Supplementary Material for BET inhibition modifies melanoma infiltrating T cells and enhances response to PD-L1 blockade

Supplemental Materials and Methods

Mice and tumor generation

Male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed in pathogen-free conditions in the Thomas Jefferson University animal facility. Melanoma tumors were generated in male C57BL/6 mice by subcutaneous injection of YUMM1.7 cells into flanks of healthy hosts. Male hosts were used since YUMM cells were derived from male mice and female hosts can reject the tumor due to foreign proteins encoded by the Y-chromosome. Age-matched mice that were housed in the same room of the same facility were used for each experiment. Tumors were measured by caliper and volumes were calculated based on the (length X (Width^2)) X 0.52 equation. All animal experiments were performed according to the approved protocols of the Thomas Jefferson University Institutional Animal Care and Use Committee.

Tumor digestion

Tumors for flow cytometry were minced using gentleMACS™ Octo Dissociator (Miltenyi Biotec, Cambridge, MA) in HBSS with 0.5 mg/ml Collagenase A and 30 mg/ml DNase I, digested in a 37°C incubator for 30 min, minced again and filtered to remove debris. The filtrate was washed, and re-suspended in complete RPMI.

Flow cytometry and cell sorting

Single-cell suspensions of tumor cells or splenocytes were prepared. Cells ($1–2 \times 10^6$) were incubated in 96-well plates in PBS with fixable viability dye Zombie Aqua™ (BioLegend, San Diego, CA) and subsequently stained in FACS buffer with combinations of the following antibodies and reagents: FITC-conjugated anti-CD4 (BioLegend, San Diego, CA, clone RM4.4), PE-conjugated anti-CD8 (BioLegend, San Diego, CA, clone 53-6.7), PE-Cyanine5.5-conjugated anti-CD3 (eBioscience, Waltham, MA, clone 145-2C11), BV421-, PE-Cy7-, or AF-700-conjugated anti-CD45.2 (Biolegend and eBioscience, clone 104), APC-eFluor 780-conjugated anti-PD1 (eBioscience, Waltham, MA, clone J43), biotin-conjugated anti-PDL1 (Biolegend, San Diego, CA, clone 10F.9G2). For nuclear FoxP3 staining, cells were fixed and permeabilized using Transcription Factor Staining Buffer Set (eBioscience, Waltham, MA) and stained with APC-conjugated anti-FoxP3 (eBioscience, Waltham, MA, clone FJK-16s). Biotin conjugates were detected using streptavidin-conjugated FITC (Biolegend, San Diego, CA). All samples were acquired on flow cytometers (LSRII and LSRFortessa; BD Biosciences, San Jose, CA) and analyzed with FlowJo (Tree Star, Ashland, OR). For sorting, tumor cells were suspended in MACS buffer and twice sorted for tdTomato-positive cells using Aria II cell sorter.

***In vivo* treatments**

BET inhibition was accomplished using the PLX51107-laced chow (108 ppm) provided by Plexxikon Inc (Berkeley, CA). For PD-L1 blockade, mice were treated with 400 μg of anti-PD-L1 (clone 10F.9G2) by intraperitoneal injection on the first day of PLX51107 treatment, followed by an additional 200 μg anti-PD-L1 given every third day for a total of six treatments. Control animals were treated with the IgG2b isotype (clone LTF-2),

following the same schedule. All antibodies were purchased from Bio-X-Cell (West Lebanon, NH).

Lentivirus preparation and infection

YUMM1.7 tdTomato cell lines were created by culturing YUMM1.7s with a lentivirus (CMV:tdTomato, provided by Invitrogen, San Jose, CA) for 3 days. Subsequently, tdTomato expressing YUMM1.7 cells were selected via cell sorting to achieve a purity of >95%.

