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

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## ABSTRACT

**Background** Advanced or metastatic prostate cancer (PCa) is still an incurable malignancy with high lethality and a poor prognosis. Despite the remarkable success of immunotherapy against many types of cancer, most patients with PCa receive minimal benefit from current immunotherapeutic strategies, because PCa is an immune cold tumor with scarce T-cell infiltration in the tumor microenvironment. The aim of this study was to develop an effective immunotherapeutic approach for immune cold PCa tumors.

**Methods** The therapeutic efficacy of androgen deprivation therapy (ADT) and zoledronic acid (ZA) plus thymosin α1 (Tα1) therapy was analyzed retrospectively in patients with advanced or metastatic PCa. The effects and mechanisms by which ZA and Tα1 regulated the immune functions of PCa cells and immune cells were evaluated by a PCa allograft mouse model, flow cytometric analysis, immunohistochemical and immunofluorescence staining assays, and PCR, ELISA, and Western blot analyses.

**Results** In this study, clinical retrospective analysis revealed that ADT combined with ZA plus Tα1 improved the therapeutic outcomes of patients with PCa, which might be associated with an enhanced frequency of T cells. ZA and Tα1 treatment synergistically inhibited the growth of androgen-independent PCa allograft tumors, with increased infiltration of tumor-specific cytotoxic $CD8^+$ T cells and enhanced tumor inflammation. Functionally, ZA and Tα1 treatment relieved immunosuppression in PCa cells, stimulated pro-inflammatory macrophages, and enhanced the cytotoxic function of T cells. Mechanistically, ZA plus Tα1 therapy blocked the MyD88/NF-κB pathway in PCa cells but activated this signaling in macrophages and T cells, altering the tumor immune landscape to suppress PCa progression.

**Conclusions** These findings uncover a previously undefined role for ZA and Tα1 in inhibiting the disease progression of immune cold PCa tumors by enhancing antitumor immunity and pave the way for the application of ZA plus Tα1 therapy as an immunotherapeutic strategy for treating patients with immunologically unresponsive PCa.

### WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Studies have shown that zoledronic acid (ZA) and thymosin α1 (Tα1) have immunoregulatory effects on macrophages and T cells, and that they have been commonly used as adjuvants in the clinical treatment of patients with cancer.

### WHAT THIS STUDY ADDS

⇒ This study elucidates the effective antineoplastic efficacy of ZA and Tα1 treatment in inhibiting tumor progression in patients with prostate cancer (PCa) by altering the tumor microenvironment from immune cold to T-cell-inflamed. This study also uncovers the new mechanisms by which ZA and Tα1 modulate the immune plasticity of PCa cells, macrophages, and T cells through the MyD88/NF-κB pathway, which enhances antitumor immunity in PCa tumors.

### HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study provides the basis for developing ZA plus Tα1 therapy as a immunotherapeutic strategy for patients with immune cold PCa.

## INTRODUCTION

Prostate cancer (PCa) is one of the most commonly diagnosed malignancies and a major cause of cancer-related death in men in Western countries.[1] Due to the importance of androgen receptor in the development and progression of PCa,[2 3] androgen deprivation therapy (ADT) is still the primary treatment for advanced or metastatic PCa. However, most patients with PCa frequently develop acquired resistance to ADT during the course of clinical treatment, and this resistant disease is more likely to develop into a more aggressive state with bone metastases, also called castration-resistant prostate cancer[4] or neuroendocrine prostate cancer.[5] These patients have a poor prognosis, and their disease remains incurable and lethal.

Therefore, there is an urgent need to develop innovative and more effective therapeutic approaches for patients with aggressive PCa.

Currently, immunotherapy has been evolving, and various immune checkpoint inhibitors based on programmed cell death protein-1/programmed death-ligand 1 and cytotoxic T-lymphocyte antigen 4 have been approved for cancer treatment and have achieved clinical benefit across a wide range of tumor types. However, many patients with cancer show a limited response or are almost unresponsive to immunotherapy, which is associated with an absence or low presence of lymphocytes in the tumor microenvironment.[6–9] These immunologically unresponsive tumors, such as glioblastoma and pancreatic cancer, are called as 'cold' tumors, in contrast to hot and T-cell-inflamed tumors that have a high level of infiltrated T cells at the tumor site, such as melanoma and bladder cancer. Studies have revealed that patients with PCa also derive little therapeutic benefit from current immunotherapies, indicating that PCa might also be considered an immune cold tumor.[10 11] Emerging evidence demonstrates that turning up the heat on immune cold tumors, which is predominantly characterized by increased infiltration of tumor-specific cytotoxic T cells and enhanced inflammation within tumors, is capable of promoting a response to immune checkpoint inhibitors in non-immunoreactive tumors.[6–9] Hence, converting immunologically unresponsive tumors into T-cell-inflamed tumors may be a promising strategy to inhibit the disease progression of patients with immune cold PCa.

Zoledronic acid or zoledronate (ZA), a third-generation bisphosphonate, is primarily used to treat Paget's disease, osteoporosis, and skeletal-related events in patients with cancer with bone metastases.[12] Many studies have reported that ZA also participates in immune regulation by promoting the biological functions of macrophages and T cells.[13–15] Thymosin α1 (Tα1), a thymic hormone, has been commonly used as an immune adjuvant in the clinical treatment of patients with chronic hepatitis B and C and cellular immunodeficiency, which is associated with its immunomodulatory activity on T cells, natural killer (NK) cells, and macrophages.[16 17] Additionally, Tα1 is commonly used as an immunotherapeutic tool in combination with chemotherapy for treating many types of cancer in preclinical settings and clinical practice.[18 19] However, the effects and underlying mechanisms of combination treatment with ZA and Tα1 on immunoregulation in PCa tumors are largely unknown.

In this study, we find that combination therapy of ADT and ZA plus Tα1 improves therapeutic outcomes in patients with advanced or metastatic PCa and suppresses PCa tumor progression in mouse models, which might be attributed to enhanced frequency of cytotoxic $CD8^+$ T cells. Notably, ZA and Tα1 treatment reprograms the tumor immunosuppressive microenvironment and enhances antitumor immunity by modulating the MyD88/NF-κB pathway at the tumor site. Our study provides the basis for testing ZA plus Tα1 therapy as a potentially effective immunotherapeutic option for patients with immunologically unresponsive PCa.

## METHODS

### Retrospective analysis of patients with PCa receiving ADT with or without ZA and Tα1

A cohort of 43 patients with advanced or metastatic PCa treated with ADT with or without ZA and Tα1 from June 2010 to June 2022 at the First Affiliated Hospital of Jinan University (Guangzhou Overseas Chinese Hospital) were enrolled, and their therapeutic outcomes were analyzed retrospectively. Patients with advanced or metastatic PCa receiving ADT in combination with ZA and Tα1 were defined as the 'ZA+Tα1' group (n=20), and the other patients with PCa receiving ADT alone were classified as the 'Control' group (n=23). The serum levels of prostatic acid phosphatase (PAP), prostate-specific antigen (PSA), and free PSA in all patients, and the frequency of T cells in the peripheral blood and tumor volume detected by MRI in some patients with PCa at different time points during the treatment period were also analyzed retrospectively. Patient demographics and baseline disease characteristics were well-matched between the study groups and retrospectively collected independently from the hospital electronic patient record system. The detailed clinical information of patients with advanced or metastatic PCa enrolled in this retrospective analysis was summarized in online supplemental tables 1-3.

### Cells and cell culture

RM-1 cells (murine PCa cells), RAW264.7 cells (murine macrophages), THP-1 cells (a human monocytic cell line derived from patient with an acute monocytic leukemia), and Jurkat cells (an immortalized line of human T lymphocytes) were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). PC-3 cells (human PCa cells) were obtained from American Type Culture Collection (ATCC; USA). PC-3 and RAW264.7 cells were grown in Dulbecco's modified eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS) (ExCell Bio) and 1% penicillin–streptomycin (HyClone). RM-1, THP-1 cells, and normal human prostate epithelial RWPE-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) containing 10% FBS and 1% penicillin–streptomycin. These cells were maintained in a humidified environment containing 5% $CO_2$ at 37°C and routinely confirmed to be negative for Mycoplasma.

### Animal study

Male C57BL/6 mice aged 6–8 weeks were purchased from GemPharmatech (Nanjing, China) and raised in a specified pathogen free (SPF) environment. Briefly, RM-1-luc cells ($3\times10^5$ cells/mouse) suspended in diluted Matrigel (Corning) were orthotopically injected into the prostate of mice anesthetized with isoflurane. After tumors were established for 15 days, the tumor-bearing mice

were grouped randomly and administered ZA (Aclasta, 0.52 mg/kg, intravenous injection, every 3 days) and/or Tα1 (SciClone Pharmaceuticals, 0.208 mg/kg, subcutaneous injection, daily). Tumor growth was monitored with a Caliper IVIS Lumina II on Days 0, 7, and 15 after treatment initiation as previously described.[20] On Day 15, the tumors were resected, weighed, and photographed. Then, the tumors were subjected to flow cytometric analysis, or were fixed, embedded, and sectioned for immunohistochemical (IHC) and immunofluorescence (IF) staining analyses. For the antineoplastic effects of ZA and Tα1 on the transgenic adenocarcinoma mouse prostate (TRAMP) mouse model, the TRAMP mice aged from 18 to 22 weeks were treated with ZA and Tα1 for 30 days. Then, the mice were sacrificed, and the genitourinary tract, including the bladder, prostate, seminal vesicles, and urethra, were harvested, weighed, and stained with H&E following standard procedures.

