁺ cells were subjected to leukemic stem/progenitor cell expansion culture in the presence of imatinib (0.5 μ M), increasing concentrations of IRAK1/4 inhibitor or the combination of these for 7 days (Figure 4A). The absolute number of total cells, CD34⁺CD38⁻ cells and CD34⁺CD38⁻CD45RA⁻CD90⁺ cells were compared after 7 days culture. A dose-dependent impairment on the proliferation of total cells, CD34⁺CD38⁻ cells and CD34⁺CD38⁻CD45RA⁻CD90⁺ cells was observed in the presence of IRAK1/4 inhibitor, but the impairment was more effective in combination with imatinib (Figures 4B-D and Figures S3A-D). Additionally, the increasing concentration of IRAK1/4 inhibitor in the presence of imatinib increased the proapoptotic activity (Figures 4E and 4F). Next, we transplanted CML CD34⁺ cells into sublethally irradiated immunocompromised NOG mice constitutively producing human interleukin-3 (IL-3) and granulocyte/macrophage-colony stimulating factor (GM-CSF) (NOG hIL-3/GM-CSF Tg;⁴⁸) to assess the efficacy of the combination of IRAK1/4 inhibitor and imatinib on the eradication of CML LSCs in a xenograft model with CML-CP patient samples (Figure 4G). The combination eradicated CD45⁺ cells more effectively than imatinib alone (Figure 4H). IRAK1/4 inhibitor alone and the combination exhibited a

potent efficacy on the eradication of CD34⁺CD38⁻ cells and CD34⁺CD38⁻ CD90⁺CD45RA⁻ cells compared with imatinib alone (Figures 4I and 4J). There was a tendency, though not statistically significant, for the combination to be more potent than IRAK1/4 inhibitor alone. These results indicated that IRAK1/4 inhibitor has strong efficacy in the eradication of human CML-CP LSCs.

PD-L1 blockade together with imatinib is effective at eliminating CML LSCs

Our RNA-Seq analysis showed that the expressions of immune checkpoint molecules such as PD-L1, CD80/86, and TIM-3 were relatively higher in IMDP cells than other cell types (Figures 5A and S4A-C), with PD-L1 mRNA expression highest among them. DP and IMDP cells showed a higher protein level of PD-L1 than SP and IMSP cells, respectively, based on the flow cytometry analysis (Figure 5B). These data indicated the possibility that PD-L1 is involved in the survival of CML LSCs. PD-L1 expression is regulated by NF- κ B via inflammatory signaling such as IL1R, TLR and IFN signals ⁴⁹, which were linked to the imatinib-insensitivity of CML LSCs in our RNASeq analysis. The combination of imatinib and IRAK1/4 inhibitor, which deactivated NF- κ B, attenuated PD-L1 expression on mouse and human CML LSCs compared with imatinib or IRAK1/4 inhibitor alone (Figures S4D-F). These observations encouraged us to set up a combination therapy of imatinib and anti-PD-L1 antibody. We treated CML mice with imatinib, anti-PD-L1 antibody, or the combination of these. The combination greatly prolonged the survival of CML mice compared with imatinib or anti-PD-L1 antibody alone (Figure 5C). The combination also reduced the absolute number of DP cells and

splenomegaly compared with imatinib alone (Figures 5D-F). In particular, the combination therapy induced apoptosis in CML LSCs (Figure 5G). Further, PD-L1 expression on CML LSCs from CML mice with the combination treatment was significantly attenuated compared with imatinib alone (Figure S4G). The combination efficacy was abolished when Rag2KO mice⁵⁰, which lack T cell immunity, were used as recipients in the mouse CML-like model. However, CD8 T cell transfer together with the combination successfully recovered the efficacy in these mice (Figure 5H). Intriguingly, none of the treatments without CD8 T cell transfer prolonged the survival of CML mice, indicating that T cell immunity was crucial for the eradication of CML LSCs. We also assessed the efficacy of other blocking antibodies for immune checkpoint molecules. Both anti-PD-1 and anti-CTLA4 antibodies exhibited comparable efficacy with anti-PD-L1 antibody in combination with imatinib (Figure S4H). The efficacy of blocking the immune checkpoint molecules was equivalent to that of IRAK1/4 inhibitor. These results indicate that CML LSCs express immune checkpoint molecules to protect themselves from T cell antitumor immunity. Thus, inducing T cell antitumor immunity by the blockade of immune checkpoint molecules on CML LSCs together with TKIs is potentially effective for the eradication of CML LSCs. Moreover, since the combination of IRAK1/4 inhibitor and imatinib attenuates PD-L1 expression on CML LSCs, the combination also induces T cell antitumor immunity in addition to its proapoptotic activity.

