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Engineering a BCR-ABL–Activated Caspase for the Selective Elimination of Leukemic Cells

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Increased understanding of the precise molecular mechanisms involved in cell survival and cell death signaling pathways offers the promise of harnessing these molecules to eliminate cancer cells without damaging normal cells. Tyrosine kinase oncoproteins promote the genesis of leukemias through both increased cell proliferation and inhibition of apoptotic cell death. Although tyrosine kinase inhibitors, such as the BCR-ABL inhibitor imatinib, have demonstrated remarkable efficacy in the clinic, drug-resistant leukemias emerge in some patients because of either the acquisition of point mutations or amplification of the tyrosine kinase, resulting in a poor long-term prognosis. Here, we exploit the molecular mechanisms of caspase activation and tyrosine kinase/adaptor protein signaling to forge a unique approach for selectively killing leukemic cells through the forcible induction of apoptosis. We have engineered caspase variants that can directly be activated in response to BCR-ABL. Because we harness, rather than inhibit, the activity of leukemogenic kinases to kill transformed cells, this approach selectively eliminates leukemic cells regardless of drug-resistant mutations.

dimerization | phosphorylation

The activated fusion kinase BCR-ABL that causes chronic myeloid leukemia (CML) both increases cell proliferation and inhibits apoptosis (1, 2). Current therapies for tyrosine kinase-driven leukemias are based on inhibiting the aberrant tyrosine kinase activity with small molecule tyrosine kinase inhibitors (TKIs), such as imatinib. However, TKIs can lose efficacy because of point mutations or amplification of the tyrosine kinase, resulting in relapse and a poor long-term prognosis (3, 4). Additionally, because CML stem cells are innately resistant to TKIs, these drugs are not curative (5, 6). Therefore, there is a clinical need for an alternative approach to selectively targeting CML cells to treat resistant disease and eradicate residual leukemia.

Many chemotherapeutic agents trigger apoptotic cell death by activating a family of cysteine proteases called caspases (7). Upon receipt of apoptotic stimuli, initiator caspases are activated. For instance, the initiator caspase-8 is activated through the formation of death-inducing signaling complexes (DISC) in response to engagement of extracellular ligands by cell surface death receptors such as CD95 or TNFR1. Likewise, activation of caspase-9 is mediated by the assembly of the caspase-activating complex apoptosome after the release of cytochrome *c* from the mitochondrial intermembrane space to the cytoplasm. Once activated, initiator caspases can cleave and activate the zymogenic forms of executioner caspases (e.g., caspase-3), leading to the proteolytic cleavage of specific apoptotic substrates and, ultimately, the dismantling of the cell. Importantly, activation of initiator caspases is driven by "induced proximity"; initiator caspases-8 and -9 are activated when individual molecules are brought into close proximity with one another through the formation of the DISC or apoptosome platform, respectively (8–11). Accordingly, artificial dimerization of initiator caspases can also trigger their activation without assembly of the DISC or apoptosome (12). Because multiple apoptotic pathways that lead to caspase activation are inhibited in CML, it would be optimal to activate caspases outside of the normal cell death pathways; we hypothesized that forcible dimerization of initiator caspases that can be induced in a BCR-ABL–dependent manner regardless of drug resistance would circumvent these loci of inhibition, providing a valuable tool for selectively killing leukemic cells.

Results and Discussion

We embarked on the design of a caspase variant that could be activated specifically in response to BCR-ABL activity. As shown in Fig. 1*A*, this construct, termed "iCaspase-8" (inducible caspase-8), consists of the adaptor protein Crk flanked by two copies of caspase-8 (protease domain without the prodomain). In this caspase variant, phosphorylation of Tyr-221 on Crk (Tyr-222 on chicken Crk used in the present study) by BCR-ABL was expected to cause a conformational change in Crk resulting from intramolecular binding of pTyr-221 to the Crk SH2 domain (13). We anticipated that the caspase-8 moieties would then be juxtaposed in close proximity, thereby triggering the intramolecular dimerization of caspase-8 (13). It has been reported that BCR-ABL exhibits different substrate preferences than its normal cellular counterpart, c-ABL (14, 15). Thus, although every cell contains tyrosine kinases, we expected that the significantly higher level of BCR-ABL kinase activity in transformed cells, combined with the slightly altered substrate specificity, would trigger selective activation of the caspase constructs in transformed cells. In normal cells, construct dimerization would be very inefficient and the proteins should be innocuous. As predicted by this model, expression of iCaspase-8 triggered massive apoptotic death in the BCR-ABL–positive patient-derived human cell line K562 (Fig. 1*B*). iCaspase-8 also specifically killed Ba/F3 cells stably expressing BCR-ABL (Fig. 1 *C* and *D* and Fig. S1 *A* and *B*). Importantly, iCaspase-8 did not induce apoptosis in normal Ba/F3 cells (Fig. 1 *C* and *D*). Moreover, because Crk is not phosphorylated on Y221 in cells expressing the leukemic oncoproteins, $FLT3^{D835Y}$ (found in acute myeloid leukemia; ref. 16) or TEL-PDGFRβ (found in chronic myelomonocytic leukemia; ref. 17), iCaspase-8 did not kill Ba/F3 cells expressing these activated tyrosine kinases (Fig. 1 *C–E* and Fig. S1 *D* and *E*). The subcellular localization of iCaspase 8 was

