NKG2A⁺CD8⁺ T cells infiltration determines immunosuppressive contexture and inferior response to immunotherapy in clear cell renal cell carcinoma

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ABSTRACT

Background Immunotherapy is gaining momentum, but current treatments have limitations in terms of beneficiaries. Clear cell renal cell carcinoma (ccRCC) harbors the highest expression of human leukocyte antigen E (HLA-E), ligand of NKG2A, among all solid tumors. In this study, we aim to investigate the role of NKG2A⁺CD8⁺ T cells in tumor microenvironment and its potential as a novel target in ccRCC.

Methods This study included four independent cohorts, including 234 patients from Zhongshan cohort (ZSHC) who underwent partial or radical nephrectomy at Zhongshan Hospital, and 117 metastatic patients from metastatic Zhongshan cohort (ZSHC-metastatic renal cell carcinoma) who were treated with immune checkpoint inhibitor or tyrosine kinase inhibitor alone. We also incorporated a cohort of 530 patients diagnosed with ccRCC from The Cancer Genome Atlas (referred to as TCGA-kidney renal clear cell carcinoma) and 311 patients from CheckMate cohort for bioinformatics exploration and hypothesis validation. Fresh surgical specimens from 15 patients who underwent ccRCC surgery at Zhongshan Hospital were collected for flow cytometry analysis. Another 10 fresh surgical specimens were used to investigate the therapeutic potential of NKG2A blockade after in vitro intervention. The infiltration of NKG2A⁺CD8⁺ T cells was assessed using immunohistochemical staining, flow cytometry, and immunofluorescence staining in ZSHC cohort.

Results Patients with higher infiltration of NKG2A⁺CD8⁺ T cells in ccRCC exhibited shorter overall survival and resistance to immunotherapy. NKG2A⁺CD8⁺ T cells expressed upregulated checkpoint molecules and displayed impaired effector functions, along with tissue-residency characteristics. Combination of programmed cell death protein-1 (PD-1) blockade and NKG2A blockade demonstrated an enhanced capability in reactivating CD8⁺ T cells effector functions.

Conclusion Intense infiltration of NKG2A⁺CD8⁺ T cells were associated with poorer prognosis and response to immunotherapy. NKG2A blockade combined with current immunotherapy exhibited a robust ability to reactivate CD8⁺ T cells effector functions.

INTRODUCTION

Renal cell carcinoma (RCC) is one of the three major malignant tumors of the urinary system.¹ In 2020, there were over 400,000 new cases of RCC globally,² among which clear cell renal cell carcinoma (ccRCC) accounts for about 75% and serves as the most common pathological type.³ Compared with high 5-year survival probability (over 80%) of patients with non-metastatic localized
Immunotherapy has revolutionized the treatment of mRCC over the past decade. Compared with tyrosine kinase inhibitors (TKI) alone, latest results from phase III trials demonstrated that combination therapy based on immune checkpoint blockade (ICB) plus TKI significantly improved the outcomes of patients with mRCC. However, durable responses only achieved in a subset of patients with mRCC. Treatment options after progression are limited and usually far from efficacious. Therefore, exploration of new treatment approaches for patients with mRCC are urgently needed.

Interestingly, unlike other tumors, intense infiltration of CD8+ T cells correlated with poorer prognosis in ccRCC, which is likely due to functional subdivision of distinct CD8+ T cells subtypes. Our group previously also found that in ccRCC, TNFRSF9+CD8+ T cells possessed both exhaustion and effector phenotype and CXCL13+CD8+ T cells possessed exhausted phenotype with impaired antitumor function. NKG2A is a C-type lectin family member that can form dimers with CD94 on the cell surface. Unlike the programmed cell death protein-1 /programmed cell death ligand 1 (PD-1/PD-L1) pathway, NKG2A acts independently of the T-cell receptor (TCR). The non-classical major histocompatibility complex-I molecule human leukocyte antigen-E (HLA-E) is the main ligand for NKG2A-CD94. And among solid tumors, ccRCC represents the highest level of HLA-E expression. NKG2A is reported to be mainly expressed in CD8αβ T cells, CD56hi natural killer (NK) cells and natural killer T (NKT) cells. NKG2A-CD94 binding to HLA-E can inhibit the activation of T cells and NK cells. Previous studies suggested that NKG2A antagonism primarily functions through NKG2A+CD8+ T cells rather than NKG2A- NK cells in tumor vaccine therapy resistant individuals. However, the role of NKG2A+CD8+ T cells in tumor microenvironment (TME) and the therapeutic effect of targeting NKG2A in ccRCC remained unknown.

