Shi et al. BMC Cancer (2025) 25:645
https://doi.org/10.1186/s12885-025-14075-2

BMC Cancer

RESEARCH

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# Targeting DKK3 to remodel tumor immune microenvironment and enhance cancer immunotherapy

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**Abstract**
Cancer immunotherapy such as immune checkpoint blockade (ICB) therapy has made important breakthroughs in cancer treatment, however, currently only parts of cancer patients benefit from ICB therapy. The suppressive tumor immune microenvironment (TIME) impedes the treatment response of immunotherapy, indicating the necessity to explore new treatment targets. Here, we reported a new potential immunotherapeutic target, Dickkopf-3 (DKK3), for cancer treatment. DKK3 expression is up-regulated in the tumors from multiple cancer types, and high DKK3 expression is associated with worse survival outcome across different cancers. We observed that DKK3 directly inhibits the activation of $CD8^+$ T cells and the Th1 differentiation of $CD4^+$ T cells ex vivo. Also, by establishing four different mouse cancer models, we found that DKK3 blockade triggers effective anti-tumor effects and improve the survival of tumor-bearing mice in vivo. DKK3 blockade also remodels the suppressive TIME of different cancer types, including the increased infiltration of $CD8^+$ T cells, IFN-$\gamma^+$$CD8^+$ T cells, Th1 cells, and decreased infiltration of M2 macrophages and MDSCs in the TIME. Moreover, we found that combined blockade of DKK3 and PD-1 induces synergistic tumor-control effect in our mouse cancer model. Therefore, our study reveals the impact of DKK3 in the TIME and cancer progression, which suggests that DKK3 is a novel and promising immunotherapeutic target for enhanced cancer immunotherapy.

**Keywords** DKK3, Tumor immune microenvironment, T cell, PD-1 Blockade, Cancer immunotherapy

## Introduction

Cancer immunotherapy has brought revolutionary treatment breakthroughs of various malignancies by fueling the human immune system to recognize and destroy tumor cells [1, 2]. There have been notable successes in the clinical application of immune checkpoint inhibitors (ICIs), in particular those targeting programmed death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) in cancers including melanoma, lung cancers, and breast cancers [3–5]. However, despite these advancements, only a limited subgroup of patients is responsive to immune checkpoint blockade (ICB) therapy [6]. As a result, ongoing research aims to identify novel targets and strategies to improve the efficacy of immunotherapies and reverse resistance.

One key reason for the limited response of cancer immunotherapy in solid cancers is the complicated and suppressive tumor immune microenvironment (TIME), which significantly hampers anti-tumor immune responses [7, 8]. The TIME is composed of various inhibitory cell types and factors, such as M2 macrophages, myeloid-derived suppressor cells (MDSCs), type 2 helper

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T cells (Th2 cells), and suppressive cytokines, all of which make contributions to the suppression of effective anti-tumor immune activity [9, 10]. These factors create a barrier that restricts immune cells' ability to eliminate the cancer cells. As a result, overcoming constraints of the suppressive TIME remains a critical challenge for enhancing the application of immunotherapy for tumor treatment.

Dickkopf-3 (DKK3) is a member of the Dickkopf family, known for modulating Wnt signaling pathways, which are essential for embryonic development, cellular differentiation, and tissue homeostasis [11–14]. In cancer biology, DKK3 exhibits a complex role with varied expression patterns depending on the tumor type. While initially identified as a potential tumor suppressor, recent research has revealed its pro-tumorigenic effects in certain cancers. For instance, elevated DKK3 expression has been related to poor prognosis in gastric, oral, and breast cancers, where it is associated with enhanced tumor growth, metastasis, and treatment resistance [15–17]. Additionally, emerging evidence suggests that the DKK3 expression is associated with immune cell recruitment and function in different disease settings [18–20]. However, the exact impact of DKK3 in anti-tumor immunity, and if DKK3 may be a possible immunotherapeutic target for cancer treatment remain unknown.

Here, we reported about the expression of DKK3 and its association with patient survival and immune state across cancer types. Also, by both exploring the impact of DKK3 in immune responses ex vivo and in vivo of different mouse cancer models, we provided preclinical evidence that DKK3 can be a novel target for enhanced cancer immunotherapy.

## Results

### High *DKK3* expression is associated with worse patient survival across cancer types

To analyze the DKK3 expression and its prognostic value for cancer patients, we first accessed the patient tumor mRNA data from TCGA database. As shown in Fig. 1A, we found that *DKK3* expression is significantly increased ($p < 0.05$) in patient tumor tissues compared to paired adjacent tissues in patients from TCGA-PAAD (Pancreatic Adenocarcinoma), THYM (Thymoma), HNSC (Head and Neck Squamous Cell Carcinoma), and DLBC (Diffuse Large B-Cell Lymphoma). Also, we found that patients with higher tumoral *DKK3* expression have significantly worse survival outcomes ($p < 0.05$) than patients with low *DKK3* expression among all TCGA samples from 33 cancer types (pan-cancer), including STAD (Stomach Adenocarcinoma), BLCA (Bladder Urothelial Carcinoma), GMB (Glioblastoma Multiforme), HNSC and MESO (Mesothelioma) (Fig. 1B). Thus, these results indicate that *DKK3* expression is increased and related to poor patient prognosis across different cancer types.

### DKK3 expression is associated with immunosuppressive tumor microenvironment (TME) across cancer types

We next analyzed whether DKK3 expression has impact in the immune composition in the TME of multiple cancer types, including $CD8^+$ T cells, Th1 cells, Tregs, M2 macrophages and MDSCs. The results showed that DKK3 expression is significantly negatively associated with increased intratumoral $CD8^+$ T cells infiltration in tumors from COAD (Colon Adenocarcinoma), BRCA (Breast Carcinoma), ESCA (Esophageal Carcinoma), LUSC (Lung Squamous Cell Carcinoma) and PAAD ($p < 0.05$, Fig. 2A), and increased intratumoral infiltration of Th1 cells in tumors from BRCA, COAD, KICH (Kidney Chromophobe Carcinoma), LGG (Low-Grade Glioma) and STAD ($p < 0.05$, Fig. 2B). Meanwhile, DKK3 expression is significantly negatively associated with increased Treg infiltration in BLCA, COAD, LUAD (Lung Adenocarcinoma), PAAD and PRAD (Prostate Adenocarcinoma) ($p < 0.05$, Fig. 2C), increased M2 macrophage infiltration in BLCA, COAD, KICH, TGCT (Testicular Germ Cell Tumors), and READ (Rectum Adenocarcinoma) ($p < 0.05$, Fig. 2D), and increased MDSC infiltration in CESC (Cervical Squamous Cell Carcinoma), ESCA, HNSC, MESO and SKCM (Skin Cutaneous Melanoma) ($p < 0.05$, Fig. 2E). Together, these results demonstrate that DKK3 expression is very closely linked to the suppressive TIME across cancer types, which may impede the anti-tumor immune responses and have a pro-tumor role.

