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Radiotherapy enhances the anti-tumor effect of CAR-NK cells for hepatocellular carcinoma

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Abstract

Background Chimeric antigen receptor (CAR)-NK cell therapy has shown remarkable clinical efficacy and safety in the treatment of hematological malignancies. However, this efficacy was limited in solid tumors owing to hostile tumor microenvironment (TME). Radiotherapy is commonly used for solid tumors and proved to improve the TME. Therefore, the combination with radiotherapy would be a potential strategy to improve therapeutic efficacy of CAR-NK cells for solid tumors.

Methods Glypican-3 (GPC3) was used as a target antigen of CAR-NK cell for hepatocellular carcinoma (HCC). To promote migration towards HCC, CXCR2-armed CAR-NK92 cells targeting GPC3 were first developed, and their cytotoxic and migration activities towards HCC cells were evaluated. Next, the effects of irradiation on the anti-tumor activity of CAR-NK92 cells were assessed in vitro and in HCC-bearing NCG mice. Lastly, to demonstrate the potential mechanism mediating the sensitized effect of irradiation on CAR-NK cells, the differential gene expression profiles induced by irradiation were analyzed and the expression of some important ligands for the NK-cell activating receptors were further determined by qRT-PCR and flow cytometry.

Results In this study, we developed CXCR2-armed GPC3-targeting CAR-NK92 cells that exhibited specific and potent killing activity against HCC cells and the enhanced migration towards HCC cells. Pretreating HCC cells with irradiation enhanced in vitro anti-HCC effect and migration activity of CXCR2-armed CAR-NK92 cells. We further found that only high-dose (8 Gy) but not low-dose (2 Gy) irradiation in one fraction could significantly enhanced in vivo anti-HCC activity of CXCR2-armed CAR-NK92 cells. Irradiation with 8 Gy significantly up-regulated the expression of NK cell-activating ligands on HCC cells.

Conclusions Our results indicate the evidence that irradiation could efficiently enhance the anti-tumor effect of CAR-NK cells in solid tumor model. The combination with radiotherapy would be an attractive strategy to improve therapeutic efficacy of CAR-NK cells for solid tumors.

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Introduction

According to the Global Cancer Statistics 2022, liver cancer ranks as the sixth most common cancer globally and the third leading cause of cancer-related mortality, with an estimated 865,000 new cases and 757,948 deaths in 2022. Hepatocellular carcinoma (HCC), the predominant form of primary cancer, accounts for 75–85% of all cases [1]. A multidisciplinary approach to treatment is crucial for extending the survival of HCC patients. Despite a five-year survival rate of approximately 60% for those who underwent resection of HCC lesions, tumor recurrence affects up to 80% of these patients [2]. Moreover, due to the difficulty in diagnosing early-stage hepatocellular carcinoma, most HCC patients are diagnosed with advanced stage at the time of initial diagnosis, thus missing the opportunity for curative treatments such as hepatectomy or radiofrequency ablation [2, 3]. Chronica infections with the hepatitis B virus and hepatitis C virus are the main risk factors for HCC development [3]. Long-term inflammation and antigen stimulation foster an immune-tolerant tumor microenvironment (TME) in liver cancer, facilitating the immune evasion of tumor cells and their resistance to immune checkpoint inhibitors (ICIs) [4].

Chimeric antigen receptor (CAR) engineered NK (CAR-NK) cells, as a novel immunotherapy technology, have dual anti-tumor effects and better safety, and are easy to be generated as off-the-shelf and universal products compared to current CAR-T cells [5]. CD19-targeting CAR-NK cells have exhibited remarkable clinical efficacy and safety in CD19-positive hematological malignancy [6]. Nevertheless, for solid tumors, the treatment with CAR-NK cells is still struggling due to immunosuppressive TME which not only hinders the infiltration of CAR-NK cells into tumor, but also attenuates the cytotoxic effect of intratumoral CAR-NK cells [7].

Radiotherapy (RT) is a commonly used treatment modality for solid tumors, including HCC. Numerous studies have shown that radiotherapy significantly improves the tumor immune microenvironment and enhances anti-tumor immune response through mechanisms such as releasing tumor antigens, damaging tumor stromal cells, and altering cytokine expression profiles [8, 9]. The combination of radiotherapy and ICIs has been extensively investigated and shown a strong synergistic effect in preclinical and clinical studies. Furthermore, previous studies have shown that DNA damage caused by radiotherapy significantly induced the expression of NKG2D ligands on tumor cells, which enhanced the anti-tumor activity of NK cells [10]. Therefore, radiotherapy may enhance the anti-tumor effect of CAR-NK cells by improving the tumor microenvironment and increasing the expression of NK cell-activating ligands in tumor cells. Here, we provided the preclinical evidence supporting the potential of radiotherapy in enhancing the anti-tumor effect of CAR-NK cells in HCC solid tumor.

Materials and methods

Cell lines and culturing conditions

Huh-7, HepG2, Hep3B, HEK293T, L-02 and U87 cell lines were purchased from Nation Collection of Authenticated Cell Cultures in China. NK92 and Jurkat cell lines were purchased from Guangzhou Cellcook Biotech. Huh-7, HepG2, Hep3B, U87, L-02 and HEK293T cell lines were maintained in DMEM medium (Gibco) supplemented with 10% FBS (Excell Bio), 100 IU/mL of penicillin–streptomycin (Hyclone). Jurkat cells were cultivated in RPMI-1640 (Gibco) complete medium. NK-92 cells were cultured in PRIME-XV NK cell CDM (FUJIFILM Irvine Scientific) with 400 IU/mL IL-2 (Peprotech) and 100 IU/mL of penicillin–streptomycin (Hyclone). All cells were cultured in a humidified atmosphere of 5% CO2 at 37 °C.

Generation of CAR-expressing NK92 cells

The Glypican-3 (GPC3)-specific scFv sequence was derived from anti-glypican-3 antibody (Pub No. US 20070087005A1). Lentiviral particles (LVP) were produced by transfecting 293 T cells with pHIV-CAR-GFP (or pHIV-CAR-CXCR2), pCMV-VSVG, pCMV-Δ8.9 and pAdv plasmids. Culture supernatant was collected at 48 and 72 h, and filtered through a 0.45-um filter to remove cell debris and were pelleted by ultracentrifugation at 25,000 rpm at 4 ℃ for 2 h. Lentivirus particles were suspended in 100 µl serum-free DMEM media and frozen at − 80 °C. NK92 cells were transduced with lentivirus (MOI = 10), and CAR-NK92 cells were purified by a FACS sorter (BD Aria III).

