γδ T cell-mediated individualized immunotherapy for hepatocellular carcinoma considering clinicopathological characteristics and immunosuppressive factors

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Abstract. Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer. γδ T cells have been revealed to be promising candidates for immunotherapy in patients with HCC. However, the use of these cells in clinical practice has been demonstrated to be challenging. In the present study, γδ T cells isolated from the peripheral blood of patients with HCC (n=83) and healthy donors (n=15) were characterized. Flow cytometry was used to analyze the proportion, phenotype, tumor-killing capacity and cytokine secretion of regulatory T cells (Tregs) and γδ T17 cells in peripheral blood samples prior to and following amplification. Interleukin (IL)-17A levels in the supernatant was analyzed using an ELISA on days 3, 7, 10 and 14. The *in vitro* cytotoxicity of γδ T cells was measured using an MTT assay. It was revealed that zoledronate with IL-2 may efficiently expand γδ T cells sourced from the peripheral blood of patients with HCC. The amplification capacity of γδ T cells was associated with the clinicopathological characteristics of patients (clinical stage, levels of AFP and albumin, duration of disease, size and number of tumors, numbers of Tregs and γδ T17 cells, and levels of IL-17A). The proportion of γδ T cells positive for interferon-γ, tumor necrosis factor-α, granzyme B, perforin, and lysosome-associated membrane protein 1 was almost unchanged prior to and following amplification. Following amplification, the *in vitro* cytotoxicity of γδ T cells also remained unchanged. γδ T17 cells, Tregs and IL-17A levels were not altered during amplification. In summary, following *in vitro* amplification, circulating γδ T cells were revealed to possess features that may make them suitable for immunotherapy for HCC without increasing immunosuppressive factors. However, immunotherapy should be individualized according to the clinicopathological features of patients.

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Introduction

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer and the third and fifth main cause of cancer-associated mortality in men and women respectively in China, 2015 (1). In previous years, γδ T cells have been revealed to be feasible candidates for immunotherapy in the treatment of various types of cancer, including melanoma, breast cancer and lung cancer. In addition, a number of studies have demonstrated that γδ T cells may recognize and lyse numerous types of HCC cell and are involved in the immunotherapeutic mechanism against HCC (2-7). Zoledronate may activate and induce the selective amplification of Vγ9Vδ2 T cells *in vitro* from peripheral blood mononuclear cells (PBMCs) taken from patients, making it suitable for clinical adoptive immunotherapy (8,9). However, the use of this type of cell in clinical trials has revealed that numerous challenges to be overcome remain (10).

Human Vγ9Vδ2 T cells comprise 50-95% of peripheral blood γδ T cells and may be divided into four subsets: $CD45RA^+CD27^+$ naïve ($T_{naïve}$) cells, $CD45RA^-CD27^+$ central memory cells, $CD45RA^-CD27^-$ effector memory (T_{EM}) cells and $CD45RA^+$ $CD27^-$ effector memory (T_{EMRA}) cells (11). Furthermore, Vγ9Vδ2 T cells may express natural killer receptor group 2, member D (NKG2D) and recognize major histocompatibility complex (MHC) class I-related chain A/B and UL16-binding proteins, which are induced or upregulated on the surface of numerous types of tumor cell (10). A number of studies have suggested that γδ T cells may be activated and regulated by NKG2D (10,12).

Vγ9Vδ2 T cells also exert marked cytotoxic effects through the perforin/granzyme signaling pathway dependent on cell-to-cell contact, resulting in the release of interferon

(IFN)-γ and tumor necrosis factor (TNF)-α which enhance antitumor activity (2-4). A number of studies have demonstrated that the cytotoxicity of Vγ9Vδ2 T cells primarily depends on the perforin/granzyme signaling pathway (13,14). Therefore, the expression levels of perforin and granzyme B, which are essential in this signaling pathway, may indirectly reflect the cytotoxicity of Vγ9Vδ2 T cells.

$CD4^+$, $CD25^+$ and $FoxP3^+$ regulatory T cells (Tregs), which are involved in the formation of the immunosuppressive network, suppress antitumor immunity and are the main obstacles faced by cancer immunotherapy. *In vivo* and *in vitro* studies have revealed that Tregs may suppress the proliferation and function of cytotoxic T cells (15-17), and impair the function of HCC-infiltrating γδ T cells (18). Wu *et al* (19) demonstrated that the main innate source of interleukin (IL)-17A was γδ T17 cells and that these cells may also suppress antitumor immunity in human colorectal cancer. Furthermore, Ma *et al* (20) suggested that IL-17A produced by γδ T cells promoted tumor growth in HCC. However, the effect of *in vitro* amplification of circulating γδ T cells in patients with HCC on the levels of Tregs, γδ T17 cells and IL-17A have yet to be fully clarified.

On the basis of previous research, the association between the change in immunosuppressive factors during *in vitro* γδ T cell amplification and factors determining the suitability of patients for immunotherapy remains unclear. Therefore, the aim of the present study was to characterize the proportions and functions of circulating γδ T cells, and levels of immunosuppressive factors in patients with HCC prior to and following amplification *in vitro* using zoledronate with IL-2. In addition, the association between the amplification ability of γδ T cells and the clinicopathological characteristics of patients with HCC was investigated.