### DKK3 inhibits $CD8^+$ T cell activation and Th1 differentiation

As indicated by our bioinformatic data about the potential impact of DKK3 expression in the TIME, we next explored if DKK1 can directly modulate T cell phenotype or function ex vivo. We isolated $CD4^+$ and $CD8^+$ T cells from the spleen tissues of C57B6/J mice and incubated them anti-CD3&CD28 T cell activation beads and / or DKK3 murine recombinant protein (Fig. 3A). As shown in Fig. 3B-E, we found obviously decreased expression of CD25, CD69, CD107a and IFNγ in $CD8^+$ T cells co-cultured with DKK3 protein compared to those treated with anti-CD3&CD28 beads alone. Moreover, the expression of T-bet is decreased while GATA3 is increased in $CD4^+$ T cells co-cultured with DKK3 protein (Fig. 3F, G). Therefore, DKK3 can directly inhibit $CD8^+$ T cell activation and the type 1 (Th1) differentiation of $CD4^+$ T cells.

### DKK3 blockade controls tumor growth in different mouse cancer models

Considering the inhibitory effect of DKK3 protein in T cell activation, we next explored whether DKK3 could

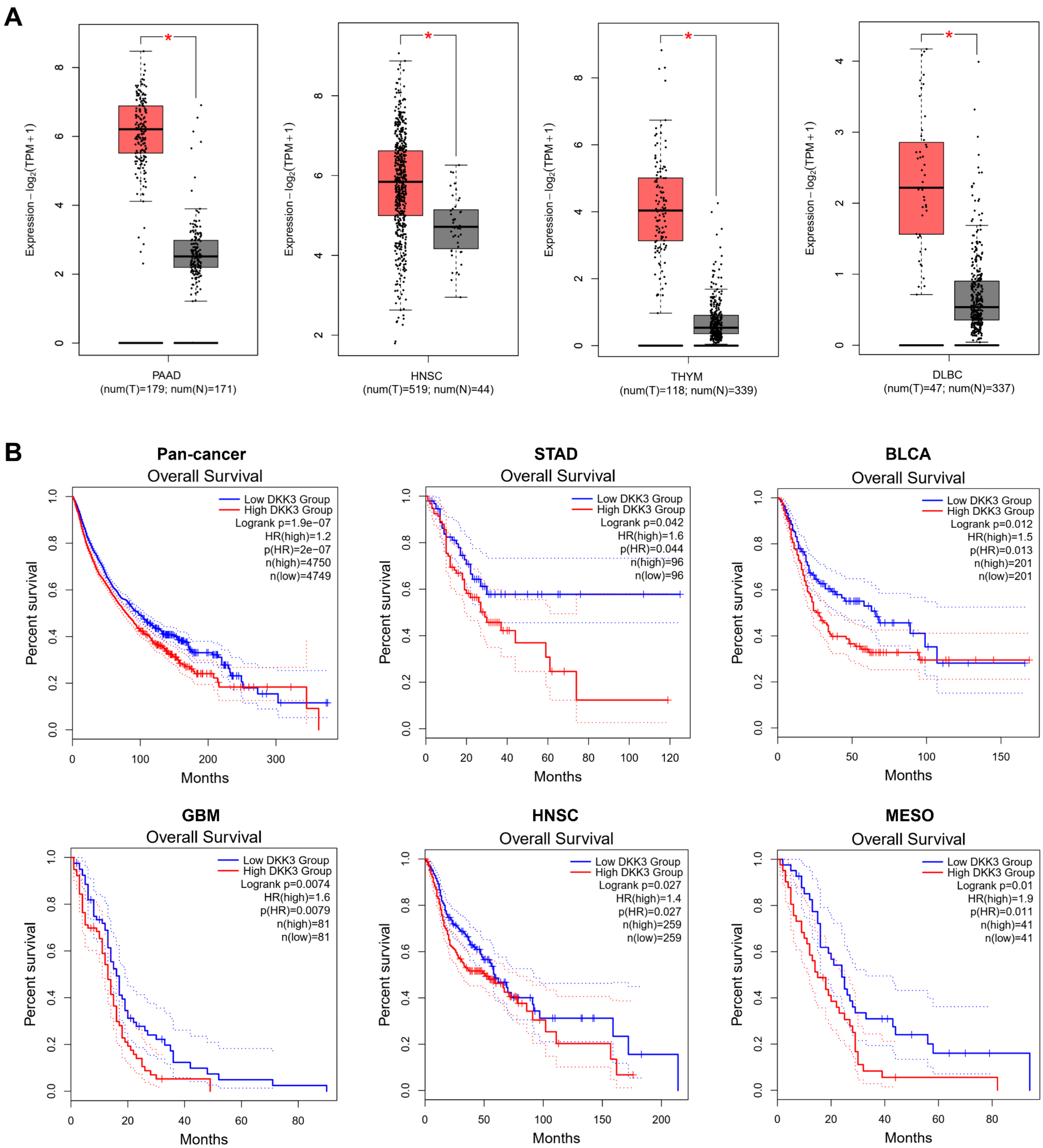


**Fig. 1** High DKK3 expression is associated with worse patient survival across different cancer types. (**A**) DKK3 expression in normal adjacent tissues and tumors of patients from TCGA-PAAD, HNSC, THYM and DLBC. (**B**) The overall survival analysis of patients with high or low DKK3 expression from TCGA (pan-cancer), STAD, BLCA, GMB, HNSC and MESO. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ as calculated by log-rank test or unpaired Student's t-test

be a possible immunotherapeutic target for cancer treatment. The syngeneic mouse colon, lung, pancreatic, and gastric cancer models were generated by subcutaneously (s.c.) challenge with mouse LLC, MC38, MFC, and Pan02 cancer cell lines, followed by treatment with mouse functional DKK3 antibody (DKK3-4.33, 10 mg/kg). As shown in Fig. 4, we found that DKK3 blockade controlled tumor growth in all of these four cancer models, and the survival of mice was also prolonged with anti-DKK3 treatment. Taken together, these in vivo results demonstrated that DKK3 can be a possible and novel treatment target for different cancers.

