PD-1 blockade in combination with dasatinib potentiates induction of anti-acute lymphocytic leukemia immunity

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ABSTRACT
Immunotherapy, in the form of hematopoietic stem cell transplantation (HSCT), has been part of the standard of care in the treatment of acute leukemia for over 40 years. Trials evaluating novel immunotherapeutic approaches, such as targeting the programmed death-1 (PD-1) pathway, have unfortunately not yielded comparable results to those seen in solid tumors. Major histocompatibility complex (MHC) proteins are cell surface proteins essential for the adaptive immune system to recognize self versus non-self. MHC typing is used to determine donor compatibility when evaluating patients for HSCT. Recently, loss of MHC class II (MHC II) was shown to be a mechanism of immune escape in patients with acute myeloid leukemia after HSCT. Here we report that treatment with the tyrosine kinase inhibitor, dasatinib, and an anti-PD-1 antibody in preclinical models of Philadelphia chromosome positive B-cell acute lymphoblastic leukemia is highly active. The dasatinib and anti-PD-1 combination reduces tumor burden, is efficacious, and extends survival. Mechanistically, we found that treatment with dasatinib significantly increased MHC II expression on the surface of antigen-presentation cells (APC) in a tumor microenvironment-independent fashion and caused influx of APC cells into the leukemic bone marrow. Finally, the induction of MHC II may potentiate immune memory by impairing leukemic engraftment in mice previously cured with dasatinib, after re-inoculation of leukemia cells. In summary, our data suggests that anti-PD-1 therapy may enhance the killing ability of dasatinib via dasatinib driven APC growth and expansion and upregulation of MHC II expression, leading to antileukemic immune rewiring.

INTRODUCTION
The BCR-ABL gene fusion is known to promote two distinct types of leukemia: chronic myeloid leukemia (CML) and Philadelphia chromosome positive (Ph+) B-cell acute lymphoblastic leukemia (B-ALL). Tyrosine kinase inhibitors (TKI) like imatinib and dasatinib, target ABL and other tyrosine kinases, and are an essential part of treating patients with Ph+ B-ALL.1-3 The current strategy for treating Ph+ B-ALL is a combination of a TKI and chemotherapy, and the median overall survival (OS) of patients treated with the combination of chemotherapy and dasatinib remains just 47 months.4 Blinatumomab is a bispecific T-cell engager approved by the US Food and Drug Administration (FDA) to treat patients with relapsed/refractory B-ALL. Blinatumomab links CD8 on the surface of T cells, with CD19 on B-ALL. The efficacy data for blinatumomab in B-ALL suggests that the immune system can be harnessed to fight ALL.5 Dasatinib combined with blinatumomab for Ph+ B-ALL is a highly active, very promising chemotheraphy free regimen for the treatment of B-ALL.6 While the addition of PD-1 inhibition to blinatumomab has preclinical rationale in B-ALL,7 to our knowledge there is only preclinical rationale of adding PD-1 inhibition to TKIs in other disease types.8 9 Even though immunotherapy, specifically anti-Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and anti-programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) therapy, has revolutionized the treatment of solid tumors and some hematologic malignancies, like Hodgkin’s lymphoma,10 11 these agents have had less success in most other hematologic malignancies, including B-ALL.12 Here, we provide evidence that dasatinib may sensitize B-ALL to the effects of PD-1 blockade.

This study was designed to test the hypothesis that inhibition of anti-PD-1 in combination with dasatinib, would enhance therapeutic effects against Ph+ B-ALL. In a syngeneic mouse model of Ph+ B-ALL, dasatinib eliminated leukemic cells, prolonged survival, and induced anti-B-ALL immunogenic memory in mice when they were re-challenged with B-ALL cells, the addition of an anti-PD-1 antibody appeared to potentiate some of these
effects. Additionally, we showed that dasatinib induces class II transactivator (CIITA), a master regulator of major histocompatibility complex (MHC) II genes, leading to upregulation of MHC II in both Ph+ B-ALL and normal antigen-presenting cells (APC).

