Supporting Information

Sagiv-Barfi et al. 10.1073/pnas.1500712112

SI Materials and Methods

Western Blots for Btk Expression. Western blots were prepared on nitrocellulose using standard techniques. Briefly, cell lysates were separated on 4–12% SDS–polyacrylamide gel electrophoresis (SDS/PAGE) under reducing conditions and were transferred to nitrocellulose (Schleicher and Schuell). The blot was then stained with antiBtk antibody (Cell Signaling) followed by rabbit anti-mouse IgG-HRP (Southern Biotech) or with GAPDH (14C10) Rabbit mAb (HRP Conjugate, Cell Signaling) and was developed using the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

BTK and ITK Occupancy in Mouse Frozen Spleen and Thymus. Spleen and thymus tissues were removed, flash frozen in liquid nitrogen and stored at –80 °C until analysis. Frozen tissues were homogenized in lysis buffer (Cell Lytic M, Sigma C2978) supplemented with protease inhibitor mixture (Pierce Protease and phosphatase inhibitor, Thermo Scientific #88669) in a high-speed electronic homogenizer (Polytron). Lysates were incubated for 15–20 min on ice and centrifuged at 10,600 × *g* for 15 min at 4 °C. 100 μg of total protein was incubated with either the BODIPY-labeled BTK (spleen sample) or ITK (thymus sample) probes (synthesized by Principia Biopharma) at 1 μM and incubated for 1 h at room temperature. Lysates were evaluated by SDS/PAGE, on a Typhoon image scanner (GE Healthcare) using the Fluorescein 526 Emission Filter, PMT:800, and Green Laser for probe binding. Gels were transferred to nitrocellulose for total BTK and ITK, membranes were probed overnight with anti-BTK for spleen (BD Biosciences 611117, 1:500) or anti-ITK for thymus (BD Biosciences 51–6979 1N) and blotted with goat anti-mouse Ab conjugated to Alexafluor 647 (Invitrogen A21236, 1:1,000) for 1 h, and evaluated using the typhoon scanner with an emission filter of Cy5 670 BP30, and Red Laser. Band intensities were quantified using Imagequant software (GE Healthcare). For each sample, the fluorescent signal was normalized for total protein (either BTK or ITK) and the percent occupancy was calculated by subtracting the normalized ratio from 100%.

In Vitro Assays. Growth and viability of cells was measured using Prestoblue Cell Viability reagent (Life Technologies) according to the manufacturer's protocol.

Flow Cytometry. Gr1-FITC, CD11b-PE, CD8-FITC, CD-4 APC, CD25-Pe, FITC-rat IgG2a, and PE-rat IgG2a were from BD Pharmingen. Cells were surface stained in PBS, 1% FBS, and 0.01% sodium azide, fixed in 2% (wt/vol) paraformaldehyde, and analyzed by flow cytometry on an FACSCalibur (BD Biosciences). Data were stored and analyzed using Cytobank (www.cytobank.org).

Ibrutinib and Anti–PD-L1 in J558 Tumor Mouse Model.

Cell line. J558, a BALB/c plasmacytoma line, was obtained from ATCC. J558 cells were cultured in complete Roswell Park Memorial Institute 1640 medium (cRPMI; Invitrogen) containing 10% (vol/vol) FBS (FBS; Thermo Scientific), 100 U/mL penicillin, 100 μg/mL streptomycin (both from Invitrogen).

Tumor transplantation and treatment. Tumor cells in exponential growth phase were washed three times and resuspended in RPMI medium without serum. Six- to 8-week-old female BALB/c (jaxmice.jax.org) were inoculated with 5×10^6 J558 cells by s.c. injection to the right hind thigh. Tumor growth was monitored with a caliper every 2–3 d and expressed as volume ($D \times d^2 \times 0.4$). Mice were euthanized when s.c. tumor size reached 1,000 mm^3. Therapy was started when tumors reached a volume of 80 mm^3. Anti–PD-L1 (10F.9G2, Bio X cell) or rat IgG2b isotype control (LTF-2, Bio X cell) (200 μg per injection) were given intraperitoneally (IP) every other day. Ibrutinib dissolved in vehicle (0.5% methylcellulose, 0.1% sodium lauryl sulfate) or vehicle alone was given by oral gavage daily on day 12–20 after tumor inoculation at a dose of 24 mg/kg body weight. Mice were housed in the vivarium of Pharmacyclics. All experiments were approved by the Pharmacyclics IACUC committee and conducted in accordance with IACUC protocols.

