Supplemental Information

Targeting EZH2 Reprograms
Intratumoral Regulatory T Cells
to Enhance Cancer Immunity

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

**Cell culture.** C57BL/6 derived MC38 colon adenocarcinoma expressing luciferase and GFP, B16F10 melanoma cells expressing GFP, and TRAMPC2 cells were maintained in DMEM with Glutamax, penicillin, streptomycin, and 10% BSA (B16F10), RPMI with Glutamax, penicillin, streptomycin, and 10% BSA (MC38), or DMEM with penicillin, streptomycin, bovine insulin, dehydroisoandrosterone, Nu-Serum IV, and BSA (TRAMPC2). Treatment of cells with CPI-1205 prepared in DMSO was performed at indicated concentrations and durations. RNA was isolated by TRIzol homogenization and extraction (Invitrogen).

**Chemokine expression.** Supernatants of tumor cell lines were harvested after >6 days of treatment with CPI-1205 and bead-based flow cytometry chemokine assays were performed using the LEGENDplex immunoassay for CXCL9 and CXCL10 as instructed (BioLegend). For qPCR, RNA was converted to cDNA using SuperScript III First-Strand Synthesis (Invitrogen), and measured using Taqman probes (Life Technologies) CXCL9 (Mm00434946) and CXCL10 (Mm00445235) normalized to HPRT (Mm01324427) in Taqman Fast Universal mix. Samples were run in triplicate on the Applied Biosystems 7500/7900 Fast Real-Time PCR System.

**In vivo cell depletion.** Depletion of CD4+ and CD8+ T cells was achieved by intraperitoneal injection of 250 µg per mouse GK1.5 (anti-CD4) and YST-169.4 (anti-CD8) monoclonal antibodies (BioXcell, West Lebanon), 6 days after tumor inoculation and twice weekly thereafter.

**In vitro T cell culture and RNA isolation.** T cells were activated with anti-CD3 and anti-CD28 coated beads (Dynabeads Mouse T-Activator CD3/CD28, Invitrogen) at a ratio of 1:1 (cell:bead) and kept at a concentration of 10⁶ cells/ml for 96 hours in DMEM medium supplemented with 10% FBS, non-essential amino acids, sodium pyruvate, L-glutamine, HEPES, β-ME and 2,000 IU/ml recombinant human IL-2 (Chiron Corp). RNA was isolated by TRIzol homogenization and extraction (Invitrogen).

**RNA sequencing.** Pre-processed and normalized RNA-seq datasets for primary human breast cancer were analyzed comparing Ezh2 expression in T effector and T regulatory cells isolated from normal tissues and tumor (Plitas et al., 2016). For murine T cell analysis, RNA sequencing libraries were generated using the Illumina TruSeq stranded mRNA kit, according to the manufacturer’s protocol (Illumina). Library fragment size distributions were assessed using the Bioanalyzer 2100 and the DNA high-sensitivity chip. Sequencing libraries were tested for sequencing efficiency on a MiSeq, and subsequently pooled for sequencing on a HiSeq 4000 (Illumina). Sequence alignment and splice junction estimation was performed using STAR (doi:10.1093/bioinformatics/bts635). Mappings were restricted to those that were uniquely assigned to the mouse genome, as provided by Ensembl (GRCm38.78) and aggregated on a per-gene basis.

**Gene Set Enrichment Analysis.** Gene set enrichment analysis (GSEA) was performed using the BROAD javaGSEA standalone version (http://www.broadinstitute.org/gsea/downloads.jsp) with the default setting of 1,000 permutations, gene-set permutation mode, and the curated hallmark gene set collection (BROAD molecular signature database, MSigDbv5.1).
Figure S1. No impact on tumor growth or chemokine production with EZH2 inhibition in vitro, related to Figures 1 and 2

(A) Number of MC38 cells over time grown in vitro with varying concentrations of the EZH2 inhibitor CPI-1205 or no inhibitor (NI). Growth of cells was not affected at any dose of the EZH2 inhibitor.

(B) Histogram plots of staining for the levels of H3K27me3, H3 (total), and EZH2 with varying concentrations of the EZH2 inhibitor CPI-1205 or no inhibitor (NI). Only the levels of H3K27me3 are reduced in a dose-dependent manner with increasing amounts of the EZH2 inhibitor.

(C) Plot of EZH2 inhibitor dosage versus mean fluorescent intensity (MFI) of H3K27me3 staining by flow cytometry in (B) shows dose-dependent reduction in H3K27me3 in MC38 tumor cells.

(D) CXCL10 and CXCL9 protein in the supernatants of MC38 tumor cells treated in vitro >6 days with indicated concentrations [μM] of CPI-1205, or untreated (no inhibitor, NI). CXCL9 was below the level of detection of the assay (<3.09 pg/mL), therefore not detected (ND). Data is mean ± SD of technical duplicates.

(E) Relative mRNA expression of CXCL10 with indicated concentrations [μM] of CPI-1205 compared to untreated. CXCL9 not shown as all C_T values were >35 cycles and thus below accurate detection. Data is mean ± SD of technical triplicates.

(F) Anti-EZH2 monoclonal antibody (D2C9) is specific by flow cytometry. Tregs sorted from wild type or FP3;Ezh2fl/fl mice were compared for staining intensity with anti-EZH2 (D2C9).

(G) Tumor-infiltrating (TI) Tregs and Teff in human melanoma are antigen experienced by comparison of CD44 staining.
Figure S2. Ezh2 deficiency in Tregs promotes enhanced CD4 and CD8 immunity, which is required for tumor rejection, related to Figure 3

(A) Spider plots of syngeneic tumor models in control or FP3;Ezh2^{fl/fl} mice.