Finally, we experienced a 60 year-old male with CML which developed during sunitinib therapy against relapsed renal cell carcinoma for 10 months. The Sokal score was in a

middle risk category (Sokal score: 1.02). The patient was treated successfully with dasatinib. In addition to dasatinib, the patient received continuous nivolumab treatment against relapsed renal cell carcinoma 4 months after the dasatinib commencement (Figure 5I). The patient achieved a molecular response with a 4.5-log reduction in BCR-ABL1 transcripts from baseline (MR4.5; BCR-ABL1 transcript level $\leq$ 0.0032% [International Scale]) at 6 months and an undetectable minimal residual disease (UMDR) at 12 months, whereas none of 14 patients treated with dasatinib alone at Juntendo University Hospital achieved a MR4.5 at 6 months, and two with low risk (Sokal score < 0.8) did at 12 months (Figure 5J and Table S2). Additionally, only less than 2.0% of all patients who received dasatinib achieved a MR4.5 at 6 months in the phase III Dasatinib Versus Imatinib Study in Treatment-Naïve Chronic Myeloid Leukemia Patients (DASISION) trial⁵¹. These results suggest that the combination of dasatinib and nivolumab in this patient happened to result in an early achievement of the deep molecular response (> 4 log reduction) in CML.

Discussion

CML LSCs are thought to be quiescent. This is one of the properties that make them insensitive to TKIs^{52,53}. However, the full portrait of CML LSCs is still enigmatic mainly due to the lack of appropriate methods to identify quiescent CML LSCs. In this study, using a novel G₀ marker (G₀M;⁴³, we found that G₀M⁺CD27⁺CML LSK cells (DP cells) contain CML LSCs, whereas G₀M⁻CD27⁺CML LSK cells (SP cells) do not (Figures 1C

and 1E-G). A transcriptome analysis revealed that inflammatory signaling, IL2/STAT5 signaling, IFN γ response and hypoxia pathways were enriched in DP cells (Figures 2E and 2F), which is consistent with features of CML LSCs^{17,18,46,54,55,56,57}.

The identification of CML LSCs by G₀M and CD27 enabled us to directly characterize CML LSCs. We demonstrated that the number of CML LSCs was increased by imatinib treatment in a mouse CML-like model (Figure 1D), and imatinib-treated DP cells exhibited more HSC-like features than did DP cells (Figures 2A and 2B). These results indicate that imatinib induces the quiescence of CML LSCs. Of note, TLRs and IL1 β signaling pathways, which activate NF- κ B, were linked to the imatinib insensitivity (Figure 2E). Consistent with this finding, pharmacological blockade of NF- κ B activation by an IKK2 inhibitor was reported to be effective on imatinib-resistant CML cells including a T315I mutant in culture⁵⁸. Taken together, the upregulation of NF- κ B signaling pathways via an inflammatory response could be responsible for the insensitivity to imatinib.

Imatinib-insensitive CML stem/progenitor cells highly expressed TLR1 and TLR6 (Figure S2), which are also overexpressed in MDS BM CD34⁺ cells⁵⁹. Since both CML and MDS are myeloid leukemias originating from HSCs, the dysregulation of TLRs may be a common feature of CML and MDS, implicating TLRs as good therapeutic targets for these diseases. In relation to this hypothesis, as depicted in Figure 3A, TLRs except for TLR3 and IL1Rs share a downstream signaling cascade leading to NF- κ B activation. Indeed, we have found that IL1R1 was highly expressed in imatinib-insensitive CML

