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IL-21 reinvigorates exhausted natural killer cells in patients with HBV-associated hepatocellular carcinoma in STAT1-depedent pathway

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ABSTRACT

Hepatocellular carcinoma (HCC) is the most common liver malignancy with dismal prognosis and limited treatment options. Natural killer (NK) cells are critical components of antitumor immunity due to their capacity to eliminate MHC class I-deficient cells. To evaluate the function of NK cells in HCC patients, circulating $CD3^{-}CD56^{+}$ NK cells were collected from HBV-associated HCC patients and healthy control individuals. Compared to NK cells from healthy controls, NK cells from HCC patients presented functional impairment, characterized by significantly reduced cytotoxicity, degranulation, and cytokine production. Exogenous IL-21 could reinvigorate NK cells from HCC patients, resulting in significantly increased levels of cytotoxicity, degranulation, and cytokine expression. However, IL-21-treated NK cells from HCC patients still presented lower response than IL-21-treated NK cells from healthy controls. IL-21 resulted in increased phosphorylation of both STAT1 and STAT3 in NK cells. Inhibition of STAT1, but not STAT3, significantly reduced IL-21-mediated reinvigoration of NK function. Together, this study demonstrated that NK cells in HBV-associated HCC patients presented functional impairments that could be reverted by IL-21 in a STAT1-mediated mechanism.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common liver malignancy worldwide. Chronic hepatitis B virus (HBV) infection is associated with over 50% of HCC cases [1]. Antiviral regimens can substantially reduce but do not completely eliminate the risk of HCC development [2]. Early stage HCC may be treated with surgical resection, liver transplantation, and local ablation. For patients with advanced or recurrent HCC, no effective treatment is available. Very early-stage cancers are difficult to diagnose and frequently overlooked, further complicating HCC treatment. Immunotherapeutic strategies that harness the cytotoxicity of immune cells may improve the prognosis of HCC patients.

A major obstacle in cancer immunotherapy is that lack of tumor antigen presentation. This is a two-part problem, with one part being that tumor-specific antigens tend to be derived from mutated self, and the other part being that tumor cells can downregulate the expression of major histocompatibility complex class I (MHC I) [3,4]. Tumor cells that lose the expression of MHC-I are selected by the immune system, resulting in the development of MHC-I-negative tumors that can escape cytotoxic T cells. These MHC-I-negative tumors may be susceptible to natural killer (NK) cell-mediated cytotoxicity, which does not require MHC-I-mediated antigen presentation for activation [5–7]. In HCC patients, infusion of cytokine-activated lymphocytes, with the majority being NK cells, in combination with conventional treatments, resulted in better overall survival [8,9], suggesting that NK cells may be harnessed in immunotherapy against HCC.

However, NK cells in cancer patients display a number of dysfunctions that limit their efficacy in tumor immunosurveillance. Intratumoral NK cells displayed decreased IFN-γ production compared to circulating or peritumoral NK cells [10]. Inhibitory receptors, including PD-1 and Tim-3, were overexpressed by NK cells in ovarian cancer and advanced melanoma [11,12]. Suppression of PD-1 and Tim-3 signaling pathways enhanced NK cell cytotoxicity, demonstrating that

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Table 1
Characteristics of study participants.

Characteristics	HCC patients	Healthy volunteers	*P*
N	20	20	
Sex (female), *N* (%)	5 (25)	5 (25)	
Age (years), median (range)	47 (38–55)	46 (35–58)	> 0.05*
Stage, *N* (%)			
I + II	7 (35)		
III + IV	13 (65)		

Staging was performed using the TNM classification system by the American Joint Committee on Cancer.
* Unpaired *t*-test.

these inhibitory receptors negatively regulated NK cells [13,14].

The cytokine IL-21 is a member of the IL-2 family with previously identified roles in promoting cytotoxicity and reverting exhaustion in $CD8^+$ T cells [15]. NK cells express the IL-21 receptor (IL-21R), which is made up of the common γ chain that is shared with other IL-2 family members and the IL-21R chain that is specific to IL-21. IL-21 signaling was found to promote NK maturation and tumor rejection *via* NKG2D, a molecule expressed by both NK cells and $CD8^+$ T cells [16,17]. Recently, IL-21 was shown to reactivate Tim-3^+PD-1^+ NK cells, which then mediated cytotoxicity against tumor cells in mice and human colorectal, melanoma, and bladder cancer cells [18].

NK cells upon *in vitro* expansion presented high cytotoxicity against HCC cell lines [19], granting grounds for further research of NK cell functionality in HCC patients. In this study, the circulating NK cells were characterized in patients with HBV-associated HCC. We found that in these patients, the NK cells presented marked functional exhaustion, which could be partially reverted by IL-21 stimulation.