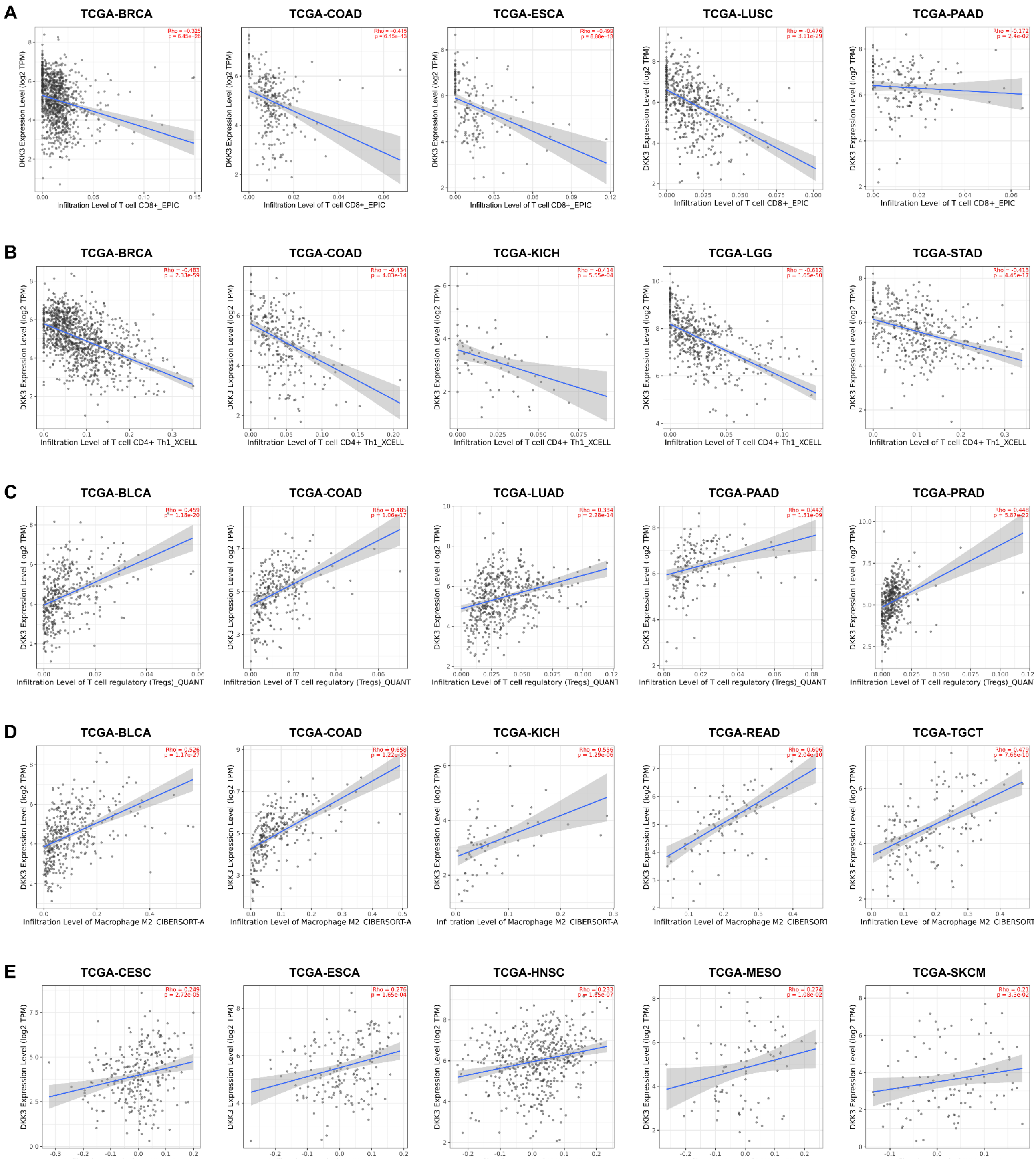


**Fig. 2** DKK3 expression is associated with immunosuppressive TME across different cancer types. (**A**) The interrelation of *DKK3* expression with the infiltration percentage of $CD8^+$ T cells in tumor samples from TCGA-BRCA, COAD, ESCA, LUSC and PAAD. (**B**) The interrelation of *DKK3* expression with the infiltration percentage of Th1 cells in tumor samples from TCGA-BRCA, COAD, KICH, LGG and STAD. (**C**) The interrelation of *DKK3* expression with the infiltration percentage of Treg cells in tumor samples from TCGA-BLCA, COAD, LUAD, PAAD and PRAD. (**D**) The interrelation of *DKK3* expression with the infiltration percentage of M2 macrophages in tumor samples from TCGA-BLCA, COAD, KICH, READ and TGCT. (**E**) The interrelation of *DKK3* expression with the infiltration percentage of MDSCs in tumors from TCGA-CESC, ESCA, HNSC, MESO and SKCM