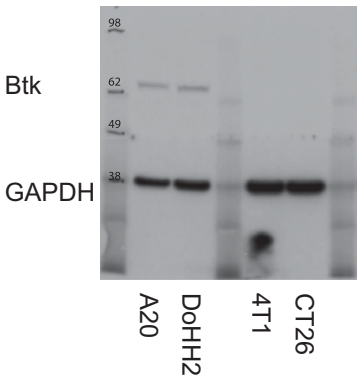

Fig. S1. BTK expression. Detection of BTK protein expression in A20 cells. Western blotting was performed using an anti-BTK antibody. Anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The molecular weights (in kDa) are shown on the left.

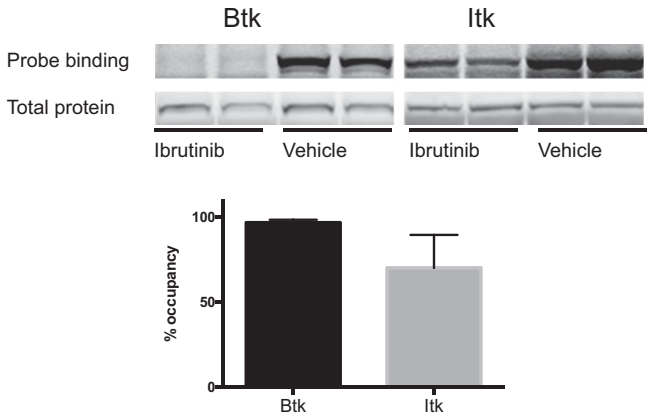

Fig. S2. Ibrutinib occupies both BTK and ITK. Mice splenocytes and thymocytes were collected 1 h following ibrutinib IP dosing. Tissues were incubated with BODIPY-labeled probes and evaluated by SDS/PAGE. (*Left*) BTK occupancy in the spleen. (*Right*) ITK occupancy in the thymus. (*Lower*) Results demonstrating the percent occupancy of BTK and ITK.

