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ORIGINAL ARTICLE

Sirtuin2 enhances the tumoricidal function of liver natural killer cells in a mouse hepatocellular carcinoma model

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Abstract

Hepatocellular carcinoma (HCC) is the third most lethal cancer in the world. Natural killer (NK) cell-mediated immunity is crucial for tumor surveillance and therapy. Characterization of the regulatory mechanisms of NK cell function is important for developing novel immunotherapies against HCC. In this study, we used a chemical-induced mouse HCC model to identify the upregulation of Sirtuin2 (SIRT2) in liver NK cells. In particular, SIRT2 was predominantly expressed in liver $CD94^+$ NK cells. The HCC liver microenvironment induced SIRT2 expression in NK cells. In addition, overexpression of exogenous SIRT2 significantly upregulated the production of cytokines and cytotoxic mediators in activated NK cells. Consistently, SIRT2-overexpressing NK cells showed a stronger tumoricidal effect on hepatoma cells. Moreover, SIRT2 remarkably promoted the phosphorylation of Extracellular-signal-regulated kinase 1/2 (Erk1/2) and p38 Mitogen-activated protein kinases (MAPK) in activated NK cells. SIRT2 knockdown in liver $CD94^+$ NK cells impaired their cytotoxic effect on hepatoma cells. Our study indicates that SIRT2 enhances the tumoricidal activity of liver NK cells in HCC.

Keywords Sirtuin2 · NK cells · Erk1/2 · p38 · Hepatocellular carcinoma

Abbreviations

ACK	Ammonium–chloride–potassium
APC	Allophycocyanin
Erk	Extracellular-signal-regulated kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
HCC	Hepatocellular carcinoma
JNK	Jun N-terminal kinase
MAPK	Mitogen-activated protein kinases
MOI	Multiplicity of infection
NAD	Nicotinamide adenine dinucleotide
NK cells	Natural killer cells
NKG2	Natural killer group 2
PBS	Phosphate-buffered saline
RIPA	Radioimmunoprecipitation assay
SIRT	Sirtuin

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Introduction

The immune response against hepatocellular carcinoma (HCC) is a persistent research interest all over the world [1]. The cellular immune reactions, which involve the activation of specific and non-specific immune cells such as T cells and natural killer (NK) cells, play a significant role in the surveillance, confinement, and removal of malignant cells [2]. Therefore, researchers are trying to elucidate the mechanisms of tumor immunity, and looking for effective ways to mobilize and enhance anti-HCC immunity as an efficacious strategy to control the initiation and recurrence of HCC. Natural killer (NK) cells are a group of lymphocytes

possessing tumoricidal activity through the signals mediated by their activating and inhibitory surface receptors. These receptors can sense the abnormal changes in several surface markers on malignant cells [3, 4]. Through carefully regulating or resetting the tumoricidal mechanisms, NK cells can be engineered into a powerful tool to remove malignant cells [5, 6]. Therefore, it is critical for immunologists to thoroughly understand the details of the modulatory mechanisms of NK cell activity.

The Sirtuin (SIRT) family contains seven members (SIRT1-7), which act as nicotinamide adenine dinucleotide $(NAD)^+$-dependent protein deacetylases and/or ADP-ribosylases [7]. SIRTs participate in the modulation of intracellular pathways including metabolic programming, stress, and genome stability. The roles of SIRTs especially SIRT1 in the immune system have been recently disclosed. Particularly, SIRT1 blocks T cell activation, perpetuates $CD4^+$ T cell tolerance and inhibits Th17-related autoimmune disorders [8, 9]. Additionally, SIRT1 reduces Foxp3 expression and impairs the suppressive function of regulatory T cells [10–12]. However, the expression and functions of SIRTs in other immune cells have not been elucidated. In particular, whether SIRTs modulate the functions of anti-tumor NK cells remains unknown.

In this research, we used a chemical-induced mouse HCC model to identify the upregulation of SIRT2 in NK cells in HCC livers. In particular, SIRT2 was predominantly expressed in liver $CD94^+$ NK cells. The HCC liver microenvironment induced SIRT2 expression in NK cells. In addition, SIRT2 significantly upregulated the expression of cytokines and cytotoxic mediators in activated NK cells. Consistently, SIRT2-overexpressing NK cells showed a stronger tumoricidal effect on hepatoma cells. Moreover, SIRT2 remarkably promoted the phosphorylation of Erk1/2 and p38 MAPK. SIRT2 knockdown in $CD94^+$ NK cells impaired their cytotoxic effect on hepatoma cells. Taken together, our study indicates that SIRT2 enhances the tumoricidal activity of liver NK cells in HCC.

Materials and methods

HCC model

6-week-old male C57BL/6J mice and green fluorescent protein (GFP) transgenic mice (C57BL/6J background) were used for the study. To induce HCC in mice, each mouse was treated as follows: intraperitoneal injection of *N*-nitrosodiethylamine (80 μg/g body weight, Sigma-Aldrich) once. 48 h later, 10 μl/g body weight 10% CCl_4 (Lingfeng Chemical, in olive oil) was intraperitoneally injected twice a week for 4 weeks. 40 μg/g body weight *N*-nitrosodiethylamine was then intraperitoneally ministered once, and then 10 μl/g body weight 10% CCl_4 once a week for 14 weeks. HCC formation was confirmed by histological changes such as the presence of hyperplastic nodules and initiation foci. Only the mice with HCC were used. Control mice were injected with phosphate-buffered saline (PBS) and olive oil.

Enrichment of immune cells

The mouse liver was ground on a 200-gauge steel mesh until a single cell suspension was made. The single cells were then resuspended in 30% percoll (GE Healthcare) and overlaid onto an equal volume of 70% percoll. The mixture was then centrifuged at 500*g* for 20 min. The mononuclear cells were harvested from the interface of 30% percoll and 70% percoll. To isolate splenocytes, the mouse spleen was pressed through a 70-μm cell strainer. Red blood cells were removed using the Ammonium–Chloride–Potassium (ACK) lysis buffer (Thermo Fisher Scientific). In some cases, mononuclear cells were pooled from 2–4 spleens or livers.