In the present study, we sought to investigate the specific phenotype of NKG2A+CD8+ T cells in ccRCC and tried to uncover its relationship with immune contexture. We further explored its predictive value for prognosis and responses to ICB. Ultimately, we sought to evaluate the potential of combining NKG2A antagonism with existing immunotherapy as a new treatment modality for patients with ccRCC.

MATERIALS AND METHODS

Study cohorts
Two independent in-house cohorts were enrolled in this study, namely Zhongshan Hospital cohort (ZSHC, n=234), and Zhongshan Hospital mRCC cohort (ZSHC-mRCC, n=117).

Zhongshan Hospital cohort (ZSHC) included 290 patients with ccRCC who underwent radical nephrectomy or partial nephrectomy from the Department of Urology, Zhongshan Hospital, Fudan University (Shanghai, China) between February 2005 and June 2007. The inclusion criteria for this study were as follows: (1) signed informed consent; (2) diagnosed with ccRCC pathologically; (3) age ≥18 years; (4) never received any systematic antitumor therapy before the surgery; and (5) with available clinicopathological data. 21 patients were excluded due to tissue microarray point detachment. 35 patients were excluded due to unassessable NKG2A or CD8 staining. Finally, 234 patients were recruited for further study.

Zhongshan Hospital mRCC cohort (ZSHC-mRCC) enrolled 128 patients with RCC (62 patients treated with ICB and 66 patients treated with TKI alone) from the Department of Urology, Zhongshan Hospital, Fudan University (Shanghai, China) between April 2012 and July 2023. The inclusion criteria for this cohort were as follows: (1) informed consent, (2) patients with RCC suffering metastasis, (3) available for formalin fixed paraffin embedded specimens. 11 patients were excluded due to their pathology type not being ccRCC. Finally, 117 patients were recruited for further study (58 patients treated with ICB and 59 patients treated with TKI alone).

Therapeutic regimens of patients in ZSHC-mRCC (ICB) were shown in online supplemental table 1. Response to ICB and TKI alone were assessed using Response Evaluation Criteria in Solid Tumors V.1.1.

Public data processing
The Cancer Genome Atlas kidney renal clear cell carcinoma cohort (TCGA-KIRC, n=530) and the CheckMate cohort (CheckMate, n=311) were included for external validation.

TCGA-KIRC cohort included 530 patients, with both clinicopathological and RNA-sequencing data available. Data was downloaded from UCSC Xena (https://xenabrowser.net/datapages/).

CheckMate cohort including three cohorts (CheckMate-025, CheckMate-009, CheckMate-010), are randomized phase III trials to compare nivolumab with everolimus in patients with advanced RCC. 8 20 21 31 11 patients with both clinical information and RNA-sequencing data were included for further investigation. Data was acquired from previous study.

Immunohistochemistry and immunofluorescence
This study conducted immunohistochemical (IHC) staining on NKG2A, NKG2A+CD8+ T cells and 13 types of tumor-infiltrating immune cells in the ZSHC and ZSHC-mRCC cohort. The antibody information was shown in online supplemental table 2. The specific experimental procedures were consistent with our previous study. The infiltration level of NKG2A+ cells and immune cells were evaluated under microscopy by two independent pathologists with relevant pathological reading abilities without knowledge of patient clinical information.
Immunofluorescence staining was used to evaluate the infiltration level of NKG2A⁺CD8⁺ T cells in the ZSHC cohort. The specific experimental procedures followed previous research. Primary antibodies included NKG2A (ab260035, Abcam) and CD8 (ab17147, Abcam), then using Bond Polymer Refine Detection (DS9800, Leica). The infiltration level of NKG2A⁺CD8⁺ T cells was assessed by two independent pathologists in the 3DHISTECH-VIEWER software without knowledge of patients’ clinical information. The average of the results from these two pathologists was calculated as the final value.

In our study, two pathologists (Dr Yunyi Kong and Dr Lingli Chen) who were blinded to the clinico-pathological data and scored all samples separately. The mean count derived from their respective evaluations was used as the basis for analysis. Instances of enumeration discrepancies exceeding five cells were subject to individual re-evaluation by both pathologists, with the objective of reaching a consensus.