Flow cytometry

Cells were stained at 4 ℃ using the indicated antibodies in flow cytometry buffer made of PBS (Gibco) supplemented with 2% FBS for 20 min. The cells were washed with FACS buffer, and then resuspended in FACS buffer containing 4′,6-diamidino-2-phenylindole (DAPI). Flow cytometry analysis was performed by a FACS system (BD LSRFortessa).

The expression of GPC3-targeting CAR on NK92 cell was determined using biotinylated human glypican-3 protein (Acrobiosystems) and APC-Streptevidin (BD Biosciences). CXCR2 expression on NK92 cells was detected using FITC anti-human CXCR2 antibody (Biolegend). GPC3 surface expression on cancer cells were determined by APC-labeled anti-human GPC3 antibody (SinoBiological). For CD107a staining,

CAR-NK92 cells were co-cultured with target cells (1:1 ratio) for 4 h, adding 1uM monensin (Biolegend) in culture media. After that, cells were washed and stained with APC-labeled anti-human CD107a antibody (Biolegend) and DAPI.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using Trizol reagent according to the manufacturer's instructions. Some 500 ng of total RNA was used as a template for complementary DNA synthesis with 1st strand cDNA synthesis supermix (Yeasen). Primers were designed using National Center for Biotechnology Information (NCBI) GeneBank sequences (for primer sequences, see supplementary Table 1). The GAPDH gene and β-actin gene were utilized as an endogenous control. The LightCycler 480 system (Roche Diagnostics) was used to perform qPCR.

Irradiation in vitro and in vivo

For in vitro experiments, tumor cells were seeded in a 6-well plate and then radiated by Precise X-Ray (Elekta) at 500 cGy/min at room temperature. After 6 h, 1×10^4 radiated cells and control cells were seeded with effector cells in a 96-well plate for subsequent assays.

For in vivo experiments, we used the ARRIVE1 reporting guidelines [11]. Subcutaneous tumor was locally irradiated using Precise X-RAY (Elekta). Tumor-bearing mice were anesthetized with 50 mg/kg pentobarbital by intraperitoneal injection and placed 100 cm away from the radiation source with the radiation beam being sharply collimated to only cover the right thighs. A dose of 2 or 8 Gy was delivered.

Transwell migration assay

NK92 cells (2×10^5) were placed in the upper chamber (5.0-μm pore size) of a well in a 24-well transwell plate (Biofil), and the lower chamber was filled with the supernatant from cancer cell culture. After 6 h incubation at 37 ℃, cells from the lower chamber were collected and determined by flow cytometry with precise count beads (Biolegend).

Cytotoxicity of CAR-NK92 cells in vitro

A total of 1×10^4 luciferase-expressing target cells were co-cultured with untransduced, CAR-GFP or CAR-CXCR2 NK92 cells, respectively, at the indicated effector-to-target ratios in triplicate wells of V-bottomed 96-well plates at 37 ℃. The viability of target cells was detected 18 h later by adding D-luciferin (potassium salt, Promega). The specific cell lysis was calculated the following formula: specific lysis (%) = 100 × {1 − [(luminescence in CAR-NK92 group /luminescence in target cells alone)/(luminescence in untransduced NK92 group /luminescence in target cells alone)]}.

CAR-NK92 cells killing of target cells in vivo

Four- to six-week-old female NCG (NOD/ShiLtJGpt-Prkdc^{em26Cd52}IL2rg^{em26Cd22}/Gpt) mice were purchased from GemPharmatech Co. A total of 2×10^6 Huh-7-luc cells in 100 μl PBS were subcutaneously injected at the right-thigh site of every mouse. After confirmation of engraftment by bioluminescence imaging (BLI) 4 d post-injection (Day 0), tumor received irradiation with 2 Gy or 8 Gy, and 4 h later, mice were intravenously injected with CXCR2-armed CAR-NK92 cells (1×10^7 per mouse) or untransduced NK92 cells as a control. Every mouse was intraperitoneally treated with 4000 IU IL-2 (SinoBiological) every 2 days. Tumor growth was monitored by bioluminescence imaging (BLI) using the IVIS imaging system.

RNA sequencing and analysis

HCC tumors in mice were firstly irradiated using 0, 2, or 8 Gy. Tumors were then resected 24 h later, and RNA was extracted using a Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture's protocol. RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and checked using RNase-free agarose gel electrophoresis. After total RNA was extracted, eukaryotic mRNA was enriched by Oligo (dT) beads. Then the enriched mRNA was fragmented into short fragments using fragmentation buffer and reversely transcribed into cDNA by using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB #7530, New England Biolabs, Ipswich, MA, USA). The purified double-stranded cDNA fragments were end repaired, A base added, and ligated to Illumina sequencing adapters. The ligation reaction was purified with the AMPure XP Beads (1.0X). And polymerase chain reaction (PCR) amplified. The resulting cDNA library was sequenced using Illumina Novaseq6000 by Gene Denovo Biotechnology Co. (Guangzhou, China).

Statistical analysis

All qRT-PCR results (calculated by the change-in-cycling-threshold (2-ΔCt) method) are nomalized to internal control gene β-actin or GAPDH. The FACS data were analysed with FlowJo version 10.8.1. Data are shown as mean ± SEM of three independent experiments. For comparison between two groups, an unpaired t-test was used. For comparisons of three or more groups, the values were analyzed by one-way ANOVA. Tumor size and mice weight statistical analysis were performed using two-way repeated-measures analysis of variance. The overall survival were calculated using the long-rank

test. Grahpad prism v8.0 and SPSS Statistics were utilized for the statistical calculation. *p < 0.05, **p < 0.01, and ***p < 0.001 were considered to be statistically significant.

Results

GPC3-targeting CAR-NK92 cells effectively killed GPC3-positive HCC cells

Glypican-3 (GPC3) is a member of the heparan sulfate proteoglycan family and specifically expressed on the surfaces of most HCC cells [12]. It has been demonstrated that GPC3 could be used as an important prognostic marker for HCC, and higher expression of GPC3 in HCC was associated with worse prognosis [13]. The expression level of GPC3 antigen on HCC cell lines (Huh-7, HepG2, and Hep3B), human normal liver cell line (L02), and glioblastoma cell line (U87) was firstly detected using flow cytometry (Fig. 1A). The results showed that GPC3 was commonly expressed on HCC cell lines, but not L02 or U87 cells.