2. Methods

2.1. Subjects

Peripheral blood samples were collected from 20 newly diagnosed and untreated HBV-related HCC patients and 20 healthy controls, whose demographic and clinical characteristics were listed in Table 1. Diagnosis was based on biopsy evidence following the guidelines by the American Association for the Study of Liver Diseases [20]. All study protocols and use of human samples were approved by the ethics

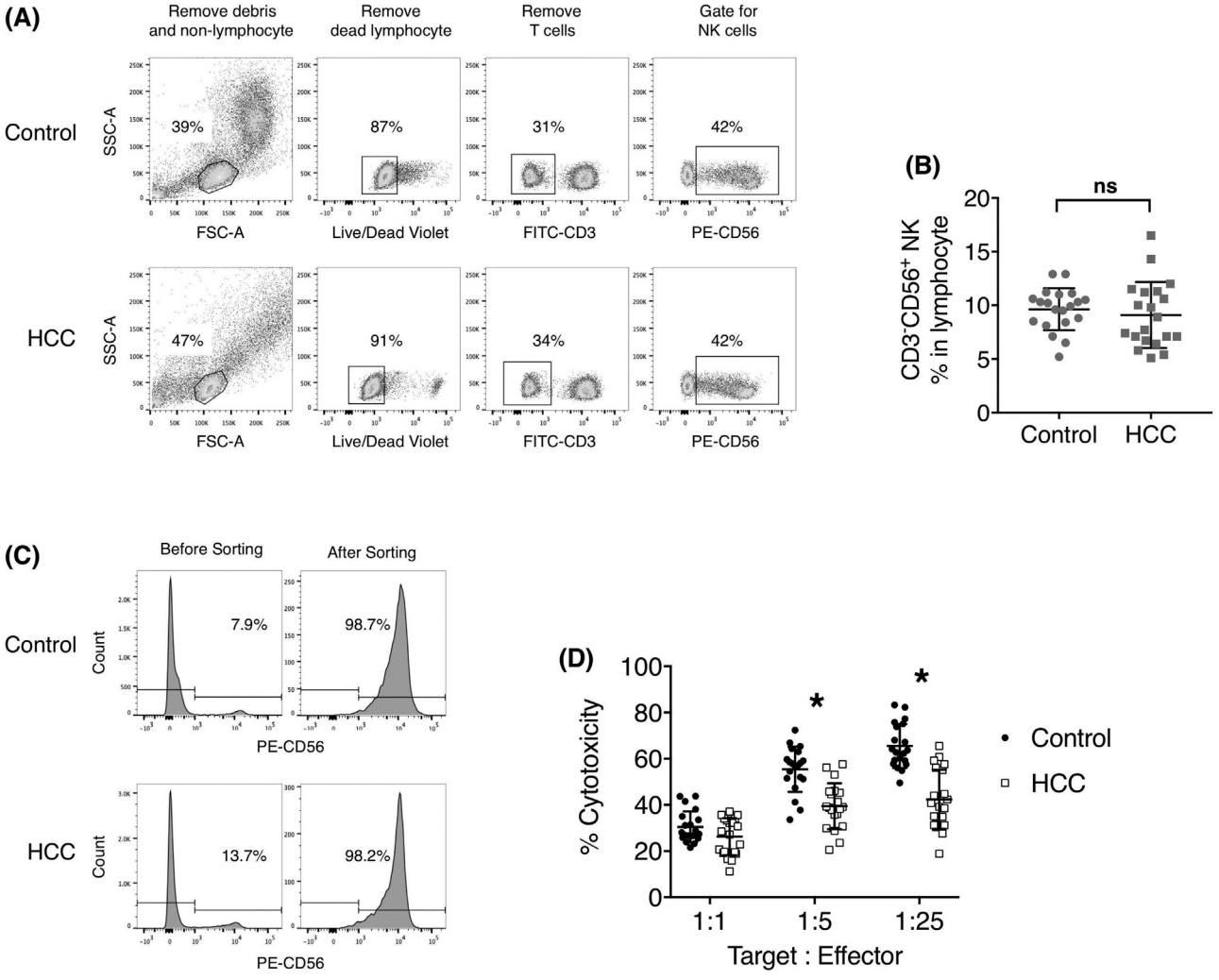

Fig. 1. NK cells from HCC patients presented significantly reduced cytotoxicity than NK cells from healthy controls.
(A) Representative gating strategy of $CD3^-CD56^+$ NK cells in total PBMCs from one control individual and one HCC individual. (B) The frequency of $CD3^-CD56^+$ NK cells in live circulating lymphocytes in 20 healthy control donors and 20 HCC subjects. Unpaired *t*-test. ns, not significant. (C) The frequency of $CD56^+$ cells before and after FACSAria-sorting, in one representative control and one representative HCC patient. (D) The cytotoxicity of the NK cells in 20 healthy control donors and 20 HCC subjects. Target cells (K562) and effector cells ($CD3^-CD56^+$ NK cells) were incubated at 1:1, 1:5, and 1:25 ratios for 12 h. Two-way ANOVA followed by Tukey's post-test. $*P < 0.05$.

