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Original Article

Sorafenib plus memory like natural killer cell combination therapy in hepatocellular carcinoma

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Abstract: Sorafenib, FDA-approved therapy for patients with advanced hepatocellular carcinoma (HCC), leads to limited improvement in overall survival. However, it may indirectly impact the expansion and activity of natural killer (NK) cells. While NK cell-based immunotherapies generally exhibit favorable safety profiles, their effectiveness in controlling solid tumor growth is constrained, primarily due to the absence of antigen specificity and suboptimal expansion and persistence within the tumor microenvironment. In this study, we postulated that enhancing NK cell functionality via cytokine activation could bolster their viability and cytotoxic capabilities, leading to an improved therapeutic response when combined with sorafenib. Memory-like (ML)-NK cells were generated through the supplementation of optimal concentrations of interleukin (IL)-12 and IL-18 cytokines. Following a single day of treatment, cytotoxicity against rat and human HCC cells was evaluated via flow cytometry analysis. A rat HCC model was developed in 30 animals via subcapsular implantation and assigned to control, NK, sorafenib, ML-NK, and combination groups. Sorafenib was administered orally, and NK cells were delivered via the intrahepatic artery. Tumor growth was measured one week after treatment evaluation. Therapeutic efficacy during in-vitro and in-vivo analysis was investigated through a one-way ANOVA test, followed by pairwise two-tailed Student t-tests, considering $P < 0.05$ statistically significant. The in-vitro experiment results demonstrated that sorafenib and conventional NK cell therapies induced more substantial cell death than the control group ($P < 0.01$). ML NK cells significantly improved cell death compared to conventional NK cell immunotherapy. Furthermore, sorafenib in combination with ML-NK cells significantly decreased the viability of HCC cells ($P < 0.05$) compared to sorafenib plus conventional NK cell combination therapy. In vivo experiments have shown that sorafenib and ML-NK cell immunotherapy reduced the growth rate of HCC tumors compared to conventional NK immunotherapy and control groups. Notably, a combination of sorafenib and ML-NK cell immunochemotherapy resulted in the most significant suppression of tumor growth when compared to other therapies. In conclusion, our experimental findings demonstrate that the concurrent administration of sorafenib and ML-NK immunotherapy enhances cytotoxicity against HCC by optimizing the therapeutic response through cytokine activation, resulting in a significant decrease in tumor growth.

Keywords: Chemoimmunotherapy, combination therapy, hepatocellular carcinoma, memory-like natural killer cell immunotherapy, sorafenib

Introduction

Hepatocellular carcinoma (HCC) is the predominant form of liver cancer, accounting for 75%-85% of all cases and being a leading cause of cancer-related deaths worldwide. According to Global Cancer Statistics, HCC causes approximately 905,677 new cases and 830,180 deaths annually [1], and its prevalence continues to rise [2]. Sorafenib, FDA-approved first-line treatment for intermediate and advanced HCC patients, provides only a modest extension of median survival and time to radiologic progression while leading to severe

adverse events that may lead to treatment termination [3, 4]. To enhance the clinical benefits for patients diagnosed with advanced-stage HCC, therefore, novel therapeutic strategies and combination therapies are urgently required.

Natural killer (NK) cells, which make up 25% to 50% of the total number of liver lymphocytes, hold great promise for the treatment of HCC tumors due to the strong correlation between the amount of NK cells in the peripheral blood of HCC patients and their prognosis [5-7]. At advanced stages of HCC, NK cells often exhibit decreased infiltration and impaired functional activities, which may be correlated with increased $CD4^+CD25^+$ T regulatory cells in the tumor microenvironment [8]. Previous studies demonstrated that dysfunction or exhaustion of NK cells in advanced HCC tumors contributes to the pathogenesis of liver cancer and promotes the potential benefits of NK cell-based therapy for HCC [9, 10]. Recently, FDA has granted clearance for allogeneic NK cell therapy as an investigational new drug, intended to treat solid tumors [11-13]; however, mixed or minor responses to adoptive therapy infusions of NK-92 immunotherapy have been observed in clinical trials with cancer patients [11, 14]. Previous studies suggested that NK cells can differentiate in response to human cytomegalovirus in vivo [15] or through combined Interleukin (IL)-12, IL-15, or IL-18 activation showcasing improved functionality [16, 17]. Especially, cytokine-activated memory-like (ML) NK cells have exhibited favorable safety profiles and have proven to be efficacious in initiating clinical remissions among patients grappling with hematological malignancies [18]. However, the ability of human ML NK cells to respond to cancer target cells has received little attention.

