Supplementary Materials for

Zoledronic acid and thymosin α1 elicit antitumor immunity against prostate cancer by enhancing tumor inflammation and cytotoxic T cells

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Supplementary materials and methods

Isolation and characterization of macrophages and T cells from PCa allograft tumors.

RM-1 tumor-bearing mice were treated with or without ZA and Tα1 for 15 days. Then, mouse peripheral blood was harvested, and red blood cells were lysed with RBC lysis buffer (00-4333-57, eBioscience), followed by centrifugation for 10 min at 700 ×g and two washes in PBS (HyClone). Untreated mice without tumor served as a control. For evaluation of the inflammatory phenotype of macrophages, the mononuclear cells were incubated with an anti-mouse CD16/32 antibody on ice for 15 min and then stained with rat anti-mouse/human CD11b-PerCP (clone M1/70, BioLegend) and anti-mouse F4/80 (clone BM8, BioLegend) on ice in dark for 1 h. Next, the sorting of $CD11b^{+}/F4/80^{+}$ macrophages were performed with a FACSAria II (BD Biosciences). The expression of M1 and M2 macrophage markers were evaluated by qPCR assay. For assessment of cytotoxic T cells, the mononuclear cells were subjected to fixation and permeabilization using Intracellular Fixation & Permeabilization Buffer Set (88-8824-00, eBioscience) according to the manufacturer's instructions. After centrifugation, wash, and block, the cells were incubated with rat anti-mouse CD3-FITC (clone 17A2, BioLegend), anti-CD8a-PE (clone 53-6.7, BioLegend), anti-granzyme B-Alexa Fluor® 647 (clone GB11, BioLegend), and anti-IFN-γ-Alexa Fluor® 647 (clone XMG1.2, BioLegend) on ice in dark for 1 h. Then, flow cytometric analysis was performed with a FACS Canto™ (BD Biosciences), and the data were analyzed by FlowJo software (Tree Star).

Isolation and characterization of T cells derived from peripheral mononuclear blood cells (PMBCs) of human healthy donors and treatments.

Human PBMCs were isolated from healthy donors using Histopaque®-1077 and -1199 (Sigma) density gradient centrifugation for 30 min at 700 × g, followed by wash in PBS. Informed consent was obtained for all subjects. After lysis with RBC lysis buffer, wash, and block, the mononuclear cells were incubated with mouse anti-human CD45-PerCP (clone 30-F11, BioLegend), anti-CD11b-FITC (clone ICRF44, BioLegend), and anti-CD3-FITC (clone UCHT1, BioLegend) on ice in dark for 1 h. Then, the sorting of $CD11b^+$ monocytes and naive $CD3^+$ T cells were performed with a FACSAria II. The cells were cultured in RPMI 1640 (Gibco) supplemented with 10% FBS (ExCell Bio) and 1% penicillin-streptomycin (HyClone) and maintained in a humidified environment containing 5% CO_2 at 37℃. For evaluation of the immune plasticity of monocytes and T cells, isolated monocytes (purity >98% by staining with anti-CD11b-PerCP) were induced to differentiate into macrophages by treatment with PMA (100 ng/mL) for 24 h prior to treatment with ZA (25 μM) and Tα1 (60 μg/ml) for 6-12 h. Isolated $CD3^+$ T (purity >98%) were stimulated with Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation (11161D, Gibco) in the absence and presence of ZA and Tα1. Next, the inflammatory phenotype of macrophages and the expression of cytotoxic effector molecules of T cells were evaluated by qPCR. The expression of MyD88, NF-κB, and p-NF-κB (Ser536) was assessed by Western blotting.

Cell viability, cell proliferation, and cell apoptosis assays

The effects of ZA and Tα1 on the viability, proliferation, and apoptosis of PCa cells (PC-3 and RM-1) were evaluated by an MTT assay, an EdU cell proliferation assay, and Annexin V-FITC/PI dual staining assay, respectively, which were performed as previously described [1, 2].

Animal study

For the effect of ZA and Tα1 on the overall survival time of mice, RM-1 tumor-bearing mice were treated with ZA and/or Tα1 until sacrifice, and Kaplan-Meier estimate was performed to evaluate the overall survival of mice in each group. For the effect of ADT (enzalutamide) with or without ZA and Tα1 on the growth of RM-1 allograft tumors, tumor-bearing C57BL/6 mice were grouped randomly and treated with vehicle, enzalutamide, or enzalutamide + ZA + Tα1. After treatment for 15 days, the tumors were harvested, weighed, and photographed. For the effect of ZA and Tα1 on the growth of RM-1 allograft tumors in immunodeficient mice, male BALB/c nude mice aged 6-8 weeks were purchased from GemPharmatech, and RM-1 cells were orthotopically injected into the prostate of mice anesthetized with isoflurane. Then, RM-1 tumor-bearing mice were grouped randomly and treated with vehicle or ZA + Tα1. After treatment for 15 days, the tumors were harvested, weighed, and photographed.

References

1. Ye, G. *et al.* The FAP alpha -activated prodrug Z-GP-DAVLBH inhibits the growth and pulmonary metastasis of osteosarcoma cells by suppressing the AXL pathway. *Acta Pharm Sin B* **12**, 1288-1304 (2022).
2. Mai, W. *et al.* Targeting platelet-derived growth factor receptor beta inhibits the proliferation and motility of human pterygial fibroblasts. *Expert Opin Ther Targets* **23**, 805-817 (2019).