**Construction of immune cells signature**

We also used a single-cell sequencing data of ccRCC to construct signatures of NKG2A⁺CD8⁺ T cells and NKG2A⁺ NK cells. The expression matrix obtained from the single-cell sequencing was further clustered to define CD8⁺T-cell subgroups. Subsequently, NKG2A⁺ cells (KLRC1: counts >0) were further clustered within the CD8⁺ T-cell group. FindMarkers from “Seurat” package was then employed to define the marker gene set for the NKG2A⁺CD8⁺ T cells subpopulation. Ultimately, 14 genes with significant differences were identified as the signature of NKG2A⁺CD8⁺ T cells. Similarly, NKG2A⁺ NK cells were also classified using the FindMarkers function to obtain the signature of NKG2A⁺ NK cells. Gene list of NKG2A⁺CD8⁺ T cells signature and NKG2A⁺ NK cells signature were shown in online supplemental table 3.

**Flow cytometry**

Twenty-five patients who received radical nephrectomy or partial nephrectomy from March 2022 to January 2023 were included in this study at Zhongshan Hospital, Fudan University. After complete removal of the tumor specimens, fresh tumor tissues were selected by cutting along the longest axis of the tumor at its base, while avoiding necrotic tissue. None of the patients received systemic antitumor treatment before surgery. All patients signed informed consent forms for the donation of biological samples and the use of related information. Among them, four specimens were excluded due to contamination, and six cases were excluded because the follow-up pathology reports indicated non-ccRCC. The final cohort for flow cytometry (FCM) experiments on ccRCC at Zhongshan Hospital consisted of 15 remaining specimens.

Specimens were digested with collagenase IV (0.05 g/40 mL prepared with penicillin–streptomycin antibodies) and Golgi blockade agent (1 µL/1 mL). The digestion was performed at 37°C for about 2 hours to obtain a single-cell suspension. Erythrocytes were removed by adding a red blood cell lysis buffer. Then Fc-receptor-blocking antibody (BD Bioscience) was used. Fixable Viability Stain 510 cell viability staining reagent was added and incubated for 20 minutes, followed by cell membrane antibody staining. If intracellular antibody staining or transcription factors were required simultaneously, a Fixation/Permeabilization Solution Kit or Transcription Factor Fixation/Permobilization Buffer was used. FCM analysis was performed using BD FACSCelesta, and the results were analyzed using FlowJo V.10.8.1. online supplemental table 4 provides a list of FCM antibodies.

**In vitro intervention assay**

Tumor tissues from 10 patients with pathologically diagnosed ccRCC were included for in vitro intervention. Tumor single-cell suspensions were cultured with anti-NKG2A antibody (IM2750, BECKMAN COULTER, 3 µg/mL), or anti-PD-1 antibody (nivolumab, A2002, Selleck, 5 µg/mL), or IgG4B κ isotype control antibodies (A1101, Biovision, 10 µg/mL) for 12 hours in Roswell Park Memorial Institute medium 1640 (RPMI-1640 medium) with a concentration of around 10% fetal bovine serum. After overnight culture, the suspensions were performed FCM analysis as described above.

**Statistical analysis**

For each sample, the average expression of all genes in a given signature was computed. The involved genes for signature calculation were listed in online supplemental table 5. The results will be presented as mean±SD or as proportions, as needed. For the comparison between grouped continuous variables, Student’s t-test and Mann-Whitney U test would be used; Wilcoxon signed-rank test was used for paired samples comparison and Spearman’s test would be employed for correlation analysis of continuous variables. The comparison of rates or proportions between groups was used the χ² test, and if T<1 or n<40, Fisher’s exact test would be used. Kaplan-Meier curves and log-rank test were used to analyze the survival differences between subgroups. Cox regression analysis was employed to assess whether the infiltration level of NKG2A⁺CD8⁺ T cells could independently impact prognosis. All hypothesis tests considered p<0.05 as statistically significant, and all tests would be two-tailed.

**RESULTS**

**NKG2A⁺CD8⁺ T cells are associated with poor prognosis in ccRCC**

First, we sought to compare the expression of NKG2A between tumor and adjacent normal tissues. In ZSHC, IHC staining confirmed overexpression of NKG2A in tumor tissues (figure 1A, figure 1B and online supplemental figure 1A; p<0.001). Furthermore, in TCGA-KIRC, NKG2A messenger RNA (mRNA) expression was also significantly upregulated in tumor tissues compared with adjacent normal tissues (figure 1C; p<0.001).
Previous studies indicated that NKG2A is predominantly expressed both in CD8+ T cells and NK cells. Therefore, we employed FCM to determine its expression in ccRCC. In compliance with the previous studies, we identified the presence of both NKG2A+CD8+ T cells and NKG2A+ NK cells in ccRCC (online supplemental figure 1B). And the average expression of NKG2A on CD8+ T cells is 12.8%, while NK cells exhibit an average NKG2A expression of 29.7% (online supplemental figure 1C).

