**Supplemental Materials**

**Intra-tumoral STING and Flt3 agonists differ in their capacity to potentiate local versus systemic T cell checkpoint modulation and abscopal immunity**

Casey R. Ager1,2, Matthew J. Reilley3, Courtney Nicholas2, Todd Bartkowiak1,2, Ashvin R. Jaiswal1,2, and Michael A. Curran1,2

1Immunology Program, University of Texas Graduate School of Biomedical Sciences at Houston, Houston, TX, USA

2Department of Immunology, University of Texas MD Anderson Cancer Center, Houston, TX, USA

3Department of Cancer Medicine, University of Texas MD Anderson Cancer Center, Houston, TX, USA

**Table of Contents**

**Supplemental Table S1 –** Flow cytometry antibody catalog

**Supplemental Figure S2** – Individual tumor growth curves; IP antibody + IT agonists

**Supplemental Figure S3** – Individual tumor growth curves; IT antibody + IT agonists

**Supplemental Figure S4** – Tumor relapse rates

**Supplemental Figure S5** – Asymmetric TRAMP-C2 tumor growth kinetics

**Supplemental Figure S6** – TIL flow analysis experimental setup and gating strategy

**Supplemental Figure S7** – TIL flow population quantifications

**Supplemental Table S1 Flow cytometry antibody catalog.** Fluorescently labeled antibodies used for flow cytometric analysis of the TRAMP-C2 tumor microenvironment. Includes antibody target, clone name, corresponding fluorophore, staining concentration, commercial source, and catalog number. The SPAS tetramer was synthesized by Baylor MHC Tetramer Core facility and the Arginase-A594 antibody was conjugated in lab using Alexa 594 dye conjugation kits (ThermoFisher).

**Supplemental Figure S2 Individual tumor growth curves; IP antibody + IT agonists.** Individual tumor growth kinetics at the injected (right) and contralateral (left) flanks of mice treated with systemic (IP) antibody cocktail and/or intratumoral (IT) myeloid agonists. Corresponds to data presented in Fig 1 and Fig 2 of the manuscript.

**Supplemental Figure S3 Individual tumor growth curves; IT antibody + IT agonists.** Individual tumor growth kinetics at the injected (right) and contralateral (left) flanks of mice treated with intratumoral (IT) antibody cocktail and/or IT myeloid agonists. Corresponds to data presented in Fig 3 and Fig 5 of the manuscript.

**Supplemental Figure S4 Tumor relapse rates.** Overall comparison of tumor relapse rates between mice exposed to CDG vs Flt3L treatments. Relapse was defined as any tumor that initially grew to at least 25mm3 in volume that regressed below 25mm3, then relapsed to grow again above 25mm3. (A) Number of relapses across all mice treated with CDG vs Flt3L. (B) Comparison of relapse rate between CDG-treated and Flt3L-treated mice. Relapse rate is defined elapsed time between the day of regression (when initial tumor shrunk below 25mm3) and the day at which the relapsed tumor mass passed the indicated volume. (C) Raw data used to calculate relapse rates. Statistical significance was calculated using Student’s T test**.**

**Supplemental Figure S5 Asymmetric TRAMP-C2 tumor growth kinetics.** (A)To obtain large (~200mm3) right flank tumors for IT injection, mice were implanted on day -14 with 1x106 TRAMP-C2 on the right flank, were implanted on day 0 with 1x106 TRAMP-C2 on the left flank, and treatment occurred on days 14, 18, and 22. (B) Tumor growth kinetics on the injected right flank and uninjected left flank are shown. Due to some variation in tumor size at the right flank during the long 28-day engraftment period, below is shown normalized tumor growth as a percentage of tumor size at day 14.

**Supplemental Fig S6 TIL flow analysis experimental setup and gating strategy.** As described in the treatment schedule, 31-day established subcutaneous TRAMP-C2 were exposed to 3 IT injections 4 days apart, and tumors were harvested for flow analysis 3 days following the final therapy. Sample gating strategy displays all analyzed cell populations from a fully-stained naïve spleen sample. 18-color compensation was performed using single stained spleen controls and the compensation matrix from TreeStar FlowJo ® version 7.6.5, and all gating was performed in FlowJo ® version 10.1.

**Supplemental Figure S7 TIL Flow Population Quantifications.** Cell populations harvested from TRAMP-C2 tumors as described in Supplemental Fig S6 were enumerated using tumor suspension cell counts and relative percentages defined by flow cytometry, and densities were calculated by dividing population cell count by tumor volume. (A) Ratio of CD8 T cells to CD11b+Gr-1+ MDSC. Individual cell populations shown are (B) CD8 cytotoxic T cells (CD45+ CD3+ CD8+), (C) CD4 effector T cells (CD45+ CD3+ CD4+ FoxP3-), (D) CD4 regulatory T cells (CD45+ CD3+ CD4+ FoxP3+), (E) myeloid derived suppressor cells (CD45+ CD3- CD11b+ Gr-1+), (F) dendritic cells (CD45+ CD3- CD11c+ CD11b-), and (G) tumor associated macrophages (CD45+ CD3- CD11b+ F4/80+ Gr-1-). Data is cumulative of 2 independent experiments with 5 mice per group. Statistical significance was calculated using Student’s T test. ns = not significant, \* = p<0.05, \*\* = p<0.01, \*\*\* = p<0.001, \*\*\*\* = p<0.0001.