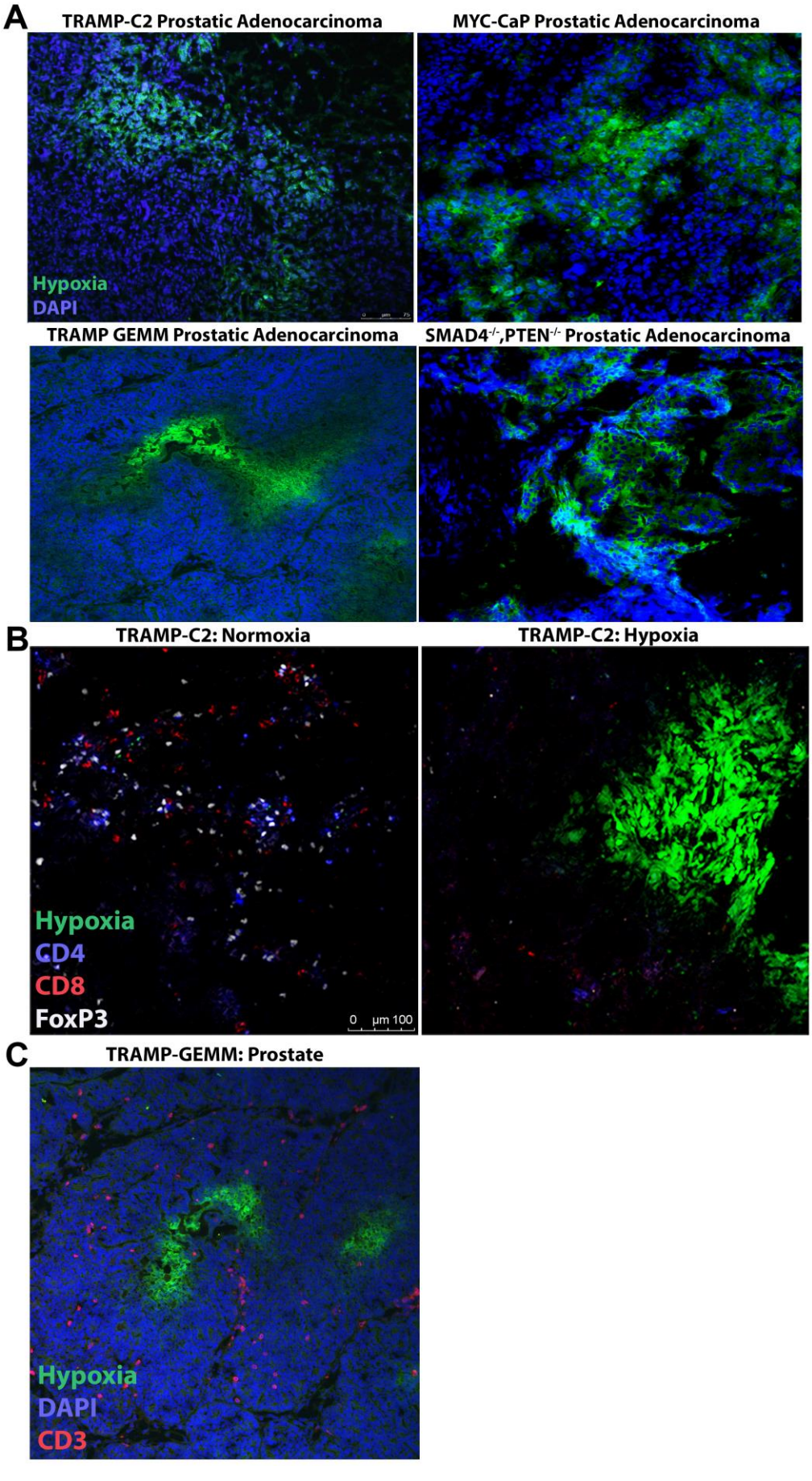
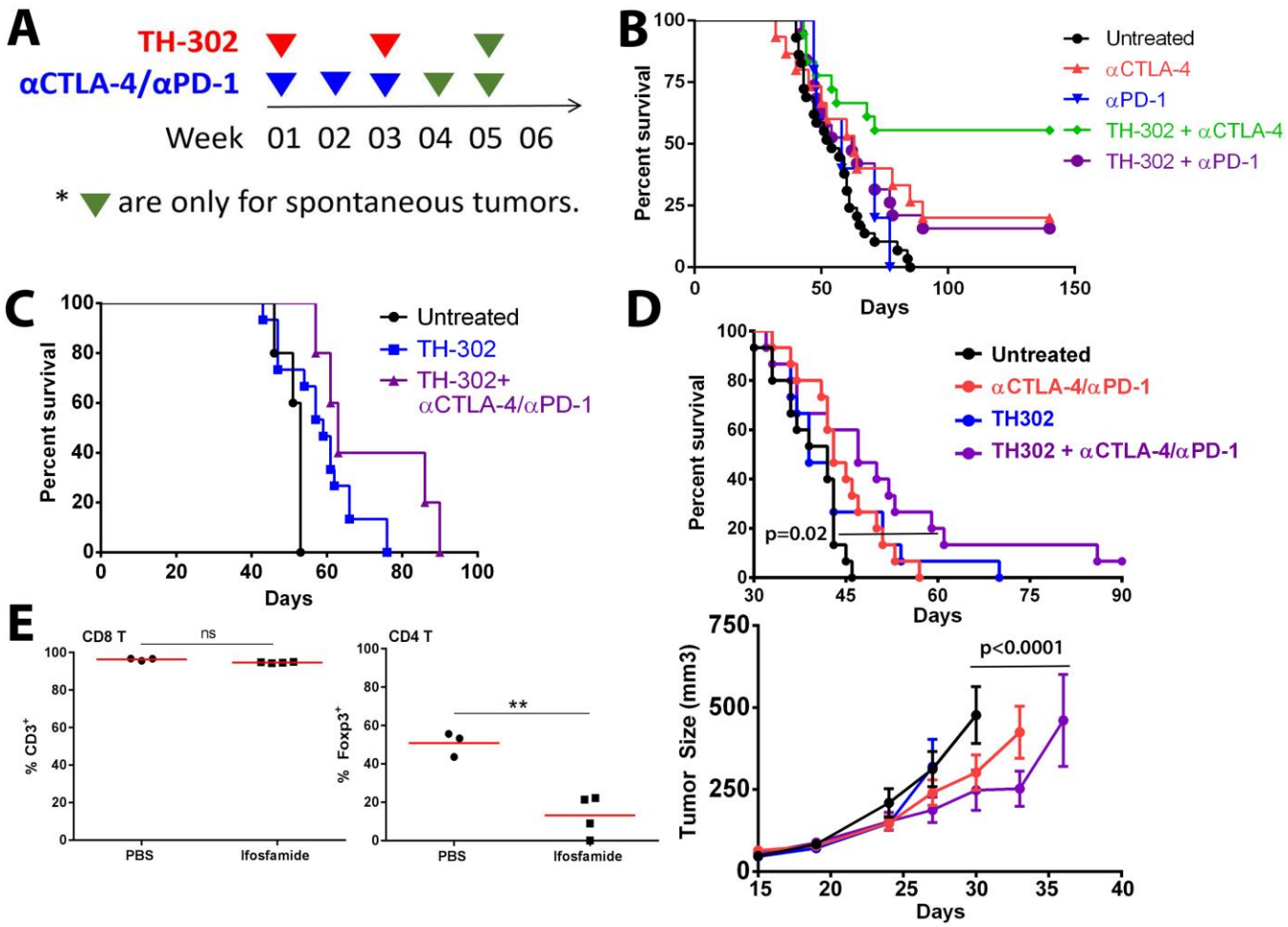
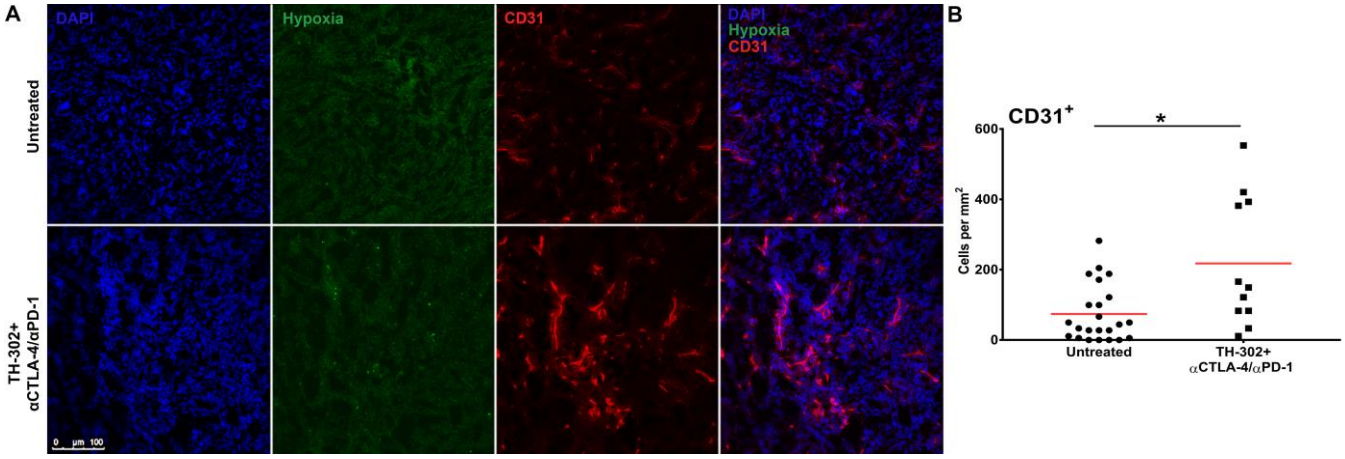
Supplementary Figures:


A
TRAMP-C2 Prostatic Adenocarcinoma
MYC-CaP Prostatic Adenocarcinoma
Hypoxia
DAPI
TRAMP GEMM Prostatic Adenocarcinoma
SMAD4-/-,PTEN-/- Prostatic Adenocarcinoma
B
TRAMP-C2: Normoxia
TRAMP-C2: Hypoxia
Hypoxia