### IHC and IF staining

PCa allograft tumors were fixed, embedded in paraffin, and sectioned at a thickness of 4 µm for IHC and IF staining analyses as previously described.[21 22] The tumor sections were deparaffinized, dehydrated, and then subjected to antigen retrieval. Next, the slides were permeabilized in 0.1% Triton X-100 for 15 min and blocked with 5% bovine serum albumin for 1 hour. For IHC analysis, slides were incubated with Ki67 (GB111499, Servicebio), PCNA (13110, Cell Signaling Technology), Cleaved caspase-3 (9664, Cell Signaling Technology), CD3 (16669, Abcam), CD8 (217344, Abcam), MyD88 (AF5195, Affinity Biosciences) and p-NF-κB (Ser536) (AF2006, Affinity Biosciences) antibodies overnight at 4°C. The slides were then washed with phosphate buffered saline (PBS) and incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies, such as HRP-conjugated anti-rabbit (7074, Cell Signaling Technology), anti-mouse (7076, Cell Signaling Technology), and anti-goat (HAF019, R&D Systems) antibodies, followed by staining using a DAB kit (Pierce). Images were acquired under an inverted microscope (IX70, Olympus, Japan). For IF analysis, the slides were incubated with F4/80 (30325, Cell Signaling Technology), Arg-1 (11285, Servicebio), CD206 (64693, Abcam), iNOS (9502, R&D Systems), CD8, and MyD88 antibodies overnight at 4°C, followed by incubation with the corresponding secondary antibodies (Invitrogen) at room temperature in the dark for 1 hour. 4',6-diamidino-2-phenylindole (DAPI) was used for nuclear staining. IF images were acquired under a Zeiss LSM 800 confocal microscope (Zeiss). The IHC and IF staining images were quantified using ImageJ software.

### Flow cytometric analysis

The effect of ZA and Tα1 on the infiltration of immune cells in the tumor microenvironment was determined by flow cytometric analysis according to our previous study.[23] Briefly, tumor tissues were minced and digested with type I collagenase (1 mg/mL, Sigma) and hyaluronidase (200 U/mL, Sigma) at 37°C for 30 min. Then, the digested tumors were filtered through 30 µm mesh filters, and single-cell suspensions were obtained by centrifugation at 500×g for 5 min. The cells were incubated with anti-mouse CD16/32 (clone 93, BioLegend) on ice for 10 min, followed by staining with fluorochrome-conjugated antibodies on ice in the dark for 30 min. To identify mouse T cells, the cells were stained with anti-mouse CD3-APC and anti-mouse CD8-PE. To identify macrophages, the cells were incubated with anti-mouse CD11b-PE and anti-mouse F4/80-FITC. Next, the cells were stained with DAPI for 5 min and analyzed on an FACSCanto II flow cytometer (BD Biosciences). The data were analyzed with FlowJo VX software.

### Quantitative PCR assay

Total RNA was extracted from PCa cells, RAW264.7 cells, THP-1 cells, and Jurkat cells using Total RNA Kit I (OMEGA) according to the manufacturer's protocol. Total RNA was reverse transcribed into complementary DNA (cDNA) with All-in-One cDNA Synthesis SuperMix (Bimake). Quantitative PCR (qPCR) analysis was performed using 2×SYBR Green qPCR Master Mix (Bimake) on a Roche LightCycler 480 real-time PCR instrument (Roche). The relative messenger RNA (mRNA) expression of target genes was normalized to that of the housekeeping gene *ACTB* or *Actb*, and the results were presented as the fold changes compared with control groups. The relative mRNA expression levels were calculated by the $2^{-\Delta\Delta CT}$ method.[24] The primer sequences are listed in online supplemental table 4.

### Western blotting

Briefly, PCa cells, RAW264.7 cells, THP-1 cells, and Jurkat cells were treated with ZA and/or Tα1 for the indicated times. The cells were then harvested and lysed in an ice-cold radio immunoprecipitation assay (RIPA) lysis buffer (Thermo Scientific) containing protease and phosphatase inhibitors (Roche) on ice for 20 min. Next, protein levels were assessed by Western blotting as previously described.[25] The following antibodies were used: Arg-1, CD206, iNOS, MyD88, p-NF-κB (Ser536), and β-actin (4970, Cell Signaling Technology).

### T-cell-mediated tumor cell killing assay

The effects of ZA and Tα1 on T-cell-mediated cytotoxicity to PCa cells were determined with Jurkat cells. Briefly, Jurkat cells were activated with Dynabeads Human T-Activator CD3/CD28 (Gibco) in the absence or presence of ZA and/or Tα1 for 72 hours according to the manufacturer's instructions. PCa cells were seeded in 96-well plates and cultured overnight. Then, the activated T cells were co-cultured with the adhered PCa cells at a ratio of 5:1 for 24 hours. Cell debris and T cells were removed, and PCa cells were stained with propidium iodide (PI; 500 µg/mL, Beyotime Biotechnology) at room temperature in the dark for 5 min. Subsequently, cell nuclei were

stained with Hoechst 33,342 for 3 min. Apoptotic PCa cells with positive PI staining were observed under an inverted fluorescence microscope, and images were acquired. The number of PI-stained apoptotic cells was quantified with ImageJ software.

### Cell transfection

For small interfering RNA (siRNA) transfection, RAW264.7 and Jurkat cells were transfected with MyD88-specific siRNAs (online supplemental table 5) by Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. After 24 hours, the cells were treated with ZA (25 µM) and/or Tα1 (60 µg/mL) for 8 hours and then subjected to qPCR and Western blotting. For plasmid transfection, PCa cells were transfected with an MyD88 overexpression plasmid (MG56953-UT, Sino Biological) or empty vector using Lipofectamine 3000. After an 8 hours transfection, the medium was refreshed, and the cells were further cultured for 48 hours. The cells were treated with ZA and Tα1 and then used for PCR assay and western blotting.

### Statistical analysis

In the clinical retrospective analysis, the data for the relative area of tumors, the proportional change in T cells in the peripheral blood and the serum levels of PAP, PSA, and free PSA of patients with PCa in each group were analyzed with GraphPad Prism V.8.4.3, and significant differences were evaluated by a paired two-tailed t-test. All in vitro experiments were repeated at least three times independently. Data were presented as mean±SEM, and statistical analyses were performed with GraphPad Prism V.8.4.3. Significant differences between two groups were evaluated by an unpaired two-tailed t-test, and those among more than two groups were determined by one-way analysis of variance followed by Tukey's multiple comparison test. P value<0.05 was considered to indicate a significant difference.

## RESULTS

### ADT and ZA plus Tα1 improve therapeutic outcomes in patients with advanced or metastatic PCa

ZA is commonly used to prevent skeletal-related events in patients with cancer with bone metastases, and Tα1 is also commonly used to improve immune function in patients with cancer with immunodeficiency caused by chemotherapy. Since most patients with advanced or metastatic PCa eventually develop bone metastases, become immunocompromised, and respond poorly to ADT, various adjuvant therapies are added to ADT to improve therapeutic outcomes. Thus, a retrospective analysis of 43 patients with advanced or metastatic PCa treated with ADT (control) or receiving ADT in combination with ZA and Tα1 (ZA+Tα1) was performed, and the changes in tumor volume and serum biomarker levels in these patients before and after treatments were compared. The results of MRI revealed that the relative area of tumors was decreased in 9 of 10 enrolled patients with advanced or metastatic PCa after treatment with ADT and ZA plus Tα1 compared with treatment initiation (figure 1A,B). PSA and PAP are two critical serum biomarkers for PCa diagnosis, and their serum levels in patients with PCa after different treatments were also retrospectively analyzed. For 14 of 20 patients with PCa, the serum levels of PSA and PAP were decreased after combination treatment with ADT and ZA plus Tα1, and their levels remained relatively low for more than 4 months (figure 1C and online supplemental figure S1). Statistically, 17 of 20 (85.0%) patients with PCa receiving treatment with ADT and ZA plus Tα1 and 15 of 23 (65.2%) patients with PCa in the control group exhibited decreases in serum PAP levels (figure 1D and online supplemental table 1). The levels of serum PSA and serum free PSA were reduced in 18 of 20 (90.0%) patients with PCa treated with ADT, ZA, and Tα1 (figure 1E,F and online supplemental table 1), whereas only 12 of 23 (52.2%) and 11 of 23 (47.8%) patients with PCa in the control group exhibited decreases in the serum levels of PSA and free PSA (figure 1E,F and online supplemental table 1). These results indicated that combination treatment with ADT and ZA plus Tα1 improved therapeutic outcomes in patients with advanced or metastatic PCa.

To further evaluate whether ADT plus ZA and Tα1 improved therapeutic outcomes in patients with advanced or metastatic PCa was associated with increased lymphocyte abundance, the frequency of T cells in the peripheral blood of patients with PCa was analyzed. We found that the numbers of $CD3^+$ and $CD8^+$ T cells were increased in 2 of 6 (33.33%) and 4 of 6 (66.67%) patients with PCa after treatment with ADT compared with those in patients with PCa before treatment (online supplemental figure S2A). However, the numbers of $CD3^+$ and $CD8^+$ T cells were significantly increased in 6 of 6 (100%) patients with PCa after combined treatment with ADT and ZA plus Tα1 compared with those in patients with PCa before treatment (online supplemental figure S2B). These differences demonstrate that the addition of ZA and Tα1 to ADT increases the frequency of T cells in the peripheral blood of patients with PCa. Taken together, this retrospective analysis indicates that ADT and ZA plus Tα1 therapy could inhibit tumor progression in patients with advanced or metastatic PCa, which might be associated with enhanced antitumor immunity.