Flow cytometry and cell sorting

Allophycocyanin (APC) anti-IFN-γ (XMG1.2), PE/Cy7 anti-TNF-α (MP6-XT22), APC anti-Granzyme B (GB11), PE anti-TCRβ (H57-597), APC/Cy7 anti-NK1.1 (PK136), PE/Cy7 anti-CD94 (18d3), FITC anti-CD27 (LG.3A10) were ordered from Biolegend. APC anti-Ly49C/I (5E6), PE anti-Natural Killer Group 2A/C/E (NKG2A/C/E, 20d5), PE/Cy7 anti-Ly49A (YE1/48.10.6), APC anti-NKG2D (CX5), PE/Cy7 anti-CD43 (1B11), PE anti-Ly49D (4E5), and PE/Cy7 anti-Ly-49G (4D11) were bought from BD Biosciences. PE/Cy7 anti-perforin antibody (eBioOMAK-D) was purchased from eBioscience. To stain cell surface markers, 1×10^5 cells in 50 μl PBS were incubated with antibodies (5 μg/ml each) at 4 °C for 30 min. To stain intracellular proteins, cells were subject to fixation with 4% paraformaldehyde at room temperature for 15 min, followed by permeabilization in 90% methanol-PBS on ice for 30 min. 10 μg/ml each antibody was then used to incubate cells at room temperature for 1 h. To evaluate apoptosis, cells were stained with the Apoptosis Detection Kit (Biolegend) based on the manufacturer's instructions. The samples were loaded on a BD LSR II flow cytometer for analysis. Cell sorting was done on a BD FACSAria sorter.

Quantitative RT-PCR

Cellular RNAs were purified using Trizol® (Invitrogen), and then reversely transcribed into cDNAs using the SuperScript™ cDNA Synthesis Kit (Invitrogen). Quantitative PCR was performed using SYBR™ Green PCR Master Mix (Invitrogen) on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Primer sequences are shown in the

Supplementary Table 1. The mRNA levels of target genes were normalized to β-actin. Relative gene expression was analyzed via the $2^{-\Delta\Delta Ct}$ calculation.

Adoptive transfer

Liver NK cells were sorted from normal GFP transgenic mice using the MojoSort™ Mouse NK Cell Isolation Kit (Biolegend). 2×10^6 NK cells in 100 µl of PBS were retro-orbitally injected into each control or HCC mouse. 1 or 3 weeks later, donor-derived GFP^+ NK cells were sorted from the livers of recipient mice.

Lentiviral transduction

SIRT2 Lentivector (#LV513623) and SIRT2 shRNA Lentivector (#i036352c) were purchased from Abm Inc. 4×10^5 HEK293T cells were incubated for 12 h in a culture dish, and then incubated with 15 µM chloroquine diphosphate (Sigma-Aldrich) for 4 h. After that, HEK293T cells were transfected with 0.8 pmol pRSV-Rev (Addgene), 1.5 pmol pMDLg/pRRE (Addgene), 2.0 pmol lentiviral vector and 100 µg polyethylenimine (Sigma-Aldrich) for 16 h. The medium was then replaced with 10 ml of fresh medium. 48 and 72 h after transfection, the lentiviral particles in the supernatants were enriched using the Lenti-X™ Maxi Purification Kit (Clontech). The lentiviral titers were evaluated using the Lenti-X qRT-PCR Titration Kit (Clontech). The SIRT2-encoding lentivirus was named SL, and the control lentivirus was named CL. To transduced NK cells, liver NK cells were sorted from control mice or HCC mice as described above. 1×10^6/ml liver NK cells were incubated with lentiviruses at the multiplicity of infection (MOI) of 40 in the presence of 8 µg/ml of polybrene (Sigma-Aldrich). The whole culture was centrifuged at 2000 rpm for 1 h at 32 °C. NK cells were then incubated in fresh medium supplemented with 1000 U/ml of rmIL-2 (R&D Systems) for 2 consecutive days.

To co-transduce normal liver NK cells with SIRT2 Lentivector and SIRT2 shRNA Lentivector, 1×10^6/ml liver NK cells were incubated with the mixture of SIRT2 Lentivector (MOI = 40) and SIRT2 shRNA Lentivector (MOI = 40) in the presence of polybrene during centrifugation at 2000 rpm for 1 h at 32 °C. The following steps were the same as above.

Cytotoxicity assay

The mouse hepatoma cell line Hepa1-6^{RAE1}, which expresses RAE1 (an NKG2D ligand) to activate NK cells [13], was a gift from Dr. Kai Dai in Wuhan University. 1×10^5/ml Hepa1-6^{RAE1} cells were co-cultured with 1×10^6/ml or 5×10^5/ml lentivirus-transduced NK cells (effector:target = 10:1 or 5:1) for 4 h in each well of a V-bottom 96-well microplate (Corning). Hepa1-6^{RAE1} cell apoptosis was then tested using the Apoptosis Detection Kit (Biolegend). Lentivirus-transduced NK cells were easily distinguished from Hepa1-6^{RAE1} cells due to the GFP expression.

To check the expression of cytotoxic mediators in co-cultured NK cells, the cell mixture was treated with 5 µg/ml brefeldin A and 5 µg/ml monensin (Sigma-Aldrich) for 2 h prior to the end of co-culture. The cells were then stained with antibodies against NK1.1 and intracellular molecules.

In vitro activation of NK cells

Liver NK cells were sorted from normal GFP mice using the MojoSort™ Mouse NK Cell Isolation Kit (Biolegend). 5×10^5/ml NK cells were cultured in RIMI1640 supplemented with 10% fetal bovine serum in the presence of rmIL-2 (50 U/ml), rmIL-15 (10 ng/ml), rmIL-12 plus rmIL-18 (10 ng/ml & 100 ng/ml), or equal amount of Hepa1-6^{RAE1} cells overnight. The cytokines were purchased from R&D Systems. The NK cells co-cultured with Hepa1-6^{RAE1} cells were then sorted by flow cytometry based on their GFP expression. NK cells were lysed and subject to Immunoblotting assay for SIRT2 expression.

Immunoblotting

Total cellular proteins were extracted using the radioimmunoprecipitation assay (RIPA) buffer (Abcam) in the presence of protease inhibitors and phosphatase inhibitors (both from Sigma-Aldrich). SIRT1, SIRT2, and SIRT5 antibody were purchased from Abcam (ab110304, ab51023 & ab105040). Phospho-Erk1/2 (Thr202/Tyr204, #8544), phospho-p38 (Thr180/Tyr182, #4511), Erk1/2 (#4695), Jun N-terminal kinase (JNK, #9252), p38 (#8690), phospho-JNK (Thr183/Tyr185, #4668) antibody were purchased from Cell Signaling Technology. The optical densities of the protein bands were scanned on a Biospetrum 300 system (UVP Ltd). To evaluate the expression of the proteins of interest, the optical densities of specific proteins were first normalized to the corresponding optical densities of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The normalized protein expression relative to the CL-transduced NK cell group or scramble shRNA lentivirus-transduced NK cell group was then calculated.