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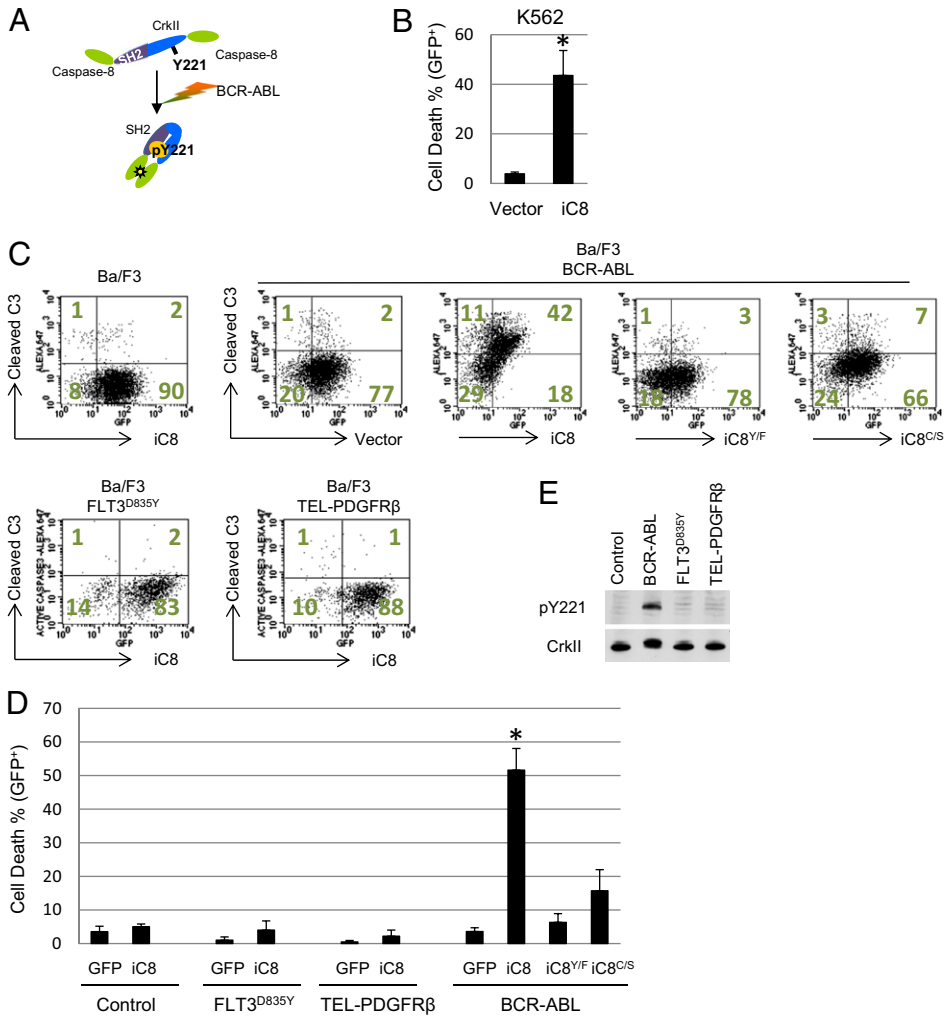

Fig. 1. iCaspase-8 specifically triggers apoptosis in cells expressing BCR-ABL. (*A*) iCaspase-8 becomes active after phosphorylation of Crk at Tyr-221 by BCR-ABL. (*B*) iCaspase-8 (MSCV-IRES-GFP-WRE) was expressed in K562 cells. Forty-eight hours after infection, cell death % (with SEM) was analyzed by PI staining (*$P < 0.05$ by Student *t* test). (*C* and *D*) Normal Ba/F3 cells, or Ba/F3 cells expressing BCR-ABL, $FLT3^{D835Y}$, or TEL-PDGFRβ, were infected with a retroviral vector (MSCV-IRES-GFP-WRE) encoding vector alone (GFP), iCaspase-8 (iC8), iCaspase-$8^{Y/F}$ ($iC8^{Y/F}$), or iCaspase-$8^{C/S}$ ($iC8^{C/S}$). Forty-eight hours after infection, the cells were fixed, stained with anti-cleaved caspase 3-antibody (Alexa 647), and analyzed by flow cytometry. Shown are representative flow cytometry plots (*C*) and average cell death % with SEM (% cells with cleaved-caspase-3 signal) in the GFP-positive population (*D*; *$P < 0.05$ GFP vs. iC8 by Student's *t* test). (*E*) Ba/F3 cell lysates were subjected to immunoblotting with anti–pan-Crk and anti–phospho-Tyr-221 antibodies.

the same (cytoplasmic) in both the Ba/F3 cells and Ba/F3 cells transformed with activated tyrosine kinases (Fig. S1*F*). Importantly, iCaspase-8 carrying a Y221F mutation (iCaspase-$8^{Y/F}$) did not cause cell death in BCR-ABL–expressing cells, and, when one of the caspase-8 moieties was replaced with a catalytically inactive mutant (C/S), the mutant iCaspase-8 (iCaspase-$8^{C/S}$) was significantly reduced in its ability to kill BCR-ABL–transformed Ba/F3 cells (Fig. 1 *C* and *D* and Fig. S1*C*). Moreover, expression of the inactive iCaspase-8 mutants affected neither cell proliferation (Fig. S2*A*) nor clonogenic survival (Fig. S2*B*) of BCR-ABL–positive cells.

