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Targeting HLA-E-NKG2A/CD94 to reverse pyroptosis-induced immunosuppression in HCC enhances NK cell antitumor activity

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Abstract:

Background: The efficacy of adoptive cell therapy in hepatocellular carcinoma is limited by the immunosuppressive tumor microenvironment, yet the underlying mechanisms remain incompletely understood.

Methods: Clinical data from hepatocellular carcinoma patients receiving NK cell therapy were analyzed alongside tumor specimens and mouse xenograft models. Immune cell composition and spatial distribution were evaluated using immunohistochemistry and immunofluorescence. In *vitro* assays, including flow cytometry, qPCR, Western blot, ELISA, and real-time cytotoxicity analysis, were performed to investigate macrophage polarization and NK cell function. RNA sequencing was used to identify key regulatory molecules, followed by functional validation and pharmacological blockade in *vitro* and in *vivo*.

Results: NK cell therapy was associated with improved patient outcomes. However, tumor progression may resume upon treatment discontinuation. Tumor infiltrating NK cells positively correlated with CD163⁺ M2 macrophages. Mechanistically, NK cell mediated tumor killing induced tumor cell pyroptosis and triggered the release of interleukin-10, which promoted macrophage polarization toward an M2 phenotype via a STAT3-dependent positive feedback loop. These M2 macrophages exhibited high expression of HLA-E and suppressed NK cell cytotoxicity and cytokine production through a contact-dependent HLA-E-NKG2A/CD94 axis. Blockade of this pathway effectively restored NK cell function and enhanced antitumor efficacy both in *vitro* and in *vivo*.

Conclusions: This study identifies a negative feedback loop in which NK cell induced tumor pyroptosis promotes IL-10 mediated M2 macrophage polarization, leading to subsequent suppression of NK cell function. Targeting the HLA-E-NKG2A/CD94 axis represents a promising strategy to overcome immune suppression and improve the efficacy of NK cell-based immunotherapy in hepatocellular carcinoma.

51 **Keywords:** Hepatocellular carcinoma; M2 macrophage; Natural killer cell; tumor
52 microenvironment; pyroptosis

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Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies worldwide and remains the third leading cause of cancer-related mortality[1-3]. Despite advances in surgical resection, locoregional therapies, targeted agents, and immune checkpoint inhibitors, the overall prognosis of advanced HCC remains poor due to high recurrence rates and limited response to systemic therapies[4, 5]. These challenges are largely attributed to the highly immunosuppressive tumor microenvironment (TME), which plays a critical role in tumor progression and therapeutic resistance.

The HCC TME is a complex ecosystem composed of tumor cells, stromal components, and diverse immune cell populations, including T cells, natural killer (NK) cells, tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells[6, 7]. While T cell exhaustion and immune checkpoint pathways such as PD-1/PD-L1 have been extensively studied, increasing evidence suggests that innate immune cells, particularly NK cells and TAMs, are also key regulators of antitumor immunity. NK cells are crucial effectors of the innate immune system with potent cytotoxic activity against tumor cells. However, in HCC, their function is frequently impaired, characterized by reduced cytotoxicity and cytokine production[8-10]. Among the immunosuppressive components of the TME, TAMs represent a dominant population and exhibit remarkable functional plasticity[11-13]. M2-like TAMs, in particular, are associated with tumor progression, immune suppression, and poor clinical outcomes across multiple solid tumor types[14-18]. These cells can inhibit NK cell activity through the secretion of immunosuppressive cytokines and the expression of inhibitory ligands, thereby contributing to immune evasion and limiting the efficacy of immunotherapy. Although adoptive NK cell therapy has shown promising antitumor activity, its clinical efficacy in solid tumors such as HCC remains suboptimal, largely due to the suppressive TME[19].

Emerging evidence indicates that the crosstalk between NK cells and TAMs plays a critical role in shaping the immune landscape[20, 21]. However, the dynamic interactions and underlying mechanisms following adoptive NK cell therapy remain poorly defined. Whether NK cell-mediated tumor killing can reshape macrophage polarization and subsequently influence NK cell function has not been fully elucidated.

In this study, we investigated the interplay between NK cells and TAMs in HCC following adoptive NK cell therapy. We demonstrate that NK cell-mediated tumor cell killing induces macrophage polarization toward an M2 phenotype, which in turn suppresses NK cell function through the HLA-E-NKG2A/CD94 axis. Furthermore, blockade of this pathway restores NK cell activity and enhances antitumor efficacy. These findings reveal a previously unrecognized negative feedback loop within the TME and provide a rationale for combinational strategies integrating NK cell therapy with immune checkpoint blockade to improve therapeutic outcomes in HCC.

Material and Method

Study approval

This study was approved by the Institutional Review Board of Sun Yat-Sen University Cancer Center (Guangzhou, P.R. China). The approval number for human studies is SZR2020-075, and the approval number for animal studies is 22070C. All experiments involving humans were carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki), and written informed consents were obtained from all patients before inclusion. All animal procedures complied with the *Guide for the Care and Use of Laboratory Animals* criteria of the National Academy of Sciences.

