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γδ T cells in hepatocellular carcinoma patients present cytotoxic activity but are reduced in potency due to IL-2 and IL-21 pathways

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ABSTRACT

Hepatocellular carcinoma (HCC) is the most common primary liver carcinoma and has one of the highest mortality rates of all cancers. The γδ T cells could infiltrate HCC and have demonstrated potent tumor-killing capacity. Here, we found that in peripheral blood, the vast majority of γδ T cells were Vδ2 T cells. In HCC patients, the frequency of Vδ2 T cells was significantly lower than in controls. γδ T cells that were harvested directly *ex vivo* possessed very limited capacity to eliminate Zol-loaded HCC cell lines, even at a high effector to target ratio. *In vitro* expansion with Zol could significantly increase the capacity of γδ T cells to eliminate HCC cell lines. But even with *in vitro* expansion, the γδ T cells from HCC patients presented significantly lower cytotoxic capacity than the γδ T cells from healthy individuals. The expression of IL-2 and IL-21 by γδ T cells was significantly lower in HCC patients than in control volunteers. Supplementing recombinant human IL-2 and IL-21 in the *in vitro* expansion culture increased the cytotoxic capacity of γδ T cells. In addition, the frequency of PD-1$^+$ γδ T cells was significantly higher in HCC patients than in controls *ex vivo*, and was significantly elevated after *in vitro* expansion. Hep3B and HepG2 did not express PD-L1, while a small fraction of SNU-398 expressed PD-L1. Interestingly, co-incubation with γδ T cell elevated PD-L1 expression in HCC cell lines. Blocking PD-1 during *in vitro* expansion stage significantly elevated cytotoxicity toward all the HCC cell lines, while blocking PD-1 during the cytotoxicity assay significantly elevated cytotoxicity toward HepG2 and SNU-398, but not toward Hep3B. Overall, these results demonstrated that the circulating γδ T cells in HCC patients were reduced in cytotoxic capacity, possibly associated with the lack of IL-2 and IL-21 production and PD-1 upregulation.

1. Introduction

γδ T cells are defined by the expression of the Vγ and the Vδ T cell receptor (TCR) chains, which are distinct from the conventional TCRs expressing the Vα and Vβ chains. Based on the specific Vδ chain, human γδ T cells are divided into two main subsets, including the Vδ1 T cells, which are predominantly found in the thymus and mucosal tissues and recognize stress-induced antigens, and the Vδ2 T cells, which are always associated with the Vγ9 chain in adults, are enriched in the peripheral blood, and recognize phosphorylated non-peptide antigens as well as stress-induced antigens [1].

A number of direct antitumor roles are identified in γδ T cells. γδ T cells can lyse breast cancer cells, renal cancer cells, and head and neck squamous carcinomas *in vitro* through perforin and granzyme secretion [2–4]. γδ T cells can also induce apoptosis of tumor cells by expressing TRAIL and FasL [5,6]. In addition, γδ T cells can express CD16 (FcγR III), through which γδ T cells may initiate antibody-dependent cell-mediated cytotoxicity [7,8]. Moreover, γδ T cells are important sources of IFN-γ and TNF-α, both of which enhance antitumor immunity through various mechanisms. In *in vitro* studies, γδ T cells have demonstrated tumor-killing capacity and, when adoptively transferred in murine tumor models, γδ T cells have been able to induce tumor regression [9,10].

Hepatocellular carcinoma (HCC) is the most common primary cancer in the liver and the third leading cause of cancer-related death worldwide [11]. Occult hepatitis B virus (HBV) infection is a major risk factor for HCC in East Asia [12,13]. Although significant advances in the treatments have been made to increase the survival time, no cure is yet available. Novel strategies that utilize the immune system, such as cytokine-induced autologous killer cells and PD-1 inhibitors, are thought to enhance antitumor immune responses that are durable in time and provide survival benefits, with promising trial results [14,15].

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Further research is needed to identify new candidates to be used in immunotherapy.

Zoledronate (Zol) is a nitrogen-containing bisphosphonate currently approved for the treatment of various bone diseases. It has been shown that Zol-treated tumor cells, including HCC cell lines, are susceptible to γδ T cell-mediated elimination [16,17]. Using Zol, we activated Vδ2 T cells from HCC patients. Their functional characteristics were then examined.

2. Methods

2.1. Study approval and samples

The participant recruitment, sample collection, and experimental procedures were approved by the Ethics Committee of the Third People's Hospital of Yunnan Province. Informed consent was received from all study subjects. Fifteen subjects with HBV-related primary hepatocellular carcinoma were recruited at the Third People's Hospital of Yunnan Province, together with 15 age and sex matched healthy volunteers. All patients presented stage III cancer according to the tumor/node/metastasis classification system by the American Joint Committee on Cancer. Potential participants with alcoholic liver disease and cirrhosis were excluded.

All study subjects provided peripheral blood samples, collected in EDTA-K3 anticoagulant-containing tubes. Peripheral blood mononuclear cells (PBMCs) were then harvested from peripheral blood *via* the standard Ficoll (GE Healthcare) gradient centrifugation procedure, and stored at −80 °C in 90% heat-inactivated FBS (Gibco) and 10% DMSO (Sigma). All HCC cell lines were purchased from ATCC and maintained in a incubator with 37 °C, 5% CO_2 and 100% humidity.