CD4
CD8
FoxP3
0 µm 100
C
TRAMP-GEMM: Prostate
Hypoxia
DAPI
CD3

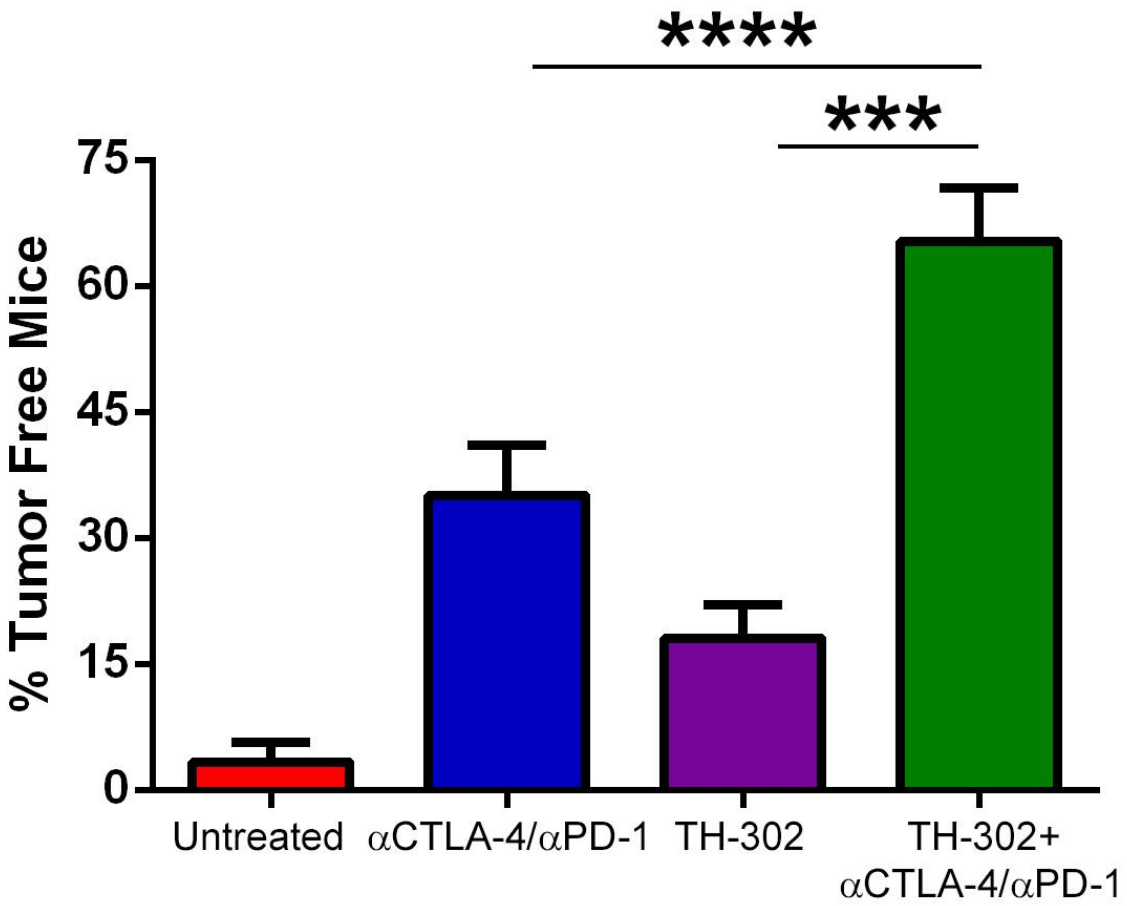
Supplementary Figure 1. *Hypoxic zones of prostate tumors are prevalent and poorly infiltrated by T cells.* (**A**) Immunofluorescence staining of s.c. implanted TRAMP-C2 and MyC-CaP tumors (top), as well as spontaneous prostate tumors from a 36-week old TRAMP mouse and a 26-week old Pb-Cre4, $Pten^{pc-/-}Smad4^{pc-/-}$ mouse (bottom) are shown **(B)** T cell infiltration under normoxia and hypoxia in an untreated TRAMP-C2 tumor and a TRAMP prostate from a 36 week old mouse (**C**) are shown. Pimonidazole was used to detect hypoxia. Hypoxia (green), CD4 (blue), CD8 (Red), and FoxP3 (White) are shown.