In that iCaspase-8 did not kill Ba/F3 cells expressing endogenous c-ABL, it seemed likely that resting levels of c-ABL could not phosphorylate and activate iCaspase-8 sufficiently to induce cell death. However, because the ultimate goal of our approach is to eliminate leukemic cells while sparing normal hematopoietic cells, it was important to confirm that the transient activation of c-ABL associated with normal cell signaling could not activate iCaspase-8. T-cell activation is well known to result in c-ABL activation (18, 19). To model the effects of this process on iCaspase-8 activation, Jurkat cells stably expressing empty vector or iCaspase-8 were treated with anti-CD3 and anti-CD28 antibodies. Although CD3/CD28 stimulation resulted in rapid c-ABL activation, it did not lead to cell death mediated by iCaspase-8 (Fig. S3 *A* and *B*). Taken together, these findings show that iCaspase-8 functions as a BCR-ABL–specific cell death activator.

To explore this approach further, we also created a caspase-9 version of the construct, named iCaspase-9 (Fig. S4*A*). iCaspase-9 also triggered apoptosis in BCR-ABL–expressing cells (Fig. S4*B*). Nevertheless, compared with iCaspase-8, the efficacy of iCaspase-9 was rather low (Fig. S4 *B* and *C*). This result is consistent with previous reports that caspase-9 requires docking on the Apaf-1 apoptosome for full activity (20, 21). Given that the initiator caspases-8 and -9 are activated by assembly of the DISC and apoptosome, respectively, we also sought to test an alternative approach to using the iCaspase strategy, in which endogenous initiator caspases would be forcibly activated in a BCR-ABL–dependent manner. We hoped that this approach would further safeguard against inappropriate activation in normal cells where unplanned dimerization of the exogenous caspase constructs might occur. We engineered constructs where Crk is flanked on

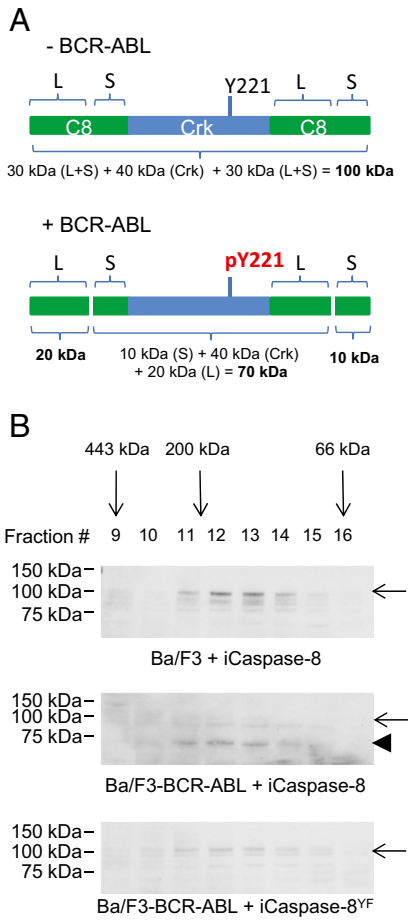

Fig. 2. iCaspase-8 activation is caused by intramolecular dimerization. (*A*) Schematic diagram of iCaspase-8. Each of the two caspase-8 moieties is composed of the small (S) and large (L) subunits. The linker connecting the two subunits will be autoprocessed upon activation of iCaspase-8, resulting in 10-, 20-, and 70-kDa proteolytic fragments. (*B*) iCaspase-8 or iCaspase-8$^{Y/F}$ was expressed in Ba/F3 cells (control or BCR-ABL). Forty-eight hours after infection, cell lysates were prepared and loaded onto a Supedex-200 column. Each chromatographic fraction was collected and subjected to immunoblotting with anti-human Caspase-8 antibody to detect iCaspase-8. Intact and cleaved iCaspase-8 is shown by an arrow and an arrowhead, respectively.

either side by the caspase recruitment domain (CARD) of the adaptor protein Apaf-1 (iApoptosome) or the death effector domain (DED) of the adaptor protein FADD (iDISC). Analogous to the scenario with iCaspase, phosphorylation of Tyr-221 on Crk by BCR-ABL would be expected to juxtapose the two CARD or DED moieties, which would, in turn, recruit and activate endogenous caspase-9 or caspase-8, respectively (Fig. S5*A*). However, we found that the iApoptosome was not as effective as iCaspase-8 or iCaspase-9 for reasons we do not fully understand and the iDISC triggered nonspecific cell death even in normal Ba/F3 cells (Fig. S5*B*). We therefore focused on iCaspase-8 as the most promising BCR-ABL–dependent inducer of cell death.