stem/progenitor cells (Figure S2). Moreover, an IL1RA in combination with a TKI inhibits the growth of CML LSCs and induces their apoptosis compared with the TKI alone ³³. These results suggest that IL1R/TLR signaling pathways have crucial roles in the maintenance of CML LSCs. However, TLR signaling pathways in CML LSCs are not well understood. We hypothesized that the blockade of both IL1R and TLR signaling pathways by IRAK1/4 inhibitors could be more effective at eradicating CML LSCs than IL1R alone. Consistent with this notion, an IRAK1/4 inhibitor can block signals from both TLRs and IL1R and is effective in a xenograft model of human MDS ⁶⁰. In the present work, we demonstrate that an IRAK1/4 inhibitor in combination with imatinib effectively eradicated CML LSCs in both a mouse CML-like model and xenograft model with newly diagnosed CML-CP patient samples compared with imatinib alone. Intriguingly, neither imatinib nor the IRAK1/4 inhibitor alone deactivated NF-κB, suggesting that BCR-ABL1 activates NF-κB in the absence of imatinib, whereas IRAK1/4-mediated signals activates NF-κB in the presence of imatinib. Consistently, IL1R/TLR signal pathways were upregulated in CML LSCs in the presence of imatinib. These results indicate that the IL1R/TLR-IRAK1/4-NF-κB signaling axis is crucial for the survival of quiescent CML-LSCs in the presence of imatinib. Therefore, IRAK1/4 inhibitors in combination with imatinib are a powerful strategy for the eradication of CML LSCs.

PD-L1 expression on CML LSCs (DP cells) was higher than that on CML progenitors (SP cells) (Figure 5A and 5B), consistent with a previous study ⁴¹. PD-L1 expression on

human CML-LSCs (Lin-CD34⁺CD38^{-/low}) is also higher than that on the same population of healthy controls ^{23,61}. In addition, blocking the PD-1/PD-L1 interaction by PD-L1 or PD-1 antibody together with cytotoxic T cell (CTL) transfer eradicates CML LSCs ^{41,42}, indicating that PD-L1 protects CML LSCs from CTL-mediated elimination. Indeed, we experienced one clinical case who received dasatinib for newly diagnosed CML-CP and nivolumab for the existing relapsed renal cell carcinoma. This patient exhibited an early MR4.5 achievement compared with dasatinib alone at Juntendo University Hospital (Figure 5J) and in the DASSISION trial. Consistent with this, in the present study, we identified that the combination of imatinib and anti-PD-L1 antibody effectively eradicated CML LSCs in a mouse CML-like model. In addition, the efficacy of the blockade of the PD-L1/PD-1 interaction was abolished in the absence of T cell immunity. Moreover, both anti-PD-1 and anti-CTLA4 antibodies in combination with imatinib were effective at prolonging the survival of CML mice (Figure S4H), suggesting that immune checkpoint molecules are crucial for the protection of CML LSCs against T cell antitumor immunity. These results indicate that blocking the interactions of immune checkpoint molecules, PD-1/PD-L1 or CTLA4/CD80/CD86 interactions, together with TKIs is effective at eradicating human CML LSCs in the presence of T cell immunity. These results also suggest that both PD-L1 and CD80/CD86 are required for the protection of CML LSCs. However, it is not practical to use checkpoint inhibitors for patients with CML-CP, because TKI alone is able to efficiently extend the survival of the patients without severe side effects and checkpoint inhibitors have potentially severe side effects. Therefore, safer strategies for the blockade of PD-1/PD-L1 other than PD-1 or PD-L1

antibody are needed.

Intriguingly, the IRAK1/4 inhibitor in combination with imatinib attenuated PD-L1 expression on mouse and human CML LSCs (Figures S4D-F). Since PD-L1 expression is regulated by NF- κ B^{62,63}, the deactivation of NF- κ B by the combination resulted in the attenuation of PD-L1 expression on CML LSCs. Therefore, a part of the efficacy of the IRAK1/4 inhibitor is mediated by activation of T cell antitumor immunity via the attenuation of PD-L1 expression. On the other hand, the IRAK1/4 inhibitor induced the apoptosis of human CML LSCs in the absence of T cell immunity (Figure 4F), indicating that the inhibitor itself has a proapoptotic function on CML LSCs. Moreover, PD-L1 has been reported to directly enhance cancer cell survival and tumor progression, in addition to its immunosuppressive function^{64,65,66}, indicating that the PD-L1 blockade itself is detrimental to CML LSCs. However, because PD-L1 blockade was not effective at eradicating CML LSCs in the absence of T cell immunity (Figure 5H), we concluded that the proapoptotic function of the IRAK1/4 inhibitor is independent of the attenuation of PD-L1.