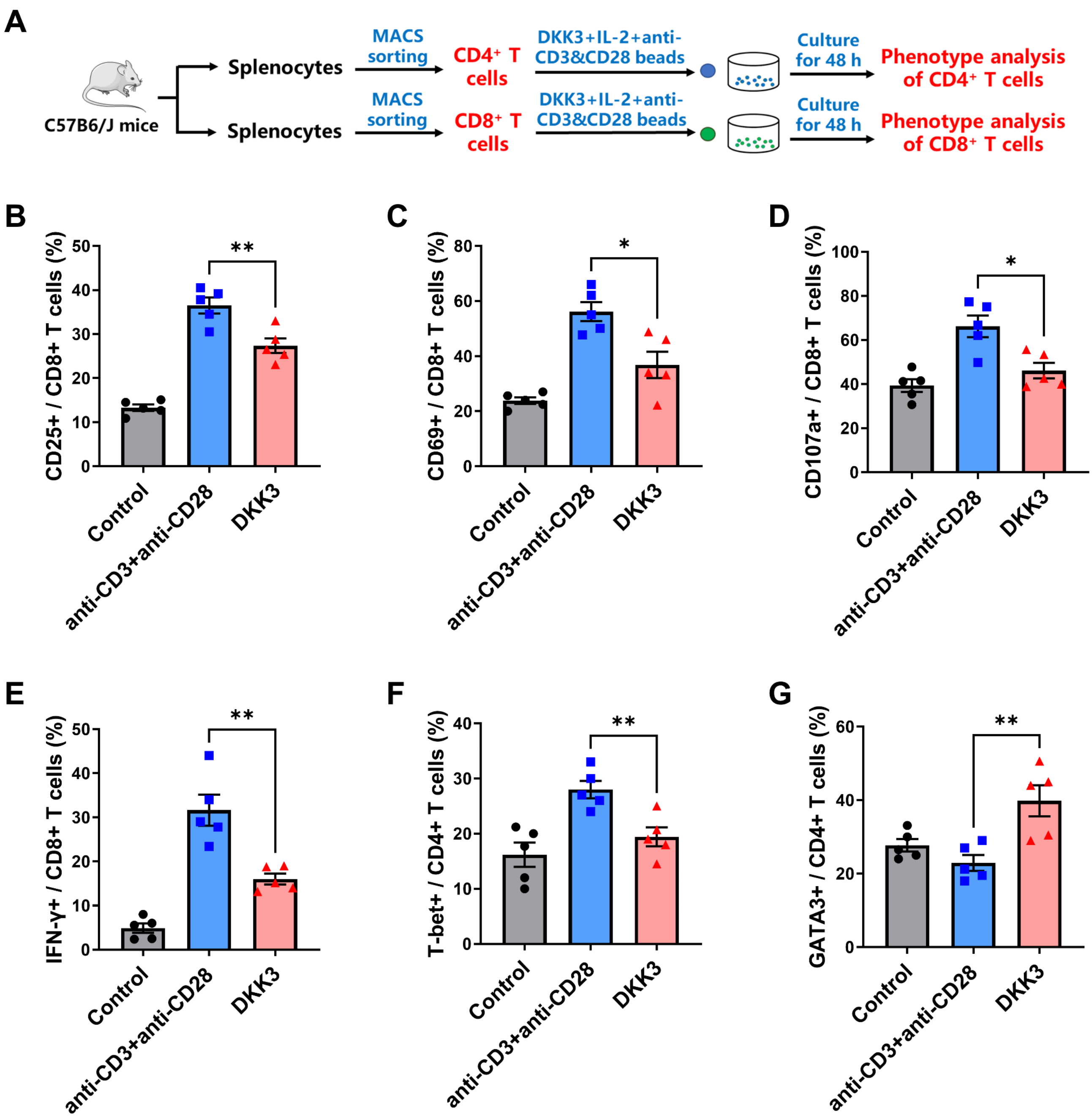


**Fig. 3** DKK3 inhibits $CD8^+$ T cell activation and Th1 differentiation ex vivo. (**A**) Primary $CD4^+$ or $CD8^+$ T cells isolated and sorted by MACS were treated with IL-2 (20 ng/mL) and IL-15 (20 ng/mL), and co-cultured with DKK3 recombinant protein (50 ng/mL). After 48 h co-culture, the activation level of $CD8^+$ T cells was measured by the expressions of (**B**) CD25, (**C**) CD69, (**D**) CD107a and (**E**) IFN-γ by flow cytometry. And the expression of (**F**) T-bet and (**G**) GATA3 in $CD4^+$ T cells were measured by flow cytometry. The unpaired student's t-test is used to determine whether data with error bars are significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$, and ****$P<0.0001$)

### DKK3 blockade remodels the tumor immune microenvironment of different cancers

The considerable tumor-control effects prompted us to investigate the impact of DKK3 blockade in the TIME. The tumor samples from each group were collected at treatment endpoint for flow cytometry detection. As presented in Fig. 5A-C, for adaptive immune response, the proportions of $CD8^+$ T cells, IFN-$\gamma^+$ / $CD8^+$ T cells, and Th1 cells (T-bet$^+$ / $CD4^+$) were evidently increased after DKK3-4.22 treatment in the TIME of LLC, MC38, MFC, and Pan02 mouse cancer models. Also, for innate immune response, the proportions of M2 macrophages ($CD163^+$ / F4/80$^+$) and MDSCs (Gr-1$^+$ / $CD11b^+$) were decreased in LLC, MC38, MFC, and Pan02 tumors

