Supplementary Information for

Original research: Androgen receptor blockade resistance with enzalutamide in prostate cancer results in immunosuppressive alterations in the tumor immune microenvironment

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Supplementary Information Text

Supplementary Methods

Cell transfection

For small interfering RNA (siRNA) transfection, cells were seeded at a density of 0.5×10^5 cells per well in 12-well plates or 2×105 cells per well in 6-well plates and transfected with 20nM of siRNA targeting the AR exon7 sequence (UCAAGGAACUCGAUCGUAU), AR-V7 sequence (GUAGUUGUGAGUAUCAUGA) or siControl (Invitrogen Catalog# 12935300) using Lipofectamine-iMAX (Invitrogen). The effect of siRNA-mediated gene silencing was examined using qRT-PCR and western blot 2-3 days after transfection. The cells were transiently transfected with plasmids expressing PD-L1 luciferase using Lipofectamine 2000 (Invitrogen).

Western blot analysis

Whole cell protein extracts were resolved on SDS-PAGE and proteins were transferred to nitrocellulose membranes. After blocking for 1 h at room temperature in 5% milk in PBS/0.1% Tween-20, membranes were incubated overnight at 4 ℃ with the indicated primary antibodies AR (441), AR (N-20), 1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA; PD-L1 antibody (13684, 1:1000 dilution, Cell Signaling Technology, Danvers, MA); CXCL-10 antibody (14969, 1:1000 dilution, Cell Signaling Technology); GAPDH antibody (5174, 1:1000 dilution, Cell Signaling Technology); tubulin (T5168, Monoclonal Anti-α-Tubulin antibody, 1:5000 dilution, Sigma-Aldrich, St. Louis, MO). Tubulin was used

as a loading control. Following incubation with secondary antibodies (W401 and W402, 1:5000 dilution, Promega, Madison, WI), immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Millipore, Billerica, MA).

Real-Time quantitative RT-PCR

Total RNA was extracted using a RNeasy Mini Kit (Qiagen). cDNA was prepared after digestion with RNase-free RQ1 DNase (Promega) and subjected to real-time reverse transcription-PCR (RT-PCR) using SsoFast Eva Green Supermix (Bio-Rad) according to the manufacturer's instructions as described previously [1]. Each reaction was normalized by the co-amplification of actin. Triplicate samples were run using the default settings of a Bio-Rad CFX-96 real-time cycler. The primer sequences are in **Table S1**.

Luciferase assay

C4-2B cells were transfected with pGL3-PD-L1-Luc reporters (Add gene Plasmid #107003) along with different concentration of DHT with or without enzalutamide in CS-FBS medium. Cell lysates were subjected to luciferase assays using the Luciferase Assay System (Promega) as described previously [1].

Cell growth assay

Myc-CaP and Myc-CaP MDVR cells were seeded in 12-well plates at a density of 0.2×10^5 cells/well in DMEM containing 10% FBS and treated with different concentration of enzalutamide or apalutamide for 3 days. The total cell numbers were counted.

Clonogenic Assay

Myc-CaP and Myc-CaP MDVR cells were treated with 20 and 40 μM enzalutamide, respectively. Cells were plated at an equal density (400 cells/dish) in 6-well plates for 3 weeks, and the medium was changed every 7 days. The colonies were rinsed with PBS before staining with 0.5% crystal violet/4% formaldehyde for 30 min and the number of colonies was counted.

RNA-seq data analysis

RNA was extracted from the Myc-CaP and Myc-CaP MDVR cells. RNA-seq libraries from 1 μg of total RNA were prepared using the Illumina Tru-Seq RNA Sample, according to the manufacturer's instructions. The mRNA-Seq paired-end library was prepared using Illumina NGS on a HiSeq 4000: 2 ×150 cycles/bases (150bp, PE). Around 30M reads/sample were obtained. Data analysis was performed with a Top Hat-Cufflinks pipeline and sequence read mapping/alignment using HISAT. StringTie data were mapped to and quantified for 54,532 unique genes/transcripts, and gene and transcript expression was quantified as Fragments per kilobase of transcript per million mapped reads (FPKM). Principal component analysis (PCA) was conducted on the FPKM gene-level data for all genes/transcripts passing the filter (filtered on expression > 0.1) in the raw data. The relatedness of the differentially expressed genes between Myc-CaP parental and Myc-CaP MDVR cells was depicted with a heatmap using R.