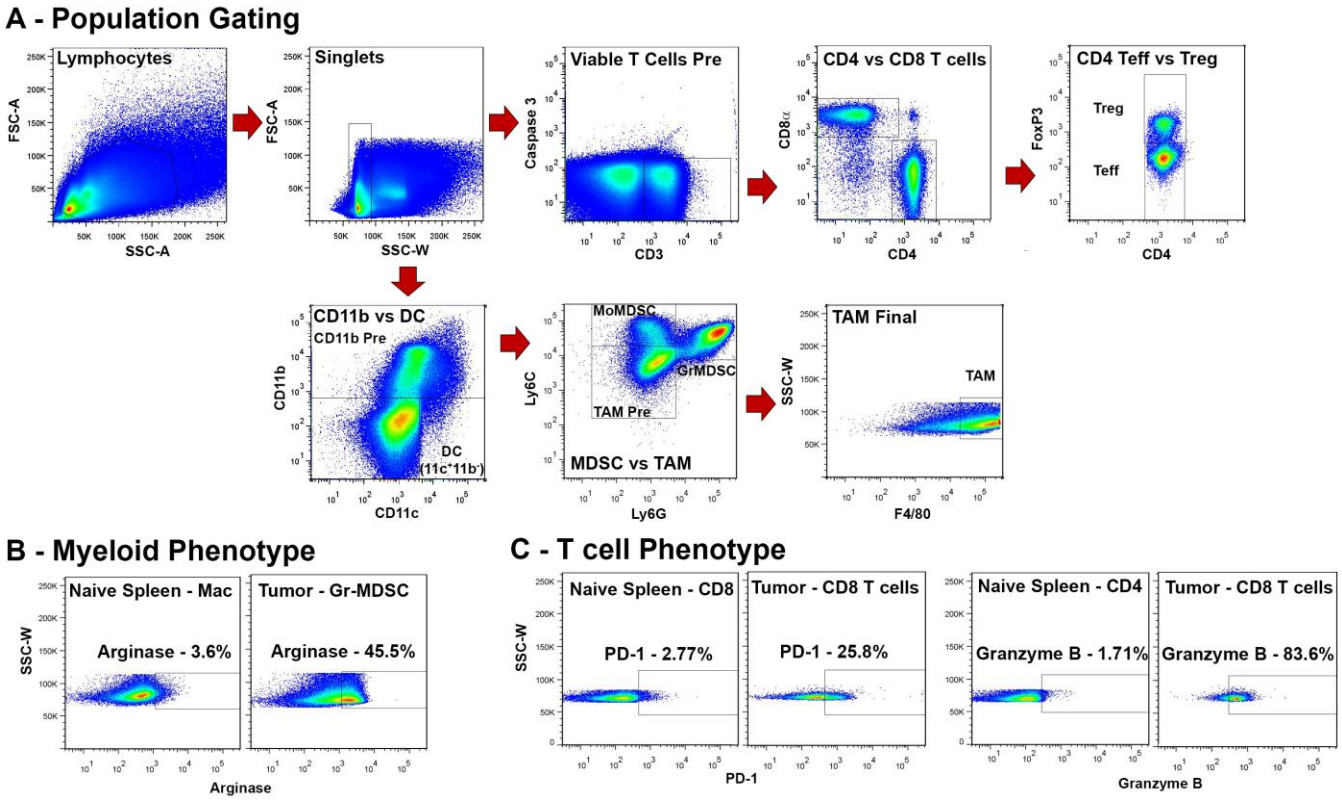
Supplementary Figure 2. *Hypoxia-targeted therapy and checkpoint blockade in the TRAMP-C2 and MyC-CaP models.* **(A)** The treatment schedule for TH-302 and checkpoint blockade is shown in blue and red for transplantable tumors (e.g. TRAMP-C2) with additions in green for spontaneous models (e.g. TRAMP mice). **(B)** C57BL/6J mice bearing 7-day pre-implanted TRAMP-C2 ($1x10^6$) tumors were treated with 2 cycles of TH-302 and/or αCTLA-4 (9H10) or αPD-1 (RMP1-14) antibody and monitored for survival for 140 days (5-10m/group, n=2-3, 5 for untreated). Survival differences were tested using the log-rank (Mantel-Cox) test. **(C)** $B6.RAG^{-/-}$ mice bearing 7-day pre-implanted TRAMP-C2 tumors were treated as in (A) and monitored for survival (5-10m/group, n=1-2). **(D)** FVB/N mice bearing 21-day pre-implanted MyC-CaP ($2x10^5$) tumors were treated with 2 cycles of TH-302 and/or αCTLA-4/αPD-1 antibody and monitored for survival and tumor growth for 90 days (5m/group, n=3). **(E)** OT-1 transgenic mice were treated with Ifosfamide (50 mg/kg) for 1 cycle of therapy. The day after conclusion of therapy, mice were euthanized and their splenocytes activated in the presence of IL-2 and OVA SIINFEKL peptide for 48 hours. Percent of $CD3^+CD8^+$ T cells and $CD3^+CD4^+Foxp3^+$ Tregs are shown (3m/group, n=1).