Next, we attempted to explore the prognostic value of NKG2A+CD8+ T cells and NKG2A+ NK cells in ccRCC. Using single-cell sequencing data, we developed NKG2A+CD8+ T cells signatures and NKG2A+ NK cells signatures. In TCGA-KIRC, patients with higher expression of NKG2A+CD8+ T cells signature had worse prognosis (figure 1D; p=0.004), while the NKG2A+ NK cells signature could not indicate patient prognosis.

Figure 1 NKG2A+CD8+ T cells are associated with poor prognosis in ccRCC. (A) Representative immunohistochemical images of NKG2A+ cells in ccRCC tissues (scale bar, 20 µm). (B–C) Boxplots comparing the expression of NKG2A in ccRCC tissues and adjacent normal tissues in ZSHC cohort (B) and TCGA-KIRC (C) cohort. Data were analyzed by Mann-Whitney U test. (D–E) NKG2A+CD8+ T cells signature predict prognosis of patients with ccRCC (D) while NKG2A+ NK cells signature could not predict prognosis of patients with ccRCC (E). Kaplan-Meier analysis and log-rank test. (F) Immunofluorescence staining of NKG2A (red), CD8 (green) and DAPI (blue) in TMA of ZSHC cohort, double-positive cells are highlighted by the yellow arrow. The green and red arrows identify CD8 and NKG2A single-positive cells, respectively (scale bar, 20 µm). (G–H) Expression quantities of NKG2A+CD8+ T cells under high magnification were compared among patients in the SSIGN scoring system (G) as well as UISS staging system (H). For the SSIGN, patients with scores of 0–1 were classified as low-grade, those with scores of 2–6 as intermediate-grade, and those with scores ≥7 as high-grade. Kruskal-Wallis test was applied. (I–J) Figures demonstrating the overall survival (I) and recurrence-free survival (J) of patients in the ZSHC cohort, stratified by infiltration of NKG2A+CD8+ T cells and median value was used as the cut-off. Kaplan-Meier analysis and log-rank test. *p<0.05, **p<0.01, ***p<0.001, ns refers to no significance. ccRCC, clear cell renal cell carcinoma; DAPI, 4',6-diamidino-2-phenylindole; HPF, high power field; KIRC, kidney renal clear cell carcinoma cohort; mRNA, messenger RNA; NK, natural killer; SSIGN, Mayo Clinic Stage, Size, Grade, Necrosis Score; TCGA, The Cancer Genome Atlas; UISS, the University of California, Los Angeles, Integrated Staging System.
NKG2A+CD8+ T cells display an exhausted phenotype

To assess the functional phenotype of NKG2A+CD8+ T cells, we found that higher infiltration of NKG2A+CD8+ T cells was associated with reduced overall survival (OS) and recurrence-free survival (RFS) in ccRCC (figure 1G and figure 1H; p=0.047 and p=0.049, respectively). Furthermore, we found that higher infiltration of NKG2A+CD8+ T cells was associated with reduced overall survival (OS) and recurrence-free survival (RFS) in ccRCC (figure 1G and figure 1H; p=0.047 and p=0.049, respectively). Moreover, multivariable analysis incorporating clinical and pathological factors indicated that NKG2A+CD8+ T cells infiltration was an independent predictor for OS (online supplemental figure 2). In summary, NKG2A expression was upregulated in ccRCC, and higher infiltration of NKG2A+CD8+ T cells correlated with poorer prognosis.