Supplementary figures and figure legends

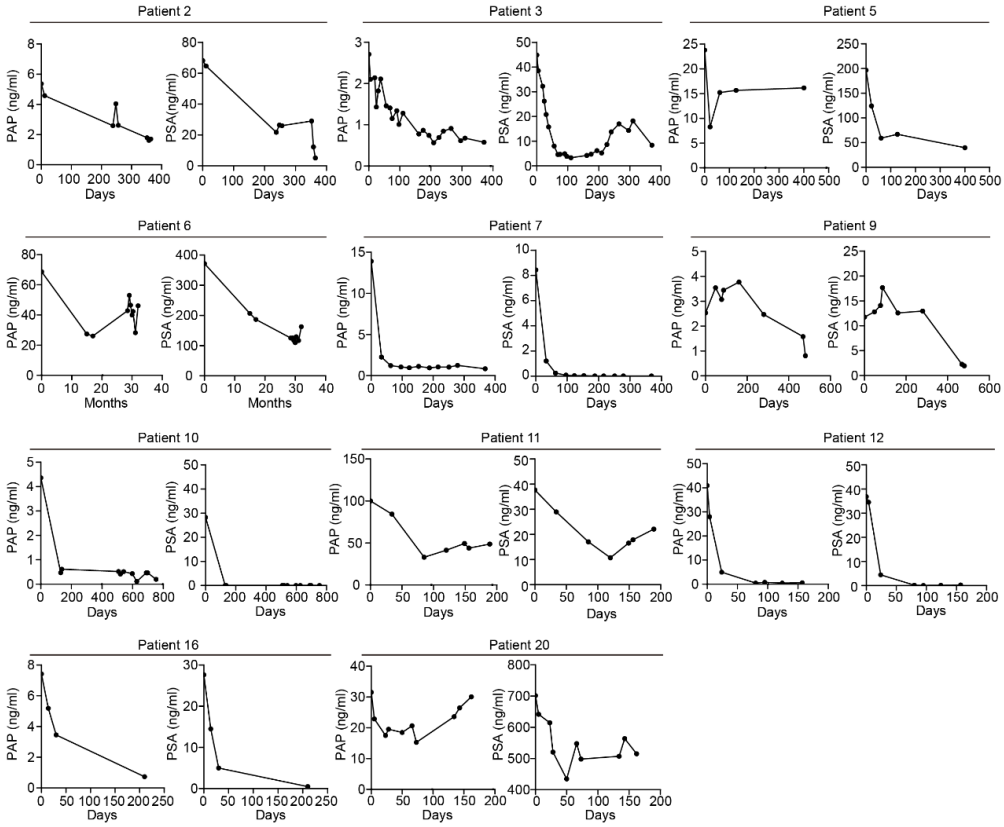

Fig. S1 The serum levels of PSA and PAP in PCa patients are decreased after treatment with ADT and ZA plus Tα1. ELISA analysis of the serum levels of PAP

and PSA in PCa patients during the treatment period of ADT and ZA plus Tα1.

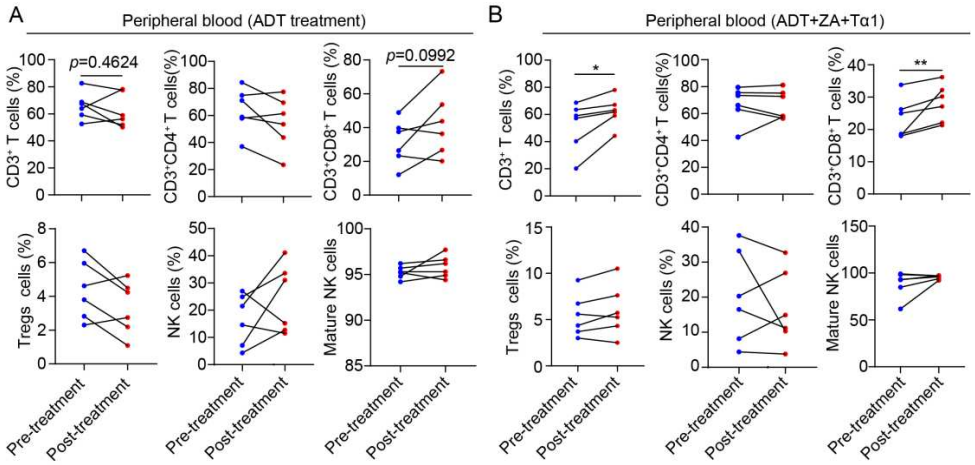

Fig. S2 ZA and Tα1 treatment increases the frequency of T cells in the peripheral blood of PCa patients. (A, B) Flow cytometric analysis was conducted to evaluate the frequency of T cells in the peripheral blood of PCa patients pre- and post-treated with (A) ADT or (B) ADT and ZA plus Tα1. Quantitation of the numbers of $CD3^+$, $CD4^+$, $CD8^+$ T cells, Treg cells, and NK cells in PCa patients in each group is shown. $^*P < 0.05$ and $^{**}P < 0.01$.