Statistics

Data are shown as mean ± standard deviation. The unpaired *t* test or one-way ANOVA with post hoc Tukey HSD test was used for comparison of the averages. Each experiment was independently repeated two or three times. $P<0.05$ was regarded as statistically significant.

Results

Liver CD94$^+$ NK cells upregulate SIRT2 expression in the HCC model

After HCC induction, mononuclear cells were enriched from spleens and livers of control and HCC mice (Supplementary Fig. 1). TCRβ$^-$NK1.1$^+$ NK cells were then sorted using flow cytometry (Fig. 1a). The mRNA abundances of SIRTs in NK cells were evaluated by quantitative RT-PCR. No change in the expression of SIRT3, SIRT4, and SIRT6 was observed (Supplementary Fig. 2), while no SIRT7 amplification was achieved (data not shown). SIRT1 expression in either liver NK cells or splenic NK cells was not altered by HCC induction (Supplementary Fig. 2). The mRNA levels of SIRT2 and SIRT5 were significantly increased in liver NK cells in HCC mice (25.45-fold increase and 1.45-fold increase, respectively), as compared with liver NK cells in control mice (Fig. 1b, c). Notably, SIRT2 expression showed the most significant change (Fig. 1b). The upregulation of SIRT2 in liver NK cells of HCC mice was confirmed by immunoblotting (Fig. 1d). To further characterize SIRT2 expression, we dissected NK cells based on the expression of CD94 and CD27, which are the markers for distinct NK subsets [14, 15]. Splenic and liver NK cells were divided into four subsets: CD94$^-$CD27$^+$, CD94$^-$CD27$^-$, CD94$^+$CD27$^-$, and CD94$^+$CD27$^+$ (Fig. 2a). The SIRT2 mRNA and protein were predominantly upregulated in CD94$^+$CD27$^-$ and CD94$^+$CD27$^+$ NK cells in HCC livers (Fig. 2b, c).

The HCC microenvironment induces SIRT2 expression in liver NK cells

To test whether the HCC microenvironment induces SIRT2 expression in NK cells, we sorted NK cells from normal GFP mouse livers and transferred them into control mice or HCC mice. 1 or 3 weeks after transfer, GFP$^+$ NK cells were sorted from the livers of recipient mice, and SIRT2 expression was analyzed by q-RT-PCR. As shown in Fig. 3, in comparison with GFP$^+$ NK cells in the livers of recipient control mice, GFP$^+$ NK cells in the livers of recipient HCC mice expressed higher SIRT2 1 week (2.61 folds) and 3 weeks (6.15 folds) after transfer. Hence, the HCC liver microenvironment induced SIRT2 expression in NK cells.

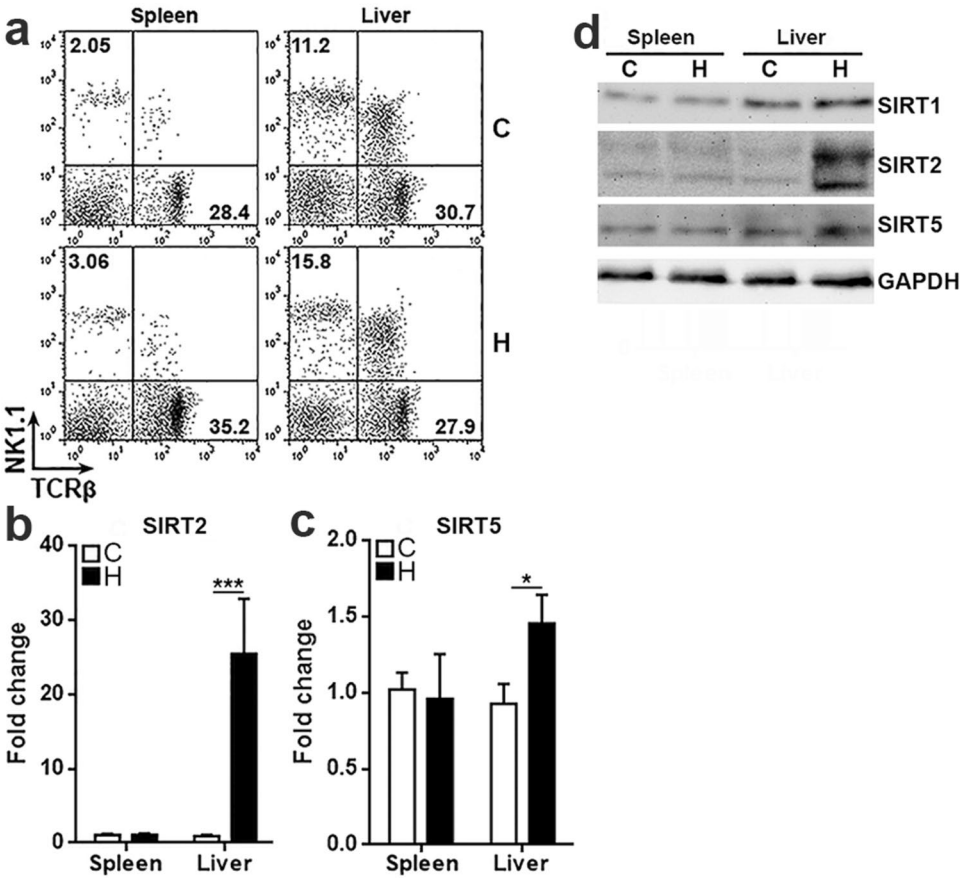

Fig. 1 SIRT2 expression in liver NK cells. **a** The gating strategy for NK cells in the spleens and livers of control and HCC mice. *C* control mice, *H* HCC mice. **b**, **c** mRNA levels of SIRT2 and SIRT5 in splenic and liver NK cells in control or HCC mice. $N=5$ per group. $^*p<0.05$; $^{***}p<0.001$. **d** Protein levels of SIRT1, SIRT2, and SIRT5 in splenic and liver NK cells in control or HCC mice. This is a representative of two independent experiments

