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Characterization of γδ T cells in patients with non-small cell lung cancer

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Abstract. Systemic immune defects that are associated with disease progression exist in a variety of malignancies. γδ T cells are innate-like lymphocytes that do not require self-major histocompatibility complex-restricted priming. *Ex vivo*-expanded circulating γδ T cells exhibit promising antitumor activity and are a potential candidate for the treatment of various malignancies, including non-small cell lung cancer (NSCLC). In the present study, flow cytometry was used as a method to study the phenotypes and characteristics of γδ T cells. A lower frequency of circulating γδ T cells was observed in NSCLC patients than in healthy controls. In advanced NSCLC patients, γδ T cells were also detected in the pleural effusion, but the frequency of γδ T cells here was significantly lower than in the peripheral blood. Vδ1$^+$and Vδ1$^-$Vδ2$^-$ T cells represented the most enriched subsets in the pleural effusion. Moreover, the present study demonstrated that Vδ1$^+$ T cells are a type of γδ T cells characterized by a cluster of differentiation (CD)3^{dim} T-cell receptor (TCR)γδ^{bright} phenotype, whereas Vδ2$^+$ T cells represent a CD3^{bright}TCRγδ^{dim} phenotype, according to the fluorescence intensity of CD3 and γδTCR using flow cytometry. Finally, the present study reported a decrease in the expression of CD27 and CD28 molecules on the surface of circulating γδ T cells in NSCLC. The present data suggest the existence of a dysregulated repertoire of γδ T cells in NSCLC, which exhibit impaired activation and a reformed cytokine-releasing profile. Although the *ex vivo* expansion of γδ T cells may be a prospective therapeutic strategy in NSCLC patients, it remains necessary to clarify which subsets (Vδ1 or Vδ2) should be expanded and the sources from which γδ T cells should be generated.

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Introduction

Unlike αβT cells, γδ T cells represent a minor subset of T cells that does not require self-majorhistocompatibility complex (MHC)-restricted priming (1). γδ T cells are believed to serve as a bridge, connecting the innate and adaptive immune responses (1). Following infection by microbial pathogens, γδ T cells appear to be the first T cells tomigrateto the lung (2). Previous studies have identified that γδ T cells can mediate antitumor activity (3).

In humans, γδ T cells are primarily identified by the V(D)J recombination of the γ and δ chain genes, particularly their Vδ chain usage (4). Vδ2 cells (mainly Vγ9/Vδ2 cells) are the predominant γδ T-cell subset (50-75%) in the peripheral blood (PB) of healthy individuals (5). Uniquely, this subset of γδ T cells responds to phosphoantigens via polymorphic γδ T-cell antigen receptors (TCR) and undergoes rapid activation (6,7). Owing to the relative abundance in the PB and ease of expansion, Vγ9/Vδ2 T-cell-based adoptive immunotherapy has been intensively investigated as the treatment for a variety of malignancies (8-10). Vδ1 T cells represent <30% of γδ T cells in the PB, but have been identified as an enriched subset in the thymus and in epithelial tissues, including the dermis, gut epithelium and spleen (11). Unlike Vδ2 cells, Vδ1 cells do not respond to phosphoantigens, but are able to recognize the MHC class I chain-related molecules A and B (12). A recent study suggested that Vδ1$^+$ T cells derived from the PB of normal donors are able to undergo *ex vivo* expansion by administration of phytohemagglutinin (PHA) and interleukin-7 (IL-7) (13); moreover, Vδ1 T cells exhibit more favorable antitumor activity than Vδ2 T cells in colon cancer (13). *Ex vivo*-expanded circulating γδ T cells represent a promising prospect in antitumor activities and are a potential candidate treatment for various malignancies, including non-small cell lung cancer (NSCLC). A phase I clinical study is currently being conducted using adoptive γδ T cell therapy in patients with advanced or recurrent NSCLC (14). However, the characterization of γδ T cells remains poorly understood in advanced NSCLC.

In the present study, the absolute count of lymphocytes and monocytes, and the subtypes and characteristics of γδ T cells in the PB and pleural effusion of advanced NSCLC patients were analyzed in order to investigate which subsets (Vδ1 or Vδ2) should be expanded *ex vivo* and used as the source from

which these γδ T cells should be generated for adoptive γδ T cell therapy.