To demonstrate that iCaspase-8 activation was caused by intramolecular, not intermolecular, dimerization of the caspase-8 protease domain, we examined the status of iCaspase-8 by size-exclusion chromatography. When expressed in normal Ba/F3 cells, iCaspase-8 was detected in the fractions corresponding to its expected molecular mass (~100 kDa) (Fig. 2 *A* and *B* and Fig. S6). When expressed in BCR-ABL–transformed Ba/F3 cells, iCaspase-8 was detected at 70 kDa by immunoblotting, indicating that activated iCaspase-8 underwent proteolytic processing between the small and large subunits of the protease domains (Fig. 2 *A* and *B* and Fig. S6). Nevertheless, upon size-exclusion chromatography, the cleaved iCaspase-8 was detected in the same column fractions as the full-length iCaspase-8, as would be expected because the subunits should remain associated in the mature enzyme. When iCaspase-8$^{Y/F}$ was expressed in BCR-ABL–transformed Ba/F3 cells, the cleavage was not observed (Fig. 2*B*). These data are fully consistent with the idea that iCaspase-8 is activated through intramolecular dimerization.

We next tested whether iCaspase-8 could eliminate BCR-ABL–positive cells from a heterogeneous population without impeding the growth and survival of normal cells. iCaspase-8 was expressed bicistronically with GFP in a mixed population of normal (90%) and BCR-ABL–expressing (10%) Ba/F3 cells. GFP-positive cells were sorted by FACS 48 h after infection with iCaspase-8, and quantitative real-time PCR (qPCR) was performed on day 7 to detect the BCR-ABL message present in the population (Fig. 3). Remarkably, by day 7, the BCR-ABL mRNA decreased to nearly undetectable levels in the iCaspase-8–treated population (Fig. 3). These results indicate that iCaspase-8 can eliminate BCR-ABL–positive cells from a cell mixture without markedly decreasing the viability/proliferation of normal cells.

To further examine the efficacy of iCaspase-8, we performed colony forming assays by using primary hematopoietic stem cells (HSCs). Mouse HSC-enriched populations (c-Kit$^+$ Lin$^-$ Sca-1$^+$ or KLS) were isolated from bone marrow and were cotransduced with BCR-ABL and iCaspase-8 bicistronically expressed with GFP and YFP, respectively. GFP-YFP double-positive cells were selected by FACS and plated in methylcellulose medium. BCR-ABL–positive cells formed greatly reduced colony numbers when coinfected with iCaspase-8 compared with control vector (YFP) (Fig. 4*A* and Fig. S7*A*). Most importantly, however, iCaspase-8 significantly diminished leukemia cell colony formation even when BCR-ABL carried kinase domain mutations commonly arising in the setting of imatinib resistance (Fig. 4*A* and Fig. S7 *A* and *B*). Furthermore, expression of iCaspase-8 in naïve KLS cells did not affect cell growth in vitro relative to controls (Fig. 4*B* and Fig. S7*C*). Note that iCaspase-8 and imatinib were slightly synergistic in the presence of WT BCR-ABL. This result is likely due to residual BCR-ABL activity that persisted at the dose of 2 μM (Fig. S7*D*). Indeed, colonies still formed even in the presence of imatinib (approximately 10% compared with control; Fig. 4A and Fig. S7*E*). It is possible that any residual

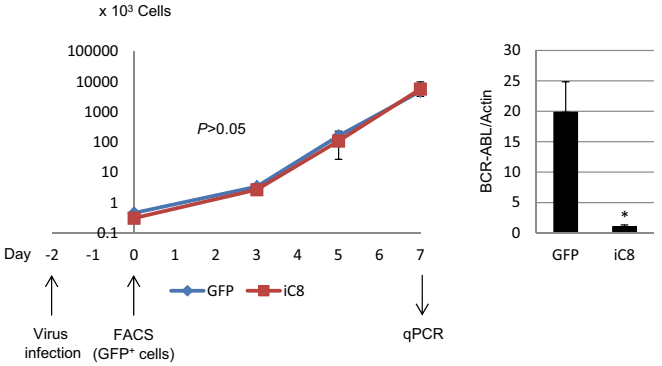

Fig. 3. iCaspase-8 eliminates BCR-ABL–positive cells while sparing normal cells. The mixture of Ba/F3 cells (normal:BCR-ABL = 9:1) was infected with an empty vector or iCaspase-8 (MSCV-IRES-GFP-WRE), and FACS sorted to select GFP-positive cells. The sorted cells were further cultured for 7 d and analyzed by qPCR for the BCR-ABL message. qPCR results were normalized against actin (*Right*; *$P < 0.05$ by Student's *t* test). The experiments were repeated four times, and there was no significant difference in cell growth between the two groups (*Left*; $P > 0.05$ by Student's *t* test). Error bars are SEM.

