Lysosomal acid lipase A modulates leukemia stem cell response to venetoclax/tyrosine kinase inhibitor combination therapy in blast phase chronic myeloid leukemia

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Abstract

The treatment of blast phase chronic myeloid leukemia (bpCML) remains a challenge due, at least in part, to drug resistance of leukemia stem cells (LSC). Recent clinical evidence suggests that the BCL-2 inhibitor venetoclax in combination with ABL-targeting tyrosine kinase inhibitors can eradicate bpCML LSC. In this study, we employed preclinical models of bpCML to investigate the efficacy and underlying mechanism of LSC-targeting with combinations of venetoclax/tyrosine kinase inhibitors. Transcriptional analysis of LSC exposed to venetoclax and dasatinib revealed upregulation of genes involved in lysosomal biology, in particular lysosomal acid lipase A (LIPA), a regulator of free fatty acids. Metabolomic analysis confirmed increased levels of free fatty acids in response to treatment with venetoclax/dasatinib. Pretreatment of leukemia cells with bafilomycin, a specific lysosome inhibitor, or genetic perturbation of LIPA, resulted in increased sensitivity of leukemia cells to venetoclax/dasatinib, implicating LIPA in treatment resistance. Importantly, venetoclax/dasatinib treatment did not affect normal stem cell function, suggesting a leukemia-specific response. These results demonstrate that venetoclax/dasatinib is a LSC-selective regimen in bpCML and that disrupting LIPA and fatty acid transport enhances the response to venetoclax/dasatinib when targeting LSC, providing a rationale for exploring lysosomal disruption as an adjunctive therapeutic strategy to prolong disease remission.

Introduction

Chronic myeloid leukemia (CML) is caused by reciprocal translocation of chromosomes 9 and 22, resulting in the generation of a BCR-ABL fusion protein and constitutive activation of the ABL tyrosine kinase.[1] The existence of leukemia stem cells (LSC) in both chronic phase CML (cpCML) and blast phase CML (bpCML) is supported by previous studies.[2,3] ABL tyrosine kinase inhibitors (TKI) are to date one of the most successful targeted therapies for malignant disease.[4] Unfortunately, TKI do not always eliminate disease-initiating LSC, as evidenced by an inability to discontinue TKI therapy in at least 50% of patients who achieve and maintain a deep molecular response for a significant period.[5]

CML is classified into three phases: chronic, accelerated and blast phase. bpCML, characterized by the acquisition of secondary mutations in addition to t(9;22) and transition to an often treatment-refractory acute leukemia, remains a formidable challenge in the management of CML.[6] In bpCML, emergence of TKI resistance frequently occurs through development of tyrosine kinase domain mutations in BCR-ABL. These include the "gatekeeper" T315I mutation, which results from a threonine to isoleucine substitution at position 315 of ABL, leading to resistance to first- and second-generation

TKI.[7-9] Ponatinib and asciminib are next-generation TKI that can overcome resistance to T315I mutations.[10,11] However, both have the potential for considerable toxicity[12] and do not always allow for durable responses in bpCML. More effective and safer therapeutic interventions are necessary to improve the cure rate in cpCML.[13]

The anti-apoptotic protein BCL-2 has recently been validated as a target in acute leukemia. The BCL-2 family of proteins are key regulators of mitochondrial-mediated apoptosis.[14] Work from several groups has demonstrated that inhibition of BCL-2 is cytotoxic to acute myeloid leukemia (AML) cells.[15] In addition, we have previously shown that BCL-2 is overexpressed in AML LSC and is important for their survival.[16] The expression of BCL-2 has been reported to be higher in CML than in normal hematopoietic stem cells and is further increased in bpCML.[17] CML cell survival is in part also mediated by upregulation of other BCL-2 family proteins including BCL-X_L and MCL-1.[18,19] We have previously demonstrated that BCL-2 inhibition with venetoclax can eradicate LSC populations in the context of AML via a mechanism involving perturbation of energy metabolism[20] suggesting that similar activities may be relevant to bpCML. Exciting preclinical studies by Carter *et al*. demonstrated *in vivo* targeting of LSC in a mouse model of cpCML, as well as *in vitro* targeting of primitive human bpCML cells, using a combination of venetoclax and the TKI nilotinib.[21] Subsequent clinical testing of venetoclax with TKI in heavily pretreated bpCML patients has shown promising results.[22]

Metabolic aberrancies appear to underlie much of venetoclax resistance in AML. One potential culprit could be the enzyme lysosomal acid lipase (LAL), encoded by the lipase A (LIPA) gene, the only known intracellular lipase active at an acidic pH that hydrolyzes cholesteryl ester and triglyceride in the lysosome.[23] LIPA-mediated lipid catabolism releases fatty acids for use as an energy source. Recent work has revealed that fatty acids derived from LAL-mediated lipolysis have important functional impacts on macrophage alternative activation,[24] metabolic reprogramming of $CD8^+$ memory T cells,[25] and lipid mediator synthesis.[26] It has also been demonstrated that increased fatty acid metabolism may be a universal mechanism for therapeutic resistance in AML, relevant to conventional chemotherapy[27] and venetoclax.[28,29] Similarly, metabolic shifts may be important in the development of therapy resistance in bpCML. In the present study, we evaluated the activity of the combination of venetoclax and dasatinib (ven/dasa) in bpCML mouse models and primary bpCML patients' samples. Studies were designed to identify pathways that could potentially enhance the relative efficacy of the drug combination in functionally defined LSC.

Methods

Cell culture

Base medium of Iscove modified Dulbecco medium with 10% fetal bovine serum and 1% penicillin/streptomycin was used, supplemented with 10 nM of human cytokines, stem cell factor, interleukin-3, and FLT3.

Human primary blast phase chronic myeloid leukemia samples

Primary bpCML samples were obtained from patients after informed consent for sample procurement. All specimen acquisition was approved by the University of Colorado Institutional Review Board.

Mouse strains and husbandry

Wild-type C57BL/6J mice, breeders of B6 Cd45.1, Pep Boy mice were purchased from Jackson Laboratory. All mice were housed at the University of Colorado Anschutz Medical Campus Animal Facility in a specific pathogen-free facility with individually ventilated cages. Mice were provided *ad libitum* access to rodent chow diet. All animal experiments were approved by the Office of Laboratory Animal Resources at the University of Colorado Anschutz Medical Campus.

Mouse studies

The genetically induced "GY" mouse model of bpCML, driven by the dual translocations *Bcr-Abl* (positive for green fluorescent protein, GFP^+) and *Nup98-Hoxa9* (positive for yellow fluorescent protein, YFP^+), was developed as previously described.[30] A T315I version of this syngeneic mouse model was also generated for this study, using the same protocol as described previously,[30] except using the T315I mutant version of the *Bcr-Abl* gene. In parallel, patient-derived xenograft studies were performed as previously described.[31] Briefly, NSG-S mice were first engrafted with a human primary bpCML sample through tail vain injection. After 4 weeks, they were treated by oral gavage with either venetoclax alone (100 mg/kg/day) or dasatinib (20 mg/kg/day) or the combination.

RNA sequencing

Bcr-Abl^{wt} "GY" syngeneic mice were treated with ven/dasa or vehicle for 4 h on day 11 after transplant of the leukemia cells. Mice were sacked after the treatment, bone marrow was isolated and sorted for LSC (Lin^-Sca1^+), and total RNA from LSC was isolated using the RNeasy Plus kit (Qiagen) with the manufacturer's protocol. Library construction and sequencing were performed according to a previously described protocol.[29] Single-end reads of 100 nucleotides were generated for each sample on the Illumina HiSeq2500 platform. Methods for RNA-sequencing analysis can be found in the *Online Supplementary Materials*.