Cell lines and cultures

Human HCC cell lines huh7, human NK lymphocyte cell line NK-92MI and human macrophage cell line THP-1 were purchased from the American Type Culture Collection. All cell lines were used within 15 passages. All cell line authentication had been carried out by short tandem repeat method through using Short Tandem Repeat Multi-Amplification Kit (Microreader 21 ID System), which was used for PCR amplification and PCR products were detected by ABI 3130xl DNA Analyzer (Applied Biosystems). All cell lines had been confirmed without Mycoplasma contamination by using Mycoplasma Stain Assay Kit (Beyotime Institute of Biotechnology). Tumor cells were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco), 100 U/mL penicillin, and 100 U/mL streptomycin in 5% CO₂ at 37°C. NK-92MI cells were cultured in GT551 H3 (TAKARA). THP-1 cells were cultured in RPMI1640 medium (Gibco) supplemented with 10% FBS (Gibco), 100 U/mL penicillin, 100 U/mL streptomycin and 0.05mM β-mercaptoethanol in 5% CO₂ at 37°C.

Immunohistochemistry and immunofluorescence

The IHC and IF staining protocol was described elsewhere[8]. The following anti-human primary antibodies were used: anti-CD163 (Abcam,1:500), anti-CD57 (ZSGB-Bio), anti-CD3 (ZSGB-Bio), anti-Granzyme B (CST,1:100), anti-Caspase-3 (CST,1:200), anti-GSDME (Abcam,1:100), anti-MLKL (Abcam,1:400), anti-HLA-E (STARTER,1:500).

Multiplex immunohistochemistry staining

The multiplex immunohistochemistry staining protocol was described elsewhere[22].The following anti-human primary antibodies were used: anti-CD163(Abcam,1:500), anti-HLA-E (STARTER,1:500).

Western blot (WB) analysis

The Western blot protocol was described elsewhere[8]. Nucleus proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology) according to the manufacturer's instructions. TATA-box-binding protein was used as a control for nucleus proteins. The following anti-human primary antibodies were used: anti-STAT3 (CST, 1:500), anti-p-STAT3 (CST,1:1000), anti-GSDMD (CST,1:1000), anti-GSDME (CST,1:1000), anti-NLRP3 (Proteintech,1:1000), anti-ERK 1/2 (CST,1:1000),anti-p-ERK 1/2 (CST,1:1000), anti-Granzyme B (CST,1:2000), anti-HLA-E (STARTER,1:1000), anti-MLKL (Abcam,1:1000), anti-Caspase-3 (CST,1:1000), anti-bax (Abcam,1:1000), anti-bcl-2 (Abcam,1:1000), anti-GAPDH (CST,1:2000).

Enzyme-linked immunosorbent (ELISA) assay

Secretion of the cytokine IL-10 was examined using a commercially available ELISA kit (Dakewe Biotech Co., Ltd) according to the manufacturer's protocol.

Cytotoxicity assay

The xCELLigence RTCA assay used to evaluate cytotoxic activity has been described in our previous study[8].

Flow cytometry

The flow cytometry protocol was described elsewhere[8]. The primary anti-human antibodies utilized for flow cytometry analysis are listed below: Fixable Viability Stain 780, PE anti-human TIGIT, PE anti-human TIM3, PE anti-human CD56, PE/Cy7 anti-human CD56, and BV421 anti-human granzyme B PE anti-human

CD163, APC anti-human CD206 antibodies (BD Biosciences), as well as FITC anti-human perforin, APC anti-human TNF α , APC anti-human IFN γ antibodies (BioLegend). Mitochondrial membrane potential was measured by flow cytometry with JC-1 dye (Yeasen) per the manufacturer's instructions. Apoptosis was analyzed by flow cytometry in accordance with the kit manufacturer's protocol (GOONIE). The stained cells were analyzed using a CytoFlex LX flow cytometer (Beckman Coulter), and the data were analyzed using FlowJo (version 10.4, TreeStar).

Animal experiments

All mice were obtained from Guangdong Medical Laboratory Animal Center (Guangdong, China) and maintained under specific pathogen-free conditions. All animal experiments were approved by the Institution Animal Care and Use Committee of the Sun Yat-sen University Cancer Center (Approval NO. 22070C). All animal procedures complied with the *Guide for the Care and Use of Laboratory Animals* criteria of the National Academy of Sciences. The maximum allowable tumor burden for animal experiments was referenced to the guidelines of the Canadian Council on Animal Care. The authors confirm that the maximum tumor burden of animals used in this study did not exceed the recommended limit.

A total of 5×10^6 Hep huh7 cancer cells were cultured, resuspended in 0.1 mL of PBS/Matrigel mixture (1:1), and then subcutaneously injected into the right flank of 4-week-old female NCG mice. Tumor volume (V) was measured using a caliper every 3 days for the duration of the experiment and calculated with the formula $V = (\text{length} \times \text{width}^2)/2$.

The mice were randomized and divided into three groups (n=4 for each group). NK cells were infused into mice via tail-vein injection at a dose of 1×10^7 cells/mouse. Mice receiving Monalizumab (Selleck) treatment were given tail-vein injections every three days at a dose of 200 mg/mouse.