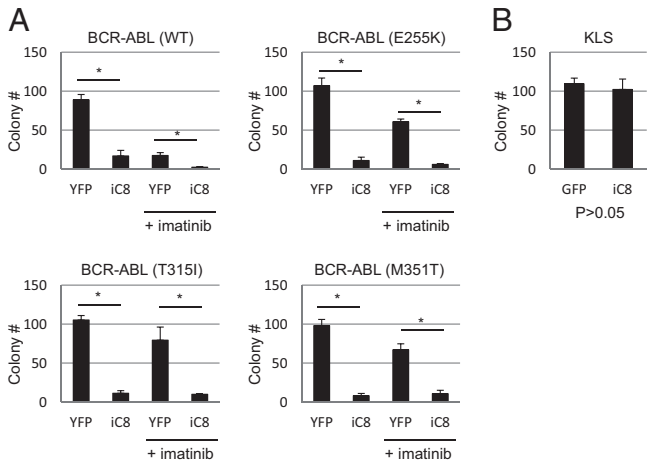

Fig. 4. iCaspase-8 selectively kills BCR-ABL–positive mouse HSCs. (*A*) Mouse KLS cells were cotransduced with BCR-ABL (MSCV-IRES-GFP) and iCapsase-8 (MSCV-IRES-YFP-WRE) or empty vector. BCR-ABL was either wild type (WT) or carried an imatinib-resistant mutation (E255K, T315I, M351T). GFP-YFP double-positive cells were further selected by FACS and plated in methylcellulose medium with or without 2 μM imatinib. The colony numbers were counted 7 d after plating (*$P < 0.05$ YFP vs. iC8 by Student *t* test). (*B*) Mouse KLS cells were transduced with iCapsase-8 (MSCV-IRES-GFP-WRE) or empty vector. GFP positive cells were selected by FACS and plated in methylcellulose medium. The colony numbers were counted 7 d after plating. Averages with SEM are shown.

BCR-ABL activity results in activation of iCaspase-8, leading to the observed synergy. In line with this hypothesis, no such synergy was observed in the presence of the imatinib-resistant BCR-ABL mutants (Fig. 4*A*).

iCapsase-8 was next tested most stringently in the setting of blast crisis CML. We generated mice with blast crisis CML driven by BCR-ABL and NUP98-HOXA9 (22, 23). The lineage-negative fraction (Lin^-) of BCR-ABL^+ NUP98-$HOXA9^+$ cells were isolated from the mouse spleens and transduced with control vector or iCaspase-8 expressed bicistronically with GFP. iCaspase-8, but not control vector, triggered massive apoptosis in BCR-ABL–expressing cells (Fig. S8 *A* and *B*). GFP-positive cells were further sorted by FACS for colony forming assays. As shown in Fig. 5*A* (also Fig. S7*F*), expression of iCaspase-8 significantly impaired clonogenic growth of the leukemic cells in vitro. We also transplanted the KLS cells into congenic mouse recipients after iCaspase-8 transduction. iCaspase-8 significantly suppressed blast crisis CML development in vivo (Fig. 5*B*; 96% control versus 40% iCaspase-8, $P < 0.05$) and its associated splenomegaly (Fig. 5 *C* and *D*). These results indicate that iCaspase-8 is highly effective in the established blast crisis form of disease.

We further tested whether iCaspase-8 could also kill human leukemia cells. We expressed iCaspase-8 in primary $CD34^+$ cells isolated from five independent patients with blast crisis CML (according to the preparation scheme shown in Fig. 6*A*). As shown in Fig. 6*B*, iCaspase-8 caused significant cell death in the primary cells, compared with vector control (GFP). To analyze the efficacy of iCaspase-8 in both stem and progenitor populations, $CD34^+$ cells were also loaded with CellTrace Violet. Importantly, expression of iCaspase-8 resulted in apoptosis in both dividing and nondividing populations, indicating that iCaspase-8 can induce apoptosis in quiescent CML stem cells, which is resistant to TKIs (7) (Fig. 6*C*).

In this study, we took a unique approach to selectively killing leukemia cells through the forcible induction of apoptosis. iCaspase-8 can be activated in a manner dependent on the kinase activity of BCR-ABL. Therefore, BCR-ABL–positive cells were selectively eliminated regardless of the presence of imatinib-resistant mutations. This approach may be particularly important in autologous bone marrow transplantation, where contamination of the graft with leukemia cells contributes to relapse and defines a need for more effective and selective purging strategies. Although autologous transplant is less frequently used in CML than before the introduction of tyrosine kinase inhibitors, it remains a salvage option for patients who have failed other therapies. Toward optimizing the purging effect, it still remains to be fully determined why a small proportion of BCR-ABL–positive cells survived in vitro and in vivo even after iCaspase-8 expression (Fig. 5). Because these "escapers" were associated with low iCaspase8 expression (Fig. S9), more robust delivery and expression system might further strengthen the efficacy of iCaspase-8. In addition, because it would be best to restrict iCaspase-8 expression to the time required to kill leukemic cells in vitro before transplantation, our procedure could ultimately be modified for use with less stable, non-integrating vectors or direct "protein" delivery, decreasing the chances of unwanted side effects. Moreover, although the constructs described here were tailored for BCR-ABL–positive leukemias, they establish proof of principle. Thus, it should be possible to alter the tyrosine phosphorylation site and SH2 within the linker separating the two capsase-8 moieties to design similar constructs for other tyrosine kinase-driven leukemias.

