Am J Cancer Res 2022;12(5):2203-2215
www.ajcr.us /ISSN:2156-6976/ajcr0142704

Original Article

Expansion of $CD3^+CD8^+PD1^+$ T lymphocytes and TCR repertoire diversity predict clinical responses to adoptive cell therapy in advanced gastric cancer

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Received March 5, 2022; Accepted April 19, 2022; Epub May 15, 2022; Published May 30, 2022

Abstract: The adoptive cell therapy (ACT) and delivery of ex vivo activated cellular products, such as dendritic cells (DCs), NK cells, and T cells, have shown promise for the treatment of gastric cancer (GC). However, it is unknown which cells can improve patient survival. This study was focused on the antitumour activity of a subset of these cellular products and their relationships with clinical outcomes. Nineteen patients were enrolled at the Capital Medical University Cancer Center, Beijing Shijitan Hospital, from June 1, 2013, to May 30, 2016. $CD8^+PD1^+$ T-cell sorting was carried out using flow cytometry, and the T-cell receptor (TCR) repertoire during ex vivo expansion for 15 days was analyzed by next-generation sequencing. After 15 days of culture, the number of $CD8^+$ T cells had increased significantly, and the number of $CD4^+$ T cells had increased correspondingly. After ex vivo expansion, $CD8^+$ T cells exhibited significantly enhanced expression of PD-1, LAG-3, and TIM-3 but not 4-1BB. Survival analysis showed that patients with a pro/pre value of $CD8^+PD\text{-}1^+$ T cells >2.4 had significantly favorable overall survival (OS) (median OS time, 248 days versus 96 days, P=0.02) and progression-free survival (PFS) (median PFS time, 183 days vs. 77 days, P=0.002). The sorted $CD8^+PD\text{-}1^+$ T cells displayed enhanced antitumor activity and increased IFN-γ secretion after coculture with autologous tumor cell lines. TCR repertoire diversity was decreased after ex vivo expansion, which decreased the Shannon index and increased the clonality value. The prognosis of patients was significantly improved and was associated with the extent of $CD8^+PD\text{-}1^+$ T-cell expansion. In summary, this study showed that after ex vivo expansion for 15 days, $CD8^+PD\text{-}1^+$ T cells could be identified as tumor-reactive cells in patients treated for GC. Changing TCR species can predict the extent of $CD3^+CD8^+PD1^+$ T-cell growth and the effect of ACT treatment.

Keywords: $CD8^+PD\text{-}1^+$ T cell, T-cell receptor repertoire, advanced gastric cancer

Introduction

Gastric cancer (GC), the third leading cause of cancer mortality worldwide, with more than 800,000 deaths annually, has its greatest incidence in east Asia [1]. Unfortunately, the majority of newly diagnosed cases are in the advanced stage [2], for which outcomes are extremely poor, with median survival ranging from 3 to 5 months with the best supportive care [3, 4]. Our study revealed that treatment with dendritic cell-cytokine-induced killer cell (DC-CIK) therapy combined with chemotherapy alone can significantly prolong progression-free survival (PFS) and overall survival (OS), which proves that DC-CIK therapy can play a beneficial role in the treatment of GC. Adoptive cell therapy (ACT) and delivery of ex vivo activated

cellular products, such as dendritic cells (DCs), NK cells, or T cells, have shown activity against various cancers [5, 6]. However, it is unknown which cells can improve patient survival.

$CD4^+$ and $CD8^+$ T lymphocytes play decisive roles in antitumor therapy [7-9]. $CD4^+$ T cells can enhance the immune response by secreting cytokines [10], while $CD8^+$ T cells kill tumor cells directly by releasing granzymes [11]. However, many molecular signaling pathways on the surface of $CD4^+$ and $CD8^+$ T cells play different biological roles when binding to tumors. Some of them, such as CD3 and CD28, can promote lymphocyte expansion and enhance the killing effect, while others, such as CTLA-4 and PD1, can inhibit the immune response [12]. In normal tissues, the inhibitory signaling pathway prevents lymphocytes from killing normal cells and protects normal cells, but in tumors, it inhibits the killing of tumor cells. Although many studies have reported that the survival time of patients is reduced when lymphocytes with high PD1 expression are increased [13, 14], Rosenberg recently found that in vitro, $CD8^+$ and $PD1^+$ T lymphocytes had a much stronger killing effect than $CD8^+PD1^-$ T lymphocytes on tumor-infiltrating lymphocytes (TILs) [15]. The main reason for the difference between the in vitro and in vivo experiments is that the combination of PD1 and PDL1 leads to the activation of inhibitory pathways downstream of PD1 [16]. In fact, lymphocytes with high PD1 expression had a better killing effect than lymphocytes with low PD1 expression. When pancreatic cancer was cured with DC-CIK therapy, we also showed that more $CD8^+PD1^+$ T lymphocytes obtained through cultivation could significantly increase the survival of patients upon transfusion. Therefore, compared with pancreatic cancer, GC has a higher frequency of gene mutations and is more suitable for DC-CIK therapy with immunotherapy [17, 18]. We hypothesized that transfusing ACT with more $CD8^+PD1^+$ T lymphocytes would have a better therapeutic effect.

The diversity of the T-cell receptor (TCR) repertoire correlates with the presence of tumor neoantigen-specific T cells. Moreover, the tumor antigen-specific TCR repertoires from the circulating blood and those of tumor-infiltrating $CD8^+PD1^+$ cells are highly similar [19]. At present, our clinical center has integrated TCR sequencing with effective tumor therapy to evaluate patient condition [20, 21]. The TCR is produced after the formation of the TCR β chain, which guides the differentiation of pre-T cells into functional $CD4^+$ and $CD8^+$ T cells [22, 23]. When a tumor is formed, many new antigens are produced by gene mutations, which can stimulate T cells with TCRs that recognize these neoantigens to amplify themselves and kill tumor cells. By sequencing the V(D)J region in the TCR ecosystem, we can indirectly reveal the immune microenvironment around the tumor [20]. Then, after the culture of peripheral blood mononuclear cells (PBMCs) in vitro, by comparing changes in the TCR mutation frequency of lymphocytes before and after culture and evaluating patient survival time, we can better understand whether T cells targeted at killing tumor cells are sensitive to our culture methods, that is, whether T cells targeting tumor antigens are expanded after in vitro culture. If so, such a result would expand the advantages of ACT.