NKG2A+CD8+ T cells infiltration is associated with immunosuppressive tumor microenvironment in ccRCC

In the intricate milieu of the tumor TME, tumor-infiltrating immune cells play diverse roles.25 The functionality of CD8+ T cells, in particular, can be modulated by the presence and interactions with other immune cells.26 To understand the mechanisms underlying the exhausted phenotype of NKG2A+CD8+ T cells, we then scrutinized the relationship between NKG2A+CD8+ T cells and other immune cells infiltration. First, in the TCGA-KIRC and CheckMate cohort, the differences of immune cells infiltration were assessed between two groups with different expressions of NKG2A+CD8+ T-cell signature. The results revealed that patients with higher infiltration of NKG2A+CD8+ T cells had higher infiltration of CD8+ T cells, regulatory CD4+ T (Treg) and T follicular helper (Tfh) cells compared with patients with lower expression of NKG2A+CD8+ T cells signature (figure 3A; p<0.001, p<0.001 and p<0.001, respectively). On the other hand, patients with higher infiltration of NKG2A+CD8+ T cells had lower infiltration of CD4+ T cells, Mast cells, monocyte and M2 macrophages (figure 3A; p<0.001, p<0.001, p<0.001 and p<0.001, respectively). Additionally, the total infiltration of macrophages was higher in patients with lower infiltration of the NKG2A+CD8+ T cells group (figure 3A; p<0.001). Analysis data of CheckMate cohort confirmed that patients with intense infiltration of NKG2A+CD8+ T cells had high infiltration of T cells such as CD8+ T cells, whereas myeloid cells such as macrophages showed lower infiltration (figure 3B).

We also evaluated the expression of immune checkpoints between two groups. Patients with higher infiltration of the NKG2A+CD8+ T cells showed higher expression of immune checkpoints such as PD-1, CTLA-4 and LAG-3 at the mRNA level (figure 3AB, C; p<0.001, p<0.001 and p<0.001, respectively).

In order to evaluate the overall immune microenvironment, we employed a commonly used immune score.27 The results demonstrated that patients with higher infiltration of the NKG2A+CD8+ T cells had significantly higher immune scores (figure 3AB; p<0.001 and p<0.001, respectively), indicating a stronger immune response of these patients, characterized by increased immune cells infiltration and elevated cytokine levels. However, simultaneously, patients with higher infiltration of the NKG2A+CD8+ T cells also exhibited significantly higher suppression scores (figure 3AB; p<0.001 and p<0.001, respectively),28 and immune checkpoint score,29 suggesting these patients harbored an immunosuppressed microenvironment (figure 3AB; p<0.001 and p<0.001, respectively).

Subsequently, we also conducted IHC in the ZSHC, the infiltration levels of 13 types of immune cells were identified and evaluated. The results showed that patients with higher infiltration of NKG2A+CD8+ T cells had more CD8+ T cells and Treg cells infiltration (figure 3C and online supplemental figure 3A; p=0.011 and p=0.006, respectively), while lower infiltration of M2 macrophages...
Figure 2  NKG2A⁺CD8⁺ T cells display an exhausted phenotype. (A–E) The expression of immune checkpoints (PD-1⁺, LAG-3⁺, CTLA-4⁺, TIM-3⁺, CD39⁺) were significantly higher in NKG2A⁺CD8⁺ T cells, compared with NKG2A⁻CD8⁺ T cells (gated on CD3⁺CD8⁺ T cells). (F–H) The expression of IFN-γ was decreased in NKG2A⁺CD8⁺ T cells, while the expression of TNF-α and GZMB remained unaffected (gated on CD3⁺CD8⁺ T cells). (I) The expression of CD107α was increased in NKG2A⁺CD8⁺ T cells, compared with NKG2A⁻CD8⁺ T cells (gated on CD3⁺CD8⁺ T cells). (J) Tissue-resident marker (CD103) was upregulated in NKG2A⁺CD8⁺ T cells (Gated on CD3⁺CD8⁺ T cells). (K–L) No significant difference of Ki-67 (K) and TCF-7 (L) were observed between NKG2A⁺CD8⁺ T cells and NKG2A⁻CD8⁺ T cells. Above: representative flow cytometry plots; below: quantitative data. Mann-Whitney U test. *P<0.05, **p<0.01, ***p<0.001, ns refers to no significance. APC, Allophycocyanin; CTLA4, T-lymphocyte-associated antigen 4; GZMB, granzyme B; HA-VCR2, hepatitis A virus cellular receptor 2; IFN, interferon; Ki-67, Kiel-67 protein; LAG-3, lymphocyte activation gene 3; MFI, median fluorescence intensity; PD-1, programmed cell death protein-1; TIM-3, T-cell immunoglobulin and mucin domain-containing protein 3; TCF-7, transcription factor 7; TNF, tumor necrosis factor.
Figure 3  NKG2A+/CD8+ T cells infiltration is associated with immunosuppressive tumor microenvironment in clear cell renal cell carcinoma. (A–B) Heatmap representative of diverse immune cell populations, checkpoint molecule expression, and overall immune status (immune checkpoints, immune score, suppression score) among NKG2A+/CD8+ T cells infiltration subgroups in TCGA-KIRC cohort (A) and CheckMate cohort (B). (C) Distribution of various immune cells in different infiltration groups of NKG2A+/CD8+ T cells in ZSHC cohort; Green dots represent patients with low infiltration of NKG2A+/CD8+ T cells, while red dots represent patients with high infiltration. (D) Boxplots illustrating the abundance of TCR-related scores across two subgroups within the TCGA-KIRC cohort. Mann-Whitney U test. *p<0.05, **p<0.01, ***p<0.001, ns refers to no significance. CTLA4, T-lymphocyte-associated antigen 4; HA VCR2, hepatitis A virus cellular receptor 2; IMDC, international metastatic renal cell carcinoma database consortium; KIRC, kidney renal clear cell carcinoma; NK, natural killer; TCGA, The Cancer Genome Atlas; TNM, Tumor-node-metastasis staging; TMB, tumor mutation burden; Treg, regulatory CD4+ T; Tfh, T-follicular helper; TCR, T-cell receptor.