Materials and methods

Patients and peripheral blood specimens. Written informed consent was obtained from all patients prior to the study. Peripheral blood samples (10 ml) from 83 patients with HCC and from 15 healthy donors used as the control group were collected in the present study. The present study was approved by the Ethics Committee of Shanxi Medical University (Taiyuan, China). The inclusion and exclusion criteria of the patients were as follows: i) patients having a confirmed diagnosis of HCC according to the National Comprehensive Cancer Network clinical practice guidelines in Oncology: Hepatobiliary Cancers (version 2; https://www.nccn.org/professionals/physician_gls/default.aspx); and ii) patients without other malignancies, autoimmune diseases or other immune-associated diseases. The clinicopathological characteristics of the patients are presented in Table I. The clinical stage of the tumors was confirmed according to the Barcelona-Clinic Liver Cancer system (21).

Isolation and amplification of γδ T cells and culture of HCC cell lines. PBMCs were isolated from the fresh peripheral blood of patients and healthy donors using Ficoll density gradient to centrifuge at 453 x g for 15 min at room temperature (GE Healthcare, Chicago, IL, USA). As described previously (5), in order to amplify γδ T cells from fresh PBMCs (mean viability: 94.4%), 5 μM zoledronate (Zometa; Novartis International AG, Basel, Switzerland) was added to GT-T551 medium (Takara Bio, Inc., Otsu, Japan) supplemented with 10% heat-inactivated autologous plasma, 80 U/ml gentamicin and 1,000 IU/ml recombinant human IL-2 (Proleukin®; Chiron Therapeutics, Suresnes, France) at the onset of cultivation. Every 3 days, 10 ml GT-T551 and 1,000 IU/ml IL-2 were added to the cultures. After 12-14 days, γδ T cells were harvested (mean viability, 96.83±6.81%) which were cultured at 37˚C in a 5% CO_2 humidified incubator during this period. The human HCC cell lines HuH7, PLC, and SMMC-7721 supplied by Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences, Shanghai, China) were cultured at 37˚C in a 5% CO_2 humidified incubator.

Flow cytometry. Prior to and following amplification, normal mouse serum (cat. no. S-I-000004, EarthOx Life Sciences, Millbrae, CA, USA) was diluted using PBS (1:50 dilution; cat. no. 10010023, eBioscience; Thermo Fisher Scientific, Inc. Waltham, MA, USA) and mixed with cells for 1 min at room temperature in order to block non-specific binding. Following this, cells were stained (either intracellularly or on the surface) at 4˚C in dark with fluorochrome-conjugated monoclonal antibodies for 20 min in order to analyze the proportion, phenotype, tumor-killing capacity and cytokine secretion of Tregs and γδ T17 cells. Anti-NKG2D-fluorescein isothiocyanate-FITC (cat. no. 11-5878-41), anti-cluster of differentiation (CD)3-phycoerythrin (PE)-cyanine (Cy)5, (cat. no. 15-0038-42), anti-CD27-PE-Cy7, (cat. no. 25-0279-41), anti-TNF-α-FITC (cat. no. 11-7349-82), anti-forkhead box P3 (FoxP3)-PE (cat. no. 12-4777-42) and anti-IL-17A-PE antibodies (cat. no. 14-7179-82) were purchased from eBioscience; Thermo Fisher Scientific, Inc.; anti-Vγ9TCR-PE (cat. no. 555733), anti-perforin-FITC (cat. no. 556577), anti-granzyme B-FITC (cat. no. 560211) and anti-CD107a-FITC (cat. no. 555800) antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA); and anti-IFN-γ-FITC (cat. no. IM2716U), anti-T cell receptor (TCR) -pan-γδ-FITC (cat. no. IM1571U), anti-CD45-proprotein convertase subtilisin/kexin type (PC) 7 (cat. no. IM3548U), anti-CD25-PC5 (cat. no. IM2646U), anti-CD4-FITC (cat. no. 6603862) and anti-CD45RA-FITC (cat. no. IM0584U) antibodies were purchased from Beckman Coulter, Inc. (Brea, CA, USA). The dilutions used for different experiments are detailed in the relevant protocols. Prior to staining for CD107a, cells were stimulated using phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (500 ng/ml) for 4-6 h in incubator at 37˚C. Immunofluorescence was determined using a Cytomics FC500 flow cytometer with CXP software (version 2.1; Beckman Coulter, Inc.).

ELISA. Culture supernatants from γδ T cells were collected on days 3, 7, 10 and 14. The IL-17A content in the supernatants were determined using a direct ELISA. Briefly, 200 μl 0.25% gelatin (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) was added to each well, and the plates were incubated for 2 h at room temperature. Then each well of a 96-well plate was coated with 50 ml supernatants from patients with HCC or healthy donor cells overnight at 4˚C. Following washing with PBS with Tween-20 (PBST; Beijing Solarbio Science

& Technology Co., Ltd., Beijing, China), 50 μl primary anti-IL-17A antibodies were diluted by a factor of 1:100 and added to the wells. The plates were then incubated for 1 h at room temperature and washed with PBST to remove excess primary antibodies. A 50 μl volume of horseradish peroxidase (HRP) -labeled secondary antibody (rabbit anti-mouse IgG; cat. no. 61-6520; eBioscience-Thermo Fisher Scientific, Inc.) was added to the wells and plates were further incubated for 45 min at 37˚C. Excess secondary antibodies were removed and HRP enzyme activity was determined by adding o-phenylenediamine for o-phenylenediamine dihydrochloride reaction at room temperature for 20-30 min in darkness, which was terminated by adding 1 M H_2SO_4 after 10 min at room temperature. The concentration of IL-17A was calculated using CurveExpert 1.4 software (Hyams Development; https://www.curveexpert.net/).