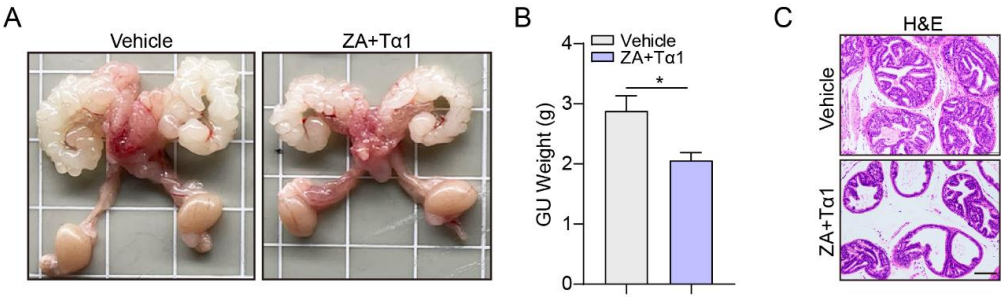

Fig S3 ZA and Tα1 treatment suppresses tumor growth in the TRAMP mice. (A) Representative images of the gross appearance of the genitourinary (GU) tracts from the TRAMP mice treated with vehicle and ZA + Tα1 for 30 days. (B) The weight of

the GU tracts from the TRAMP mice treated as indicated was evaluated (n = 4). Data are presented as mean ± SEM. $^{*}P < 0.05$. (C) Representative H&E images of dorsal prostate tissues from the TRAMP mice in each group. Scale bar: 100 μm.

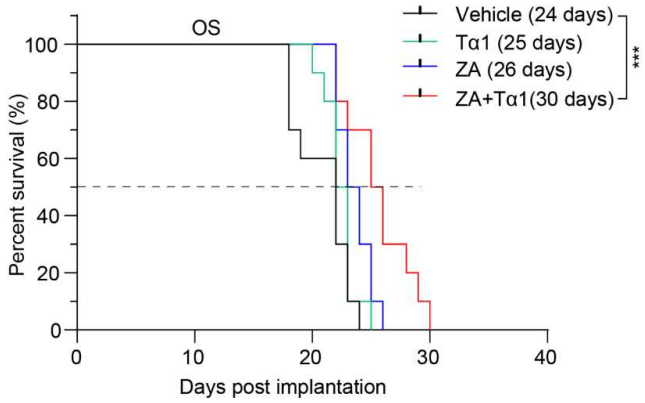

Fig S4 ZA and Tα1 treatment prolongs the survival time of PCa tumor-bearing mice. RM-1 tumor-bearing mice were treated with vehicle and ZA + Tα1 until sacrifice, and Kaplan-Meier estimates of overall survival time for mice in each group (n = 10) were performed. OS, overall survival. $^{***}P < 0.001$ by Log-rank (Mantel-Cox) test.

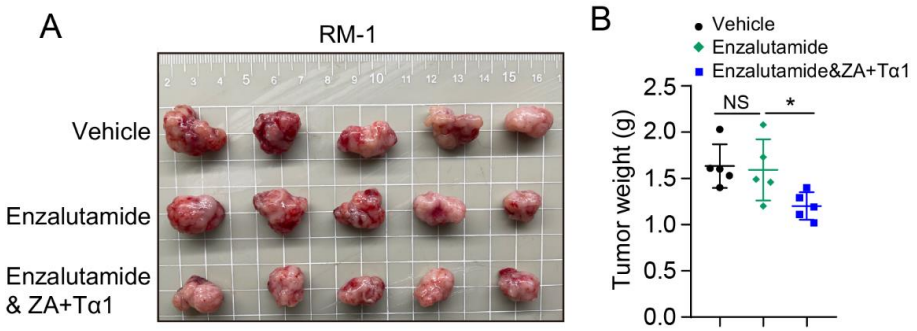

Fig. S5 ZA plus Tα1 therapy and enzalutamide suppress the progression of RM-1 tumors. (A, B) Mice bearing RM-1 allograft tumors were treated with vehicle,

enzalutamide, or enzalutamide + ZA + Tα1 for 15 days. (A) Representative images of tumors and (B) the weight of RM-1 tumors in each group are shown. Scale bar: 1 cm. Data are presented as mean ± SEM. n = 5. NS, no significance. $^{*}P < 0.05$.