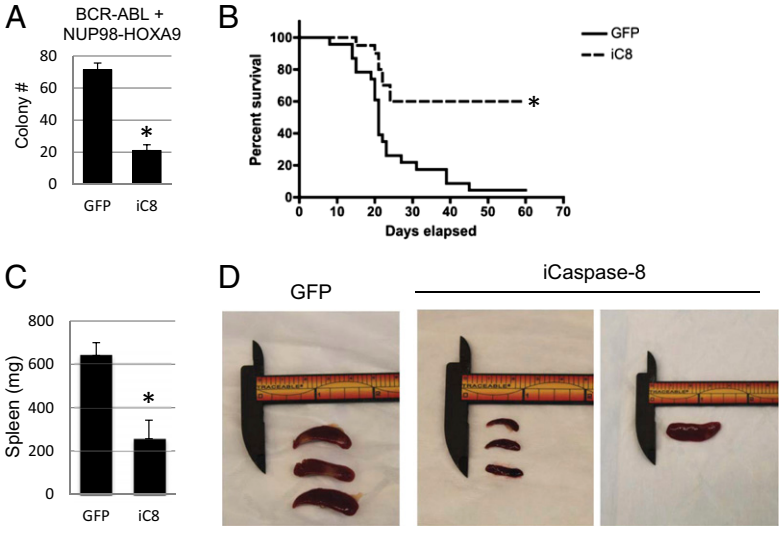

Fig. 5. iCaspase-8 selectively kills blast crisis CML cells in mouse. (*A*) Lin^- BCR-ABL^+ NUP98-$HOXA9^+$ cells isolated from mice with blast crisis CML were infected with control vector or iCaspase-8 (MSCV-IRES-GFP-WRE). Forty-eight hours after infection, GFP and YFP (BCR-ABL) coexpressing cells were selected and plated in methylcellulose medium for colony forming assays. The colony numbers were counted 7 d after plating. Average colony numbers with SEM are shown (*$P < 0.05$ by Student's *t* test). (*B*) Cells infected with BCR-ABL, NUP98-HOXA9, and either control vector or iCaspase-8 were transplanted and survival was monitored (control, $n = 23$; iCaspase-8, $n = 20$; *$P < 0.05$ by Kaplan–Meier analysis with log-rank test). (*C* and *D*) Spleens were collected from each mouse and weighed (*C*). Average weights and SEM are shown (*$P < 0.05$ by Student's *t* test). Representative spleens are shown in *D*: spleens from control (*Left*) and iCaspase-8 mice [surviving mice (*Center*) and leukemic mice (*Right*)].