RESULTS AND DISCUSSION

We first aimed to examine whether anti-PD-1 therapy could improve the efficacy of BCR-ABL directed therapy in Ph+ B-ALL in vivo. Following intravenous inoculation of C57BL/6j mice with a luciferase-labeled mCherry+ BCR-ABL+ B-ALL syngeneic murine model, we confirmed successful B-ALL engraftment (figure 1A,B) and began treatment as described in the methods. All three regimens statistically significantly extended survival when compared with vehicle-treated mice (figure 1C,D, online supplemental table 1). Anti-PD-1 therapy alone modestly improved median overall survival (OS) compared with vehicle, with a median OS of 11.5 days versus 10 days in vehicle-treated group. Dasatinib was substantially better at improving the median OS compared with vehicle as dasatinib treated mice had a median OS of 19.5 days compared with the vehicle-treated mice’s 10 days. The combination of dasatinib and anti-PD-1 was the best at extending median OS with a median survival that had not been reached at day 69 after inoculation (figure 1C). At day 21 after inoculation, there were no mice surviving in either the vehicle and anti-PD-1 treated arm, while 4/10 (40%) mice and 8/10 (80%) mice in the dasatinib and the anti-PD-1/dasatinib were alive. While not strictly statistically significant (p=0.0674), the superior efficacy of the combination is evident when comparing the median OS and the proportion of long-term surviving mice in the anti-PD-1/dasatinib combination treated mice to the dasatinib alone treated mice (figure 1C,D, online supplemental figure 1A–J and table 1).

Next, to understand the mechanism of action of the previously described observation, we repeated the above experiment, and on day 9 post-inoculation, the murine bone marrow (BM) was harvested. We then performed a detailed immune phenotypic analysis of the tumor-infiltrating lymphocytes (TILs) and APCs. Analysis by multicolor flow cytometry indicated both an influx of cells into the TIL compartment and a significant recruitment of APCs on all three administered therapies, compared with the mouse cells. This suggested a strong therapy-driven immunological rewiring of the BM landscape. First, we saw as expected a decrease in the leukemia burden in all the treatments compared with control mice (figure 1E, online supplemental figure 2A,B). Further, we identified a statistically significant influx of CD3+ T-cells into the BM after treatment with dasatinib (p=0.0043) and dasatinib plus anti-PD-1 respectively (p=0.002) (figure 1F). Specifically, within the TILs we observed a significant increase in CD8+, CD4+ effector, and CD4+ regulatory cells after treatment with dasatinib (p=0.0092; p=0.0044; p=0.0018 respectively) and dasatinib plus anti-PD-1 (p=0.0052; p=0.0038; p=0.0001) (figure 1G–I, online supplemental figure 2A–C). Of note, the cytotoxic T-cells in the treatment groups had less granzyme B than cells in the control group, although likely enough to mediate cytotoxicity (online supplemental figure 2D). Because of the previously described changes in granzyme B in the TILs, we looked for alternative pathways to explore. Next, we turned our attention to the APC compartment. Besides the aforementioned significant increase in the frequency of CD4+ T cells, we found a significant increase in the cells of the APC compartment, such as dendritic cells (DCs) (figure 1J,K, online supplemental figures 2A,B and 3A), together with an increase in frequency of mCherry+ B-cells (online supplemental figures 2A,B and 3B) in dasatinib containing regimens compared with control. This observation supports recent data showing that CD4+ T-cell mobilization targeting MHC II antigens is necessary to support CD8+ T-cell responses, as well as responses to immune checkpoint blockade (eg, anti-PD-1). Interestingly, while we found an increase in APCs in general, the percentage of tumor associated macrophages (TAMs) in anti-PD-1 treated BM was significantly increased compared with control mice. The TAMs treated with anti-PD-1 had relatively low levels of MHC II. In contrast, the percent of TAMs in the combination treatment group was reduced compared with treatment with anti-PD-1 and had significantly increased MHC II expression when compared with anti-PD-1 or vehicle-treated mice (online supplemental figure 3C,D). In fact, in multiple APC subsets, an elevated expression of MHC II in dasatinib containing treatment groups compared with vehicle was observed (online supplemental figure 3D–F). Furthermore, a significant increase in the expression of MHC II was observed in the dasatinib treated mCherry+ BCR-ABL+ B-ALL cells and mCherry− B-cells. However, this effect was not sustained on mCherry+ B-ALL cells in the dasatinib/anti-PD-1 combination group (online supplemental figure 3G,H).

Not only did we observe a statistically significant increase in the frequency of DCs in the dasatinib (p=0.0414) and the dasatinib plus anti-PD-1 treated group (p=0.0119) (figure 1K). DCs also showed an increased expression of MHC II on the cell surface compared with the vehicle treated mice (online supplemental figure 3F). Moreover, dasatinib increased the frequency of B-cells (p=0.0337), TAMs (p=0.0228), and monocytic myeloid-derived suppressor cells (MDSCs) (p=0.0006) among all MHC II positive cells (online supplemental figure 4A–C).