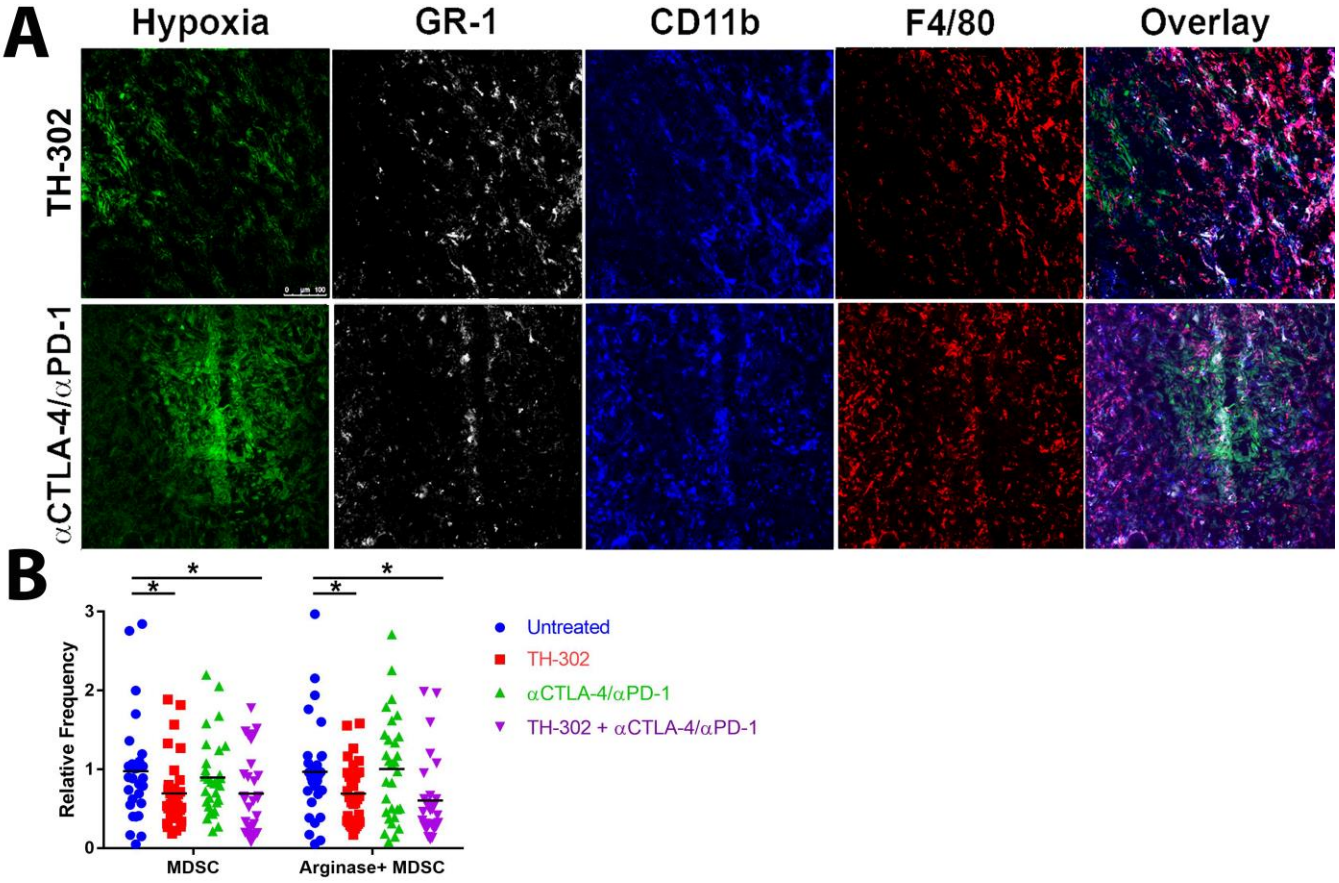
Supplemental Figure 3. *Hypoxia ablation and checkpoint blockade together promote accumulation of CD31⁺ endothelial cells.* (**A**) Mice bearing 14-day pre-implanted TRAMP-C2 tumors were treated with one cycle of TH-302 and antibody therapy. Tumors were isolated, OCT mounted, frozen, sectioned, fixed, and stained for nuclei (DAPI), hypoxia by pimonidazole (FITC), and CD31(Alexa 647). (**B**) Quantification of $CD31^+$ cells per mm^2. Statistical significance between groups was determined by unpaired t test. ns = not significant * = P<0.05, ** = P<0.01, *** = P<0.001, **** = P<0.0001.