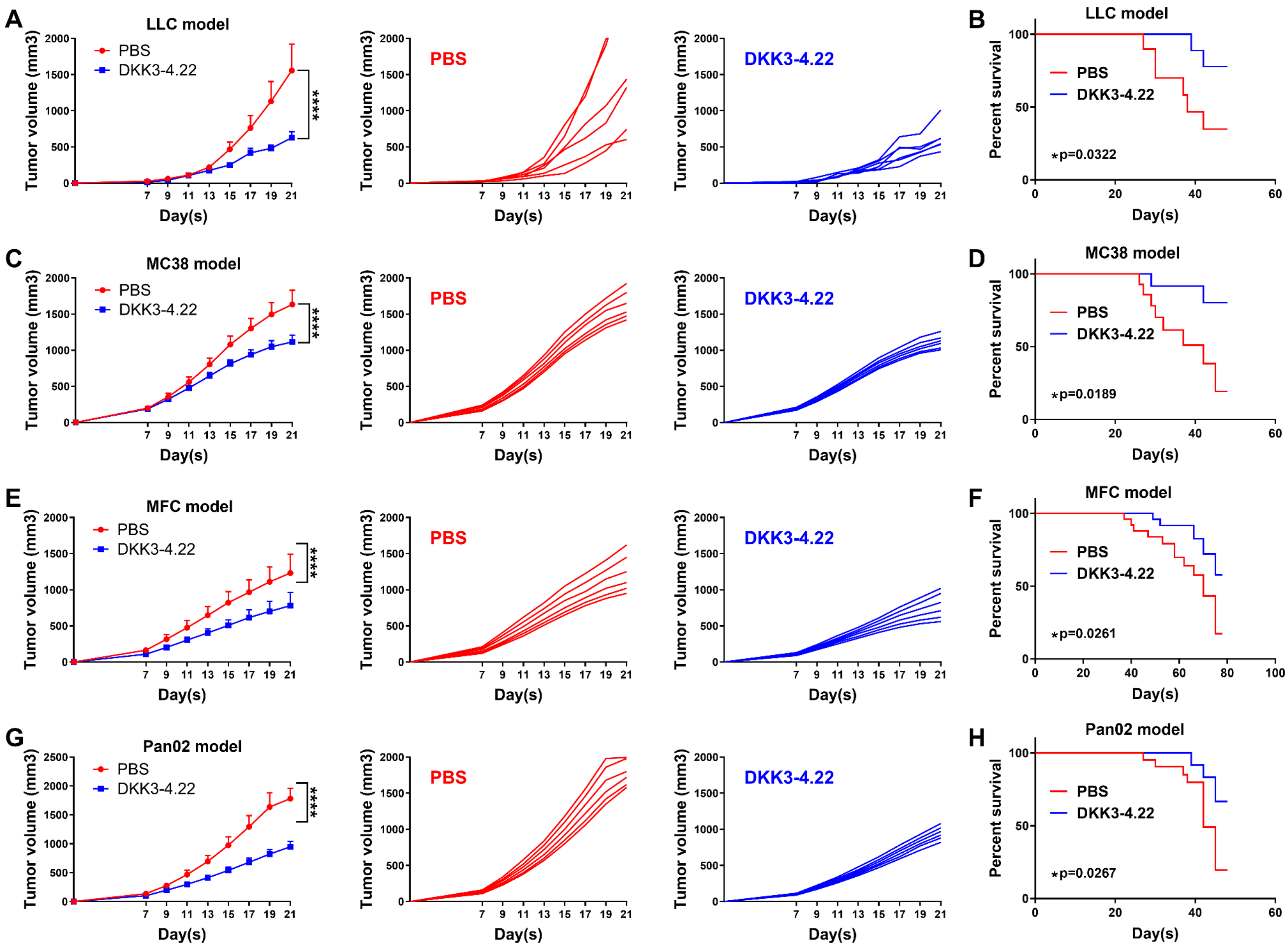


**Fig. 4** DKK3 blockade controls tumor growth in different mouse cancer models. (**A-B**) The tumor volume and overall survival of LLC-challenged mice with or without DKK3-4.22 treatment (10 mg/kg). (**C-D**) The tumor volume and overall survival of MC38-challenged mice with or without DKK3-4.22 treatment (10 mg/kg). (**E-F**) The tumor volume and overall survival of MFC-challenged mice with or without DKK3-4.22 treatment (10 mg/kg). (**G-H**) The tumor volume and overall survival of Pan02-challenged mice with or without DKK3-4.22 treatment (10 mg/kg). The unpaired student's t-test is used to determine whether data with error bars are significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$)

after DKK3-4.22 treatment (Fig. 5D-E). Therefore, these results indicate that DKK3 blockade remodels the TIME of multiple cancer types, including the improvement of both adaptive and innate anti-tumor responses.

### Combined blockade of DKK3 and PD-1 triggers synergistic anti-tumor effects

Finally, we further explored whether DKK3 blockade can enhance the treatment response of anti-PD-1 therapy in the MC38 mouse cancer model. As shown in Fig. 6, while DKK3 or PD-1 blockade alone had anti-tumor effect, the dual blockade of DKK3 and PD-1 brought remarkedly synergistic anti-tumor effect. Thus, DKK3 and PD-1 dual blockade has the possibility to be a new combinational immunotherapy strategy for cancer control.

## Discussion

In this study, we first analyzed and observed that DKK3 expression is abnormally up-regulated in multiple solid cancer types, and high DKK3 expression is also associated with poorer patient overall survival across different cancers. As for the TIME, we observed that DKK3 expression is negatively correlated with $CD8^+$ T cell, Th1 cell infiltration, while positively related to Treg, M2 macrophage, and MDSC infiltration in different tumors. Results from the ex vivo co-cultures demonstrated that DKK3 directly inhibits $CD8^+$ T cell activation and Th1 differentiation. Moreover, by using multiple syngenetic mouse cancer models, we found that DKK3 blockade induces considerable anti-tumor treatment effects in various solid cancers, and both the adaptive and innate anti-tumor immunity are improved after DKK3 blockade. Finally, DKK3 blockade can synergize with anti-PD-1 therapy to bring enhance therapeutic responses for cancer treatments.