Interestingly, we saw a significant decrease in the intensity of PD-L1 expression on the mCherry+ B-cells when comparing vehicle or the anti-PD-1 treated group to the combination treatment of dasatinib plus anti-PD-1 (p=0.024 and p=0.0025, respectively). A similar trend was observed in the mCherry+ BCR-ABL+ B-ALL cells (online supplemental figure 4D,E).

In addition to its direct antitumor activity as a BCR-ABL inhibitor, this data suggests that dasatinib may enhance
Figure 1  Dasatinib plus anti-PD-1 potentiates effective tumor control in a Ph+ ALL mouse model and induces immune activation via CD4/MHC II pathway in vivo. Mice injected and engrafted with Ph+ ALL were randomized into four groups and treated with vehicle (n=5), dasatinib (n=10), anti-programmed death 1 protein (anti-PD-1) (n=10), and dasatinib+anti-PD-1 (n=10). (A) Shows total flux values on day 3 (prior to treatment) and day 7 after treatment was started. On day 7 vehicle-treated mice have a significantly higher engraftment than all other treatment groups, dasatinib treated mice had significantly less leukemia than anti-PD-1 treated mice on day 7, and dasatinib plus anti-PD-1 treated mice had statistically significant less leukemia than mice in anti-PD-1 therapy group; (B) shows total tumor burden expressed as total flux values over time through day 69 of the four treatment groups. This graph shows that only the dasatinib and the dasatinib plus anti-PD-1 treated groups had long-term survivors; (C) shows a Kaplan-Meier survival curve of the experiment described in (B) as well as a table below that describes the median overall survival of each regimen; (D) summary of BLI changes shown for five representative mice from each group at day 3, 7, 11, 13, 17, and 69; (E) frequency of mCherry+ Ph+ B-ALL cells in the bone marrow (BM) harvested from mice treated with vehicle, dasatinib, anti-PD-1 and combination at day 9 after injection of leukemic cells; (F) frequency of CD3+ T cells in the BM harvested from mice treated with vehicle, dasatinib, anti-PD-1 and combination at day 9 after injection of leukemic cells; (G) frequency of CD8+ T cells in TILs harvested from bone marrow of mice treated with vehicle, dasatinib, anti-PD-1 and combination at day 9 after injection of leukemic cells; (H) the frequency of CD4+ effector T cells (CD3+, CD4+, Foxp3-) in the TIL with the indicated treatment; (I) the frequency of CD4+ regulatory T cells (CD3+, CD4+, Foxp3+) in the TIL harvested from murine BM at day 9 post injection, following the indicated treatment; (J) percent of APCs in the murine bone marrow; (K) percent of DCs (CD3-, CD19-, CD11c+, MHC II) in the TIL murine bone marrow with the indicated treatment. Values were presented as mean±SD. Statistically significant differences were indicated as, *p<0.05, **p<0.001, ***p<0.0001, ****p<0.0001.

ALL, acute lymphoblastic leukemia; APC, antigen-presenting cells; B-ALL, B-cell ALL; BLI, bioluminescence imaging; DC, dendritic cell; MHC II, major histocompatibility complex class II; Ph+, Philadelphia chromosome positive; PD1, programmed death-1; PDL-1, programmed cell death ligand 1; TILs, tumor infiltrating lymphocytes.
immunogenic killing through growth stimulation and an induction of MHC II on multiple APC subsets. To prove our in vivo observation, we used peripheral blood mononuclear cells (PBMCs) derived from healthy donor buffy coats and investigated the effect of dasatinib on MHC II expression in PBMCs alone or in a co-culture system with a Ph+ B-ALL patient derived xenograft (PDX) model, ICN1. In line with our in vivo observations, dasatinib increased CD3+ cell number in vitro, which was rescued after incubation with MHC II blocking antibody (figure 2A–C). However, in our in vitro experiments, we did not observe any significant impact of dasatinib on the growth/expansion of CD4+ or CD8+ cells (online supplemental figure 5A–E), which is likely due to the use of low doses of dasatinib or due to the experimental design, as we used naive, not stimulated T-cells.

A short course of dasatinib at the dose of 100 nM led to an increase in viability of MDSCs, and DCs respectively, particularly after co-culture with Ph− B-ALL cells (figure 2A,B and D,E, online supplemental figure 5F,G). These effects seem to be MHC II dependent, as the preincubation with MHC II blocking antibody did significantly reduce these effects (figure 2A,B and D,E). Dasatinib treatment strongly induced expansion of a monocytic cell population, in particular CD11b MDSCs/DCs, in both the presence and absence of leukemic cells (figure 2F). After co-culture with B-ALL PDX cells, MDSCs had decreased MHC II expression (figure 2G) while CD11b+ DCs had a significant enhancement of MHC II expression (figure 2H).