In addition to orchestrating various types of immune cells, sorafenib synergistically and directly inhibits the growth of hepatoma cells [19]. Sorafenib can enhance NK cell function in a low-dose manner without leading to NK cell exhaustion [20]. Previous studies highlighted that sorafenib elucidates an increase in NK cell cytotoxicity [21], sensitivity to NK cells [22], and activation of NK cells [23] which also expresses the potential combination of sorafenib and NK cell immunotherapy against solid tumors [24-26]. Therefore, the administration of sorafenib in combination with ML NK cell adoptive immune therapy in HCC treatment requires further attention to potentially improve the therapeutic response.

In this study, we developed ML NK cells via activation of IL-12 and IL-18 cytokine activation, investigated the therapeutic effects of the combination of sorafenib and ML NK cell chemoimmunotherapy in two different rodent and human HCC cell lines and performed an in-vivo study for evaluation of tumor growth one week after treatment.

Materials and methods

Cell culture

McA-RH7777 and N1-S1 rat epithelial hepatoma and HepG2 human epithelial-like HCC cell lines were utilized as target cells for assessment of the therapeutic response. Rat NK (RNK-16) and human NK cell lines (NK-92 MI) were examined to differentiate NK cell sensitivity via cytokine activation.

Rodent and human HCC and human NK cell lines were acquired from ATCC (Manassas, VA) and tested for mycoplasma before experiments. The HCC cell lines were cultured according to ATCC guidelines in which rat HCC cell lines (McA-RH7777 and N1-S1) were cultured in DMEM and IMDM (Gibco, Waltham, MA), and HepG2 cell line in EMEM (Corning, Manassas, VA). The rat NK cell line (RNK-16) was developed by Wang et al. [27] and generously provided by Thomas L. Olson (University of Virginia, Charlottesville, VA). Both effector cell lines were cultured in RPMI medium 1640 (Life Technologies, Waltham, MA). All the growth mediums were supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 1.25% GlutaMAX (Gibco, Waltham, MA), and 1% penicillin/streptomycin (Gibco, Waltham, MA). For effector cell lines, 2-Mercaptoethanol (2-ME) at a working concentration of 25 mM was added to the corresponding medium.

The target cells were cultured in 37°C, 95% air, and 5% CO_2 humidified incubator at least for 48 hours before they were used for in-vitro experiments. Effector cells were cultured in a freshly changed medium 24 hours before each experiment. ML NK cells were generated by culturing

NK cells with optimal concentrations of the IL-12 and IL-18 cytokines for 24 hours before treatment. During the experiments, NK cells were cultured with different doses of cytokines overnight and then washed with PBS. The activated cells were kept in fresh medium for 24 hours prior to experiments. The concentration of the IL-12 and IL-18 cytokines is optimized by evaluating viability, and cytotoxic function via flow cytometry analysis. A ratio of 10:1 effector to target cell ratio was kept with 0.2-0.3 million target cells depending on the plate size utilized in the experiments. For each cell line, a total of 6 groups, negative/control group, sorafenib group, conventional NK cell group, sorafenib plus conventional NK cell group, ML NK cell group, and sorafenib plus ML NK group, were generated to evaluate the therapeutic response of engineered NK cells via cytokine activation, and sorafenib plus ML NK cell chemoimmunotherapy against HCC.

Labeling target cells with CFSE

Target cells were labeled with CFSE according to the CFSE Fluorescent Cell Labeling Kit (Abcam, Fremont, CA) and CellTrace™ CFSE Cell Proliferation Kit (Thermo Fisher, Waltham, MA) manufacturer guidelines. Briefly, after target cells were completely detached from each other by pipetting or by digestion with 0.05% trypsin without/with EDTA (Gibco, Life Technologies Corporation, Waltham, MA), we harvested a certain number of target cells and resuspended the cell pellet with phosphate-buffered saline (PBS) to prepare the target cell-PBS solution. Then, CFSE was diluted with PBS to prepare CFSE-PBS solution and mixed with target cell-PBS with CFSE-PBS quickly (final concentration of CFSE was 10 mM) and incubated the mixture at 37°C for 20 minutes. Afterward, 10% FBS-containing medium was added to the mixture and quivered, and mixture was incubated at 37°C for 5 minutes. The cells were washed one time with a medium of an equal volume of PBS and cell pellet was resuspended with the same medium, seeding the cells in their corresponding wells and culturing them in an incubator for at least 10 minutes. HepG2 cells were first labeled with CFSE; then detached and seeded in the corresponding wells. Lastly, either or both sorafenib and NK cells were added to the corresponding wells for treatment in upcoming experiments.

Labeling cells with fluorescence dye

After 24 hours of the treatment, all the cells were harvested and stained with Calcein Violet^{AM} or live/dead^{TM} far-red fluorescence dye according to the manufacturer's guidelines. The cells were subsequently collected and washed twice with PBS, by adding dye stock solution and keeping them in an ice bath for 30 minutes. Lastly, the emitted fluorescence signals by target cells were detected with a flow cytometry cell analyzer (BD LSR Fortessa™ X20) within 1 hour of treatment.