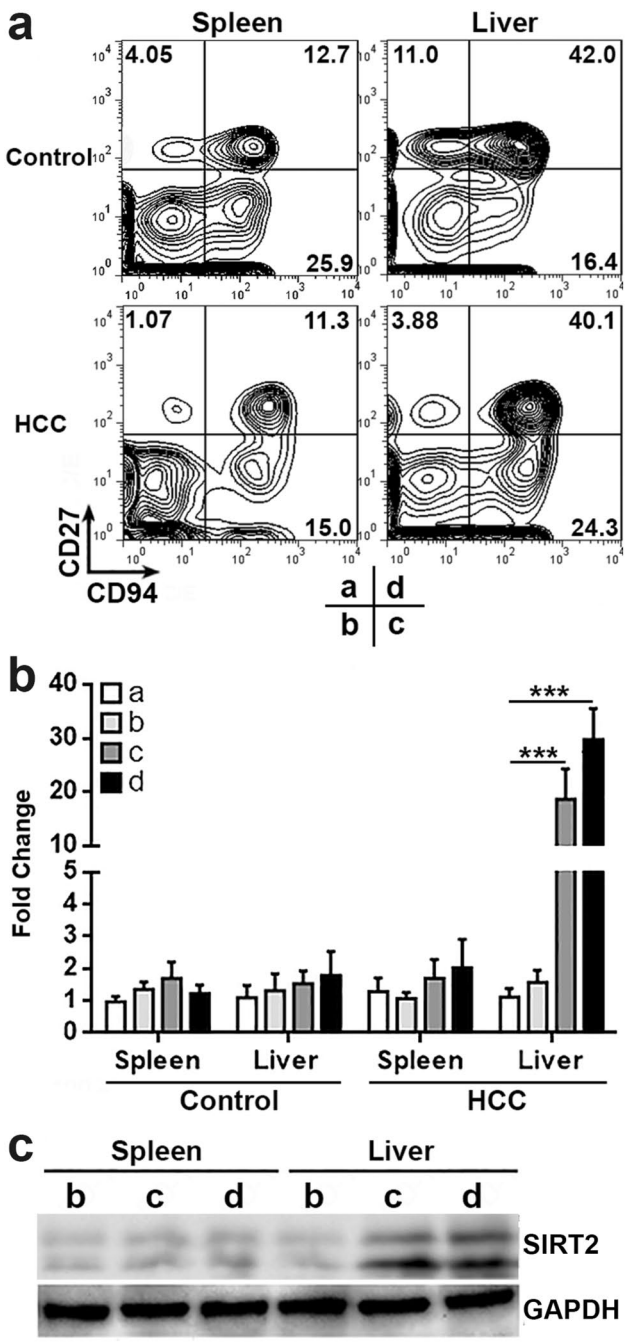

Fig. 2 SIRT2 expression in NK subsets. **a** Splenic and liver NK subsets based on the expression of CD94 and CD27 in control and HCC mice. **b** SIRT2 mRNA levels in NK subsets in spleens and livers. a: $CD94^{-}CD27^{+}$ cells. b: $CD94^{-}CD27^{-}$ cells. c: $CD94^{+}CD27^{-}$ cells. d: $CD94^{+}CD27^{+}$ cells. $N=4$ per group. $^{***}p<0.001$. **c** SIRT2 protein in the three NK subsets in spleens and livers of HCC mice. Note that the subset "a" ($CD94^{-}CD27^{+}$ cells) is not shown due to insufficient cellularity

SIRT2 promotes NK cell function

To assess the effects of SIRT2 on NK cell function, we transduced normal liver NK cells with lentivirus encoding SIRT2 and GFP. Over 60% of NK cells were GFP^{+} after transduction (Fig. 4a), suggesting that the transduction was successful. SIRT2 expression was remarkably elevated in SL-transduced NK cells in comparison with CL-transduced NK cells (Fig. 4b). We co-cultured these NK cells with Hepa1-6^{RAE1} cells for 4 h and then conducted intracellular staining to analyze the expression of cytokines and cytotoxic mediators in $NK1.1^{+}$ NK cells via flow cytometry. Exogenous SIRT2 did not alter the basal expression of these molecules when NK cells were cultured alone (data not shown). However, exogenous SIRT2 upregulated the expression of IFN-γ (40.64 ± 3.35% vs 20.66 ± 2.06%), TNF-α (24.36 ± 2.97% vs 13.15 ± 1.31%), perforin (22.78 ± 1.64% vs 16.37 ± 0.69%) and granzyme B (36.15 ± 2.13% vs 26.78 ± 1.94%) in co-cultured (i.e., activated) NK cells (Fig. 4c, d). To check the tumoricidal activity of NK cells, we co-cultured lentivirus-transduced NK cells with Hepa1-6^{RAE1} cells at the ratio of 5:1 or 10:1 for 4 h. The apoptosis of Hepa1-6^{RAE1} cells (GFP-negative cells in the co-culture as shown in Supplementary Fig. 3) was then evaluated by flow cytometry. As illustrated in Fig. 4e, f, SL-transduced NK cells induced more apoptosis of Hepa1-6^{RAE1} cells in comparison with CL-transduced NK cells at the ratio of either 5:1 (22.11 ± 1.58% vs 13.74 ± 1.19%) or 10:1 (39.43 ± 2.64% vs 24.41 ± 1.49%). Exogenous SIRT2 did not trigger NK cell apoptosis (Fig. 4g).

The NK cell activity is modulated by the engagement of activating or inhibitory receptors with their ligands. To check if SIRT2 regulates expression of these receptors, we conducted surface staining of several receptors on CL-transduced and SL-transduced NK cells. As shown in Supplementary Fig. 4, CL-transduced and SL-transduced NK cells expressed comparable levels of NKG2D, NKG2A/C/E, Ly49A, Ly49C, Ly49D, Ly49G, and CD43, respectively. Therefore, it is unlikely that SIRT2 functions by altering the expression of inhibitory or activating receptors.