Statistical analysis

All statistical analyses were performed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA). Graphs were generated using GraphPad Prism version 9.0 (GraphPad Software, La Jolla, CA, USA), and flow cytometry data were analyzed with FlowJo software (version 10). Data are presented as mean $\pm$ standard deviation (SD). Comparisons among multiple groups were performed using one-way ANOVA, followed by post hoc multiple comparisons. Groups sharing the same letter were considered not significantly different, whereas groups with different letters indicated statistically significant differences ($P < 0.05$). Two-way ANOVA was used for comparisons involving two independent variables. Comparisons between two groups were conducted using an unpaired two-tailed Student's t-test. Correlation analyses were performed using Pearson or Spearman correlation coefficients, as appropriate. Survival curves were estimated using the Kaplan-Meier method, and differences between groups were assessed using the log-rank test. For clinical data analysis, univariate Cox proportional hazards regression analysis was initially applied to identify potential factors associated with outcomes. A P value < 0.05 was considered statistically significant. Statistical significance levels were defined as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$; "ns" indicates no statistically significant difference.

Data availability

All data analyzed are included in this article, and additional information is available upon request.

Results

NK cell therapy improves prognosis and reshapes TME in HCC

To systematically evaluate the impact of NK cell therapy on the prognosis of HCC, we retrospectively analyzed clinical data from patients treated at the Sun Yat-sen University Cancer Center between 2005 and 2014. A total of 40 patients who received

at least six cycles of NK cell infusion in addition to standard therapy were included as the treatment group, while 80 matched patients who did not receive any form of cellular immunotherapy were selected as controls. Baseline clinical characteristics were well balanced between the two groups (all $P > 0.05$), indicating good comparability (Table 1).

Table 1. Demographics and disease characteristics of patients in the control group and NK group patients.

Variables	NK group (n=40)	Control group (n=80)	P value
Age	53.26±10.49 (28-83)	53.51±11.72 (28-81)	0.333
Gender			
Male	33(82.5)	68(85)	0.793
Female	7(17.5)	12(15)	
CNLC Stage			0.369
I	11(27.5)	28(35)	
II	26(65)	45(56.25)	
III	3(7.5)	7(8.75)	
HBV (+)	29(72.5)	60(75)	0.826
Vascular Invasion			0.432
Yes	3(7.5)	7(8.75)	
No	37(92.5)	73(91.25)	
Tumor Differentiation			0.352
Poor	6(15)	9(11.25)	
Moderate-Poor, Moderate	31(77.5)	61(76.25)	
Moderate-Well, Well	3(7.5)	10(12.5)	
Extrahepatic Spread			1.000
Yes	3(7.5)	5(6.25)	
No	37(92.5)	75(93.75)	
Child-Pugh class			0.616
A	39(97.5)	79(98.75)	
B	1(2.5)	1(1.25)	
Therapy			
Surgery	40(100)	80(100)	1.000
TACE	10(25)	17(21.25)	0.649
RFA	19(47.5)	39(48.75)	1.000
Radiotherapy	1(2.5)	3(3.75)	0.618
Chemotherapy	10(25)	18(22.5)	0.820
Targeted therapy	11(27.5)	28(35)	0.536
Immunotherapy	5(12.5)	8(10)	0.758

Abbreviations: CNLC, China Liver Cancer Staging; HBV, hepatitis B virus; NK, natural killer; RFA, radiofrequency ablation; TACE, transarterial chemoembolization.

Table 2. Univariate Cox proportional hazards regression analysis of prognostic factors in patients with hepatocellular carcinoma

Variables	Univariate analysis	
	HR (95%CI)	<i>P</i> value
Age (≥ 50 vs. < 50)	0.741 (0.317---1.729)	0.487
Gender	2.086 (0.623---6.989)	0.233
Therapy (NK vs. Control)	0.226 (0.058---0.877)	0.032*
CNLC Stage (I、 II vs. III)	1.741 (0.427---7.089)	0.439
HBV (pos vs. neg)	0.689 (0.263---1.808)	0.449
Vascular Invasion	1.965 (0.403---9.575)	0.403
Tumor Differentiation	1.585 (0.424---5.933)	0.494
Extrahepatic Spread	0.719 (0.121---4.277)	0.717
Child-Pugh class (A vs. B)	0.178 (0.008---4.159)	0.283
TACE	1.368 (0.367---5.096)	0.640
RFA	0.533 (0.203---1.399)	0.201
Radiotherapy	1.240 (0.111---13.876)	0.861
Chemotherapy	2.334 (0.985---5.531)	0.054
Targeted therapy	1.912 (0.655---5.587)	0.236
Immunotherapy	3.779 (0.740---19.301)	0.110

Abbreviations: CI, confidence interval; CNLC, China Liver Cancer Staging; HBV, hepatitis B virus; HR, hazard ratio; RFA, radiofrequency ablation; TACE, transarterial chemoembolization. *Statistically significant, $P < 0.05$.