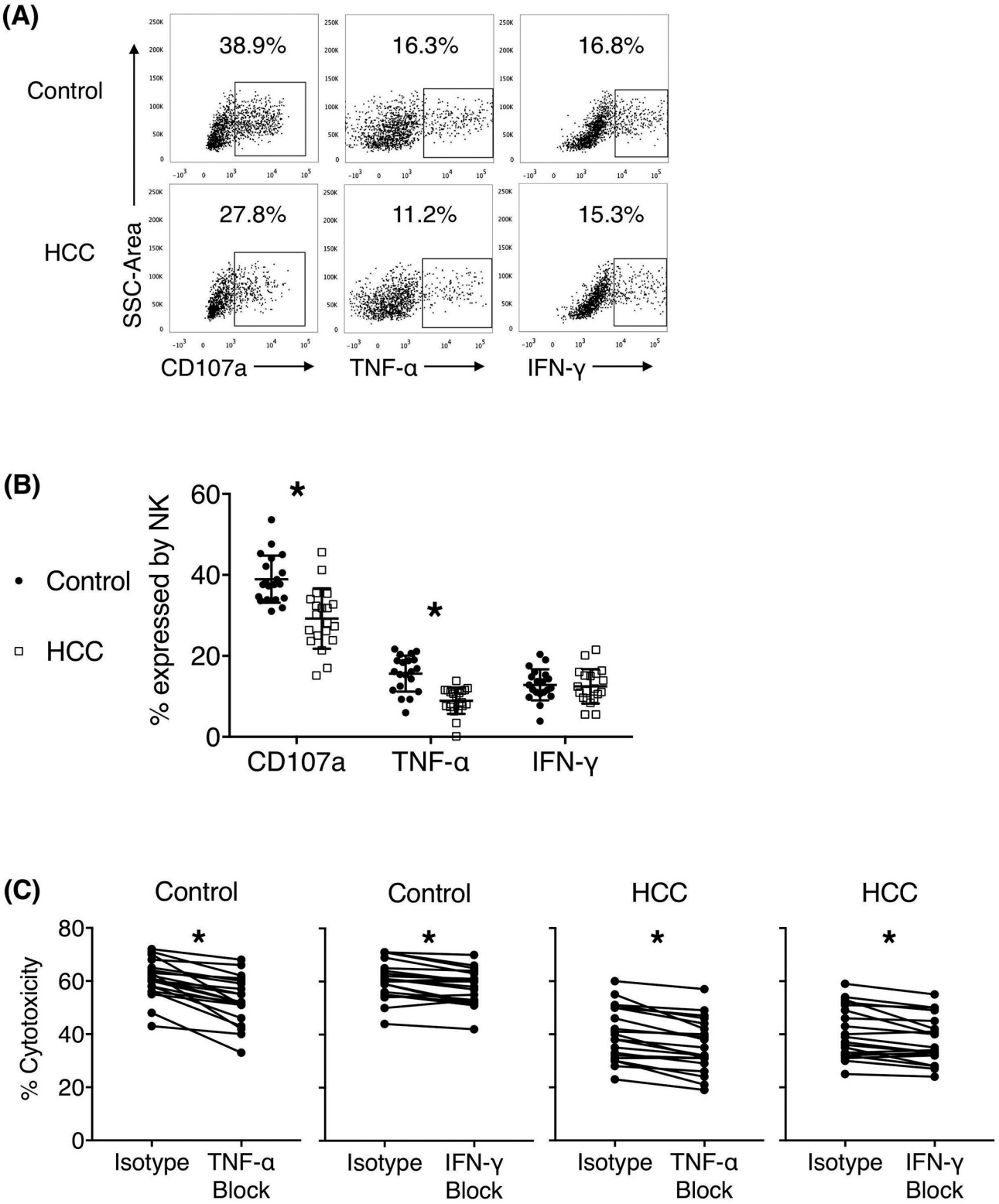

Fig. 2. NK cells from HCC patients presented significantly lower degranulation and cytokine expression than NK cells from healthy controls.
(A) The intracellular expression of CD107a, TNF-α, and IFN-γ in one representative healthy control and one representative HCC patient. NK cells from healthy controls and HCC patients were stimulated with K562 cells (1:1 ratio) for 12 h in the presence of anti-human CD107a antibody and GolgiPlug for the final 5 h. (B) The percentages of $CD107a^+$, $TNF\text{-}\alpha^+$, and $IFN\text{-}\gamma^+$ NK cells in 20 healthy donors and 20 HCC patients. Two-way ANOVA followed by Tukey's post-test. (C) The cytotoxicity of NK cells (1:5 target:effector ratio) in the presence of TNF-α or IFN-γ blocking antibodies. Paired *t*-test. $^*P < 0.05$.