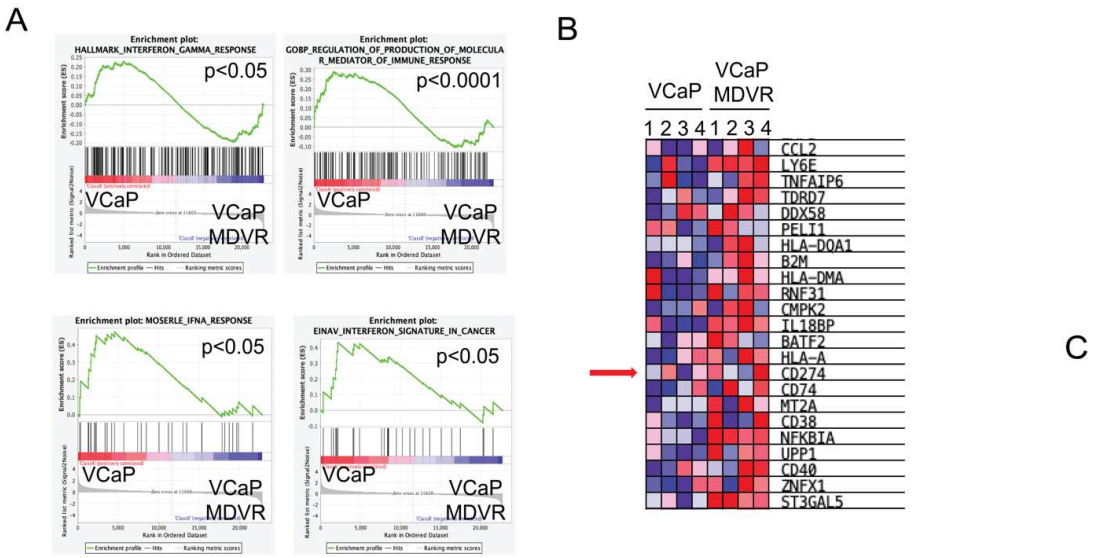
Gene Set Enrichment Analysis (GSEA)

GSEA was performed using Java desktop software (http://software.broadinstitute.org/gsea/index.jsp), as described previously [2]. Genes were

ranked according to the shrunken limma log2 fold changes, and the GSEA tool was used in the 'pre-ranked' mode with all default parameters. Immune-related signaling pathways were used for GSEA analysis.

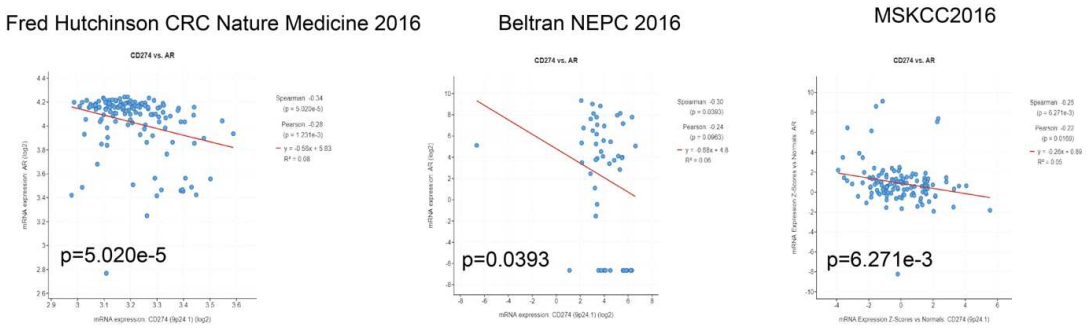
Datasets and Patient cohorts

The Stand Up 2 Cancer/Prostate Cancer Foundation-funded West Coast Prostate Cancer Dream Team (SU2C/PCF) dataset, Memorial Sloan-Kettering Cancer Center (MSKCC), Beltran NEPC, and Fred Hutchinson CRC data set were downloaded from the cBioPortal for Cancer Genomics (www.cbioportal.org). The expression levels of AR, KLK3, NKX3-1, FKBP5, and CD274 were examined and correlated.