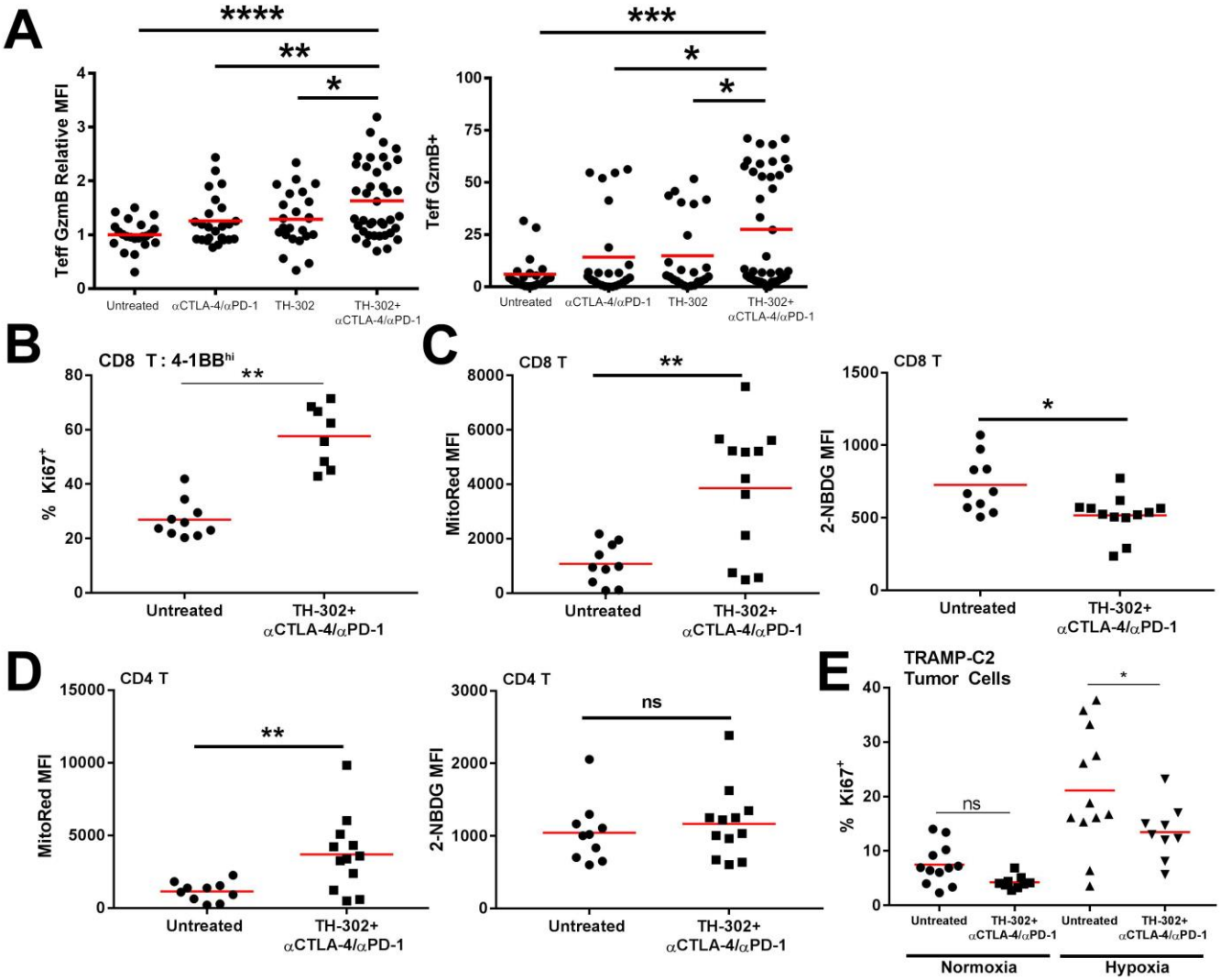
Supplementary Figure 4. *Therapeutic response of two-week pre-implanted TRAMP-C2 tumors to hypoxia-targeted therapy and checkpoint blockade.* C57BL/6J mice bearing 14-day pre-implanted TRAMP-C2 tumors were treated with two cycles of TH-302 and/or dual checkpoint antibody therapy. Percent of mice tumor free following 2 cycles of therapy (Day 32) is shown. Statistical significance between groups was determined by ANOVA. ns = not significant * = P<0.05, ** = P<0.01, *** = P<0.001, **** = P<0.0001.