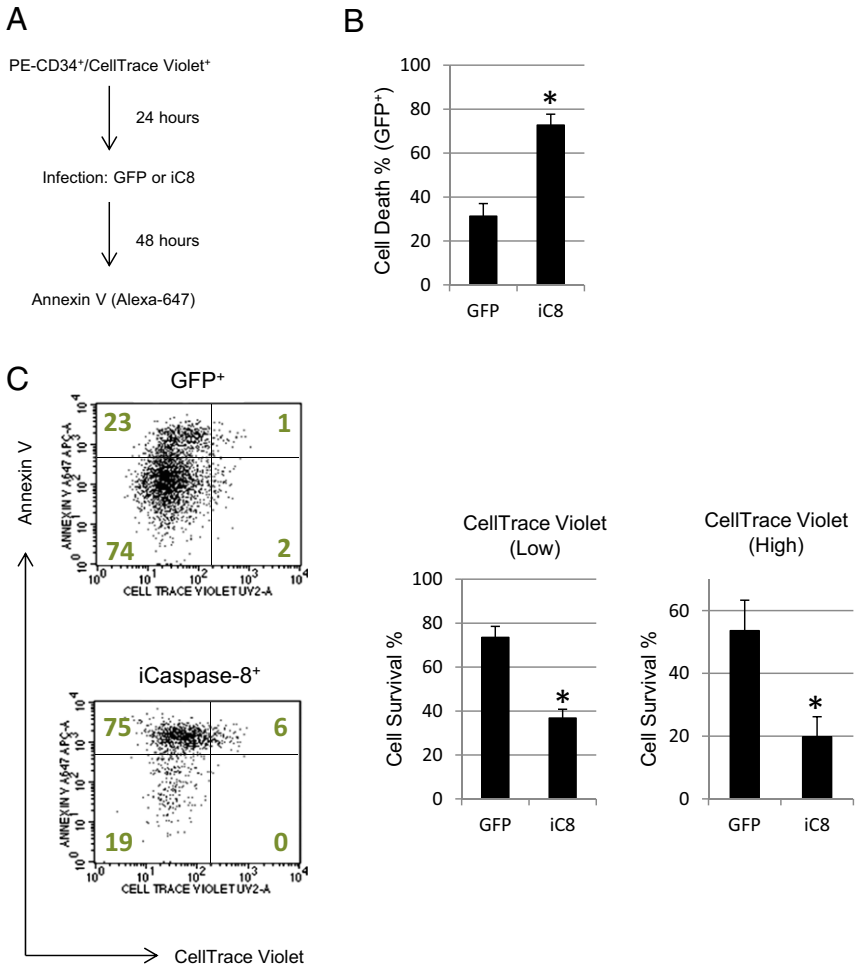

Fig. 6. iCaspase-8 selectively kills patient-derived CML cells in vitro. (*A*) Schematic diagram of the experimental process. Ficoll-purified white blood cells were isolated from patients with blast crisis stage of CML. The cells were stained with PE-conjugated anti-CD34 antibody and CellTrace Violet. CD34/CellTrace Violet double-positive cells were selected by FACS. $CD34^+$ /CellTrace $Violet^+$ cells were transduced with vector control or iCaspase-8 (pCDH-EF1-IRES-GFP). Forty-eight hours later, the cells were stained with Annexin V (conjugated with Alexa 647). (*B*) Average cell death % with SEM (% cells with Annexin V signal) in the GFP-positive population are shown (*$P < 0.05$ by Student's *t* test) (*C*). Representative flow cytometry plots (with % cell population) are shown (*Left*): Annexin V vs. CellTrace Violet in GFP^+ or iCaspase-8^+ populations. Average cell survival % with SEM in CellTrace $Violet^{high}$ and CellTrace $Violet^{low}$ populations are shown (*Right*; *$P <$ 0.05 by Student *t* test).

Materials and Methods

Plasmid Construction iCaspase-8, iCaspase-9, iApoptosome, and iDISC were cloned into MSCV vectors that contain the WRE element and a bicistronic IRES-GFP or -YFP (MSCV-IRES-GFP-WRE or MSCV-IRES-YFP-WRE) (Addgene plasmid 12282; ref. 24). For human primary cell experiments, iCaspase-8 was cloned into the lentiviral vector pCDH-EF1-IRES-GFP (System Biosciences). iCaspase-8 consists of human caspase-8 protease domain (residues 206–479) and full-length chicken Crk. The C-terminal end of the caspase-8 protease domain was connected to the N-terminal end of Crk by a linker sequence (*N*-GGGGG-GSGGGGGG-C); the C-terminal end of Crk was connected to the N terminus of the caspase-8 protease domain by a linker (*N*-GGGGGGRGGGGGGGGGG-C). Similarly, iCaspase-9 consists of human caspase-9 protease domain (residues 139–416) and chicken Crk. iApoptosome and iDISC were created by replacing the protease domain with the CARD of human Apaf-1 (residues 1–90) and the DED of mouse FADD (residues 1–85), respectively. All point mutations (iCaspase-$8^{Y/F}$ and iCaspase-$8^{C/S}$) were generated with the QuikChange mutagenesis kit (Stratagene).

BCR-ABL and its kinase domain mutants were cloned into MSCV vectors (MSCV-IRES-GFP or -YFP; ref. 25). NUP98-HOXA9 was cloned into the MSCV-IRES-t-hNGFR vector (23).

Reagents and Antibodies. The antibodies used were as follows: anti-Crk antibody (BD Transduction Laboratories), anti–phospho-Crk (phospho Y221) antibody, anti-Lamin B1 antibody (Abcam), anti-cleaved caspase-3 antibody, anti-human caspase-8 antibody, Streptavidin-APC (BD Biosciences), biotin-conjugated anti-human CD271 (hNGFR) (BD Pharmingen), anti–caspase-8 antibody, anti-CrkL antibody, anti–phospho-CrkL (phospho Y207) antibody (Cell Signaling), anti-mouse c-Kit, Sca-1, Ter119, Mac-1, Gr-1, B220, CD3, CD4, CD8, anti-human CD3, and CD28 (eBiosciences), anti-IκB-α antibody (Santa Cruz Biotechnology), and goat anti-rabbit Alexa Fluor 647 (Molecular Probes).

Interleukin-3 (IL-3) and imatinib mesylate were purchased from Peprotech and LC Laboratories, respectively.

NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) were used for isolation of the nuclear and cytocplasmic fractions. z-VAD were purchased from Enzo Life Sciences.

Cell Culture. Control and Ba/F3 cells expressing BCR-ABL, $FLT3^{D835Y}$, or TEL-PDGFRβ were generous gifts from A. M. Pendergast (Duke University, Durham, NC) and D. G. Gilliland (Harvard Medical School, Boston, MA). Ba/F3 cells were maintained in RPMI1640 medium supplemented with 10% (vol/vol) FBS and 1 ng/mL IL-3. K562 cells (ATCC) and Jurkat T-cells (ATCC) were cultured in RPMI medium supplemented with 10% (vol/vol) FBS.

Mice. BA (C57BL/Ka-*Thy1.1*) mice were used as transplant donors, and B6-CD45.1 (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) mice were used as transplant recipients (23). All mice were 8–16 wk of age. Mice were bred and maintained in the animal care facility at Duke University Medical Center. All animal experiments were performed according to protocols approved by the Duke University Institutional Animal Care and Use Committee.

Apoptosis Assays. Cells were fixed by 4% formaldehyde and membrane permeabilized by 90% methanol. The cells were stained with cleaved caspase-3 antibody and with goat anti-rabbit Alexa Fluor 647 and subjected to flow cytometric analysis. Apoptosis assays were also performed by staining live cells with Annexin-V (conjugated to Alexa Fluor 647; Molecular Probes) and propidium iodide (PI; Molecular Probes).