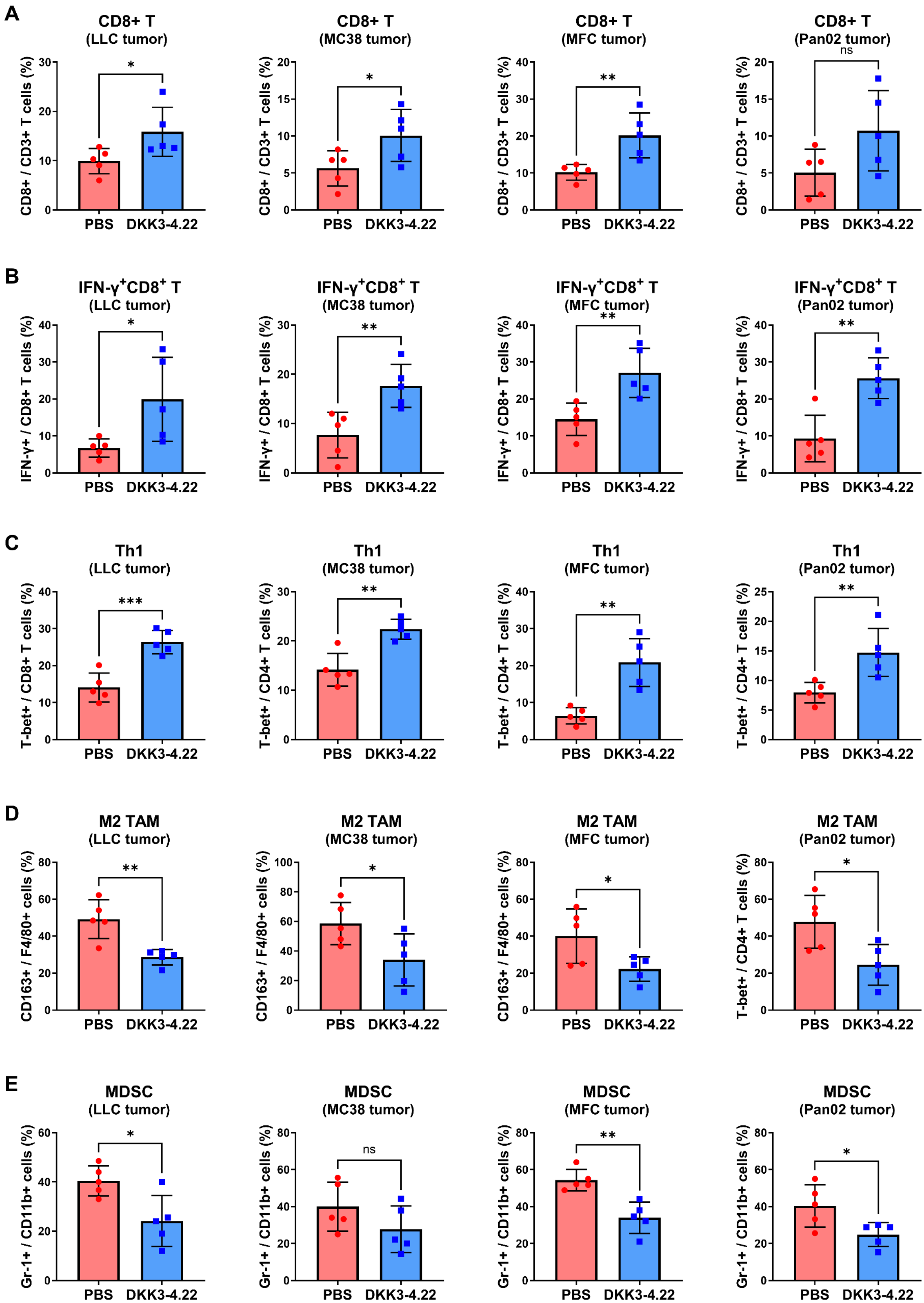


**Fig. 5** DKK3 blockade remodels the tumor immune microenvironment of different cancers. At the treatment endpoint, the percentages of (**A**) CD8+/CD3+ cells, (**B**) IFNγ+/CD8+ T cells, (**C**) T-bet+/CD8+ cells, (**D**) CD164+/F4/80+ cells, and (**E**) Gr-1+/CD11b+ cells in tumors of the syngeneic LLC, MC38, MFC and Pan02 models were detected by flow cytometry ($n=5$ per group). The unpaired student's t-test is used to determine whether data with error bars are significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$, and ****$P<0.0001$)

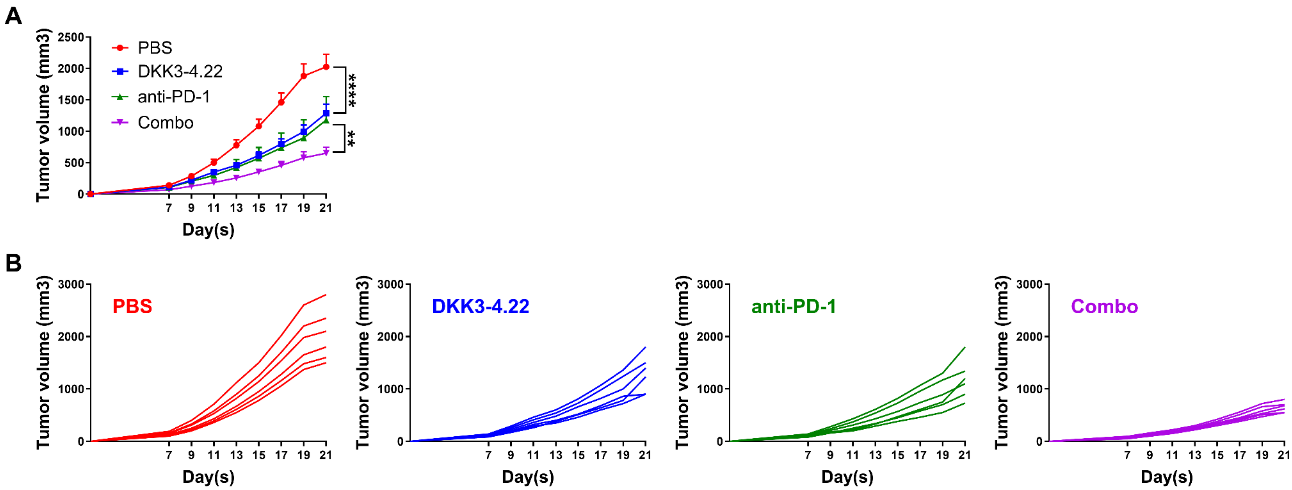


**Fig. 6** Combined blockade of DKK3 and PD-1 triggers synergistic anti-tumor effects. (**A**) The tumor volume of LLC-challenged mice with DKK3-4.22 (10 mg/kg) and / or anti-PD-1 treatment (10 mg/kg). (**B**) The tumor volume in each group of the LLC mouse model. The unpaired student's t-test is used to determine whether data with error bars are significant (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001)

Identifying reliable biomarkers for cancer prognosis and immunotherapy response remains a significant challenge in oncology. Current biomarkers, for example PD-L1 immunohistochemistry (IHC) score and the tumor mutational burden (TMB), often fail to predict responses consistently across different cancer types and patient populations [21–23]. These limitations highlight the need for more precise biomarkers that can capture the complexity of the tumor immune microenvironment. Our analysis revealed that DKK3 is not only upregulated in multiple cancer types but also strongly correlates with poor patient survival and an immunosuppressive tumor microenvironment. This positions DKK3 as a potential biomarker that could provide more nuanced insights into both prognosis and immune status. By assessing DKK3 expression, clinicians may be able to better predict patient outcomes and identify those who are more likely to benefit from immunotherapies, particularly in combination with DKK3-targeted treatments. Thus, DKK3 offers a promising avenue for refining biomarker-based approaches in cancer treatment.

Tumor immunity consists of both adaptive and innate anti-tumor immune responses, which both have critical impact in the TIME and immunotherapy response. T cells, particularly cytotoxic $CD8^+$ T cells and type I helper $CD4^+$ T cells, play crucial roles in tumor immunotherapy by mediating effector or cytotoxic anti-tumor immune responses [24–28]. $CD8^+$ T cells are primarily responsible for directly killing tumor cells by releasing cytotoxic factors like perforin and granzyme [29], while $CD4^+$ T cells, particularly Th1 cells, enhance the immune response by producing cytokines like interferon-gamma (IFN-γ), which support $CD8^+$ T cell activation and sustain immune surveillance against tumors [30]. The effectiveness of many immunotherapies, including ICB, depends on the successful activation and infiltration of these T cell populations into the tumor microenvironment [1, 31, 32]. Our study demonstrated that DKK3 has a direct inhibitory effect on both $CD8^+$ and $CD4^+$ T cells. Specifically, we found that DKK3 suppresses $CD8^+$ T cell activation by reducing the expression of key activation markers such as CD25, CD69, and IFN-γ. Additionally, DKK3 impairs the differentiation of $CD4^+$ T cells into the Th1 subset by downregulating T-bet expression while increasing GATA3 expression. These findings suggest that DKK3 hinders the adaptive anti-tumor response, making it a potential target for enhancing T cell-based cancer immunotherapy.