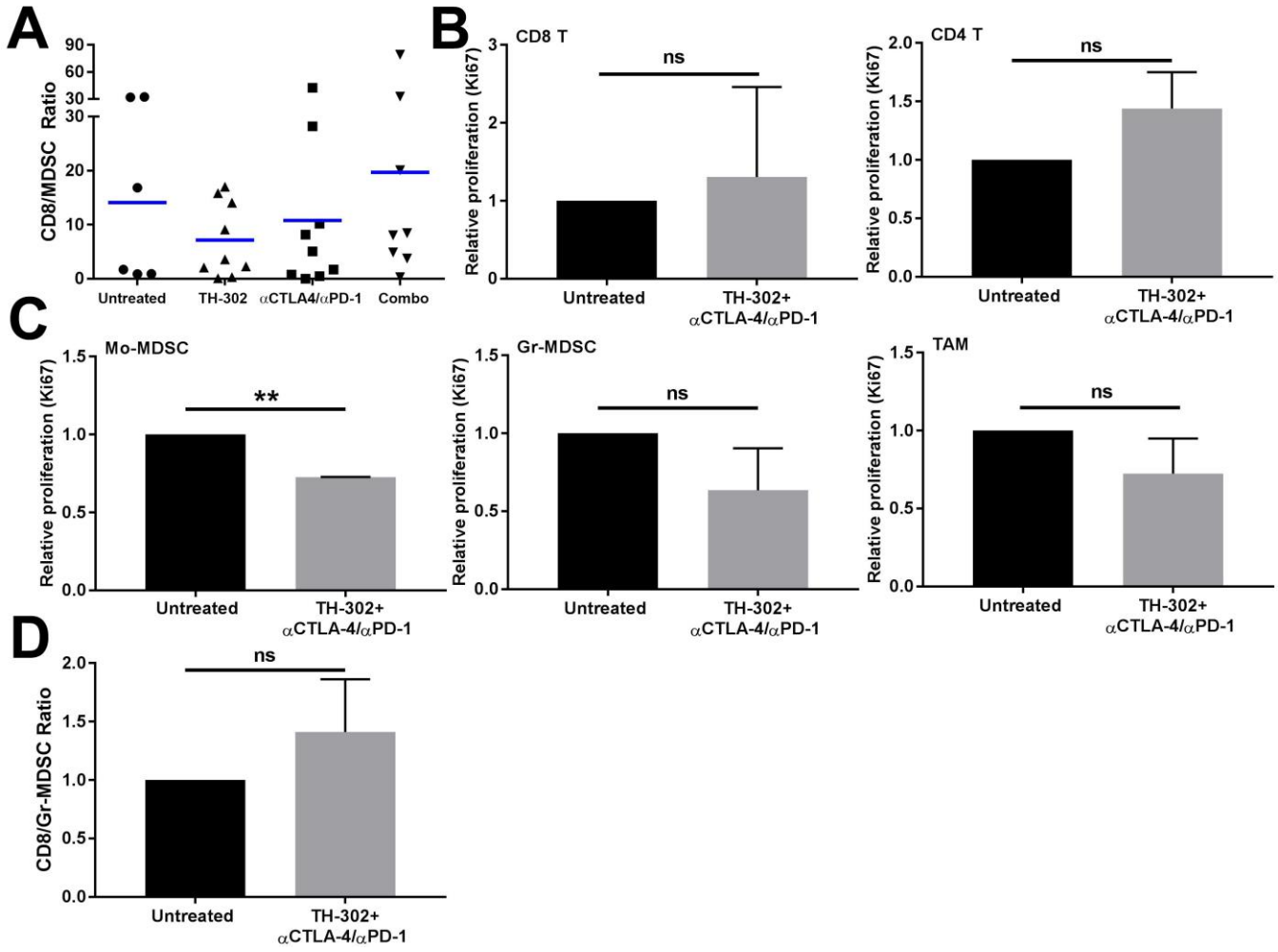
Supplemental Figure 5. *Flow cytometry gating schema and example controls.* (**A**) Debris and doublets/aggregates were removed. Viable $CD3^+$ T cells were further divided into $CD4^+CD8^-$ and $CD8^+CD4^-$ T cells. $CD4^+CD8^-$ T cells were divided into $CD4^+Foxp3^-$ Teffs and $CD4^+Foxp3^+$ Tregs. Mo-MDSC were defined as $CD11b^+Ly6C^+Ly6G^-$, DC as $CD11c^+$ $CD11b^-$, Gr-MDSC as $CD11b^+Ly6C^-Ly6G^+$, and TAM as $CD11b^+Ly6C^{-/low}Ly6G^-F4/80^+$. (**B**) Representative flow plot showing Arginase gating in Gr-MDSC and (**C**) Representative flow plot showing gating of PD-1 and Granzyme-B in CD8 T cells versus control populations from naïve mouse spleen.