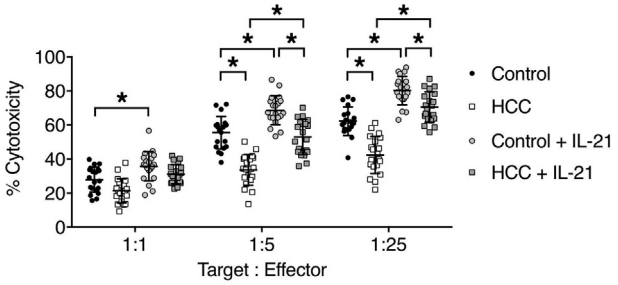

Fig. 3. IL-21 significantly increased the cytotoxicity of NK cells in healthy controls and HCC patients.
The cytotoxicity of the NK cells in 20 healthy control donors and 20 HCC subjects. Effector cells ($CD3^-CD56^+$ NK cells) were incubated in the absence or presence of 20 ng/mL IL-21 for 72 h, and then with target cells (K562) for 12 h. Two-way ANOVA followed by Tukey's post-test. *$P < 0.05$.

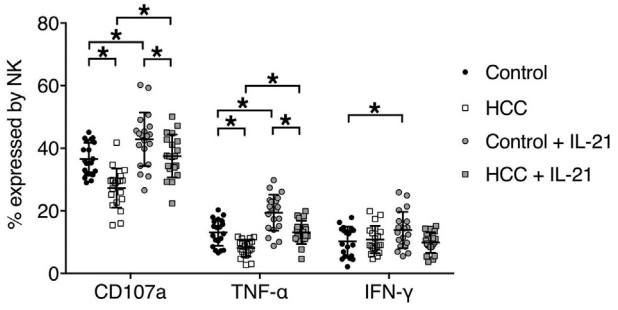

Fig. 4. IL-21 increased the degranulation and cytokine expression of NK cells from healthy controls and HCC patients.
NK cells from healthy controls and HCC patients were incubated in the absence or presence of 20 ng/mL IL-21 for 72 h, and then stimulated with K562 cells (1:1 ratio) for 12 h. The percentages of $CD107a^+$, $TNF\text{-}\alpha^+$, and $IFN\text{-}\gamma^+$ NK cells in 20 healthy donors and 20 HCC patients were shown. Two-way ANOVA followed by Tukey's post-test. *$P < 0.05$.

committee of The First People's Hospital of Yunnan Province. All participants provided written informed consent prior to sample collection.

2.2. Samples

About 35 mL of peripheral blood was obtained from each subject. Peripheral blood mononuclear cells (PBMCs) were harvested from blood using Ficoll-Hypaque (Sigma-Aldrich) centrifugation technique, washed twice, and cryopreserved in 90% heat-inactivated fetal bovine serum (FBS; Invitrogen) and 10% DMSO (Sigma). Cells were cooled step-wise to −80 °C and then to −150 °C. All experiments were performed using frozen and thawed samples within 2 months of sample collection. For thawing, frozen PBMCs were briefly placed in 37 °C water bath and then in complete medium (RPMI 1640 solution supplemented with 10% heat-inactivated FBS, 1 × GlutaMax, 1 × penicillin and 1 × streptomycin; Invitrogen) supplemented with 1% DNase (Sigma), and washed in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 2 mM EDTA (Invitrogen). Thawed cells were then rested before experiments, by incubating in complete medium overnight. The incubation condition was 100% humidity and 5% CO_2 at 37 °C. K562 cells were obtained from ATCC.

2.3. Isolation of NK cells

Thawed PBMCs were incubated with FITC-conjugated anti-human CD3 and PE-conjugated anti-human CD56 for 30 min in dark in cold PBS supplemented with 2% FBS. Excess antibodies were removed *via* washing and the NK cells were sorted as $CD3^-CD56^+$ cells in BD FACSAria instrument, with purity consistently above 95%.

2.4. Cytotoxicity assay

K562 cells were labeled with Calcein AM (Invitrogen), plated in 96-well flat-bottom plate at 5×10^4 cells per well, and were rested for 4 h such that the cells settle to the plate bottom. FACS-sorted NK cells were counted using Trypan Blue solution (Sigma) with viability > 85%, and were then added to the plates at various targets: effector ratios as specified in the experiments. All plates were topped up with culture medium to 200 µL per well and incubated for 12 h. Maximum lysis assay was performed using pure sterile water to replace culture medium. Spontaneous lysis assay was performed in the absence of NK cells. After incubation, the supernatant was removed and replaced with sterile PBS, and the cell viability after cytotoxicity assay was measured in an FLx800LBS Reader (Lonza). Percent cytotoxicity was calculated by (spontaneous − experiment) / (spontaneous − maximum) × 100%.