Cell culture

YUMM1.7, YUMM1.1, YUMM4.1, YUMM3.3, and YUMM1.7ER cells (donated by Dr. Marcus Bosenberg, Yale University, 2014-2017) were cultured in DMEM/F12 media containing 10% Fetal Bovine Serum (FBS) and supplemented with 1% penicillin–streptomycin (PenStrep) and 1% nonessential amino acids. D4M3.A cells (donated by Dr. Constance E. Brinckerhoff, Dartmouth University, 2016) were cultured in DMEM/F12 media containing 5% FBS, 1% PenStrep, and 1% L-Glutamine. All cells were grown at 37°C in a humidified incubator supplemented with 5% CO_2. All Cell lines were treated with plasmosin for 2 weeks before beginning experiments to clear possible mycobacterial contamination. Cells were routinely assayed for mycoplasma contamination with MycoScope Kit (Genlantis, San Diego, CA). Cell line authentication via short tandem repeat (STR) analyses were completed in 2015 and 2016.

Statistics

Unless noted otherwise, significant values were considered to have $P \leq 0.05$ as determined by a two-tailed Student t test assuming unequal variance and error bars are ± SEM.

For analysis of tumor growth, repeated over time log-transformed tumor volumes were modeled as a low order polynomial function of day using a linear mixed effects (LME) model adjusting for the random effects of animal. The fixed effects in the LME model included the treatment group, Day, Day^2, and Day^3, as well as the interaction between treatment group and Day, Day^2, and Day^3. That is, the time-dependent trends were modeled as 3th degree (cubic) polynomials in Day. The random animal effects included only intercept, linear and quadratic coefficients. The residuals were evaluated to validate the assumptions of the models. Based on the fitted LME model, the overall comparisons of treatment groups were performed in terms of the growth rates (testing the global null hypotheses that all coefficients in the polynomial function of day are equal for the compared treatment groups).

Western Blotting

Cells were washed twice in cold PBS and lysed with Laemmli sample buffer with β-mercaptoethanol. Proteins were resolved by SDS-PAGE, and proteins were transferred to PVDF membranes. After blocking in 5% BSA, membranes were incubated with the indicated primary antibodies overnight at 4°C, followed by incubation with peroxidase-coupled secondary antibodies. Immunoreactivity was detected using HRP-conjugated secondary antibodies (CalBioTech, Spring Valley, CA) and chemiluminescence substrate (Thermo Scientific, Rockford, IL) on the Versadoc imaging system (Bio-Rad). The primary antibodies used were as follows: BRD3 (#61489, 1:1000) from Active Motif (Carlsbad, CA) and BRD2 (#5848, 1:1000), BRD4 (#13440, 1:500) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (#2118, 1:2000) from Cell Signaling Technology (Danvers, MA).

Blood levels of PLX51107

Blood (100 μl) was collected via retro-orbital route on two separate time points on day 8 and day 30. Plasma was separated immediately by centrifugation at 1000 RCF for 10 minutes and stored at -80°C. Analyses of PLX51107 levels in plasma samples were carried out by Integrated Analytical Solutions (Berkeley, CA) using LC-MS/MS.

***In vitro* IFN-γ treatment**

YUMM 1.7 cells were cultured in DMEM/F12 media containing 10% FBS and supplemented with 1% penicillin–streptomycin and 1% nonessential amino acids. When indicated, 10 ng/ml of recombinant mouse IFN-γ (Thermo Fisher, San Jose, CA) was added to culture medium.