In summary, we developed a new method for detecting quiescent CML LSCs using G₀M in a mouse CML-like model. The identification of quiescent CML LSCs enabled drug screening directly against quiescent CML LSCs. Using this system, we found that an IRAK1/4 inhibitor in combination with imatinib eradicated CML LSCs by dual functions. One function is proapoptotic and the other is the induction of antitumor immunity by

attenuating immune check point molecules. Therefore, IRAK1/4 inhibitors are attractive drugs for eradicating CML LSCs with TKIs. However, since IRAK1/4 are central mediators of inflammatory responses, optimization of the drug dose is crucial for clinical use.

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Author contributions

Conceptualization and Methodolog, Y.T. and T.K.; Investigation, K.M., T.F., K.A., M.T., T.U., H.T. and Y.T.; Writing – Original Draft, Y.T. and T.K.; Writing – Review & Editing, Y.T., S.G. and T.K.; Funding Acquisition, Y.T. and T.K.; Resources, S.T., N.W., S.M., M.I., M.A., M.N., T.T., N.K., R.I., C.L. and E.S.M.; Supervision, S.G., T.F. and T.K.

Competing Interests

The authors declare no competing interests.

Methods

Reagents

Imatinib was purchased from LC Laboratories. IRAK1/4 inhibitor (R568 for in vitro experiments and R930259 Diet (100 ppm) for in vivo experiments) was provided by Rigel Pharmaceuticals, Inc.

Mice

C57BL/6J mice were purchased from Japan SLC. G₀M mice (*Vav1-Cre*; *Rosa*^{R26R-G0M/R26R-wt} [B6.Cg-Gt(ROSA)26Sor<tm1(mVenus/Cdkn1b<*>)Toki>, BRC No. 109200]) mice were obtained by in-house breeding. Rag2 knockout (B6(Cg)-Rag2tm1.1Cgn/J) mice were purchased from Sankyo Labo Service Corporation. NOG hIL-3/GM-CSF Tg (NOD.Cg-Prkdcscid Il2rgtm1Sug Tg(SRa-IL3, CSF2)7-2/Jic) mice were provided by the Central Institute for Experimental Animals (CIEA; Japan). All animal studies were approved by the Animal Care Committee of the Institute of Medical Science at the University of Tokyo (approval number: PA13-19 and PA16-31) and were conducted in accordance with the Regulation on Animal Experimentation at the University of Tokyo based on International Guiding Principles for Biomedical Research Involving Animals.

Patient samples

Proper informed consent was obtained from all participants. All experiments were performed according to the Declaration of Helsinki and were approved by the Ethics Committee of Juntendo University School of Medicine (IRB#2019113) and the Research Ethics Review Committee of the Institute of Medical Science of the University of Tokyo (2019-8-0702). Heparin-anticoagulated BM cells were obtained from 5 newly diagnosed CML-CP patients (Table S1). Informed consent was obtained in accordance with the Declaration of Helsinki, and all procedures were approved by the Research Ethics Board at Juntendo University. Mononuclear cells were isolated using Lymphoprep (STEMCELL Technologies) density gradient separation, and CD34⁺ cells (> 85%) were enriched

immunomagnetically using an CD34 MicroBead Kit (Milteny Biotec). Purity was verified by re-staining isolated cells with an allophycocyanin-cyanine7-labeled (APC-Cy7) anti-CD34 antibody (clone 561, BioLegend), and the cells were analyzed using a FACS Aria (BD) machine.

Preparation of retrovirus

The cDNA encoding human BCR-ABL1 (a gift from H.Honda, Tokyo Women's Medical University) was cloned into the EcoRI site of the MSCV-IRES-GFP vector. Retroviral packaging cells (Plat-E) were transiently transfected with the MSCV-BCR-ABL1-IRES-GFP plasmid using polyethylenimine (Sigma-Aldrich) and used for transplantation into mice as described later.