Tumor model development and monitoring

All procedures were performed per animal protocol approved by the Institutional Animal Care and Use Committee of our institution. For HCC tumor model development, 1.5×10^6 N1-S1 cells were prepared simultaneously during tumor implantation experiments. To prevent leakage during injection, tumor cells were embedded in a 50% Matrigel solution. The injection procedure utilized a 1 ml 27G syringe with zero dead space, administered into the subcapsular liver region of thirty male SD rats (200-300 g) under basic anesthesia initiated and maintained by 2% isoflurane with 3 L/min of oxygen. Following cell injection, the syringe was held in place for approximately 10 s before removal, and a blood-stopping pad was promptly positioned. A slight pressure was applied to avert any potential leakages, a step confirmed through visual inspection. The surgical region was stitched following two-layer closure technique and covered with gauge and self-adhesive bandage wrap. To release any pain, 0.05 mg/kg of buprenorphine, and 2 mg/kg of meloxicam were provided subcutaneously. The stress levels of the subjects were closely monitored to prevent any distress. Animals were monitored with a 3T Philips MRI with a commercial wrist coil under anesthesia until the tumor diameter reached approximately 5 mm. Animals were randomly assigned to the following groups (n = 6 for each); control, NK cell, sorafenib, ML NK cell, and sorafenib plus ML NK cell combination group. For oral administration of sorafenib (10 mg/kg), animals were restrained to keep the pharynx route clear for successful delivery of the drug via a bulb-tipped gastric gavage needle. Animals were monitored for any discomfort or difficulty in breathing before returning to their cages. For NK and ML

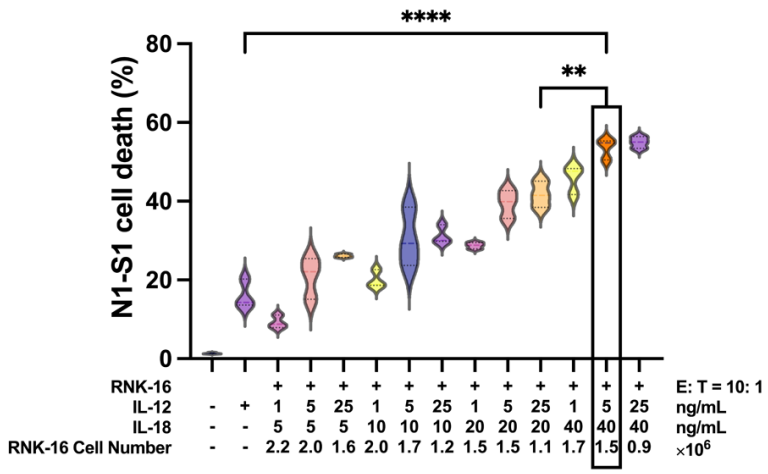

Figure 1. Optimization of concentration of IL-12 plus IL-18 cytokines to improve the efficacy of NK cells. The concentration of IL-12 plus IL-18 was optimized in terms of viability and cytotoxicity of NK cells (**: P < 0.01 and ****: P < 0.0001).

NK cell administration, a catheter was placed into the proper hepatic artery of the animals by performing the previously designed procedure by Sheu et al. [28]. Briefly, we surgically exposed the portal triad located above the first loop of the duodenum. To prevent bleeding, the common hepatic artery was temporarily ligated using a 2-0 suture, and the gastroduodenal artery was permanently ligated with a 4-0 suture to prevent the cells from flowing back into the gastrointestinal tract. A 24G microcatheter (Terumo SurFlash®, Somerset, NJ) was introduced distal to this ligation point in the gastroduodenal artery and subsequently advanced into the proper hepatic artery. A 0.1 mL bolus of heparin was intravenously administered via a catheter, 10 million NK or ML NK cells were suspended in 0.5 mL of PBS (PBS), and 0.2 mL saline flush was administered through a catheter, sequentially. Afterward, the catheter was subsequently extracted, and a 4-0 suture was employed to definitively ligate the gastroduodenal artery.

Data analysis

The information is depicted through box and whisker plots, which display the interquartile range along with the minimum and maximum values, or as bars that illustrate the mean ± SEM (standard error of the mean). FlowJo (version 10.8.0, BD® Sciences, Ashland, OR) was used for flow cytometry data analysis by an experienced immunologist with an experience of 5 years. GraphPad Prism 7 (version 7.0a, GraphPad Software, Boston, MA) was employed to perform one-way ANOVA and T tests during statistical analyses. *P*-values were determined using two-tailed tests, with significance levels denoted as follows; *: P < 0.05; **: P < 0.01; ***: P < 0.001; ****: P < 0.0001.