In vitro cytotoxicity assay. The *in vitro* cytotoxicity of γδ T cells from patients with HCC following amplification was determined using an MTT assay (Sigma Aldrich; Merck KGaA). Briefly, exponentially growing target cells (HuH7, PLC and SMMC-7721 cells) were prepared at a density of $5x10^3$ cells/well and seeded in 96-well plates with γδ T cells at effector/target ratios of 0:1, 5:1, 10:1 or 20:1. HCC cells and γδ T cells were simultaneously seeded as two control groups and were incubated at 37˚C in an atmosphere containing 5% CO_2 for 48 h. Subsequently, 20 μl MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added to each well, and cells were cultured at 37˚C in incubator for an additional 4 h, and subsequently 100 μl dimethylsulfoxide (Sigma Aldrich; Merck KGaA) was added to each well. Cells were shocked for 10 min in the dark at room temperature, and the optical density (OD) of each well was determined using a microplate reader at 570 nm. The cytotoxicity was calculated according to the following formula: Cytotoxicity (%)=(control OD-experimental OD)/control ODx100%. The assay was repeated three times.

Statistical analysis. SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. Data are expressed as the mean ± standard deviation (SD). Paired or non-paired Student's t-tests were performed as appropriate. One-way analysis of variance was used to analyze the differences among three HCC cell lines at different effector/target ratios. Further comparison of the differences between two groups was performed using least-significance difference test or Student-Newman-Keuls. Univariate analyses were performed using χ^2 tests. Multivariate analyses for factors affecting the quality of amplification were performed using logarithmic regression analysis. Spearman's correlation was used to analyze the associations between α-fetoprotein (AFP) in 10% autologous plasma and the absolute numbers of γδ T cells following amplification. P<0.05 was considered to indicate a statistically significant difference.

Results

Proliferation of γδ T cells derived from patients with HCC and healthy controls. γδ T cells derived from healthy donors and patients with HCC were cultured *in vitro* in a humidified atmosphere at 37˚C. Following culture for 240 h, the γδ T cells were amplified to form a cell mass. The morphology of the cell mass from patients with HCC and healthy donors were similar (Fig. 1A-D).

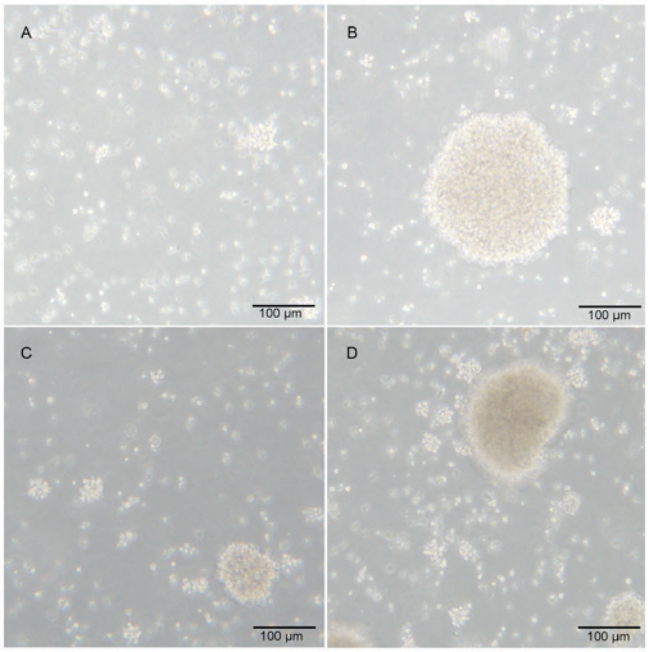

Figure 1. Proliferation of γδ T cells derived from patients with HCC and healthy donors. (A) γδ T cells derived from healthy donors cultured for 48 h. (B) γδ T cells derived from healthy donors cultured for 240 h. (C) γδ T cells derived from patients with HCC cultured for 48 h. (D) γδ T cells derived from patients with HCC cultured for 240 h. HCC, hepatocellular carcinoma.

Zoledronate and IL-2 may efficiently expand the γδ T cells from PBMCs of patients with HCC. Prior to amplification, the numbers of γδ T cells from patients with HCC and healthy donors were $(2.12±1.15)x10^4$ and $(1.78±0.91)x10^5$, respectively, and the proportion of γδ T cells out of the total number of T cells was significantly decreased in patients with HCC compared with healthy donors (3.32±1.67 vs. 5.06±1.91%, respectively; P<0.05; Fig. 2A and B). Prior to and following amplification, the proportion of Vγ9Vδ2 T cells out of the total number of γδ T cells was not significantly decreased compared with healthy donors (P>0.05; Fig. 2C and D). However, following amplification, the numbers of γδ T cells from patients with HCC and healthy donors were $(1.68±0.92)x10^7$ and $(1.05±0.65)x10^8$, respectively, and the proportion of γδ T cells out of the total number of T cells (3.32±1.67 vs. 30.27±15.25%, respectively; P<0.05) and Vγ9Vδ2 T cells out of the total number of γδ T cells (60.26±19.31% vs. 93.14±12.87%, prior to and following amplification, respectively; P<0.05) were significantly increased in patients with HCC.