### ZA and Tα1 synergistically suppress the growth of PCa allograft tumors

To further evaluate the antineoplastic effects and mechanisms of ZA and Tα1, androgen-independent RM-1 PCa allograft tumor mouse models were established, and tumor-bearing mice were treated with ZA every 3 days and/or Tα1 daily for 15 days (figure 2A). Our results showed that combination treatment with ZA and Tα1 resulted in a significant reduction in PCa tumor growth, as compared with the effect of each treatment alone (figure 2B). By Day 15, tumors were resected, and our results showed that

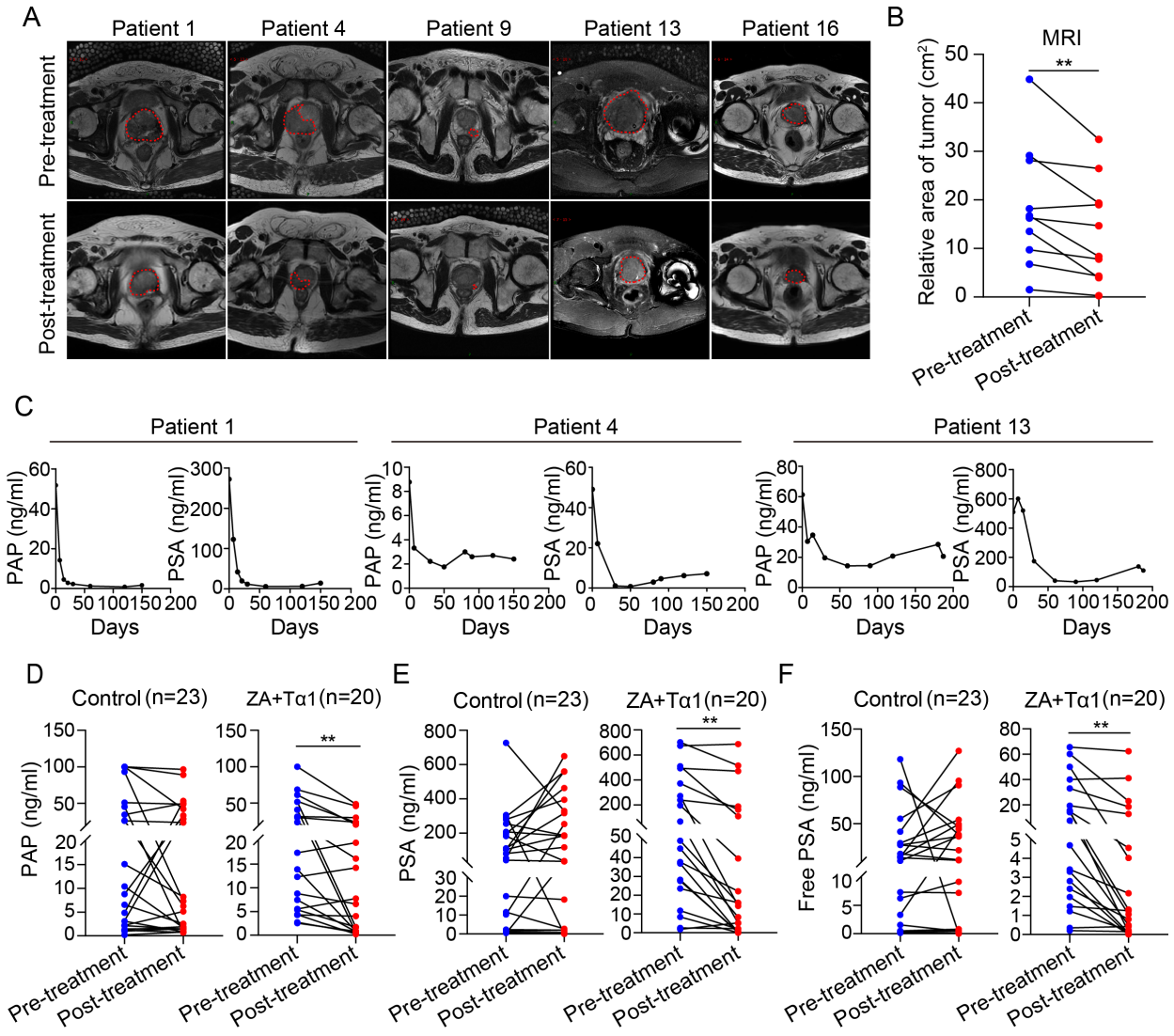


**Figure 1** ADT and ZA plus Tα1 improve therapeutic outcomes in patients with advanced or metastatic PCa. (A) The changes in tumor growth in patients with advanced or metastatic PCa receiving ADT combined with ZA and Tα1 was determined by MRI. Representative MRI images are shown, and the red circles indicate the tumor regions in the patients with PCa. (B) Quantification of the relative area of tumors from patients with PCa pretreatment and post-treatment with ADT and ZA plus Tα1. (C) ELISA analysis of the serum levels of PSA and PAP in patients with PCa during the period of ADT and ZA plus Tα1. (D–F) ELISA analysis of the serum levels of PSA, PAP, and free PSA in patients with PCa before and after treatment with ADT and ZA plus Tα1. **P<0.01. ADT, androgen deprivation therapy; PAP, prostatic acid phosphatase; PCa, prostate cancer; PSA, prostate-specific antigen; Tα1, thymosin α1; ZA, zoledronic acid.

the weight of PCa allograft tumors from mice receiving treatment with ZA and Tα1 was dramatically decreased compared with that of PCa tumors from mice treated with ZA, Tα1, or vehicle (figure 2C). We also found that the body weight of the tumor-bearing mice in each group was not significantly changed, indicating a low toxicity for ZA and Tα1 in tumor-bearing mice (figure 2D). ZA and Tα1 treatment also significantly inhibited tumor growth in the TRAMP mice (online supplemental figure S3). To investigate whether the combined drugs affect the survival time of tumor-bearing mice, mice were treated with ZA and Tα1 until sacrifice. Our results showed that compared with vehicle-treated mice (overall survival (OS) time, 24 days), ZA and Tα1 treatment significantly prolonged the overall survival time of RM-1 tumor-bearing mice (OS time, 30 days) (online supplemental figure S4). Additionally, we found that enzalutamide alone failed to inhibit the growth of RM-1 allograft tumors; however, enzalutamide and ZA plus Tα1 therapy led to a significant reduction in PCa tumor growth (online supplemental figure S5), suggesting that ZA and Tα1 increased the response to ADT in mice bearing PCa tumors.

Then, IHC staining was further used to evaluate the inhibitory effect of ZA and Tα1 on PCa progression. The numbers of Ki67-positive and PCNA-positive cells were much lower in ZA plus Tα1-treated PCa tumors than in ZA-treated, Tα1-treated, or vehicle-treated tumors (figure 2E,F). ZA and Tα1 treatment also led to a substantial increase in the number of apoptotic cells that were positive for cleaved caspase-3 or TUNEL staining

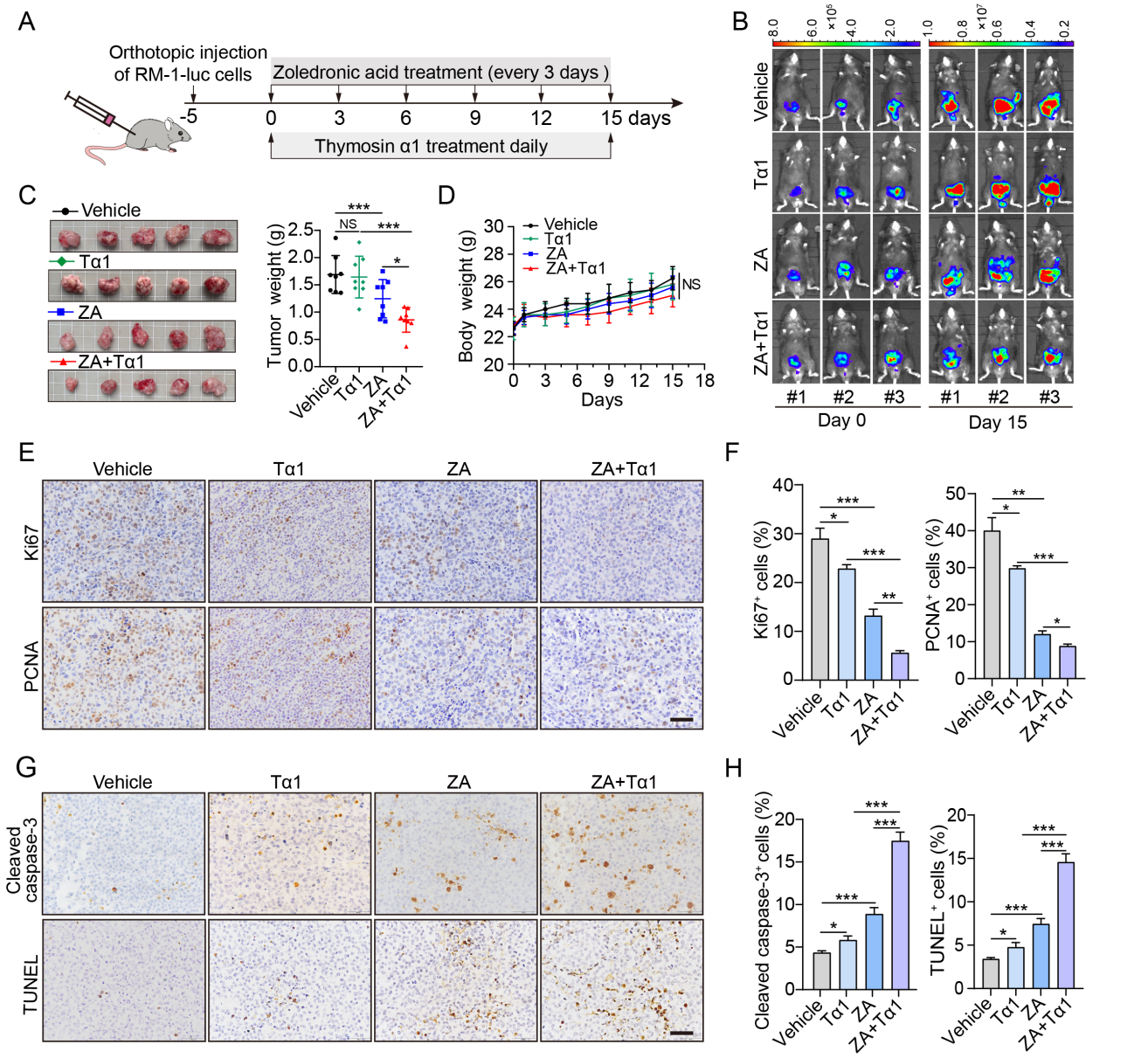


**Figure 2** ZA and Tα1 synergistically suppress the progression of PCa allograft tumors. (A) The therapeutic schedule for ZA and Tα1 treatment in mice bearing orthotopic RM-1-luc allograft tumors. (B) The volume of tumors in mice treated with vehicle, ZA, Tα1, or ZA+Tα1 was evaluated on Days 0 and 15 with an *in vivo* imaging system. (C) The weight of tumors was determined on Day 15. Representative images of tumors are shown. (D) The body weight of tumor-bearing mice was evaluated every other day. (E) IHC staining of Ki67$^+$ and PCNA$^+$ cells in RM-1 allograft tumors. Scale bar: 50 µm. (F) Quantification of the IHC staining in (E) is shown. (G) IHC staining of cleaved caspase-3$^+$ and TUNEL$^+$ cells in RM-1 allograft tumors. Scale bar: 50 µm. (H) Quantification of the IHC staining in (G) is shown. Data are presented as mean±SEM. n=5. $^*$P<0.05, $^{**}$P<0.01, and $^{***}$P<0.001. NS, no significance. IHC, immunohistochemical; PCa, prostate cancer; Tα1, thymosin α1; ZA, zoledronic acid.