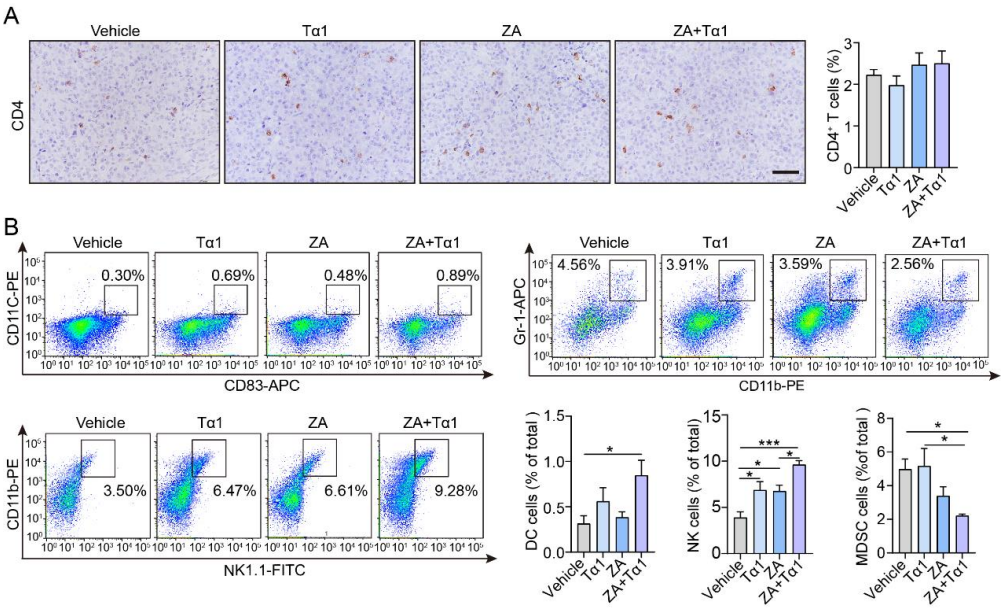

Fig. S6 ZA plus Tα1 therapy shows slight effects on the recruitment of DC cells, MDSCs and NK cells in PCa tumors. Mice bearing orthotopic RM-1 allograft tumors were treated with ZA and/or Tα1 for 15 days. (A) IHC staining of $CD4^+$ T cells in tumors in each group. Scale bar: 50 μm. Representative images and quantification of IHC staining are shown. (B) Flow cytometric analysis was performed to determine the frequency of $CD11c^+CD83^+$ dendritic cells, $CD11b^+Gr\text{-}1^+$ MDSCs, and $CD11b^+NK1.1^+$ NK cells in PC tumors treated as indicated. Data are presented as mean ± SEM. n = 5. $^{*}P < 0.05$ and $^{***}P < 0.001$.

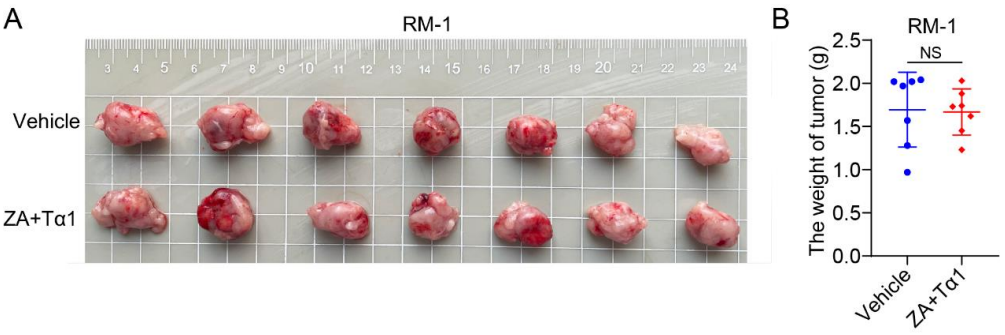

Fig. S7 ZA and Tα1 treatment negligibly affects the growth of RM-1 allograft tumors in immunodeficient mice. (A, B) BALB/c nude mice bearing RM-1 tumors were treated with vehicle and ZA + Tα1 for 15 days. (A) Representative images of tumors and (B) the weight of RM-1 tumors in each group are shown. Data are presented as mean ± SEM. n = 7. NS, no significance.

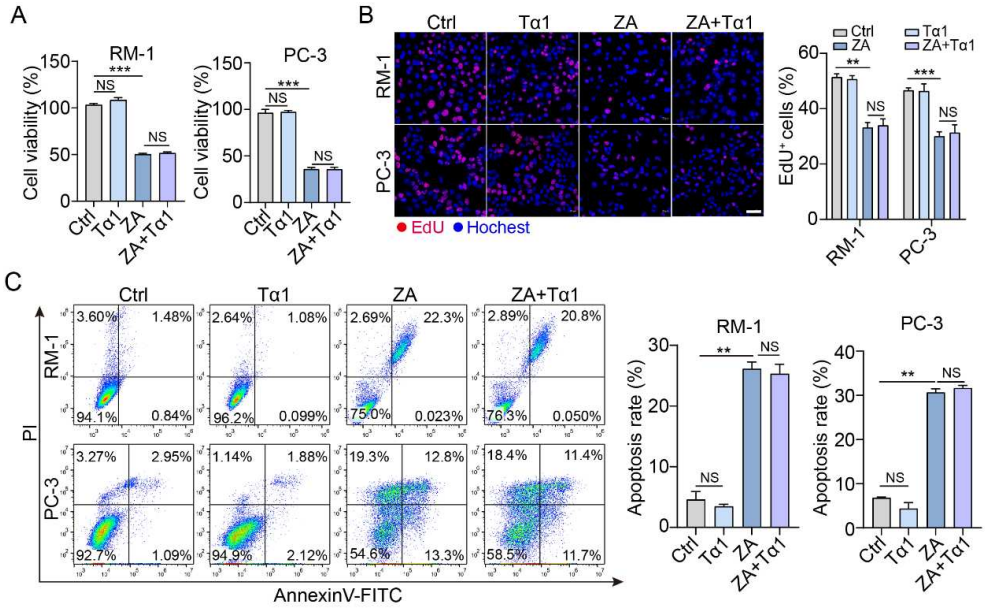

Fig. S8 ZA and Tα1 suppress the proliferation and viability of PCa cells *in vitro*. (A) An MTT assay was conducted to evaluate the effects of ZA and Tα1 on PCa cell viability. (B) The effects of ZA and Tα1 on the proliferation of RM-1 and PC-3 cells

were assessed by an EdU cell proliferation assay. Scale bar: 50 μm. (C) PCa cells were treated with ZA and Tα1 for 48 h. Then, cell apoptosis was evaluated by an Annexin V-FITC apoptosis detection kit. Data are presented as mean ± SEM. n = 3. NS, no significance. $^{**}P < 0.01$, and $^{***}P < 0.001$.