SIRT2 promotes Erk1/2 and p38 MAPK signaling in activated NK cells

SIRT2 has been shown to increase MAPK signaling in other cell types [16, 17]. To determine if this is the case in NK cells, lentivirus-transduced NK cells were incubated in the medium free of IL-2 for 4 h, followed by culture alone or co-culture with Hepa1-6^{RAE1} cells for 2 h. After that, the cell mixture was incubated with 0.25% Trypsin–EDTA (Sigma-Aldrich) for 5 min at room temperature to dissociate cell clusters. GFP^{+} cells, i.e., lentivirus-transduced NK cells were then sorted from the cell mixture via flow cytometry, and the phosphorylation of

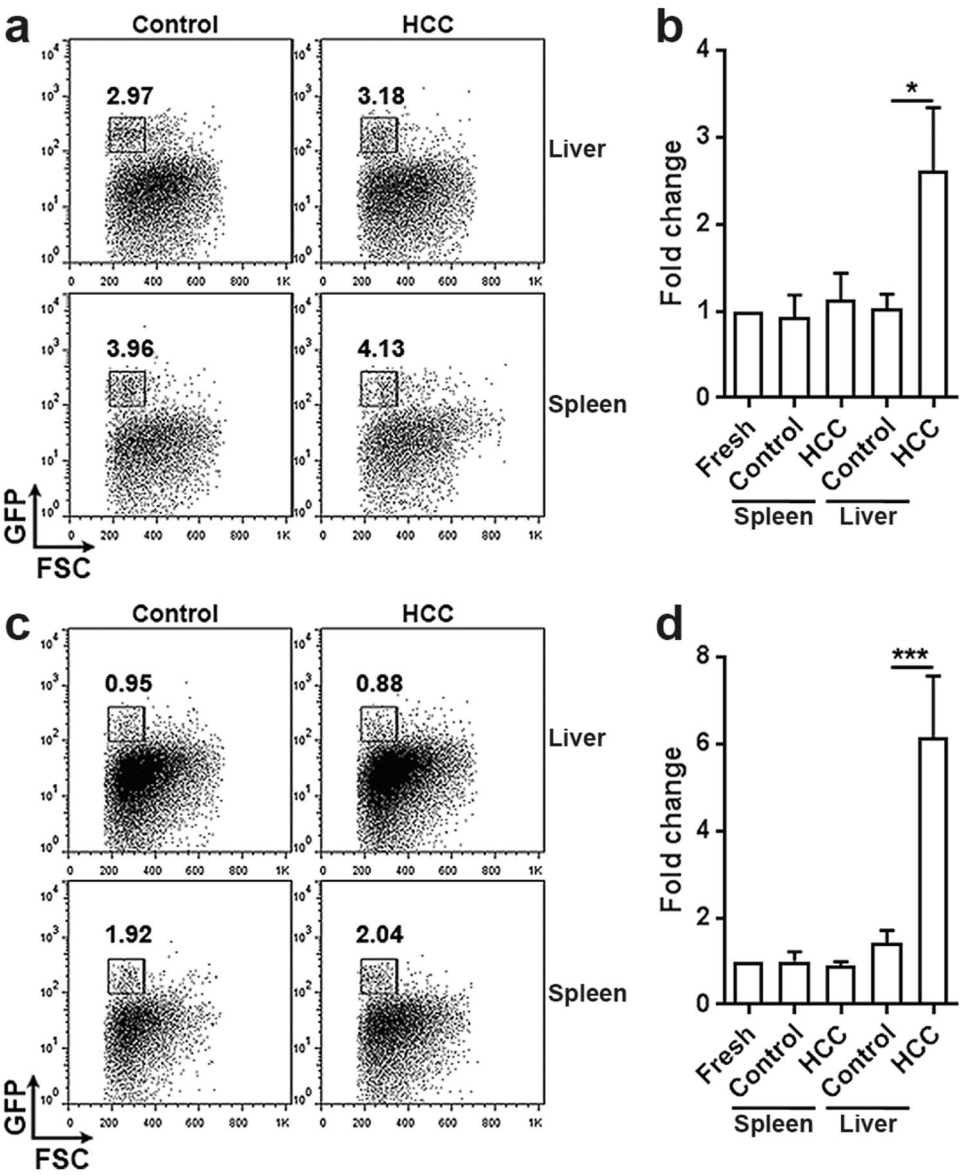

Fig. 3 The HCC liver microenvironment induces SIRT2 expression in NK cells. **a**, **c** The gating strategy for donor-derived GFP$^+$ NK cells in the livers and spleens of recipient mice 1 week (**a**) or 3 weeks (**c**) after transfer. *Control* recipient control mice, *HCC* recipient HCC mice. **b**, **d** SIRT2 mRNA levels in donor-derived GFP$^+$ NK cells 1 week (**b**) or 3 weeks (**d**) after transfer. $N=4$ per group. $^*p<0.05$. $^{***}p<0.001$

Erk1/2, p38, and JNK were subsequently detected in NK cells by immunoblotting. As shown in Fig. 5, exogenous SIRT2 did not profoundly alter the phosphorylation of Erk1/2, p38 and JNK when NK cells were cultured alone. Co-culture with target cells induced the phosphorylation of Erk1/2 (2.19-fold increase) and p38 (2.35-fold increase) in CL-transduced NK cells, and exogenous SIRT2 further increased the phosphorylation of Erk1/2 (3.75-fold increase) and p38 (5.30-fold increase) (Fig. 5a–c). Exogenous SIRT2 had no effect on JNK phosphorylation or expression in co-cultured NK cells (Fig. 5a, d, e).

SIRT2 knockdown impairs the tumoricidal activity of liver CD94$^+$ NK cells

To further explore the function of SIRT2, liver CD94$^+$ NK cells, which highly expressed SIRT2, were sorted from HCC mice and then transduced with SIRT2 shRNA lentivirus to reduce SIRT2 expression, as described in "Materials and Methods". 2 days after the transduction, these NK cells were incubated in the medium free of IL-2 for 4 h, and were subsequently incubated with or without Hepa1-6^{RAE1} cells at the ratio of 5:1 for 2 h. After incubation, the cell mixture was incubated with 0.25% Trypsin–EDTA for 5 min at room temperature to prepare the single cell suspension. GFP$^+$ cells, i.e., lentivirus-transduced NK cells were then sorted from the cell mixture via flow cytometry, and the phosphorylation of Erk1/2, p38 and JNK were subsequently detected in NK cells by Immunoblotting. As shown in Fig. 6a, after co-culture, the phosphorylation of Erk1/2 and p38 was almost reduced by half in SIRT2-knockdown NK cells as compared with that in control NK cells (Fig. 6a, b). To test the cytotoxic activity of NK cells, lentivirus-transduced NK cells were