2.2. Flow cytometry

PBMCs or HCC cell lines were incubated with human IgG (Sigma) for 5 min at room temperature to block Fc-receptors. The anti-human TCRγδ, Vδ2, CD3, PD-1, and/or PD-L1 antibodies (BioLegend) and the Aqua dead cell stain (Invitrogen) were added for 30 min at 4 °C. Excess staining agents were removed by washing, and the stained samples were acquired in a FACSCanto II cytometer (BD). The frequency of γδ T cells and Vδ2 T cells was examined using the FlowJo software (Tree Star). To examine the expression of IL-2 and IL-21, cells were incubated with 5 μg/mL brefeldin A and 5 μg/mL monensin (BD) for 6 h before staining, acquisition, and analysis.

2.3. In vitro expansion

γδ T cells were negatively selected using the human gamma/delta T cell isolation kit (Stemcell). The γδ T cells were then seeded in a 96-well plate at 5×10^4 cells per well with 5 μmol/mL Zol (Enzo Life Sciences). In preliminary experiments, we found that irradiated feeder cells could improve γδ T cell survival and proliferation following stimulation. Hence, 1×10^5 irradiated (100 Gy) autologous PBMCs were added to each well as feeder cells. All cell cultures were topped up to 200 μL per well with complete culture medium. When indicated, recombinant human IL-2 and IL-21, or polyclonal goat anti-human PD-1 antibodies or isotype controls (R&D Systems), were added in the complete culture medium. The medium was replaced every 72 h. In some experiments, the 12-day supernatant was collected for human IL-2 and IL-21 ELISA assays (BioLegend). Before using in cytotoxicity assays, the *in vitro* expanded γδ T cells were negatively selected from the expansion cell culture, and live cells were counted using Trypan Blue Dye (Thermo Fisher).

2.4. Cytotoxicity assay

The HCC cell lines were incubated with Calcein-Am (Thermo Fisher) for 30 min at 37 °C, followed by washing. The labeled target cells were then incubated with *ex vivo* γδ T cells or expanded γδ T cells at ratios indicated in the experiments for 6 h with 5 μmol/mL Zol, and the fluorescent intensity was measured before and after the incubation. Percent of specific lysis was calculated as (fluorescence in maximum control- fluorescence in experiment) / (fluorescence in maximum control - fluorescence in minimum control) x 100%. Polyclonal goat anti-human PD-1 antibodies or isotype controls were added at 10 μg/mL in PD-1 inhibition studies.

2.5. Statistical analysis

The statistics was performed in Prism version 6.0 (GraphPad Software). The statistical significance of differences between two groups was determined using unpaired *t*-test with Welch's correction, and between multiple groups using 2-way ANOVA followed by Tukey's test. Two-tailed *p* values lower than 0.05 were considered significant.

3. Results

3.1. Identification and isolation of Vδ2 T cells in HCC patients and control volunteers

The PBMCs were collected from HBV-related HCC patients and healthy control volunteers that were matched in age and sex with the patients. To identify Vδ2 T cells, anti-TCRγδ and anti-Vδ2 staining were performed on live lymphocytes (Fig. 1A, left panel). Approximately 2% to 6% of live lymphocytes could be identified as Vδ2 T cells by positive gating on both anti-TCRγδ and anti-Vδ2 axes (Fig. 1B). In HCC patients, the frequency of Vδ2 T cells was slightly lower than that in healthy control volunteers (Fig. 1B).

For functional analyses, untouched Vδ2 T cells are favorable since they are not coated with potentially activating antibodies. Hence, we used magnetic negative selection to enrich for Vδ2 T cells from total PBMCs. To examine the quality of enrichment, we performed anti-TCRγδ and anti-Vδ2 staining on cells after purification (Fig. 1A, right panel). The frequency of Vδ2 T cells was consistently above 90% after purification, and no difference between control volunteers and HCC patients were observed (Fig. 1C).

3.2. Cytotoxicity of γδ T cells from HCC patients and control volunteers

To examine whether γδ T cells presented cytotoxic activity, we used a number of HCC cell lines as target cells, including Hep3B, a hepatitis B-infected cell line with fibroblast and epithelial to mesenchymal transition features and derived from an 8-year old African male, HepG2, a line that retained hepatocyte features and derived from a 15-year old Caucasian male, and SNU-398, a hepatitis B-infected line derived from a 42-year old Asian male [18,19]. Polyclonal γδ T cells were isolated from the HCC patients and control volunteers *via* negative magnetic selection, and were co-incubated with Hep3B, HepG2, and SNU-398 in the presence of Zol (Fig. 2A). We found that even at a high effector to target (E:T) ratio of 20, the specific lysis mediated by γδ T cells was low at approximately 2% to 4% on average. Nonetheless, we found that the γδ T cells from control volunteers presented significantly higher lytic capacity toward HepG2 and SNU-398 than the γδ T cells from HCC patients.