In addition to T cells, myeloid-derived cells, such as M2 macrophages and MDSCs, also play pivotal roles in regulating the immune response within tumors [33, 34]. Tumor-associated macrophages (TAMs), which are mostly the M2-polarized population, promote tumor progression by suppressing immune activation, supporting angiogenesis, and facilitating tissue remodeling [35–38]. Similarly, MDSCs contribute to tumor growth by inhibiting T cell function and fostering an immunosuppressive microenvironment [39–41]. The accumulation of these cells within tumors is often related to poor prognosis and resistance to immunotherapy, underscoring their importance as therapeutic targets [42]. Our study revealed that blocking Dickkopf-3 (DKK3) significantly improves the innate immune response mediated by myeloid cells. Specifically, we observed a reduction in the infiltration of M2 macrophages and MDSCs following DKK3 blockade in several mouse cancer models. This shift in the myeloid compartment toward a less suppressive phenotype suggests that DKK3 inhibition not only enhances adaptive immune responses but also facilitates a more pro-inflammatory, anti-tumor microenvironment.

Thus, our findings highlight the value of DKK3 as a novel therapeutic target to modulate myeloid cell function and overcome myeloid-driven immunosuppression in cancer. Although we do not explore the exact therapeutic mechanisms of DKK3 blockade for cancer treatment, according to previous studies [43–45], DKK3 blockade may control tumor growth by improving the activation of $CD8^+$ T cells, or reversing the suppressive function of Tregs and M2 macrophages.

While PD-1 blockade has shown great success in treating certain solid cancers, the overall response rates remain suboptimal for many patients. One of the main challenges is the presence of an immunosuppressive TME, which limits the infiltration and function of effector T cells, even in the presence of PD-1 inhibitors [46–48]. Resistance to PD-1 blockade can also arise from factors such as poor T cell priming [49], inadequate antigen presentation [50], suppressive role of TAMs and MDSCs [51–53], or the presence of other immune-inhibitory molecules that dampen the immune response [54]. Our study demonstrates that blocking DKK3 exerts significant anti-tumor effects across multiple mouse cancer models, including lung, colon, gastric, and pancreatic cancer. DKK3 blockade leads to enhanced immune cell infiltration and activation, particularly among $CD8^+$ T cells and Th1 cells. Furthermore, we observed that the combination of DKK3 and PD-1 blockade produces a synergistic anti-tumor response, resulting in greater tumor control and prolonged survival in mouse models compared to either treatment alone. These findings suggest that dual blockade of DKK3 and PD-1 may overcome the limitations of PD-1 blockade monotherapy and represents a promising new immunotherapeutic strategy for cancer treatment. However, we only evaluated the effect of combined blockade of DKK3 and PD-1 treatment in MC38 mouse model, and more models and cancer types need to be explored for the future clinical evaluation.

Despite the current findings of this study, there are several limitations / challenges to consider. First, some results of our study are based on the retrospective design which lacks a control arm. Second, we only use TCGA samples and do not include their own samples or data. Third, the precise molecular mechanisms by which DKK3 influences the immune microenvironment need to be further elucidated to optimize its use in combination therapies. Forth, while our results in multiple mouse cancer models are encouraging, further clinical studies are needed to validate the predictive value of DKK3 and therapeutic potential of DKK3 blockade in cancer patients. These limitations / challenges need to be further explored in the future so that our preliminary results can be validated for clinical utility.

In summary, based on both bioinformatic, ex vivo, and in vivo explorations, this study offers novel insights into the impact of DKK3 in cancer progression and tumor immunity, and supports DKK3 as a new treatment target for enhance cancer immunotherapy.

## Methods and materials

### Patient data analysis

Gene expression profiles along with paired clinical information from patients with various cancer types, including both tumor and the adjacent normal tissues, were retrieved from the website (portal.gdc.cancer.gov) of The Cancer Genome Atlas (TCGA). DKK3 expression levels were divided into high and low (half-cut) groups, and overall survival was analyzed accordingly. Data handling and analysis were performed mainly using the R (version 4.1.1) alongside GraphPad Prism 7 for graphical visualization.

### Cell lines

Murine cell lines, including LLC, MC38, MFC, and Pan02, were obtained from the Shanghai Institute of Biochemistry and Cell Biology Cell Bank (Shanghai, China). The cells were cultured in RPMI-1640 medium (Corning), which was supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/mL, Beyotime), and streptomycin (100 μg/mL, Beyotime). Incubation was carried out at 37 °C in with 5% CO2. Routine mycoplasma testing was regularly performed using PCR. All cell lines used in the experiments were limited to fewer than 10 passages, and their authenticity was confirmed through short tandem repeat (STR) analysis in September 2020.

### Mice and animal models

For the establishment of murine cancer models, syngeneic mice were employed. Specifically, 615 mice, used for MFC-based experiments, were purchased and obtained from the Institute of Hematology, Chinese Academy of Medical Sciences (Tianjin, China). C57B6/J mice, utilized for LLC, MC38, and Pan02 cell lines, were sourced from Jicui Experimental Animal Co., Ltd (Nanjing, China). All animals were kept in pathogen-free housing conditions at Huai'an Cancer Hospital. Mice aged from 6 to 12 weeks of age, both male and female, were assigned randomly into experimental groups based on body weight and age. All mice used in this study were humanely euthanized using carbon dioxide ($CO_2$) asphyxiation, following the ethical guidelines and protocols approved by the Institutional Animal Care and Use Committee (IACUC). Mice were placed in a chamber, and $CO_2$ was introduced gradually, at a flow rate that displaces 30–70% of the chamber volume per minute, as recommended by the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals. The animals remained in the chamber until unconsciousness was confirmed by the cessation of movement, followed by the absence of a heartbeat and respiration. No additional anesthetics were administered as $CO_2$ alone was deemed sufficient for the humane and effective euthanasia of the animals. This method was chosen for its rapidity, ease of application, and minimal discomfort for the mice. Huai'an Cancer Hospital's Institutional Animal Care and Use Committee approved all the in vivo experiments using mice (protocol number: 2023AE00964).