Generation of a mouse CML-like model

G₀M mice were treated with 5FU and four days later culled to isolate whole BM cells. The cells were cultured in IMDM medium (Thermo Fisher Scientific) supplemented with 20% FBS, 1% penicillin/streptomycin, 50 μ M β -mercaptoethanol, 10 ng/ml mouse interleukin (IL)-6, 50 ng/ml human TPO and 50 ng/ml mouse stem cell factor (SCF) overnight. On the next day, the cells were infected with the above retrovirus carrying MSCV-BCR-ABL1-IRES-GFP using RetroNectin® (TaKaRa Bio Inc.). 1 x 10³ GFP-positive cells were transplanted intravenously into lethally irradiated (9.5 Gy) C57BL/6 mice together with 1x10⁵ BM mononuclear cells from non-irradiated C57BL/6 mice.

Flow cytometric analysis and antibodies

BM hematopoietic cells were isolated from the femurs and tibias by flushing and the depletion of red blood cells by ACK Lysing Buffer (Thermo Fisher Scientific). The cells were stained with the appropriate dilution of fluorochrome- and/or biotin-conjugated antibodies and 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) for dead cell exclusion and analysis using a FACSVerse flow cytometer and FASCAria (BD Biosciences). The following antibodies (all BioLegend) were used: Lineage Cocktail [CD5(53-7.3)-biotin, TER-119(TER-119)-biotin, CD11b(M1/70)-biotin, Gr-1(RB6-8C5)-biotin and B220(RA3-6B2)-biotin] with streptavidin BV605, c-Kit(2B8)-PE-Cy7, Sca-1(D7)-APC and CD27(LG.3A10)-BV421 for mouse CML LSC analysis; CD34(561)-APC-Cy7, CD38(HB-7)-PE-Cy7, CD90(5E10)-biotin with streptavidin BV605, CD45RA(HI100)-FITC, CD45(2D1)-FITC or -BV421, Lineage Cocktail [CD3(OKT3), CD14(M5E2), CD16(3G8), CD19(HIB19), CD20(2H7), CD56(HCD56)]-BV510 for human CML LSC analysis; and CD274(10F.9G2 and 29E.2A3)-PE for the detection of mouse and human PD-L1, respectively. The detection of annexin V-positive cells for apoptosis analysis was performed using either Annexin V-PE or APC.

In vivo treatment of mice with CML-like disease

All treatments were commenced 10 days after the BM transplantation. Imatinib (40 mg/kg) or vehicle was administered once daily by oral gavage. The IRAK1/4 inhibitor was administered by feed administration (R930259 Diet (100 ppm)). Anti-PD-L1 antibody (10F.9G2, BioXcell; 200 µg), anti-PD-1 antibody (29F.1A12, BioXcel; 200 µg),

anti-CTLA-4 antibody (9D9, BioXcel; 200 µg), or their isotype control antibodies were administered by intraperitoneal injection once a week. For the CD8 transfer, 2×10^6 CD8 T cells from the spleen were intravenously injected into CML mice.

In vitro drug treatment for patient samples

1×10^2 or 1×10^3 CML-CP CD34⁺ cells were cultured using the StemSpan™ Leukemic Cell Culture Kit (STEMCELL Technologies) in a well of a 96-well flat-bottom plate in the presence of DMSO, imatinib (0.5 µM), IRAK1/4 inhibitor (R568; 1, 3 and 10 µM), or the combination of for 7 days. At day 7, all cells were subjected to flow cytometric analysis to count the number of live cells and assess the proportion of CD34⁺CD38⁻ and CD34⁺CD38⁻CD90⁺CD45RA⁺ fractions. Measurements of Annexin V positive cells in those fractions were performed at the same time.

Immunodeficient murine xenograft experiments

CML CD34⁺ cells were transplanted (1×10^5 or 1×10^6 cells per mouse) via tail-vein injection into female 8-to-12-week-old sublethally irradiated (2.0 Gy) NOG hIL-3/GM-CSF Tg mice (CIEA). Human cell engraftment was monitored at 4 to 6 weeks after transplantation by FACS for the presence of human CD45⁺ cells in the blood. Mice that showed evidence of human cell engraftment were randomized and treated with vehicle (water), imatinib, IRAK1/4 inhibitor in feed (R930259 Diet (100 ppm)), or a combination of imatinib and IRAK1/4 inhibitor for 14 or 21 days commencing at 2 weeks after the transplantation. Imatinib (40 mg/kg) or vehicle were administered once daily by oral

gavage. Mice were euthanized at the end of the treatment, and marrow contents of the femurs and tibias were obtained. The presence of human CD45, CD34, CD38, CD90 CD45RA and CD274 on the engrafted cells was assessed by FACS. Engraftment data were obtained from CML CD34⁺ samples treated for 21 days from two patients.