compared with ZA or Tα1 monotherapy (figure 2G,H). Together, these results demonstrate that ZA and Tα1 produce a synergistic inhibitory effect on the progression of PCa tumors in mouse models.

### ZA plus Tα1 therapy enhances tumor inflammation and cytotoxic T-cell infiltration in PCa tumors

Since ZA and Tα1 have been shown to regulate the biological functions of macrophages and T cells, we further investigated whether the antineoplastic effects of ZA and Tα1 in PCa tumors could be attributed to the reprogramming of the tumor immunosuppressive microenvironment. Flow cytometric analysis revealed that ZA plus Tα1 treatment significantly decreased the number of CD11b$^+$/F4/80$^+$ tumor-associated macrophages (TAMs) in PCa allograft tumors (figure 3A). Studies have reported that increasing local inflammation is an approach to convert immune cold tumors into hot tumors.[6 9] As expected, ZA and Tα1 treatment markedly decreased the percentages of F4/80$^+$Arg-1$^+$ or F4/80$^+$CD206$^+$ anti-inflammatory TAMs but significantly increased the number of F4/80$^+$iNOS$^+$

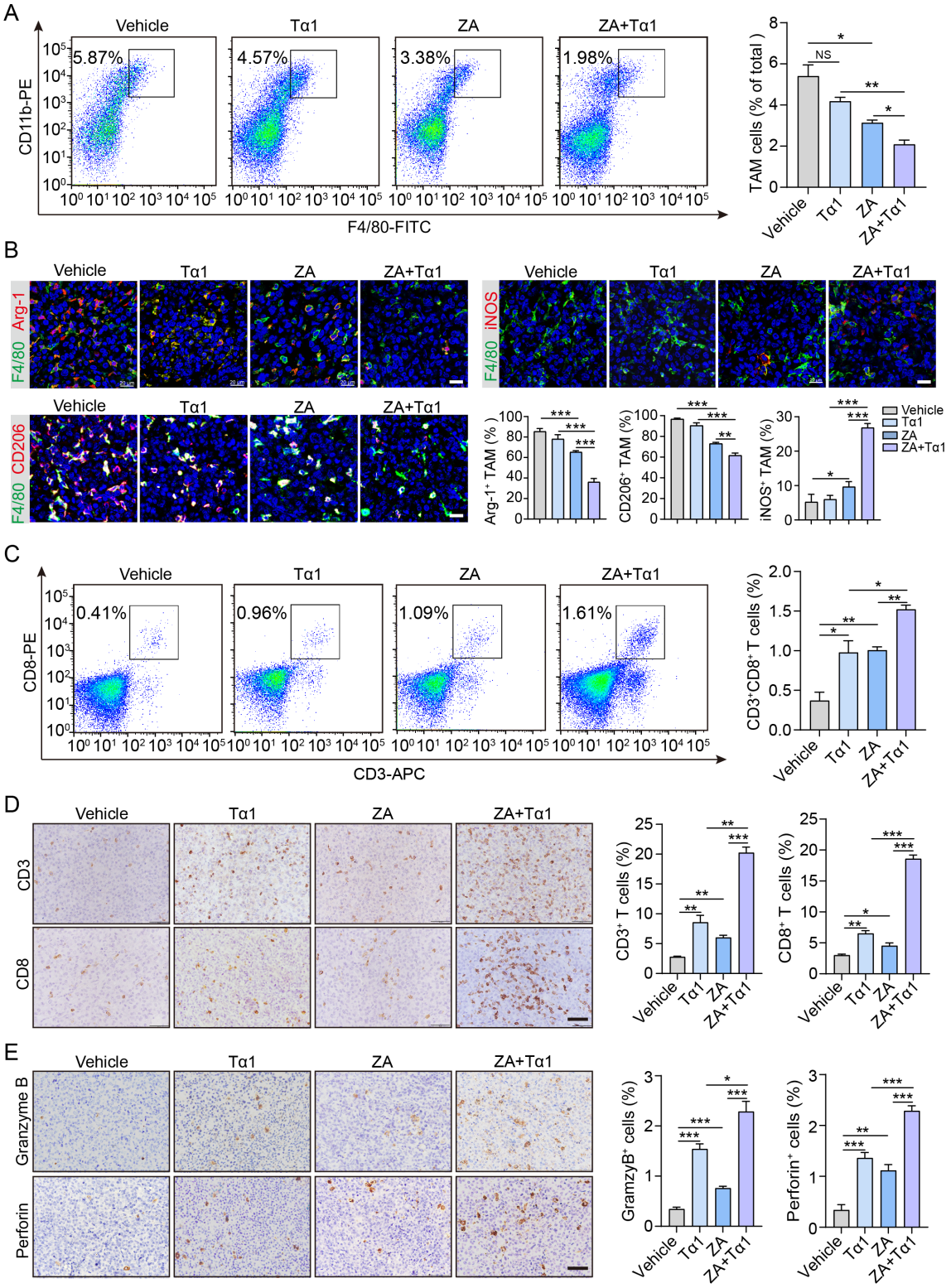


**Figure 3** ZA plus Tα1 therapy enhances tumor inflammation and cytotoxic T-cell infiltration in PCa tumors. (A) Flow cytometric analysis was performed to determine the frequency of $CD11b^+/F4/80^+$ macrophages in PCa tumors treated as indicated. (B) IF staining and the proportional changes in M2 macrophages ($F4/80^+CD206^+$ or $F4/80^+Arg\text{-}1^+$) and M1 macrophages ($F4/80^+$ $iNOS^+$) in PCa tumors receiving treatment with vehicle, ZA, Tα1, or ZA+Tα1. Scale bar: 20 µm. Quantification of the IF staining is shown. (C) Flow cytometric analysis was used to evaluate the infiltration of $CD3^+$ and $CD8^+$ T cells in PCa tumors treated as indicated. (D) IHC staining and quantification of infiltrated $CD3^+$ and $CD8^+$ T cells in PCa tumors treated with vehicle, ZA, Tα1, or ZA+Tα1. Scale bar: 50 µm. Quantification of the IHC staining is shown. (E) IHC staining and quantification of the levels of cytotoxic effector molecules (granzyme B and perforin) in PCa tumors treated as indicated. Scale bar: 50 µm. Data are presented as the mean±SEM. n=5. $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$. NS, no significance. IF, immunofluorescence; IHC, immunohistochemical; PCa, prostate cancer; TAM, tumor-associated macrophage; Tα1, thymosin α1; ZA, zoledronic acid.

pro-inflammatory TAMs in PCa tumors, as compared with those in vehicle-treated, ZA-treated, or Tα1-treated groups (figure 3B). Moreover, increasing the frequency of tumor-specific T cells is also a strategy to turn up the heat on non-immunoreactive tumors.[6 9] Remarkably, compared with the effect of each treatment alone, ZA and Tα1 treatment dramatically increased the numbers of $CD3^+$ and $CD8^+$ T cells within PCa tumors, as determined by flow cytometric analysis (figure 3C) and IHC staining (figure 3D). Compared with vehicle treatment, ZA and Tα1 treatment also increased the infiltration of dendritic cells and NK cells but decreased the recruitment of myeloid-derived suppressor cells (MDSCs) in PCa allograft tumors (online supplemental figure S6). The expression levels of cytotoxic T-cell effector molecules, including granzyme B and perforin, were also dramatically upregulated in PCa allograft tumors from mice treated with ZA and Tα1 (figure 3E). Moreover, we found that combination treatment with ZA and Tα1 failed to inhibit the growth of PCa allograft tumors in immunodeficient mice (online supplemental figure S7), indicating that T cells might be required for the antineoplastic activity of ZA and Ta1 against PCa. Collectively, these data indicate that ZA plus Tα1 treatment inhibits the growth of PCa tumors probably by enhancing pro-inflammatory TAMs and stimulating cytotoxic T cells at the tumor site.