Univariate Cox proportional hazards regression analysis (Table 2) identified NK cell therapy as a significant protective factor for patient prognosis ($P = 0.032$), whereas other variables showed no significant association. Survival analysis further demonstrated that patients receiving NK cell therapy had a significantly improved overall survival (OS) compared to controls ($P = 0.037$) (Fig.1A). The median OS was not reached in the NK cell group, while it was 124.4 months in the control group, with clearly separated Kaplan–Meier survival curves. Importantly, no severe treatment-

related adverse events were observed, and only mild, transient toxicities were reported, indicating a favorable safety profile (Table 3).

Table 3. Adverse events in patients following NK cell infusion

Adverse event type	No. of patients (n = 40)
Fever	2
Palpitation	2
Fatigue	5
Rash	NA
Pruritus	NA
Arthralgia	2
Decreased appetite	NA
Nausea/vomiting	3
Allergic reaction	NA
Elevated blood pressure	2
Pneumonia	NA
Hepatitis	NA
Colitis	NA

A representative case illustrated that adjuvant NK cell therapy after surgical resection could effectively control tumor progression in the short term. However, tumor recurrence occurred after discontinuation of therapy, suggesting that the durability of NK cell-mediated antitumor effects may be limited and potentially influenced by dynamic changes in the TME. Figure 1B and D shows liver MRI images of the patient. Figure 1B demonstrates complete resection of the liver tumor after surgery, whereas Figure 1D shows tumor recurrence post-surgery, with the lesion area marked in the red box. Figure 1C is a line graph depicting changes in serum AFP levels in this patient.

To further explore alterations in the TME, paired tumor specimens from five patients with HCC were analyzed by immunohistochemistry. Among them, four patients underwent surgical resection to obtain pathological sections both before and after NK cell therapy. One patient received surgical resection and biopsy for pathological sampling at 2 months and 14 months after NK cell therapy, respectively.

The clinical workflow of the five patients is presented in Figure 1E. Increased infiltration of CD57⁺ NK cells was observed following NK cell therapy. Notably, CD163⁺ M2 macrophages were also significantly increased in post-treatment samples (Fig.1F and H). Quantitative analysis confirmed that both NK cells and M2 macrophages were elevated after therapy, while their numbers declined over time following treatment cessation (Fig.1G and I).

Consistently, immunofluorescence staining validated increased NK cell infiltration after therapy, which decreased upon treatment withdrawal. Correlation analysis revealed that CD163⁺ M2 macrophage abundance was positively associated with both tumor necrosis area ($P = 0.0119$) and NK cell infiltration ($P = 0.0006$), suggesting coordinated changes within the TME (SFig.1A-D).

These findings were further supported by in vivo models. In both HCC and nasopharyngeal carcinoma xenograft models, adoptive cell therapies (NK-92MI or T-cell receptor T cells) led to increased infiltration of effector immune cells accompanied by the accumulation of CD163⁺ M2 macrophages within tumor tissues (SFig.1E and F). These results indicate that NK cell therapy not only improves survival outcomes in HCC patients but also induces significant remodeling of the TME, characterized by concurrent increases in NK cells and M2 macrophages, which may influence the long-term efficacy of immunotherapy.

NK cell induced IL-10 promotes CD163⁺ M2 macrophage polarization

To determine whether M2 macrophage accumulation after NK cell therapy was driven by chemotaxis or polarization, we first performed transwell migration assays using conditioned media from NK cells, huh7 tumor cells, and NK cell-mediated tumor killing. Results showed that NK CM induced the strongest THP-1 migration, whereas killing-derived CM induced minimal migration, suggesting that macrophage accumulation is unlikely to be mediated by chemotaxis (Fig.2A and B).

We next evaluated macrophage polarization after exposure to different CM. PMA-differentiated THP-1 macrophages treated with killing CM exhibited clear morphological changes (Fig.2C) and significantly increased expression of CD163 and

other M2-associated markers, whereas NK CM had little effect (Fig.2D and E). These findings indicate that NK cell-mediated tumor killing promotes macrophage polarization toward an M2 phenotype rather than simple recruitment. To more intuitively illustrate the polarization effects of different conditioned media on macrophages, we performed cellular immunofluorescence staining to detect CD163 positivity (red) on the surface of macrophages treated with different conditioned media, followed by quantitative analysis of fluorescence intensity. The results demonstrated that macrophages co-cultured with 48hrs NK-cell-mediated killing of huh7cells conditioned media (hereinafter referred to as 48H CM) exhibited the highest CD163 expression levels (Fig.2G and H).

To identify the underlying mediators, cytokine profiling of NK cells, tumor cells, and co-culture systems was performed. IL-10 was markedly upregulated in the NK–tumor killing condition compared with either cell type alone, suggesting it as a key candidate factor (Fig.3A and B). Subsequently, ELISA was performed to measure IL-10 levels in different conditioned media. The results showed that 48H CM contained the highest IL-10 concentration. Flow cytometry further confirmed that NK cells secreted only low basal levels of IL-10, whereas IL-10 was predominantly produced by huh7 cells upon NK-cell-mediated cytotoxicity (Fig.3C-E).