To assess the efficacy of GPC3-targeting CAR-NK cells for HCC cells, a CAR construct with anti-GPC3 scFv sequence was generated (Fig. 1B), based on previously reported CAR backbone containing CD8α hinge, NKG2D transmembrane domain, 2B4 co-stimulatory domain and CD3ζ activation domain, followed by self-cleavage P2A and enhanced green fluorescent protein (EGFP) [14]. GPC3 CAR-expressing lentivirus was prepared using baboon endogenous retroviral envelope (BaEV) according to our previously described protocol [15, 16]. A human NK cell line (NK92) was transduced with lentiviruses expressing CAR and EGFP, and then CAR-EGFP-expressing NK92 (CAR-GFP NK92) cells were purified by flow cytometry basing on both CAR and EGFP expression (Fig. 1C). To investigate the potency and specific cytotoxicity of GPC3-targeting CAR-NK92 cells, untransduced NK92 cells and CAR-GFP NK92 cells were co-cultured with different luciferase-expressing GPC3-positive HCC cells, including HepG2, Huh-7, and

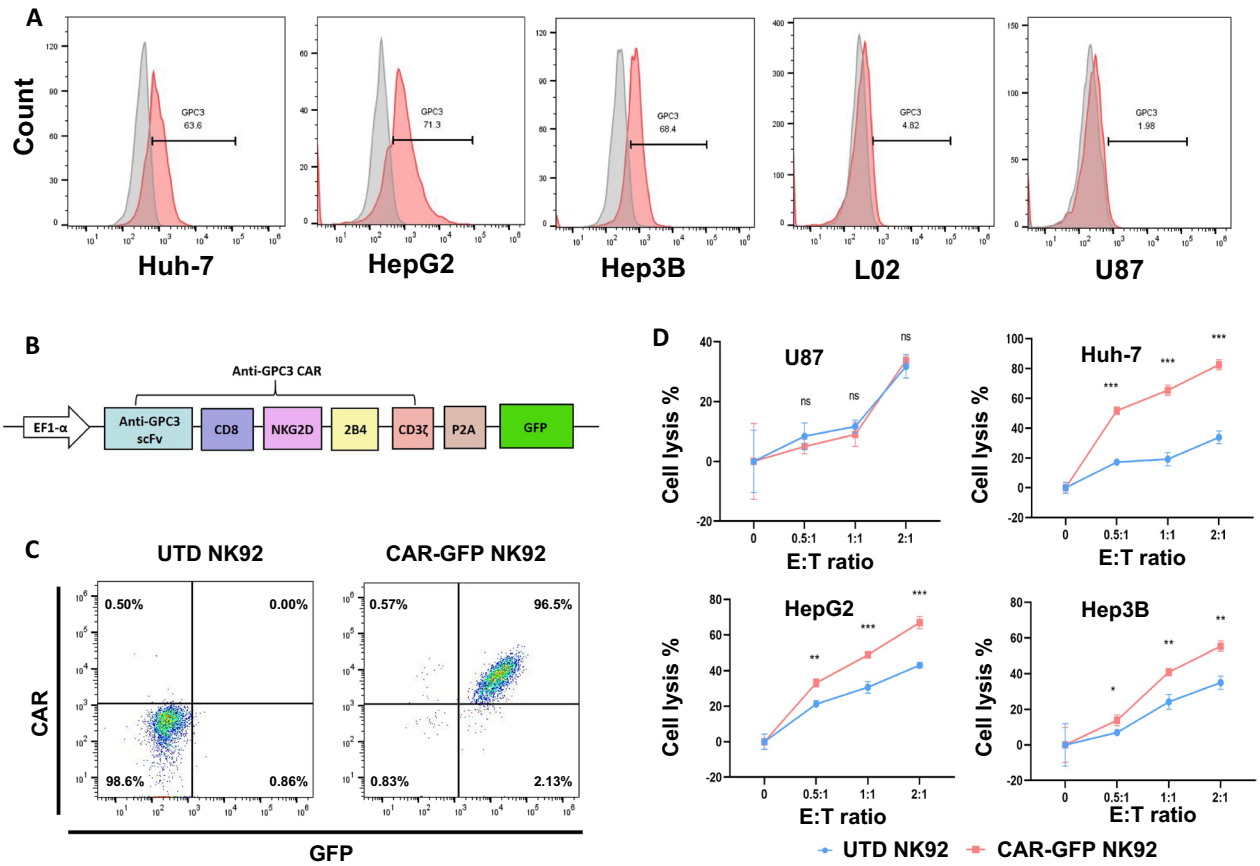

Fig. 1 Development and validation of GPC3-targeting CAR-NK92 cells. **A** The expression of GPC3 antigen on HCC cell lines (Huh7, HepG2, and Hep3B), human normal liver cell line (L02), and glioblastoma cell line (U87) was detected using flow cytometry. **B** A CAR construct with anti-GPC3 scFv sequence. **C** The purified CAR-expressing NK92 cells were analyzed by flow cytometry basing on both CAR and EGFP expression. **D** The potency and specificity of GPC3-targeting CAR-NK92 cells against GPC3-positive HCC cells. Data reflect the mean ± SEM of three separate experiments. ns: no significance, *p < 0.05, **p < 0.01, and ***p < 0.001, E:T ratio, effector-to-target cell ratio

Hep3B cells, and GPC3-negative U87 cells, at different effector-to-target (E:T) cell ratios for 18 h. As shown in Fig. 1D, GPC3-targeting CAR-NK92 cells killed $GPC3^+$ HCC cells in a dose-dependent manner, but did not kill $GPC3^-$ U87 cells, regardless of the E:T ratios. These results show that GPC3-targeting CAR-NK92 cells can efficiently and specifically kill $GPC3^+$ HCC cells in vitro ($p < 0.05$).