2.5. Degranulation and intracellular cytokine staining

1×10^5 NK cells per well were incubated with 1×10^5 K562 cells per well for 12 h. APC-conjugated anti-CD107a antibody (BD) was added at the beginning of the incubation, and 5 µg/mL GolgiPlug (BD) was added 5 h before the end of incubation. Supernatant was removed and the cells were incubated with Dead Cell Stain Violet (Invitrogen) and anti-human CD3 and CD56 antibodies (BD) for 30 min in cold PBS supplemented with 2% FBS. Excess antibodies were removed by washing, and the cells were permeabilized using CytoPerm/CytoFix buffer (BD). Cells were then stained with PE-Cy7-conjugated anti-human TNF-α and PerCP/Cy5.5-conjugated IFN-γ (BD) for 30 min in cold 1 × Perm Wash, washed, and fixed in 2% Formalin. P-STAT1 and P-STAT3 were stained using the BD Phosflow Staining kits for PerCP/Cy5.5-conjugated STAT1 (pY701) and PerCP/Cy5.5-conjugated STAT3 (pY705). All incubation steps were performed at 4 °C in dark. Samples were acquired and examined in a BD FACSCanto instrument.

2.6. Treatment with IL-21 and STAT inhibitors

Recombinant human IL-21 (R&D Systems) was incubated with NK cells for 72 h at 20 ng/mL, in the absence or presence of Fludarabine or S31-301 (Sigma-Aldrich). NK cells were then washed twice to remove excess cytokines or chemicals before adding to target cells.

2.7. Statistical analysis

One-way and Two-way ANOVA, followed by Tukey's multiple comparisons, were applied for multiple group comparisons. Unpaired or paired *t*-test was applied for comparisons between two groups. *P* values smaller than 0.05 was considered statistically significant.

3. Results

3.1. NK cells from HCC patients presented lower cytotoxicity than NK cells from healthy individuals

$CD3^-CD56^+$ NK cells were identified stepwise using flow cytometry (Fig. 1A), and represented 9.6% ± 1.9% (mean ± SD) of circulating lymphocytes in healthy control individuals, and 9.1% ± 3.1% of circulating lymphocytes in HBV-associated HCC patients (Fig. 1B). No significant difference between the control group and the HCC group was found. To evaluate the function of NK cells in HCC patients, circulating $CD3^-CD56^+$ NK cells were sorted from HBV-associated HCC patients and healthy control individuals (Fig. 1C). The cytotoxicity of NK cells was then examined. NK cells from HCC patients demonstrated significantly reduced capacity to eliminate K562 target cells than NK cells from healthy controls, at 1:5 and 1:25 target:effector ratios (Fig. 1D).