To determine the role of $CD8^+PD1^+$ T cells in the benefit of ACT to patients with GC, we performed this study to evaluate whether these cells expand in the ACT product and whether they are impaired or exhausted or retain immune function. Specifically, we analyzed the fold expansion capacity of $CD8^+PD1^+$ T cells and the TCR repertoire for predicting clinical outcome in patients with GC treated with ACT.

Methods

Source of clinical specimens

The study was approved by the Regional Ethical Review Board for Capital Medical University Cancer Center. Patients were treated according to the Declaration of Helsinki's ethical principles for medical research involving human subjects. All patients provided informed written consent prior to study enrollment. Specimens (peripheral blood, tumor cell lines and DC-CIK products) from 19 GC patients of similar stage and clinical characteristics who had been enrolled in previous studies evaluating DC-CIK therapy were chosen for analysis.

Generation of autologous tumor cell lines

Autologous tumor cell lines were established from tumor specimens of $HLA\text{-}A2^+$ patients

from Capital Medical University Cancer Center. Briefly, resected tumor fragments were enzymatically digested overnight with 0.1% collagenase type IV, 0.01% hyaluronidase type V, and 30 U/mL deoxyribonuclease I type IV in RPMI 1640. The disaggregated tumor cells were cultured in RPMI 1640 containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamicin (Life Technologies) to prepare the cell suspension for the T-cell cytotoxicity assay.

Preparation of the DC-CIK product

The DC-CIK product was generated in vitro as described previously [24]. Peripheral blood stem cells were mobilized by injection of GM-CSF 5 mcg/kg per day (Chugai Pharm Co. Ltd., Japan) until the level of mononuclear cells in peripheral blood reached 1.5×10^9/L. Then, the PBMCs were separated by a COBE Spectra cell separator (COBE BCT, Lakewood, CO, USA) until the CD34$^+$ count reached a threshold of 4.5×10^6/kg. All collections were frozen at -80°C until further analysis. Between 30-50 mL of thawed apheresis product was cocultured for 7 days with IL-4 (1,000 U/mL; R&D Systems, Inc., Minneapolis, MN), TNF-α (20 ng/mL; R&D Systems, Inc., Minneapolis, MN) and GM-CSF (800 U/mL; Amoytop Biotech Co., Ltd., Xiamen, China) to generate autologous DCs. The PBMCs were activated and expanded to produce cytokine-induced killer cells (CIKs) in complete medium consisting of AIM-V supplemented with 10% heat-inactivated human AB serum and the recombinant cytokines IL-2 (at 2,000 U/mL; Boehringer Mannheim, Germany) and IFN-γ (at 1,000 U/mL; Boehringer Mannheim, Germany) and anti-CD3 antibody (at 1.7 mL/mL; Boehringer Mannheim, Germany). Subsequently, half of the medium was replaced with fresh AIM-V medium containing IL-2 (2,000 IU/mL) every other day. After 7-10 days, the autologous DCs were mixed with cultured CIKs at a ratio of 1:100 for 7 days, and the cocultured dendritic cell-cytotoxic T lymphocytes (DC-CTLs) were then harvested and infused intravenously into patients.

Generation of CD8$^+$PD-1$^+$ T cells

CD8$^+$PD1$^+$ T cells were expanded from apheresis products. Cell counts were recorded daily from day 0 to day 15. The sorted CD8$^+$PD1$^+$ T cells were incubated for antitumor activity assays in vitro. The TCR repertoire of cultured cells was analyzed at day 0 and day 15 to determine the association with the peripheral blood lymphocyte phenotype after DC-CIK infusion and the subsequent clinical response.

Flow cytometric analysis and sorting

We used the following fluorochrome-conjugated antibodies: CD3 PerCP-Cy5.5, CD4 FITC, CD8 FITC, CD25 PE, CD28 PE, and CD56 PE (Beckman) and PD-1 PE, LAG-3 PE, 4-1BB PE, and TIM-3 PeCy-7 (Biolegend). We detected the cell subpopulation of PBMCs prior to culture and within cultured CIKs by flow cytometric analysis as described previously [24]. Briefly, cells were resuspended in staining buffer and then stained with primary antibody at 4°C for 30 min in the dark. Stained cells were centrifuged for 10 min at 1,500 rpm at room temperature and subsequently washed in staining buffer twice prior to FACS analysis. Three-color flow cytometric analysis was run to determine cell phenotypes using Cytomics FC500 and CXP analysis software (Beckman-Coulter, USA). CD8$^+$PD1$^+$ T-cell sorting was carried out using a MoFlo Astrios EQ instrument (Beckman, USA). First, CD8$^+$ T cells were enriched using CD8 microbeads (Biolegend) and then incubated with FITC-conjugated CD8, PE-Cy5.5-conjugated CD3, and PE-conjugated PD-1 at 4°C for 30 min. Cell sorting was based on the gating strategy (PI$^-$, CD3$^+$, CD8$^+$ and PD1$^+$). The sorted populations were expanded to detect their reactivity on days 13-15 with irradiated allogeneic feeder cells (5,000 rad) pooled from three donors in T-cell medium supplemented with 10% human AB serum, anti-CD3 and IL-2 (2,000 IU/mL) (Boehringer Mannheim, Germany).

Assessment of tumor recognition and cytotoxicity assay

An IFN-γ enzyme-linked immunospot (ELISPOT) assay was used to measure the recognition of targets. After 15 days of in vitro culture in cell medium supplemented with 2,000 IU/mL IL-2 at 37°C in 5% CO_2, cultured T cells were washed and then cocultured either alone or with HLA-A2$^+$ target tumor cells. In the ELISPOT assays, effector cells (1×10^5) were added to target cell lines (1×10^4) at an effector/target (E/T) ratio of 10:1 per well in a 96-well plate and incubated for 24 h according to the manufacturer's

Table 1. Demographics and baseline characteristics of patients

Variable	Median/Number
case,n	19
age	67±5.1
Sex	
Female	5
Male	14
ECOG-PS	
1	4
2	15
TNM staging	
III	6
IV	13
Site of metastasis	
liver	9
lung	3
peritoneum	2
other	0

instructions. The raw data were analyzed and plotted using CTL Immunospot software (Cellular Technology Limited, USA). The identification of greater than 40 spots and two backgrounds was required to report positive T-cell reactivity.