### ZA and Tα1 treatment relieves immunosuppression in PCa cells

Targeting tumor cells themselves, particularly their immunosuppressive pathways, is also a strategy that has the potential to turn immunologically unresponsive tumors into immunoreactive tumors.[6 8 9] We first found that ZA alone decreased the viability, proliferation of PCa cells and induced PCa cell apoptosis, whereas Tα1 poorly affected the proliferation and decreased the viability of PCa cells and negligibly synergized with the effects of ZA in vitro (online supplemental figure S8). Then, we investigated the effect of ZA plus Tα1 treatment on relieving immunosuppression in PCa cells, and the expression of cytokines regulating the biological functions of inflammatory macrophages and T cells was evaluated. Our results showed that ZA and Tα1 treatment significantly downregulated the expression of factors associated with macrophage recruitment in PCa cells including CCL2 and CCL5 (figure 4A), while this treatment increased the levels of chemokines involved in inflammatory processes and cytotoxic T-cell infiltration, such as CXCL9, CXCL10, and CXCL11 (figure 4B and online supplemental figure S9A). ELISA analysis further confirmed that the secretion of CCL2 and CCL5 was dramatically decreased, but the expression of CXCL10 was significantly increased in PCa cells treated with ZA and Tα1 compared with those treated with vehicle, ZA, or Tα1 (figure 4C). Moreover, ZA and Tα1 treatment upregulated the expression levels of factors that are capable of inducing M1 polarization of macrophages, including tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in RM-1 and PC-3 cells (figure 4D,E and online supplemental figure S9B). In contrast, this treatment dramatically decreased the levels of anti-inflammatory and immunosuppressive cytokines, such as interleukin (IL)-4, M-CSF, and transforming growth factor (TGF)-β1 (figure 4F). These changes may be responsible for the increases in pro-inflammatory TAM abundance and cytotoxic T-cell infiltration observed in PCa tumors receiving ZA plus Tα1 therapy (figure 3). Furthermore, the expression of genes associated with major histocompatibility class I antigen processing and presentation, including *HLA-A*, *PSMB9*, and *TAP1*, was found to be downregulated in PCa cells.[26] Our results showed that ZA and Tα1 increased the levels of HLA-A, PSMB9, and TAP1 in PCa cells and allograft tumors (online supplemental figure S10), which might also be responsible for the increase in cytotoxic T-cell-mediated apoptosis in PCa tumors treated with ZA and Tα1. Together, these data suggest that ZA and Tα1 synergistically suppress the immunosuppressive function of PCa cells.

### ZA and Tα1 treatment induces pro-inflammatory macrophages and promotes cytotoxic T cells

Since studies have demonstrated that ZA and Tα1 exert immunomodulatory activity on immune cells, we then investigated whether ZA and Tα1 cooperatively regulate the biological functions of macrophages and T cells. Murine RAW264.7 macrophages and human THP-1 monocytes were treated with ZA and/or Tα1, and then their pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes were determined. Our results showed that ZA and Tα1 treatment synergistically skewed macrophages toward the classically activated M1 phenotype, as indicated by the dramatic upregulation of pro-inflammatory cytokines, including TNF-α, IL-12, and IL-6 (figure 5A and online supplemental figure S11A), but significant reductions in immunosuppressive factors, such as IL-10, TGF-β1, and indoleamine 2,3-dioxygenase 1 (IDO1) (figure 5B and online supplemental figure S11B). Moreover, ZA and Tα1 treatment synergistically decreased the expression of Arg-1 and CD206 but promoted the levels of biomarkers associated with M1-macrophage polarization, such as CD86 and iNOS in macrophages, as determined by qPCR (figure 5C and online supplemental figure S11C) and Western blotting (figure 5D). This combination treatment also dramatically increased the expression of chemokines, including CXCL10 and CXCL11, in RAW264.7 and THP-1 cells (figure 5E). Moreover, macrophages were first isolated from the peripheral blood of RM1 tumor-bearing mice with or without treatment by fluorescence-activated cell sorting (online supplemental figure S12A), and our results showed that ZA and Tα1 treatment significantly decreased the expression of IL-10, TGF-β1, and Arg-1 but dramatically increased the expression of IL-12, CXCL10, and iNOS were in isolated macrophages (online supplemental figure S12B). Similarly, human monocytes were isolated from peripheral

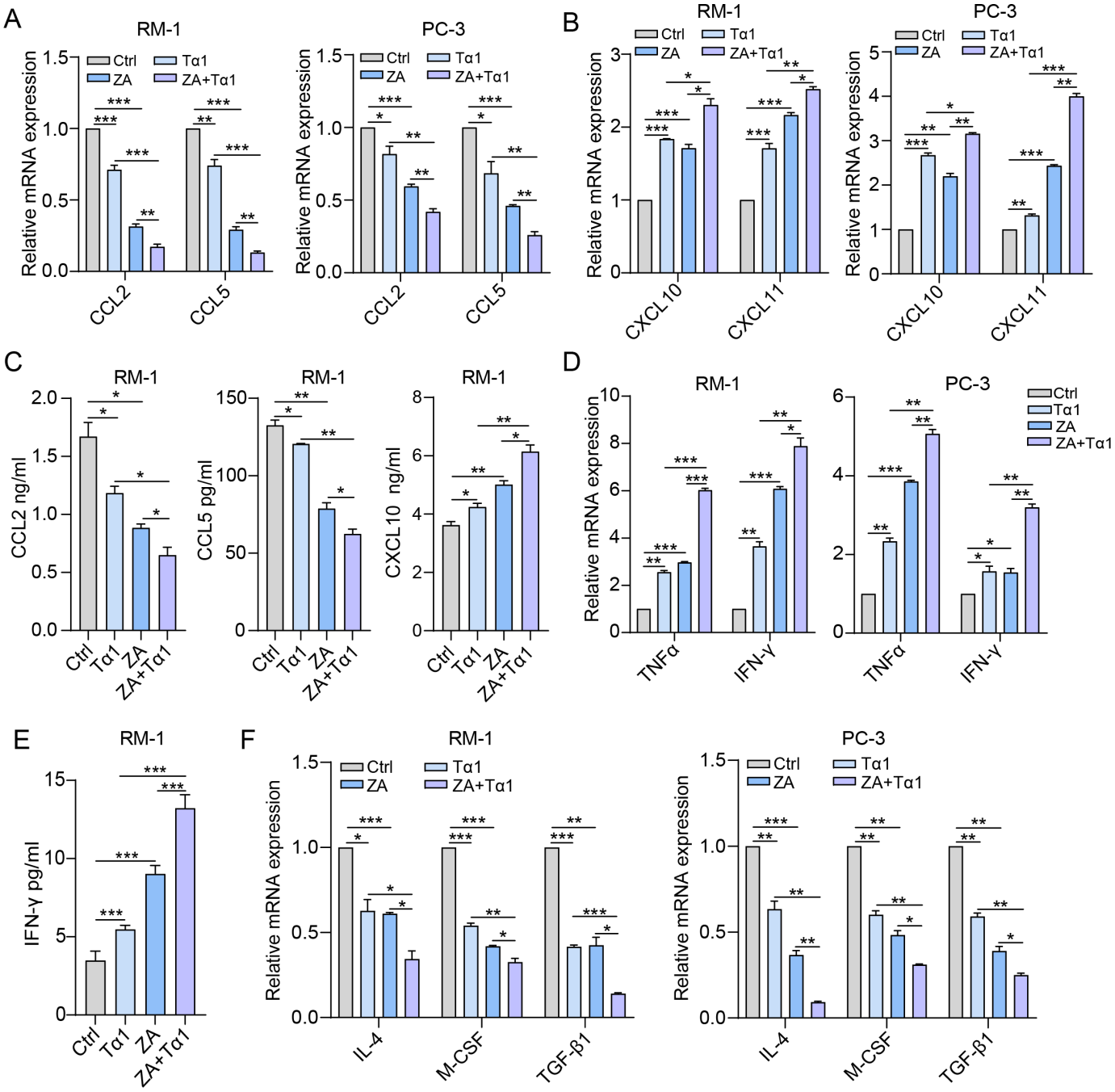


**Figure 4** ZA and Tα1 treatment relieves immunosuppression in PCa cells *in vitro*. (A, B) RM-1 and PC-3 cells were treated with ZA and/or Tα1 for 12 hours. qPCR was conducted to determine the levels of (A) CCL2 and CCL5, and (B) CXCL10 and CXCL11. (C) ELISA was performed to determine the concentrations of CCL2, CCL5, and CXCL10 in the culture supernatants of RM-1 cells treated as indicated. (D) The expression of TNF-α and IFN-γ in RM-1 and PC-3 cells treated with ZA and/or Tα1 was determined by qPCR. (E) ELISA analysis of the level of IFN-γ in RM-1 cells treated as indicated. (F) qPCR analysis of the levels of IL-4, M-CSF and TGF-β1 in PCa cells treated with ZA and/or Tα1. Data are presented as mean±SEM. n=3. $^{*}$P<0.05, $^{**}$P<0.01, and $^{***}$P<0.001. IFN, interferon; IL, interleukin; mRNA, messenger RNA; PCa, prostate cancer; M-CSF, macrophage colony-stimulating factor; TGF, transforming growth factor; TNF, tumor necrosis factor; Tα1, thymosin α1; ZA, zoledronic acid.

mononuclear blood cells of healthy donors and induced into macrophages (online supplemental figure S12C), and our results showed that ZA and Tα1 increased the expression of CD86 and iNOS but decreased the expression of Arg-1 and CD206 in human macrophages (online supplemental figure S12D). These results indicate that ZA and Tα1 directly induce an M1 phenotype in macrophages. Then, we further evaluated the effect of ZA and Tα1 on the cytotoxic function of T cells using Jurkat cells. We found that ZA and Tα1 treatment increased the expression of cytotoxic effector molecules in T cells, including granzyme B, IFN-γ, TNF-α, and perforin (figure 5F,G). Similar results were also obtained in T cells derived from peripheral mononuclear blood cells