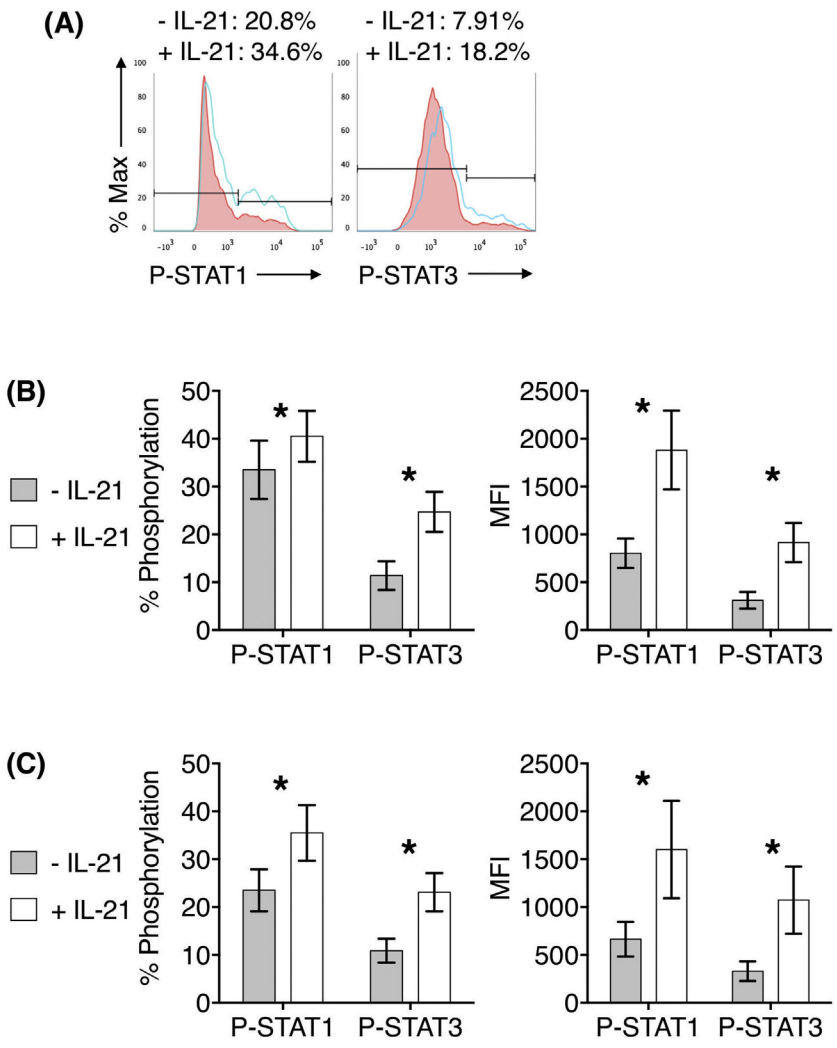

Fig. 5. IL-21 increased the phosphorylation of NK cells from HCC patients.
(A) NK cells from HCC patients without IL-21 incubation (−IL-21) and with 20 ng/mL IL-21 incubation for 72 h (+IL-21) were stimulated with K562 cells for 12 h. The frequencies of phosphorylated STAT1 (P-STAT1) and phosphorylated STAT3 (P-STAT3) in NK cells from one representative HCC patient are shown. (B) The frequencies of P-STAT1 and P-STAT3 and their MFI from 20 healthy controls patients without or with IL-21 were shown as mean ± SD. (C) The frequencies of P-STAT1 and P-STAT3 and their MFI from 20 HCC patients without or with IL-21 were shown as mean ± SD. Paired *t*-test. $^*P < 0.05$.

3.2. NK cells from HCC patients presented reduced degranulation and cytokine expression than NK cells from healthy individuals

To explain the difference in NK cell-mediated cytotoxicity between HCC patients and healthy controls, the surface presentation of CD107a, a degranulation marker, was examined in NK cells following coincubation with K562 cells (Fig. 2A). Compared to the NK cells from healthy controls, the NK cells from HCC patients presented significantly lower levels of CD107a (Fig. 2B).

The expression of intracellular cytokines, including TNF-α and IFN-γ, were examined concurrently (Fig. 2A). NK cells from HCC patients presented significantly lower levels of TNF-α, but comparable levels of IFN-γ, compared to NK cells from healthy controls (Fig. 2B).

Subsequently, we investigated whether the cytokine production capacity was associated with the cytotoxicity mediated by NK cells. K562 cells and NK cells were incubated at 1:5 target:effector ratio, in the presence of TNF-α blocking antibody, IFN-γ blocking antibody, or their corresponding isotype controls. The cytotoxicity of NK cells was then examined. In both controls and HCC patients, blocking either TNF-α or IFN-γ could significantly reduce the cytotoxicity of NK cells (Fig. 2C).

3.3. IL-21 improved NK cell function in healthy controls and HCC patients

It was demonstrated that IL-21 could enhance the effector functions of $CD8^+$ T cells and NK cells [21,22]. Here, we investigated whether IL-21 could elevate the function of NK cells in HCC patients. NK cells from healthy controls and HCC patients were isolated and incubated with recombinant IL-21 (rIL-21). The cytotoxicity assay was then performed. In both healthy controls and HCC patients, the NK cell-mediated cytotoxicity was significantly higher in the presence of IL-21 than in the absence of IL-21 (Fig. 3). However, healthy NK in the presence of IL-21 still presented stronger cytotoxicity than HCC NK in the presence of IL-21.

3.4. IL-21 increased degranulation and TNF-α expression by NK cells from HCC patients

We also examined the effect of IL-21 on the degranulation and cytokine expression by NK cells from healthy controls and HCC patients. In the absence of IL-21, HCC NK cells presented significantly lower CD107a and TNF-α expression than healthy NK cells (Fig. 4). IL-21 significantly increased the CD107a and TNF-α expression in both healthy controls and HCC patients. IL-21 was able to increase levels of CD107a and TNF-α expression of HCC NK cells to the equivalent levels by control NK cells in the absence of IL-21, but not to the level of control NK cells in the presence of IL-21. In addition, IL-21 significantly increased the IFN-γ expression in healthy control NK cells, but not in HCC NK cells.