A Cell Counting Kit-8 (CCK-8) assay was used to detect cytolytic activity. Target cells were plated with effector cells at various E/T ratios (6.25:1, 12.5:1, 25:1 and 50:1) in 96-well U-bottomed plates and incubated for 24 h at 37°C. The supernatants were harvested for absorbance measurement in a microplate reader at 450 nm.

TCR sequencing

DNA was extracted from ex vivo expanded T cells using a Qiagen DNA FFPE kit, DNA blood kit, or DNA blood mini kit (Qiagen). TCR V and CDR3 sequencing was performed using survey-resolution (cultured cells) or deep-resolution (PBMCs) ImmunoSEQ platforms. Bioinformatic and biostatistical analyses of productive clones were performed to assess the dynamics of expanded T cells. The TCR V and CDR3 sequence diversity at day 15 of expansion was compared to the initial TCR diversity.

Phenotypic analysis of peripheral blood immune cells

Peripheral venous blood was obtained from each patient at various time points after DC-CIK infusion. Whole blood (100 μl) was incubated in the dark with primary antibody at 4°C for 15 min. Anti-CD3-FITC/anti-CD56-RPE (Dako), anti-CD3-FITC (fluorescein isothiocyanate), anti-CD4-RPE, anti-CD8-RPE, anti-CD45RO, and anti-CD4-FITC/anti-CD25-PE (BD Bioscience) were used. After hemolysis for 10 min, the samples were centrifuged at 1,500 rpm for 10 min at room temperature, washed twice in PBS and subjected to flow cytometric analysis. Three-color flow cytometric analysis was performed to determine cell phenotypes using an FC500 instrument (Beckman-Coulter) and CXP analysis software (Beckman-Coulter). Lymphocytes were gated by forward scatter versus side scatter. Analysis was set to collect 5,000 gated events.

Statistical methods

Continuous variables were expressed as the means ± standard deviation (SD) and compared using two-tailed unpaired Student's t tests; categorical variables were compared using χ^2 or Fisher's tests. Life-table estimates of survival time were calculated for the evaluation of PFS and OS as the primary endpoints according to the Kaplan-Meier methodology [25]. Receiver operating characteristic (ROC) curves were used to determine the optimal cut-off values of post/pre $CD8^+PD\text{-}1^+$, $CD8^+LAG\text{-}3^+$, and $CD8^+TIM\text{-}3^+$ T cells; post/pre Shannon index; clonality; evenness; and post/pre TCR subclones. All statistical evaluations were carried out using SPSS software (Statistical Package for the Social Sciences, version 15.0, SPSS Inc., Chicago, IL) and GraphPad Prism 5 (Version 5.01, GraphPad Software, Inc., USA). A value of $P<0.05$ was considered statistically significant in all the analyses.

Results

Patient characteristics and phenotypic analysis of peripheral blood T lymphocyte subtypes after ex vivo expansion

Nineteen patients were enrolled in this study at the Capital Medical University Cancer Center, Beijing Shijitan Hospital from June 1, 2013, to May 30, 2016. The characteristics of all patients are detailed in **Table 1**. Mononuclear cells were harvested from peripheral blood and expanded ex vivo. The total number of T cells increased throughout the culture period until