Materials and methods

Subject recruitment and sample preparation. The complete blood cell count of 102 patients with stage I-IV NSCLC using seventh edition of the TNM classification for lung cancer (15) (51 male, 51 female; 64.45±1.55 years of age; range, 48-86 years) who were admitted to the Department of Oncology, The Second Affiliated Hospital of Jiaxing College (Jiaxing, China) between January 2011 and December 2015, was retrospectively analyzed. Blood cell count data was also obtained from 114 cases of aged-matched healthy controls. Of the NSCLC patients and controls, 35 patients (stage III and IV) and 25 age-matched healthy individuals underwent γδ T-cell analysis in this study. Of these 35 patients, 10 were diagnosed as having stage IV NSCLC with malignant pleural effusion. Analysis of the characteristics of the γδ T cells in the pleural effusion of these 10 patients, together with another 2 elderly NSCLC patients (>75 years of age) was conducted. The studies were approved by the Ethics Committee of The Second Affiliated Hospital of Jiaxing College and written informed consent was obtained from each individual that provided a specimen. Study subjects did not have infectious diseases and had not undergone chemotherapy or radiotherapy in the previous week; however, certain patients and healthy donors did have chronic conditions, including hypertension, high cholesterol and diabetes.

Isolation of mononuclear cells from pleural effusion. Following collection of a 50-ml specimen of pleural effusion from 12 patients, mononuclear cells were isolated by centrifugation at 1,000 x g over a Ficoll-Paque (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) density gradient.

Blood cell count. A BC-5200 Hematology Analyzer (Beckman Coulter, Inc., Brea, CA, USA) was used to examine the absolute number of lymphocytes and monocytes in the present study.

Flow cytometry staining. To determine the identity of the biomarkers on the surface of γδ T cells, multicolored immunofluorescence staining was conducted using freshly collected blood samples and mononuclear cells isolated from the pleural effusion of the subjects. The antibodies were conjugated to fluorescent markers as follows: CD3-PE-Cy5.5 (cat. no. 340949), TCR γδ-APC (cat. no. 555718), TCRγδ-FITC (cat. no. 559878), Vδ2-PE (cat. no. 3345652), CD27-PE (cat. no. 555441) and CD28-APC (cat. no. 559770). These antibodies, as well as isotype-matched control antibodies, were purchased from BD Pharmingen (dilution, ready to use; BD Biosciences, San Jose, CA, USA). Vδ1-FITC antibodies (cat. no. TCR2730) were purchased from Thermo Fisher Scientific, Inc., (Waltham, MA, USA). For extracellular staining, 50 μl of each blood sample, and the mononuclear cells isolated from the pleural effusion which were in 1X PBS with 1% bovine serum albumin, were incubated with different combinations of fluorochrome-coupled antibodies (10 μl of each antibody). After a 20-min incubation at room temperature, cells were washed twice with 1X PBS and flow cytometry was performed using a BD FACSCanto II flow cytometer (BD Biosciences). Data were collected and analyzed with DIVA software (version 6.1.3; BD Biosciences, San Jose, CA, USA).

Table I. Clinicopathological features of lung cancer patients in the present study (n=102).

Category	Value
Gender, n (%)	
Male	51 (50.0)
Female	51 (50.0)
Mean age ± SEM, years	64.45±1.55
Histology, n	
Adenocarcinoma	69
Squamous cell carcinoma	16
NSCLC (not specified)	17
TNM stage, n	
I-II	9
III	12
IV	81

SEM, standard error of the mean; NSCLC, non-small cell lung cancer; TNM, Tumor-Node-Metastasis classification system.

Statistical analysis. Data are presented as the mean ± standard error of the mean. Comparisons between groups were made using an unpaired Student's t-test. P-values <0.05 were considered to indicate statistical significance. GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used for all statistical calculation and figure generation.