Supplemental Figures

Figure S1

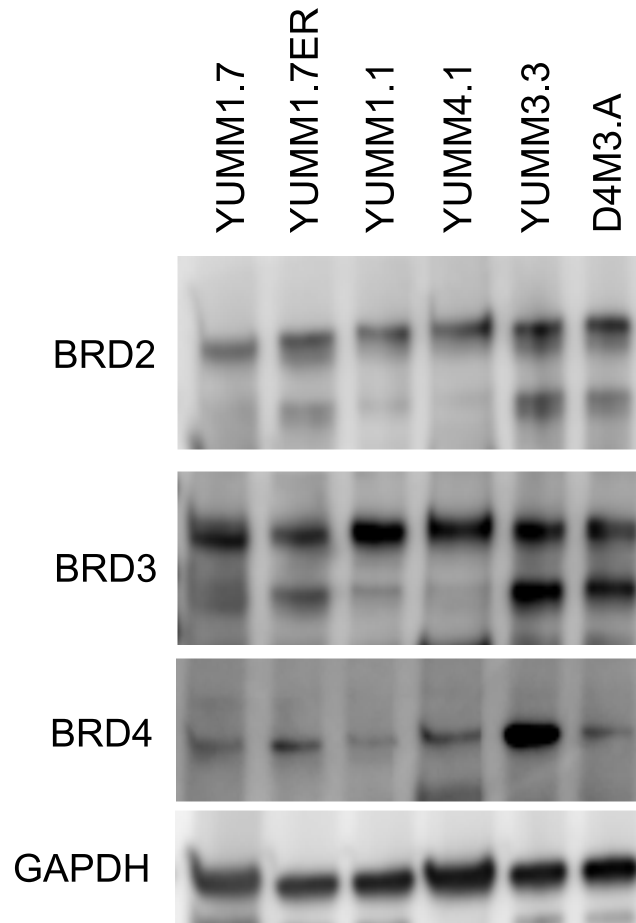

Figure S1: BRD/BET proteins expression in mouse melanoma panel.

Western blot for BRD2, BRD3 and BRD4 for the mouse melanoma cell lines expressing mutant BRAF: YUMM1.7, YUMMER1.7, YUMM1.1, YUMM3.3, and D4M3.A. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level was used as loading control.

Figure S2

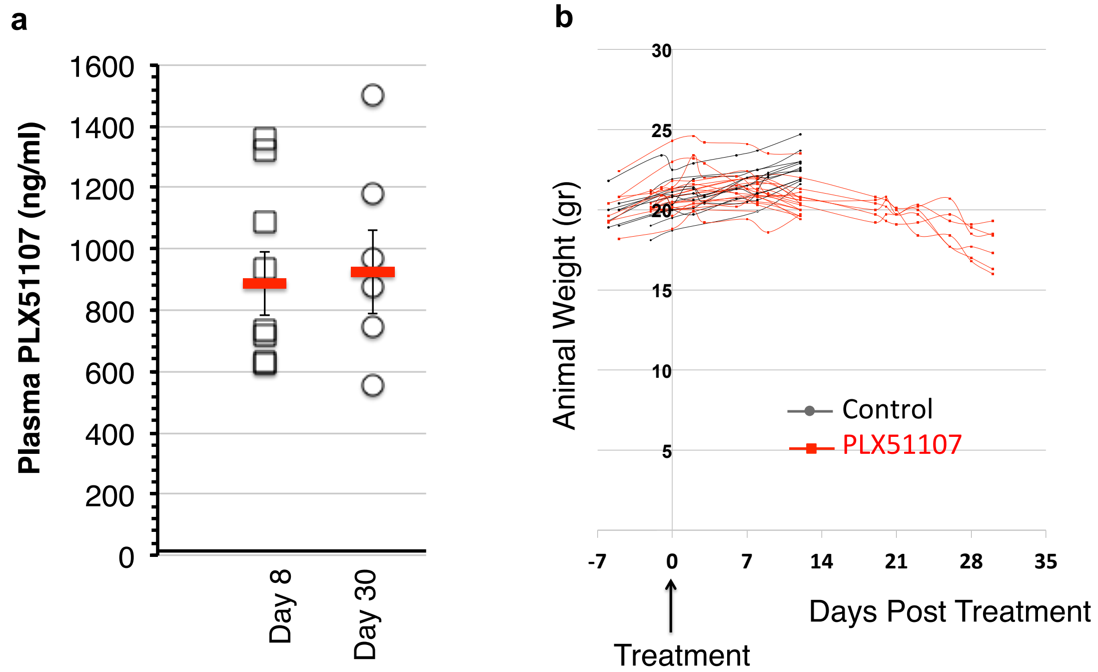

Figure S2: PLX51107 blood levels and animal weight monitoring during treatment. C57BL/6 mice were fed PLX51107-laced mouse chow for the indicated durations. (a) Serum PLX51107 levels were measured by ELISA in treated mice 8 and 30 days after initiating treatment. (b) Animal weight was monitored during the treatment period.