Functional validation demonstrated that recombinant IL-10 was sufficient to induce CD163⁺ M2 polarization, while IL-10 blockades significantly reversed the effects of killing-derived CM. Consistently, inhibition of IL-10 also suppressed downstream STAT3 activation, indicating that IL-10 promotes M2 polarization via the STAT3 signaling pathway (Fig3H and I). Importantly, macrophages polarized by killing-derived CM acquired an IL-10–secreting phenotype, suggesting a self-sustaining feedback loop in which IL-10 induces M2 polarization, and M2 macrophages further amplify IL-10 production to maintain an immunosuppressive tumor microenvironment (Fig.3J and K). Figure 3L shows IL-10 levels produced by macrophages induced by different conditioned media as measured by ELISA, and these results are consistent with the flow-cytometry findings.

Ossirene, an IL-10 inhibitor, was used for *in vivo* animal experiments. The detailed workflow of animal experiments is shown in SFig. 2A. Supplementary Figure 2B demonstrates that Ossirene does not affect the proliferation of huh7 cells. *In vivo* animal experiments showed that NK cell therapy increased M2 macrophage infiltration in tumor tissues, whereas IL-10 inhibition significantly reduced this effect without directly affecting tumor cell proliferation, confirming that IL-10 primarily acts through immune microenvironment modulation (SFig.2C and D). After NK cell adoptive transfer, the major organs of mice displayed normal tissue architecture, without obvious inflammatory infiltration, necrosis or pathological damage (SFig.2E).

Overall, these results demonstrate that NK cell-mediated tumor killing induces IL-10 release, which drives CD163⁺ M2 macrophage polarization via STAT3 activation and contributes to immunosuppressive tumor microenvironment remodeling.

NK cell mediated pyroptosis induces M2 polarization

To investigate the patterns of tumor cell death induced by NK cells and their role in regulating macrophage polarization, we first examined whether apoptosis, necroptosis, or pyroptosis predominates during NK cell-mediated tumor killing. Immunohistochemical staining and analysis were performed on paired tumor samples collected before and after NK-cell therapy. The results showed that regardless of baseline expression levels, the expression of markers for apoptosis (Caspase-3), pyroptosis (GSDME), and necroptosis (MLKL) was elevated after treatment, suggesting simultaneous activation of multiple programmed cell death pathways within the TME (Fig.4A and B).

To identify the dominant pathway linked to macrophage polarization, we analyzed gene and protein expression in NK-tumor co-culture systems. Among cell death-related pathways, pyroptosis-associated molecules (including NLRP3, GSDMD, and GSDME) were significantly upregulated at both mRNA and protein levels, indicating that NK cell-mediated tumor killing predominantly triggers pyroptotic signaling (Fig.4C and D).

NLRP3-AIM3-IN-3, a pyroptosis inhibitor, exhibited no impact on huh7 cell proliferation *in vitro* (Fig.4E). Both flow cytometry and WB assays revealed that pyroptosis blockade markedly attenuated the capacity of killing-derived CM to induce CD163⁺ M2 macrophage polarization (Fig.4F-H). In contrast, inhibition of apoptosis or necroptosis exerted minimal effects on macrophage polarization, indicating that these pathways are not the primary drivers of the observed M2 shift.

We further examined the expression of necroptosis and apoptosis related molecules in different cell groups and killing mixtures at both RNA and protein levels (SFig.3A and B). Necrostatin-1 (hereinafter referred to as Nec-1) and Z-VAD-fmk serve as inhibitors for necroptosis and apoptosis, respectively. CCK8 assays confirmed that neither inhibitor affected the proliferative activity of huh7 cells *in vitro* (SFig.3C and D). WB analysis revealed that supplementation with each inhibitor partially down-regulated the expression of corresponding proteins (SFig.3G and H). Nevertheless, flow-cytometry assays demonstrated that neither inhibitor could block the polarization of macrophages toward CD163⁺ M2 phenotype (SFig.3E and F). Importantly, while apoptosis and necroptosis pathways were activated during NK-tumor interactions, inhibition of these pathways did not significantly affect IL-10 production in the co-culture system. Consistently, ELISA results confirmed that IL-10 production is significantly diminished when pyroptosis is blocked, whereas it remains largely unchanged upon inhibition of other cell death pathways (SFig3.I).

Taken together, these results demonstrate that NK cell-mediated tumor killing induces multiple forms of programmed cell death; however, pyroptosis is the dominant pathway responsible for IL-10 release, which in turn drives CD163⁺ M2 macrophage polarization.

M2 macrophages inhibit NK cell function through the HLA-E–NKG2A/CD94 axis

To investigate how M2 macrophages regulate NK cell function, we first established indirect and direct co-culture systems. In the transwell (indirect) system, macrophages had minimal impact on NK cell function, as cytokine production and cytotoxic

molecule expression remained largely unchanged (Fig5E and SFig.4A). In contrast, in direct co-culture, NK cell function was significantly impaired in a time-dependent manner, with marked reductions in perforin, granzyme B, IFN- γ , and TNF- α after 48 hrs (Fig.5A-C). These findings indicate that M2 macrophage-mediated suppression of NK cells is primarily contact dependent.