Results

Absolute number of lymphocytes and monocytes in the PB of NSCLC patients. The complete blood cell counts of 102 patients (51 male, 51 female; 64.45±1.55 years) with stage I-IV NSCLC were retrospectively analyzed. The clinicopathological features of patients are provided in Table I. The blood cell count data were obtained from 114 cases of aged-matched healthy controls (51 male, 63 female; mean age, 63.40±1.1 years). The absolute number of lymphocytes and monocytes was assessed using an automatic hematology analyzer. The absolute value of lymphocytes (normal range, 1.10-3.20x10^9/l) in the PB was 1.285x10^9±0.049x10^9/l in the NSCLC group, significantly lower than that of the healthy controls, where it was 2.065x10^9±0.051x10^9/l (P<0.001) (Fig. 1A); however, the absolute value of monocytes (normal range is 0.10-0.60x10^9/l) was 0.484x10^9±0.022x10^9/l in the NSCLC group, significantly higher than that in the healthy controls, where it was 0.363±0.011 (P<0.001) (Fig. 1B).

Frequency of CD3$^+$γδ$^+$T cells and Vδ1Vδ2 subtypes in circulation of NSCLC patients. Flow cytometry was performed to determine the proportion of CD3$^+$γδ$^+$T cells in the PB of

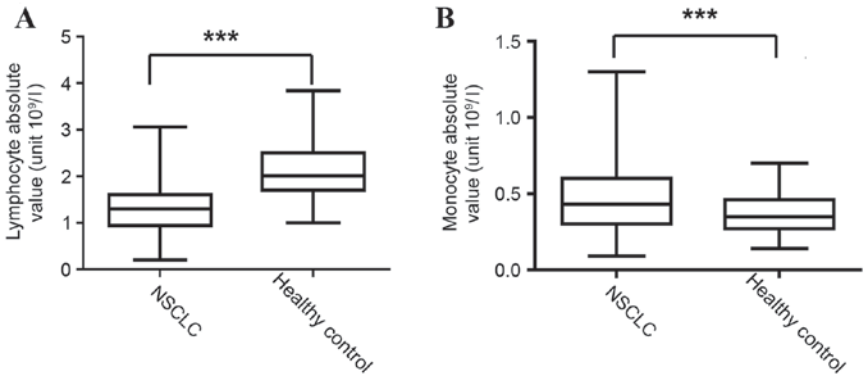

Figure 1. Retrospective analysis of the complete blood cell count in NSCLC patients. (A) A decrease was observed in the absolute number of lymphocytes in NSCLC patients ($1.285x10^9±0.049x10^9$/l) compared with healthy controls ($2.065x10^9±0.051x10^9$/l) (P<0.001). (B) An increase in the absolute number of monocytes was observed in NSCLC patients ($0.484x10^9±0.022x10^9$/l) compared with healthy controls (0.363±0.011) (P<0.001). ***P<0.001. NSCLC, non-small cell lung cancer; FSC, forward scatter; SSC, side scatter; TCR, T-cell receptor.

NSCLC patients. A total of 35 (stage III-IV) NSCLC patients (23 male, 12 female; mean age, 65.7±1.3 years) were examined in this study (2 NSCLC patients with pleural effusion were excluded owing to their old age, >75 years old). The clinicopathological features of the patients are provided in Table II. In order to make a comparison, the frequency of γδ T cells were examined in 25 cases of age-matched healthy controls (17 male, 8 female; mean age, 63.6±4.2 years). Among these subjects, 20 patients with NSCLC and 11 healthy donors were further analyzed for the percentage of Vδ1 and Vδ2 T cells, two major subsets of γδ T cells, and the expression of the co-stimulatory markersCD27 and CD28. To assess the frequency of γδ T cells present in the sample, lymphocytes were first gated on the basis of a forward scatter/side scatter (FSC/SSC) profile, followed by γδ T-cell analysis in a gated population with $CD3^+$ staining (Fig. 2A). A pan-TCRγδ antibody was used to identify γδ T cells, and Vδ1/Vδ2 antibodies were further used to determine Vδ1 and Vδ2 subsets (Fig. 2A). Fig. 2B shows that in NSCLC patients, the mean frequency of $CD3^+γδ^+$T cells was 4.16±0.44% (n=35), whereas the value in healthy individuals was 6.40±0.77% (n=25); the two groups have a statistically significant difference (P<0.05). In parallel with a previous report (16), Vδ2 T cells represented a major subset, and the Vδ2/Vδ1 ratio was >1 in the PB of all normal donors (100.0%) (11/11); however, in NSCLC patients, the $Vδ2^+$ T cells only represented a major subtype in 35.0% (7/20) of the population of γδ T cells; $Vδ1^+$ cells were counted as the priority γδ T cells in 35.0% (7/20) of patients, and in the rest of patients, $Vδ1^-Vδ2^-$ cells represented the main subset (6/20).