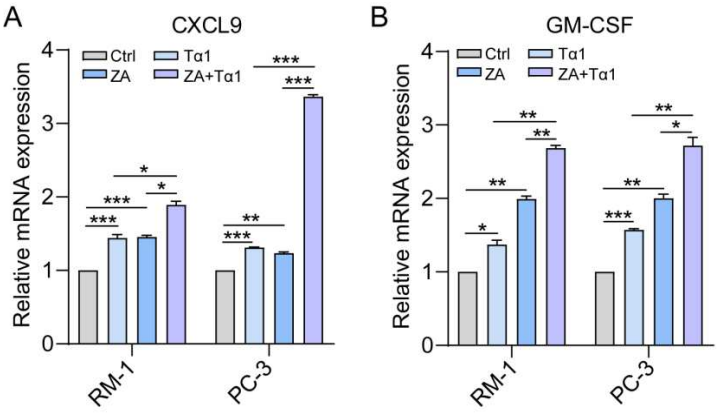

Fig. S9 ZA and Tα1 treatment inhibits the immunosuppressive function of PCa cells. RM-1 and PC-3 cells were treated with ZA and Tα1 for 12 h. Then, the expression of (A) CXCL9 and (B) GM-CSF in PCa cells was determined by qPCR. Data are presented as mean ± SEM. n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

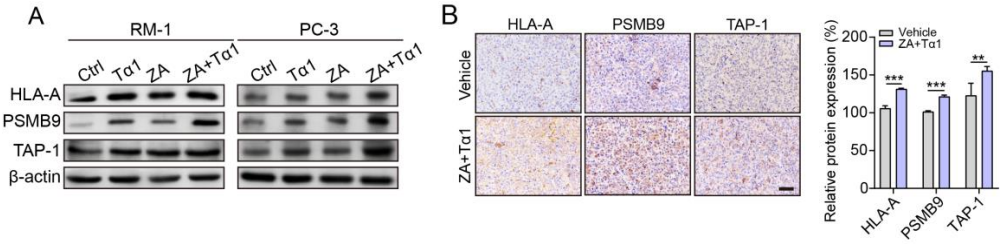

Fig. S10 ZA and Tα1 treatment upregulates the levels of HLA-A, PSMB9, and TAP1 in PCa cells. (A) RM-1 and PC-3 cells were treated with ZA and/or Tα1 for 24 h, and the expression of HLA-A, PSMB9, and TAP-1 was determined by Western

blotting. (B) IHC staining of HLA-A, PSMB9, and TAP-1 in PC tumors from mice treated with vehicle or ZA+Tα1. Scale bar: 50 μm. Representative images and quantification of IHC staining are shown. Data are presented as mean ± SEM. n = 5. $^{**}P < 0.01$ and $^{***}P < 0.001$.

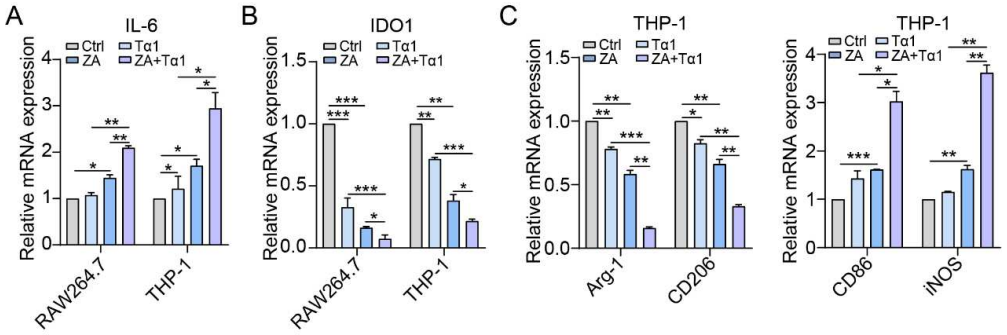

Fig. S11 ZA and Tα1 treatment enhances the pro-inflammatory function of macrophages. RAW264.7 and THP-1 cells were treated with ZA and Tα1 for 12 h. Then, the expression of (A) IL-6, (B) IDO1, and (C) Arg-1, CD206, CD86, and iNOS was evaluated by qPCR. Data are presented as mean ± SEM. n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