CAR-NK92 cells armed with CXCR2 promoted their migration towards HCC cells

Previous studies have demonstrated that some chemokines, the ligands of CXCR2, were highly expressed in HCC cells [17], and the expression levels of these chemokines were associated with prognosis of HCC patients [18–21]. To verify chemokine expression profile in HCC cells, we first detected the mRNA level of chemokines in different HCC cell lines (Huh-7 and HepG2). Among all detected chemokines, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL8, and CXCL16 were highly expressed in Huh-7 cells, and CXCL2 and CXCL8 were highly expressed in HepG-2 cells (Fig. 2A). Interestingly, all these highly expressed chemokines, except for CXCL16, had a common receptor, CXCR2 (Fig. 2B), which indicated that the over-expression of CXCR2 on CAR-NK cells could promote their migration toward HCC cells. To test this possibility, a construct co-expressing GPC3-targeting CAR and CXCR2 (CAR-CXCR2) was designed (Fig. 2C), and CAR-CXCR2 NK92 cells were produced and purified (Fig. 2D). To detect the potential impact of over-expression of CXCR2 on the proliferation of CAR-NK92 cells, cell proliferation was assessed using CCK8 assay. Similar proliferation levels were observed between CAR-GFP NK92 cells and CAR-CXCR2 NK92 cells (Fig. 2E). Consistently, the over-expression of CXCR2 did not impact the cytotoxicity of CAR-NK92 cells, which was assessed by detecting luciferase activity of targeting cells (Fig. 2F) and CD107a expression on NK92 cells using flow cytometry (Fig. 2G, H). To assess whether CXCR2 over-expression could promote the migration of CAR-NK92 cells towards HCC cells, we conducted a transwell assay (Fig. 2I), in which control medium or culture supernatant of Huh-7 or HepG2 cells was added to the lower chamber, and untransduced NK92 cells, CAR-GFP NK92 or CAR-CXCR2 NK92 cells were seeded to the upper chamber. After incubation for 6 h, migrated NK92 cells in the lower chamber were collected and counted by flow cytometry using precision count beads, and chemotaxis index was analyzed. Compared with untransduced NK92 cells and CAR-GFP NK92 cells, CAR-CXCR2 NK92 cells showed significantly increased migration efficiency under the attraction of culture supernatant of Huh-7 or HepG2 cells ($p < 0.05$, Fig. 2J).

Pretreating of HCC cells with irradiation enhanced in vitro cytotoxic effect and migration activity of CAR-CXCR2 NK92 cells

To assess the effect of irradiation on in vitro anti-tumor activity of CAR-NK cells, HCC cells expressing luciferase were firstly exposed to different doses of irradiation (0 Gy, 2 Gy, and 8 Gy) and 6 h later, co-cultured with CAR-NK92 cells at 0.5:1 of E:T ratio for 18 h. The cytotoxicity of CAR-NK92 cells to irradiation-pretreated HCC cells was detected using luciferase assay. Interestingly, irradiated HCC cells did not increase their sensitivity to cytotoxic effect of untransduced NK92 cells or CAR-GFP NK92 cells, irregardless of radiation doses. However, irradiation could significantly increase the sensitivity of HCC cells to cytotoxic effect of CAR-CXCR2 NK92 cells, and no difference was observed between 2 and 8 Gy groups (Fig. 3A). Furthermore, to determine whether pretreating HCC cells with irradiation could improve the migration activity of CAR-NK cells towards HCC cells, migration efficiency of CAR-NK92 cells towards culture supernatant of irradiation-pretreated HCC cells was evaluated by a transwell assay, and the migrated NK92 cells were collected and counted by flow cytometry using precision count beads (Fig. 3B). We found that irradiation pretreatment significantly promoted the migration of CAR-CXCR2 NK92 cells towards culture supernatant from HCC cells that had received 2 Gy- or 8 Gy-irradiation compared with that from 0 Gy control (Fig. 3C), while no similar tendency was observed for CAR-GFP NK92 cells. Next, we evaluated the influence of irradiation on the expression of chemokines in HCC

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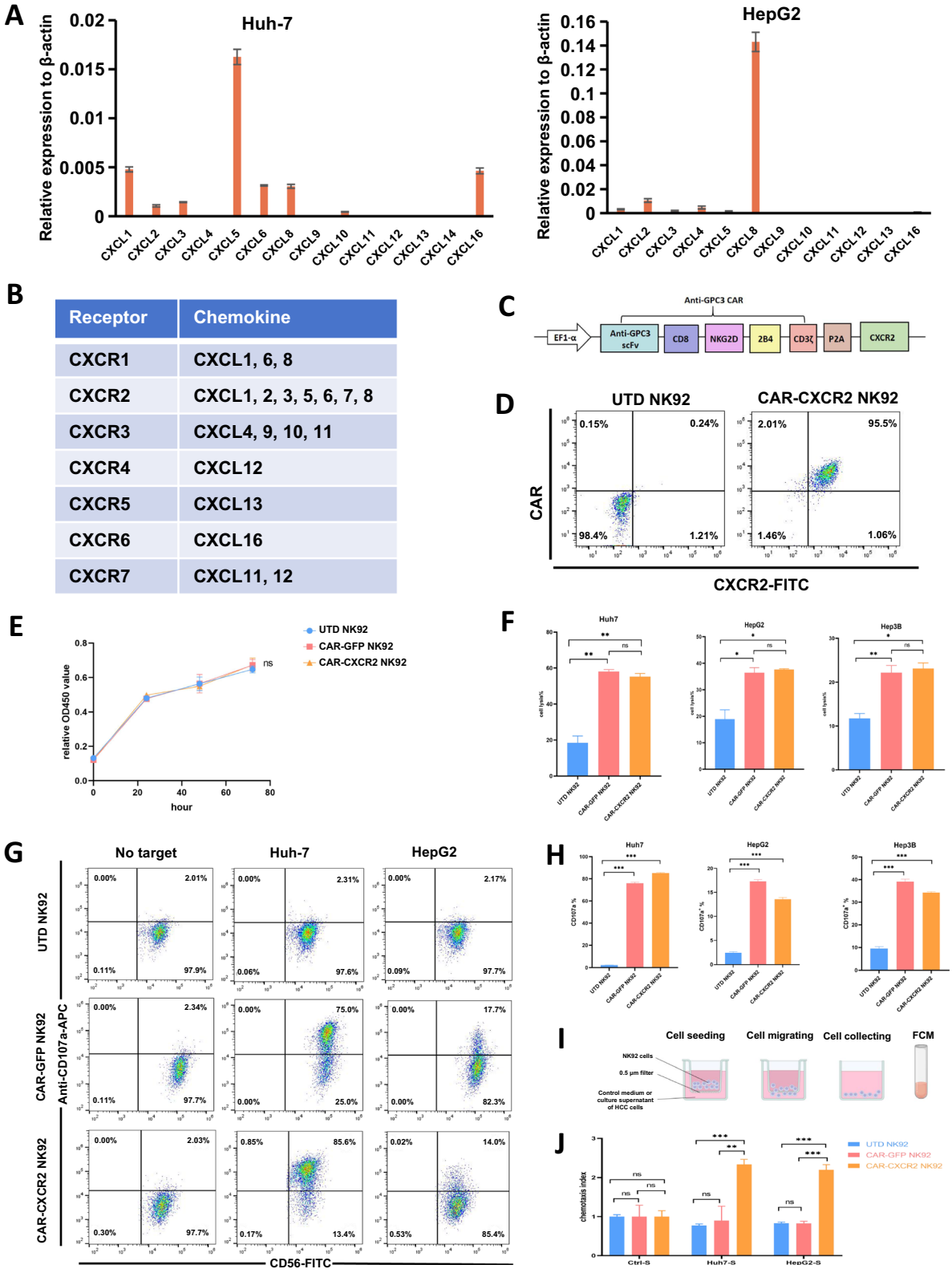
Fig. 2 CAR-NK92 cells armed with CXCR2 promoted their migration towards HCC cells. **A** The mRNA level of chemokines were detected in Huh-7 and HepG-2 cells. **B** Chemokines and their receptors. **C** A construct containing GPC3-targeting CAR and CXCR2. **D** The expression of CAR and CXCR2 on sorted CAR-CXCR2 NK92 cells. **E** Cell proliferation was assessed using CCK8 assay. **F** The cytotoxicity of CAR-NK92 cells was assessed by detecting luciferase activity at 0.5:1 of E:T ratio. **G** The cytotoxicity of CAR-NK92 cells was assessed by detecting CD107a expression on NK92 cells using flow cytometry. **H** The percentages of CD107a + NK92 cells were analyzed. **I** Schematic representation of a transwell assay. **J** The chemotaxis index was analyzed to assess the migration efficiency of NK92 cells towards culture supernatant of Huh-7 or HepG-2 cells. Data reflect the mean ± SEM of three separate experiments. ns: no significance, *p < 0.05, **p < 0.01, and ***p < 0.001