Frequency of $CD3^+γδ^+$T cells and Vδ1Vδ2 subtypes in the PB and in the pleural effusion of advanced NSCLC patients. To compare the difference between γδ T cells and Vδ1Vδ2 subtypes in the PB and pleural effusion in stage IV NSCLC patients, 12 patients (6 male, 6 female; mean age, 70.2±9.2 years) were recruited. The clinicopathological features of the patients are provided in Table III. The paired blood and pleural effusion samples were collected at the same time. The mean frequency of $CD3^+γδ^+$T cells was higher in the PB of the two groups (5.32±1.08%) than that in the pleural effusion of the two groups (2.47±0.69%), with a statistically significant difference (P<0.05) (Fig. 3A).

In the 12 NSCLC patients recruited for the current experiment, the $Vδ2^+γδ$ T cell was the predominant subtype in the PB in 5 patients (41.7%), whereas Vδ1 or $Vδ1^-Vδ2^-$ was the major $CD3^+γδ^+$T cell subtype in the remaining 7 patients (58.3%). In the pleural effusion, Vδ1 or $Vδ1^-Vδ2^-$ was the predominant subtype of $CD3^+γδ$ T cells in all 12 patients (100.0%). In comparison to the frequency of $Vδ1^+$ and $Vδ2^+$T cells between the PB and the pleural effusion, data from the present study showed that the percentage of Vδ2 T cells was significant lower in the pleural effusion, with 36.01±8.25% of cells found to be T cells in the PB vs. 8.16±2.38% in the pleural effusion (P<0.01) (Fig. 3B). However, the percentage of Vδ1 T cells in the pleural effusion was significantly higher, with 44.54±8.49 of cells in the blood found to be T cells vs. 64.78±5.50% in the pleural effusion (P<0.05) (Fig. 3C).

Vδ1 and Vδ2 have different mean fluorescence intensity (MFI) for CD3 and TCR γδ. Next, the expression of CD3 and TCRγδ on the surface of the Vδ1 and Vδ2 T-cell population was analyzed. During the study of circulating Vδ1 and Vδ2 cells in the 20 NSCLC patients and 11 healthy donors, further analysis was performed, investigating the MFI of CD3 in Vδ2 and Vδ1 γδ T cells. This analysis found that the MFI of CD3 was significant higher in Vδ2 cells than in Vδ1 cells in NSCLC patients and healthy individuals; a CD3 MFI of 4,776±691.2 in $Vδ2^+$cells vs. 2,612±319.4 in $Vδ1^+$cells (n=20; P<0.01) was observed in NSCLC patients (Fig. 4A), and a CD3 MFI 9,689±1,270 in $Vδ2^+$ cells vs. 3,454±592.6 in $Vδ1^+$ cells in healthy controls (n=11; P<0.001) (Fig. 4B). The MFI of TCRγδ was significantly lower in $Vδ2^+$ cells than in $Vδ1^+$ cells in NSCLC (858±62.49 vs. 2,614±313.10, respectively; n=20; P<0.001) (Fig. 4C), and in healthy controls (1,351±182.8 vs. 3,724±725.20, respectively; n=11; P<0.01) (Fig. 4D). Therefore, Vδ2 cells should be considered to be a population of $CD3^{bright}TCRγδ^{dim}$ T cells, and by contrast, Vδ1 cells should be considered to be a population of $CD3^{dim}TCRγδ^{bright}$ T cells.