(B) Top, number of B16F10 cells over time grown in vitro with varying concentrations of the EZH2 inhibitor CPI-1205 or no inhibitor (NI) and plot of EZH2 inhibitor dosage versus mean fluorescent intensity (MFI) of H3K27me3 staining by flow cytometry in B16F10 cells. Bottom, fraction of mice that rejected B16F10 tumors of total implanted in immunocompetent mice (left) and immunocompromised (Rag-1^-/-) mice (right) treated or untreated with EZH2 inhibitor (CPI-1205). Immunocompetent mice were treated at day -2 with B16-GVAX as in Figure 3C.

(C) Comparison of FP3;Ezh2^{fl/fl} and Ezh2^{fl/fl} controls shown here revealed no difference in immune cell frequencies between these groups.

(D) Absolute number of infiltrating CD8^+ T (top) or Treg (bottom) cells per milligram of MC38 tumor mass. Absolute numbers of cells correlates with frequency of T cells of CD45^+ cells in Figs. 2d, 2e, 2i.

(E) Frequency of MC38 tumor rejection with systemic depletion of CD4^+ or CD8^+ T cells. Twice weekly intraperitoneal treatments with 250 μg of depleting antibody was started 6 days after tumor inoculation.

Data represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 from unpaired t tests.
Figure S3. Disruption of EZH2 function in Tregs induces an inflammatory phenotype, related to Figure 5

(A) Representative flow cytometry plots of intracellular TNFα and IL-10 in Foxp3+ Tregs isolated from MC38 tumors in control and FP3;Ezh2fl/fl mice. Inset numbers represent the percentage of cells of total Foxp3+ Treg population.

(B-C) From RNA sequencing analysis of Ezh2-deficient versus wild type Tregs (sort purified CD62Lhi) after four days of in vitro activation with anti-CD3 and anti-CD28.

(B) GSEA plots of differential gene expression between Ezh2-deficient Tregs and wild type Tregs.

(C) Normalized read counts of key immunosuppressive and pro-inflammatory genes from RNA sequencing analysis. Data represent mean ± SEM, *p<0.05 and **p<0.01 from unpaired t tests.
Figure S4. Temporal disruption of EZH2 in Tregs, related to Figure 6

(A) Representative flow cytometric staining for H3K27me3 levels in RFP (filled histogram) vs. RFP+ (solid line) Tregs from control or FP3-ER;Ezh2fl/Δ mice. Tissues were obtained from mice following 12 days on tamoxifen. Quantification of data from lymph nodes from three experiments with 2-5 mice per experiment is shown by comparing the relative MFI of RFP+ compared to RFP− Tregs.

(B) Percentage of Foxp3+ Tregs exhibiting Cre activity (measured by RFP+ cells) after 2 weeks on tam in lymph node or tissues (lung).

(C) Frequencies of RFP+ Treg cells in lymph nodes and lung tissues of control or FP3-ER;Ezh2fl/Δ mice on at least 12 days of continuous tamoxifen treatment (On Tam) and 15-21 days following discontinuation of treatment (Off Tam). Only Tregs from FP3-ER;Ezh2fl/Δ mice exhibit a decline in RFP+ Tregs in tissues.

(D) Representative flow cytometric staining for H3K27me3 and total H3 protein in Treg cells (sort purified from control or FP3-ER;Ezh2fl/Δ mice) five days after stimulation in vitro with anti-CD3/anti-CD28 beads in continuous culture with or without 4-hydroxytamoxifen (500 nM). Data represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 from unpaired t tests.
Figure S5. Temporal disruption of Ezh2 in Tregs enhances effector T cell frequency and function, related to Figure 6

(A-C) Related to data presented in Figure 6A.

(A) Spider plots of TRAMPC2 and MC38 tumor growth in control or FP3-ER;Ezh2<sup>fl/+</sup> mice treated with tam from time of tumor inoculation throughout experiment (TRAMPC2) or beginning 7 days after tumor inoculation throughout the experiment (MC38).

(B-C) Percentage of CD8<sup>+</sup> T cells of viable CD45<sup>+</sup> immune cells, Foxp3<sup>+</sup> Tregs of CD4<sup>+</sup> T cells, and ratio of CD8/Treg cells in tumor draining lymph node (dLN) and tumors in TRAMPC2 (B) and MC38 (C) tumor models from (A).

(D-E) Related to data presented in Figure 6D. Quantification of IFN-γ production from CD8<sup>+</sup> (D) and eCD4<sup>+</sup> (E) T cells obtained from indicated tissues after 11 days of tamoxifen treatment in control or FP3-ER;Ezh2<sup>fl/+</sup> mice.

(F) Representative flow cytometry plots of intracellular TNFα and IL-10 in Foxp3<sup>+</sup> Tregs isolated from MC38 tumors in control and FP3-ER;Ezh2<sup>fl/+</sup> mice. Inset numbers represent the percentage of cells of total Foxp3<sup>+</sup> Treg population.

Data represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 from unpaired t tests.
Figure S6. Ezh2 deficiency in Tregs is distinct from Treg depletion in promoting anti-tumor immunity, related to Figure 7

(A) Spider plots of MC38 tumors in chimeric female mice depicted in Figure 7H. DT was administered on days -2, 0, 2, 4 after tumor inoculation.

(B) CD8$^+$ T cell frequency of lymphocyte FSC/SSC gate in peripheral blood of chimeric female mice receiving diphtheria toxin in (A). Peripheral blood was analyzed on day 21 after tumor inoculation.

(C) Representative photographs of chimeric female mice of indicated genotypes following treatment with diphtheria toxin.

(D) Cumulative MC38 tumor growth from two independent experiments. Inset numbers represent the total number of mice that rejected tumors of total inoculated from both experiments.

Data represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 from two-way ANOVA with Sidak’s multiple comparisons or by unpaired t tests.