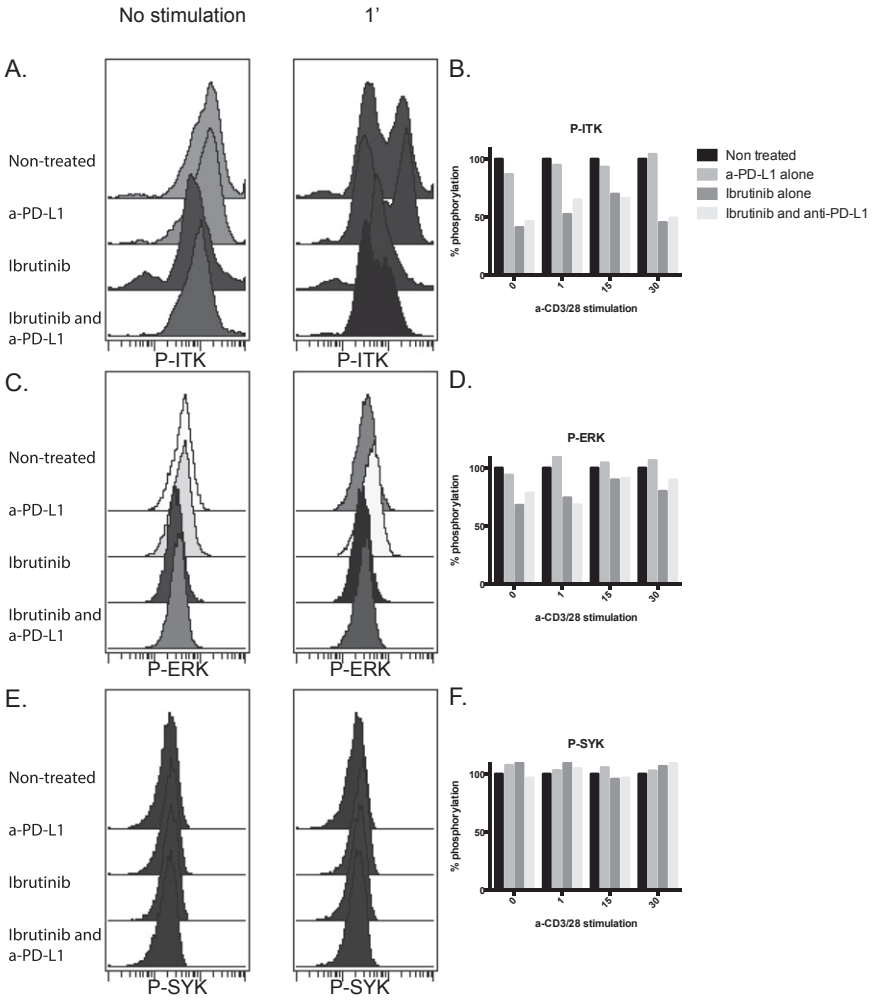

Fig. S3. Ibrutinib treatment reduces ITK and downstream phosphorylation in splenocytes T cells. One hour following treatment with ibrutinib, anti–PD-L1 or the combination of ibrutinib and anti–PD-L1 spleens were assayed by phospho-flow for the phosphorylation of ITK, ERK and SYK. Results representing phosphorylation at the basal level or following 1-, 15-, or 30-min stimulation with a-CD3/a-CD28 antibodies are shown. (*A*) FACS plots at the basal ITK phosphorylation level (*Left*) and following 1-min stimulation (*Right*). (*B*) Bar-graph summarizing the phosphorylation following the different stimulations. (*C* and *D*) ERK phosphorylation. (*E* and *F*) SYK phosphorylation.

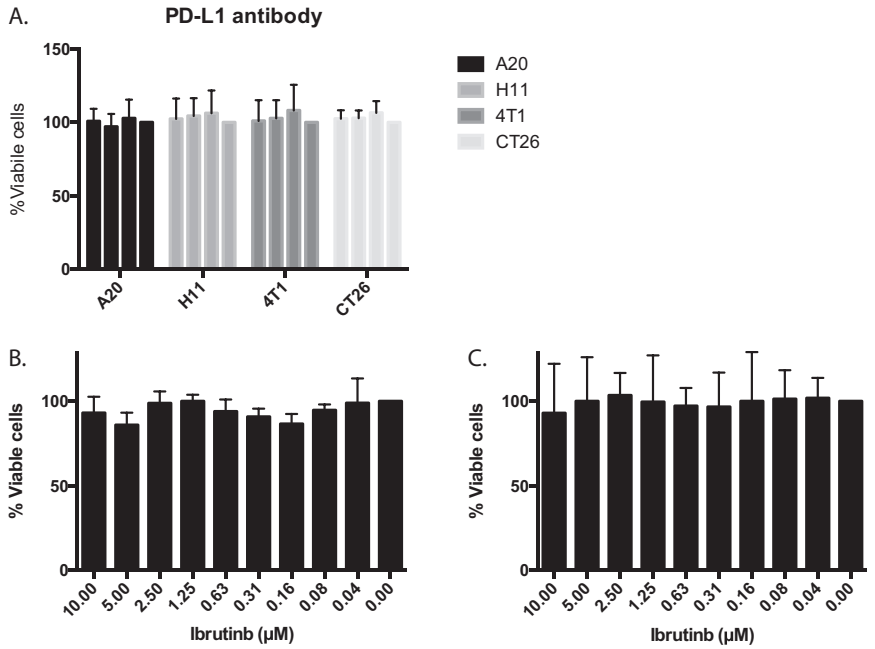

Fig. S4. Cell lines are insensitive to anti–PD-L1 antibody or ibrutinib in vitro. (*A*) A20, H11, 4T1-Luc, and CT26 cell lines were incubated with 100, 50, 10, and no anti–PD-L1 for 24 h (left to right). Cell viability was measured using Prestoblue cell viability reagent and normalized to the nontreatment control. (*B* and *C*) 4T1 (*B*) and CT26 (*C*) cell survival following 24-h incubation with ibrutinib at the indicated dose.