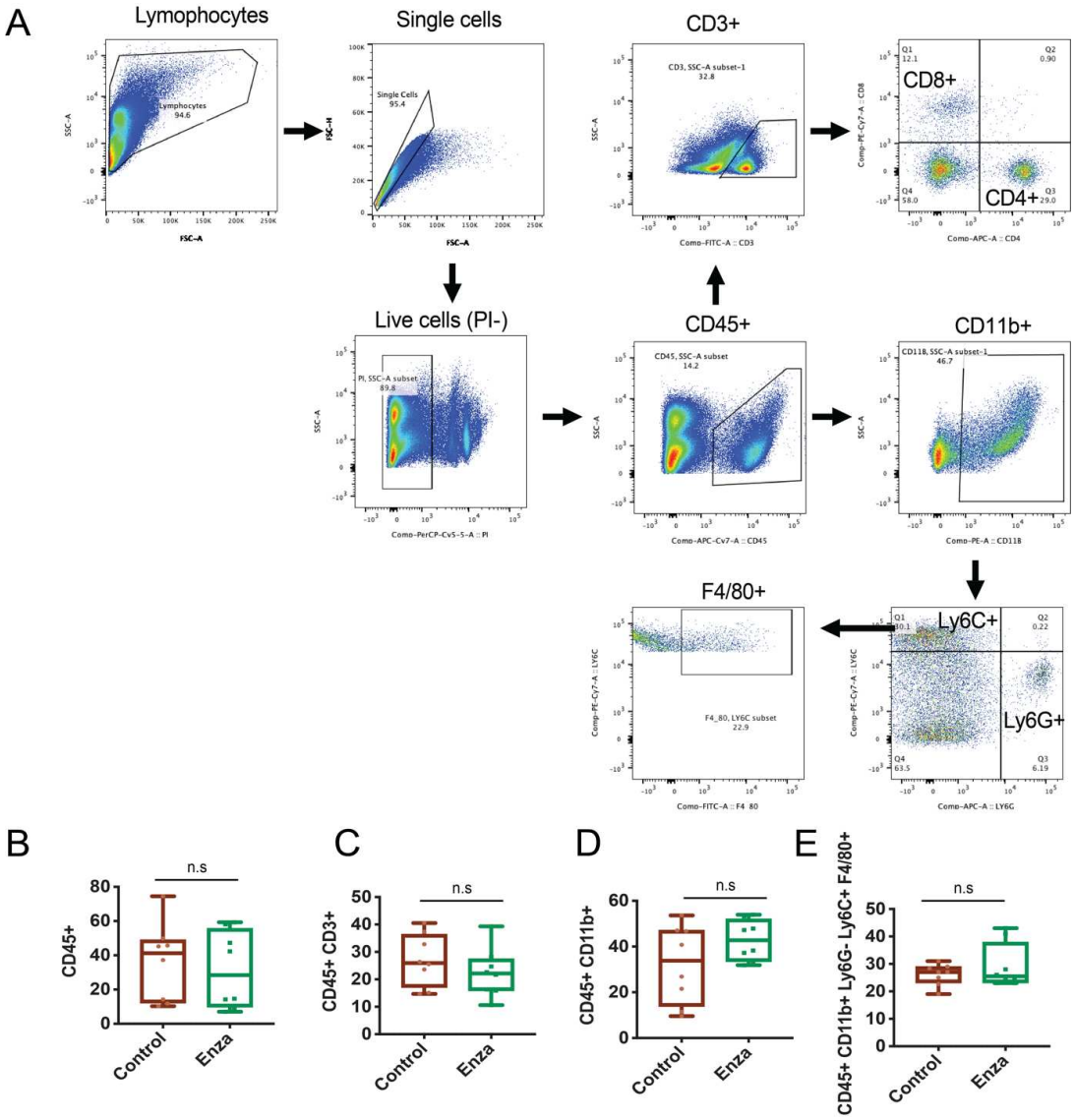
Supplemental Figure 1

Supplementary Fig.1. Immune-related signaling pathways are downregulated in enzalutamide resistant VCaP MDVR cells. RNA seq data from GSE78201 was extracted and subjected to GSEA. The GSEA plots of immune-related signaling pathways were presented.