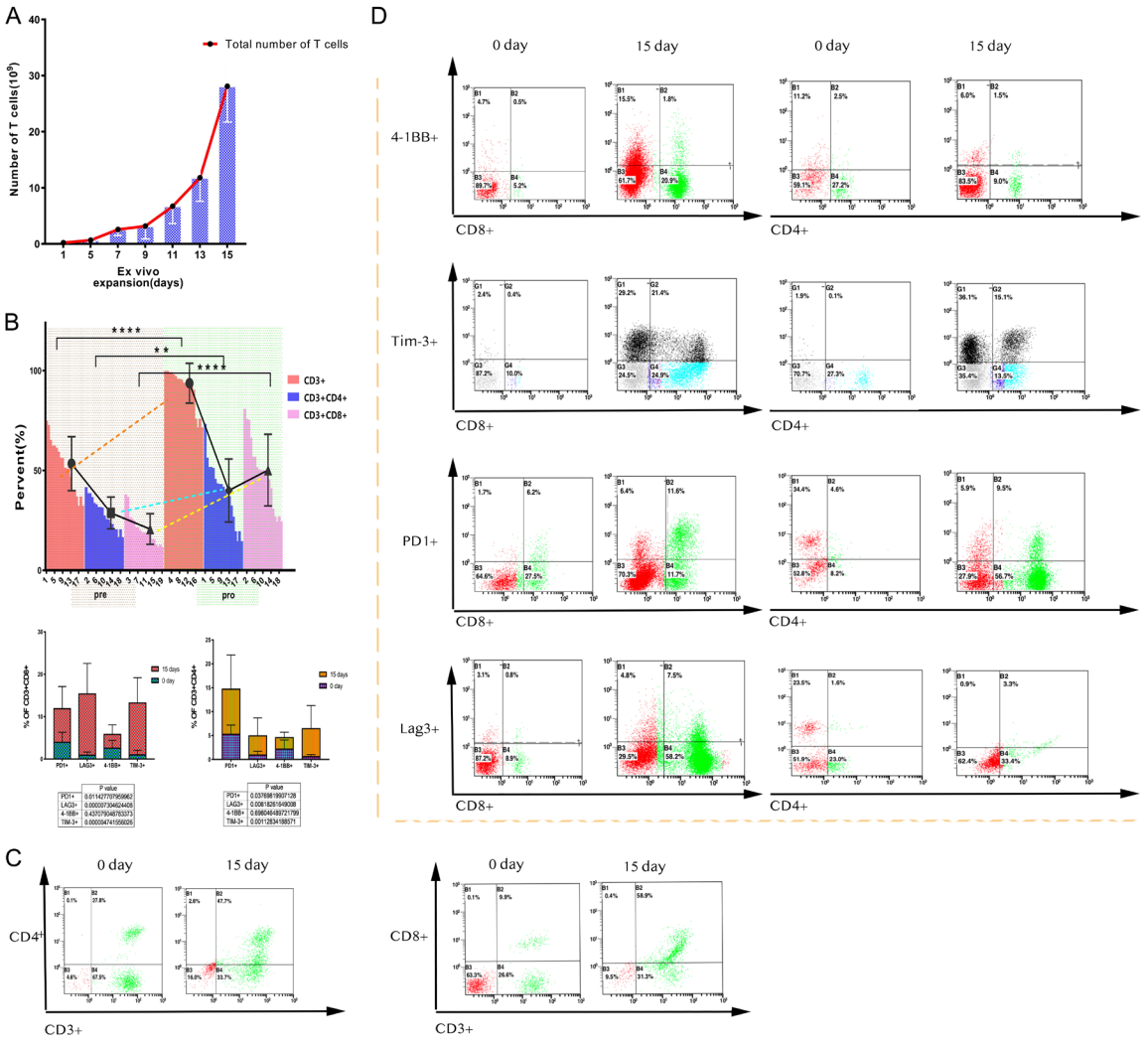

Figure 1. PBMCs exhibit unique phenotypic traits from their baseline characteristics after ex vivo expansion. A: The total number of T cells was increased after 15 days compared with 0 days. B: The numbers of $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes had increased significantly by day 15 compared with day 0. After 15 days of culture, the number of $CD8^+$ cells was higher than the number of $CD4^+$ cells (mean ± SEM). $CD8^+$ T cells and $CD4^+$ T cells exhibited enhanced expression of PD-1, LAG-3, and TIM-3 but not 4-1BB after ex vivo expansion. C: Coexpression patterns of CD3 and CD4 and of CD3 and CD8 before and after ex vivo expansion for 15 days in GC patients. D: Coexpression patterns of LAG-3, PD-1, 4-1BB and TIM-3 on $CD8^+$ and $CD4^+$ PBMCs before and after ex vivo expansion for 15 days in a representative patient. *P<0.05, **P<0.01, ***P<0.001, Mann-Whitney test.

day 15. The total number of T cells was 2.57+ 1.06*10^9 after 7 days and 28.1+6.38*10^9 after 15 days (**Figure 1A**). In different lymphoid subpopulations, we found that the $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes had increased significantly by day 15 compared with those at day 0 (**Figure 1B**, pro vs. pre $CD3^+CD4^+$ T cells, P<0.01; pro vs. pre $CD3^+CD8^+$ T cells, P<0.0001). After 15 days of culture, the number of $CD8^+$ T cells was higher than the number of $CD4^+$ T cells; on day 0, the opposite pattern was observed. The expression of some receptors in inhibitory molecular pathways, such as PD-1, LAG-3, TIM-3 and costimulatory receptor 4-1BB, was measured on T-cell subsets (**Figure 1B**). $CD8^+$ T cells and $CD4^+$ T cells exhibited enhanced expression of PD-1, LAG-3, and TIM-3 but not 4-1BB after ex vivo expansion. TIM-3 was the receptor most overexpressed by $CD8^+$ T cells after expansion for 15 days compared with the baseline expression, followed by LAG-3 and PD-1 (**Figure 1B**).

CD8+PD1+ T cells play a major therapeutic role in the treatment of GC after 15 days of culture

We found that after 15 days of culture, the number of CD8+ T cells had increased significantly and the number of CD4+ T cells had increased correspondingly. By comparing the results of lymphoid subsets before and after culture with the changes in patient survival after transfusion, we were able to determine which specific cells play key roles. Pro/pre >2.4, pro/pre >4.3 and pro/pre >6.1 were the cutoff values for CD8+PD-1+, CD8+LAG-3+, and CD8+ TIM-3+, respectively, and pro/pre >1.8, pro/pre >2.3 and pro/pre >2.1 were the cutoff values for CD4+PD-1+, CD4+LAG-3+ and CD4+TIM-3+, respectively. Survival analysis showed that patients with pro/pre CD8+PD-1+ T cells >2.4 had significantly favorable OS (median OS time, 248 days versus 96 days, P=0.02, **Figure 2A**) and PFS (median PFS time, 183 days vs. 77 days, P=0.002, **Figure 2B**). However, other lymphoid subsets had no effect on PFS or OS after 15 days of culture (**Figure 2C-L**, P>0.05). Moreover, we observed that the greater the number of CD3+CD8+PD1+ T cells, the better the tumor treatment effect, which increased patient PFS and OS.

CD8+PD1+ T cells have a strong antitumor effect

To investigate whether the CD8+PD-1+ T cells of PBMCs represented the tumor-reactive population, we isolated CD8+ and CD8+PD-1+ T cells from patient PBMCs; expanded them in vitro for 15 days with IL-2, anti-CD3 stimulation, and irradiated feeders; and tested their ability to recognize autologous tumor cell lines by IFNγ-ELISPOT using HLA-A2+ tumor cell lines as target cells. Notably, CD8+PD-1+ T cells, but not CD8+PD-1- T cells, included tumor-reactive cells, as determined by IFN-γ secretion and 4-1BB upregulation after coculture with the autologous tumor cell line (**Figure 3B**). Moreover, after expansion for 15 days, IFN-γ secretion and 4-1BB upregulation were enhanced (**Figure 3B**). Additionally, CD8+PD-1+ T cells were capable of lysing the HLA-A2+-matched tumor cell lines (**Figure 3A**). These findings indicate that PD-1 expression identifies tumor-reactive PBMC-derived CD8+ T cells. Thus, the expression of the inhibitory receptor PD-1 after ex vivo expansion could be used to prospectively identify and select a patient-specific repertoire of CD8+ tumor-reactive T cells.