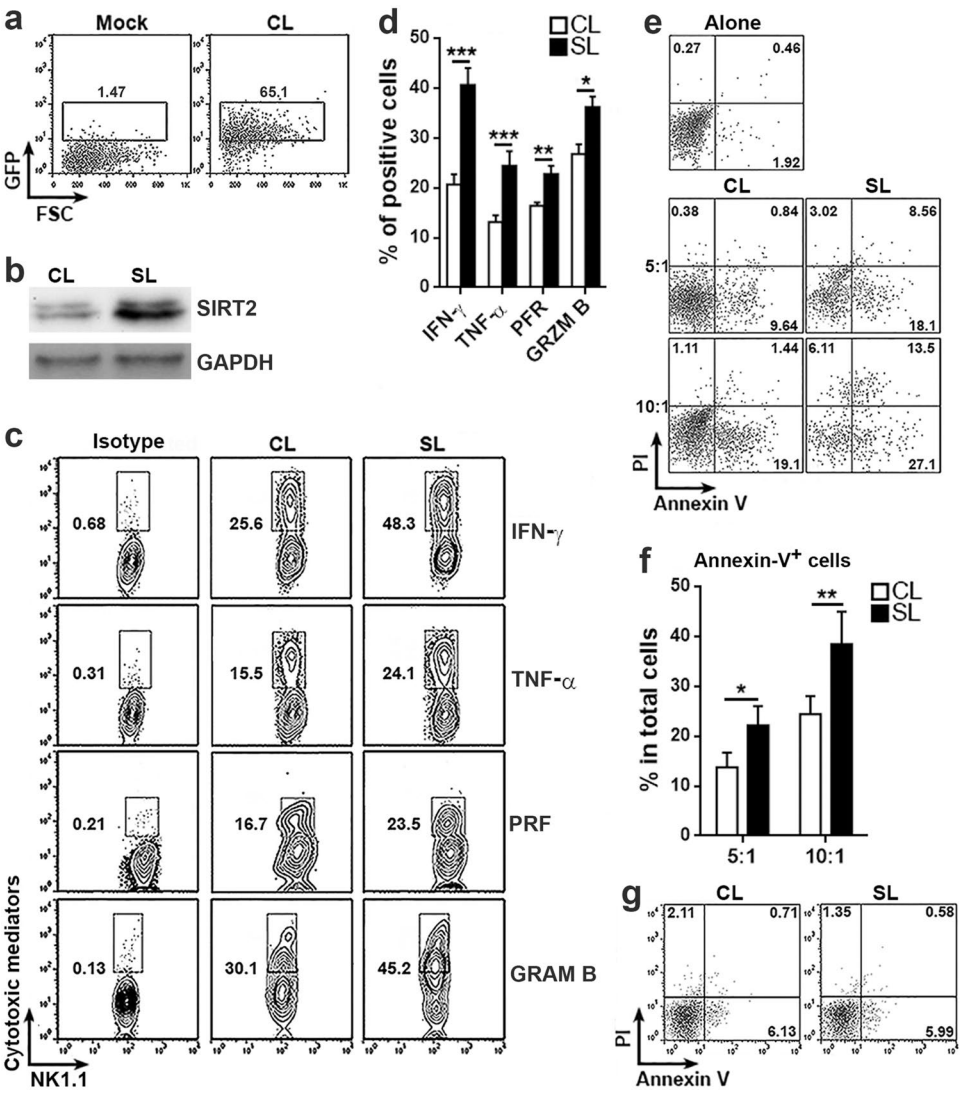

Fig. 4 SIRT2 promotes NK cell function. **a** GFP expression in NK cells at day 2 after lentiviral transduction. Mock: mock transduction. CL: transduction with control lentivirus. **b** SIRT2 protein in NK cells at day 2 after transduction. *CL* CL-transduced NK cells, *SL* SL-transduced NK cells. **c**, **d** The expression of indicated molecules in lentivirus-transduced NK cells after co-culture with Hepa1-6^{RAE1} cells. Representative contour plots are displayed in **c**, and the statistical results are shown in **d**. $N=6$ per group. **e**, **f** Apoptosis of Hepa1-6^{RAE1} cells after co-culture with lentivirus-transduced NK cells. Representative dot plots are shown in **e**, the statistical result is shown in **f**. CL: co-culture with CL-transduced NK cells. *SL* co-culture with SL-transduced NK cells. $N=6$ per group. **g** NK cell apoptosis at day 2 after lentiviral transduction. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$

co-cultured with Hepa1-6^{RAE1} cells for 4 h. At the effector/target ratio of 5:1, SIRT2-knockdown NK cells induced less apoptosis of target cells in comparison to control NK cells ($10.56\pm3.17\%$ vs $20.48\pm4.29\%$, Fig. 6c, d). Additionally, we also sorted liver $CD94^{+}$ NK cells from control mice and HCC mice, and co-cultured them with Hepa1-6^{RAE1} cells for 4 h, respectively. We found that liver $CD94^{+}$ NK cells from HCC mice induced more Hepa1-6^{RAE1} apoptosis than their counterparts from control mice at the effector/target ratio of 10 ($14.41\pm3.01\%$ vs $6.88\pm1.13\%$, Fig. 6e).

Moreover, we co-transduced normal liver NK cells with SIRT2-encoding lentivirus and SIRT2 shRNA lentivirus to test if SIRT2 knockdown diminishes the effect of exogenous SIRT2 on NK cells. As shown in Supplementary Fig. 5a, exogenous SIRT2 expression was significantly reduced by SIRT2 shRNA. In the cytotoxicity assay, SIRT2 knockdown alleviated NK cell-induced apoptosis of target cells, as compared with SIRT2-overexpressing NK cells ($12.13\pm3.33\%$ vs $23.35\pm4.14\%$, Supplementary Fig. 5b, c). Therefore, SIRT2 is a positive regulator of NK cells.

In vitro activation does not induce SIRT2 expression in NK cells

To test if NK cell activation triggers SIRT2 expression, we sorted liver NK cells-normal GFP mice and stimulated them in vitro overnight with IL-2, IL-15, IL-12 plus IL-18, or equal amount of Hepa1-6^{RAE1} cells. The SIRT2 protein was analyzed after stimulation. However, these cytokines and NKG2D signaling did not induce SIRT2 expression (Supplementary Fig. 6). Therefore, the factors responsible for SIRT2 induction remain unclear.