### Generation of tumor models in mice

To create subcutaneous tumor models, cells from the LLC (5 × 10^5 for each mouse), MC38 (5 × 10^5 for each mouse), MFC (1 × 10^6 for each mouse), and Pan02 (2 × 10^5 per mouse) cell lines were injected s.c. into 8-12-week-old sex-matched mice ($n = 6$ for each experimental group). Tumor development was monitored daily. Tumor dimensions were detected using calipers, and volumes were calculated under the formula: volume = L × W² / 2, where L represents the longest dimension and W the perpendicular shorter dimension. Tumors were harvested for analysis three weeks post-injection.

### Mouse treatments in vivo

Following tumor establishment, mice were randomly assigned to different treatment arms. They received intraperitoneal injections of 10 mg/kg of a mouse monoclonal DKK3 antibody (DKK3-4.22, BE0385, Bio X Cell), a mouse functional PD-1 antibody (BE0146, Bio X Cell), or a combination of both. Injections were administered 2–3 times weekly until the study endpoint.

### Tumor tissue single-cell suspension Preparation

Tumor tissue was excised and processed to generate single-cell suspensions. An enzymatic digestion of tissue was performed using collagenase IV (MCE) at a concentration of 1 mg/mL of MCE, and DNase I (MCE) at a concentration of 100 U/mL of MCE in serum-free RPMI-1640 medium. The digestion was carried out at 37 °C for 1 h. Suspensions were then filtered through 100-μm nylon strainers (Corning), followed by washing with PBS. Using Biosharp's red blood cell lysis buffer, red blood cells were removed, and the remaining cells were washed thoroughly before use.

### Procedures of flow cytometry

Single-cell suspensions were collected and prepared either from tumor tissues or from T cells cultured in vitro. To examine cell surface marker expression, cells were stained with fluorochrome-conjugated antibodies targeting CD45, CD3, CD4, CD8, CD11b, CD163, F4/80, and Gr-1. Staining was performed for 15–30 min at 4 °C, followed by two PBS washes. Intracellular markers such as IFN-γ, T-bet, and GATA3 were stained using the True-Nuclear™ Transcription Factor (Foxp3) Buffer Set (Beyotime) or the Leukocyte Activation Cocktail (BD Biosciences) for intracellular detection. Flow cytometric analysis was conducted on a BD Accuri C6 PLUS system (BD Biosciences), and the data were analyzed with the FlowJo software (version 10.5, Tree Star). Antibodies used included: CD45 (FITC, #157617), CD3 (PE-Cy7, #100203), CD8 (APC, #100711), CD4 (BUV615, #102457), CD11b (SB550, #101231), F4/80 (FITC, #123107), and Gr-1 (PE, #108407), CD163 (PE, #156703) from Biolegend. For intracellular markers, the antibodies used were IFNγ (APC, Biolegend, #505809), T-bet (PE, #644810), and GATA3 (APC, #653806).

### T cell culture and stimulation ex vivo

Mouse T cells including $CD4^+$ and $CD8^+$ subpopulations were collected and sorted from the spleens of 8-week-old female C57B6/J mice using magnetic cell separation kits (Miltenyi Biotec, #1301-105-075 for $CD8^+$ T cells and #132-106-454 for $CD4^+$ T cells). Cells were sorted using MACS buffer, prepared with PBS containing 0.5% bovine serum albumin (Beyotime) and 2mM EDTA. Isolated T cells were activated in RPMI-1640 medium supplemented with 10–15% FBS, 20 ng/mL murine recombinant IL-2 (Prepotech), and Dynabeads™ Mouse T-Activator (Gibco). Cultured cells were incubated for 3–7 days at 37 °C with 5% CO2. For experimental treatments, $CD8^+$ and $CD4^+$ T cells were administrated with 50 ng/mL recombinant murine DKK3 for 48 h, followed by flow cytometry analysis to assess activation markers (CD25, CD69, CD107a, IFN-γ) and differentiation markers (T-bet, GATA3).

### Statistical analysis

R (4.1.1) and GraphPad Prism 7 were used for all the statistical analyses in this study. Differences between two groups were evaluated by a two-tailed Student's t-test for data which are normally distributed, and the Wilcoxon rank-sum test was employed for data which are not non-normally distributed. Survival data were analyzed using the Kaplan-Meier curves, and comparisons between different survival groups were made using the log-rank test. Results are presented as mean ± standard error of the mean (SEM), with significance thresholds: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

#### Abbreviations

| | |
|---|---|
| TIME | Tumor immune microenvironment |
| DKK3 | Dickkopf-3 |
| TCGA | The Cancer Genome Atlas |
| TMB | Tumor mutation burden |
| PAAD | Pancreatic Adenocarcinoma |
| THYM | Thymoma |
| HNSC | Head and Neck Squamous Cell Carcinoma |
| and DLBC | Diffuse Large B-Cell Lymphoma |
| STAD | Stomach Adenocarcinoma |
| BLCA | Bladder Urothelial Carcinoma |

| | |
|---|---|
| GMB | Glioblastoma Multiforme |
| MESO | Mesothelioma |
| COAD | Colon Adenocarcinoma |
| BRCA | Breast Carcinoma |
| ESCA | Esophageal Carcinoma |
| LUSC | Lung Squamous Cell Carcinoma |
| LUAD | Lung Adenocarcinoma |
| PRAD | Prostate Adenocarcinoma |
| TGCT | Testicular Germ Cell Tumors |
| READ | Rectum Adenocarcinoma |
| CESC | Cervical Squamous Cell Carcinoma |
| SKCM | Skin Cutaneous Melanoma |
| Combo | Combination |

**Acknowledgements**
This work was funded by The Youth Development Fund of Huai'an Cancer Hospital (2023PY00264). The funding source had no role in the project design, data collection, data analysis, or writing of this study.

**Author contributions**
K.S. conducted the experiments, Y.Z., H.Y., and X.Z. made analyzes, Z.C. wrote and prepared the manuscript. All authors reviewed the manuscript.

**Funding**
The Youth Development Fund of Huai'an Cancer Hospital (2023PY00264).

**Data availability**
All data is provided within the manuscript.

## Declarations

**Ethics approval and consent to participate**
Huai'an Cancer Hospital's Institutional Animal Care and Use Committee approved all of the in vivo experiments using mice (protocol number: 2023AE00964).

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

**Conflict of interest**
There are no potential conflicts of interest among the authors.

Received: 13 September 2024 / Accepted: 2 April 2025
Published online: 09 April 2025

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