Furthermore, we explored whether dasatinib could induce expression of myeloid MHC II separate from its role in killing of BCR-ABL+ tumor cells. We therefore used MHC II positive Ph− cell lines (Koeffler and Golde 1 (KG1) and Tohoku Hospital Pediatrics-I (THP1)) and treated them with dasatinib or vehicle. In both cell lines, treatment with dasatinib led to increases in human leukocyte antigen DQb isotype (HLA DQb) RNA expression (p=0.02), as well as human leukocyte antigen-DPb isotype (HLA DPb) (p=0.03), two of genes involved in the MHC II complex of proteins (figure 3A–B). However, treatment only produced significant elevation of messenger RNA (mRNA) level for human leukocyte antigen-DRa isotype (HLA DRa) in KG1 (p=0.005) and for human leukocyte antigen-DMa isotype (HLA DMa) in THP1 (p=0.014) (online supplemental figure 6A,B). The mRNA expression of CIITA, a master regulator of MHC II expression, was increased in KG1 cells (p=0.025) (online supplemental figure 6C). However, at the protein level we observed a dose-dependent increase in both MHC II and CIITA expression in both cell lines (figure 3C,D). Because the mRNA increase was not sufficient or consistent with the increases seen at a protein level, we surmise that there is translational/post-translational regulation of CIITA by dasatinib. Most importantly, we saw increases in surface expression of MHC II with dasatinib treatment suggesting that dasatinib could induce enhanced surface expression of MHC II in MHC II expressing cells (figure 3E–H). The MHC II/CD4+ interaction is known to induce a powerful immunologic response and has been thought to play a role in optimal vaccine responses; we therefore tested whether dasatinib could induce immunologic memory in our in vivo model.14 We did this by re-inoculating the “cured” mice and age-matched controls with B-ALL cells. The presence of sentinel anti-tumor T-cell memory was validated because previously treated mice continued without disease up to 60 days post-injection, while the aged-matched controls quickly succumbed to B-ALL (figure 3I–L, online supplemental figure 6D,E).

While dasatinib has a known antitumor effect in Ph+ B-ALL, these findings suggest that in addition to its direct antitumor effect, dasatinib also has an immune enhancing effect. The underlying mechanism of dasatinib-mediated myeloid MHC II upregulation suggests potential future applications of this agent as an enhancer of various types of immunotherapies including T-cell checkpoint blockade and cancer vaccines. Interestingly, this increase in MHC II appears to be cell of origin specific. Tarafdar, et al previously reported that CML stem cells down regulate MHC II and treatment with dasatinib and the TKI imatinib had no effect on MHC II or CIITA in CML stem cells. Whereas we saw increases in MHC II in cells that were high expressers of MHC II. All of this suggests that MHC II induction by dasatinib is cell type specific and BCR-ABL independent.15