Expression of co-stimulatory markersCD27 and CD28 is decreased on the surface of $CD3^+γδ^+$T cells in patients with advanced NSCLC. The TNF receptor family member CD27 is widely expressed on natural killer cells, and on $CD4^+$ and

Table II. Clinicopathological features of stage III and IV lung cancer patients.

Category	Value
Gender, n (%)	
Male	23 (65.7)
Female	12 (34.3)
Mean age ± SEM, years	65.7±1.3
Histology	
Adenocarcinoma	19
Squamous cell carcinoma	9
NSCLC (not specified)	7
TNM stage, n	
III	9
IV	26

NSCLC, non-small cell lung cancer; TNM, Tumor-Node-Metastasis classification system; SEM, standard error of the mean.

Table III. Clinicopathological features of advanced lung cancer patients with pleural effusion.

Category	Value
Gender, n (%)	
Male	6 (50.0)
Female	6 (50.0)
Mean age ± SEM, years	70.2±9.2
Histology, n	
Adenocarcinoma	7
Squamous cell carcinoma	2
NSCLC (not specified)	3

SEM, standard error of the mean; NSCLC, non-small cell lung cancer.

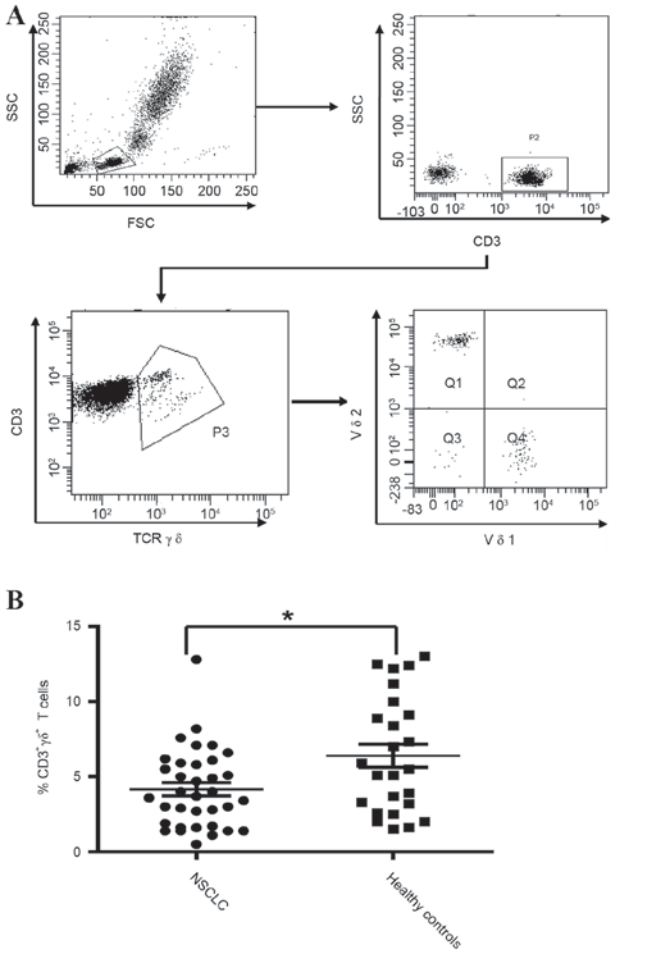

Figure 2. Identification and characterization of a population of γδ T cells and Vδ1$^+$Vδ2$^+$ subtypes in the lymphocytes. (A) Cells were gated as lymphocytes and then CD3$^+$T cells. Subsequently, the presence of TCRγδ$^+$ T cells was assessed in CD3$^+$ T cells. In the population of CD3$^+$TCRγδ$^+$T cells, Vδ1$^+$Vδ2$^+$ and Vδ1$^-$Vδ2$^-$were further gated and analyzed. (B) The percentage of CD3$^+$γδ T cells in NSCLC patients and healthy controls (4.16±0.44 vs. 6.40±0.77%, respectively; P<0.05). *P<0.05. NSCLC, non-small cell lung cancer; FSC, forward scatter; SSC, side scatter; TCR, T-cell receptor; CD, cluster of differentiation.