To increase the availability of γδ T cells and enhance their effector function, we expanded the γδ T cells in the presence of Zol and irradiated feeder cells. The *in vitro* expanded γδ T cells were then co-incubated with Zol-treated HCC cell lines at various E:T ratios (Fig. 2B). The *in vitro* expanded γδ T cells possessed significantly higher cytotoxicity than the *ex vivo* isolated γδ T cells. Also, we found that the γδ T cells from control volunteers were significantly more potent against Hep3B, HepG2, and SNU-398 than the γδ T cells from HCC patients.

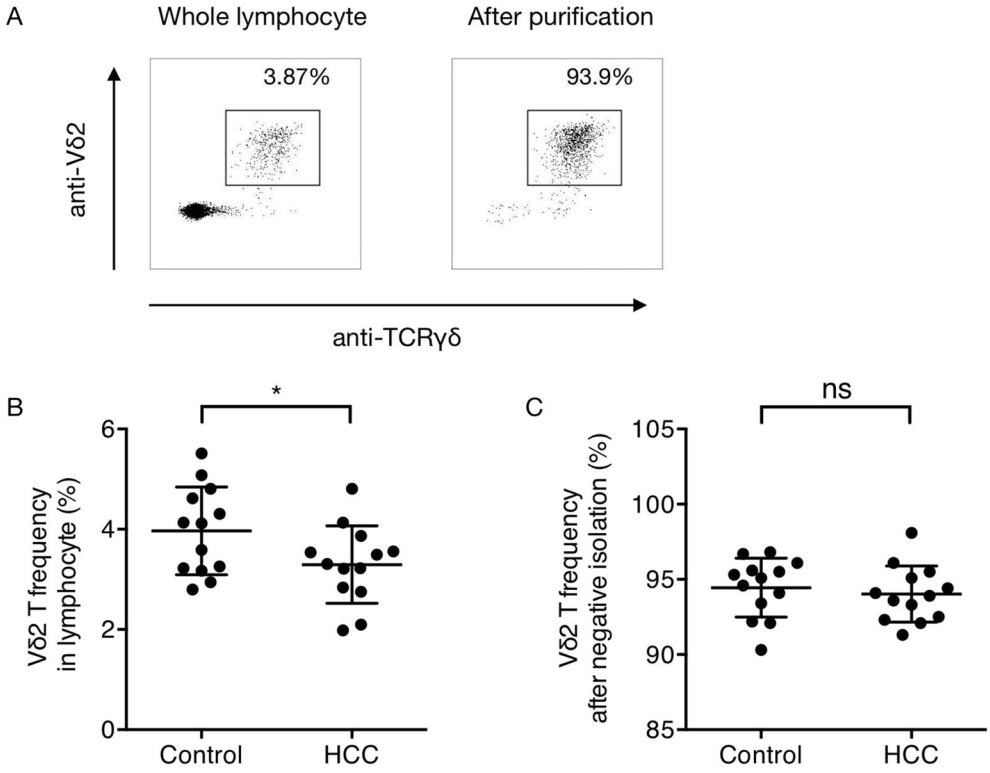

Fig. 1. Vδ2 T cells in peripheral blood.
(A) Anti-TCRγδ and anti-Vδ2 staining was performed on whole PBMCs (left panel) or γδ T cell-purified PBMCs (right panel). Figures shown were pre-gated on live lymphocytes by Dead Cell Aqua-negative gating and lymphocyte-specific forward *vs.* side scatter gating. (B) The frequency of Vδ2 cells in total live lymphocytes in healthy controls and HCC patients, without γδ T cell purification. (C) The frequency of Vδ2 cells in purified γδ T cells in healthy controls and HCC patients. Unpaired *t*-test with Welch's correction. $^{*}p < 0.05$. ns, not significant.

3.3. Differences between γδ T cells from HCC patients and control volunteers

To explain the difference in cytotoxic capacity between γδ T cells from HCC patients and control volunteers, we examined the expression of cytokines in the *in vitro* expanded γδ T cells *via* intracellular staining (Fig. 3A). We found that the frequency of Vδ2 T cells that expressed IL-2 and IL-21 was significantly lower in HCC patients than in control volunteers. To quantify the amount of IL-2 and IL-21 being produced, we examined the IL-2 and IL-21 concentrations in the supernatant of the *in*