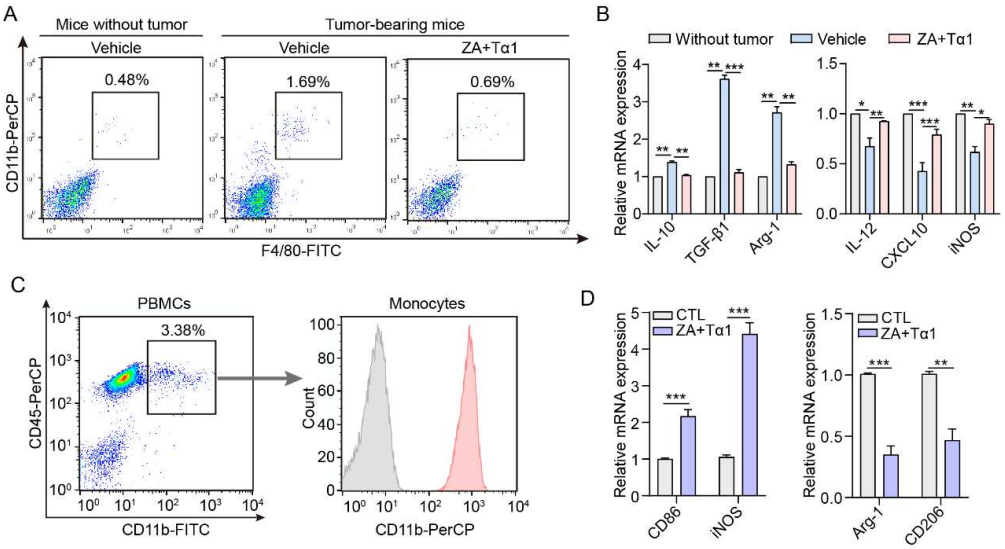

Fig. S12 ZA and Tα1 treatments stimulates an inflammatory phenotype in macrophages from RM-1 tumor-bearing mice and human healthy donors. (A) Flow cytometric analysis of macrophages isolated from the peripheral blood of mice bearing RM-1 PCa allograft tumors after treatment with or without ZA and Tα1. Untreated mice without tumor serve as a negative control. (B) qPCR assay was conducted to evaluate the expression levels of M1 and M2 macrophage markers in isolated macrophages in each group (n = 3). (C) Flow cytometric analysis of monocytes isolated from the PMBCs of healthy donors. Isolated monocytes were cultured, followed by identification and purity analysis. (D) qPCR assay was performed to evaluate the effect of ZA and Tα1 treatment on the inflammatory phenotype of isolated monocytes. Data are presented as mean ± SEM. n = 3. NS, no significance. $^{**}P < 0.01$, and $^{***}P < 0.001$.

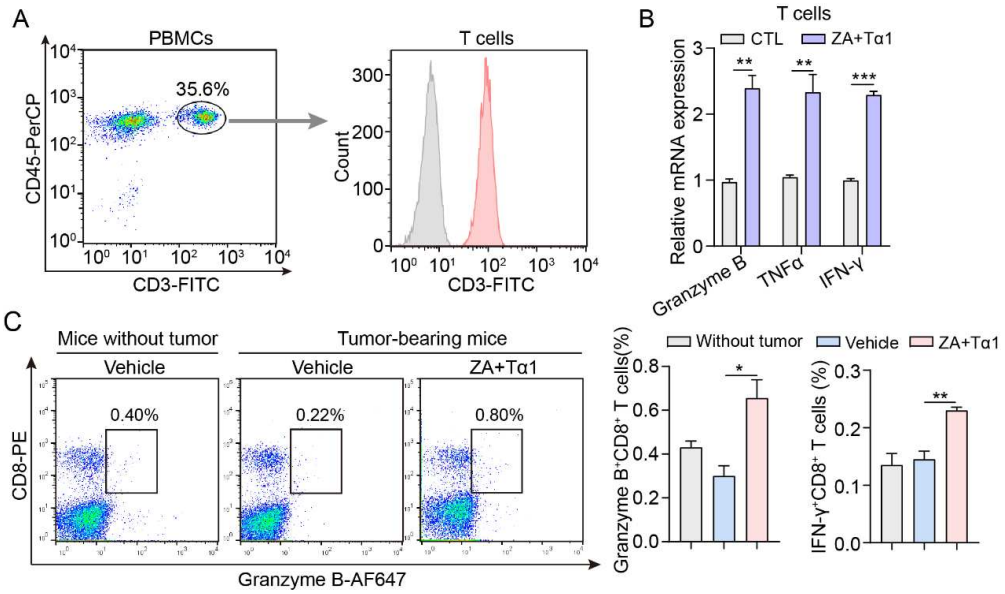

Fig. S13 ZA and Tα1 treatments increases the expression of cytotoxic effector molecules in T cells from RM-1 tumor-bearing mice and human healthy donors. (A) Flow cytometric analysis of $CD3^+$ T cells isolated from the PMBCs of healthy donors. Isolated T cells were cultured, followed by identification and purity analysis. (B) The effect of ZA and Tα1 treatment on the expression of granzyme B, TNFα, and IFN-γ in isolated T cells was evaluated by qPCR (n = 3). (C) Flow cytometric analysis of the proportions of granzyme $B^+/CD8^+$ and IFN-$\gamma^+/CD8^+$ T cells in the peripheral blood of RM-1 tumor-bearing mice treated with or without ZA and Tα1 (n = 3). Untreated mice without tumor serve as a negative control. Data are presented as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