and NK cells (figure 3C and online supplemental figure 3A; p=0.018 and p=0.046, respectively). These findings further validated the results from TCGA-KIRC and CheckMate cohort (figure 3A,B). Moreover, the intense infiltration of CD8+ T cells was accompanied by a higher abundance infiltration of Treg cells (figure 3C). TCR Shannon score reflecting TCR diversity and TCR Richness score were higher in NKG2A+/CD8+T cells highly infiltrated group (figure 3D; p<0.001 and p<0.001, respectively). However, the TCR evenness score was found to be lower in NKG2A+/CD8+ T cells higher infiltration group (figure 3D; p<0.001). Molecular features of ccRCC have been reported to be associated with immune status, and we have explored the relationship between common mutations and NKG2A+/CD8+ T cells infiltration. Our investigation has found that SETD2 mutation patients have more NKG2A+/CD8+ T cells infiltration (online supplemental figure 3B). Collectively, our data showed that patients with high infiltration of NKG2A+/CD8+ T cells had an infiltrated but suppressive immune microenvironment.

NKG2A+/CD8+ T cells infiltration predicts poor immunotherapy responses in ccRCC

Given that NKG2A+/CD8+ T cells were in an exhausted state and their infiltration was linked to a suppressive immune milieu, we assessed whether the infiltration level
of NKG2A+CD8+ T cells could impact the prognosis of ICB therapy.

In the ZSHC-mRCC cohort, we evaluated the relationship of NKG2A+CD8+ T cells and corresponding clinical annotations (figure 4A and online supplemental table 7). The clinicopathological characteristics of the patients from ZSHC-mRCC cohort are summarized in online supplemental table 7. In terms of OS and progression-free survival (PFS), patients with higher infiltration of NKG2A+CD8+ T cells showed poorer sensitivity to ICB-based therapy (figure 4B,C; \( p=0.010 \) and \( p=0.079 \), respectively), but not to TKI alone (figure 4D; \( p=0.687 \)).

We further tried to validate the above findings in the CheckMate cohort. First, we assessed the impact of CD8+ T cells and NKG2A mRNA on the OS of the nivolumab treated arm. We found that neither the CD8+ T cells infiltration level nor the NKG2A mRNA level alone could significantly predict the prognosis in both nivolumab and everolimus treatment arm (online supplemental figure 4A–D; \( p=0.732 \), \( p=0.251 \), \( p=0.799 \) and \( p=0.332 \), respectively). Furthermore, when combining CD8+ T cells infiltration with NKG2A mRNA level, we observed that patients with higher CD8+ T cells infiltration had worse OS only when their NKG2A mRNA expression was at a high level (figure 4E,F; \( p=0.594 \) and \( p=0.041 \), respectively). However, this phenomenon was not observed in the everolimus arm (figure 4G,H; \( p=0.975 \) and \( p=0.248 \), respectively).