Metabolomics

Murine LSC (GY^+ Lin^-Sca1^+) were isolated using the BD ARIA II cell sorter, treated for 4 h with ven/dasa or vehicle, and subjected to metabolomic analyses ($0.2x10^6$ cells/sample) via ultrahigh pressure liquid chromatography - mass spectrometry (Vanquish and Q Exactive, Thermo Fisher) as previously described.[32]

Results

Venetoclax and dasatinib target bulk and leukemia stem cell populations in a mouse model of blast phase chronic myeloid leukemia

To assess the therapeutic efficacy of venetoclax and dasatinib separately and in combination we performed *in vitro* and *in vivo* experiments utilizing a mouse model of bpCML. This model employs co-expression of the *Bcr-Abl* and *Nup98-Hoxa9* translocations, independently monitored using the expression of GFP and YFP, respectively (henceforth referred to as GY cells). These translocations are detected in human bpCML and the model has previously been described in detail for studies of leukemia genetics and stem cell biology.[30,33,34] To assess the effect of ven/dasa treatment *in vitro*, the GY cells were treated in cell culture with each drug alone or in combination for 24 h, and viability was assessed with annexin V flow cytometry. Bulk cells treated with the two-drug combination demonstrated a significant loss of viability compared to GY cells treated with either drug alone (Figure 1A). Furthermore, the percentage of phenotypic LSC defined by surface markers (Lin^-Sca1^+) was also significantly reduced in the ven/dasa-treated group (Figure 1B). To evaluate *in vivo* activity, syngeneic mice were transplanted with GY cells, (10^5 cells per mouse through the tail vein) and, at day 5 after transplantation, the mice were treated for 5 days with vehicle, single agents or the ven/dasa combination. The combination of ven/dasa led to a significant reduction in leukemia burden compared to either drug alone (Figure 1C). We also evaluated the effect of ven/dasa on the murine LSC population (Lin^-Sca1^+) and observed a significant decrease in the LSC population compared to that achieved with treatment with the single agents or vehicle control (Figure 1D).

To further investigate the clinical benefit of ven/dasa therapy, mice with established GY leukemic engraftment were treated for 10 days with single agents or the ven/dasa combination and monitored for survival; mice were sacrificed upon appearance of signs of leukemia-related morbidity. No survival benefit was seen with venetoclax alone relative to vehicle, whereas dasatinib alone led to significantly increased survival compared to that of controls. Animals receiving the ven/dasa combination eliminated the leukemic cells and were monitored for up to 80 days with no sign of leukemia recurrence as determined by the absence of any GY cells in their bone marrow upon sacrifice (Figure 1E, F).

Kinetics of response to the venetoclax and dasatinib combination in a mouse model of blast phase chronic myeloid leukemia

To better understand the kinetics of drug response, we also performed short-term drug treatment studies. As shown in Figure 2A-C, leukemic mice were treated for 2, 3 and 4 days and effects on leukemia burden as well as on LSC were evaluated. Leukemia burden overall and LSC percentage were significantly decreased as early as 2 days after treatment initiation. Thus, targeting of primitive populations occurs very rapidly. Notably, analysis of normal progenitor cells, ($Lin^-Sca1^+c\text{-}kit^+$, termed LSK cells), which are co-resident in the same animals, showed no detectable reduction in colony-forming assays implying a leukemia-specific response (Figure 2D).

Due to the known disseminated nature of the GY leukemia model, we also assessed the effect of ven/dasa on leukemic infiltration of extramedullary locations such as the spleen. Treatment of mice with ven/dasa resulted in significant inhibition of bulk disease as well as the LSC compartment compared to treatment with either drug alone (*Online Supplementary Figure S1A, B*). The average spleen size in the combination-treated cohort was reduced to the size of normal spleens (*Online Supplementary Figure S1C*). Overall, the bpCML mouse model showed the enhanced efficacy of the ven/dasa combination over treatment with the single drugs.

Venetoclax and ponatinib target bulk and leukemia stem cell populations in a mouse model of *BCR-ABL*^{T315I} mutation

Despite the success of TKI, resistance has been identified in some patients due to point mutations in the *BCR-ABL* kinase domain. Ponatinib was developed as a TKI that can inhibit all critical kinase domain mutations including T315I.[10] Thus, we investigated the addition of venetoclax to ponatinib in the context of T315I-mutant bpCML. We generated a mouse model of leukemia harboring T315I mutant BCR/ABL-GFP and NUP98/HOXA9-YFP (termed GY-315). As expected, *in vitro* treatment of GY-315 cells showed resistance to dasatinib. Furthermore, GY-315 cells showed no response to ven/dasa (*Online Supplementary Figure S2A*). In contrast, both GY and GY-315 cells responded to ponatinib treatment, confirming that ponatinib is active against both the wild-type as well as the mutant *Bcr*-Abl oncogene (*Online Supplementary Figure S2B)*.

To assess the effect of the venetoclax and ponatinib combination (ven/pona) *in vitro*, the GY-315 cells were treated with each drug alone or in combination for 24 h. Treatment with ven/pona resulted in a significant loss of viability of bulk leukemia cells compared to either drug alone (Figure 3A). Furthermore, the percentage of phenotypic LSC (Lin^-Sca1^+) was also significantly reduced in the ven/pona-treated group (Figure 3B). To evaluate the effect of these drug combinations *in vivo*, leukemic mice were generated by transplantation of GY-315 cells into recipient animals followed by treatment with each drug alone or the ven/pona combination. Both single agents resulted in decreased leukemia burden in bone marrow, an effect that was more pronounced in mice treated with ponatinib alone, but the combination of ven/pona led