Immunotherapy has revolutionized the treatment of cancer for some patients, but additional work is needed to evaluate how combinations can improve its efficacy. In fact, Alspach et al recently described the role MHC II neoantigens play in tumor immunity and the response to immunotherapy. Specifically, in regard to tumor immunity, MHC II restricted antigens have non-overlapping functions compared with MHC I antigens.13 This suggests that augmenting MHC II could increase response to a variety of immune-based therapies. Here, we show that by increasing MHC II on the surface of the APCs we increased the immune response to anti-PD-1. The novelty here is that dasatinib, an FDA approved agent, induced MHC II and was potentiated by anti-PD-1 therapy. This has obvious implications in BCR-ABL-driven diseases like Ph+ B-ALL where the increase of MHC II by dasatinib could help explain the remarkable activity of dasatinib plus blinatumomab in the front-line setting of patients with Ph+ B-ALL as reported by Foà et al.6 Additionally, our observation of upregulation of MHC II expression in APCs with dasatinib therapy may help explain the discrepancy in the clinical activity of dasatinib plus blinatumomab with the observations of Leonard et al who report that dasatinib inhibits T-cell proliferation.16 Like Leonard et al we also saw a reduction in granzyme B production by cytotoxic T-cells under dasatinib treated conditions but suspect that the increase in MHC II expression on APCs could account for the remarkable clinical activity of the dasatinib plus blinatumomab combination. While the increase in MHC II expression on APCs may improve the immunogenic immune killing of anti-PD-1 therapy or...
Figure 2  Dasatinib induces immune activation via APC/MHC II pathway in vitro. Representative gating strategy and flow cytometry profile of healthy donor peripheral blood mononuclear cells (PBMCs) alone (A) or (B) in co-culture with Ph⁺ B-ALL PDX model ICN1. PBMCs from five (n=5) healthy donor buffy coats were first treated with MHC II blocking antibody for 1 hour, followed by staining with CellTrace Violet, washing and resuspending in media with or without dasatinib 100 nM, and additionally challenged by ICN1 cells with effector:stimulator ratio 3:1. Cells were incubated for 5 days. Cells after collection and staining were evaluated by flow cytometry first by gating according to expression of CD45 and CD11b versus SS INT (A–B), as well as assessment of CD3⁺ cells proliferation by counting beads, CellTrace Violet retention and expression of CD4, CD8, CD19, MHC II, CD45. Gating shown on example of PBMCs analysis from Donor 895; (C) viable cell number of CD3⁺ T cells with the indicated treatments with/out ICN1 Ph⁺ B-ALL cell co-culture, normalized to control unstimulated cells, each dot represents a sum of three replicates run for n=5 independent PBMCs donors; (D) evaluation of viable MDSC cells with the indicated treatments with/out ICN1 Ph⁺ B-ALL cell co-culture, normalized to control cells from the PBMCs only setting, summarized for n=5 independent healthy PBMCs donors; (E) evaluation of viable DCs with the indicated treatments with/out ICN1 Ph⁺ B-ALL cell co-culture, normalized to control from the PBMCs only setting, as a summary of response for n=5 healthy PBMCs donors. (F) MFI of CD11b in the monocyte gate as shown in (A and B) following the indicated treatments with/out ICN1 Ph⁺ B-ALL cell co-culture, summarized for n=5 independent healthy PBMCs donors; (G) MFI of MHC II MFI in DCs with the indicated treatments with/out ICN1 Ph⁺ B-ALL cell co-culture, normalized to control from the PBMCs only setting, with the indicated treatments with/out ICN1 Ph⁺ B-ALL cell co-culture, normalized to control unstimulated cells. Each dot represents a sum of three replicates run for n=5 independent PBMCs donors. All graphs were presented as mean±SD. Statistically significant differences were indicated as: *p<0.05, **p<0.001, ***p<0.0001, ****p<0.00001. Ab, antibody; ALL, acute lymphoblastic leukemia; APC, antigen-presenting cell; B-ALL, B-cell ALL; BLI, bioluminescence imaging; DC, dendritic cell; FS INT, forward scatter integral; MDSC, myeloid-derived suppressor cells; MFI, mean fluorescence intensity; MHC II, major histocompatibility complex class II; PBMC, peripheral blood mononuclear cells; Ph⁺, Philadelphia chromosome positive; PDX, patient derived xenograft; SS INT, side scatter integral.
Dasatinib induces MHC II via CIITA in antigen presenting cells. (A–B) Relative human leukocyte antigen-DQβ isotype (HLA DQβ) mRNA expression by qPCR, in two monocytic cell lines (KG1 and THP1) treated with vehicle and dasatinib; n=3 independent experiments; (C–D) western blot analysis of MHC II and CIITA protein. (E–F) Representative histograms of MHC II expression measured by flow cytometry and summary of flow cytometric analysis for MFI of MHC II summarized for KG1 cells with DMSO or dasatinib treatment overnight. (G–H) Representative histograms of MHC II expression measured by flow cytometry and summary of flow cytometric analysis for MFI of MHC II summarized for THP-1 cells with or without dasatinib treatment overnight. (I) Tumor burden evaluation as shown by BLI for re-inoculation experiments of long-term surviving mice from experiment described in (A–D) showing engraftment in mice previously treated with dasatinib+anti-PD-1 injected with mCherry⁺ B-leukemic cells, reinjection experiment was performed with age-matched controls. (J) Kaplan-Meyer curve showing survival of mice treated prior with anti-PD-1+dasatinib, reinoculated with leukemia. (K) Tumor burden evaluation as shown by BLI for re-inoculation experiments of long-term surviving mice from experiment described in (A–D) showing engraftment in mice previously treated with dasatinib injected with mCherry⁺ B-leukemic cells, reinjection experiment was performed with age-matched controls. (L) Kaplan-Meyer curve showing survival of mice treated prior with dasatinib, re-inoculated with leukemia. All graphs were presented as mean±SD. Statistically significant differences were indicated as: *p<0.05, **p<0.01, ***p=0.001, ****p<0.0001. BLI, Bioluminescence imaging; CIITA, class II transactivator; DMSO, dimethyl sulfoxide; HLA, human leukocyte antigen; KG1, Koeffler and Golde (1) cell line; mRNA, messenger ribonucleic acid; MFI, mean fluorescence intensity; MHC II, major histocompatibility complex class II; PD-1, programmed cell death protein-1; qPCR, quantitative Polymerase Chain Reaction; THP1, Tohoku Hospital Pediatrics-1 cell line.
blinatumomab, the improvement seen with the combination is likely multifactorial. Tracy et al recently described another TKI that targets BCR-ABL, nilotinib, which was able to reverse CD4+ T-cell dysfunction and had considerable activity in combination with anti-PD-1 therapy in a different mouse model of Ph+ B-ALL.13 Furthermore, augmenting the activity of blinatumomab and/or TKIs is currently actively under investigation. Trials evaluating blinatumomab with immune checkpoint inhibitors or checkpoint inhibitors with TKIs are ongoing and include: (NCT04329325, NCT02744768, NCT03263572, NCT02143414, NCT03147612, NCT03160079, NCT03512405, NCT02879695). These findings support further investigating the role of checkpoint immunotherapy combinations in BCR-ABL+ driven diseases like Ph+ B-ALL, where anti-PD-1 therapy could be combined with dasatinib to enhance endogenous anti-B-ALL immunity which could improve initial B-ALL clearance compared with dasatinib alone. Additional investigations in other tumor models suggest that dasatinib’s ability to sensitize cancer to immune checkpoint therapy may potentially be extended to other non-hematologic indications.8,9