Results

Development of ML NK cells for enhanced NK cell efficacy

The different concentrations of IL-12 and IL-18 cytokines were examined to optimize the cytotoxicity through twelve different concentrations, 1-25 ng/ml of IL-12 and 5-40 ng/ml of IL-18 cytokines. RNK-16 cells were supplemented with IL-12 or IL-18 cytokines for 24 hours, washed twice, and incubated for 24 hours before co-culturing them with target tumor cells. Following the activation of the RNK-16 cell line, morphological changes were observed under the microscope, and cell numbers were counted for assessment of viability.

In addition, the efficacy of ML NK cell immunotherapy against N1-S1 cells was evaluated with flow cytometry (**Figure 1**). The results indicated an improvement of cell cytotoxicity associated with increasing concentration of interleukins. As the concentration of IL-18 was fixed, the cytotoxicity of RNK-16 cells co-cultured with 5 ng/mL of IL-12 was not significantly different than 25 ng/mL of IL-12 concentration. However, there was a significant increase in cytotoxicity for the RNK-16 cells activated with 40 ng/mL IL-18 as the concentration of IL-12 was set to 5 ng/mL. The viability of ML NK cells developed with 5 ng/mL of IL-12 and 40 ng/mL of IL-18 was higher than in the groups with higher concentration. Due to the good balance between cell number and cytotoxic function, 5 ng/ml of IL-12 and 40 ng/ml of IL-18 concentration were determined as the optimal selection for the development of ML NK cells.

Sorafenib and NK cell monotherapies in HCC

Following a 24-hour treatment of target cell lines with 4 μM sorafenib, cells were harvested

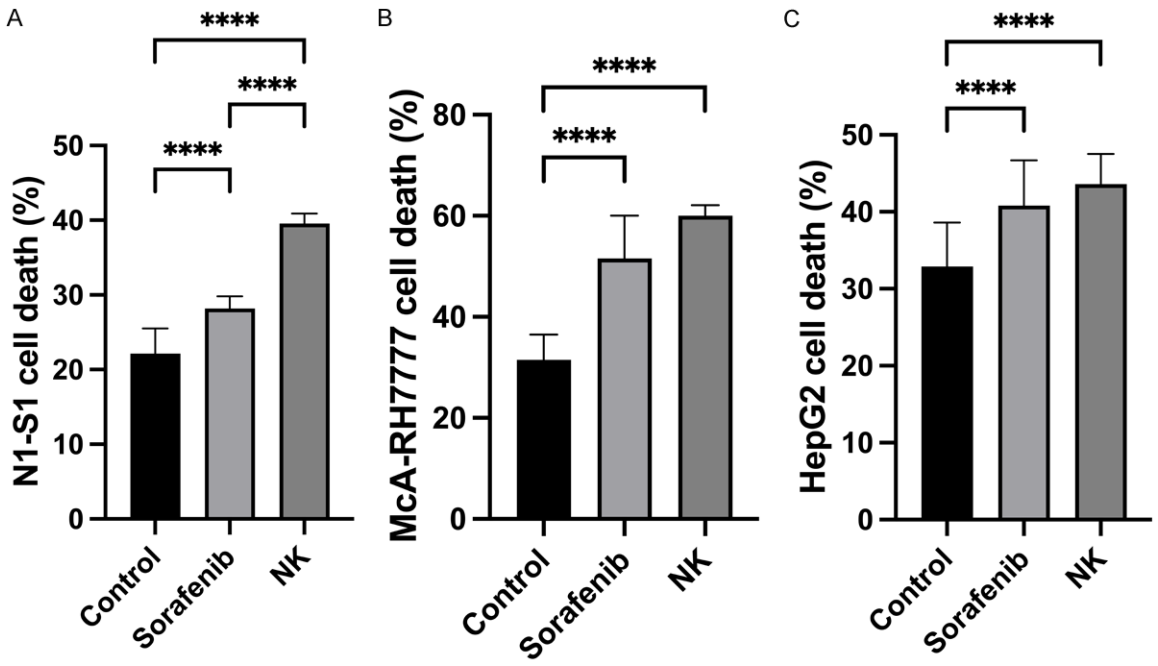

Figure 2. Sorafenib demonstrates a marked induction of tumor cell death in both rat and human HCC cell lines. The data present statistical results regarding cell death rates for N1-S1 (A), McA-RH7777 (B), and HepG2 (C) in both the control group (vehicle) and the sorafenib treatment group. Additionally, NK cell lines exhibit a significant induction of death in HCC cell lines stronger than Sorafenib. The data provide statistical results for death rates of N1-S1 (A), McA-RH7777 (B), and HepG2 (C) in the control group (vehicle) and the NK cell line treatment group (****: $P < 0.0001$).

and subjected to staining with live/dead™ far-red fluorescence dye. The observed fluorescence signals in CFSE-positive target cells provided compelling evidence of sorafenib significantly augmenting cell death across the three types of target HCC cell lines (**Figure 2**). Specifically, for the N1-S1 and McA-RH7777 cell lines, sorafenib-induced cell death ratios were 27.24 ± 0.82 ($P < 0.05$) and 51.79 ± 1.79 ($P < 0.001$), respectively. In the case of HepG2, the recorded cell death rate was 41.29 ± 0.87 ($P < 0.001$). Conversely, NK cells facilitated a substantial increase in cell death across the three types of target HCC cells (**Figure 2**). For rat HCC cells, NK cell-associated cell death rates were 39.34 ± 0.41 and 62.30 ± 0.66 ($P < 0.001$), whereas HepG2 tumors exhibited a cell death rate of 43.53 ± 0.70 ($P < 0.001$). The results strongly indicate a more pronounced cell death associated with NK cells compared to sorafenib treatment.