Figure S3

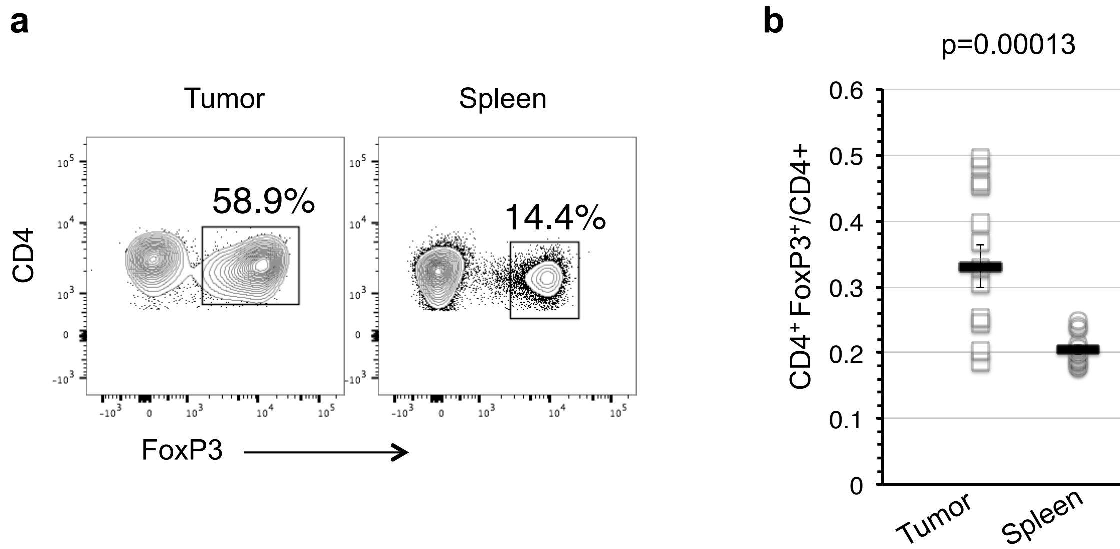

Figure S3: YUMM1.7 tumors enrichment for regulatory T cells.

YUMM1.7 cells were used to generate murine melanoma tumors in male C57BL/6 hosts. 250,000 cells were injected into right flanks of recipients. When tumor sizes reached 1500-2000 mm^3, mice were sacrificed. Spleens and tumors were isolated, homogenized, and stained with appropriate antibodies and analyzed by flow cytometry. Tregs were identified in tumors and spleens as $CD4^+$, $FoxP3^+$ cells and their frequencies within the total number of $CD4^+$ cells were compared in tumor and spleen. (a) Representative gating strategies and (b) overall distribution of Tregs in tumors and spleens are depicted ($p=0.00013$).