Further analysis showed that macrophages induced by 48H CM exerted the strongest inhibitory effects. NK cells co-cultured with these macrophages exhibited significantly reduced effector molecule expression and impaired cytotoxicity against huh7 target cells, as confirmed by both apoptosis-based assays and real-time killing analysis (Fig.5D and F-G). Together, these results demonstrate that M2 macrophages suppress NK cell function and induce functional exhaustion.

SFig.4B shows flowcytometry detected expression of activating and inhibitory receptors on the surface of NK cells co-cultured with macrophages induced by different conditioned media. The inhibitory receptors LAG-3 and TIM-3 exhibited the highest expression in NK cells co-cultured with 48H CM-induced macrophages. The inhibitory receptor TIGIT and the activating receptor CD69 were expressed at relatively low levels across all four groups.

We first performed chemotaxis assays to evaluate the chemotactic effect of macrophages induced by different conditioned media on NK cells. No significant difference was observed in the number of migrated NK cells (SFig.5A). Nevertheless, microscopic examination revealed that macrophages induced by 48H CM triggered morphological alterations in NK cells, characterized by cell swelling and large vacuole formation. Flowcytometry based apoptosis assays were further performed on these migrated NK cells. NK cells exposed to M2 macrophage-derived conditioned medium displayed increased apoptosis (SFig.5B and C), reduced mitochondrial membrane potential (SFig.5B and E), and elevated expression of pro-apoptotic proteins (SFig.5F and G), indicating early cell death and mitochondrial dysfunction.

To further explore the underlying mechanisms, transcriptomic analysis was performed. RNA-seq revealed distinct gene expression profiles among these macrophages (SFig.6A-D), with enrichment of immune regulation-related pathways,

including cytokine signaling, inflammatory response, and negative regulation of NK cell-mediated cytotoxicity (SFig.6E). Among the differentially expressed genes, HLA-E emerged as a key upregulated molecule in M2 macrophages (SFig.6F). SFig.6G assessed the overall trends of four pathways.

Both IF staining and WB assays confirmed markedly elevated HLA-E expression in 48 H CM induced M2 macrophages (Fig.6A-C). Given that HLA-E primarily modulates NK cell function by inhibiting the NKG2A/CD94 signaling axis, we further examined the relevant pathway molecules. The results revealed that NK cells co-cultured with 48H CM-induced macrophages for two days exhibited markedly decreased p-ERK expression, accompanied by reduced Granzyme B levels (Fig.6D). It should be noted that protein lysates used for WB were obtained from suspended NK cells. Since NK cells were in direct contact with adherent macrophages, the harvested samples might contain a small amount of macrophage contamination. To further exclude this interference, we separately examined purified macrophages from each group. As shown in Fig.6E, no obvious differences in ERK1/2 and p-ERK1/2 expression were observed among the four groups of macrophages, and Granzyme B was undetectable in macrophages, which is consistent with the intrinsic biological features of macrophages. Taken together, HLA-E may regulate NK cell function through ERK/p-ER associated signaling pathways.

Importantly, analysis of paired clinical tumor samples confirmed that elevated HLA-E expression also occurs in real-world clinical settings. CD163⁺HLA-E⁺ macrophages were markedly enriched in tumor tissues from patients following NK-cell therapy (Fig.6F and G).

These findings demonstrate that M2 macrophages suppress NK cell function through direct contact and induction of apoptosis, and mechanistically through upregulation of HLA-E, which engages the NKG2A/CD94 inhibitory pathway to dampen NK cell cytotoxicity and contribute to immune evasion.

Monalizumab restores NK cell function

Given that M2 macrophages highly express HLA-E and suppress NK cell function

through the HLA-E–NKG2A/CD94 axis, we investigated whether blockade of this inhibitory pathway could restore NK cell activity. Monalizumab, a humanized monoclonal antibody targeting NKG2A, was used to disrupt HLA-E–NKG2A/CD94 signaling.

First, we confirmed that Monalizumab had no direct effect on tumor cell proliferation (Fig.6H). We then established three experimental groups: NK cells co-cultured with M0 macrophages (M0), NK cells co-cultured with M2 macrophages (48H), and NK cells co-cultured with M2 macrophages in the presence of Monalizumab (48H + Monalizumab). Flow cytometry analysis showed 48H group exhibited markedly reduced expression of key effector molecules, including IFN- γ , perforin, TNF- α , and granzyme B (Fig.6I). Flowcytometry-based apoptosis detection confirmed that M2 macrophages markedly suppressed the cytotoxic activity of NK cells against huh7 target cells (Fig.6J). RTCA analysis further supported these findings. M0 group exhibited strong killing activity, whereas M2 macrophages significantly attenuated NK-mediated cytotoxicity. Importantly, Monalizumab treatment partially rescued NK cell killing capacity, although not to the level observed in the M0 group (Fig.6K and L). These results consistently indicate that blockade of the HLA-E–NKG2A/CD94 axis can reverse M2-mediated NK cell suppression.