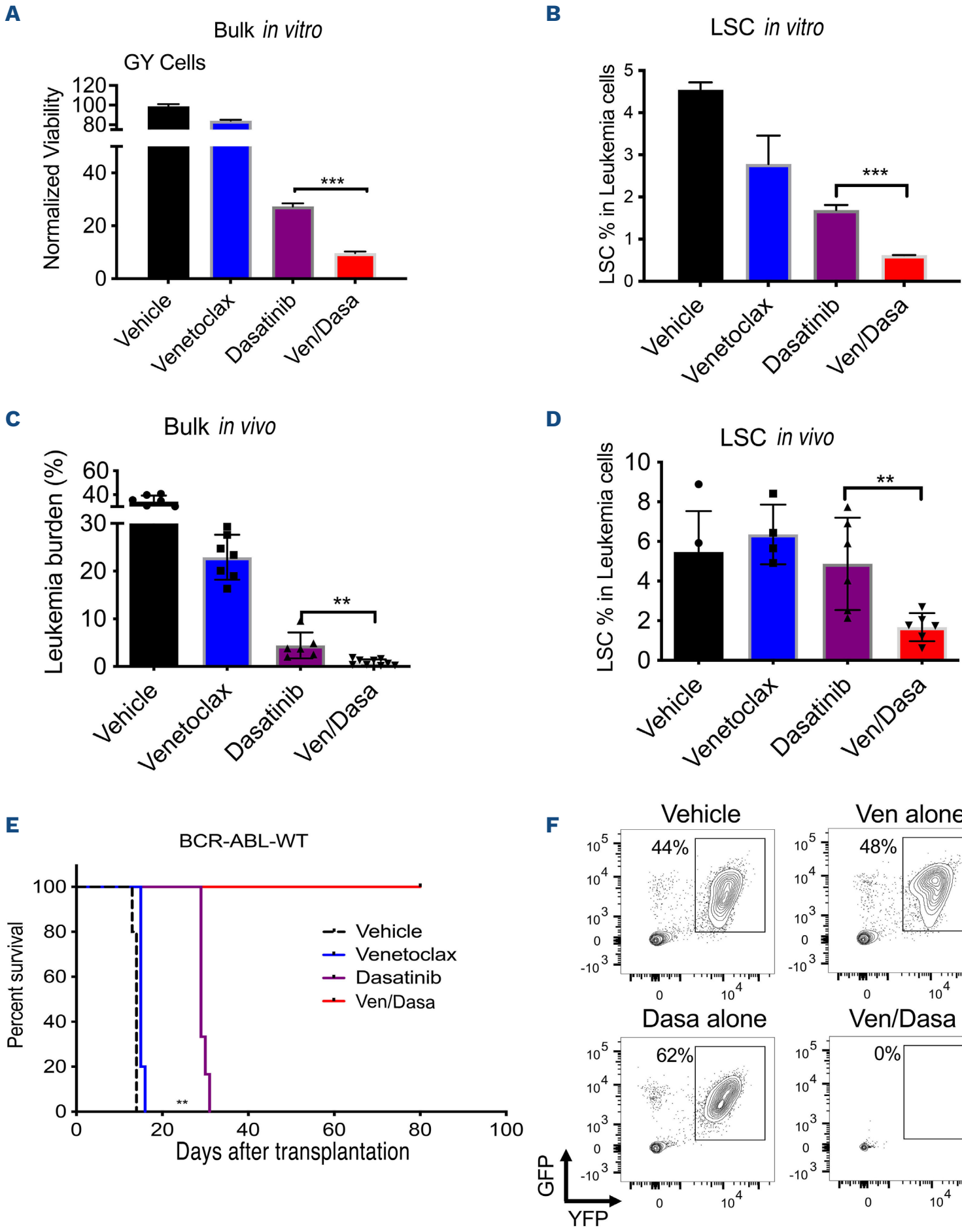

Figure 1. The venetoclax/dasatinib combination targets bulk cells and the leukemia stem cell compartment in a mouse model of blast phase chronic myeloid leukemia. (A, B) Viability of bulk cells (A) and the leukemia stem cell (B) compartment ($Lin^{-}Sca1^{+}$) of GY leukemia cells treated *in vitro* with venetoclax (100 nM), dasatinib (100 nM), or their combination after 24 hours compared to cells treated with the vehicle control. (C, D) Leukemic mice were treated with vehicle, venetoclax alone (100 mg/kg/day, oral gavage), dasatinib alone (20mg/kg/day, oral gavage) and the combination for 5 days starting at day 7 after transplantation of leukemic cells. Mice were sacrificed at day 12 after the transplantation and the tissues were harvested to determine bulk (C) and leukemic stem cell (D) leukemic burden in bone marrow. (E) Leukemic mice were treated with vehicle, venetoclax alone, dasatinib alone and the combination, as described above. Survival of control and treated leukemic mice (N=8) was monitored and mice were sacrificed upon demonstration of morbid symptoms. No such symptoms appeared in mice treated with the combination, so these mice were sacrificed 80 days after the start of the experiment. (F) Representative flow plot for engraftment status of leukemia cells (GY cells) from the survival experiment illustrated in (E). $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. Ven/Dasa: venetoclax and dasatinib combination; LSC: leukemia stem cells; WT: wild-type; Ven: venetoclax; Dasa: dasatinib.

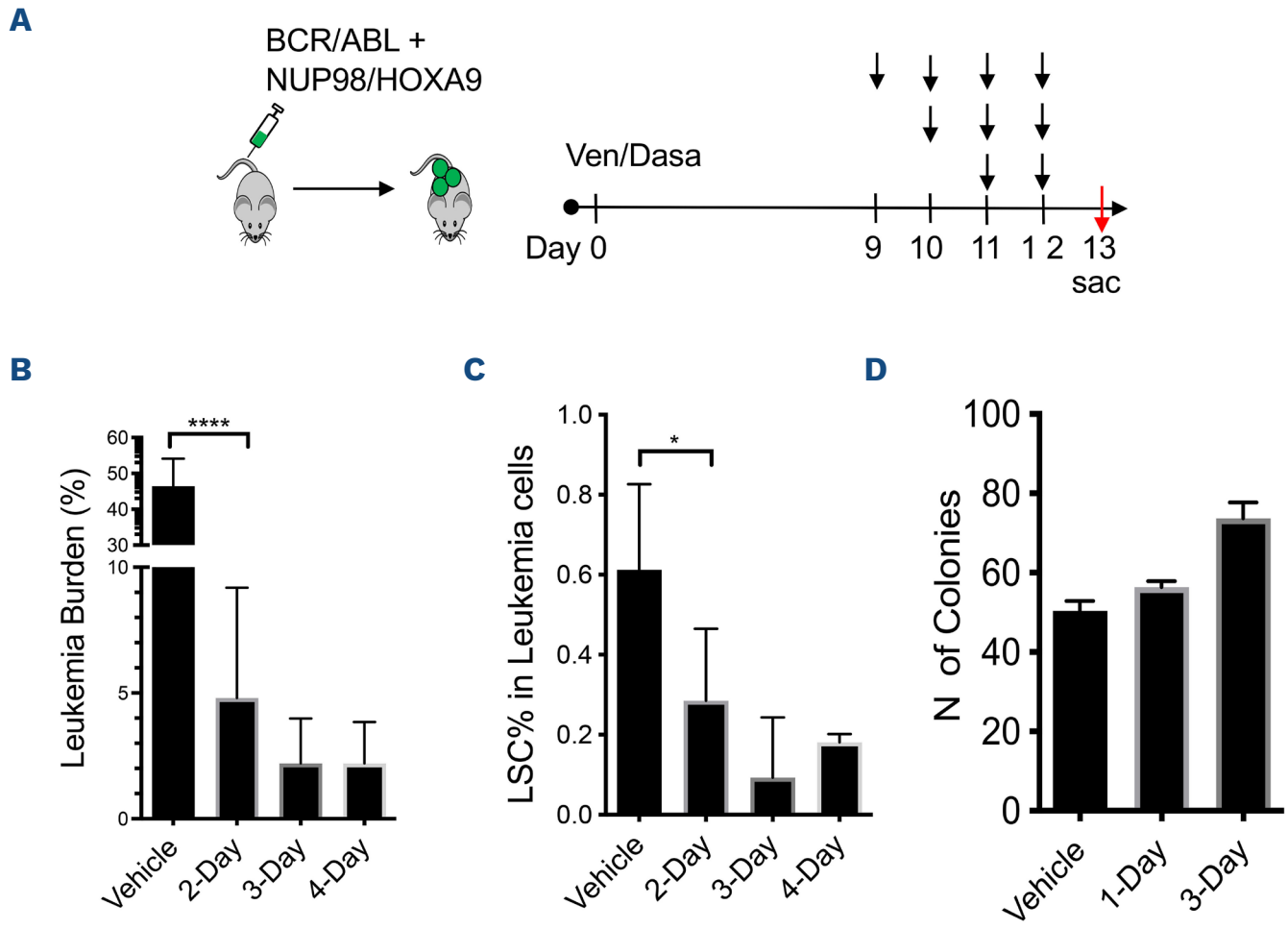

Figure 2. Kinetics of the venetoclax/dasatinib combination in a mouse model of blast phase chronic myeloid leukemia. (A) Experimental design for 2, 3, and 4 days of treatment of leukemic mice. (B, C) Effect on bulk cells (B) and the leukemic stem cell compartment (Lin^-Sca1^+) (C) after 2, 3 and 4 days of treatment with the venetoclax/dasatinib combination. (D) Colony-forming unit assay using bone marrow cells ($Lin^-Sca1^+cKit^+$) from normal mice treated for 1 or 3 days with the venetoclax/dasatinib combination. Error bars denote mean ± standard deviation from triplicate experiments. Statistical analyses were performed using a Student *t* test. *P≤0.05, ****P≤0.0001. sac: sacked; Ven/Dasa: venetoclax and dasatinib combination; LSC: leukemia stem cells.

to a significant decrease in leukemia burden relative to that achieved with the single agents (Figure 3C). Similar results were observed in the LSC (Lin^-Sca1^+) population (Figure 3D). Survival studies performed using the same experimental approach as shown in Figure 1E demonstrated a significantly prolonged survival for mice treated with the ven/pona combination (Figure 3E). Although we observed a significant survival benefit from ven/pona treatment in the T315I mutant GY mouse model we were unable to determine curative outcome due to the toxicity of ponatinib (weight loss and poor grooming). It could be useful to investigated whether lower ponatinib doses in combination with venetoclax may allow eradication of LSC without treatment-related mortality.

We also assessed the effect of ven/pona on leukemic infiltration of extramedullary locations such as the spleen. Treatment of mice with ven/pona resulted in significant inhibition of bulk disease as well as the stem cell compartment compared to either drug alone (*Online Supplementary Figure S2C, D*). The average spleen size in the combination-treated mice was reduced to the size of normal spleens (*Online Supplementary Figure S2E*). Overall, ven/pona and ven/dasa have similar effects and confirm that this approach is translatable to disease that harbors the T315I TKI resistance mutation.