RNA sequencing

We performed RNA sequencing (RNA-Seq) as described previously (Hayashi et al., 2018) with minor modification. In brief, using 100 sorted cells, the first strand of cDNA was synthesized using a PrimeScript RT reagent kit (TaKaRa Bio Inc.) and not-so random primers. Following the synthesis of the first strand, the second strand was synthesized by Klenow Fragment (3', -5', exo-; New England Biolabs Inc.) and complement chains of the not-so random primers. Using purified double-strand cDNA, the library for RNA-Seq was prepared and amplified using a Nextera XT DNA sample Prep Kit (Illumina Inc.). These prepared libraries were sequenced on a Next-Seq system (Illumina Inc.) according to the manufacturer's instruction. In addition, each obtained read was mapped to the reference sequence "GRCm38/mm10" using CLC genomic workbench v11.0.0 (Qiagen), and expression levels were normalized and subjected to statistical analyses based on EdgeR.

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519 **Statistical analyses**

520 Statistical differences were determined using the unpaired and two-tailed Student's t-test,
521 one-way ANOVA with Tukey's correction for multiple comparisons, and the log-rank
522 (Mantel-Cox) test for the survival analysis using Graphpad Prizm6 software (MDF).

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537 **Figures & Figure legends**

Figure 1

Figure 1. G0M and CD27 identify quiescent CML LSCs

(A) Experimental design. (B) Survival curves for mice treated with vehicle (n=9) and imatinib (n=11). (C) Representative FACS plots of BCR-ABL1⁺Lin⁻Sca1⁺c-Kit⁺ cells in the BM from mice treated with vehicle and imatinib. (D) Absolute number of LSCs (G0M⁺CD27⁺ cells in FACS plots in C) in the BM. (E) Survival curves for recipient mice transplanted with the indicated populations from CML mice treated with vehicle. DP (n=8), SP (n=8), G0M⁺CD27⁻ (n=8), and DN (n=7). (F) Representative FACS plots of BCR-ABL1⁺Lin⁻Sca1⁺c-Kit⁺ cells in the BM from the recipient mice transplanted with DP and SP cells in E. (G) The percentage of LSCs (G0M⁺CD27⁺ cells in FACS plots in F) in BCR-ABL1⁺ cells in the BM. (H) Survival curves for recipient mice transplanted with the indicated populations from CML mice treated with imatinib. DP (n=5), SP (n=4), G0M⁺CD27⁻ (n=3), and DN (n=3). (I) Representative FACS plots of BCR-ABL1⁺Lin⁻Sca1⁺c-Kit⁺ cells in the BM from the recipient mice transplanted with DP and SP cells in H. (J) The percentage of LSCs (G0M⁺CD27⁺ cells in FACS plots in I) in BCR-ABL1⁺ cells in the BM. Data are shown as the mean ± SEM. P values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons. ** P < 0.01.

Figure 2

Figure 2. Inflammatory signal pathways are enriched in imatinib-insensitive CML-LSCs.

(A) Principal component analysis (PCA) of the gene expression profiles of the indicated CML populations (SP, DP, IMSP and IMDP cells: n=3 each) and normal HSCs (GSE138884) (n=6). (B and C) Pathway analyses by the Enrichr web tool. Positive correlation features of PC1 (B) and negative correlation features of PC2 (C) in A. (D) PCA of the gene expression profiles of the four different CML populations. (E and F) Pathway analyses by the Enrichr web tool. Negative correlation features of PC2 (E) and PC1 (F) in D.