The decrease in TCR diversity in immune cells after 15 days of culture may affect clinical outcomes

Through the above experiments, we learned that an increase in the number of CD8+PD1+ T cells after culture could increase the clinical therapeutic effect. Neoantigens can stimulate T-cell activation of the corresponding TCR receptor, and we can reveal the diversity of TCRs through TCR sequencing of the Vβ ecological region. Regarding the estimation of TCR diversity based on the Shannon index, the more species there are, the higher the index value and the clonality on the opposite side [26]. Evenness is the accumulation of frequency from high to low, adding up to 50% of the corresponding clonotype number/total clonotype, with a larger value representing a more even distribution. We sequenced the TCRs of all 19 samples before and after culture, and the experimental results showed that the Shannon index of 15-day cultured cells was significantly reduced compared to that at day 0. The value on day 15 value was significantly increased, which indicated a decrease in the number of species (**Figure 4A**). Regarding evenness, the changes were significantly reduced on day 15, suggesting that TCR populations were more heterogeneous on day 15 than on day 0 (**Figure 4A**). When all three indicators were significantly different, we investigated which indicators directly affected the curative effect of treatment as evaluated based on patient survival. We first obtained three different cutoff values through ROC analysis. We then divided the 19 patients into groups according to their cutoff values. Survival analysis showed that compared with patients with a post/pre Shannon index value >0.7, patients with a post/pre value <0.7 had significantly favorable PFS (median PFS time, 153 days vs. 75 days, P=0.026, **Figure 4B**) and OS (median OS time 225 days versus 116 days, P=0.026, **Figure 4B**). In addition, compared with patients with a post/pre clonality value <1.7, those with post/pre clonality >1.7 had significantly favorable PFS (median PFS time, 176 days vs. 53 days, P=0.0024, **Figure 4B**) and OS (median OS time, 226 days versus 161 days, P=0.04, **Figure 4B**). However,

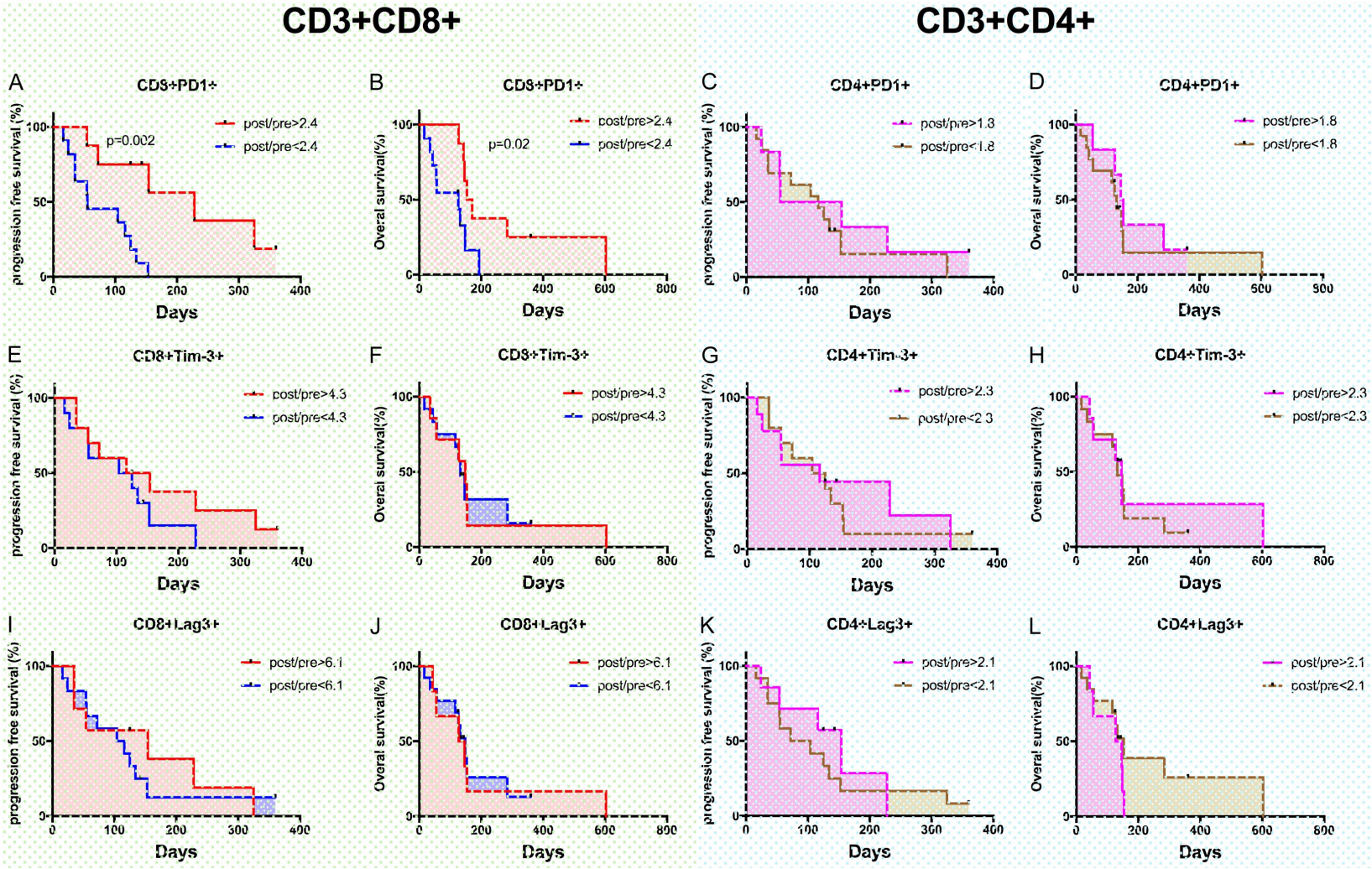

Figure 2. Survival analysis of patients divided into subgroups by the ratios of post/pre CD8⁺PD-1⁺, CD8⁺LAG-3⁺ and CD8⁺TIM-3⁺ cells and post/pre CD4⁺PD-1⁺, CD4⁺LAG-3⁺ and CD4⁺TIM-3⁺ cells. A, B: Patients with values of post/pre CD8⁺PD-1⁺ T cells >2.4 had significantly favorable OS and PFS compared with those with values ≤2.4. C, D: Patients with values of post/pre CD4⁺PD-1⁺ T cells >1.8 did not significantly differ in OS or PFS from patients with values ≤1.8. E, F: Patients with values of post/pre CD8⁺Tim-3⁺ T cells >4.3 did not significantly differ in OS or PFS from patients with values ≤4.3. G, H: Patients with values of post/pre CD4⁺Tim-3⁺ T cells >2.3 did not significantly differ in OS or PFS from patients with values ≤2.3. I, J: Patients with values of post/pre CD8⁺LAG-3⁺ T cells >6.1 did not significantly differ in OS or PFS from patients with values ≤6.1. K, L: Patients with values of post/pre CD4⁺LAG-3⁺ T cells >2.1 did not significantly differ in OS or PFS from patients with values ≤2.1.