In terms of phenotype, there were also significant differences in patients with HCC prior to and following amplification. Following amplification, the proportions and numbers of $T_{naïve}$ (24.88±13.17 vs. 6.52±4.43% prior to and following amplification, respectively; P<0.05) and T_{EMRA} (34.18±18.45 vs. 13.38±5.81% prior to and following amplification, respectively; P<0.05) cells were significantly decreased. The proportion of T_{EM} cells was significantly increased following amplification

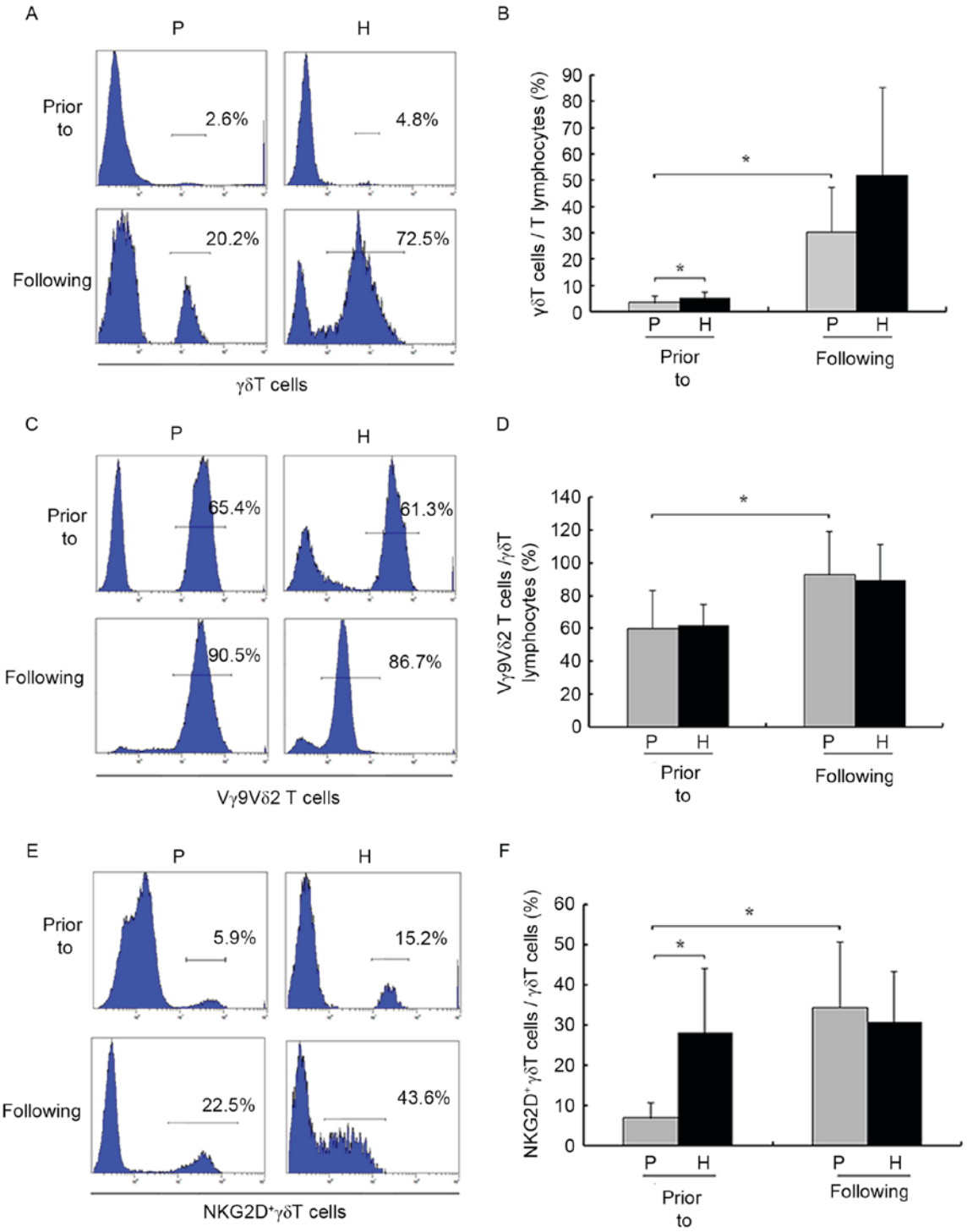

Figure 2. Proportions of γδ T cells, Vγ9Vδ2 T cells and NKG2D$^+$ γδ T cells in patients with HCC following amplification. (A) The proportions of γδ T cells out of the total number of T cells in peripheral blood samples of patients with HCC and healthy donors prior to and following amplification, and (B) quantification. (C) The proportions of Vγ9Vδ2 T cells out of the total number of γδ T cells prior to and following amplification and (D) quantification. (E) The proportions of NKG2D$^+$ γδ T cells prior to and following amplification and (F) quantification. Results are expressed as the mean ± standard deviation. *P<0.05 with comparisons indicated by lines. NKG2D, natural killer receptor group 2, member D; P, patients; H, healthy controls.

(6.76±4.07 vs. 63.16±11.16% prior to and following amplification, respectively; P<0.05). As presented in Fig. 2E and F, prior to amplification, γδ T cells were generally positive for NKG2D in healthy donors (6.93±2.89 vs. 27.93±13.48% for patients and healthy donors, respectively; P<0.05). Following amplification, numbers of NKG2D$^+$ γδ T cells were significantly increased compared with healthy donors.

Amplification capacity of γδ T cells is correlated with the clinicopathological characteristics of patients. Notably, γδ T cells from all patients did not expand equally as well. Therefore, the aim of the present study was to elucidate the factors underlying this phenomenon. The results of the univariate analysis, presented in Table I, demonstrate that the quality of amplification was significantly associated

Table I. Univariate analyses of the quality of amplification associated with clinicopathological characteristics and a number of suppressive factors.