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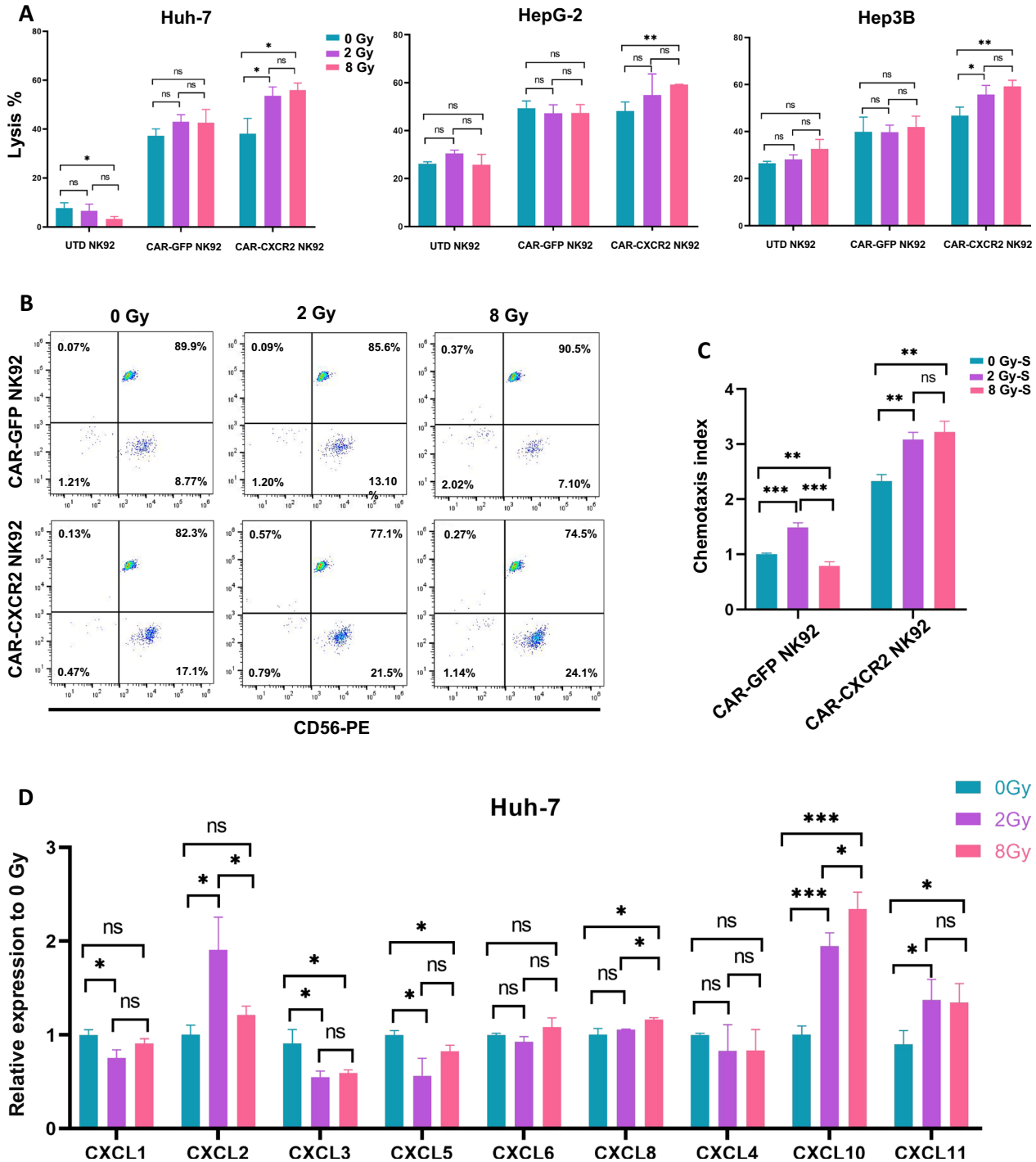


Fig. 3 The effect of pretreating of HCC cells with irradiation on the functions of CAR-NK92 cells. **A** The cytotoxicity of CAR-NK92 cells to irradiation-pretreated target cells with different doses was detected using luciferase assay at 0.5:1 of E:T ratio. **B** The migration efficiency of CAR-NK92 cells towards culture supernatant of irradiation-pretreated HCC cells was evaluated by a transwell assay and the migrated NK92 cells were collected and counted by flow cytometry using precision count beads. **C** The chemotaxis index was analyzed to assess the migration efficiency of NK92 cells towards irradiation-pretreated HCC cells. **D** The influence of irradiation on the expression of chemokines was assessed by qRT-PCR. ns: no significance, *p < 0.05, **p < 0.01, and ***p < 0.001

cells by qRT-PCR, and found that irradiation significantly up-regulated the expression of CXCR2 (CXCL2 and CXCL8) and CXCR3 ligands (CXCL10 and CXCL11) of Huh-7 cells (Fig. 3D). These results showed that pretreating HCC cells with irradiation could enhance in vitro cytotoxic effect and migration activity of CXCR2-armed CAR-NK92 cells ($p < 0.05$) but not GFP-expressing CAR-NK92 cells ($p > 0.05$).