Figure S4

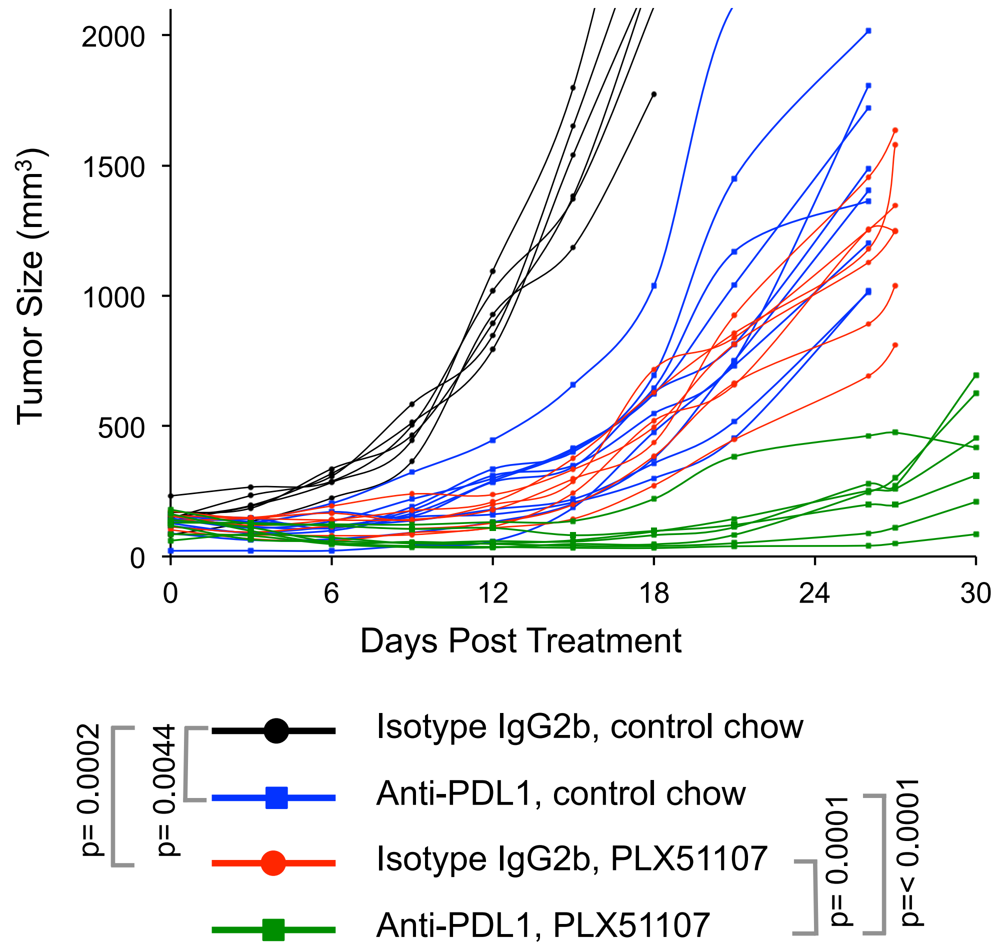

Figure S4: Effect of combined BET inhibition and anti-PD-L1 on YUMM1.7 tumor growth.

YUMM1.7 murine melanoma tumors were generated in C57BL/6 hosts. When tumor sizes reached approximately 50 mm^3, recipients were placed on either control chow or PLX51107-laced chow while receiving either 400 µg followed by 6 more intraperitoneal injections of 200 µg anti-PD-L1 antibody or isotype control. Tumor volumes were monitored at the indicated time points. Each line corresponds to an individual mouse.

Figure S5

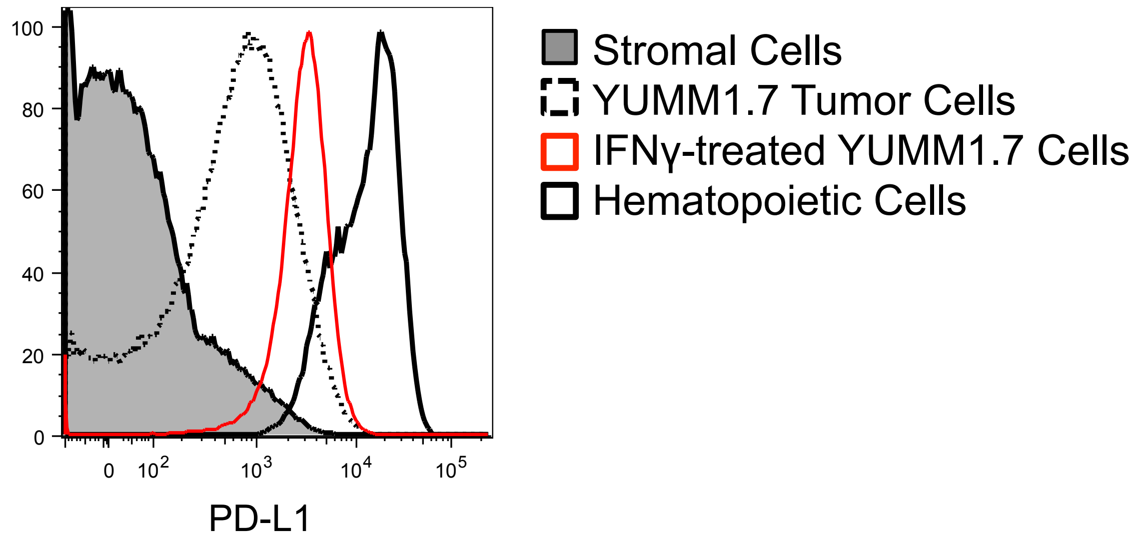

Figure S5: PD-L1 expression on YUMM1.7 cells in tumors and in IFNγ-treated cultures.

Tumors were generated by tdTtomato-expressing YUMM1.7 cells, and subsequently homogenized and stained, as described in Figure S2 legend. Three distinct populations within the tumor were resolved based on CD45.2 and tdTomato expression as described in Figure 3 legend. YUMM1.7 cells were kept in IFNγ supplemented cultures (10 ng/ml) for 3 days and analyzed along with the 3 populations described for surface PD-L1 expression.