Supplemental Figure 6. *Impact of TH-302 or checkpoint antibody therapy on TRAMP-C2 myeloid stroma.* (**A**) 2-week pre-established TRAMP-C2 tumors were treated with TH-302 or checkpoint blockade and then tumors were isolated, fixed, embedded in OCT, sectioned, and stained with anti-pimonidazole (FITC), Gr-1 (V450), CD11b (Alexa 546), and F4/80 (Alexa 647). (**B**) The frequencies of $CD11b^{+}Gr\text{-}1^{+}$ and $CD11b^{+}Gr\text{-}1^{+}Arginase^{+}$ MDSC relative to untreated animals are shown for all individual mice from 5 independent experiments. Statistical significance between groups was determined by ANOVA. ns = not significant * = P<0.05, ** = P<0.01, *** = P<0.001, **** = P<0.0001.



Supplemental Figure 7. *TH-302 in combination with checkpoint blockade augments T cell effector function and diminishesTRAMP-C2 tumor cell proliferation.* (**A**) Mice were implanted with TRAMP-C2 tumors in 30% Matrigel and treated beginning on day 14 for 2 cycles of therapy. One day following therapy, tumor infiltrating lymphocytes were purified and analyzed by flow cytometry. Expression of Granzyme B by CD4 T cells is shown for individual mice from 5 independent experiments. (**B**) $TCR\beta^+CD8^+$ T cell proliferation was assessed by Ki67 staining. (C) Mice bearing 14-day pre-implanted TRAMP-C2 tumors were treated with 1 cycle of TH-302 and αCTLA-4/αPD-1 antibody. The day after conclusion of therapy, mice were injected intravenously with 2-NBDG (Cayman Chemical) 30 minutes before they were sacrificed and tumors were harvested. Flow cytometric analysis of glycolysis and oxidative phosphorylation on CD8 and (**D**) CD4 T cells. (**E**) Proliferation of TRAMP-C2 tumor cells after treatment with TH-302 and checkpoint blockade was assessed by Ki67 staining. Statistical significance between groups was determined by ANOVA for **A** and Student's t-test for **B-E**. ns = not significant * = P<0.05, ** = P<0.01, *** = P<0.001, **** = P<0.0001.



Supplemental Figure 8. *Impact of combination TH-302 and checkpoint blockade on the immune microenvironment of TRAMP transgenic mice.* (**A**) TRAMP mice were treated at 16 weeks with 3 cycles of TH-302 and/or αCTLA-4 (9H10)/αPD-1 (RMP1-14) antibody and euthanized at 36 weeks. The intratumoral ratios of $CD3^+CD8^+$ T cells versus $CD11b^+Gr\text{-}1^+$ MDSC are shown for individual mice from 3 independent experiments of 1-5m/group. Statistical significance between groups was determined by ANOVA. ns = not significant * = P<0.05, ** = P<0.01, *** = P<0.001, **** = P<0.0001. (**B**) 30-week old TRAMP mice were treated with TH-302 (50 mg/kg) for 9 days with αCTLA-4/αPD-1 antibodies administered on days 1,3,4,7 and 9 and tumor-infiltrating cells purified for analysis on day 10. Proliferation of viable tumor-infiltrating $TCR\beta^+CD8^+$ T cells and $TCR\beta^+CD4^+Foxp3^-$ T cells as well as that of (**C**) Mo-MDSC ($CD11b^+Ly6G^-Ly6C^+$), Gr-MDSC ($CD11b^+Ly6G^+Ly6C^-$) and TAM ($CD11b^+Ly\text{-}6G^-Ly6C^{-/low}F4/80^+$) was assessed by Ki67 staining. (3m/group, n=2). (**D**) The intratumoral ratios of $CD3^+CD8^+$ T cells versus Gr-MDSC are shown for the mice in **B**. Statistical significance between groups was determined by Student's t test. ns = not significant * = P<0.05, ** = P<0.01, *** = P<0.001, **** = P<0.0001. Error bars in B-D are mean ± S.D.