Figure 3

Figure 3. IRAK1/4 inhibitor together with imatinib eradicates CML LSCs

(A) The IL1R/TLR-IRAK1/4 signaling pathway. (B) Survival curves for mice treated with vehicle, imatinib, IRAK1/4 inhibitor (IRAKi (R930259 Diet): 100 ppm) and the combination of (n=6 each). (C) Spleen weights of CML mice treated with vehicle, imatinib, IRAKi and the combination of (n=5-7). Representative spleens are shown in the bottom. (D) Representative FACS plots of BCR-ABL1⁺Lin⁺Sca1⁺c-Kit⁺ cells in the BM for all treatment conditions. (E) Absolute number of LSCs (G₀M⁺CD27⁺ cells in FACS plots in D) in the BM. (F) p65 localization in CML LSCs for all treatment conditions. (G) Quantification of nuclear p65 in CML LSCs (n= 3–4 biological replicates per group). Scale bar, 5 μ m. Data are shown as the mean $\pm$ SEM. P values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons. * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 4

Figure 4. IRAK1/4 inhibitor together with imatinib is detrimental to human CML LSCs

(A-F) CD34⁺ cells (1×10^3) from the BM of a newly diagnosed CML-CP patient were cultured in triplicates in liquid culture in the presence of imatinib (IM: 0.5 μ M) or IRAK1/4 inhibitor (IRAKi (R568): 1, 3 or 10 μ M) alone or in combination for one week (A). Similar results from two different patients are shown in Figure S3. Absolute number of total live cells (B), CD34⁺CD38⁻ cells (C) and CD34⁺CD38⁻CD90⁺CD45RA⁻ cells (D). Percentage of apoptotic cells in CD34⁺CD38⁻ cells (E) and CD34⁺CD38⁻CD90⁺CD45RA⁻ cells (F) was measured by annexin staining. (G-J) NOG-hIL-3/hGM-CSF mice were transplanted with CD34⁺ cells from the BM of a CML-CP patient (n=1) and treated with vehicle (n=3), imatinib (n=4), IRAK1/4 inhibitor (IRAKi (R930259 Diet): 100 ppm) (n=3), or the combination of these (n=4). (G) Experimental design. Absolute number of human CD45⁺ cells (H), CD34⁺CD38⁻ cells (I), and CD34⁺CD38⁻CD90⁺CD45RA⁻ cells (J) in the BM were analyzed at the end of the experiment. Data are shown as the mean $\pm$ SEM. P values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Figure 5

Figure 5. The blockade of PD-L1 together with imatinib eradicates CML LSCs

(A) RPKM values for PD-L1 among the indicated populations (n=3). (B) MFI of PD-L1 among the

indicated populations (n=3-4). (C) Survival curves for mice treated with vehicle (n=6), imatinib (n=9), anti-PD-L1 antibody (n=8) and the combination of (n=9). (D) Representative FACS plots of BCR-ABL1⁺Lin⁻Sca1⁺c-Kit⁺ cells in the BM from imatinib and the combination treated groups. (E) Absolute number of LSCs (G₀M⁺CD27⁺ cells in the FACS plots in D) in the BM from all treatment groups (n=4-6). (F) Spleen weights of CML mice from all treatment groups (n=5-7). (G) The percentage of apoptotic cells in CML LSCs from all treatment groups was measured by annexin staining (n=4-6). (H) Survival curves for Ly5.1 Rag2 knockout CML mice treated with vehicle, imatinib, anti-PD-L1 antibody (PD-L1), 2×10⁶ MACS-purified CD8⁺ T cells from Ly5.1 mice (CD8) and the combination of (n=4-5). (I) A medical history of the double cancer patient. (J) BCR-ABL1 International Scale (IS) from CML-CP patients treated with dasatinib alone (n=14) or dasatinib together with anti-PD-1 antibody (nivolumab) (n=1) at 6, 9 and 12 months after commencement of dasatinib treatment. Data are shown as the mean ± SEM. P values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P<0.0001.

Supplemental Figure Titles and Legends

FigureS1

Figure S1. Identification of quiescent CML LSCs by G_0M , related to Figure 1

(A) Colony-forming assays of the indicated populations from vehicle-treated CML mice. 500 cells were plated in the 1st round (left), and 5×10^3 cells were plated in the 2nd round (right). n=4 each. (B) Kinetic analysis of the chimerism of GFP⁺ cells in the peripheral blood of recipient mice transplanted with the indicated populations from vehicle-treated CML mice. DP (n=3), SP (n=4), $G_0M^+CD27^-$ (n=5), and DN (n=5). (C) Wright-Giemsa marrow cytopspins of the indicated populations from untreated CML mice. Scale bar, 5 μ m. (D) Kinetic analysis of chimerism of GFP⁺ cells in the peripheral blood of recipient mice transplanted with the indicated populations from imatinib-treated CML mice. DP (n=5), SP (n=5), $G_0M^+CD27^-$ (n=5), and DN (n=4). Data are shown as the mean $\pm$ SEM. P values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons. * P < 0.05, *** P < 0.001, ****P<0.0001.