Clinicopathological characteristic	High n (%)	Low n (%)	χ^2	P-value
Sex			0.232	0.656
Male	15 (44.4)	28 (53.8)		
Female	16 (51.6)	24 (46.2)		
BCLC stage			22.270	<0.001
A	24 (77.4)	13 (25.0)		
B	5 (16.1)	19 (36.5)		
C	2 (6.2)	20 (38.5)		
Tumor size, cm			7.574	0.007
>5	10 (32.3)	33 (63.5)		
≤5	21 (67.7)	19 (36.5)		
Tumor number			4.310	0.044
1	21 (67.7)	23 (44.2)		
≥2	10 (32.3)	29 (55.8)		
DOD, months			16.929	<0.001
≥20	24 (77.4)	16 (30.8)		
<20	7 (22.6)	36 (69.2)		
TBIL, μmol/l			0.361	0.646
≥17.1	17 (54.8)	32 (61.5)		
<17.1	14 (45.2)	20 (38.5)		
AFP, ng/ml			19.136	<0.001
≤20	23 (74.2)	13 (25.0)		
>20	8 (25.8)	39 (75.0)		
Albumin, g/l			3.832	0.041
≥55	20 (64.5)	22 (42.3)		
<55	11 (35.5)	30 (57.7)		
Ascites			0.066	0.824
Yes	17 (54.8)	27 (51.9)		
No	14 (45.2)	25 (48.1)		
TACE			1.745	0.263
Yes	4 (12.9)	13 (25.0)		
No	27 (87.1)	39 (75.0)		
ALT, U/l			0.148	0.819
≥40	15 (48.4)	22 (42.3)		
<40	16 (51.6)	30 (57.7)		
AST, U/l			0.086	0.819
≥40	12 (38.7)	21 (40.4)		
<40	19 (61.3)	31 (59.6)		
PT, sec			0.001	0.998
≥14	15 (48.4)	24 (46.2)		
<14	16 (51.6)	28 (53.8)		
Tregs, %			17.566	<0.001
<0.91±0.54	23 (74.2)	14 (26.9)		
≥0.91±0.54	8 (25.8)	38 (73.1)		
γδ T17 cells, %			7.961	0.006
<0.68±0.17	20 (64.5)	17 (32.7)		
≥0.68±0.17	11 (35.5)	35 (67.3)		
Age, years			0.021	0.989
<40	1 (3.2)	2 (3.8)		
40-55	12 (38.7)	20 (38.5)		
55<	18 (58.1)	30 (57.7)		

BCLC, Barcelona-Clinic Liver Cancer; DOD, duration of disease; TBIL, totalbilirubin; AFP, α-fetoprotein; TACE, transarterial chemoembolization; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PT, prothrombin time; Tregs, regulatory T cells.

Table II. Multivariate analyses of the quality of amplification associated with clinicopathological characteristics and a number of suppressive factors.

Variables	P-value	OR	95% confidence interval Lower	Upper
AFP	0.041	3.734	1.112	15.801
DOD	0.030	0.041	0.002	0.729
Tregs	0.006	4.808	2.915	17.357
γδ T17 cells	0.023	2.479	1.415	11.089

AFP, α-fetoprotein; DOD, duration of disease; Tregs, regulatory T cells; OR, odds ratio.

with clinical stage, levels of AFP and albumin, duration of disease (DOD), size and number of tumors, numbers of Tregs and γδ T17 cells and levels of IL-17A. The results of the multivariate analysis revealed that the levels of AFP and the proportions of Tregs and γδ T17 cells were independent factors associated with low-quality amplification, whereas DOD was an independent factor associated with high-quality amplification (Table II). There was no correlation between AFP in 10% autologous plasma and the amplification ability of γδ T cells (r_s=-0.396; P=0.379), indicating that exogenous AFP did not affect the amplification of γδ T cells *in vitro*.

These data indicated that amplification with zoledronate and IL-2 may increase the proportion of γδ T cells and promote the effective phenotype. However, the amplification ability was not the same in all patients, varying depending on the clinicopathological characteristics of the patients with HCC and the presence of specific suppressive factors.

Secretion and cytotoxic activity of γδ T cells. Prior to amplification, the proportion of perforin$^+$ γδ T cells in patients with HCC was not significantly decreased compared with healthy donors (P>0.05; Fig. 3A and B). However, following