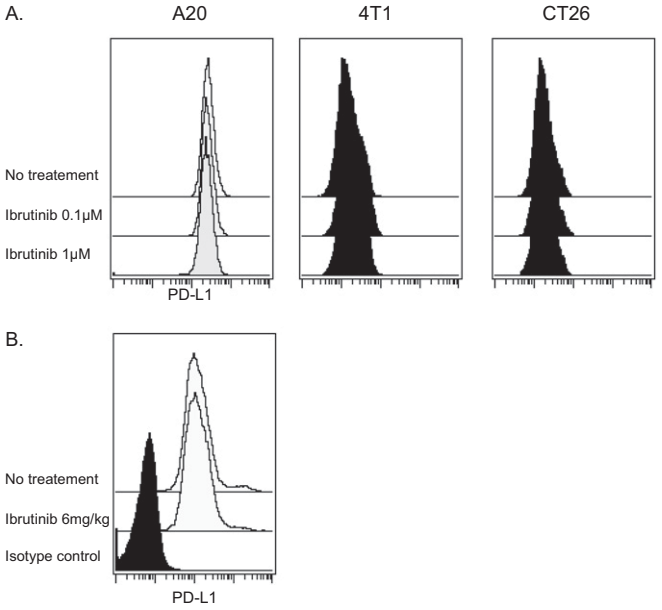

Fig. S5. Ibrutinib does not affect PD-L1 surface levels on tumor cells. (*A*) FACS plots for PD-L1 levels of surface expression in cell lines following 12h treatment with ibrutinib. (*B*) Tumors were extracted from treated mice 1 h following treatment and stained for their surface expression levels of PD-L1.

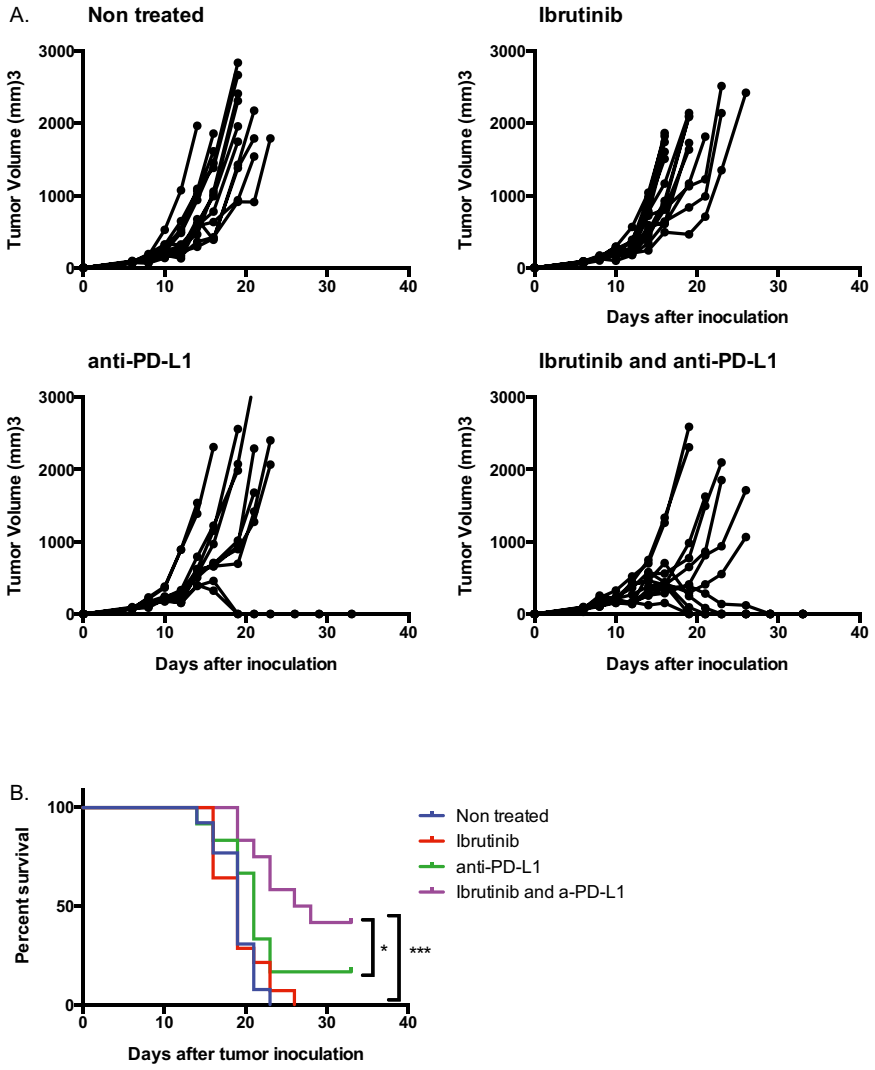

Fig. S6. The combination of ibrutinib with anti–PD-L1 improves survival and cures J558 tumor bearing mice. BALB/C mice were inoculated with 5 × 106 J558 cells s.c. on the right hind thigh, tumor growth was monitored with a digital caliper and expressed as volume (D x d^2 × 0.4). Ibrutinib was given by oral gavage daily on day 12–20 after tumor inoculation at a dose of 24 mg/kg body weight and anti–PD-L1 (200 μg) antibody was IP injected every other day. (*A*) Tumor growth curves. (*B*) Kaplan–Meier survival plots of the treated mice.