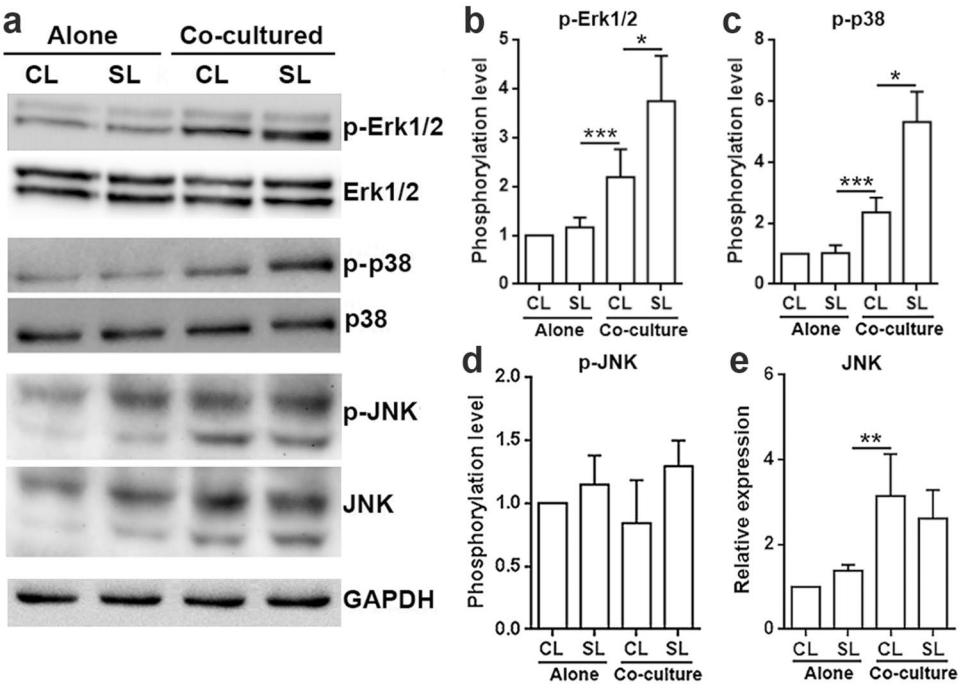

Fig. 5 SIRT2 activates Erk1/2 and p38 MAPK in NK cells. **a** Representative immunoblotting images showing the phosphorylation of Erk1/2, p38, and JNK in NK cells. Alone: NK cells cultured alone. Co-cultured: NK cells co-cultured with target cells. *CL* CL-transduced NK cells. *SL* SL-transduced NK cells. **b–d** The statistical results of phosphorylated Erk1/2, p38 and JNK. **e** Relative expression of JNK. The optical densities of target proteins are normalized to corresponding optical densities of GAPDH, followed by calculating the expression relative to the CL-transduced NK cell group. $N=4$ per group. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$

Discussion

$CD94^{+}$ NK cells are more effective than $CD94^{-}$ NK cells [14]. In our study, $CD94^{+}CD27^{+}$ NK cells expressed higher SIRT2 than $CD94^{+}CD27^{-}$ NK cells. $CD27^{+}$ NK cells are potent cytokine producers in several tissues [18], and $CD27^{-}$ NK cells are dysfunctional in patients with HCC [19]. Hence, it is likely that SIRT2 expression is associated with the activity of liver NK cells. To our knowledge, we are the first to report the significance of SIRT2 in primary NK cells.

Our data suggest that the HCC liver microenvironment is crucial for SIRT2 expression. Our findings are consistent with the notion that the basic function of SIRTs is to promote the communication between cells and their environment [20]. However, the exact SIRT2-inducing factors in HCC livers remain unidentified. SIRTs are extensively regulated in response to a wide range of stimuli, including nutritional and metabolic challenges, inflammatory signals or hypoxic and oxidative stress [21]. SIRT2 expression is shown to be dependent on p53 due to the binding of p53 to the *SIRT2* promoter [22]. A recent study indicates that p53 regulates NK cell functional maturation [23]. It is possible that HCC up-regulates p53 in liver NK cells, and p53 subsequently induces SIRT2 expression. Our ongoing study is testing this hypothesis. Moreover, because SIRT2 is highly expressed in $CD94^{+}$ NK cells, it is likely that CD94-mediated signaling mediates SIRT2 expression.

We also found that SIRT2 enhanced the tumoricidal effect of NK cells, probably through activating Erk1/2 and p38 MAPK signaling. Consistently, a previous study demonstrates that SIRT2 induces the activation of Erk1/2 and p38 MAPK in different cell types [24, 25]. Importantly, Erk1/2 is essential for NK cell activity, since many NK cell receptors activate Erk2 signaling to trigger granule polarization and cytotoxicity [26]. Crosslinking of CD28, NKG2D and other NK receptors leads to Erk2 activation in NK cells [26]. Additionally, p38 activation upregulates cytokine production within NK cells [27]. SIRT2 might activate Erk1/2 and p38 signaling to both and promote the tumoricidal effect of NK cells. SIRT2 can act as an ADP-ribosyltransferase, deacetylase, and demyristoylase [21]. These enzymic activities might result in MAPK activation.

Although we found that SIRT2 favored NK cell function, SIRT2 was only expressed in about 60% of liver NK cells ($CD94^{+}$ NK cells). It is plausible to speculate that if SIRT2 is expressed in all liver NK cells, or more SIRT2 is expressed in an active NK cell population, the tumoricidal effect would be more potent and the HCC growth would be inhibited more robustly. Therefore, our next plan is to elucidate the signal pathway that is crucial for the induction of SIRT2 in NK cells. If the pathway is disclosed, we might use chemical compounds that specifically activate or deactivate this pathway to stimulate SIRT2 expression in NK cells. Moreover, the NK-92 cell line, which is a human NK cell line highly cytotoxic to a broad spectrum of malignant cells, can be genetically modified to CAR-NK-92 cells to kill HCC [28]. If we can induce the stable expression of SIRT2 in CAR-NK-92 cells, we might promote the anti-HCC function of these cells and thus develop a new therapeutic agent