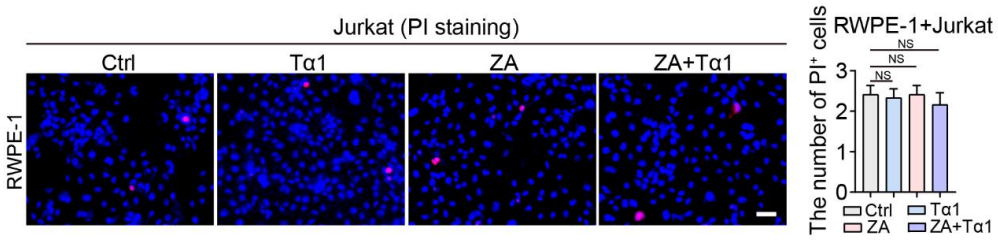

Fig S14. ZA plus Tα1-primed T cell have no significant cytotoxic effect on RWPE-1 cells. Jurkat cells activated by anti-CD3/CD28 were treated with ZA and Tα1, followed by coculture with RWPE-1 cells for 24 h. Apoptotic cells were evaluated by a PI staining assay. Representative images and quantification of PI-stained cells are shown. Data are presented as mean ± SEM. n = 3. NS, no significance.

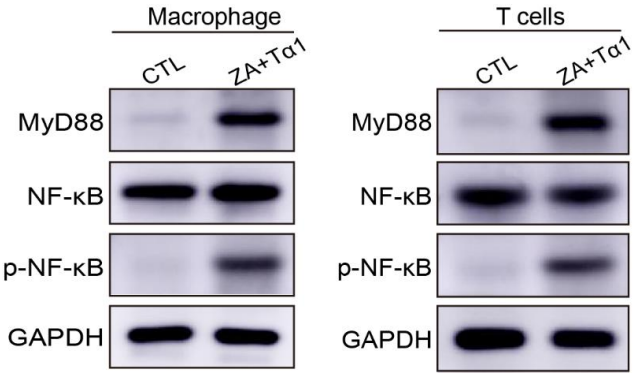

Fig. S15 ZA and Tα1 activate the MyD88/NF-κB signaling in macrophages and T cells from human heathy donors. Isolated macrophages and T cells were treated with ZA (25 μM) and/or Tα1 (60 μg/ml) for 6 h. The levels of MyD88, NF-κB, and p-NF-κB (Ser536) were determined by Western blotting. GAPDH served as a loading control.

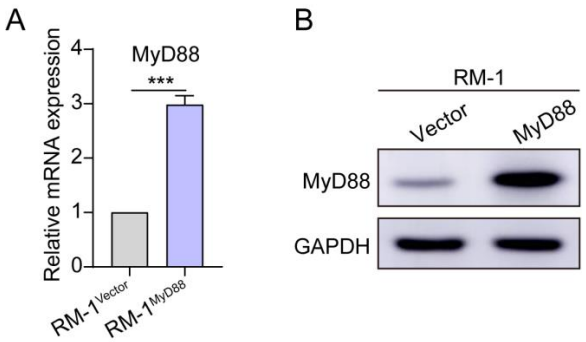

Fig. S16 The transfection efficiency of MyD88 overexpression plasmid in PCa cells. RM-1 cells were transfected with vector or MyD88-Flag plasmid for 48 h, the expression of MyD88 was determined by (A) qPCR and (B) Western blotting. Data are presented as mean ± SEM. n = 3. $^{***}P < 0.001$.

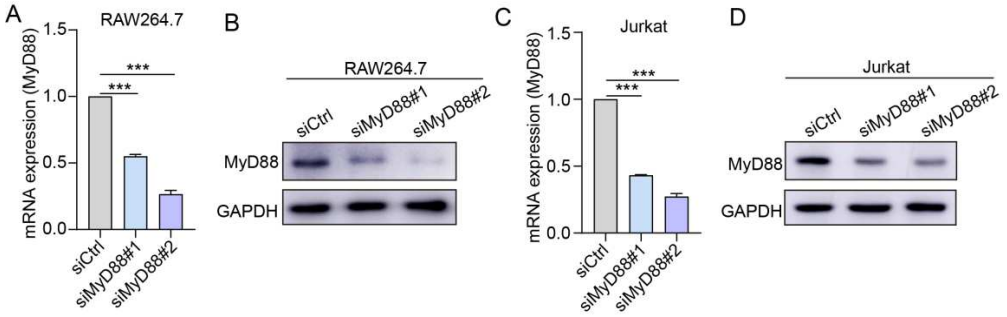

Fig. S17 The knockdown transfection efficiency of MyD88 siRNAs in macrophages and T cells. (A, B) RAW264.7 cells and (C, D) Jurkat cells were transfected with NC siRNA or MyD88 siRNA for 48 h, the expression of MyD88 was determined by qPCR and Western blotting. Data are presented as mean ± SEM. n = 3. $^{***}P < 0.001$.