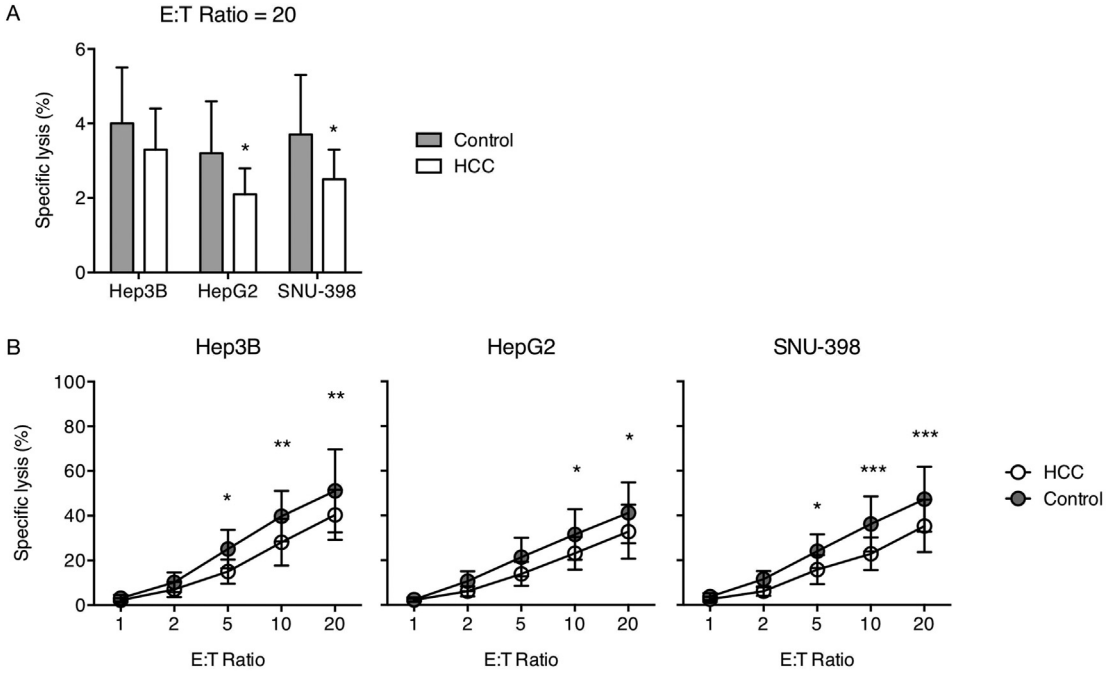

Fig. 2. Cytotoxicity of γδ T cells in HCC patients and control volunteers.
(A) Negatively isolated polyclonal γδ T cells were co-incubated with Zol-treated HCC cell lines Hep3B, HepG2, and SNU-398 at 20:1 effector (γδ T cell) to target (HCC cell line) ratio. The specific lysis by γδ T cells from 15 healthy controls and 15 HCC patients was shown as mean ± standard deviation. Unpaired *t*-test with Welch's correction. (B) Negatively isolated polyclonal γδ T cells were first expanded *in vitro* for 12 days, and then co-incubated with Zol-treated HCC cell lines at various E:T ratios. The specific lysis by γδ T cells from 15 healthy controls and 15 HCC patients was shown as mean ± standard deviation. Two-way ANOVA followed by Tukey's test. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