Evaluation of ML NK cell efficacy against HCC

Target rodent and human HCC cells were treated with ML NK cells for the same period and target/effector ratio and stained with live/dead™ far-red fluorescence dye for flow cytometry analysis. The results indicated that cytokine-induced ML NK cells could significantly facilitate a stronger HCC tumor cell death for rat and human hepatoma cell lines than conventional NK cells (**Figure 3**). For rat HCC cells, the percentage of cell death induced by ML NK cells was 45.18 ± 0.20 ($P < 0.001$) and 64.86 ± 0.67 ($P < 0.01$). Moreover, a significant improvement was observed in the death ratio of human HCC cells with the treatment of ML NK cells at 46.44 ± 0.74% ($P < 0.05$).

Therapeutic efficacy of sorafenib plus NK cells

For the comprehensive analysis of combination treatment outcomes, we exposed target cells to 4 μM sorafenib along with NK cells at an effector-to-target ratio of 10:1 for 24 hours. Subsequently, we stained the harvested cells with a fluorescence dye. Flow cytometry results revealed a significant enhancement in cell death when employing the combination of sorafenib and NK cells compared to sorafenib and NK cell monotherapies (**Figure 4A-C**). In the case of N1-S1 and McA-RH7777 cells, the percentage of cell death induced by sorafenib

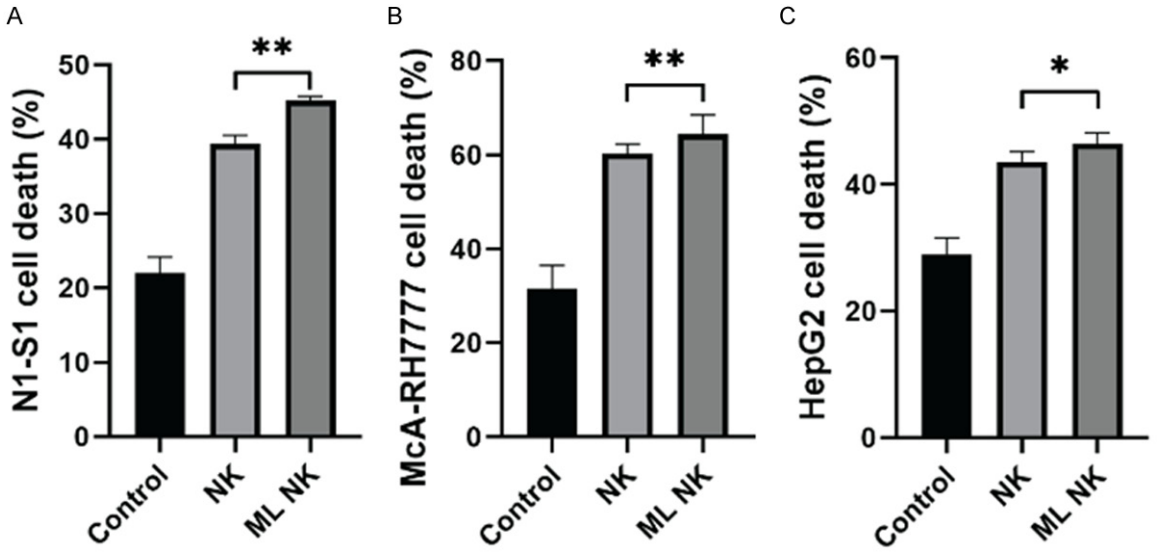

Figure 3. The addition of IL-12 and IL-18 significantly enhances NK cell cytotoxicity against N1-S1 (A), McA-RH7777 (B), and HepG2 (C) HCC cells (*: $P < 0.05$, **: $P < 0.01$).

plus NK cells reached 44.51 ± 0.75 ($P < 0.01$) and 69.30 ± 0.70 ($P < 0.001$), respectively. The ratio of human HCC cell death was measured as 47.48 ± 0.77 ($P < 0.05$) with the treatment of the sorafenib and NK cell combination therapy.