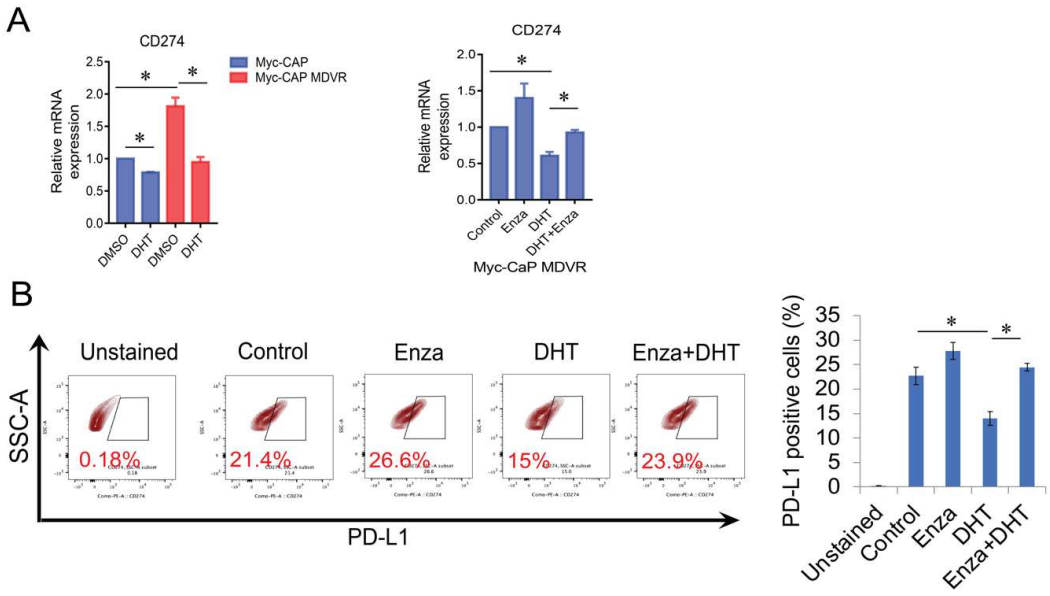
Supplemental Figure 2

Supplementary Fig.2. AR negatively correlates with CD274 in three independent CRPC patient databases. The gene expression data from four CRPC patient databases was extracted from cBioPortal, and AR and CD274 correlations were determined by Spearman correlation.