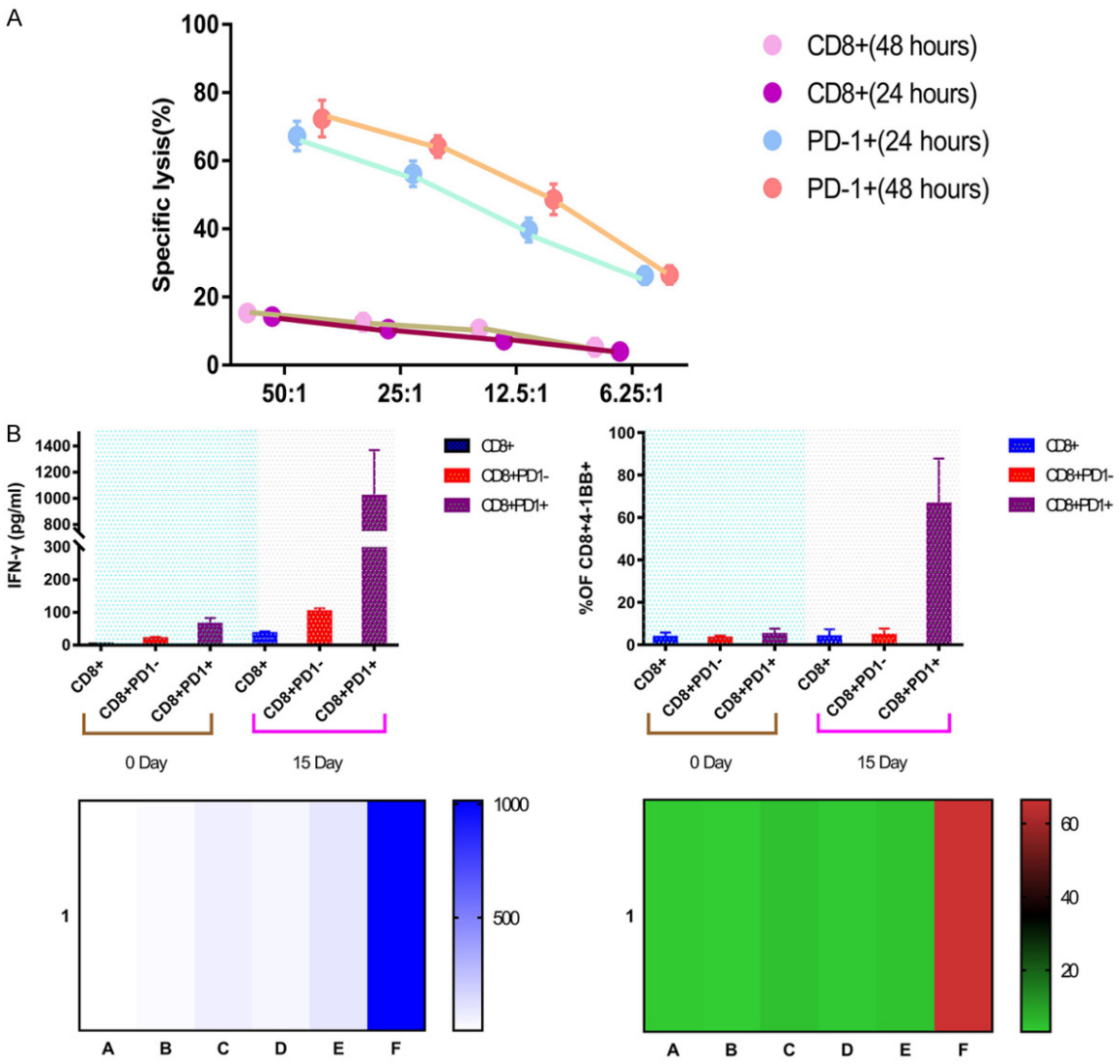

Figure 3. CD8+PD-1+ T cells could be identified as tumor-reactive CD8 T cells. A: Lysis of HLA-A2+ matched tumor cells by patient-derived CD8+ 511 T cells. B: Reactivity of PD-1+ and PD-1-CD8+ T cells derived from patients against autologous tumor cell lines. IFN-γ release and upregulation of 4-1BB (mean ± SD) are shown. IFN-γ is a phytohemagglutinin-induced virus inhibitor produced by white blood cells after they have been stimulated. 4-1BB, a tumor necrosis factor receptor superfamily (TNFRSF) member, plays an important role in activating T cells, natural killer (NK) cells, and other immune cells.

patients with evenness post/pre >0.5 had no significant difference in PFS (P=0.59, **Figure 4B**) or OS (P=0.055, **Figure 4B**) from those with post/pre evenness <0.5.

From the flow cytometry experiment, we found that after 15 days of culture, the main cells showing increases were CD3+CD8+ T lymphocytes, but the main cytotoxic cells were CD3+CD8+PD1+ T cells. Therefore, to determine whether the above TCR indicators affecting PFS and OS were related to CD3+CD8+PD1+ T cells, we conducted a preliminary correlation analysis. As shown in **Figure 4E**, the number of CD3+CD8+PD1+ T cells was increased after culture, and TCR diversity decreased accordingly (Shannon index decreased, P=0.02, R=0.28; clonality increased, P=0.008, R=0.35), whereas the number of CD3+CD8+PD1+ T cells had no relationship with TCR evenness (P=0.59, R=0.02).

Then, we classified the TCR in each cell, aiming to share sequences for the same cell and sepa-