Figure S2

Figure S2. TLRs and IL1R1 are highly expressed in imatinib-insensitive CML LSCs, related to Figure 2

The RPKMs of Toll-like receptors (TLR1, TLR2, TLR4 and TLR6) and IL1R1 among the indicated CML populations (n=3 each) and normal HSCs (n=4). Data are shown as the mean ± SEM. P values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons. * P < 0.05, ** P < 0.01.

Figure S3

Figure S3. The combination of IRAK1/4 inhibitor and imatinib is detrimental to CML-CP CML LSCs, related to Figure 4

(A-D) MACS-purified CD34⁺ stem/progenitor cells (1×10^2) from the BM of newly diagnosed CML-CP patients (n=2) were cultured in triplicates in liquid culture in the presence of imatinib (IM: 0.5 μ M), IRAK1/4 inhibitor (IRAKi (R568): 1, 3 or 10 μ M), or the combination of for one week. The absolute number of CD34⁺CD38⁻ cells (A and C) and CD34⁺CD38⁻CD90⁺CD45RA⁻ cells (B and D). Data are shown as the mean $\pm$ SEM. P values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons. * P < 0.05, ** P < 0.01, *** P < 0.001, ****P<0.0001.

Figure S4

Figure S4. Immune checkpoint molecules on CML LSCs, related to Figure 5

(A-C) The RPKMs of CD80, CD86 and Tim3 in the indicated CML populations (n=3 each) and normal HSCs (n=4). (D) The mean fluorescence intensity (MFI) of mouse PD-L1 on DP cells from CML mice treated with imatinib, IRAK1/4 inhibitor or the combination of. (E, F) The MFI of human PD-L1 on CD34⁺CD38⁻ cells (E) and CD34⁺CD38⁻CD90⁺ cells (F) in the BM of CML-CP CD34⁺ xenografts treated with

vehicle, imatinib, IRAK1/4 inhibitor (IRAKi (R930259 Diet): 100 ppm) or the combination of these. (G) The MFI of mouse PD-L1 on DP cells from CML mice treated with imatinib, anti-PD-L1 antibody or the combination of these. (H) Survival curves for CML mice treated with imatinib alone (n=9) or with one of anti-PD-L1, anti-PD-1 and anti-CTLA4 (n=6 each). IM, imatinib. Data are shown as the mean $\pm$ SEM. P values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons. * P < 0.05, ** P < 0.01.

Supplementary Tables & Table Titles

Table S1: Characteristics of CML patient samples used in this study. Related to Methods.

CML patient No.	Date of BM biopsy	Sex	Sex/Age	Samples used for experiments in
1	2019/7/10	Female	28	Figures 3A-E
2	2019/8/2	Male	59	Figure S3A and S3B
3	2019/4/1	Female	46	Figures S3A and S3B
4	2020/10/15	Male	61	Figures 3F-H

Table S2: Characteristics of CML patients who received Dasatinib

CML patient No.	Age	Treatment	Sokal Score
1	69	Dasatinib(100mg Once Daily)	0.76
2	41	Dasatinib(100mg Once Daily)	0.54
3	77	Dasatinib(100mg Once Daily)	0.81
4	41	Dasatinib(100mg Once Daily)	0.75
5	29	Dasatinib(100mg Once Daily)	0.45
6	84	Dasatinib(100mg Once Daily)	0.99
7	39	Dasatinib(100mg Once Daily)	0.52
8	52	Dasatinib(100mg Once Daily)	0.65
9	50	Dasatinib(100mg Once Daily)	0.59
10	73	Dasatinib(100mg Once Daily)	1.42
11	71	Dasatinib(100mg Once Daily)	1.11
12	41	Dasatinib(100mg Once Daily)	0.54
13	47	Dasatinib(100mg Once Daily)	0.64
14	36	Dasatinib(100mg Once Daily)	0.53
15	60	Dasatinib(100mg Once Daily)+Nivolumab	1.02

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