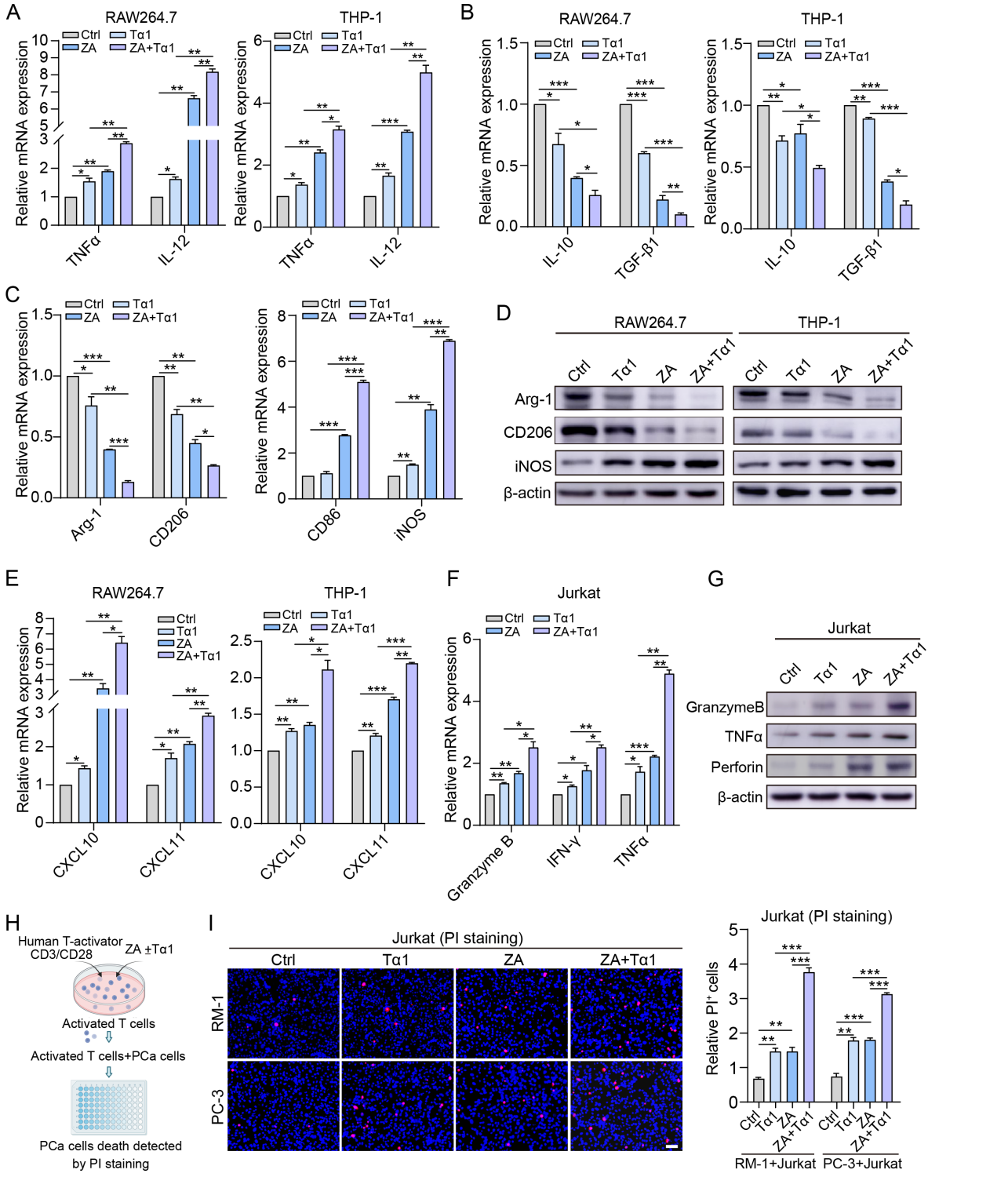


**Figure 5** ZA and Tα1 treatment induces pro-inflammatory macrophages and promotes cytotoxic T cells in vitro. (A, B) RAW264.7 and THP-1 cells were treated with ZA and/or Tα1 for 12 hours, and qPCR was performed to determine the expression levels of (A) TNF-α, IL-12, (B) IL-10 and TGF-β1. (C, D) The expression of markers associated with M2 (Arg-1 and CD206) and M1 (CD86 and iNOS) polarization in macrophages treated with ZA and/or Tα1 for 24 hours was determined by (C) qPCR and (D) Western blotting. (E) qPCR analysis of the expression of CXCL10 and CXCL11 in RAW264.7 and THP-1 cells after the indicated treatments. (F, G) The expression of T-cell cytotoxic effector molecules (granzyme B, TNF-α, IFN-γ, and perforin) in Jurkat cells treated with ZA and/or Tα1 for 24 hours was determined by (F) qPCR and (G) Western blotting. (H) Jurkat cells were activated with anti-CD3/CD28 and then treated with ZA and/or Tα1. Then, the Jurkat cells were co-cultured with RM-1 and PC-3 cells for 24 hours. (I) A PI staining assay was conducted to evaluate the cytotoxic effect of treated Jurkat cells on PCa cells. Representative images and quantification of PI-stained cells are shown. Data are presented as mean±SEM. n=3. $^{*}$P<0.05, $^{**}$P<0.01, and $^{***}$P<0.001. IFN, interferon; IL, interleukin; mRNA, messenger RNA; PCa, prostate cancer; PI, propidium iodide; TGF, transforming growth factor; TNF, tumor necrosis factor; Tα1, thymosin α1; ZA, zoledronic acid.

of healthy donors (online supplemental figure S13A,B). Moreover, we found that the proportions of granzyme B$^+$/CD8$^+$ and IFN-γ$^+$/CD8$^+$ T cells in the peripheral blood were dramatically increased in PCa tumor-bearing mice treated with ZA and Tα1 compared with vehicle-treated groups (online supplemental figure S13C). To evaluate the cytotoxic effect of T cells, activated T cells treated with ZA and/or Tα1 were further co-cultured with PCa cells (figure 5H). Our results showed that compared with vehicle-pretreated, ZA-pretreated, or Tα1-pretreated T cells, ZA plus Tα1-primed T cells exerted a stronger cytotoxic effect on RM-1 and PC-3 cells, which was indicated by an increased number of apoptotic cells with positive PI staining (figure 5I). We also found that ZA plus Tα1-primed T cells negligibly affected the viability of RWPE-1 cells (online supplemental figure S14), demonstrating the specific killing of PCa cells by T cells. Taken together, these findings suggest that ZA and Tα1 treatment cooperatively stimulate pro-inflammatory macrophages and enhance the cytotoxic function of T cells.

### The MyD88/NF-κB signaling is required for ZA plus Tα1 therapy-mediated immunostimulatory function of PCa cells, macrophages, and T cells

Next, the underlying mechanisms by which ZA and Tα1 synergistically target PCa cells themselves, stimulate pro-inflammatory macrophages, and enhance cytotoxic T cells were further investigated. The effect of ZA and Tα1 on the MyD88/NF-κB pathway, a signaling cascade critically involved in innate and adaptive immunity,[27 28] was evaluated. As expected, ZA and Tα1 treatment significantly inhibited the activation of the MyD88/NF-κB pathway, and there were dramatic decreases in the levels of MyD88 and p-NF-κB (Ser536) in PCa cells treated with the combination therapy compared with RM-1 and PC-3 cells treated with vehicle, ZA, or Tα1 (figure 6A). However, compared with each treatment alone, ZA and Tα1 treatment dramatically promoted the activation of the MyD88/NF-κB pathway in macrophages and T cells in vitro (figure 6B,C) and derived from peripheral blood of PCa tumor-bearing mice receiving treatment with ZA and Tα1 (online supplemental figure S15). Then, to investigate the role of the MyD88/NF-κB pathway in ZA plus Tα1 therapy-mediated immunostimulatory effects, PCa cells were transfected with an MyD88 overexpression plasmid (online supplemental figure S16), and macrophages and T cells were transfected with MyD88-specific siRNAs (online supplemental figure S17). Our results showed that MyD88 overexpression dramatically activated the MyD88/NF-κB pathway in PCa cells, which was significantly suppressed by ZA and Tα1 treatment (figure 6D). The expression of CCL2, CCL5, and M-CSF was increased and the levels of CXCL10 and CXCL11 were decreased in MyD88-overexpressing PCa cells, whereas these effects mediated by MyD88 overexpression were dramatically inhibited in PCa cells treated with ZA and Tα1 (online supplemental figure S18A, B). ELISA analysis further confirmed that ZA and Tα1 treatment decreased the expression of CCL2 and CCL5 but increased the level of CXCL10 in MyD88-overexpressing PCa cells (figure 6E). In contrast, silencing MyD88 inhibited the activation of the MyD88/NF-κB pathway in RAW264.7 cells, while this reduction was significantly attenuated by ZA and Tα1 treatment (figure 6F). MyD88 knockdown decreased the expression of TNF-α, IL-12, and CXCL10 but increased the levels of IL-10, TGF-β1, and IDO1 in macrophages (online supplemental figure S18C,D). MyD88 silencing also decreased the secretion of IL-12 and CXCL10 but enhanced the production of IL-10 and TGF-β1 in macrophages, and these changes were partially reversed by ZA and Tα1 treatment (figure 6G and online supplemental figure S17C,D). Moreover, the expression of iNOS and CD86 was decreased, and the levels of Arg-1 and CD206 was upregulated in MyD88-silenced RAW264.7 cells, indicating that silencing MyD88 resulted in M2 polarization of macrophages, whereas ZA and Tα1 treatment stimulated a pro-inflammatory phenotype in MyD88-silenced macrophages (figure 6H and online supplemental figure S18E). These findings suggest that ZA and Tα1-induced MyD88/NF-κB activation may contribute to enhanced inflammation in macrophages. Similarly, ZA and Tα1 treatment remarkably abrogated MyD88 knockdown-mediated inactivation of the MyD88/NF-κB pathway in T cells (figure 6I). MyD88 knockdown decreased the expression of granzyme B, TNF-α, perforin, and IFN-γ in T cells, whereas these reductions were reversed by ZA and Tα1 treatment (figure 6J–L). ELISA analysis further revealed that ZA and Tα1 increased the secretion of IFN-γ in MyD88-silenced T cells (figure 6L). Collectively, these data demonstrate that ZA and Tα1 synergistically relieve immunosuppression in PCa cells, stimulate pro-inflammatory macrophages, and enhance cytotoxic T cells by modulating the MyD88/NF-κB pathway.