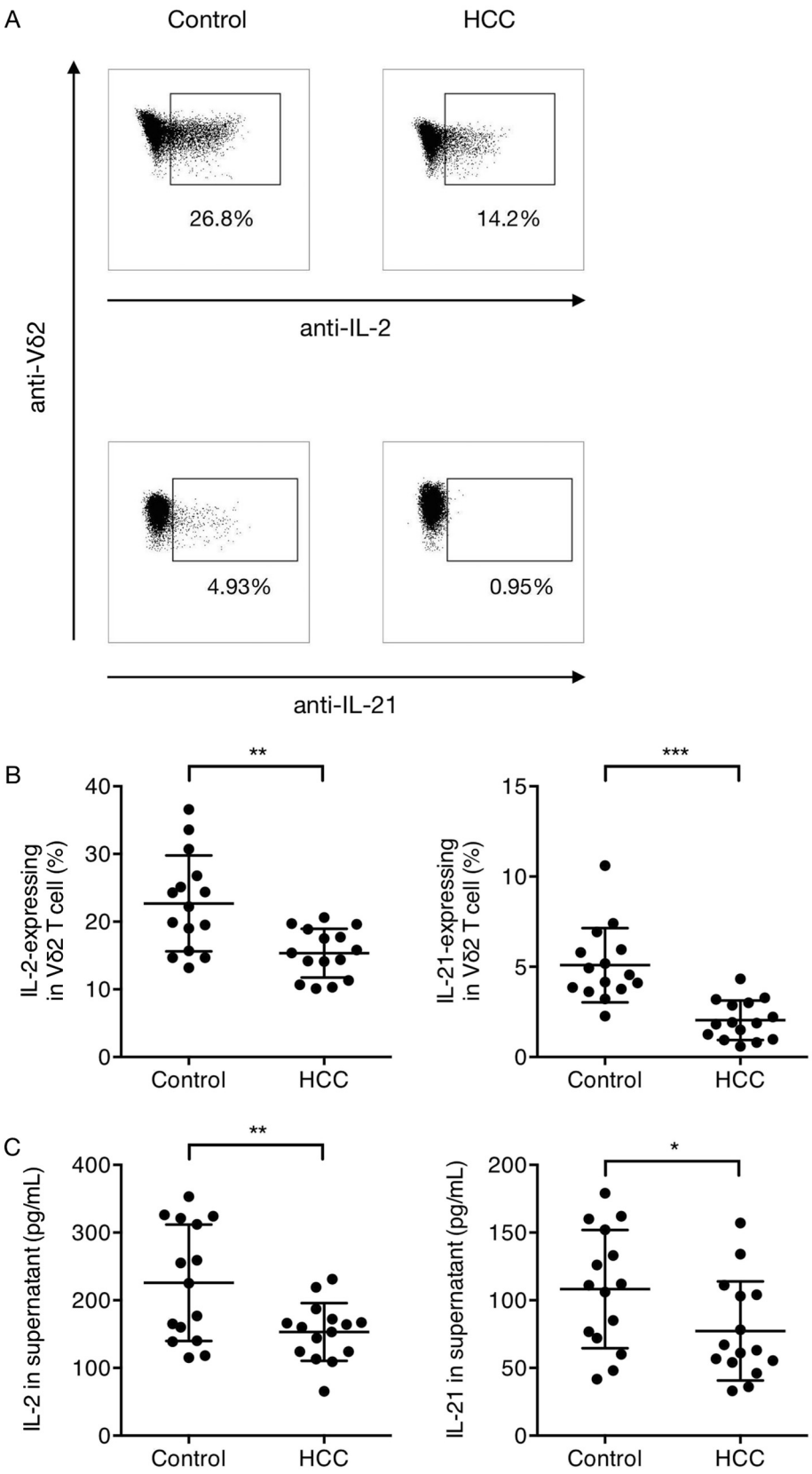

Fig. 3. Expression of IL-2 and IL-21 by *in vitro* expanded γδ T cells.
(A) *In vitro* expanded γδ T cells were incubated with brefeldin A and monensin for 6 h, and the IL-2 and IL-21 expression was then evaluated by intracellular staining. One example each from control volunteers and HCC patients was shown. (B) The frequency of IL-2-expressing and IL-21-expressing Vδ2 T cells from 15 healthy controls and 15 HCC patients. (C) The concentration of IL-2 and IL-21 in the supernatant from γδ T cell *in vitro* expansion culture. Unpaired *t*-test with Welch's correction. $^{**}p < 0.01$. $^{***}p < 0.001$.

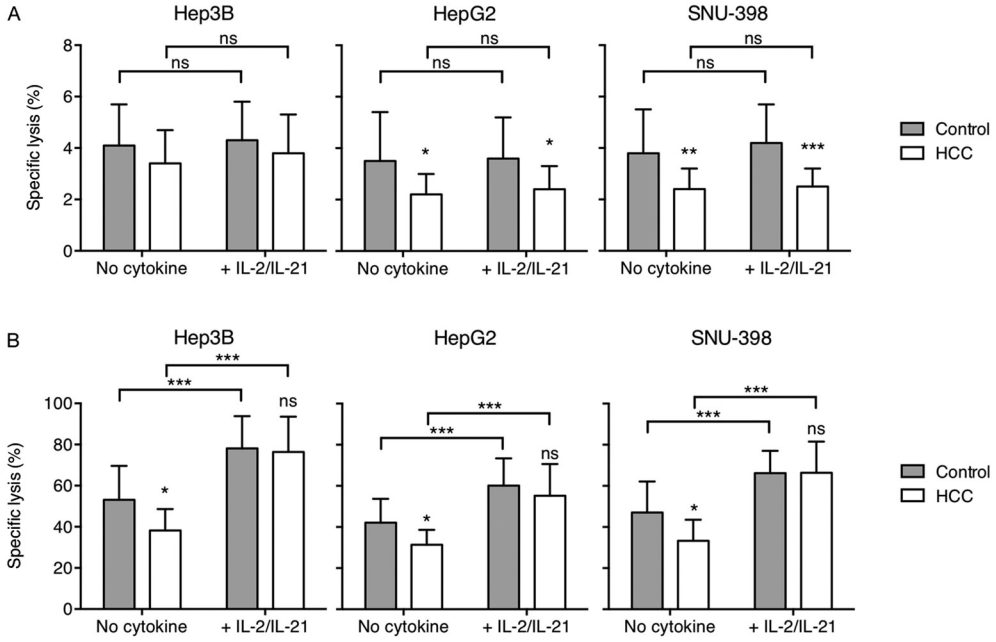

Fig. 4. Cytotoxicity of γδ T cells with exogenous IL-2 and IL-21.
(A) Negatively isolated γδ T cells were incubated with were co-incubated with Zol-treated HCC cell lines Hep3B, HepG2, and SNU-398 at 20:1 effector to target ratio, with or without 20 U/mL IL-2 and 20 ng/mL IL-21. (B) Negatively isolated γδ T cells were expanded with or without 20 U/mL IL-2 and 20 ng/mL IL-21. Expanded γδ T cells were then co-incubated with Zol-treated HCC cell lines Hep3B, HepG2, and SNU-398 at 20:1 effector to target ratio. The specific lysis by γδ T cells from 15 healthy controls and 15 HCC patients was shown as mean ± standard deviation. 2-way ANOVA followed by Tukey's test. $^{*}p < 0.05$. $^{**}p < 0.01$. ns, not significant.

vitro expansion culture. The supernatant from HCC patients contained significantly lower levels of IL-2 and IL-21 than the supernatant from control volunteers (Fig. 3C).

3.4. Addition of IL-2 and IL-21 significantly improved the cytotoxicity of in vitro expanded γδ T cells

To evaluate whether a lack of IL-2 and IL-21 was associated with the reduction in γδ T cell function, we added exogenous IL-2 and IL-21 to the cytotoxicity assay of γδ T cells that were harvested *ex vivo*. The *ex vivo* γδ T cells from HCC patients presented significantly lower cytotoxicity against HepG2 and SNU-398 than the *ex vivo* γδ T cells from control volunteers, either with or without IL-2 and IL-21 (Fig. 4A). In neither control volunteers nor HCC patients, IL-2 and IL-21 applied directly *ex vivo* could enhance the cytotoxicity of γδ T cells.