3.5. IL-21 mediated NK enhancement depended STAT1 but not STAT3 phosphorylation

IL-21 signals through Jak1/Jak3 and induces the phosphorylation of

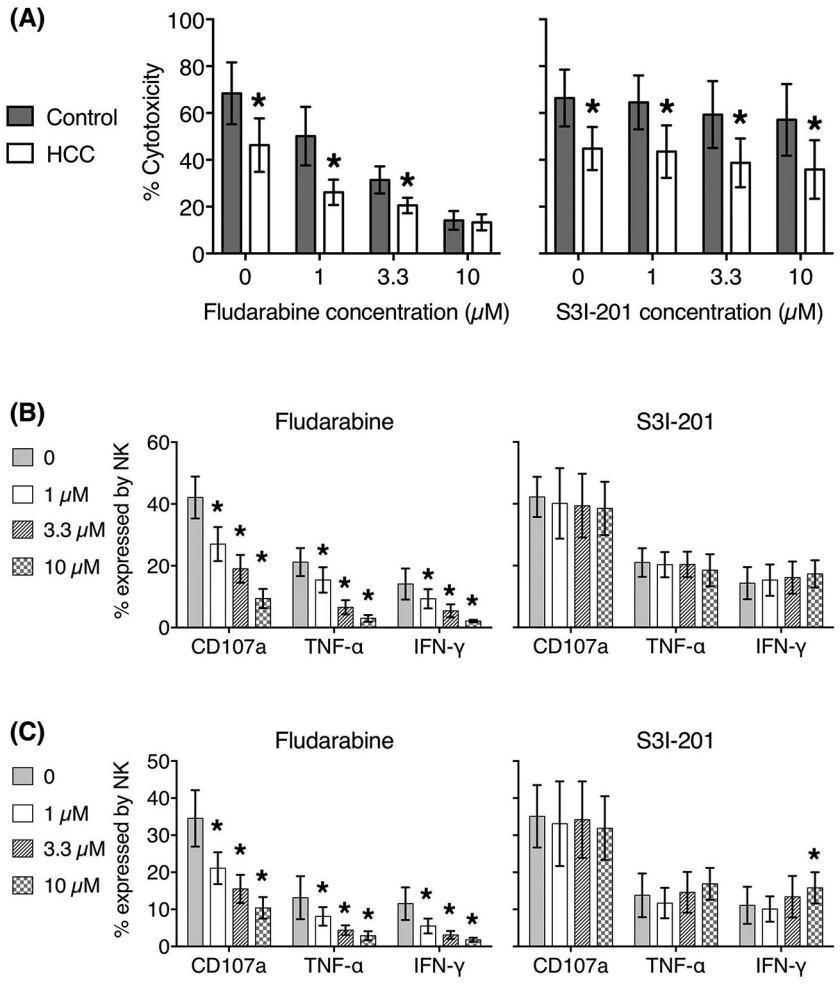

Fig. 6. Inhibition of STAT1, but not STAT3, compromised IL-21-mediated stimulation of NK cells.
NK cells from HCC patients or healthy controls were incubated with 20 ng/mL IL-21 for 72 h, in the absence or the presence of Fludarabine or S31-201. NK cells were then stimulated with K562 for the measurement of cytotoxicity, degranulation, and cytokine expression. (A) The cytotoxicity of IL-21-treated NK cells in the absence or presence of Fludarabine or S3I-201 at the indicated concentrations. Two-way ANOVA followed by Tukey's post-test. (B) The degranulation and cytokine expression of IL-21-treated healthy control NK cells ($N = 20$) in the absence or presence of Fludarabine or S3I-201 at the indicated concentrations. (C) The degranulation and cytokine expression of IL-21-treated control NK cells ($N = 20$) in the absence or presence of Fludarabine or S3I-201 at the indicated concentrations. One-way ANOVA followed by Tukey's post-test. $^*P < 0.05$.

STAT1, STAT3, and STAT5 [23,24]. To investigate the downstream effects of IL-21 signaling in NK cells from HCC patients, we examined the STAT1 and STAT3 phosphorylation in NK cells before and after incubation with exogenous IL-21 using flow cytometry (Fig. 5A). In both healthy controls and HCC patients, the phosphorylation levels of STAT1 and STAT3 were significantly elevated, both in terms of the frequency of P-STAT1 and P-STAT3 in NK cells and in terms of the mean fluorescence intensity (MFI) of P-STAT1 and P-STAT3 (Fig. 5B and C).

Subsequently, the STAT1 inhibitor Fludarabine and STAT3 inhibitor S3I-201 were added to the experiments, such that whether the IL-21-mediated stimulation of NK cells depended on STAT1 and STAT3 phosphorylation. NK cells were treated with IL-21 in the absence or presence of various concentrations of Fludarabine or S3I-201. The cytotoxicity of NK cells was then examined. In both healthy controls and HCC patients, the STAT1 inhibitor Fludarabine, but not the STAT3 inhibitor S31-201, significantly reduced the cytotoxicity of IL-21-treated NK cells in a dose-dependent manner (Fig. 6A). Fludarabine also suppressed the CD107a, TNF-α, and IFN-γ expression in IL-21-treated NK cells (Fig. 6B and C). S3I-201, on the other hand, did not significantly change the CD107a or TNF-α expression in IL-21-treated NK cells (Fig. 6B and C). Interestingly, S3I-201 at high concentration (10 μM) significantly increased the level of IFN-γ expression by IL-21-treated NK cells from HCC patients (Fig. 6B).