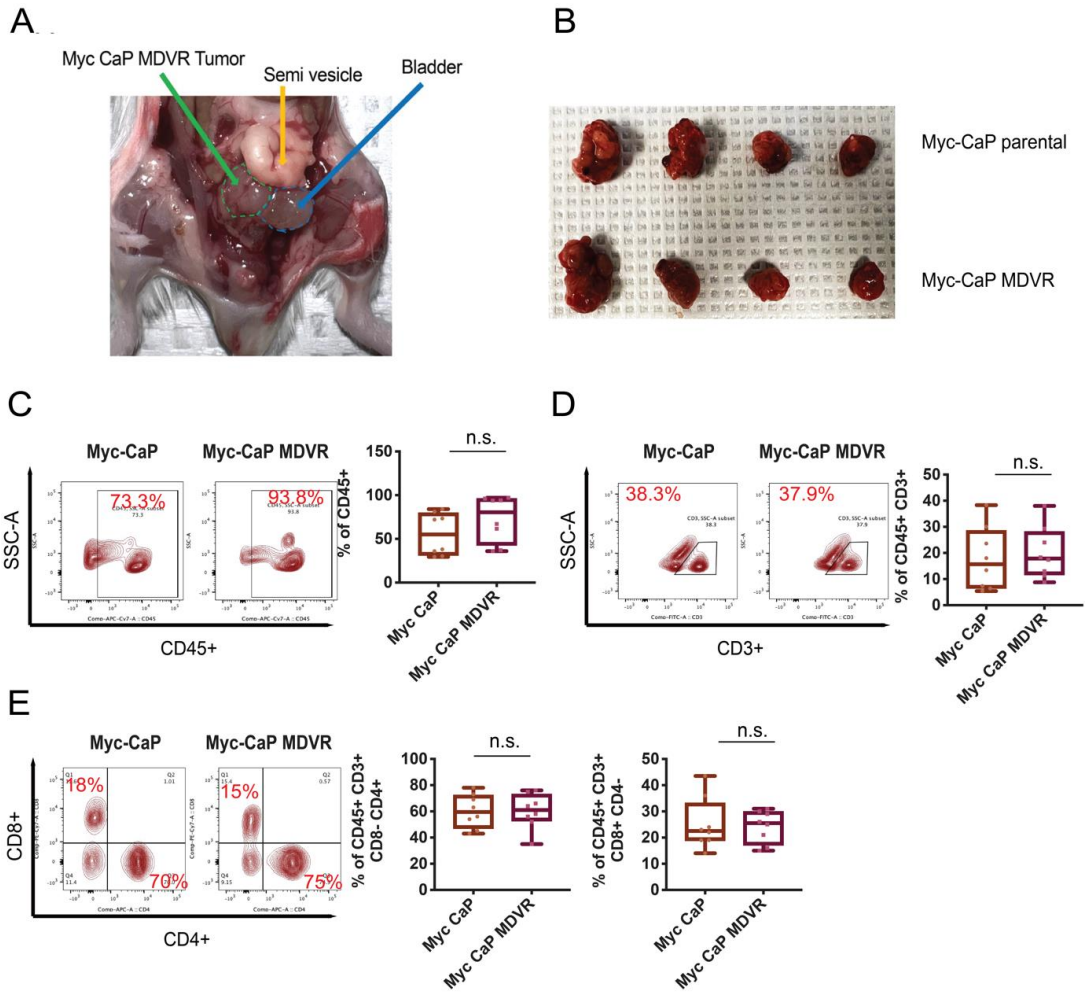
Supplemental Figure 3

Supplementary Fig.3. Enzalutamide treatment suppresses Myc-CaP tumor growth. A. Gating strategy of Myc CaP tumors to determine the population of T cells ($CD45^+$, $CD3^+$, $CD4^+$ $CD8^+$), PMN-MDSC ($CD45^+CD11b^+Ly6G^+Ly6C^{low}$), M-MDSC ($CD45^+CD11b^+Ly6G^-Ly6C^{high}$), and macrophage ($CD45^+CD11b^+Ly6G^-Ly6C^{high}F4/80^+$). **B-E.** The lymphocytes ($CD45^+$), total T cells ($CD45^+$, $CD3^+$), $CD11b^+$ cells, and macrophages ($CD45^+CD11b^+Ly6G^-Ly6C^{high}F4/80^+$) were determined in tumors using flow cytometry. The output histograms were analyzed by FlowJo software and the percentage of population between sublines were plotted. n.s.: not significant.