Venetoclax and dasatinib target bulk and primitive populations in blast phase chronic myeloid leukemia patients' samples

To translate these findings to human models of disease, we repeated *in vitro* and *in vivo* drug treatment experiments using primary specimens from bpCML patients. Primary leukemia samples (N=4) were treated with single agents or their combination *in vitro* for 24 h and then their viability assessed using annexin V flow cytometry. As shown for a representative specimen (Figure 4A, B), both the bulk and primitive compartments showed a significant decrease in viability compared to controls, with the most pronounced effect seen in the ven/dasa-treated group. Data from three additional primary samples showed a similar response (*Online Supplementary Figure S3A-D*), confirming the efficacy of the venetoclax tyrosine kinase inhibitor combination, as reported previously.[21] To directly test the impact of ven/dasa on LSC function *in vivo*, primary human bpCML cells were transplanted into immunocompromised NSG-S mice.[35] At 4 weeks after transplantation (at 10% to 45% human cell engraftment depending on the primary sample) mice were treated for 10 days with venetoclax, dasatinib or the combination. A significant reduction in tumor burden was observed in ven/dasa-treated mouse cohorts derived from two bpCML patients' samples (Figure 4C), supporting loss

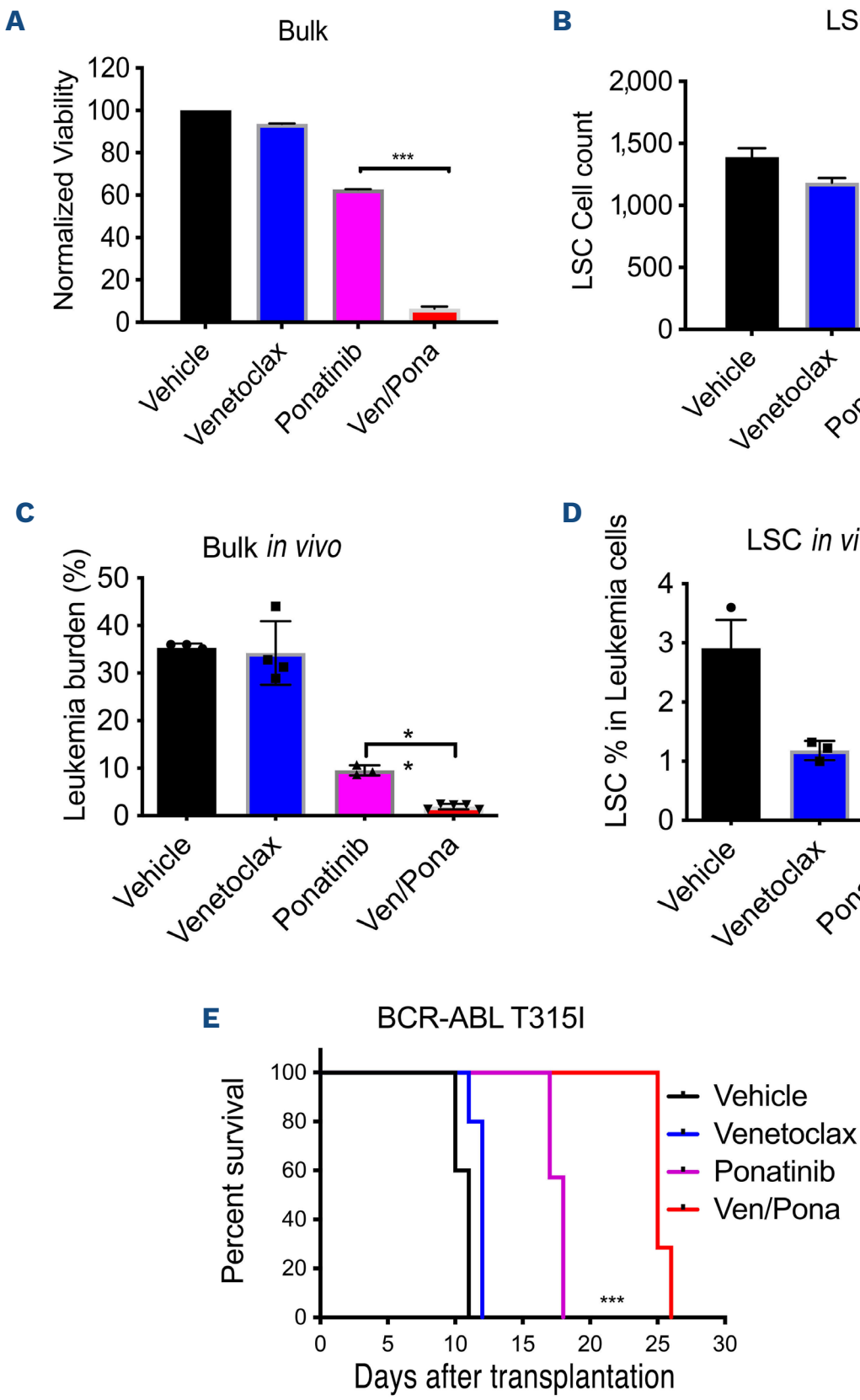

Figure 3. The venetoclax/ponatinib combination targets bulk cells and the leukemia stem cell compartment in a mouse model of blast phase T315I-mutant chronic myeloid leukemia. (A, B) T315I-mutant GY$^+$ leukemia cells were treated *in vitro* with venetoclax (100 nM), ponatinib (100 nM) and their combination for 24 hours and viability measured relative to vehicle control in bulk leukemia cells (A) and the leukemia stem cell compartment (Lin$^-$Sca1$^+$) (B). (C, D) Similarly, leukemic mice were treated with vehicle, venetoclax alone (100 mg/kg/day, oral gavage), ponatinib alone (10 mg/kg/day, oral gavage) and their combination for 5 days starting at day 7 after transplantation of leukemia. Mice were sacrificed at day 12 after the transplantation and their tissues were harvested to determine bulk leukemia burden (C) and quantitation of the leukemia stem cell compartment (Lin$^-$Sca1$^+$) (D) in the bone marrow. (E) Leukemic mice were treated with vehicle, venetoclax alone, ponatinib alone and their combination, as described above. Survival of control and treated leukemic mice (N=8) was monitored and mice were sacrificed upon demonstration of morbid symptoms. Error bars denote mean ± standard deviation from triplicate experiments. Statistical analyses were performed using a Student *t* test. *P≤0.05, **P≤0.01, ***P≤0.001. Ven/Pona: venetoclax/ponatinib combination; LSC: leukemia stem cells.

of LSC functionality as a result of dual drug treatment, compared to either drug alone. We also performed secondary engraftment from the cohort injected with bpCML1. The cells from primary engrafted mice, normalized to the percent of human cells from each treated group, were injected into the tail veins of secondary recipient mice. Mice were sacrificed after 12 weeks, and human hematopoietic engraftment was evaluated by flow cytometry. As can be seen in Figure 4D, E, the cells from the ven/dasa-treated group failed to engraft in secondary recipients, thereby demonstrating eradication of the LSC compartment. To further test the impact of ven/dasa on LSC function *in vivo*, primary human bpCML cells from one more case were transplanted into immunocompromised NSG-S mice; as shown in Figure 4F, a significant reduction in tumor burden was observed in the ven/dasa-treated cohort. In contrast, treatment of CD34-enriched normal hematopoietic stem cells with similar doses of single agents or ven/dasa did not result in significant cell death and had no effect on colony formation (*Online Supplementary Figure S3E, F*), demonstrating a strong LSC-specific effect.

Lysosomal mechanisms influence the response to venetoclax and dasatinib in murine blast phase chronic myeloid leukemia