To further assess the therapeutic efficacy of ML NK cells in combination with sorafenib, target cells were co-cultured with sorafenib and ML NK simultaneously. Following 24 hours of treatment, cells were washed, harvested, and stained with a fluorescent dye. Flow cytometry detected emitted fluorescent signals of CFSE-positive target cells. The results indicated that sorafenib plus ML NK cells could significantly enhance the death rate in the three HCC tumor cells compared to NK cells alone or sorafenib plus NK cells (**Figure 4D-F**). For rat HCC cells, combination therapy (sorafenib plus NK) facilitated cell death of 44.51 ± 0.75% and 69.30 ± 0.70% ($P < 0.001$), while ML NK immunotherapy resulted in 45.18 ± 0.20% and 64.86 ± 0.67%. Sorafenib plus ML NK cell immunotherapy significantly improved the death ratio for N1-S1 (48.59 ± 1.06%, $P < 0.05$) and McA-RH7777 (74.02 ± 1.45%, $P < 0.05$) compared to sorafenib plus NK cell therapy. For human HCC, the sorafenib plus NK group was associated with 47.48 ± 0.77% of tumor cell death, while the combination of sorafenib and ML NK raised the cell death ratio to 52.06 ± 0.66% ($P < 0.05$).

HCC tumor growth and therapeutic response

Morphological changes in HCC tumors resulting from sorafenib and NK cell immunotherapy were investigated using a rat tumor model created by implanting 1.5 million N1-S1 cells intrahepatically. HCC tumors were allowed to grow for five days until they reached a diameter of approximately 5 mm, guided by MRI, and were then treated according to their respective groups. In brief, animals in the sorafenib group and the combination group received a daily oral sorafenib drug solution for seven days after the baseline scan. Conversely, rats in the control, NK, and ML NK groups were administered saline, NK, and ML NK solutions via intrahepatic arterial catheter, respectively, four days after the baseline assessment. MRI scans were performed on the animals seven days after the baseline scans to assess morphological changes, and representative T2w MRI data are shown in **Figure 5A-E**. All treatment strategies substantially inhibited tumor growth compared to conventional NK cell immunotherapy and the untreated control group, as illustrated in **Figure 5F**. Subjects treated with sorafenib (2.36 ± 1.71 mm^2) exhibited more pronounced tumor growth in comparison to animals treated with ML NK immunotherapy (2.25 ± 0.81 mm^2). No significant differences were observed between the combination and monotherapy groups, possibly due to the slower changes in tumor mor-