### ZA plus Tα1 therapy alter the tumor immune landscape by modulating the MyD88/NF-κB pathway *in vivo*

To further investigate whether ZA and Tα1 elicit antitumor immunity by regulating the MyD88/NF-κB pathway *in vivo*, the expression levels of MyD88 and p-NF-κB (Ser536) in PCa cells, F4/80$^+$ macrophages, and CD8$^+$ T cells at the tumor site were determined. Our results showed that ZA and Tα1 treatment dramatically decreased the expression of MyD88 and p-NF-κB (Ser536) in PCa allograft tumors, as compared with the effect of each treatment alone (figure 7A). Conversely, the numbers of F4/80$^+$MyD88$^+$ and F4/80$^+$p-NF-κB$^+$ TAMs were dramatically increased in ZA plus Tα1-treated PCa allograft tumors compared with PCa tumors from mice treated with vehicle, ZA, or Tα1 (figure 7B). Additionally, the numbers of CD8$^+$MyD88$^+$ and CD8$^+$p-NF-κB$^+$ T cells were remarkably increased in PCa tumors from mice receiving ZA and Tα1 treatment (figure 7C). Together, these data suggest that ZA and Tα1 turn immune cold PCa tumors to T-cell-inflamed tumors by inhibiting the MyD88/NF-κB pathway in PCa cells but activating this signaling axis in TAMs and CD8$^+$ T cells.

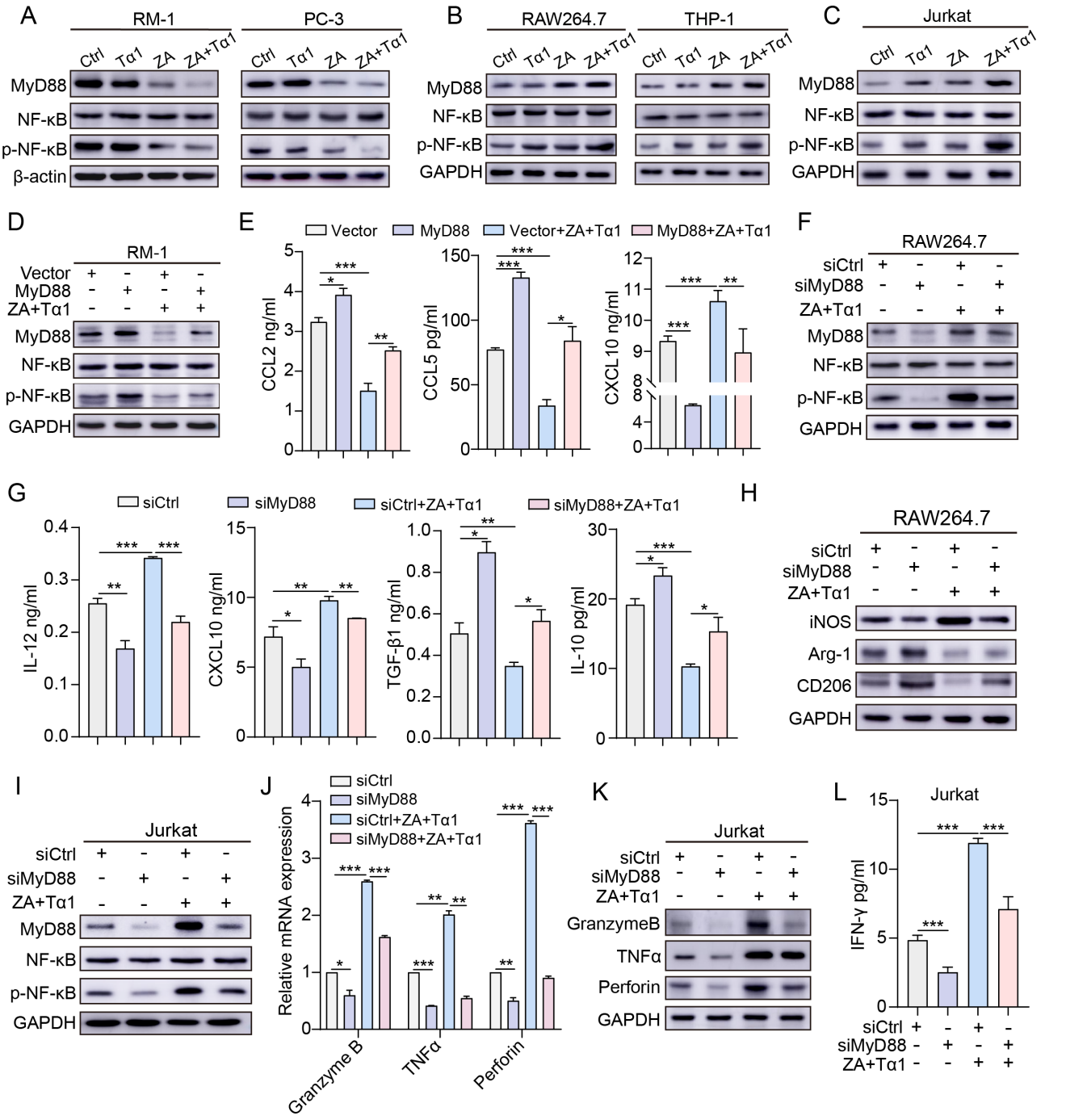


**Figure 6** The MyD88/NF-κB signaling is required for ZA plus Tα1 therapy-mediated immunostimulatory functions of PCa cells, macrophages, and T cells. (A) RM-1 and PC-3 cells, (B) RAW264.7 and THP-1 cells, and (C) Jurkat cells were treated with ZA (25 μM) and/or Tα1 (60 μg/mL) for 6 hours. The levels of MyD88, NF-κB, and p-NF-κB (Ser536) were determined by Western blotting. GAPDH served as a loading control. (D, E) RM-1 cells were transfected with a vector or MyD88 overexpression plasmid for 48 hours, followed by treatment with ZA and Tα1 for 6 hours. Then, (D) the expression of MyD88, NF-κB and p-NF-κB (Ser536) was evaluated by Western blotting, and (E) the expression of CCL2, CCL5, and CXCL10 in RM-1 cells was determined by ELISA. (F) RAW264.7 cells were transfected with NC siRNA (siCtrl) or MyD88-specific siRNA (siMyD88) for 24 hours, followed by treatment with ZA and Tα1 for 6 hours. Then, the expression of MyD88, NF-κB and p-NF-κB (Ser536) was determined by western blotting. (G) ELISA analysis of the levels of IL-12, CXCL10, TGF-β1, and IL-10 produced by RAW264.7 cells in each group. (H) Western blot analysis of the expression of iNOS, Arg-1, and CD206 in macrophages treated and transfected as indicated. (I) Jurkat cells were transfected with NC siRNA or MyD88-specific siRNA for 24 hours, followed by treatment with ZA and Tα1 for 6 hours. The expression of MyD88, NF-κB and p-NF-κB (Ser536) was determined by Western blotting. (J, K) The expression of granzyme B, TNF-α, and perforin was determined by (J) qPCR and (K) Western blotting. (L) The expression of IFN-γ by T cells from each group was evaluated by ELISA. Data are presented as mean±SEM. n=3. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$. IFN, interferon; IL, interleukin; mRNA, messenger RNA; PCa, prostate cancer; siRNA, small interfering RNA; TGF, transforming growth factor; TNF, tumor necrosis factor; siCtrl, negative control siRNA; Tα1, thymosin α1; ZA, zoledronic acid.