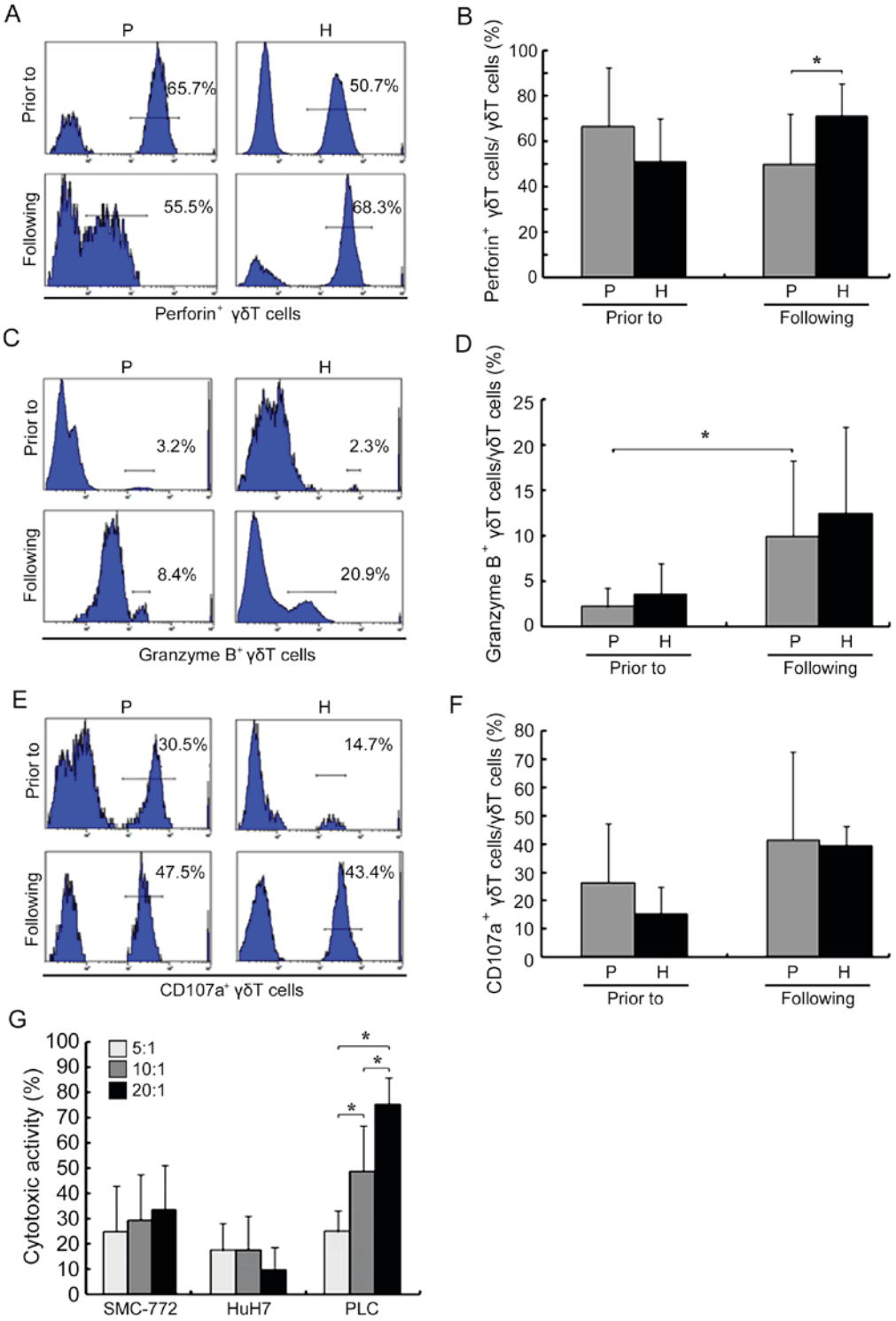

Figure 3. Proportions of perforin+, granzyme B+ and CD107a+ γδ T cells following amplification. (A) The proportions of perforin+ γδ T cells in the peripheral blood of patients with HCC and healthy donors prior to and following amplification and (B) quantification. (C) The proportions of granzyme B+ γδ T cells prior to and following amplification and (D) quantification. (E and F) The proportions of CD107a+ γδ T cells prior to and following amplification. (G) The cytotoxic activity of γδ T cells against four HCC cell lines was assessed using an MTT assay. Results are expressed as the mean ± standard deviation. *P<0.05 with comparisons indicated by lines. HCC, hepatocellular carcinoma; P, patients; H, healthy controls; CD, cluster of differentiation.

amplification, the proportion in patients with HCC decreased (66.61±20.87 vs. 49.97±15.97% prior to and following amplification, respectively; P<0.05), becoming significantly lower when compared with healthy donors (71.25±14.06%; P<0.05). The proportion of granzyme B+ γδ T cells in patients with HCC significantly increased following amplification (2.17±1.62 vs. 9.96±6.22% prior to and following amplification, respectively; P<0.05; Fig. 3C and D); however, there was no significant difference when compared with that in healthy donors (P>0.05). Amplification also did not significantly affect CD107a (26.41±15.66 vs. 41.52±26.17% prior to and following amplification, respectively; P>0.05)