By further simulating the infiltration levels of NKG2A+CD8+ T cells using the NKG2A+CD8+ T cells signature, we found that patients with higher NKG2A+CD8+ T cells signature had worse OS with nivolumab treatment (figure 4I; \( p=0.037 \)), but not with everolimus treatment (figure 4J; \( p=0.818 \)). Interestingly, within the cohort characterized by high infiltration of NKG2A+CD8+ T cells, there was an upregulation in the expression of T-effector, myeloid inflammation and IFN-\( \gamma \)-related features, concomitant with a discernible downregulation in the expression of angiogenesis feature (online supplemental figure 5; \( p<0.001 \), \( p<0.001 \), \( p<0.001 \) and \( p=0.021 \), respectively). This suggested that high NKG2A+CD8+ T cells infiltration could lead to resistance to immunotherapy, and thus new treatment approaches are urgently needed.

**DISCUSSION**

TME assumes a crucial role in orchestrating the dynamics of tumor proliferation, metastasis as well as response to therapeutic modalities. Within this complex milieu, adaptive immune responses triggered by adaptive immune cells holds paramount significance in the context of immunotherapy for ccRCC, exerting an influence on responsiveness and prognosis of patients. Among these immune orchestrators, CD8+ T cells are regarded as the most powerful effectors in the anti-tumor immune response, stand as the very backbone of cancer immunotherapy. Interestingly, in contrast to other tumors, a pronounced infiltration of CD8+ T cells is correlated with less favorable prognosis in ccRCC, which is likely due to functional subdivision of distinct CD8+ T cell subtypes. Thus, further clustering of CD8+ T cells in ccRCC holds significant implications. In light of these intricate immune dynamics within the ccRCC microenvironment, our investigation delves further into the specific phenotype of NKG2A+CD8+ T cells and its potential role in treatment resistance.

Single agent immunotherapy could induce treatment resistance in a portion of patients. In the current study, we have discovered that this might be associated with the specific phenotype of NKG2A+CD8+ T cells. Using FCM, we discerned that NKG2A+CD8+ T cells express higher...
NKG2A+CD8+ T cells infiltration predicts poor immunotherapy responses in ccRCC. (A) Heatmap showed clinical features of the ZSHC-mRCC (ICB) cohort. (B–D) Kaplan-Meier analysis of patients with RCC in ZSHC-mRCC (ICB) (B and C) cohort and ZSHC-mRCC (TKI) cohort (D). (E–H) Combination of CD8+ T cells infiltration and NKG2A expression could predict response to nivolumab in CheckMate cohort. (I–J) NKG2A+CD8+ T cells infiltration could predict prognosis of patients with ccRCC treated with nivolumab (I), but not everolimus (J). Kaplan-Meier analysis and log-rank test. ccRCC, clear cell renal cell carcinoma; CR, complete response; ISUP, international society of urological pathology; ICB, immune checkpoint blockade; mRCC, metastatic renal cell carcinoma; NA, not available; ORR, objective response rate; PR, partial response; PD, progressive disease; SD, stable disease; TKI, tyrosine kinase inhibitors.
levels of various immune checkpoints compared with NKG2A\(^{+}\)CD8\(^{+}\) T cells, including PD-1, LAG-3, TIM-3, and CD39. Besides, we observed an impaired effector function of NKG2A\(^{+}\)CD8\(^{+}\) T cells in ccRCC with reduced secretion of IFN-\(\gamma\). Previous researches indicated that NKG2A\(^{+}\)CD8\(^{+}\) T cells exhibit an activated phenotype and represent a subset of transiently exhausted CD8\(^{+}\) T cells.\(^{36}\) An elevated infiltration of NKG2A\(^{+}\)CD8\(^{+}\) T cells might potentially correlate with enhanced immunotherapy response.\(^{36}\) Our research delineated that patients with higher infiltration of NKG2A\(^{+}\)CD8\(^{+}\) T cells manifest suboptimal responses to anti-PD-1 therapy. This could be explained by the exhausted phenotype of NKG2A\(^{+}\)CD8\(^{+}\) T cells in ccRCC presented in this study. An additional rationale may be attributed to the functional status of NKG2A\(^{+}\)CD8\(^{+}\) T cells being contingent on the expression of its corresponding ligand (HLA-E). Considering the elevated expression levels of HLA-E observed in ccRCC relative to other solid tumors,\(^{16}\) it is plausible to hypothesize that HLA-E might be another contributor to the discrepant effector function state of NKG2A\(^{+}\)CD8\(^{+}\) T cells.