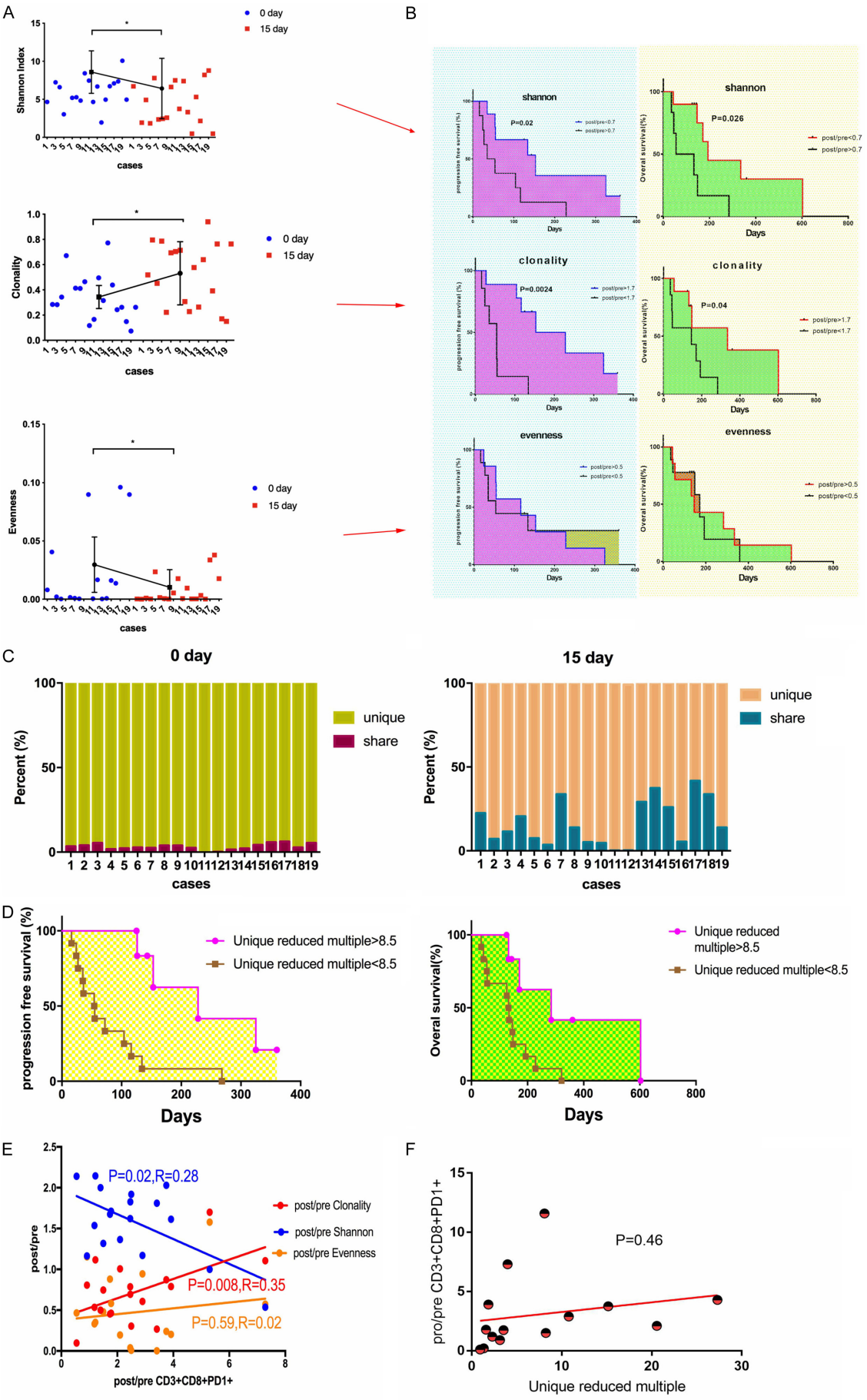
A
0 day
15 day
Shannon Index
Clonality
Evenness
cases
B
shannon
P=0.02
P=0.026
clonality
P=0.0024
P=0.04
evenness
progression free survival (%)
Overal survival(%)
Days
post/pre<0.7
post/pre>0.7
post/pre>1.7
post/pre<1.7
post/pre>0.5
post/pre<0.5
C
0 day
15 day
Percent (%)
unique
share
cases
D
Unique reduced multiple>8.5
Unique reduced multiple<8.5
progression free survival (%)
Overal survival(%)
Days
E
P=0.02,R=0.28
P=0.008,R=0.35
P=0.59,R=0.02
post/pre Clonality
post/pre Shannon
post/pre Evenness
post/pre
post/pre CD3+CD8+PD1+
F
P=0.46
pro/pre CD3+CD8+PD1+
Unique reduced multiple

Figure 4. Variable TCR diversity after expansion is observed and is associated with outcomes in different GC patients. A: Alterations in the Shannon index, clonality and evenness after day 15 ex vivo. B: Patients with a post/pre Shannon index value of <0.7 had significantly favorable OS and PFS compared with those with a value ≥0.7; patients with a post/pre clonality value >1.7 had significantly favorable OS and PFS compared with those with a value ≤.7; patients with a post/pre evenness value did not significantly differ in OS or PFS from those with a value ≤0.5. C: The proportion of TCR clones increased and the proportion of unique TCR clones decreased after T-cell expansion ex vivo in GC patients. D: Survival analysis showed that the number of patients with a unique reduced multiple >8.5 had significantly longer PFS (P=0.0018) and OS (P=0.027) than those with values <8.5. E: Correlation analysis showed that the value of post/pre $CD8^+PD\text{-}1^+$ T cells was significantly associated with post/pre Shannon index and post/pre clonality but not post/pre evenness. F: Correlation analysis showed that the unique reduced multiple had no significant association with the value of post/pre $CD8^+PD1^+$ T cells.

rate unique sequences found in only a single cell. By comparing the shared sequences and unique sequences before and after culture, we found that after 15 days of culture, the percentage of shared sequences had increased significantly. Therefore, TCR sequences that were specific for tumor killing were shared. After 15 days of culture, as the number of tumor-specific $CD8^+PD1^+$ T cells increased, the percentage of shared sequences also increased (**Figure 4C**). In cases 9, 11 and 12, there were few to no changes because of the heterogeneity of the tumor and immune microenvironment. Some individuals had little response to the stimulation according to the activation of other pathways or immunocytes for our further study to explore. By comparing the proportion of unique sequences before and after culture, we obtained the reduced multiple. Through ROC curve analysis, we obtained the cutoff value, namely, the unique reduction multiple=8.5. Comparisons of the PFS (median PFS time 208 days vs. 79 days, P=0.0018, **Figure 4D**) and OS (median OS time 260 days versus 143 days, P=0.0279, **Figure 4D**) of patients revealed that unique sequences were reduced and that patient survival improved. Then, we investigated whether the decline in the unique sequence occupancy ratio was influenced by the increase in the number of $CD8^+PD1^+$ T cells by conducting a correlation analysis. As shown in **Figure 4F**, we found no relationship between these variables (P=0.46).