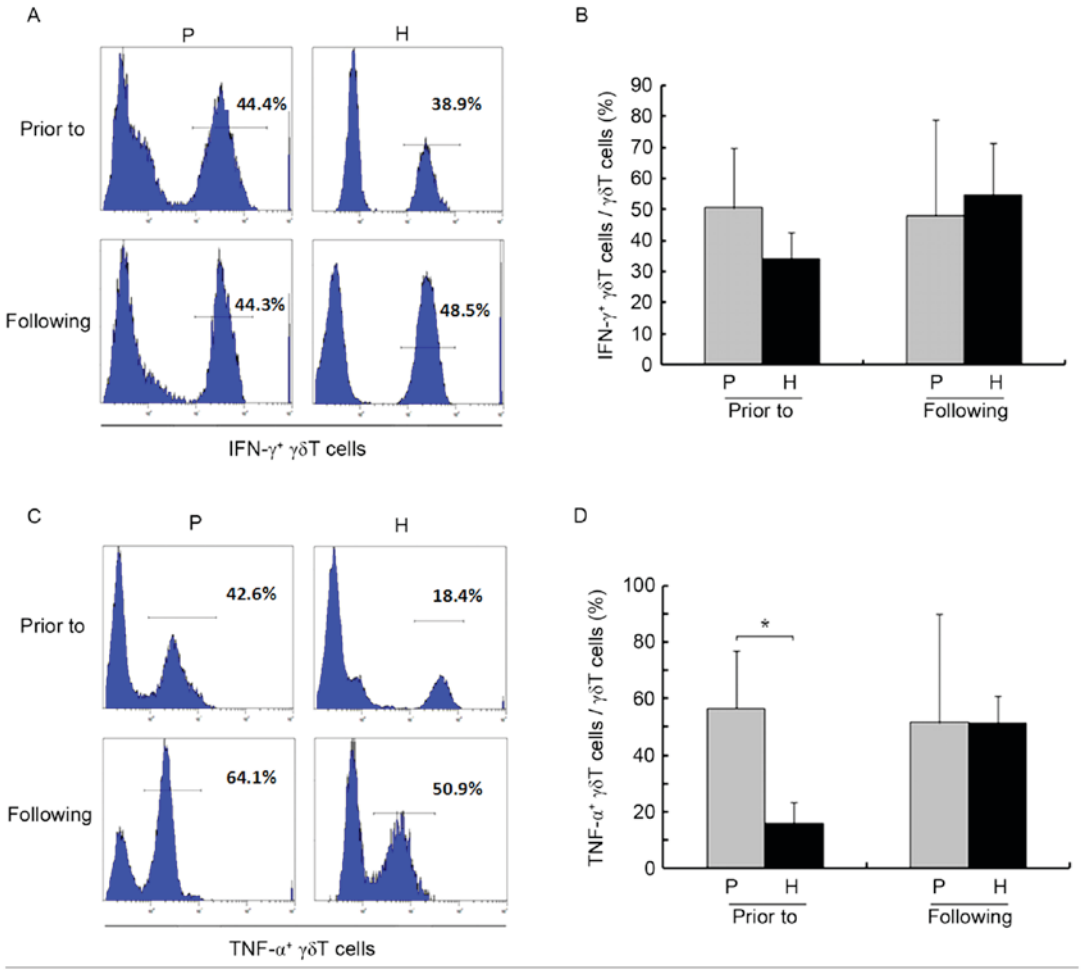

Figure 4. Proportions of IFN-γ+ and TNF-α+ γδ T cells following amplification. (A) The proportions of IFN-γ+ γδ T cells in peripheral blood of patients with HCC and healthy donors prior to and following amplification and (B) quantification. (C) The proportions of TNF-α+ γδ T cells prior to and following amplification and (D) quantification. Results are expressed as the mean ± standard deviation. *P<0.05 with comparisons indicated by lines. IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; P, patients; H, healthy controls.

and IFN-γ (50.61±15.25 vs. 48.07±25.10% prior to and following amplification, respectively; P>0.05; Figs. 3E and F, 4A and B). In addition, prior to amplification, the proportion of TNF-α+ γδ T cells was higher in patients compared with healthy controls (56.70±16.43 vs. 15.74±5.71%, respectively; P<0.05; Fig. 4C and D), and amplification had almost no effect on this parameter (56.70±16.43 vs. 51.62±30.67% prior to and following amplification, respectively; P>0.05).

The results of the MTT assay, as presented in Fig. 3G, revealed that γδ T cells exerted significant cytotoxic effects on four HCC cell lines at differing effector/target ratios. In addition, for the PLC cells, cytotoxicity was significantly increased when the effector/target ratio was increased.

γδ T17 cells, Tregs and IL-17A were not altered during amplification. Immunosuppressive cells and factors were examined during amplification. Amplification had almost no effect on the levels of Tregs and γδ T17 cells (P>0.05; Fig. 5A-D). The levels of IL-17A in the supernatants were assessed using an ELISA. As presented in Fig. 5E, the levels were not significantly altered on days 3, 7, 10 and 14 (P>0.05).

Discussion

Previously, numerous immunotherapeutic methods have been developed in an attempt to induce tumor-specific adaptive immune responses. Adaptive immunotherapy with γδ T cells represents a novel, safe and effective approach to inducing immunological and clinical responses (22-24). However, few studies have examined these parameters in HCC. In the present study, it was concluded that circulating γδ T cells in patients with HCC expanded by the use of zoledronate and IL-2 *in vitro*, and may lyse HCC cells effectively, without increasing immunosuppressive factors during amplification. Additionally, the amplification ability of γδ T cells was associated with the clinicopathological features of patients with HCC.

A γδ T-cell proliferation of at ≥70% was considered the threshold for therapy (25). A real-time cell analyzer may be used for monitoring the absolute cell numbers and cytotoxicity of circulating γδ T cells from patients with cancer, in order to provide a more comprehensive assessment for personalized tumor treatment (26). In the present study, the absolute numbers and proportion of γδ T cells in patients with HCC increased significantly following amplification; however, this was not consistently