We have also revealed that individuals with higher NKG2A\(^{+}\)CD8\(^{+}\) T cells infiltration exhibited increased infiltration of Treg cells. Additionally, there is an overall

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**Figure 5** NKG2A blockade synergizes with anti-PD-1 therapy in reinvigorating CD8\(^{+}\) T cells cytotoxicity. (A) An in vitro intervention model was established using fresh ccRCC tissues from 10 patients to assess the ability of PD-1 monoclonal antibody and NKG2A monoclonal antibody to reactivate CD8\(^{+}\) T cells. (B–D) 10 ccRCC tissue samples were collected and divided into two subgroups based on the expression of NKG2A on CD8\(^{+}\) cells. (B) In tumors with NKG2A\(^{low}\) CD8\(^{+}\) T cells infiltration group (C) the application of the PD-1 inhibitor nivolumab significantly increased the expression of IFN-\(\gamma\) and GZMB in CD8\(^{+}\) T cells; however, the efficacy of nivolumab diminished in tumors with NKG2A\(^{high}\) CD8\(^{+}\) T cells infiltration group (D). Statistical significance was determined using paired t-test or Wilcoxon signed-rank test. Each point represents an individual patient. (E) After nivolumab treatment, NKG2A expression on CD8\(^{+}\) T cells was upregulated. (F–H) The NKG2A and PD-1 dual intervention group exhibits a stronger ability to reactivate CD8\(^{+}\) T cells, with a certain advantage in improving both the degranulation (F) and cytotoxicity (G–H) functions of CD8\(^{+}\) T cells. (I) No significant difference of TCF-7 was observed among four groups. Significance values were determined using RM one-way analysis of variance and Tukey’s multiple comparison test. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\). ns refers to no significance. ccRCC, clear cell renal cell carcinoma; GZMB, granzyme B; IFN, interferon; PD-1, programmed cell death protein-1; RM, repeated measures; TCF-7, transcription factor 7.
higher expression of immune checkpoints in microenvironment enriched with NKG2A+CD8+ T cells infiltration. Immune score analysis indicates that individuals with higher infiltration of NKG2A+CD8+ T cells display stronger immune response. Interestingly, meanwhile, this occurrence is accompanied by a generalized augmentation in the expression of immune checkpoints, contributing to the formation of a relatively suppressive TME in these patients. TCR Shannon score reflects the diversity of TCR repertoire and has been related to positive immunotherapeutic responses in various cancers. Indeed, previous study of the RCC TME reported that the TCR diversity was lower than in normal tissue. Intriguingly, in our study, we found TCR diversity, reflected by TCR Shannon score, was enriched in patients with high NKG2A+CD8+ T cells infiltration, and ICB resistant subgroup lack of specific TCR amplification for tumor antigens. Consequently, individuals with higher infiltration of NKG2A+CD8+ T cells would tend to experience relatively unfavorable prognosis and resistance to anti-PD-1/PD-L1-based immunotherapy.

ICBs have advanced the treatment landscape of mRCC. However, there exist individuals who remain unresponsive to contemporary immunotherapy. Furthermore, most patients may inevitably acquire resistance after a period of treatment where treatment options remain limited following immunotherapy resistance. Patients with higher infiltration of NKG2A+CD8+ T cells were observed to suffer diminished overall prognosis when treated with anti-PD-1 therapy. Notably, existing guidelines endorse limited alternative treatment options for this group of patients. Recently, a phase II clinical trial of NKG2A inhibitors reported combination of durvalumab and monalizumab (NKG2A monoclonal antibodies) outperformed durvalumab monotherapy (objective response rate(ORR): 35.5% vs 17.9%) in lung cancer, underscored the significant promise of combined immunotherapy. We speculate that an amalgamation of NKG2A and PD-1 immune checkpoint inhibitors may proffer prospective benefits in ccRCC. Through an in vitro intervention model, we have validated for the first time in human tissue-derived samples that the combination of PD-1 and NKG2A blockade exhibited enhanced capacity to reactivate CD8+ T cells, outperforming the efficacy of single ICB therapy in ccRCC.

Our study held retrospective nature with limited samples included. For example, in vitro intervention experiments did not obtain metastatic sample tissues for validation. More in-depth research and clinical trials are needed in the future to confirm the potential of NKG2A as a new target in ccRCC.

In conclusion, we demonstrated NKG2A+CD8+ T cells were associated with unfavorable prognosis and response to immunotherapy. The synergistic interplay of NKG2A blockade and current immunotherapy exhibited a formidable capacity to reactivate CD8+ T cells effector functions. These findings herald fresh perspectives for the advancement of ccRCC immunotherapy.

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