**METHODS**

**Generation of luciferase+ mCherry+ Ph+ murine B-ALL model**

C57BL/6j mice (C57BL6, stock # 000664) were obtained from the Jackson Laboratory. All mice were bred and housed in a specific pathogen-free animal facility at the University of Texas MD Anderson Cancer Center. Bone marrow (BM) cells from 4 weeks old mice was isolated by using a flushing technique.18 A single cell suspension was then enriched for pro-B-cells by using magnetic-activated cell sorting (MACS) columns and B220 beads (CD45R (B220) MicroBeads, cat # 130-049-501). OP9 and pro-B cells co-culture was established as described elsewhere19 and pro-B cells were cultured for week in alpha minimum essential medium (αMEM) (Cellgro) supplemented with 20% fetal bovine serum (FBS) (American Type Culture Collection (ATCC)), 2 mM glutamine (Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco) 50 μM β-mercaptoethanol (Sigma), and 10 ng/ml IL-7 (Miltenyi Biotec)]. The pro-B cells were transduced by spinoculation with MIG-p190 BCR-ABL-mCherry retroviral supernatant in the presence of 5 μg/ml polybrene for 45 min at 450 g and 37 °C with Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) culture medium supplemented with 10% heat-inactivated FBS (Atlanta Biologicals), 100 units/ml penicillin, and 100 μg/ml streptomycin. Later these transformed cells were labeled with luciferase in a subsequent virus transduction. Preparation of the viral vector and viruses were performed as previously described.16 Transduced cells were then expanded, and 1 million cells were transplanted by tail vein injection into sub-lethally irradiated (700 cGy) recipient mice. This resulted in the development of leukemia disease and animal death within 28 days. BM cells of the primary recipient mouse were collected on day 21 post reconstitution and sorted based on mCherry fluorescence. These mCherry+ cells (B220- CD19- CD4- CD8- CD11b-) were cultured for 7 days and upon transplantation into secondary immunocompetent C56BL6 recipients, triggered ALL development with animal life span of 10-17 days.

**Bioluminescence imaging**

D-luciferin (100 μL, 30 mg/mL, sodium salt, Gold Biotechnology, St Louis, Missouri, USA) was injected intraperitoneal (IP) into mice, the image was acquired 10 min after substrate injection using In Vivo Imaging System (IVIS; PerkinElmer).20

**In vivo study of dasatinib and anti-PD-1 therapy in a Ph+ B-ALL mouse model**

Six-week-old, C57BL6 mice, were inoculated with mCherry+ B-ALL cells (0.25×10⁶ per mouse) by tail vein injection (day 0). On day 3 post-injection, engraftment was confirmed via bioluminescence imaging (BLI) and mice were distributed into four groups (5 mice in the vehicle group and 10 mice in the other three groups) with comparable tumor burden. Vehicle mice were treated with 80μM of citric acid via oral gavage (OG) for 5 days on days 4–8, dasatinib mice were treated with dasatinib 10mg/kg in 80μM of citric acid OG on days 4–8, anti-PD-1 mice were treated with 200ug in 200uL of phosphate buffered saline (PBS) via IP injections every 3 days starting on day 4 for five doses, and the anti-PD-1 plus dasatinib mice were treated with a combination of anti-PD-1 mouse regimen with the dasatinib mouse regimen.21–27. BLI to determine the extent of engraftment and flow cytometry to monitor circulating leukemic mCherry+ cells in peripheral blood were performed twice weekly. Following the completion of treatment with the last dose of anti-PD-1 on day 17, mice were monitored for any signs of disease and mouse survival was recorded as an endpoint, additionally long-term surviving mice were saved and used for immunological memory studies.