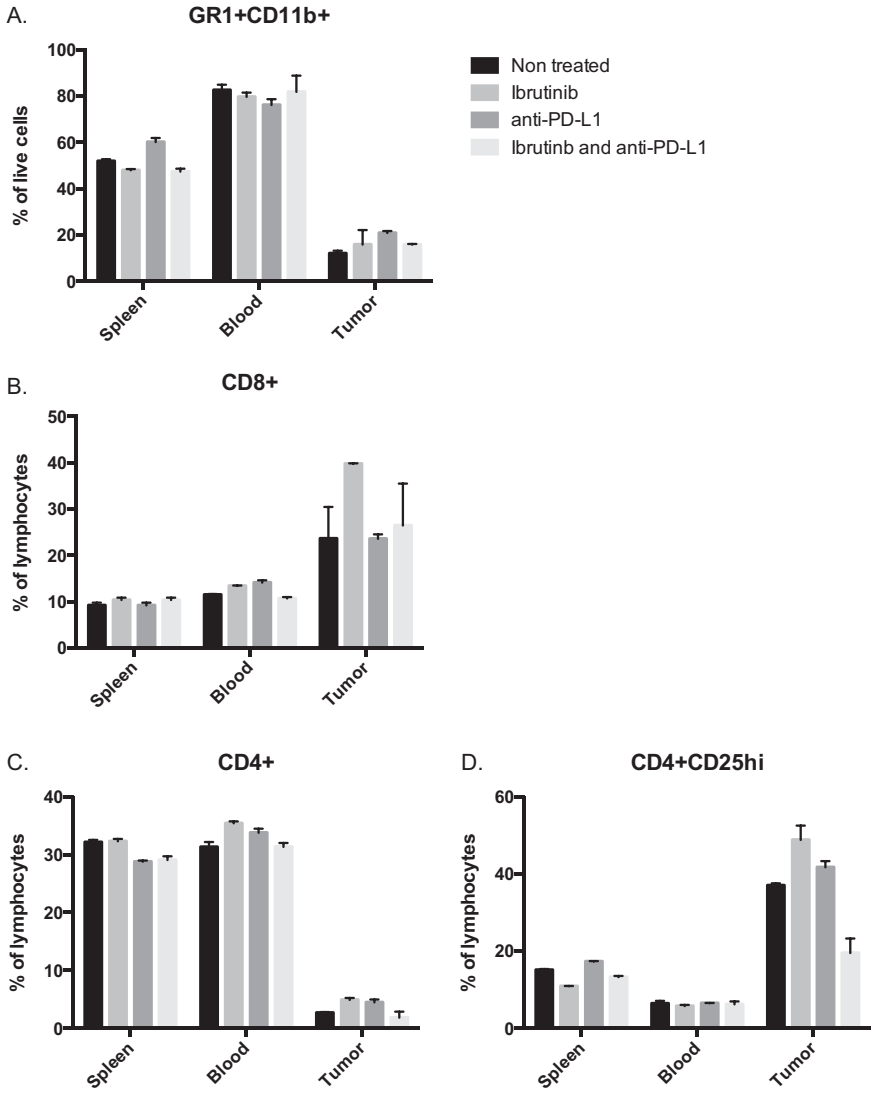

Fig. S7. Spleen, blood and tumor were collected from 4T1-Luc-tumor bearing mice on the seventh day of treatment. Samples were stained with the corresponding antibodies and were subjected to FACS analysis. (*A*) MDSCs are presented as the GR1+CD11b+ cells. CD8+ (*B*), CD4+ (*C*), and the CD25+ (*D*) population of the CD4+ subtype are stained with the corresponding antibodies. Results are presented as percentages of positive cells for the indicated marker.

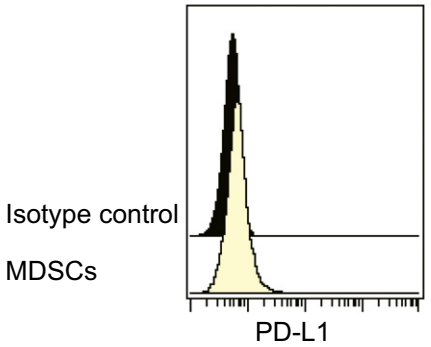

Fig. S8. PD-L1 expression on MDSCs from mice bearing 4T1-Luc tumors. MDSCs from blood of mice bearing 4T1-Luc tumors were stained for PD-L1 expression. MDSCs PD-L1 surface staining is shown in comparison with rat IgG2a isotype control.