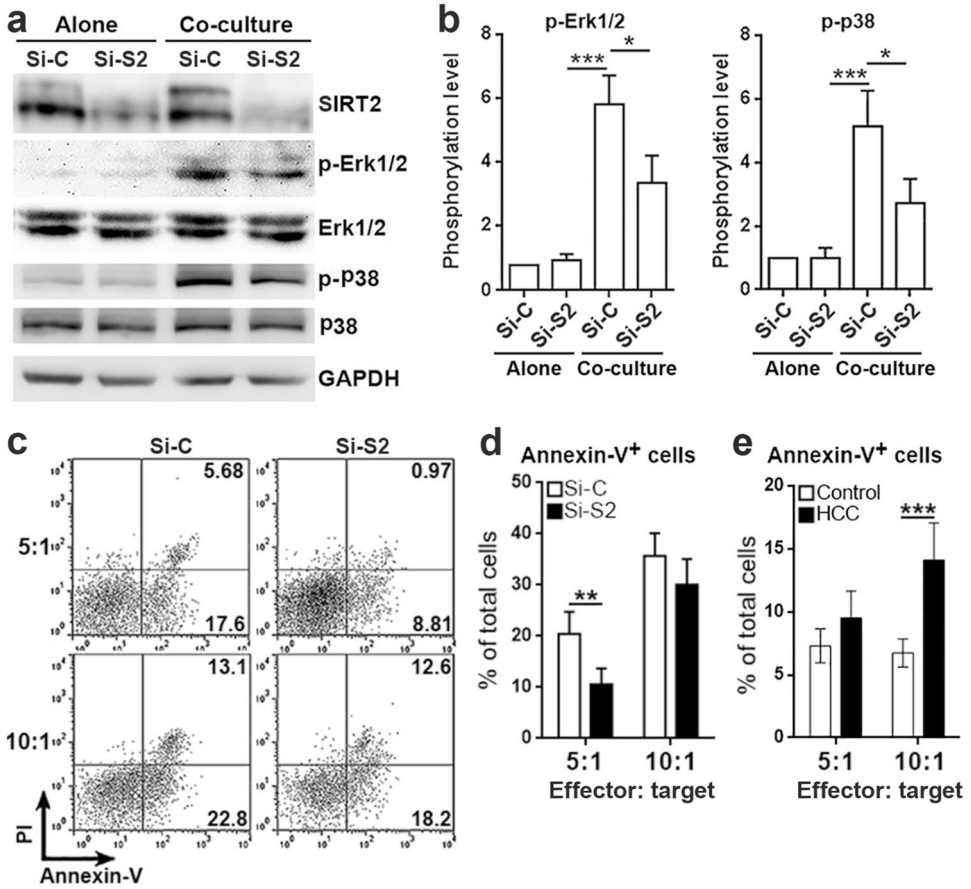

Fig. 6 SIRT2 knockdown impairs the tumoricidal activity of CD94$^+$ NK cells. **a**, **b** SIRT2 expression and the phosphorylation of p38 and Erk1/2 in CD94$^+$ liver NK cells after lentiviral transduction and co-culture with target cells. Si–C: NK cells transduced with scramble shRNA lentivirus. Si-S2: NK cells transduced with SIRT2 shRNA lentivirus. Representative immunoblotting images are shown in **a**, and the statistical results of phosphorylated Erk1/2 and p38 are shown in **b**. The optical densities of target proteins are normalized to the corresponding optical densities of GAPDH, followed by calculation of the expression relative to the scramble shRNA lentivirus-transduced NK cell group. $N=5$ per group. **c**, **d** Apoptosis of Hepa1-6^{RAE1} cells after co-culture with lentivirus-transduced NK cells. Representative dot plots are shown in **c**. The statistical result is shown in **d**. Si–C: co-culture with NK cells transduced with scramble shRNA lentivirus. Si-S2: co-culture with NK cells transduced with SIRT2 shRNA lentivirus. $N=5$ per group. **e** Apoptosis of Hepa1-6^{RAE1} cells after co-culture with liver CD94$^+$ NK cells sorted from control mice or HCC mice, respectively. *Control* NK cells from control mice, *HCC* NK cells from HCC mice. $N=3$ per group. $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$

for HCC. In addition, enhanced SIRT2 expression might promote the tumoricidal function of NK-92 cells against other tumors.

In the RNA interference assay, the knockdown of SIRT2 reduced the cytotoxic effect of NK cells when the effector/target ratio was 5:1. At the ratio of 10:1, SIRT2 knockdown only insignificantly downregulated the cytotoxic effect. This could be explained by the fact that SIRT2 only partially impacts NK cell activity. When NK cell number is limited, the effect of SIRT2 is easy to be shown. However, when NK cells are abundant relative to target cells, the NK cell number compensates for the SIRT2 knockdown-induced reduction of cytotoxicity.

Taken together, our study highlights the role of SIRT2 in the regulation of NK cell-mediated tumoricidal function. SIRT2 could be a novel therapeutic target to promote anti-tumor immunity in the HCC immunotherapy.

Conclusions

1. SIRT2 is upregulated in liver CD94$^+$ NK cells in mice with HCC.
2. The HCC liver microenvironment induces SIRT2 expression in NK cells.
3. SIRT2 promotes the cytotoxic effect of NK cells on HCC cells.
4. SIRT2 functions by upregulating the phosphorylation of Erk1/2 and p38 MAPK.

Author contributions MC conducted the animal model, cell sorting, quantitative RT-PCR, adoptive transfer, and in vitro culture. MX prepared the lentiviruses and performed the lentiviral transduction. CZ conducted the immunoblotting analysis. HW did the intracellular staining and flow cytometry analysis. QZ conducted the statistical analysis. FZ designed the experiments, analyzed the data and composed the manuscript.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval The animal study was approved by the Wuhan University Animal Care and Use Committee (animal research approval number: 11400700245566). The animal experiments were performed following the Wuhan University Animal Use Guidelines.

Informed consent Not applicable because no human subjects were included.

Animal source All mice were obtained from SBS Genetech Co, Ltd.

Cell line authentication Not applicable because primary NK cells were used. The mouse hepatoma cell line Hepa1-6^{RAE1} was a gift from another lab (from Dr. Kai Dai, Wuhan University) so no authentication information was gained. The HEK293T cell line was purchased from Procell Life Science & Technology Co, Ltd. (Wuhan, China) and was not authenticated, because this cell line was only used for lentivirus packaging in the current study.

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