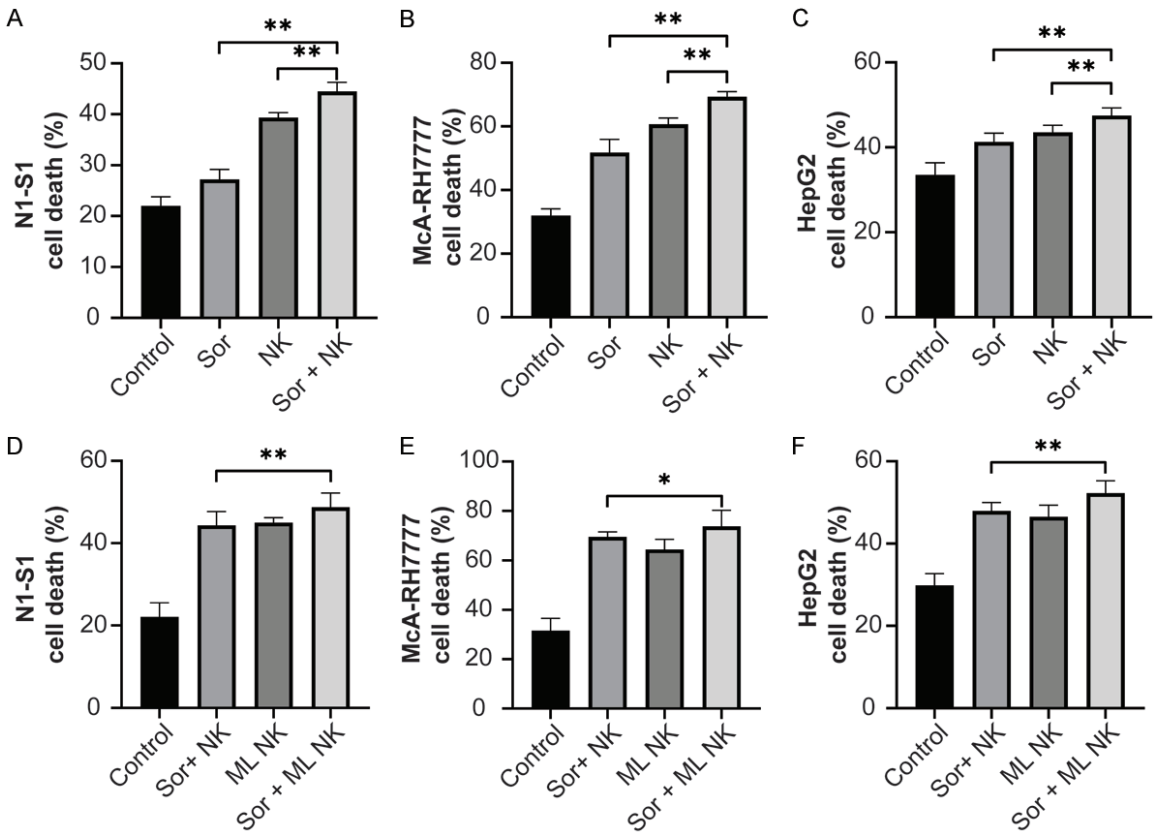

Figure 4. The combination of sorafenib and NK cells significantly induces a higher death rate in N1-S1 (A), McA-RH7777 (B), and HepG2 (C) HCC cells compared to either treatment alone (**: $P < 0.01$). Furthermore, the cytotoxic function of sorafenib plus NK cell chemoimmunotherapy is enhanced through cytokine activation (Sorafenib plus ML NK), and the cell death ratio for (D) N1-S1, (E) McA-RH7777, and (F) HepG2 HCC cells significantly improve with this novel combination therapy (*: $P < 0.05$, **: $P < 0.01$).

phology following immunotherapy. However, the combination of sorafenib and ML NK cell therapy resulted in the slowest tumor growth, reducing the tumor size to 1.35 ± 0.32 mm^2 (**Figure 5F**).

Discussion

In this investigation, we cultured ML NK cells from both rat and human subjects through the activation of IL-12 and IL-18 cytokines. The study delved into the synergistic potential of the newly developed NK cells when combined with sorafenib treatment against HCC tumor cells, scrutinizing their performance through rigorous in-vitro and in-vivo experiments. The findings from our experimentation revealed a marked enhancement in the cytotoxic function of ML NK cells in comparison to their conventional counterparts. Notably, the amalgamation of sorafenib with ML NK cell chemoimmunotherapy exhibited substantial promise, leading to a noteworthy increase in HCC tumor cell death. Additionally, outcomes from preclinical studies indicated that the combined therapy of sorafenib and ML NK cells effectively curtailed early tumor growth, presenting visible results within the initial seven days of treatment.

The multifaceted nature of HCC necessitates a diverse treatment approach to enhance both efficacy and safety. As per the Barcelona Clinic Liver Cancer (BCLC) staging system, sorafenib stands as the primary treatment for patients with intermediate and advanced HCC, particularly in cases where the atezolizumab plus bevacizumab treatment strategy is contraindicated [29]. However, the limitations of sorafenib are apparent, extending the median survival time for patients with advanced HCC by less than three months and bringing about significant clinical adverse events [5]. The imperative task

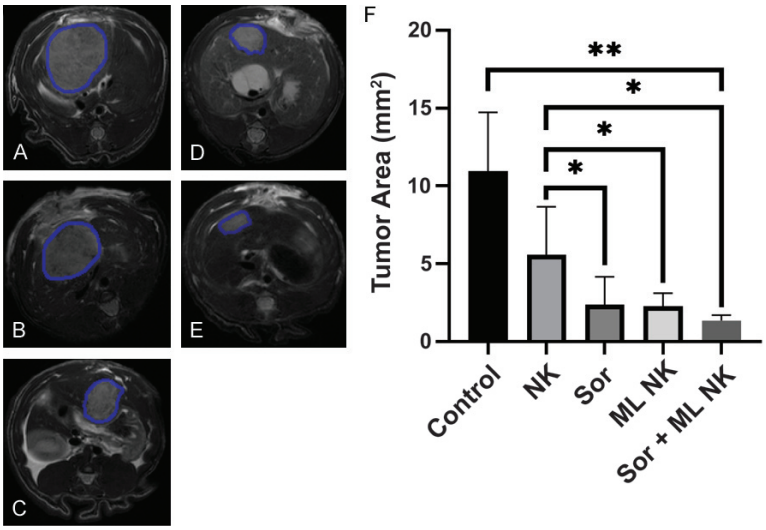

Figure 5. Representative T2-weighted MRI data illustrate tumor size variations in distinct groups: control group (A), NK group (B), sorafenib group (C), ML NK group (D), and sorafenib plus ML NK combination group (E). Quantitative results emphasize a reduction in tumor burden with the combination of sorafenib and ML NK cell immunotherapy (F) (*: $P < 0.05$, **: $P < 0.01$).

at hand is the improvement of sorafenib treatment efficacy while concurrently addressing the associated adverse events. Promisingly, adoptive transfusion of NK cells has emerged as an alternative treatment strategy for advanced HCC, demonstrating efficacy without causing significant cytotoxicity to normal cells [30, 31]. This approach gains further merit when considering the capacity of sorafenib to orchestrate various immune cells in the tumor microenvironment (TME) of HCC, particularly enhancing NK cell function in a low-dose manner without inducing exhaustion [19].