4. Discussion

This study demonstrated that, compared to NK cells from healthy individuals, the NK cells from HCC patients presented reduced functional capacity, characterized by reduced cytotoxicity, lower degranulation, and lower TNF-α expression. We further found that IL-21 significantly enhanced the cytotoxicity, degranulation, and cytokine production of NK cells. Both STAT1 and STAT3 phosphorylation were upregulated in the presence of IL-21, but only STAT1 phosphorylation was required for the IL-21-mediated stimulatory effects on NK cells.

Previous studies demonstrated that the engagement of MHC class I molecules with inhibitory receptors on NK cells inhibits NK function, while downregulation of MHC class I in tumor cells could activate cytotoxicity mediated by NK cells [25]. The MHC class I-related chain A (MICA) and chain B (MICB), on the other hand, could potently activate NK cells [26], and were rarely found in normal cells but could be expressed by tumor cells, including malignant hepatocytes [27]. Hence, it seems paradoxical that NK cells presented lower functional capacity in HCC patients than in healthy controls. The molecular mechanisms responsible for mediating NK cytotoxicity should be investigated. Previously, it was shown that Tim-3 and PD-1, two molecules expressed by exhausted $CD8^+$ T cells, could also induce NK cell exhaustion [11,13]. Interestingly, the Tim-3 ligand galectin 9 and the PD-1 ligand PD-L1 are both enriched in the intrahepatic environment [28,29]. Whether these molecules are responsible for the downregulation of NK function in HCC patients need to be examined. Also, the NK cells can be further distinguished into a major $CD16^{bright}CD56^{dim}$ subset and a minor $CD16^{+/-}CD56^{bright}$ subset, with the former exhibiting significantly higher cytotoxic activity than the latter. In the liver, the resident NK cells were enriched with a $CD56^{bright}$ subset that was scarcely found in the peripheral blood [30,31]. In future studies, the composition of NK subsets in the peripheral blood and in the tumor, and whether the low

cytotoxicity in HCC NK cells could be attributed to a potential upregulation in the $CD56^{bright}$ population, should be investigated. Currently, NK cells are regarded as promising candidates for cancer immunotherapy due to their ability to recognize MHC class I-deficient tumor cells. *In vitro* expanded and activated NK cells have been shown to mediate effective killing of HCC cell lines with high potency [19]. To overcome the impairment in NK function, several cytokines have been used to stimulate NK cells. IL-2, IL-12, and IL-15-treated NK cells demonstrated significantly elevated cytolytic activity against tumor cells, characterized by elevated expression of effector molecules and increased proliferation, while the addition of IL-21 synergistically increased NK activity [32–34]. Also, it was shown that IL-15 was more effective at inducing NK proliferation, while IL-21 preferentially activated effector function [34]. Hence, it has been proposed that IL-15 may be used during *in vitro* expansion of NK cells, followed by a brief exposure with IL-21 to boost the cytolytic activity. Our research showed that IL-21 alone was effective at increasing the cytotoxicity, degranulation, and cytokine release from NK cells. In future studies, the individual and combinatory effects of these cytokines on the expansion and function of NK cells, as well as on the expression of inhibitory receptors, should be studied in further detail.

This study has several limitations that should be addressed in future investigations. First, in order to standardize the measurement of NK function across different individuals, the cytotoxicity and stimulation of NK cells were performed using K562 cells, which was a leukemia line that were frequently used as a gold-standard for measuring NK cytotoxicity. The effect of NK cells on the primary tumor cells from each cancer individual is still unclear. Second, we observed that IL-21 activated the phosphorylation of both STAT1 and STAT3. In previous studies and this investigation, STAT1 phosphorylation was responsible for IL-21-mediated functional activation in exhausted NK cells [16,18], while STAT3 abrogated NK cell-mediated tumor surveillance [35]. These results suggest that the actions of STAT3 may oppose STAT1, even though exogenous IL-21 resulted in a pro-activation effect in NK cells overall. Therefore, the specific actions of STAT3 in NK cell exhaustion and reinvigoration by IL-21 should be further investigated. Third, the reinvigoration of NK cells was investigated under *in vitro* setting and in isolation of other cells that might affect NK function, such as malignant hepatocytes, antigen-presenting cells, and other tumor-infiltrating lymphocytes. Further research is necessary to examine IL-21 in NK cells in an *in vivo* setting. Fourth, we found that the inhibition of TNF-α and IFN-γ could reduce the cytotoxic effects of NK cells. It has been shown that TNF-α could directly mediate cell apoptosis in the absence of cytolytic molecules [36]. In future studies, the indirect effect of TNF-α and IFN-γ on the expression of effector molecules from NK cells, as well as the direct effect on K562 cells, should be studied independently.

Conflict of interest

None.

Acknowledgments

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