The collective results from murine models of bpCML show

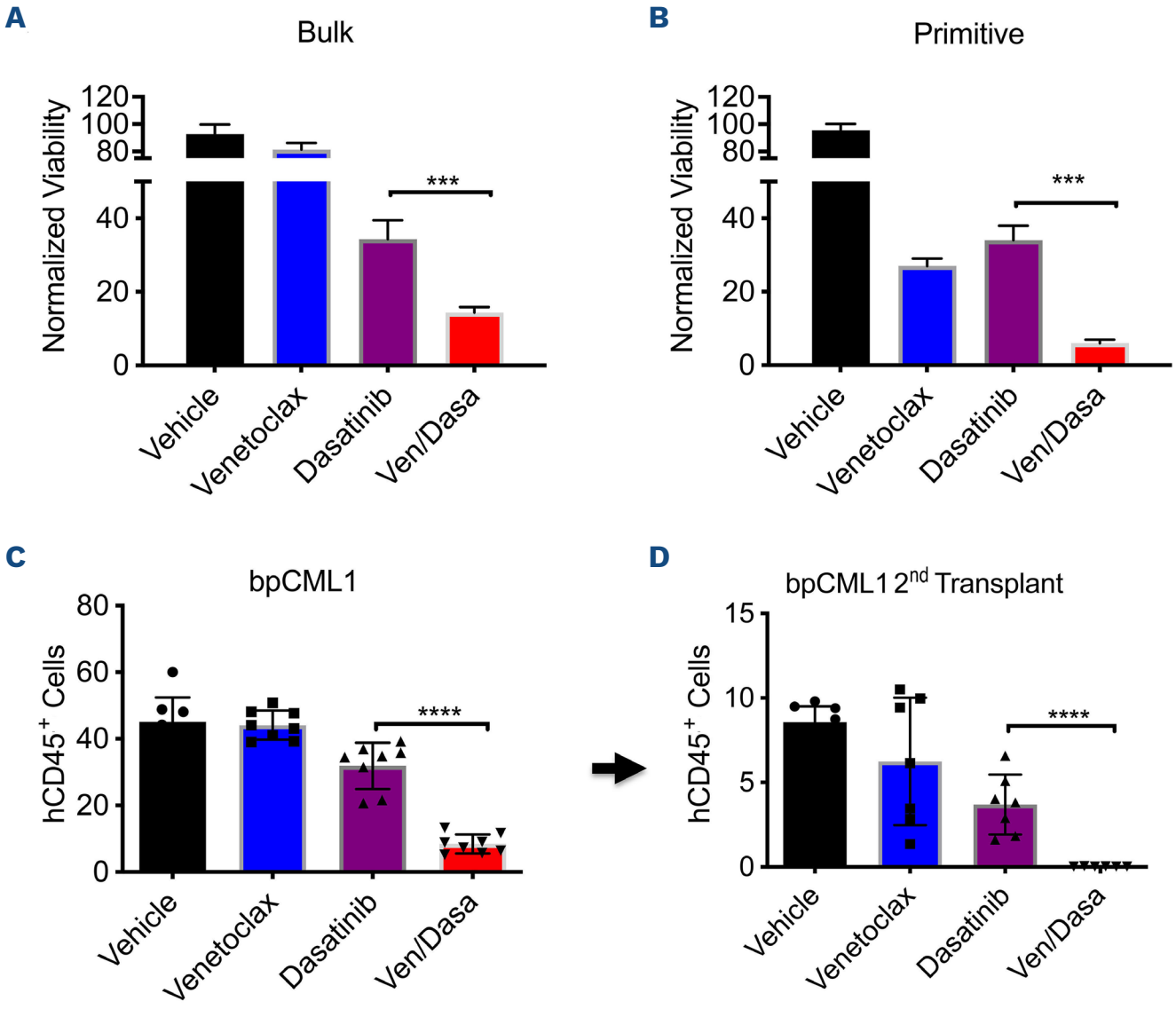

Figure 4. The venetoclax/dasatinib combination targets bulk cells and the leukemia stem cell compartment in human primary blast phase chronic myeloid leukemia patient's samples. Viability of samples from a representative patient with blast phase chronic myeloid leukemia (bpCML) treated *in vitro* with venetoclax (100 nM), dasatinib (100 nM) and their combination after 24 hours compared to vehicle control. (A) Bulk cells. (B) Primitive compartment ($CD34^+CD38^+$). (C and F) Leukemic NSG-S mice transplanted with human bpCML patient-derived xenografts (bpCML1 and bpCML2) were treated with vehicle, venetoclax alone (100 mg/kg/day, oral gavage), dasatinib alone (20 mg/kg/day, oral gavage) or their combination, starting at day 25 for 10 days. Mice were sacrificed at day 35 after transplantation. (D) Cells from primary engrafted mice were injected into secondary recipient mice and monitored for 12 weeks and sacrificed after 12 weeks. (E) Flow plot from the secondary engraftment experiment. Bone marrow leukemia burden, quantified as percentage human CD45 ($hCD45^+$) population, is shown. Error bars denote mean ± standard deviation from triplicate experiments. Statistical analyses were performed using a Student *t* test. ***$P \leq 0.001$, ****$P \leq 0.0001$. Ven/Dasa: venetoclax and dasatinib combination; Ven: venetoclax; Dasa: dasatinib.

that ven/dasa treatment selectively and directly targets the LSC compartment. Thus, we sought to further define mechanisms of LSC targeting to optimize the efficacy of the ven/dasa regimen. To this end, we performed an analysis of the immediate transcriptional response to ven/dasa treatment in the murine bpCML model. As outlined in Figure 5A, leukemic mice were treated with ven/dasa for 4 h, bone marrow was harvested after the treatment, flow sorted for Lin^-Sca1^+ cells (LSC-enriched populations) and subjected to RNA sequencing. For comparison, dasatinib alone and vehicle controls were also included in the transcriptional analyses. Venetoclax conferred no survival advantage over vehicle so was not included in the study. Our rationale in designing this experiment was that genes upregulated by drug treatment, prior to the onset of overt apoptosis, will include mechanisms of protection. As shown in Figure 5B, principal component analysis indicated distinct gene signatures in the LSC for each treatment group. Gene set enrichment analyses suggested strong upregulation of genes involved in lysosomal biology in ven/dasa-treated cells when compared to the expression patterns observed in vehicle controls (Figure 5C, D).

To investigate the role of lysosomal activity, we used bafilomycin, a specific inhibitor of lysosome function. As shown in Figure 5E, *in vitro* pre-treatment of GY cells for 1 h with bafilomycin followed by treatment for 24 h with

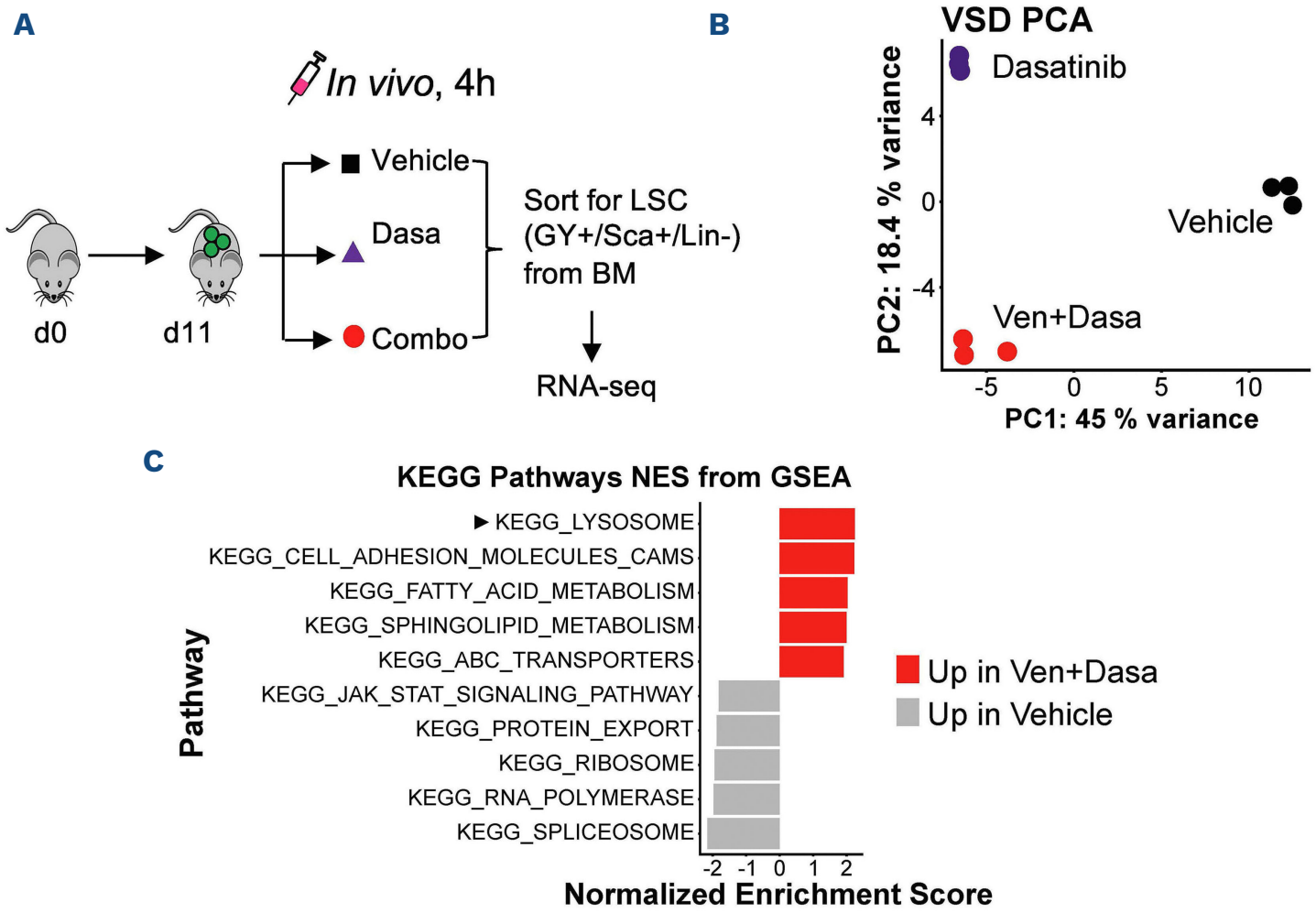

Figure 5. Upregulation of genes related to lysosome biology protects leukemia stem cells from venetoclax/dasatinib combination therapy. (A) Experimental design for treatment, flow sorting, and RNA isolation from leukemia stem cells (LSC) for total RNA sequencing. (B) Principal component analysis plot for gene expression profile in groups treated with vehicle, dasatinib, and the