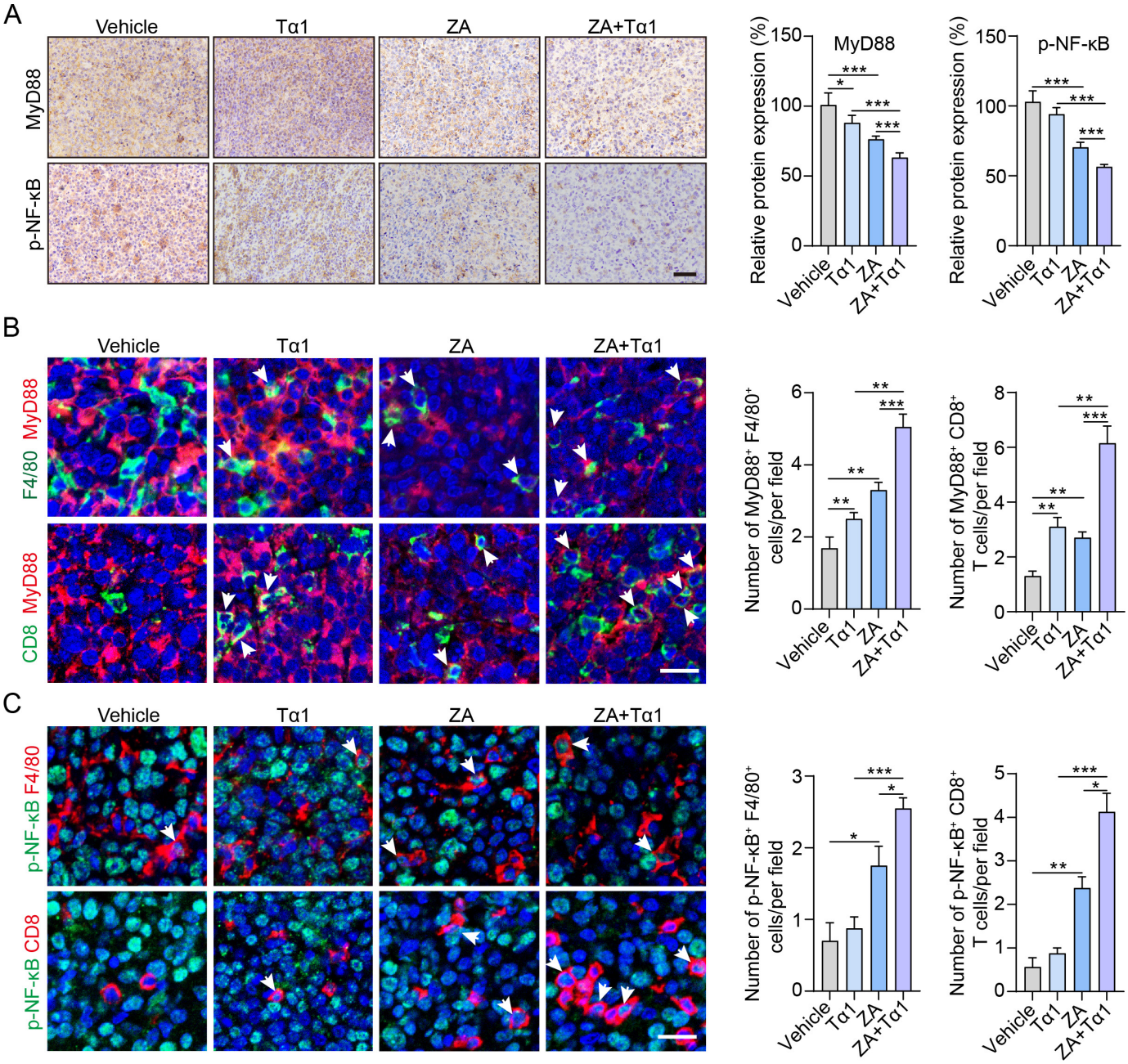


**Figure 7** ZA and Tα1 treatment alters the tumor immune landscape by modulating the MyD88/NF-κB pathway *in vivo*. (A) IHC staining of MyD88 and p-NF-κB in PCa RM-1 tumors from mice treated with vehicle, ZA, Tα1, or ZA+Tα1. Quantification of the IHC staining is shown. Scale bar: 50 µm. (B, C) IF staining of (B) F4/80$^+$MyD88$^+$ and F4/80$^+$p-NF-κB$^+$ TAMs and (C) CD8$^+$MyD88$^+$ and CD8$^+$p-NF-κB$^+$ T cells in PCa RM-1 tumors from mice treated with ZA and/or Tα1. Scale bar: 20 µm. Quantification of the IF staining is shown. Data are presented as mean±SEM. n=5. $^*$P<0.05, $^{**}$P<0.01, and $^{***}$P<0.001. IF, immunofluorescence; IHC, immunohistochemical; PCa, prostate cancer; TAM, tumor-associated macrophage; Tα1, thymosin α1; ZA, zoledronic acid.

## DISCUSSION

Advanced or metastatic PCa remains incurable and lethal because patients with this disease easily develop resistance to ADT. Therefore, adjuvant therapies or other more effective therapeutic strategies capable of circumventing ADT resistance may produce better therapeutic outcomes in patients with incurable PCa. To date, immunotherapy has been revolutionarily developed for cancer treatment and has achieved great therapeutic benefits in patients with immunoreactive tumors, also called hot or T-cell-inflamed tumors, such as melanoma, lung cancer, renal cancer, urothelial carcinoma, hepatocellular carcinoma, and gastric carcinoma.[9] However, patients with PCa, a kind of so-called cold tumor, do not achieve satisfactory survival outcomes through treatment with immune checkpoint inhibitors, such as ipilimumab and nivolumab.[10] Thus, effective immunotherapeutic approaches for patients with immune cold PCa are urgently needed. In this study, we found that the addition of ZA and Tα1 promoted the response to ADT in patients with advanced

or metastatic PCa, which might be associated with an enhanced antitumor immune response. ZA plus Tα1 therapy also inhibited tumor growth in mice bearing androgen-independent PCa allograft tumors, and these effects might be attributed to increases in cytotoxic T-cell infiltration and inflammation in the tumor microenvironment. Our study unraveled the underlying mechanisms by which ADT combined with ZA and Tα1 improved therapeutic outcomes in patients with advanced or metastatic PCa and might provide an effective and promising therapeutic approach for non-immunoreactive PCa tumors.

Regarding the immunostimulatory effects and mechanisms of ZA and Tα1 therapy in PCa tumors, the MyD88/NF-κB signaling pathway, an axis related to host immunity and the inflammatory response, was investigated. Studies have revealed that toll-like receptors (TLRs) play a critical role in immunity and inflammation, and all TLRs except TLR3 can signal through the MyD88/NF-κB cascade. There have been 11 TLRs identified in human and 13 TLRs identified in mouse, among which TLR1, TLR2, TLR4, TLR5, TLR6, TLR10, and TLR11 are localized to the cell surface and TLR3, TLR7, TLR8, and TLR9 are present on endosomal vesicles. Studies have also reported that TLR2, 4, and 9 are associated with enhanced tumor progression in PCa, while TLR3, TLR4, TLR5, and TLR7 are involved in progression inhibition,[29] indicating a complex and elusive role for the TLRs/MyD88/NF-κB axis in PCa progression. Furthermore, previous research has shown that Tα1 regulates the biological functions of macrophages and dendritic cells through TLR7[30] and TLR2/TLR9,[31] respectively. ZA was also reported to induce the TLR4-mediated M1 polarization of macrophages.[32] In our study, ZA and Tα1 inhibited the activation of the MyD88/NF-κB pathway in PCa cells, which decreased the expression of anti-inflammatory and immunosuppressive cytokines but increased the levels of pro-inflammatory cytokines and chemokines associated with lymphocyte infiltration. In contrast to their effects on PCa cells, ZA and Tα1 promoted the activation of the MyD88/NF-κB pathway in macrophages and T cells, which induced the polarization of macrophages into a pro-inflammatory phenotype and increased the cytotoxic function of T cells. These results suggested that ZA and Tα1 elicited antitumor immune effects by differentially modulating the MyD88/NF-κB signaling pathway, which might be associated with different mechanisms related to different TLRs as well as other mechanisms involving the MyD88/NF-κB pathway and beyond. Hence, we should further deeply investigate the molecular mechanisms by which ZA and Tα1 regulate TLRs/MyD88/NF-κB signaling and other related signaling molecules in PCa cells and immune cells, which could help better elucidate the immunostimulatory effects of ZA and Tα1 on immune cold PCa tumors.

In addition to the effects on macrophages and T cells, our results showed that the recruitment of dendritic cells and NK cells were increased but the number of MDSCs in PCa allograft tumors after treatment with ZA and Tα1. These effects might also contribute to growth inhibition in PCa allograft tumors. Concerning the chemokines involved in lymphocyte recruitment into tumors, CXCL9, CXCL10, and CXCL11 are essential for the trafficking and infiltration of $CD8^+$ T cells, Th1 cells, and NK cells through the corresponding receptor CXCR3.[33] A previous study showed that these three chemokines were epigenetically silenced by hypermethylation in tumor cells.[34] In our study, we found that the levels of CXCL10 and CXCL11 were decreased in MyD88-overexpressing PCa cells and that CXCL10 expression was downregulated in MyD88-silenced macrophages, whereas these reductions were attenuated by ZA and Tα1 treatment. These results indicated that the MyD88/NF-κB pathway differentially regulated the expression of CXCL10 and CXCL11 in tumor cells and macrophages, which probably did not occur through epigenetic regulation and thus remains to be further investigated. In regard to macrophage inflammation, ZA and Tα1 directly induced a pro-inflammatory phenotype in macrophages, as indicated by increases in IL-12, CXCL10, iNOS, and CD68 and decreases in IL-10, TGF-β1, Arg-1, and CD206. This treatment also increased the expression of pro-inflammatory cytokines (TNF-α, IFN-γ, and GM-CSF) and decreased the levels of cytokines that induce M2-macrophage polarization (IL-4, M-CSF, and TGF-β1) in PCa cells. These findings suggested that ZA and Tα1 stimulated pro-inflammatory macrophages directly and indirectly through relief of immunosuppression in PCa cells. In regard to T cells, ZA and Tα1 promoted the expression of granzyme B, IFN-γ, TNF-α, and perforin as well as increased the number of apoptotic PCa cells in a T-cell/PCa cell co-culture system. The levels of cytokines related to cytotoxic T-cell exhaustion and suppression were also downregulated in ZA and Tα1-treated macrophages and PCa cells. These data demonstrated that ZA and Tα1 stimulated cytotoxic T cells either directly or indirectly via the immune plasticity of macrophages and PCa cells. Collectively, these findings preliminarily revealed and elucidated the mechanisms underlying the complex and integrated interplay among PCa cells, macrophages, and T cells mediated by ZA plus Tα1 therapy that turned immune cold PCa tumors into T-cell-inflamed tumor; these findings also provided deeper insights into the immunomodulatory effects of ZA and Tα1 on tumor cells and immune cells.

In conclusion, our study demonstrates that ADT and ZA plus Tα1 therapy inhibits disease progression in patients with advanced or metastatic PCa and suppresses tumor progression in a PCa allograft mouse model. The antineoplastic mechanisms of ZA and Tα1 are associated with blockade of MyD88/NF-κB signaling in PCa cells but activation of this axis in macrophages and T cells, leading to increased infiltration of cytotoxic T cells and enhanced tumor inflammation (online supplemental figure S19). Our findings shed new light on the synergistic efficacy of ZA and Tα1 as immunotherapeutic strategy for treating non-immunoreactive patients with advanced or metastatic

PCa and indicate the potential of ZA plus Tα1 therapy in turning up the heat on immune cold tumors.

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**Contributors** DZ, JZ, and WY designed and supervised the experiments and revised the manuscript. SW and MH draft the manuscript. SW and MC performed animal experiments. YJ and GY performed flow cytometric analysis and analyzed the data. MH, GY, and YJ performed immunohistochemical and immunofluorescent assays and image acquisition. SW, GY, and YJ performed cell line studies. JZ, JP, and ZS performed clinical retrospective analysis of patients with prostate cancer. DZ, JZ, and WY acted as guarantors.

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