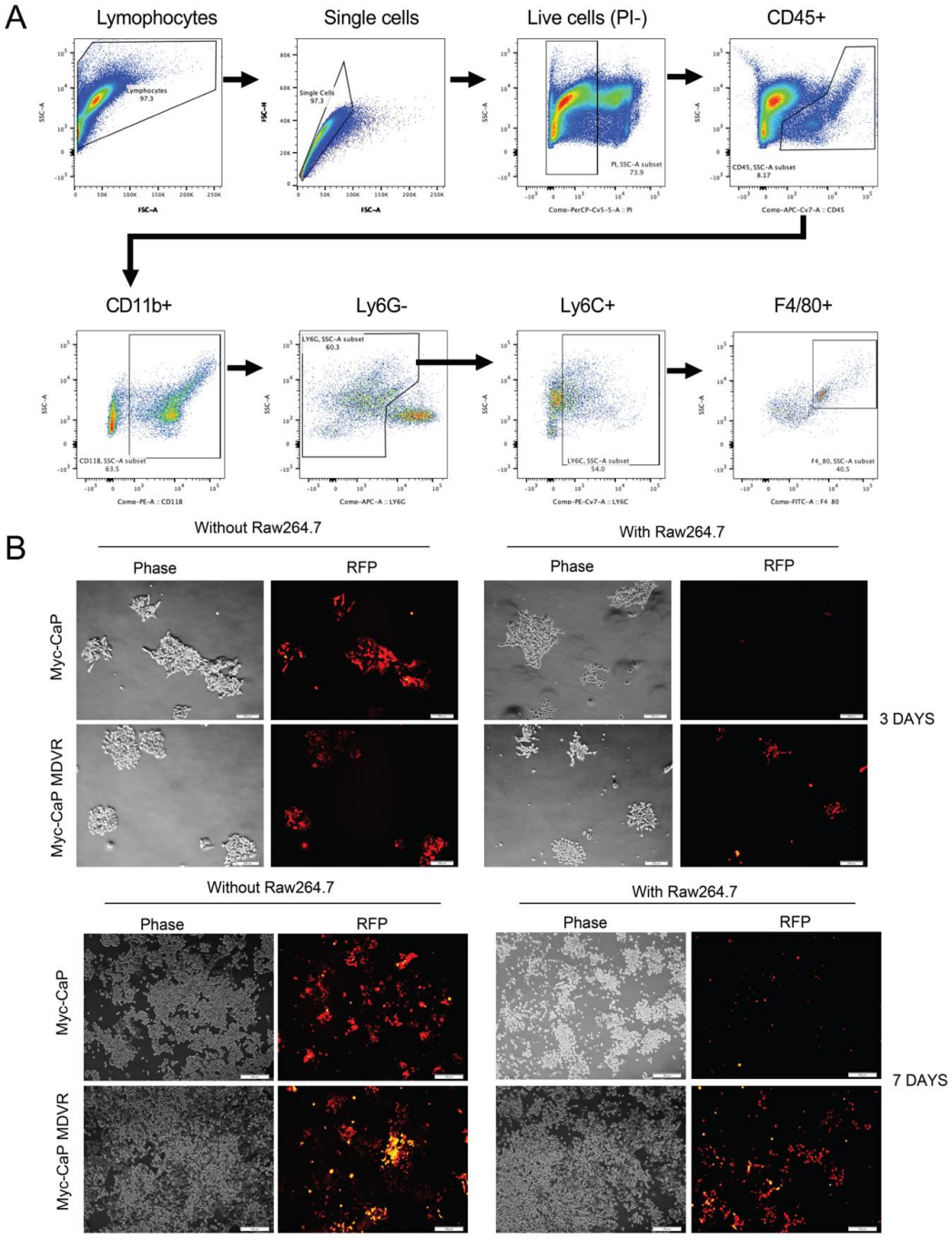
Supplemental Fig.4

Supplemental Fig.4. PD-L1 expression in Myc CaP MDVR cells. A. RNA expression levels of PD-L1 in Myc CaP parental and MDVR cells and the response of the latter ones to enzalutamide, DHT or the combined treatment. **B.** Detection of the PD-L1 expressing Myc CaP MDVR cells in response to enzalutamide, DHT or the combined treatment by flow cytometry. The percentages of the PD-L1 positive cells were compared among treatments. * $p < 0.05$



Supplemental Fig.5

Supplementary Fig.5. The population of tumor infiltrating cells in orthotopic Myc-CaP and Myc-CaP MDVR tumors were determined. A-B. The orthotopic tumor model was established in FVB mouse with injecting Myc-CaP parental and Myc-CaP MDVR cells (n=4). **C-E.** Tumor infiltrating cells were isolated from Myc-CaP and Myc-CaP MDVR orthotopic tumors respectively and incubated with fluorophores-conjugated antibodies to determine the total lymphocytes ($CD45^+$), total T cells ($CD45^+$, $CD3^+$), $CD4^+$ T cells, $CD8^+$ T cells, and gated by flow cytometry. The output histograms were analyzed by FlowJo software and the percentage of population between sublines were plotted. n.s.: not significant.



Supplemental Fig.6

Supplemental Fig.6. A. Gating strategy for the co-culturing experiments to determine the macrophage ($CD45^{+}CD11b^{+}Ly6G^{-}Ly6C^{high}F4/80^{+}$) population. **B.** RAW264.7 inhibited Myc-CaP cell growth but had minimal effects on Myc-CaP MDVR cells. 0.1x 10^4 RAW264.7 was mixed with 0.5 x 10^4 Myc-CaP-RFP or 0.5 x 10^4 Myc-CaP MDVR-RFP cells and the fluorescence confluency was monitored under fluorescence microscope at 3 days and 7 days.