Continued on following page.

venetoclax/dasatinib combination. (C, D) Gene set enrichment analysis showing enrichment of genes related to lysosome biology in venetoclax/dasatinib-treated LSC. (E) Leukemia cells (GY^+; lin^-) were pretreated with bafilomycin (10 nM) for 1 h and then treated *in vitro* for 24 h with venetoclax alone (100 nM), dasatinib alone (100 nM), or their combination. Cell viability was measured compared to that of cells treated with the vehicle control. (F) Heatmap showing expression of representative lysosome-related genes in LSC untreated *versus* treated with the venetoclax/dasatinib combination. (G) LIPA expression in response to dasatinib alone and the venetoclax/dasatinib combination. Error bars denote mean ± standard deviation from triplicate experiments. Statistical analyses were performed using a Student *t* test. NS: not statistically significant, ***$P \leq 0.001$, ****$P \leq 0.0001$. d: day; Combo: venetoclax/dasatinib combination; BM: bone marrow; RNA-seq: RNA sequencing; PCA: principal component analysis; Ven: venetoclax; Dasa: dasatinib; KEGG: Kyoto Encyclopedia of Genes and Genomes; NES: normalized enrichment score; GSEA: gene set enrichment analysis; FDR: false discovery rate; Baf: bafilomycin.

venetoclax and/or dasatinib strongly increased sensitivity to the ven/dasa treatment, implying a protective role for lysosome activity. Of the genes that were upregulated in the lysosome pathway specifically due to treatment with both venetoclax and dasatinib, we found that lysosomal acid lipase (LIPA or LAL) was consistently elevated (Figure 4F, G). This enzyme is also referred to as cholesterol ester hydrolase and is involved in hydrolysis and recycling of cholesterol and free fatty acids for cellular energy.[23] The upregulation of LIPA in the ven/dasa-treated LSC population was confirmed by quantitative polymerase chain reaction analysis (*Online Supplementary Figure S4I*).

Metabolomic analysis of GY cells treated with venetoclax plus dasatinib reveals upregulation of free fatty acids

Since LIPA is known to positively regulate free fatty acid levels, we performed metabolomic analyses of LSC isolated after 4 h of treatment with ven/dasa (Figure 6A). We found that several fatty acids were upregulated in the ven/dasa-treated group compared to control (Figure 6B). More importantly, supplementing the medium with these same fatty acids (e.g., α-linolenic acid and dihomo-γ-linolenic acid) resulted in partial rescue from ven/dasa-mediated cell death, implicating upregulation of fatty acid levels as a protective response to ven/dasa challenge (Figure 6C, D).

Modulation of LIPA expression results in altered response to venetoclax and dasatinib treatment in a mouse model of blast phase chronic myeloid leukemia

To examine the role of endogenous LIPA in leukemia cells, we utilized CRISPR technology and electroporated Cas9-sgRNA complexes into murine GY leukemia cells to knockout LIPA.[36] As shown in Figure 7A, B, this approach achieved strong knockdown of LIPA at both the mRNA and protein levels. Treatment of LIPA knockout cells with ven/dasa resulted in increased sensitivity (Figure 7C), further corroborating the concept that upregulation of lysosomal activity serves as a protective response to ven/dasa challenge. We also overexpressed the gene in murine GY leukemia cells (Figure 7D, E) and found that treatment of LIPA-overexpressing leukemia cells with ven/dasa resulted in increased resistance compared to cells transduced with vector alone (Figure 7F). These data suggest that increased free fatty acids due to higher expression of LIPA results in diminished cytotoxicity of ven/dasa. This implies that utilization of fatty acids by leukemic cells may be important for diminished drug response. Thus, we hypothesized that inhibiting the expression of carnitine palmitoyltransferase 1A (CPT1A), an important free fatty acid mitochondrial membrane transporter, would be able to modulate the response to ven/dasa. Indeed, knockdown of CPT1A using siRNA (Figure 7G) resulted in increased sensitivity of the GY cells to ven/dasa treatment compared to that of cells transfected with scrambled siRNA (Figure 7H).

Modulation of LIPA expression results in altered response to venetoclax and dasatinib treatment in human blast phase chronic myeloid leukemia samples

To determine whether the findings from GY mouse cells were also evident in human bpCML cells, we examined the effect of both LIPA knockdown and CPT1A knockdown in primary human bpCML using an siRNA approach. As shown in Figure 8A, B and *Online Supplementary Figure S4C, D*, we confirmed significant knockdown of LIPA expression at mRNA and protein levels and found that treatment of LIPA knockdown cells with ven/dasa resulted in increased drug sensitivity compared to that of scrambled control cells (Figure 8C, *Online Supplementary Figure S4E*). Furthermore, after confirming knockdown of CPT1A expression in primary human samples (Figure 8D, *Online Supplementary Figure S4F, G*) we similarly demonstrated increased sensitivity to ven/dasa compared to that of scrambled control cells (Figure 8E, *Online Supplementary Figure S4H*). Together these results suggest that treatment with ven/dasa results in upregulation of genes associated with lysosome biology, in particular LIPA, as a protective response. LIPA is known to increase production of free fatty acids that can be used as a fuel source, which in part may be responsible for the diminished response to ven/dasa.

Discussion

The primary goal of this study was to investigate the molecular mechanisms that mediate the LSC-targeting activity of venetoclax in combination with a TKI in bpCML. In agreement with the studies of Carter *et al.*,[21] we observed that venetoclax in combination with a TKI is a highly effective LSC-targeting regimen in both xenograft studies using