**Pharmacodynamic studies**

To test the pharmacodynamics of combination anti-PD-1 with dasatinib, five naïve wild-type recipient mice were used for each treatment group (vehicle treated, dasatinib, dasatinib+anti-PD-1, and anti-PD-1) mice were injected with mCherry+ B-ALL cells and treated as described above. On day 9, when the vehicle group of mice became terminally ill, all the mice were euthanized, and the BM was collected for multicolor flow cytometry. In addition to the four treatment groups listed above, naïve mice who were never exposed to tumor cells were also sacrificed and analyzed as a separate control group.

**Tumor isolation and flow cytometric analysis of TILs**

Briefly, BM was flushed and then filtered through a 70 μm cell strainer.19 Viable immune cells were enriched through density gradient separation over Ficoll-Histopaque 1119
(Sigma-Aldrich) with a short spin (10 min) at 1000 g to minimize loss of myeloid populations. Samples were then fixed using the Foxp3/Transcription Factor Staining Buffer Set (Thermo) and stained with up to 12 antibodies at a time from BioLegend, BD Biosciences, Affymetrix, Santa Cruz Biotechnology, and Thermo Fisher Scientific as previously described,28 (online supplemental table 2). Flow data was collected on a 5-laser, 18-color BD LSR II cytometer and analyzed using FlowJo V.7.6.5 (Tree Star).

**Immunological memory**

To test immune memory, three long-term survivor mice from the dasatinib only treated group and the dasatinib plus anti-PD-1 groups, respectively, were re-injected with 0.25×10⁶ leukemia cells per mouse via tail-vein. Additionally, three age-matched control (previously unexposed, C57BL/6j) mice were injected with the same number of cells via tail vein injection. These mice were monitored for engraftment via IVIS imaging as previously described.

**Mixed lymphocyte reactions and MHC II expression assay**

PBMCs (responder cells) were isolated from n=5 buffy coats obtained from healthy blood donors by density centrifugation, using lymphocyte-separation media (Corning), followed by red cell lysis with ammonium chloride. PBMCs were then used directly for co-culture experiments (PBMCs-ICN1). All experiments were prepared in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with L-glutamine and 10% heat-inactivated fetal calf serum (Life Technologies). PBMCs were labeled with CellTrace Violet (CTV) (Life Technologies) before co-culture with stimulator/target (Ph⁺ B-ALL ICN1 PDX) cells. Further to investigate effects of MHC II blocking antibody (BD Bioscience), PBMCs were incubated with the purified mouse anti-human HLA-DR, DP, DQ (BD Biosciences) blocking antibody at the concentration of 10 µg/mL for 1 hour, washed and resuspended in fresh RPMI-1640 medium. Further PBMCs alone or in co-culture with ICN1 cells were treated with dasatinib at the concentration of 100 nM. PBMCs were co-cultured with stimulator cells for up to 5 days at a ratio of 3:1 responder:stimulator. Proliferation was measured by flow cytometry as a loss of CTV mean fluorescence intensity, after 5 days of incubation, using lymphocyte – separation media (Corning), followed by red cell lysis with ammonium chloride.

**Quantitative real-time PCR**

Cells were washed twice with PBS and subjected to total RNA extraction using RNeasy Mini Kit (QIAGEN). The sequences of quantitative PCR (qPCR) primers are listed in the online supplemental table 3. qPCR was performed using iQ5 real-time PCR machine (Bio-Rad). Data analysis was performed using the comparative cycle threshold (Ct) method. Results were first normalized to internal control β-actin mRNA, and then the relative expression of genes in response to dasatinib was estimated by normalized expression level after exposure to dasatinib relative to the corresponding normalized expression under no treatment. Results are presented as mean±SD of n=3 independent RNA preparations.