Next, we examined the effect of IL-2 and IL-21 on expanded γδ T cells, by adding IL-2 and IL-21 in the *in vitro* expansion culture. A cytotoxicity assay was then performed on the expanded γδ T cells. We found that IL-2 and IL-21 could significantly improve the γδ T cell cytotoxicity in both control volunteers and HCC patients (Fig. 4B). Of note, whereas the expanded γδ T cells from HCC patients presented reduced capacity to eliminate HCC cell lines, in the presence of exogenous IL-2 and IL-21, no significant differences in the cytotoxicity of expanded γδ T cells between HCC patients and healthy controls were found.

3.5. The PD-1/PD-L1-mediated inhibition of γδ T cell cytotoxicity toward HCC cell lines

To further investigate the dysfunction of γδ T cells in HCC patients, the PD-1 expression was examined using surface staining (Fig. 5A). The frequency of PD-1^{+} cells in γδ T cells was significantly higher in HCC patients than in controls *ex vivo* (Fig. 5B). *In vitro* expansion in the presence of Zol and feeder cells resulted in significantly higher frequencies of PD-1^{+} γδ T cells in both controls and HCC patients ($p < 0.001$ for both groups). The frequency of PD-1^{+} γδ T cells after *in vitro* expansion was still higher in HCC patients than in controls.

Subsequently, we examined the PD-L1 expression in the HCC cell lines. Neither Hep3B nor HepG2 were PD-L1^{+} (Fig. 5C). A small proportion of SNU-398 cells were PD-L1^{+} (Fig. 5C). Since these cells were used as target cells in cytotoxicity assay, we examined the PD-L1 expression in these cells after co-incubation with γδ T cells (Fig. 5C). Interestingly, co-incubation with γδ T cells, from both controls and patients, resulted in increased frequencies of PD-L1^{+} cells in HCC cell lines (Fig. 5D).

Due to the expression of PD-1 in γδ T cells and PD-L1 in HCC cell lines, we investigated the effect of PD-1/PD-L1 blocking on γδ T cell-mediated cytotoxicity, using polyclonal anti-human PD-1 antibodies during the Zol-mediated expansion of γδ T cells, or during the co-incubation/cytotoxicity assay of HCC cells. Anti-PD-1 applied at the *in vitro* γδ T cell expansion stage resulted in strongly and significantly elevated cytotoxicity toward all the HCC cell lines tested in this study (Fig. 6A). Anti-PD-1 applied at the co-incubation stage, on the other hand, significantly elevated cytotoxicity toward HepG2 and SNU-398, but not toward Hep3B, the cell line with the lowest PD-L1 expression (Fig. 6B). On average, PD-1 inhibition at the *in vitro* expansion stage resulted in higher cytotoxicity than PD-1 inhibition at the co-incubation/cytotoxicity assay stage.

4. Discussion

In this study, we investigated the characteristics of the γδ T cells from HCC patients. The vast majority of γδ T cells in the peripheral blood were Vδ2 T cells, which represented approximately 2% to 6% of lymphocytes in various individuals, and this frequency was significantly lower in HCC patients. It was previously shown that γδ T following 14-day stimulation with PHA, IL-2, and IL-15 had 50% to nearly 100% specific lysis rate against Zol-treated HCC cell lines [17]. However, we found that γδ T cells that were harvested directly *ex vivo* had a very low specific lysis rate of only about 2% to 6% toward Zol-treated HCC cell lines, suggesting that the circulating γδ T cells were not fully activated. γδ T cells are known to infiltrate liver and HCC [20], and whether γδ T cells in tumor still present low activity *ex vivo* and *in vivo* should be examined in tumor samples and in animal models, respectively.

In vitro expansion is a frequently used strategy to activate and prepare lymphocytes for adoptive transfer. γδ T cells expanded with Zol potently eliminated HCC cell lines. Interestingly, the *in vitro* expanded γδ T cells from HCC patients presented significantly lower cytotoxic capacity than the *in vitro* expanded γδ T cells from healthy individuals, suggesting that the lower cytotoxic capacity we observed in *ex vivo* γδ T cells from HCC patients was not due to a lack of activation. We also found that the γδ T cells from HCC patients, when stimulated, presented