Discussion

We previously found that DC-CIK therapy combined with S-1 plus cisplatin in patients with advanced GC could obtain favorable PFS and OS. Clinical efficacy was associated with decreases in ctDNA mutational profiles and the TCR repertoire. There were many types of cells among the DC-CIKs, but we did not know which had the greatest antitumor effect. Through flow cell sorting, we examined the changes in lymphoid subgroups before and after culture and found that $CD8^+$ T cells and $CD4^+$ T cells were significantly increased, with the increase in $CD8^+$ T cells being the most obvious; these cells played a major role in killing the tumor cells. Many signaling pathways operate on the surface of $CD8^+$ T cells. The most studied pathways are the CD28, PD1, TIM-3, LAG-3, and 4-1BB pathways; whereas the CD28 pathway can enhance the killing effect of T cells, the other four pathways all inhibit the killing function of T cells [5]. By comparing the expression of these receptors before and after culture, we found that TIM-3, LAG-3 and PD1 expression was significantly increased. However, analysis of the survival time of transfusion patients showed that only $CD8^+$ $PD1^+$ T cells significantly influenced survival time, which indicated that, unlike other signaling pathways, the PD1 signaling pathway can inhibit the antitumor immune response and enhance the cytotoxic role of $CD8^+$ T cells.

When tumors occur, mutated genes can encode many new antigens that stimulate T-cell activation. In response to other cytokines, neo-Ag-specific $CD8^+$ TILs can be attracted to the tumor microenvironment, and immune cells generate high levels of *CD8A* gene expression and rapidly express high levels of the programmed cell death 1 (*PDCD1*) and T-lymphocyte-associated protein 4 (*CTLA-4*) genes to achieve balance [27]. This process explains why increased numbers of tumor-mutated genes lead to the expression of more PD1 signals and a better corresponding anti-PD1 treatment effect [28]. In the tumor microenvironment, the components of the PD1 signaling pathway can bind PDL1 and PDL2 to activate the downstream ITIM protein and thereby suppress the immune response [27, 29]. However, without PDL1 and PDL2 signals, the killing effect of PD1-positive cells is significantly higher than that of PD1-

negative cells in vitro [15]. Our experiments confirmed this idea. We used $CD8^+PD1^+$ T cells in killing tests and found that these cells produced more IFN-γ than other cells and that the cells expressing PD1 killed tumors more effectively than did other cells. IFN-γ is primarily secreted by activated T cells and natural killer (NK) cells and can promote macrophage activation, mediate antiviral and antibacterial immunity, enhance antigen presentation, orchestrate activation of the innate immune system, coordinate lymphocyte-endothelium interactions, and regulate Th1/Th2 balance [30, 31]. Furthermore, we found that after culture, the expression of 4-1BB in $CD8^+PD1^+$ T cells had also increased. 4-1BB is a surface glycoprotein that belongs to the tumor necrosis factor receptor superfamily member 9 (TNFRSF9). Its expression is induced upon activation in a number of leucocyte types. Interestingly, for cancer immunotherapy, 4-1BB becomes expressed on primed T and NK cells, which upon ligation provides powerful costimulatory signals [32]. In summary, $CD8^+PD1^+$ T cells have strong immune activity. After transfusion of $CD8^+PD1^+$ T cells into patients, we found that compared with a value <2.4, a pro/pre $CD8^+PD1^+$ T cell value >2.4 was associated with significantly improved PFS and OS in patients. From this finding, we deduced that the expression of PD1 was similar to that of PDL1, with PD1 thus acting as an immunosuppressant. The PD1 signal in transfusion cells is much stronger than PDL1 expression in tumors after expansion in vitro, which could kill tumors. To verify this possibility, we will continue to purify $CD8^+PD1^+$ T cells, and we will compare the expression of PDL1 in tumor tissues between before and after expansion in vitro and observe the tumor killing effect.

In the above experiments, we observed that $CD8^+PD1^+$ T cells played a significant role in killing tumors and thereby improved patient prognosis. The identification of $CD8^+$ T-cell tumors mainly relies on DCs to deliver antigens to MHC-I molecules so that $CD8^+$ T cells can become activated [27, 33, 34]. We wondered if we could use the frequency of TCR mutations to obtain a more detailed view of tumor treatment. By comparing the distribution of TCR before and after transfusion, we found that a decrease in TCR species, that is, a decreased Shannon index, and an increase in clonality value led to a significantly improved prognosis. Considering the previous results of the lymphoid subgroup, we conducted a correlation analysis of the TCR type decrease and the number of $CD8^+PD1^+$ T cells, which are mainly used to kill tumors; we found that they are closely related. On the one hand, while the number of $CD8^+PD1^+$ T cells recognizing tumor antigens may increase, some other immune cells tend to undergo apoptosis, which eventually leads to the selective expansion and survival of TCR species targeting tumor antigens. To verify the above conclusions, we continued to compare the changes in TCR species diversity before and after culture. The proportion of total TCR sequences was significantly increased after 15 days of culture; in contrast, TCR sequences appeared on only a single T cell, which means that the proportion of unique sequences was significantly decreased. However, the downward trend from before to after cultivation of the proportion of unique sequences was not associated with the increase in $CD8^+PD1^+$ T cells. This result might be related to our small sample size and the other T lymphoid subpopulations affected.

Conclusion

In summary, this study showed that after ex vivo expansion for 15 days, a $CD8^+PD\text{-}1^+$ T-cell subgroup contained a population of tumor-reactive cells. A post/pre $CD8^+PD\text{-}1^+$ T-cell value >2.4 after ex vivo expansion was associated with significantly favorable OS and PFS compared with those corresponding to a post/pre $CD8^+PD\text{-}1^+$ T-cell value ≤2.4. TCR diversity and subclones were significantly associated with the outcomes of GC patients and changes in $CD8^+PD\text{-}1^+$ T-cell subpopulations. Subsequent work will amplify the sample size and isolate $CD8^+PD\text{-}1^+$ T cells for use as an anticancer therapeutic.

Acknowledgements

This work was supported by the Key Science & Technology Project of Beijing Educational Committee and the Beijing Municipal Natural Science Foundation (KZ202110025029).

Disclosure of conflict of interest

None.

Abbreviations

GC, gastric cancer; ACT, adoptive T-cell therapy; TCR, T-cell receptor; PD-1, Programmed death receptor 1; OS, overall survival.

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