To directly assess the role of HLA-E, we generated macrophages overexpressing HLA-E (Fig.6M). NK cells co-cultured with HLA-E overexpressing macrophages showed significantly reduced effector function compared to controls, confirming that elevated HLA-E expression alone is sufficient to suppress NK cell activity (Fig.6N).

These results demonstrate that targeting the NKG2A checkpoint with Monalizumab effectively alleviates HLA-E-mediated inhibition and restores NK cell function, highlighting the HLA-E-NKG2A/CD94 axis as a critical therapeutic target in the immunosuppressive tumor microenvironment.

Monalizumab combined with NK cell infusion enhances antitumor immunity

To evaluate whether blockade of the NKG2A checkpoint enhances the antitumor efficacy of NK cell therapy in vivo, a huh7 xenograft model was established in NCG

mice. Mice were randomly assigned to three groups: PBS control (NC), NK cell infusion (NK), and NK cell infusion combined with Monalizumab (NK + Monalizumab). NK cells were administered at two time points, and Monalizumab was given periodically throughout the treatment course (Fig.7A).

Tumor growth was significantly inhibited in the NK-treated group compared to controls, while the combination therapy group exhibited the most pronounced tumor suppression. Both tumor volume and tumor weight were lowest in the NK + Monalizumab group, with consistent trends observed in tumor growth curves, indicating a synergistic antitumor effect (Fig.7B-D).

Immunohistochemical analysis further demonstrated enhanced NK cell infiltration and function in tumor tissues following combination therapy. Specifically, increased numbers of CD57⁺ NK cells and elevated granzyme B expression were observed in the NK + Monalizumab group compared to NK treatment alone, suggesting improved NK cell persistence and cytotoxic activity within the tumor microenvironment (Fig.E-G). Importantly, safety evaluation revealed no significant histopathological abnormalities in major organs, and no evident off-target NK cell infiltration was detected, indicating a favorable safety profile under the experimental conditions (SFig.7).

These in vivo results demonstrate that Monalizumab enhances the therapeutic efficacy of NK cell infusion by improving NK cell infiltration and function. These findings supporting the strategy of combining NK cell therapy with HLA-E-NKG2A/CD94 axis blockade to overcome immunosuppression and enhance antitumor immunity.

Discussion

This study uncovers a previously unrecognized feedback mechanism in which NK cell-induced tumor pyroptosis promotes IL-10-driven M2 macrophage polarization, leading to subsequent suppression of NK cell function via the HLA-E-NKG2A/CD94 axis. Targeting this immunosuppressive circuit provides a potential strategy to improve the efficacy of NK cell-based immunotherapy in HCC.

517 In recent years, immunotherapy has gradually become an important modality for
518 cancer treatment. Among them, NK cell based adoptive cell therapy has demonstrated
519 certain antitumor potential in multiple solid tumors[23-27]. Previous studies have
520 reported that the abundance of infiltrated NK cells in hepatocellular carcinoma is
521 positively correlated with patient prognosis. NK cells are functionally inactivated
522 within the HCC tumor microenvironment, and restoring NK-cell function via
523 metabolic reprogramming can reduce tumor burden[8]. Nevertheless, clinical studies
524 have shown that although some patients receiving NK cell therapy achieve partial
525 tumor control in the short term, long-term therapeutic efficacy is still constrained by
526 tumor recurrence, immune evasion and other factors[28, 29]. Therefore, in depth
527 exploration of tumor microenvironment alterations following NK cell therapy and
528 their impacts on NK cell function is of great significance for further optimizing
529 cellular immunotherapeutic strategies.

530 Combined immunohistochemical staining and cellular functional experiments
531 revealed that macrophages underwent robust CD163 M2-like polarization following
532 NK cell-mediated tumor cell killing. Macrophages actively interact with multiple
533 types of immune cells. We further demonstrate that NK cell-mediated antitumor
534 activity paradoxically triggers a negative feedback loop that impairs its own
535 therapeutic efficacy. Specifically, NK cell cytotoxicity induces tumor cell pyroptosis
536 and stimulates IL-10 secretion, which drives macrophage polarization toward an
537 immunosuppressive CD163⁺ M2 phenotype. These M2 macrophages inhibit NK cell
538 function through the HLA-E-NKG2A/CD94 axis, ultimately facilitating tumor
539 immune evasion.

540 In further mechanistic investigations, we found that NK-cell-mediated tumor cell
541 killing triggers not only conventional apoptosis and necroptosis but also tumor cell
542 pyroptosis. Pyroptosis is an inflammatory form of programmed cell death, which
543 represents caspase-dependent programmed cell death governed by multiple signaling
544 cascades including the canonical, non-canonical, other caspase-dependent and