CD8$^+$ T lymphocytes, as well as on γδ T cells (17). CD27 and its ligand, CD70, are involved in a signaling pathway that promotes the survival of primed T cells (18). CD28 is a well-documented co-receptor of αβT cells, and activation of the CD3/CD28 pathway will enhance the proliferation of αβT cells (19). Therefore, the expression of CD27 and CD28 on the surface of γδ T cells was investigated in the PB of the same 20 NSCLC patients and 11 healthy donors. The gating strategy is shown in Fig. 5A. In brief, lymphocytes were first gated based on their FSC/SSC profile, followed by analysis of CD27/CD28 expression in a gated population of CD3$^+$γδ$^+$ cells (Fig. 5A). There was a significantly increased frequency of CD27$^-$CD28$^-$CD3$^+$γδ$^+$ T cells in the PB of NSCLC patients (40.2±5.7%) compared with the PB of the healthy control group (11.9±4.8%) (P<0.01) (Fig. 5B). An increased population of CD27$^-$CD28$^-$CD3$^+$γδ$^+$T cells was also observed in the pleural effusion of NSCLC patients compared with that in the PB, but the difference was not statistically significant (data not shown).

Discussion

In the present study, decreased numbers of lymphocytes and increased numbers of monocytes were observed in the PB of NSCLC patients. This observation supports previous reports concerning the systemic immune defects that exist in malignancies, which include low numbers of circulating T cells and low chemokine levels, and enhance the number of anti-tumor T cells that are likely to partially reverse immunosuppressive activities (20). A low lymphocyte-to-monocyte ratio (LMR) has been shown to be an independent unfavorable prognostic factor for predicting survival in SCLC patients (21). A lower LMR most likely have decreased cytotoxic T lymphocytes and higher tumor-associated macrophages (22). A previous study reported that the monocyte count in the peripheral blood was higher in the patients with cervical and endometrial cancer compared with a control group of healthy blood donors (23). An elevated number of peripheral monocytes has also been associated with a poor prognosis for patients with lung adenocarcinoma (24).

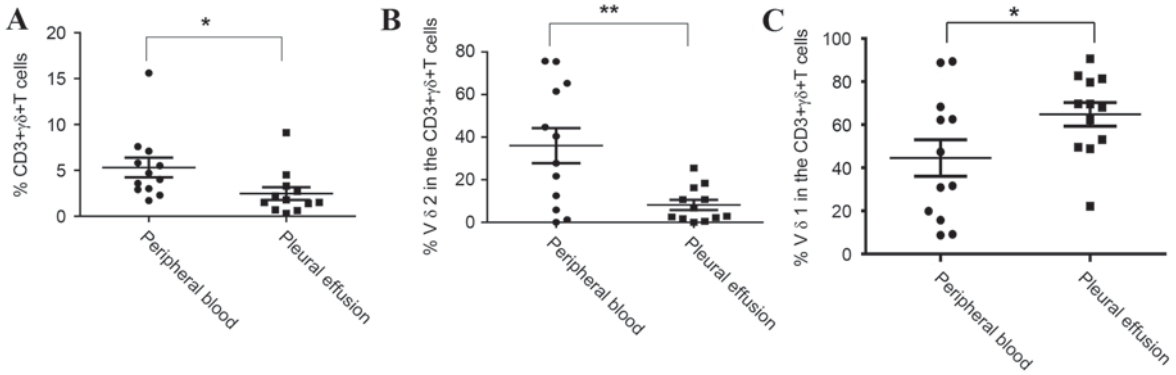

Figure 3. Comparison of γδ T cells and Vδ1Vδ2 subsets between the PB and pleural effusion in paired samples derived from advanced non-small cell lung cancer patients. (A) There was a decreased percentage of $CD3^+\gamma\delta^+$T cells in the pleural effusion compared with the PB (2.47±0.69 vs. 5.32±1.08%, respectively; P<0.05). (B) A higher percentage of Vδ2 cells were present in the population of $CD3^+\gamma\delta^+$T cells in the PB compared with the pleural effusion (36.01±8.25 vs. 8.16±2.38%, respectively; P<0.01). (C) There was a lower percentage of Vδ1 T cells in the PB than in the pleural effusion (44.54±8.49 vs. 64.78±5.50%, respectively; P<0.05). *P<0.05. **P<0.01. PB, peripheral blood; CD, cluster of differentiation.