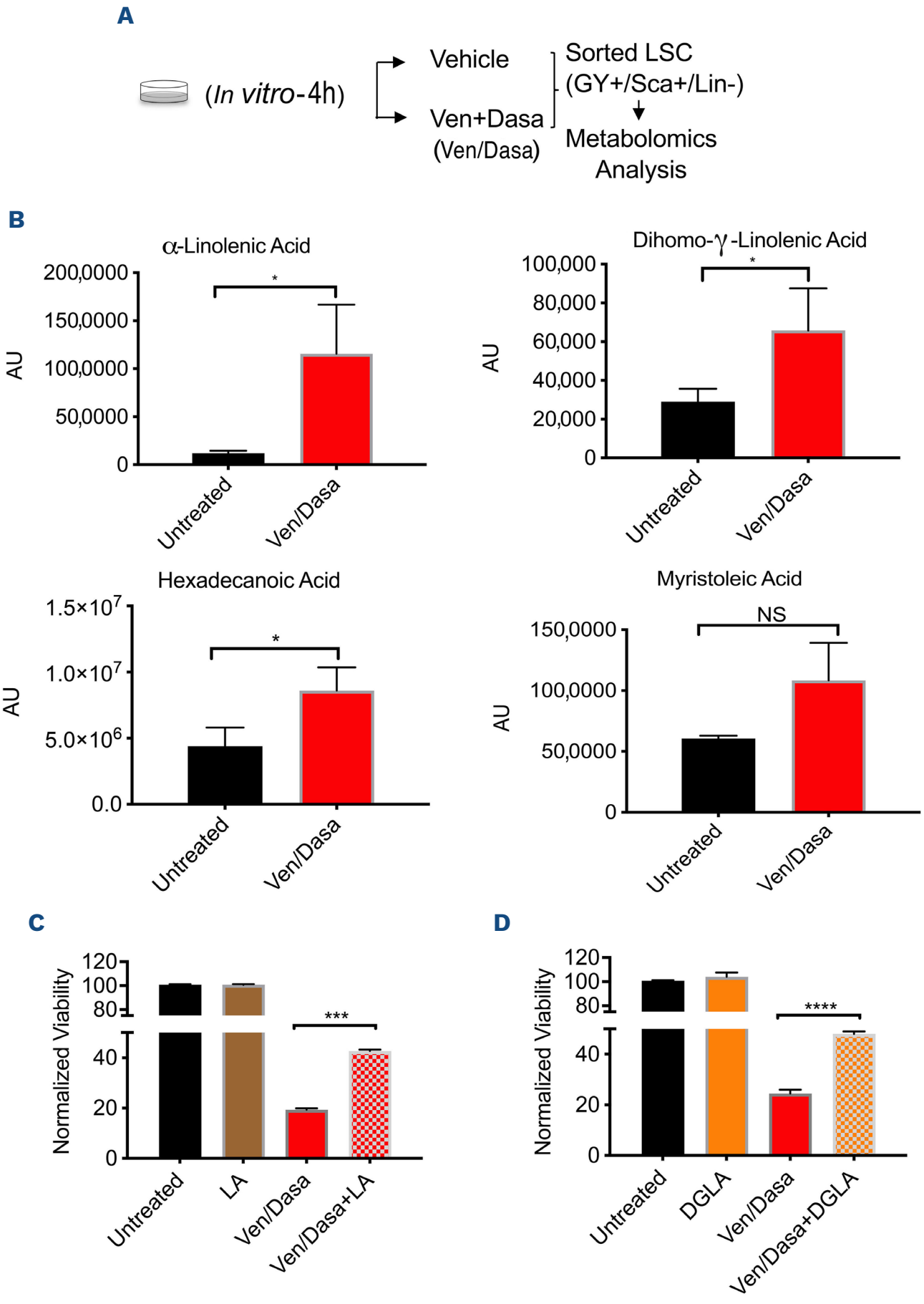

Figure 6. Venetoclax/dasatinib treatment results in an increase of free fatty acids in leukemia stem cells. (A) Experimental design for treatment followed by metabolomic analysis of free fatty acids in leukemia stem cells. (B) Relative amounts of different fatty acids in leukemia stem cells in response to treatment with α-linolenic acid, myristoleic acid, hexadecanoic acid or dihomo-γ-linolenic acid. (C, D) Leukemia cells (GY^+, lin^-) were pre-treated *in vitro* with linolenic acid (10 μM) (C) or dihomo-γ-linolenic acid (10 μM) (D) for 1 h and then treated for 24 h with the venetoclax/dasatinib combination (100 nM). Viability was measured compared to vehicle control. Error bars denote mean ± standard deviation from triplicate experiments. Statistical analyses were performed using a Student *t* test. NS: not statistically significant, **P*≤0.05, ****P*≤0.001, *****P*≤0.0001. LSC: leukemia stem cells: Ven/Dasa: venetoclax and dasatinib combination; AU: arbitrary units; LA: linolenic acid; DGLA: dihomo-γ-linolenic acid.

primary human bpCML samples as well as in a previously described mouse model of bpCML. Survival experiments showed that the ven/dasa combination effectively cured mice of leukemia induced with wild-type *Bcr-Abl*, whereas untreated mice typically succumbed to disease within 10-15 days. We also showed the efficacy of venetoclax and ponatinib in a newly created mouse model harboring T315I mutant *Bcr-Abl* in combination with *Nup98/HoxA9*. Although we observed a significant survival benefit of ven/pona in the T315I mutant GY mouse model we were unable to determine curative outcome due to the toxicity of ponatinib. Lower ponatinib doses in combination with venetoclax may allow eradication of LSC without treatment-related mortality. Our data further identify a compensatory upregulation of genes associated with lysosome biology upon treatment with ven/dasa. The functional relevance of this finding is clearly evident upon addition of bafilomycin to the ven/dasa regimen, which significantly increased eradication of primitive bpCML cells. In particular, upregulation of *LIPA* seems to be a protective mechanism activated by these

cells to attempt survival. Since LIPA is known to regulate free fatty acid levels, we performed metabolomic analysis of LSC isolated after treatment with ven/dasa for 4 h. We found that several fatty acids were upregulated in the ven/dasa-treated cells. Recent work has demonstrated that increased fatty acid metabolism may be a mechanism for AML resistance to conventional chemotherapies such as cytarabine[27,37] and our laboratory has previously reported that increased expression of genes involved in fatty acid metabolism correlates with a poor response of AML to venetoclax/azacitidine.[28,29] We hypothesized that activation of fatty acid processing through enhanced LIPA

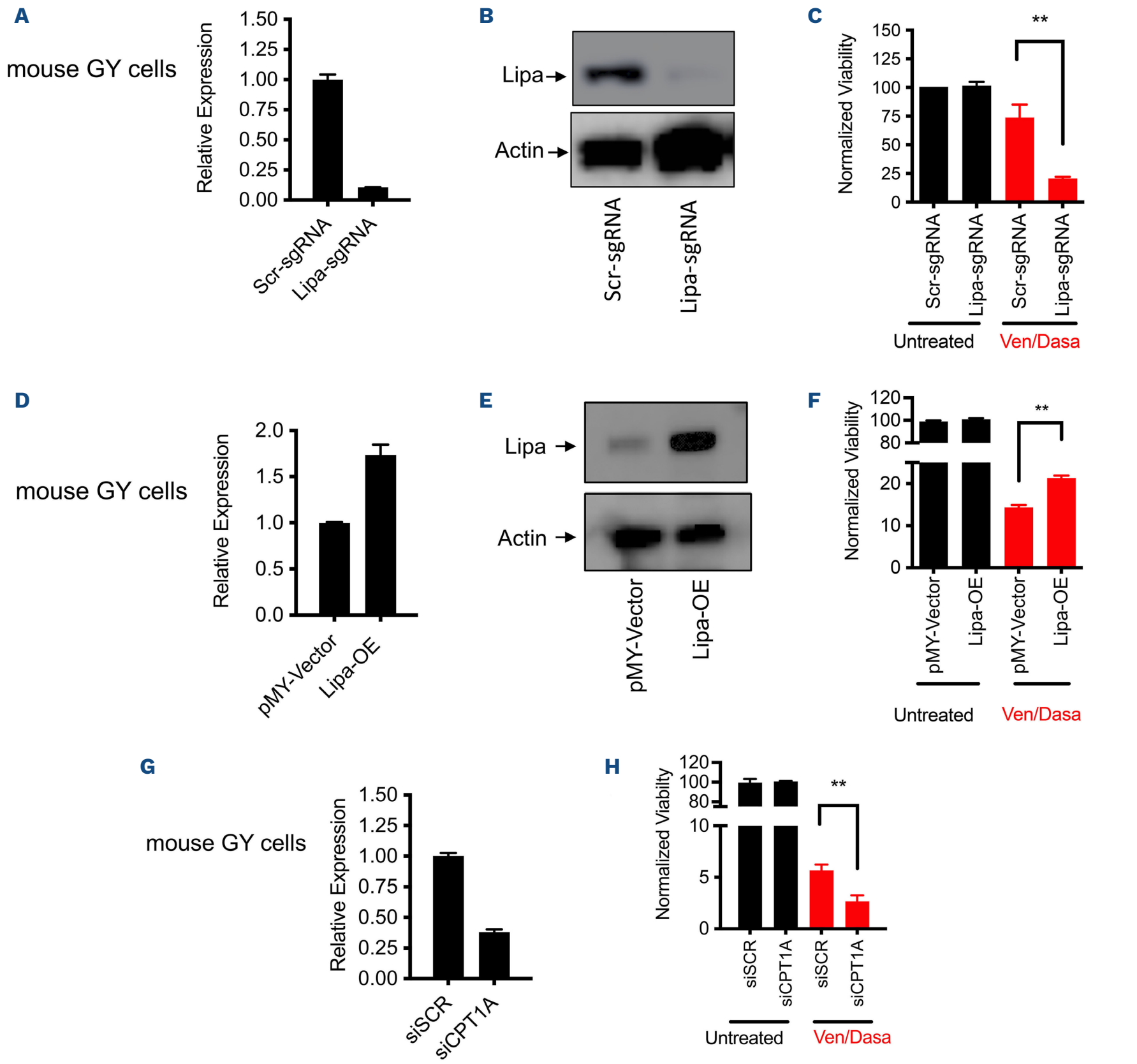

Figure 7. Endogenous LIPA and CPT1A are critical for resistance to venetoclax/dasatinib in mouse leukemia cells. (A) mRNA level of LIPA in LIPA knockout (sgRNA-LIPA) and scramble control (Scr-sgRNA) GY leukemia cells using the CRISPR/CAS9 method. (B) Western blot of LIPA in LIPA-knockout and scramble control leukemia cells. (C) Viability of LIPA knockout and scramble control GY leukemia cells treated with the venetoclax/dasatinib (Ven/Dasa) combination (100 nM) relative to the viability of untreated cells. (D) mRNA level of LIPA in LIPA overexpressing (OE) and vector control GY leukemia cells. (E) Western blot of LIPA in LIPA OE and vector control GY leukemia cells. (F) Viability of LIPA OE cells treated with Ven/Dasa (100 nM) compared to vector control GY leukemia cells, normalized to untreated cells. (G) mRNA level of CPT1A after 48 h of siRNA knockdown compared to scramble control in GY leukemia cells. (H) Viability of siRNA-mediated CPT1A knockdown and scramble control GY leukemia cells after treatment with Ven/Dasa (100 nM) relative to the viability of untreated cells. **$P \leq 0.01$.