enzyme-induced pathways[30]. Pyroptosis exerts dual regulatory effects within the tumor microenvironment. On one hand, pyroptosis induced by radiotherapy, chemotherapy and other interventions can not only directly suppress tumor growth and metastasis, but also elicit antitumor immunity via cytokine release[31-34]. On the other hand, interleukin-1 β recruited monocyte-macrophages into the tumor microenvironment and facilitates breast cancer progression, indicating a functional crosstalk between pyroptosis and monocyte-macrophages[35]. Recent studies have demonstrated that during immune-cell-driven antitumor responses, granzymes secreted by cytotoxic lymphocytes are capable of inducing not only tumor cell apoptosis but also pyroptosis through activating gasdermin family proteins, which further amplifies immune responses[36, 37]. Using pyroptosis inhibitors and a series of functional assays, our study confirms that NK cells induce tumor cell pyroptosis during target-cell killing, accompanied by the release of multiple cytokines, among which IL-10 is markedly elevated. These findings suggest that in NK-cell-triggered tumor cell death, pyroptosis may serve as more than merely a tumor-cell-killing mechanism; it also plays a critical role in shaping the immune landscape of the tumor microenvironment.

In addition to soluble factors, we demonstrate that cell-cell contact-dependent signaling plays a crucial role in NK cell inhibition. RNA sequencing identified HLA-E as a key molecule upregulated in M2 macrophages, which suppresses NK cell cytotoxicity via engagement of the inhibitory receptor NKG2A/CD94. Previous studies have demonstrated that macrophages can express HLA-E and modulate the surface expression of NKG2A/CD94 on T cells and NK cells, and the HLA-E-NKG2A/CD94 axis is critically involved in immune suppression[38]. In addition, M2-polarized macrophages impair the cytotoxic function of CD8 T cells and NK cells by secreting immunosuppressive cytokines to upregulate inhibitory molecules such as PD-L1[39, 40]. Targeting NKG2A/CD94 can potentiate the antitumor cytotoxic

function of both T cells and NK cells[41-44]. Therapeutic blockade of this pathway using an anti-NKG2A antibody Monalizumab effectively restored NK cell activity in vitro and in vivo. Monalizumab is a novel NKG2A-targeted inhibitor that is currently under phase I-II clinical investigations. It has exhibited promising therapeutic efficacy in several cancer types including head-and-neck squamous cell carcinoma, non-small-cell lung cancer, cervical cancer and endometrial cancer[45-48]. Nevertheless, its actual clinical benefits in solid tumors remain to be validated in phase III clinical trials.

These findings provide potential implications for optimizing NK cell based immunotherapeutic strategies. On the one hand, blocking the formation or function of M2 macrophages, such as by targeting the IL-10 signaling pathway or inhibiting M2 polarization, may help reverse the immunosuppressive state of the tumor microenvironment. On the other hand, therapeutic intervention targeting the HLA-E–NKG2A/CD94 immune checkpoint, such as the combination of NK cell adoptive transfer with Monalizumab treatment, represents a promising strategy to improve the antitumor efficacy of NK cells. Therefore, future studies are warranted to further explore the clinical potential of adoptive NK cell therapy combined with immune checkpoint blockade for the treatment of hepatocellular carcinoma.

Several limitations should be acknowledged. First, the clinical sample size was relatively limited, and larger cohorts are required to validate the observed associations. Second, although our mechanistic findings were supported by both in vitro and in vivo models, the use of immunodeficient mouse models and xenogeneic systems may not fully recapitulate the complexity of the human TME. Additionally, the NK-92MI cell line, while advantageous for experimental consistency, differs from primary NK cells in cytokine dependence, receptor repertoire, and functional regulation, which may affect the generalizability of our findings. Future studies incorporating primary or patient-derived NK cells, as well as humanized models, will be essential to strengthen clinical relevance. Finally, the downstream signaling events

of the HLA-E-NKG2A/CD94 axis in NK cells require further investigation using multi-omics and single-cell approaches.

Conclusion

We demonstrate that NK cell-mediated tumor killing induces tumor cell pyroptosis and subsequent release of IL-10, which drives macrophage polarization toward a CD163⁺ M2 phenotype. These M2 macrophages, in turn, suppress NK cell cytotoxicity through the HLA-E-NKG2A/CD94 axis, forming a self-limiting regulatory circuit within the tumor microenvironment. Importantly, targeting the HLA-E-NKG2A/CD94 checkpoint effectively restores NK cell function and enhances antitumor activity, highlighting a promising combinational therapeutic strategy.

List of abbreviations

CNLC	China Liver Cancer Staging
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
NK	natural killer
RFA	radiofrequency ablation
TACE	transarterial chemoembolization
TME	microenvironment
OS	overall survival

Ethics approval and consent to participate

The study was approved by the Institutional Ethics Committee of the Sun Yat-sen University Cancer Center, and consent for participation and publication was obtained from all the patients included in the study and is available for review at any time.

Consent for publication

Not applicable.

629

630 **Availability of data and materials**

631 All data analyzed are included in this article and additional information is available
632 upon request.

633

634 **Competing interests**

635 The authors declare no conflict of interest.

636

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642

643 **Authors' contributions**

644 D.S.W. and Y.Y.C. contributed to the conception and design; X.Y.Y., Y.Y.C and J.L
645 conducted the experiments. X.Y.Y., J.L and analyzed and interpreted the data. X.Y.Y.
646 and D.S.W. drafted the manuscript. All authors read and approved the manuscript.

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