Irradiated HCC enhanced the in vivo sensitivity to CXCR2-armed CAR-NK92 cells

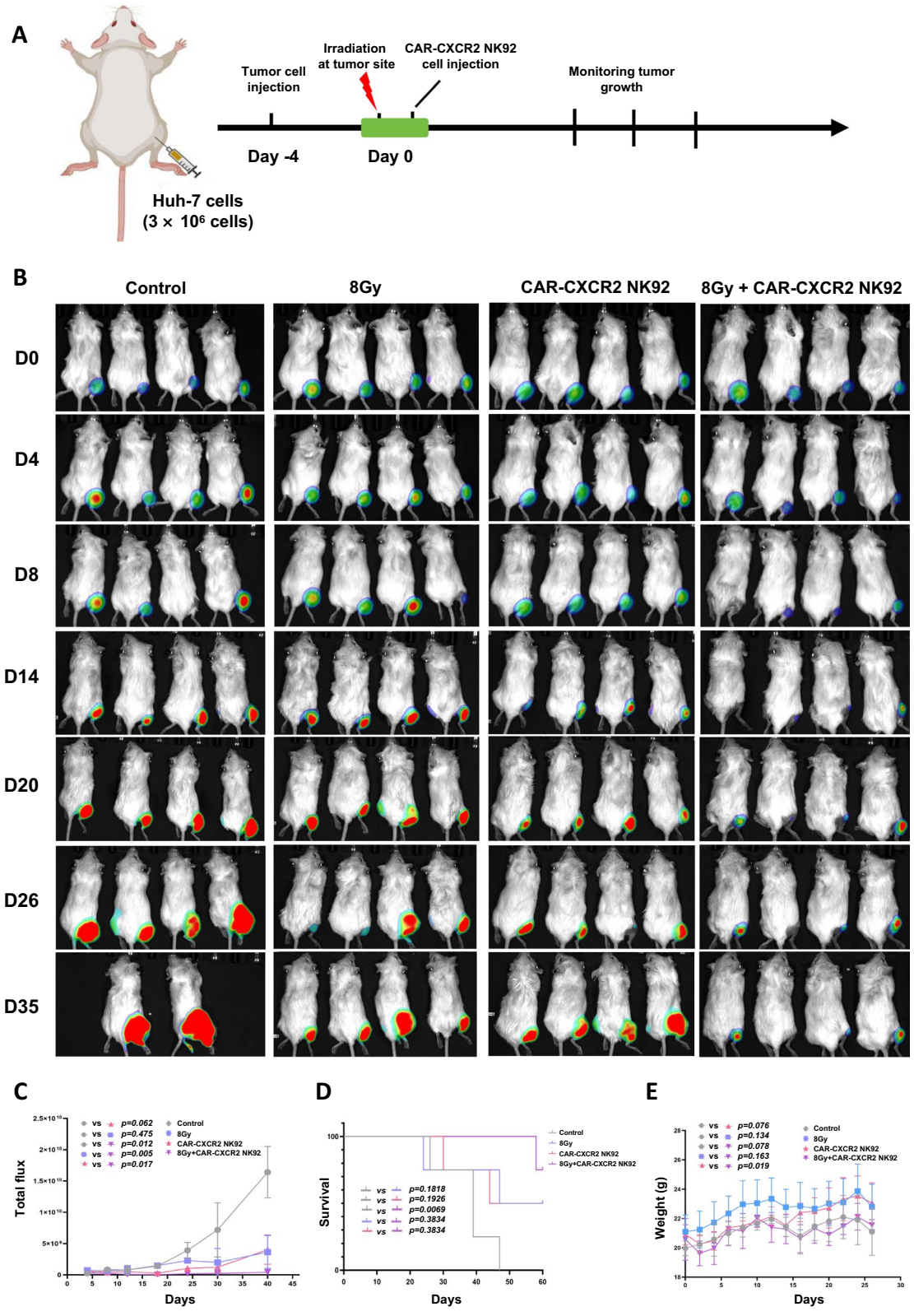
To further assess whether irradiated HCC could enhance in vivo sensitivity to CXCR2-armed CAR-NK92 cells, NOD-scid IL2rg^{null} (NCG) mice were injected subcutaneously with luciferase-expressing Huh-7 HCC cells (3×10^6 per mouse) at the right-thigh site (Fig. 4A). After confirmation of engraftment by bioluminescence imaging (BLI) 4 d post-injection, tumor received irradiation with 2 Gy or 8 Gy (n = 4), and 4 h later, mice were given a single injection of CXCR2-armed CAR-NK92 cells (1×10^7 per mouse) intravenously and 4×10^3 IU of recombinant human IL-2 (rhIL-2) by intraperitoneal injection once every other day. The results showed that CXCR2-armed CAR-NK92 cells could inhibit, but not significantly, tumor growth in vivo, compared with control mice which were injected with untransduced NK92 cells (Fig. 4B and C, Fig. S1), 2 Gy-irradiation alone at tumor site did not inhibit tumor growth compared with control mice, and 2 Gy-irradiation pretreatment at tumor site also did not improve anti-tumor efficacy of CXCR2-armed CAR-NK92 cells (Fig. S1). Furthermore, we found that 8 Gy-irradiation alone only slightly inhibited tumor growth, while 8 Gy-irradiation pretreatment at tumor site remarkably improved anti-tumor efficacy of CXCR2-armed CAR-NK92 cells, resulting in prolonged survival compared with mice treated with CXCR2-armed CAR-NK92 cells alone (Fig. 4B and D). Additionally, no significant difference was observed in body weight between the four groups of mice (Fig. 4E). These results show that irradiated HCC with high-dose but not low-dose could enhance in vivo sensitivity to CXCR2-armed CAR-NK92 cells ($p < 0.05$).