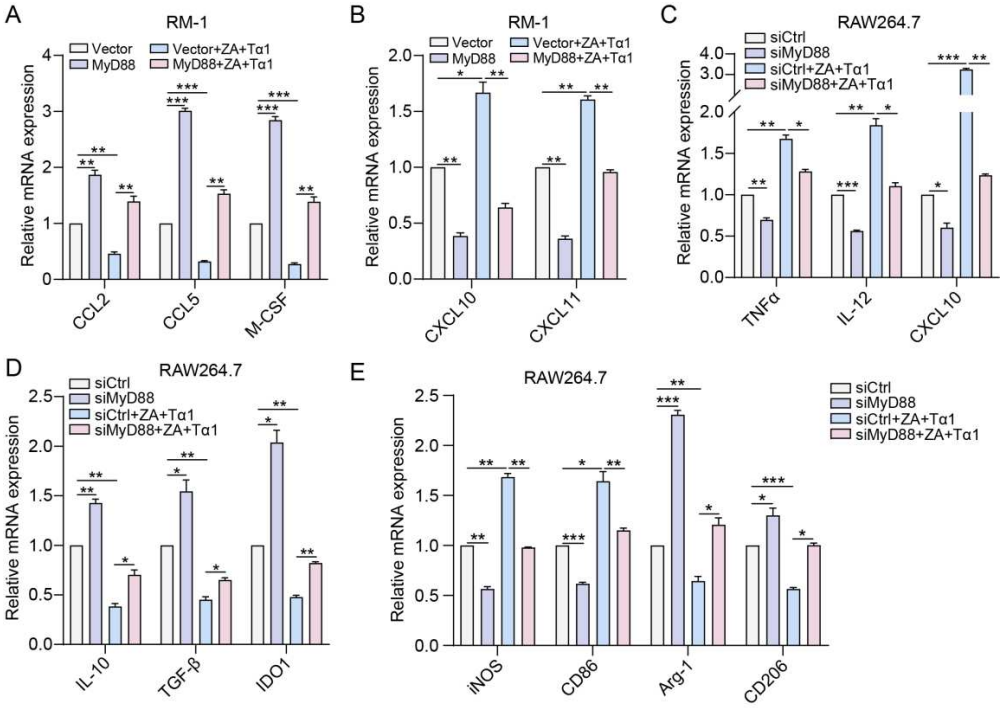

Fig. S18 ZA and Tα1 treatment stimulates pro-inflammatory macrophages and cytotoxic T cells by regulating the MyD88/NF-κB pathway. (A, B) RM-1 cells were transfected with vector or MyD88-Flag plasmid for 48 h, followed by treatment with ZA and Tα1 for 6 h. Then, the expression of (A) CCL2, CCL5, and M-CSF, and (B) CXCL10 and CXCL11 was determined by qPCR. (C-E) RAW264.7 cells were transfected with NC siRNA or MyD88 siRNA for 24 h, followed by treatment with ZA and Tα1 for 6 h. Then, the expression of (C) TNFα, IL-12, and CXCL10, (D) IL-10, TGF-β, and IDO1, and (E) iNOS, CD86, Arg-1, and CD206 was evaluated by qPCR. Data are presented as mean ± SEM. n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

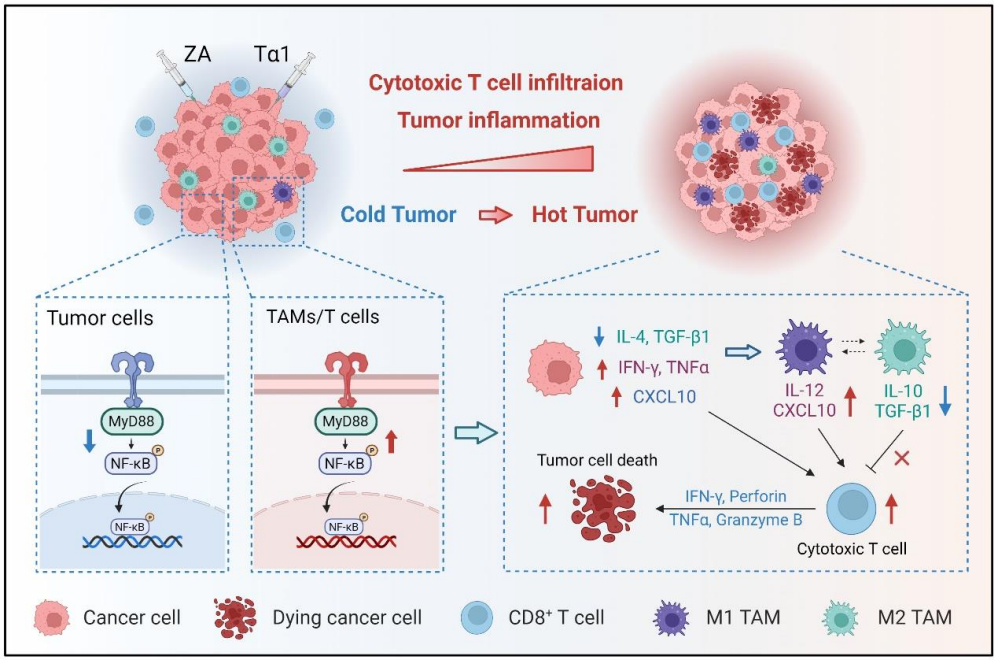

Fig. S19. Diagram depicting the mechanisms by which ZA plus Tα1 therapy inhibits PCa tumor progression. ZA and Tα1 treatment inhibits the activation of the MyD88/NF-κB pathway and relieves immunosuppression in PCa cells. In contrast, this treatment promotes the activation of the MyD88/NF-κB pathway in TAMs and T cells, which stimulates pro-inflammatory macrophages and enhances the infiltration and cytotoxic function of $CD8^+$ T cells at the tumor site. The ZA and Tα1-mediated immunoregulatory effects convert immunologically unresponsive PCa tumors into T-cell-inflamed tumors through the integrated interplay and immune plasticity of PCa cells, macrophages, and T cells, which alters the immune landscape to suppress the progression of PCa tumors.