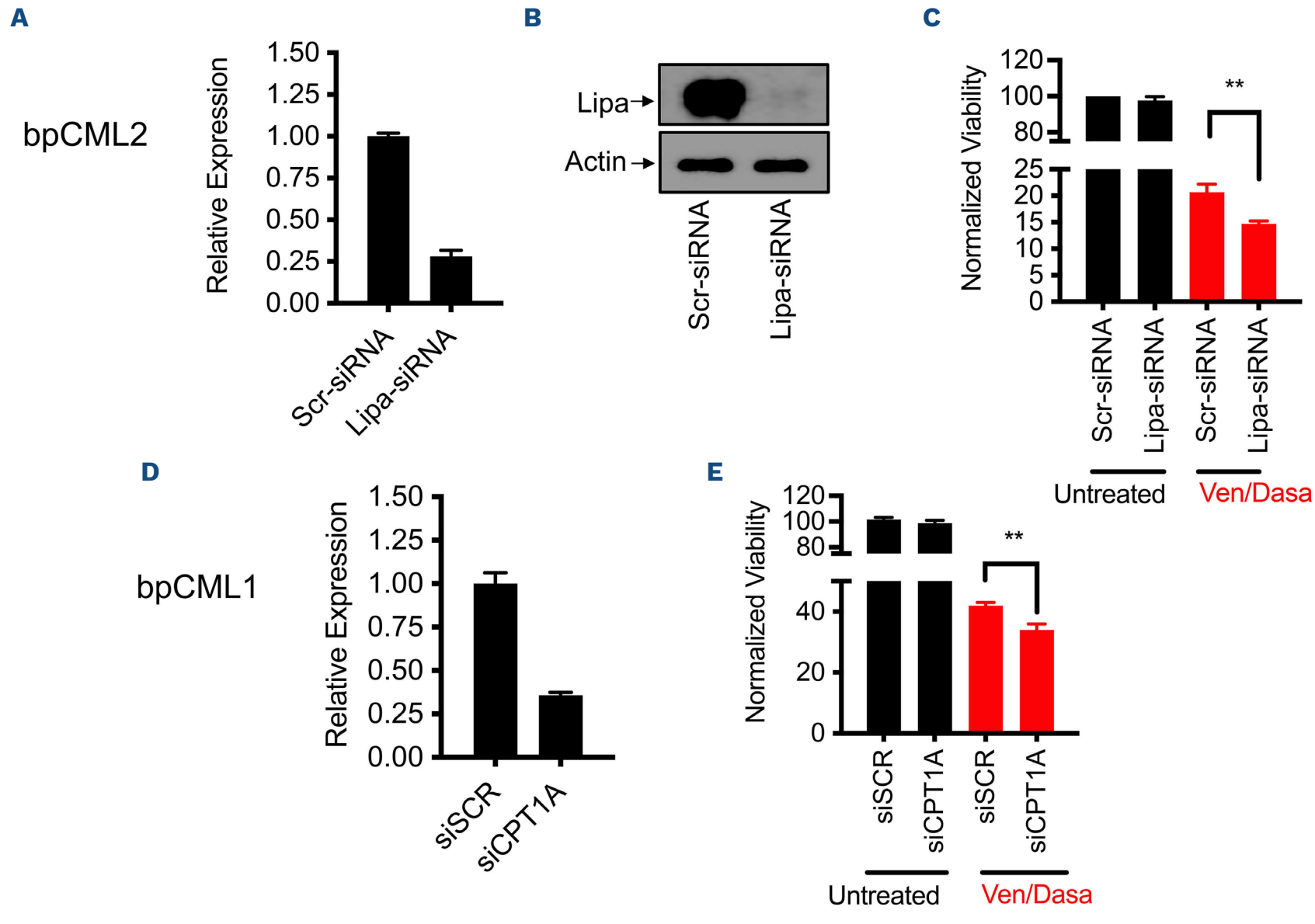

Figure 8. Endogenous LIPA and CPT1A are critical for resistance to the venetoclax/dasatinib combination in human blast phase chronic myeloid leukemia samples. (A) mRNA level of LIPA after 48 h of siRNA knockdown compared to scrambled control in human blast phase chronic myeloid leukemia cells (bpCML2). (B) Western blot of LIPA in LIPA knockdown and scramble control in bpCML2 cells. (C) Viability of LIPA knockout and scramble control human bpCML2 cells after treatment with the venetoclax/dasatinib (Ven/Dasa) combination (100 nM) relative to untreated cells. (D) mRNA level of CPT1A after 48 h of RNA knockdown compared to the scrambled control in human bpCML cells (bpCML1). (E) Viability of CPT1A knockdown and scramble control human bpCML1 cells after treatment with Ven/Dasa (100 nM) relative to the viability of untreated cells. Error bars denote mean ± standard deviation from triplicate experiments. Statistical analyses were performed using a Student *t* test. ***P*≤0.01.

activity may also represent a compensatory response to venetoclax-based therapies that employ TKI. Accordingly, knockdown of CPT1A, the main mitochondrial transporter of free fatty acids,[38] resulted in increased sensitivity to the ven/dasa combination, confirming the role of free fatty acids in attenuating the response to ven/dasa.

The clinical implications of our findings are significant. Given that TKI do not eliminate LSC in all patients with CML,[39,40] most must receive lifelong therapy. ABL-class TKI are known to have side effects, ranging from endocrine dyscrasias to arterial thromboses,[41] and are poorly tolerated in some patients. TKI resistance also develops over time in about 15% of patients.[42] One future direction of this work would be to evaluate venetoclax/TKI combinations in newly diagnosed cpCML to see whether more effective LSC targeting can be achieved with lower doses of TKI in the presence of venetoclax, as well as whether addition of venetoclax can reduce evolution of ABL kinase domain resistance mutations. Notably, only 40-50% of patients with cpCML are able to stop treatment with single-agent TKI after prolonged durations of molecular remission, as demonstrated by the prospective STIM1 and TWISTER trials.[43,44] It would be intriguing to repeat these trials after initial treatment with venetoclax/TKI combinations to see whether a higher percentage of cpCML patients are able to discontinue therapy altogether. Furthermore, for bpCML in particular, the only curative treatment available at present is allogeneic hematopoietic stem cell transplantation,[13] in which graft-*versus*-leukemia activity can be effective.[45,46] However, graft-*versus*-leukemia often co-occurs with severe graft-*versus*-host-disease which compounds the toxicity of stem cell transplantation for patients.[47] With more effective LSC targeting, stem cell transplantation may be required in fewer patients with associated reductions in transplant-related morbidity and mortality; furthermore, patients may be able to go into transplantation with lower burdens of disease, which could

enhance the post-transplant survival rates.
Finally, an important extension of our current work will be to expand our evaluation of combination therapies with venetoclax/TKI and fatty acid transport inhibitors or by extension autophagy inhibitors. Triple therapy combinations may enhance LSC targeting and extend durations of response given our preclinical data that blocking these pathways enhances efficacy of ven/dasa against bpCML. Understanding the mechanisms of synergy between venetoclax and TKI in myeloid malignancies will be important to optimizing clinical use of this regimen.
In summary, we confirm and extend the preclinical utility of venetoclax/TKI combination therapies for targeting bpCML LSC. Furthermore, our data suggest that blocking upregulation of free fatty acids through mechanisms such as inhibition of LIPA activity might synergize with venetoclax/TKI combinations to eradicate LSC, allowing for more durable responses. Our findings support a therapeutic rationale for blocking pathways involved in free fatty acid generation as a potential strategy for increasing disease-free survival in patients affected by bpCML.

Disclosures

DAP has received research funding from and served as a consultant to AbbVie. The other authors declare no conflicts of interest.

Contributions

MM and CTJ designed research. AW, HY, SP, BS, MA, AI, MG, MJA, RM, IS, SP, and AK performed and/or analyzed experiments. RC-H and AD'A performed the metabolomic analysis. AG, KE, SG, and MR performed the RNA sequencing analysis. MM prepared the figures. MM and CTJ wrote the manuscript with input from AW, SP, DWS, DAP, and CS.

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Data-sharing statement

Original data, primer/probe sequences, and protocols are available upon request by contacting the corresponding author.

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