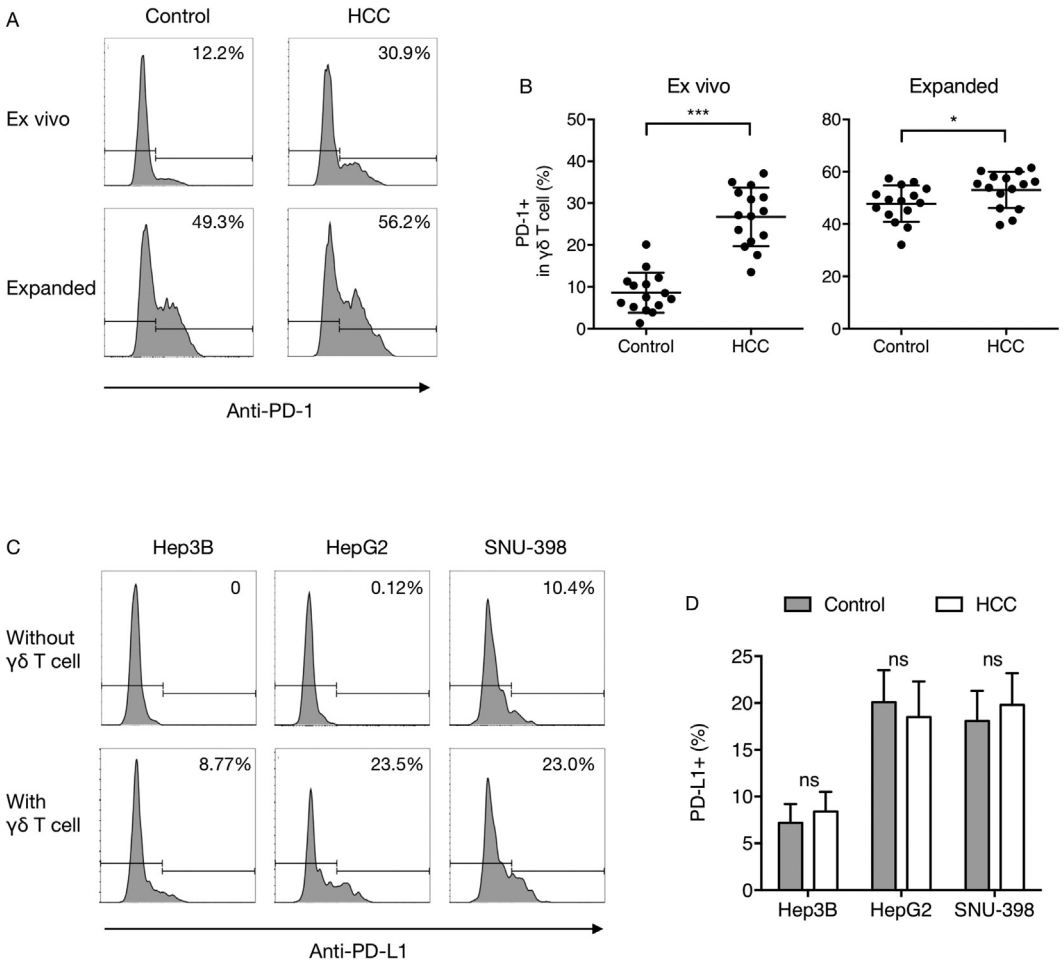

Fig. 5. PD-1/PD-L1 expression on γδ T cells and HCC cell lines.
(A) PD-1 expression in γδ T cells from one representative control and one representative HCC patient. Figures shown were pre-gated on live γδ T cells. (B) The frequencies of PD-1$^+$ cells in γδ T cells from 15 healthy controls and 15 HCC patients, directly *ex vivo* or after *in vitro* expansion with Zol. (C) PD-L1 expression in each of the HCC cell lines, without γδ T cells or with Zol-expanded γδ T cells from one HCC patient. (D) The frequencies of PD-L1$^+$ cells in each of the HCC cell lines, following incubation with Zol-expanded γδ T cells from 15 controls and 15 HCC patients, shown as mean ± standard deviation. Unpaired *t*-test with Welch's correction. *$p < 0.05$. ***$p < 0.001$. ns, not significant.

significantly lower capacity to express IL-2 and IL-21. Supplementation of recombinant human IL-2 and IL-21 in the *in vitro* expansion culture significantly increased the cytotoxic capacity of γδ T cells. Moreover, Zol-stimulated and IL-2 and IL-21-treated γδ T cells from HCC patients were as potent as their counterparts from healthy individuals. In addition, the PD-1 expression in γδ T cells and PD-L1 expression in HCC cell lines were examined. The frequency of PD-1$^+$ cells in γδ T cells was significantly higher in HCC patients than in controls *ex vivo*, and was significantly elevated after *In vitro* expansion. Blocking PD-1 during *in vitro* expansion stage significantly elevated cytotoxicity toward all the HCC cell lines. Interestingly, In a previous study by Iwasaki et al. [21], Zol treatment of tumor cell lines, including PC-3 (metastatic prostate adenocarcinoma), EJ-1 (B cell lymphoma), and MRK-nu-1 (breast carcinoma), seemed to override the PD-1/PD-L1-mediated inhibition, as the γδ T cell-mediated specific lysis was comparable between PD-L1-blocked and PD-L1-unblocked experiments. For unknown reasons, this was not the case in our study.