High-dose irradiation upregulates the expression of NK cell-activating ligands on HCC cells

To demonstrate the mechanism of high-dose irradiation on enhancing in vivo anti-HCC activity of CXCR2-armed CAR-NK92 cells, Huh-7 xenograft tumors (300–350 mm^3) were irradiated with different doses (0 Gy, 2 Gy, and 8 Gy), tumors were collected 24 h later and gene expression profiles of tumors were determined through RNA sequencing (RNA-Seq). We found that 8 Gy-irradiation but not 2 Gy-irradiation induced the expression of HLA-A, B, C, and E (Fig. 5A). Importantly, ULBP1, a ligand for the NK-cell activating receptor NKG2D, was remarkably upregulated by 8 Gy-irradiation compared with 0 Gy- or 2 Gy-irradiation (Fig. 5A). Furthermore, 8 Gy-irradiation could inhibit the expression of TGF-β2 and TGF-βR2 (Fig. 5A) which mediate an important immunosuppressive signal for immune evasion in cancer [22]. To further verify the impact of irradiation on the expression of activating ligands for NK cells, we determined the mRNA levels of ULBP1 and MICA/B, two important ligands of NKG2D, on irradiated HCC cells with different doses in vitro using qRT-PCR. We found that irradiation upregulated the mRNA expression of both ULBP1 and MICA/B, and 8 Gy-irradiation induced the highest expression of ULBP1 compared to 2 Gy and 0 Gy (Fig. 5B). Although 2 Gy-irradiation induced the higher expression of MICA/B mRNA than 8 Gy-irradiation (Fig. 5C), MICA/B protein level on the surface of Huh-7 cells was significantly upregulated by 8 Gy-irradiation compared to 2 Gy- and 0 Gy-irradiation, which was detected by flow cytometry (Fig. 5D and E). Gene set enrichment analysis (GSEA) of the RNA-seq data showed that gene signatures induced by 8 Gy- but not 2 Gy-irradiation positively correlated with those reported for NK cell-mediated cytotoxicity, in line with our in vivo observations (Fig. 5F). These data indicate that high-dose irradiation enhances the anti-HCC effect potentially due to increasing the expression of NK cell-activating ligands on tumor cells and improving the tumor microenvironment.

(See figure on next page.)

Fig. 4 Irradiated HCC enhanced in vivo sensitivity to CXCR2-armed CAR-NK92 cells. **A** Schematic of the experimental process. NCG mice were injected with Huh-7 HCC cells (3×10^6 per mouse) at the right-thigh site. After confirmation of engraftment by bioluminescence imaging (BLI) 4 d post-injection (Day 0), tumor received irradiation with 8 Gy (n = 4), and 4 h later, mice were intravenously injected with CXCR2-armed CAR-NK92 cells (1×10^7 per mouse). **B** Mice were imaged by BLI on the day of injection with CAR-CXCR2 NK92 cells (day 0) and at subsequent indicated time point. **C** Comparison of the total flux (luciferase signals from Huh-7 tumor) in the mice treated with control (untransduced NK92 cells), 8 Gy-irradiation, CAR-CXCR2 NK92 or 8 Gy-irradiation combined with CAR-CXCR2 NK92 cells (n = 4). **D** Kaplan–Meier survival curves of mice received different treatments. **E** Weight of mice received different treatments



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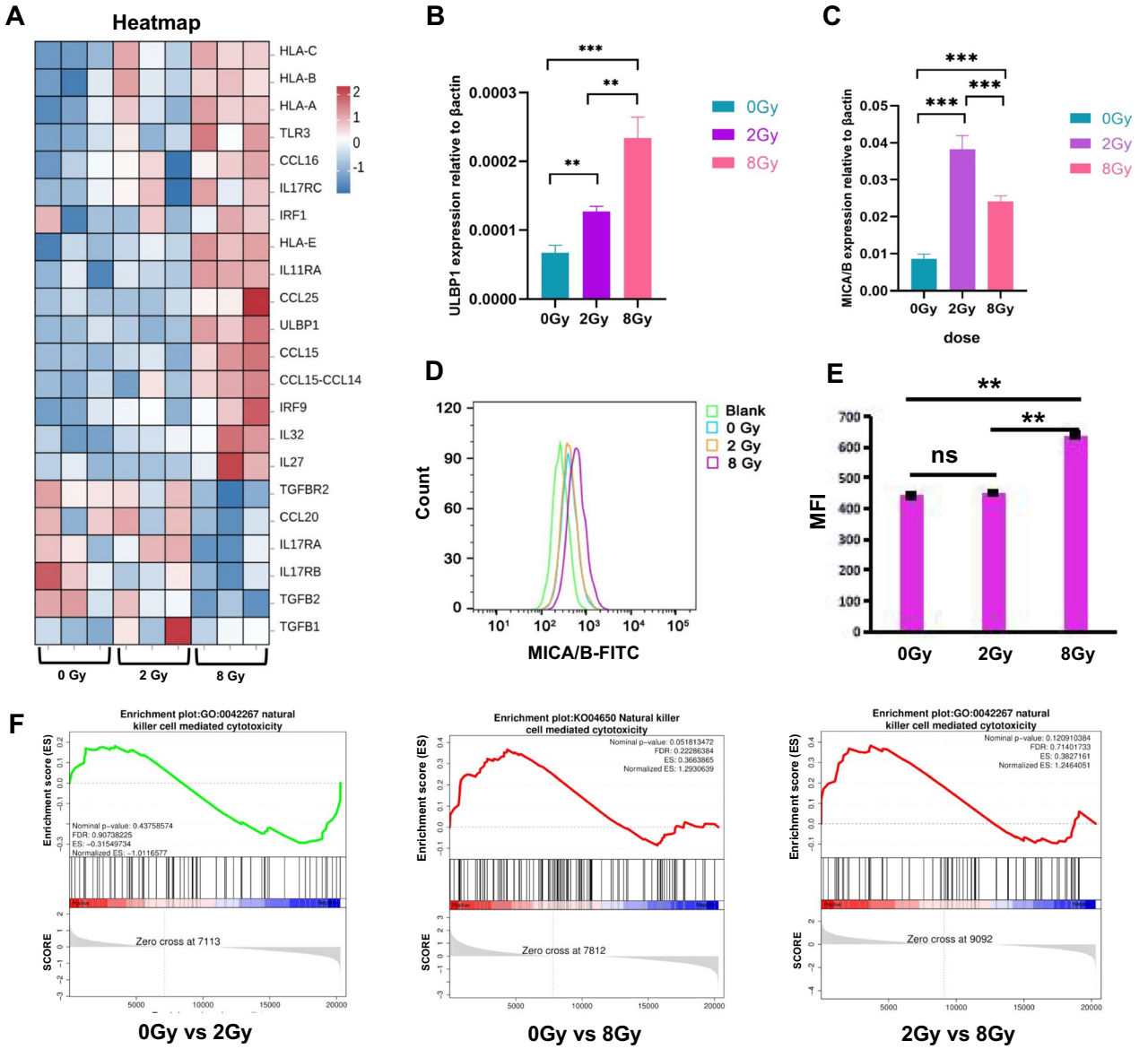