The interaction between expanded NK cells and sorafenib has previously been investigated [23, 32]. Shi et al. [23] demonstrated that the combination of sorafenib and NK cells effectively enhances the suppression of HCC. This enhancement is attributed to the heightened NK cell cytotoxicity resulting from the inhibition of the androgen receptor (AR) signaling pathway by sorafenib. Additionally, Kamiya et al. [32] disclosed that NK cells significantly augment the anti-HCC effects of sorafenib. Importantly, they observed that the presence of sorafenib does not impact the cytotoxic capacity of NK cells. In most studies, NK cells utilized were derived from peripheral blood, posing a considerable challenge in obtaining sufficient NK cells for treating HCC patients, especially those who may have undergone multiple cycles of chemotherapy and/or radiotherapy. Addressing this limitation, the NK-92 cell line emerges as a promising source of NK cells. Approved by the FDA as an investigational new drug for experimental use in patients, NK-92 offers the advantage of large-scale production under good manufacturing practice conditions. This makes it a viable candidate as an "off-the-shelf" therapeutic agent for cell-based cancer immunotherapy [33].

NK cells harbor significant potential in harnessing the host's immune system, in conjunction with sorafenib, to combat HCC. Despite their demonstrated capacity for engraftment, expansion, and migration to tumor sites following adoptive transfer, clinical trials have yielded varied outcomes. These range from negligible impacts on patient survival or metastasis occurrence to marginal enhancements [34]. Gill et al. suggested that the limited efficacy of NK cell adoptive transfer, as evidenced by NK exhaustion, may result from prolonged exposure to tumors, as observed in murine models [35]. Differentiation into a memory-like state presents a distinct avenue for enhancing in vivo proliferation, persistence, and anti-tumor responses. Ongoing clinical trials are currently assessing the effectiveness of infusing cytokine-induced memory-like NK cells, primarily for hematological malignancies. Previous studies revealed a mechanism by which the HCV core protein exploits receptors within liver cells to activate NF-κB, a signaling pathway involved in various cellular processes [36, 37]. Although NF-κB activity was elevated in HCCs, HCV RNA levels were surprisingly lower in tumor tissue compared to healthy liver [38, 39]. While the precise mechanism of NF-κB activation in HCC remains elusive, Asakawa et al. suggested a potential role for IL-18 and its receptor in its constitutive activation [40]. IL-12 acts as a conductor of the anti-tumor orchestra, driving Th0 to Th1 differentiation, boosting T and NK cell numbers, triggering IFN-γ and NO production,

hindering tumor blood supply, and sharpening tumor recognition by immune cells, all culminating in enhanced antigen presentation and potent tumor cell targeting [41, 42]. In a previous study, Zhou et al. reported that treatment with 9597/IL-12 significantly elevated levels of key immune players compared to both control groups, including $CD3^+$ T cells, $CD4^+$ Th cells, IFN-γ^+ Th1 cells, S-100^+ DCs, and cytokines like IL-2, IFN-γ, and IL-12 which aligns with the established role of Th1 cells and DCs in orchestrating antitumor responses [43]. Moreover, Zhuang et al. expressed that combination of IL-12/15/18 with IL-2 in a cytokine cocktail proves to be an efficacious method for in vitro activation of human NK cells, eliciting anti-HCC activity in which activated NK cells exhibited migration to the liver, leading to a reduction in the incidence of spontaneous HCC formation in a spontaneous HCC model [44]. In our recent study, we focused on evaluating the potential of briefly preactivated ML NK cells with IL-12 and IL-18 individually and in combination with sorafenib against HCC tumor cells. Experimental findings revealed that the combination of sorafenib and cytokine-induced ML NK cells elicited robust cytotoxicity against human and rodent HCC tumor cells compared to monotherapies (**Figure 4D-F**). Furthermore, the preformed preclinical study substantiated the efficacy of combination sorafenib plus ML NK cell chemo-immunotherapy in impeding tumor growth, inducing significant morphological changes even at early treatment stages (**Figure 5**).

In this investigation, we developed ML NK cell lines and assessed their cytotoxic function alone and in combination with sorafenib against HCC tumors in in-vitro experiments. Moreover, we demonstrated the efficacy of this treatment strategy based on tumor growth patterns. In-vitro experimental outcomes indicated that ML NK cells induced significantly higher rates of cell death compared to conventional (rat and human) NK cell lines. The combination of sorafenib and ML NK cells exhibited a synergistic enhancement in treating HCC cell lines, providing a promising alternative for HCC treatment. Additionally, our preclinical study demonstrated that sorafenib plus ML NK cell combination chemoimmunotherapy notably inhibits tumor growth starting from the first week. These findings lay the groundwork for considering the application of targeted molecular agents and adoptive cell transfusions in clinical settings for patients with advanced HCC.

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Disclosure of conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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