Interestingly, Hep3B and HepG2 did not express PD-L1, while a small fraction of SNU-398 expressed PD-L1. Co-incubation with γδ T cell elevated the PD-L1 expression in HCC cell lines. One possible explanation is that the γδ T cells may secrete proinflammatory cytokines that stimulate the expression of PD-L1. Indeed, IFN-γ, IL-17, TNF-α, and IL-27 have all been shown to promote PD-L1 by cancer cells [22]. Future studies should address whether the expansion process of γδ T cells could increase the expression of cytokines that negatively impacts cytotoxicity by elevating PD-L1 expression, and devise better expansion strategies. Another possibility is that PD-L1-expressing tumor cells were preferentially "selected" by the PD-1-expressing γδ T cells during the cytotoxicity assay. An indirect piece of evidence is that, PD-1 inhibition during the cytotoxicity assay significantly elevated cytotoxicity toward HepG2 and SNU-398 but not toward Hep3B, the cell line with the lowest PD-L1 expression. These results demonstrated the PD-1/PD-L1-mediated protection of HepG2 and SNU-398.

Overall, our study demonstrated that circulating γδ T cells possessed cytotoxicity against HCC and could potentially be used as candidates in immunotherapies. However, optimal response likely requires additional cytokines and PD-1 inhibition. In the future, other aspects of γδ T cells in HCC should be investigated. It has been shown that γδ T cell selectively mediate the elimination of primary renal carcinoma cells but not healthy renal cells in a TCR-dependent and NKG2D-dependent manner [4]. The involvement of NKG2D ligands in the interaction between γδ T cells and HCC should be further investigation. Also, γδ T cells may promote immune suppression *via* IL-17-mediated infiltration of myeloid-derived suppressor cells and small peritoneal macrophages into the liver [23,24], in addition to expressing regulatory and pro-angiogenesis cytokines [25]. Hence, the γδ T cell gene expression in the HCC microenvironment should be further investigated.

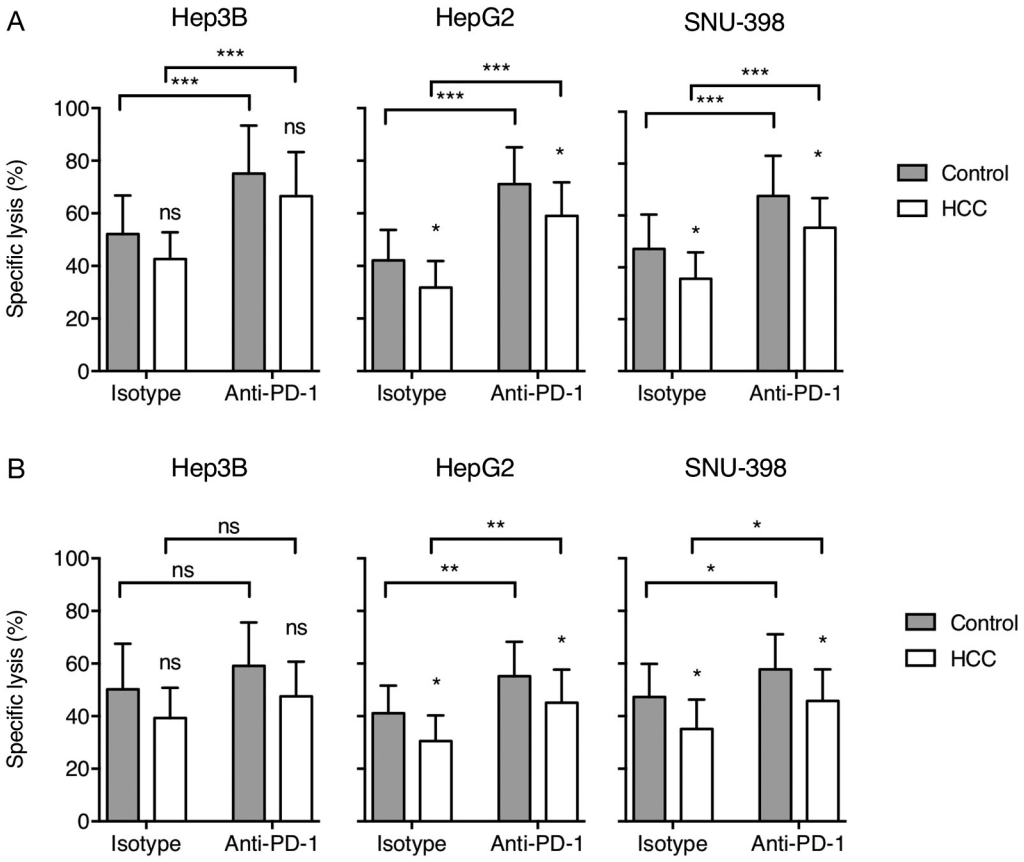

Fig. 6. PD-1 inhibition on γδ T cell-mediated cytotoxicity.
(A) PD-1 was inhibited using 10 μg/mL polyclonal anti-PD-1 antibody during Zol-stimulated γδ T cell expansion. Cytotoxicity assay of Hep3B, HepG2, and SNU-398 was then performed at 20:1 effector to target ratio. (B) *In vitro* Zol-expanded γδ T cells were co-incubated with Hep3B, HepG2, and SNU-398 at 20:1 effector to target ratio, in the presence of 10 μg/mL polyclonal anti-PD-1 antibody. 2-way ANOVA followed by Tukey's test. $*p < 0.05$. $**p < 0.01$. $***p < 0.001$. ns, not significant.

Conflict of interest

The authors declare no conflict of interest.

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