Gel filtration was performed as described (26). Control or BCR-ABL-expressing Ba/F3 cell were infected with the retroviruses expressing iCaspase-8 or its inactive mutant iCaspase-$8^{Y/F}$. Forty-eight hours after infection, the cell lysate (in a volume of 250 μL) was made and loaded onto a Superdex-200 column (GE Healthcare) at a flow rate of 0.3 mL/min.

HSC Isolation. KLS cells were isolated from mouse bone marrow as described (23). In brief, cells enriched with c-Kit beads (Miltenyi Biotec) were stained

with c-Kit-APC, Sca1-PE-Cy5.5, and lineage (Lin) markers conjugated with phycoerythrin (PE). As lineage markers, antibodies were used against murine Ter119, Mac-1, Gr-1, B220, CD3, CD4, and CD8.

Methylcellulose Colony Formation Assays. The isolated KLS cells were cultured overnight in X-Vivo15 media (Lonza) supplemented with 50 μM β-mercaptoethanol, 10% (vol/vol) FBS, stem cell factor (SCF, 100 ng/mL; R&D Systems) and thrombopoietin (TPO, 20 ng/mL; R&D Systems). Twenty-four hours after isolation, KLS cells were infected with the retroviruses and cultured for additional 48 h. YFP and GFP double-positive cells were selected by FACS and plated in methylcellulose medium (M3434; Stem Cell Technologies) with or without 2 μM imatinib mesylate. Colonies were counted 7 d after plating.

Mouse Leukemia Model. For generation of blast crisis CML in mice, KLS cells were infected with MSCV-BCR-ABL-IRES-YFP and MSCV-NUP98-HOXA9-IRES-t-hNGFR (23). Forty-eight hours after infection, cells coexpressing YFP and t-hNGFR were sorted and transplanted retroorbitally into B6-CD45.1 recipient mice, which had been sublethally irradiated (7 Gy). After transplantation, recipient mice were maintained on antibiotic water (sulphamethoxazole and trimethoprim) and evaluated daily for signs of morbidity, weight loss, failure to groom, and splenomegaly. Premorbid animals were killed, and splenocytes were harvested.

The isolated splenocytes were stained with lineage markers conjugated with PE-Cy5. Donor-derived Lin^- leukemia cells were selected by FACS and cultured in X-Vivo medium as described above. Twenty-four hours later, the cells were infected with MSCV-iCaspase-8-IRES-GFP-WRE or MSCV-IRES-GFP-WRE. Forty-eight hours after infection, GFP^+ cells were selected by FACS and transplanted retroorbitally into sublethally irradiated (7 Gy) B6-CD45.1 mice (3,000 cells per mouse). After transplantation, recipient mice were monitored for signs of morbidity as described above.

Jurkat T-Cell Stimulation. Jurkat T cells were infected with retrovirus expressing iCaspase8 or GFP alone. GFP-positive cells were sorted and then stimulated for 18 h on plates coated with 5 μg/mL anti-CD3 and anti-CD28 antibodies. Cell death was then assayed by PI exclusion.

qPCR Analysis. RNA was isolated by using RNAqueous-Micro (Ambion). Equal amounts of RNAs were converted to cDNA by using SuperScript II reverse transcriptase (Invitrogen). qPCRs were performed by using an iCycler (BioRad) by mixing cDNAs, iQ SYBRGreen Supermix (Bio-Rad), and gene-specific primers. Results were normalized to the level of β-actin (Actb, mouse) or β2 microglobulin (B2m, mouse). Primer sequences are as follows: BCR-ABL-F, 5′-TCCACTCAGCCACTGGATTTAA-3′; BCR-ABL-R, 5′-TGAGGCTCAAAGTCAGATGCTACT-3′; iCaspase-8-F (human caspase-8), 5′-AAATGAAAAGCAAACCTCGGG-3′; iCaspase-8-R (human caspase-8), 5′-CTTCAAAGGTCGTGGTCAAAG-3′; Actin-F, 5′-TTCGTTGCCGGTCCACA-3′; Actin-R, 5′-ACCAGCGCAGCGATATCG-3′; B2m-F, 5′-ACCGGCCTGTATGCTATCCAGAA-3′; B2m-R, 5′-AATGTGAGGCGGGTGGAACTGT-3.

Human CML Experiments. Studies on primary human CML samples were carried out with approval from the Duke University Institutional Review Board. The Ficoll-Paque-purified cells were stained with anti-CD34 conjugated with PE (Miltenyi Biotec) and CellTrace Violet (Life Technologies). CD34/CellTrace double-positive cells were selected by FACS and cultured overnight in X-Vivo15 media supplemented with 50 μM β-mercaptoethanol, 10% FBS, 10 ng/mL SCF, 20 ng/mL TPO, and 10 ng/mL FLT3L (R&D Systems). Twenty-four hours later, the cells were infected with the lentiviruses (pCDH) and cultured for additional 48 h.

Statistical Analysis. The statistical analysis was carried out by using the R language version 2.6.2 (www.r-project.org) and GraphPad Prism software version 4.0c (GraphPad Software).

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