Table S1. Primer list for q-PCR

	Forward	Reverse
AR-FL	AAGCCAGAGCTGTGCAGATGA	TGTCCTGCAGCCACTGGTTC
AR-V7	AACAGAAGTACCTGTGCGCC	TCAGGGTCTGGTCATTTTGA
KLK3	GCCCTGCCCGAAAGG	GATCCACTTCCGGTAATGCA
CXCL9	GGAAGCAGCCAAGTCGGTTA	AGGAGGTTTCCACATCCTGC
CXCL10	TGAATCCAGAATCGAAGGCCA	TGCATCGATTTTGCTCCCCT
CXCL11	AGGTGGGTGAAAGGACCAAA	GACTCCTTTGGGCAGTGGAA
CCL20	CGAATCAGAAGCAGCAAGCAA	AGCATTGATGTCACAGCCTTC
CD274	TGCCGACTACAAGCGAATTACTG	CTGCTTGTCCAGATGACTTCGG
IL15	TGTGGCTCTTTGGAGCAATG	CGATCTTGTATGGGCTGGCT
IRF1	ACCCTGGCTAGAGATGCAGA	TCCTTGTTGATGTCCCAGCC
IFI44	AGACAGAGCAGCTACCCTCA	CTAAGCCGCTTCCCTCCAAA
ACTIN	AGAACTGGCCCTTCTTGGAGG	GTTTTTATGTTCCTCTATGGG
mCXCL9	AACCTCCCACGTAGCTTTCG	GCCACTAAGCTACAGCCACA
mCXCL10	ATGACGGGCCAGTGAGAATG	TCAACACGTGGGCAGGATAG
mCXCL11	AGTAACGGCTGCGACAAAGT	CTTGCTTGGATCTGGGGTCC
mCD80	ACAGTCGTCGTCATCGTTGT	CCCGAAGGTAAGGCTGTTGT
mCD86	TGCCGTGCCCATTTACAAAG	CTGTGCCCAAATAGTGCTCG
mCD68	ACACTTCGGGCCATGTTTCT	GGGGCTGGTAGGTTGATTGT
mH2-EB1	TGTCACGGTCGAGTGGAAAG	AAGTAGATGAACAGCCCCGC
mARG1	GGTCTGTGGGGAAAGCCAAT	CAGTGTGAGCATCCACCCAA
mCD206	GGAGTGATGGAACCCCAGTG	CTGTCCGCCCAGTATCCATC
mTGM2	GGTGTCCCTGAAGAACCCAC	GGAGGCCAATATCAGTCGGG
mCD274	GGGCGTTTACTATCACGGCT	CCCAGTACACCACTAACGCA
mAR	AGGATTCCCAAAGAAGCCGA	TGGGGTCAACCTGCTCTTTA
mGAPDH	GTATGACTCCACTCACGGCA	GCCTCACCCCATTTGATGTT

Table S2. List of flow antibodies used in this study.

Antigen	Type	Clone	Fluorophore	Company	Catalog#	Dilution
CD274 (Mouse)	Rat IgG2b, k	10F.9G2	PE	Biolegend	124308	1：100
CD45	Rat IgG2b, λ	I3/2.3	APC-Cyanine7	Invitrogen	A15395	1：100
CD3	Rat IgG2b, k	17A2	FITC	Invitrogen	11-0032-82	1：200
CD4	Rat IgG2a, k	RM4-5	APC	Invitrogen	17-0042-82	1：200
CD8a	Rat IgG2a, k	53-6.7	PE-Cyanine7	Invitrogen	25-0081-82	1：100
CD11b	Rat IgG2a, k	M1/70	PE	Invitrogen	12-0112-82	1：200
Gr-1	Rat IgG2b, k	RB6-8C5	PE-Cyanine7	Invitrogen	25-5931-82	1：200
F4/80	Rat IgG2a, k	BM8	FITC	Invitrogen	11-4801-82	1：200
Ly6C	Rat IgG2c, k	HK1.4	PE-Cyanine7	Invitrogen	25-5932-82	1：200
Ly6G	Rat IgG2a, k	1A8-Ly6g	APC	Invitrogen	17-9668-82	1：100
FXOP3	Rabbit IgG	1054C	PE	R&D	IC8214P	1：100
IFNγ	Rat IgG1, κ	XMG1.2	PE	Biolegend	505808	1：100
CD274 (Human)	Mouse IgG2b, κ	29E.2A3	APC	Biolegend	329708	1：100

References

1. Liu C, Lou W, Zhu Y, et al. Intracrine Androgens and AKR1C3 Activation Confer Resistance to Enzalutamide in Prostate Cancer. *Cancer Res* 2015;75(7):1413-22. doi: 10.1158/0008-5472.CAN-14-3080
2. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005;102(43):15545-50. doi: 0506580102 [pii]
10.1073/pnas.0506580102 [published Online First: 2005/10/04]