Fig. 5 High-dose irradiation upregulates the expression of NK cell-activating ligands on HCC cells. **A** Gene expression profiles of Huh-7 xenograft tumors irradiated with 0, 2, 8 Gy were determined through RNA sequencing, and heatmap of differential genes associated with NK cell activation and tumor microenvironment were shown. **B** Increased expression of ULBP1 mRNA induced by irradiation was confirmed by qRT-PCR. **C** Increased expression of MICA/B mRNA induced by irradiation was confirmed by qRT-PCR. **D** MICA/B protein level on the surface of tumor cells was significantly upregulated by 8 Gy-irradiation compared to 2 Gy- and 0 Gy-irradiation, which was detected by flow cytometry. **E** Mean fluorescence intensity (MFI) was compared between the three groups of Huh-7 cells irradiated in vitro by 0-, 2-, or 8-Gy doses. **F** GSEA of the RNA-seq data showed that gene signatures induced by only 8 Gy-irradiation positively correlated with those reported for NK cell-mediated cytotoxicity. ns: no significance, $^{**}p < 0.01$, and $^{***}p < 0.001$

Discussion

GPC3 is considered to be a promising therapeutic target for HCC. GPC3-targeting CAR-T cells have been developed to treat HCC in clinical trial setting and showed efficacy and a good safety profile [23]. Nevertheless, the efficacy of CAR-T cells is largely limited due to the heterogeneity of antigen expression on tumor cells and immunosuppressive TME in HCC. NK cells, as important innate lymphacytes, are able to directly and rapidly kill tumor cells without antigen-restriction. CAR-modified NK cells obtain remarkable clinical efficacy and safety in CD19-positive hematological malignancy [6]. However, current CAR-NK cell therapy is still confronting with

an insurmountable challenge from immunosuppressive TME in solid tumors [7].

Radiotherapy is a well-established anti-cancer treatment that exerts its effects primarily through the induction of DNA damage in tumor cells. DNA double-strand breaks are the most lethal lesions induced by ionizing radiation, which instigate a cascade of cellular DNA damage responses (DDRs), leading to genomic instability if not properly repaired [24]. When the damage is too severe to be repaired, it can lead to apoptosis and necrosis. The necrotic death of tumor cells releases damage-associated molecular patterns (DAMPs), which are able to trigger cancer cells and other cells within TME [25]. Moreover, tumor necrosis can also modulate the TME by altering the levels of cytokines, chemokines, and other factors that influence the behavior of immunogenic cells and tumor blood vessels [9, 26]. Additionally, radiotherapy may induce "abscopal effect", which could synergize immunotherapy such as immune checkpoint inhibitors [27]. These effects of RT make combinations with adoptive cell therapy attractive to potentially enhance tumour responses and improve outcomes. Herein, our results demonstrate the potential of radiotherapy in enhancing the anti-tumor effect of CAR-NK cells in HCC solid tumor. Interestingly, our results showed that irradiation enhanced in vitro anti-HCC effect and migration activity of only CAR-CXCR2-NK92 cells but not CAR-GFP-NK92 cells. This results were partially explained by the finding that irradiated HCC cells could significantly up-regulated the expression of chemokines for CXCR2 and CXCR3. CXCR2-armed CAR-NK92 cells over-expressed CXCR2 that could bind to CXCR2-related chemokines secreted by irradiated HCC cells. GFP-expressing CAR-NK92 cells expressed GFP, instead of CXCR2, so that they could not be attracted by CXCR2-related chemokines.

Previous studies suggested that stereotactic body radiotherapy (SBRT), a precise radiotherapy with high-doses per fraction, is superior to the conventionally fractionated RT (CFRT) with lower doses per fraction for eliciting anticancer immune responses [8]. In line with this previous finding, our results showed that only high dose (8 Gy) in one fraction amazingly enhanced in vivo anti-tumor efficacy of CXCR2-armed CAR-NK92 cells, which was supported by the induction of immune-activating gene expression pattern, especially for some ligands associated with NK cell activation, by 8 Gy but not 2 Gy. Notably, TGF-β is often upregulated in tumor tissues, mediates the formation of the immunosuppressive tumor microenvironment [22, 28]. Additionally, TGF-β has been shown to directly inhibit the activation and functions of NK cells, potentially through the repression of the mTOR pathway [29]. Our study showed that high-dose irradiation inhibited the expression of TGF-β and its receptors compared to 0 Gy- or 2 Gy-irradiation, indicating high-dose irradiation might improve intratumoral immune microenvironment, and to some extent, promote in vivo anti-tumor effect of CXCR2-armed CAR-NK92 cells. Additionally, the greater tumor lysis by 8 Gy irradiation could cause greater secretion of antigens and therefore activation of the immune system, which may further enhance in vivo anti-tumor effect of CXCR2-armed CAR-NK92 cells when used in an immunocompetent model.

In conclusion, our results provide, for the first time, the evidence that irradiation could efficiently enhance the anti-tumor effect of CAR-NK cells in solid tumor model. It is important to note that, in our study, irradiation with high-dose per fraction achieved a surprising effect in enhancing anti-tumor effect in vivo of CAR-NK cells compared to that with low-dose per fraction. Nevertheless, our findings were based on a preclinical model of specific to HCC. Further research is needed to explore the potential of this therapeutic approach in a broader range of cancer types. In addition, this difference caused by different irradiation patterns requires further investigation, including a larger sample size and a more detailed analysis of the dynamic immune response.

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Author contributions

GX conceptualized and designed this study. XL, ZL and XD performed most experiments. KW, YS, YC and FW performed partial experiments. XL, JL, YG, and HX executed acquisition and analysis of data. XL, RS and GX prepared figures, performed statistical analysis, and wrote the manuscript. GX, YZ and ZG provided administrative, technical, or material support. All authors read and approved the final manuscript.

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Availability of data and materials

RNA-seq data are available in the TCGA database (PCAWG Firehose). All other data are available in the main text or in the supplementary materials.

Declarations

Ethics approval and consent to participate
All experimental procedures were approved and overseen by the Southern Medical University Institutional Animal Care and Use Committee and were performed in accordance with the guidelines and regulations for animal experiments set down by Southern Medical University Nanfang Hospital under registration number IACUC-LAC-20230703-001.

Competing interests
The authors declare no competing interests.

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