**Western Blotting**

Cells were treated as indicated overnight in serum-free media and subjected to lysis in the lysis buffer (5 M Urea, 10% sodium dodecyl sulfate (SDS), water in a 1:1:1 ratio). The lysates were then sonicated, sample buffer was then added to the cell lysates and proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Germany). The membranes were blocked in Tris-buffered saline containing 0.2% Tween 20 and 5% fat-free dry milk and incubated first with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies. Specific proteins were visualized with enhanced chemiluminescence detection reagent according to the manufacturer’s instructions (Pierce Biotechnology, Waltham, Massachusetts, USA). Antibodies included CIITA (Cat. 3793) and MHC Class II (LGII-612.14; Cat. 68258) from presented as a summary of means obtained for each individual PBMCs donor and presented as mean±SD. Ordinary one-way analysis of variance (ANOVA) was used for statistical significance calculations between all treatment conditions or two-way ANOVA was used to obtain statistical significance between conditions in groups with and without co-culture.

**Cell lines**

KG1 and THP-1 cell lines were purchased from the ATCC (Manassas, Virginia, USA) or Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Cells were validated by short tandem repeat (STR) analysis of variance (ANOVA) was used for statistical significance calculations between all treatment conditions or two-way ANOVA was used to obtain statistical significance between conditions in groups with and without co-culture.
Cell Signaling (Danvers, Massachusetts, USA), and alphatubulin (B-5-1-2; Cat. T5168) from Sigma-Aldrich (Burlington, Massachusetts, USA).

Statistical analysis
Data are expressed as means±SD. Statistical significance was set at p<0.05 using one-way or two-way ANOVA, where appropriate and verified with the unpaired t-test with Welch’s correction. Statistical analysis including the mouse survival data was carried out using the log-rank test in GraphPad Prism (V.9.0.0, GraphPad Software, San Diego, California, USA), and the HRs with CIs were calculated with soft R (V.4.2.2) available at r-project.org.

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Contributors
M-CH and MK supervised the study. PK, NB, KH, and MK designed the experiments. KH and SM established the mouse acute lymphoblastic leukemia cell lines. KH and AC performed the animal studies. RLC and MAC performed the multicolor flow. ZJ performed the qPRC. XH helped design the statistical analysis and performed the HR and CI calculations. XS performed the Western Blotting. NB and PK performed the remaining experiments. H-HL and PW provided scientific input. PK, NB, JH, M-CH, and MK wrote and revised the manuscript.

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Competing interests
PK reports advisory board fees from Novartis, Daiichi Sankyo, BMS, and Ascentage, the speaker’s bureau of Takeda and Novartis, reports participating in the data safety monitoring board with Treadwell Therapeutics, and honoraria with Clinical Care Targeted Communications. PW reports employment from Genentech. MAC reports grants and personal fees from Immunogenesis, Inc., personal fees from Cellzome, Inc., personal fees from ImmunoMet, Inc., personal fees from ImmunoMet, Inc., personal fees from Oncoreponse, Inc., personal fees from Pieris, Inc., personal fees from Nuvirx, Inc., personal fees from Aptevo, Inc., personal fees from Servier, Inc., personal fees from Kineta, Inc., personal fees from Salaris, Inc., personal fees from Xencor, Inc., personal fees from Agenus, Inc., personal fees from Mereo, Inc., personal fees from Amunix, Inc., personal fees from Adagene, Inc., outside the submitted work. In addition, MAC has a patent Methods and Composition for Localized Secretion of Anti-CTLA-4 Antibodies with royalties paid to multiple licensees, a patent Dual specificity antibodies which bind both PD-L1 and PD-L2 and prevent their binding to PD-1 with royalties paid to Immunogenics, Inc., and a patent Cyclic Dinucleotides as Agonists of Stimulation of Interferon Gene Dependent Signaling licensed to Immunogenics, Inc. MK reports grants from Abylnx, Agios, Ascentage, AstraZeneca, Calithera, Cellectis, Eli Lilly, Rafael Pharmaceutical, and Sanofi; and grants and other support from Abbvie, F. Hoffman La-Roche, Forty–Seven, Genentech, and Stemline Therapeutics; and other support from Amgen, Kisoji, Reata Pharmaceutical outside the submitted work; and has a patent (US 7,795,305 B2: “CDDO-Compounds and Combination Therapy”) with royalties paid to Reata Pharm, a patent ("Combination Therapy With a Mutant IDH1 Inhibitor and a BCL-2") licensed to Eli Lilly, and a patent (62/993,166: Combination of a MCL-1 Inhibitor and Midostaurin, Uses and Pharmaceutical Compositions Thereof) pending to Novartis. Funding for this work was provided by the Advanced Scholar Program at MD Anderson (2016-2019, Koller), the University of Texas MD Anderson Cancer Center support Grant (CA016672), and the University of Texas MD Anderson—China Medical University and Hospital Sister Institution Network Fund.

Patient consent for publication
Not applicable.

Ethics approval
Experiments were conducted and approved by the University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee.

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