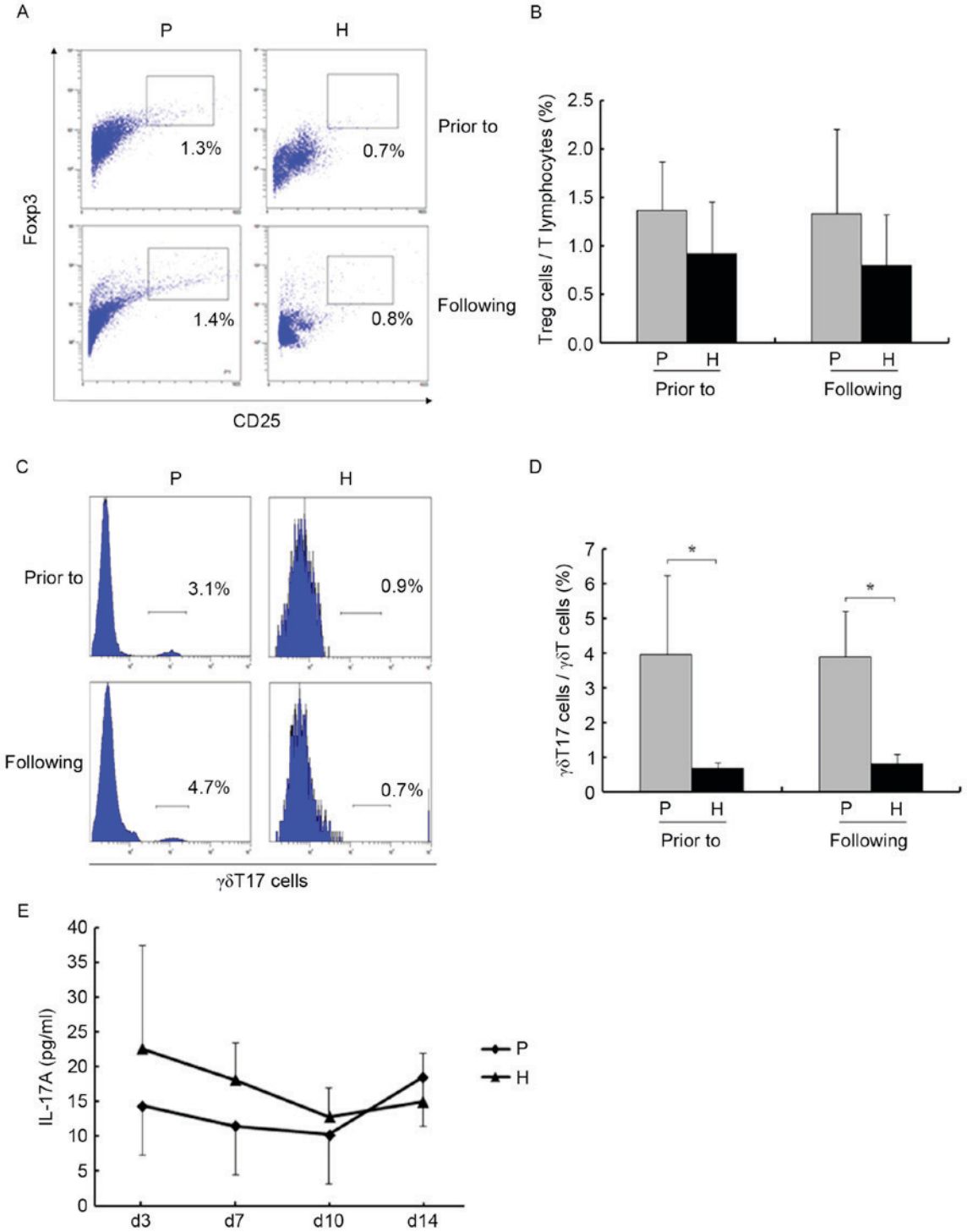

Figure 5. Proportions of Tregs and γδ T17 cells did not change significantly following amplification. (A) The proportion of Tregs in the peripheral blood of patients with HCC and healthy donors prior to and following amplification and (B) quantification. (C) The proportion of γδ T17 cells in the peripheral blood of patients with HCC and healthy donors prior to and following amplification and (D) quantification. (E) The levels of supernatant IL-17A on days 3, 7, 10 and 14. Results are expressed as the mean ± standard deviation. *P<0.05 with comparisons indicated by lines. HCC, hepatocellular carcinoma; Foxp3, forkhead box 3; CD, cluster of differentiation; Treg, regulatory T cells; IL, interleukin; P, patients; H, healthy controls.

observed in all patients, and this effect may be associated with various clinicopathological characteristics and suppressive factors. It was revealed that the quality of amplification was negatively associated with the serum AFP level, proportion of γδ T17 cells and proportion of Tregs, but positively associated with the DOD. These results suggested that optimized immunotherapy of γδ T cells in patients with HCC should be individualized.

In order to further explore the feasibility and efficacy of immunotherapy, the phenotype, secretion and cytotoxicity of Vγ9Vδ2 T cells were examined. Encouragingly, the results of the present study suggested that there was substantial differentiation of Vγ9Vδ2 T cells towards the effective phenotype of secretion and lysis following amplification, which was consistent with other studies (24,27). Previous studies have

revealed that activated Vγ9Vδ2 T cells are a primary source of IFN-γ and TNF-α, which have direct cytotoxic activity against tumor cells and indirect cytotoxic activity via the stimulation of macrophages and dendritic cells (28-30). In the present study, although the proportions of IFN-γ^+ and TNF-α^+ γδ T cells were not significantly altered, the absolute numbers and proportions of T_{EM} cells were significantly increased following amplification. It was revealed that the secretion of γδ T cells was increased following amplification. Collectively, the results of the present study revealed that the cytotoxic activity of γδ T cells was also increased following amplification.

Immunosuppressive factors are the main obstacles for the anticancer immunity effects of γδ T cells *in vivo*. The accumulation of Tregs in a number of tumors mediate tumor-promoting effects through the suppression of antitumor immunity (31). Furthermore, IL-17A has been revealed to promote metastasis and is associated with a poor prognosis in patients with HCC (32). Immunosuppressive cells and factors should not be expanded during the amplification of effective cells. To the best of our knowledge, the present study is the first to explore the changes in Tregs, γδ T17 cells and IL-17A during the amplification of circulating γδ T cells in patients with HCC *in vitro*. The results of the present study revealed that these immunosuppressive cells and factors were not increased following amplification, which suggested that γδ T cells expanded by zoledronate and IL-2 *in vitro* may be safe for immunotherapy in patients with HCC.

A number of studies have demonstrated that Tregs express immune checkpoint proteins, including programmed cell death-1 (PD-1) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) (33,34), and impair the function of HCC-infiltrating γδ T cells (18). Additionally, activated T cells upregulate CTLA-4 and PD-1, which act to increase T-cell responses, and antibody blockade of immune checkpoints enhances T-cell responses (35). Adoptive γδ T-cell immunotherapy combined with checkpoint inhibitors may be a promising therapeutic strategy for the treatment of HCC.

In summary, circulating γδ T cells from patients with HCC expanded using zoledronate and IL-2 *in vitro* may be used for immunotherapy in patients with HCC without increasing immunosuppressive factors. However, this immunotherapy should be individualized according to the specific clinicopathological features of the patients.

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