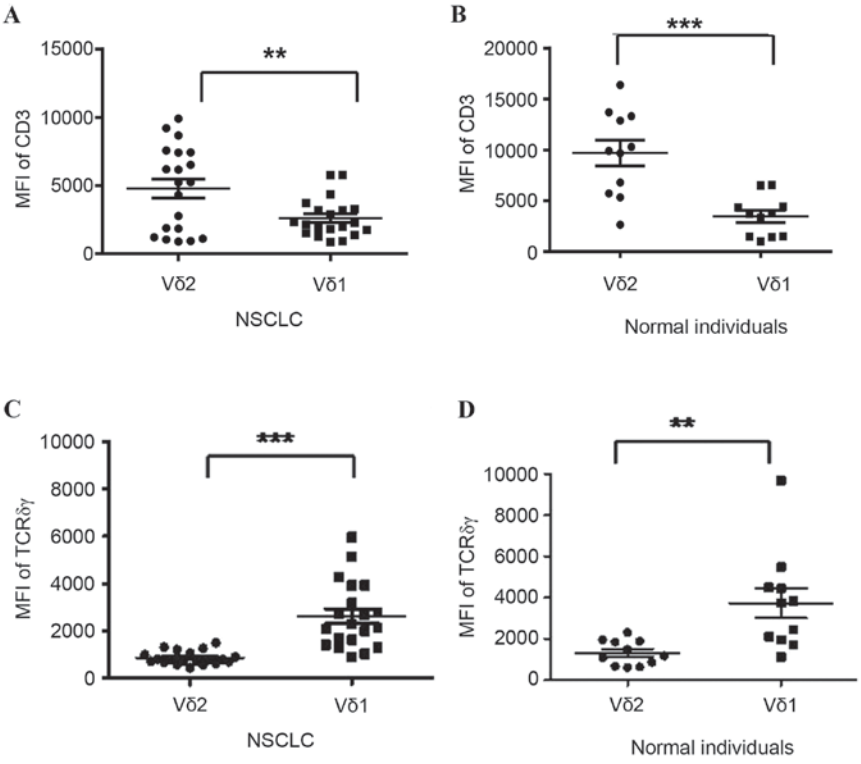

Figure 4. Expression of CD3 and TCRγδ molecules on the surface of Vδ1 and Vδ2 T cell subsets in the circulation of NSCLC patients and healthy individuals. (A) The MFI of CD3 was decreased in Vδ1 cells compared with that in Vδ2 cells in the group of NSCLC patients (2,612±319.4 vs. 4,776±691.2 AU, respectively; P<0.01). (B) The MFI of CD3 in $V\delta1^+$ cells was decreased compared with that in Vδ2 cells in the group of normal individuals (3,454±592.6 vs. 9,689±1,270 AU, respectively; P<0.001). (C) The MFI of TCRγδ in Vδ1 cells was increased compared with Vδ2 in the group of NSCLC patients (2,614±313.10 vs. 858±62.49 AU, respectively; P<0.001). (D) The MFI of TCRγδ in Vδ1 T cells was increased compared with that in Vδ2 cells in the group of normal individuals (3,724±725.20 vs. 1,351±182.8, respectively; P<0.01). **P<0.01. ***P<0.001. NSCLC, non-small cell lung cancer; MFI, mean fluorescence intensity; TCR, T-cell receptor; CD, cluster of differentiation.

The number of γδ T cells is lower in elderly individuals than in the young population (25). The present study also observed a decrease in the frequency of γδ T cells in the PB of NSCLC patients. Circulating Vδ2 γδ T cells appeared to lose their predominance in NSCLC patients compared with healthy individuals; Vδ1, and to a lesser extent $V\delta1^-V\delta2^-\gamma\delta$, T cells became a major subset and the ratio of Vδ2:Vδ1 T cells inverted in one-third of all studied patients; a similar observation has been reported in a study of γδ T cells in gastric cancer (26). On the basis of previous studies, Vδ1 T cells are less susceptible to activation-induced cell death and exhaustion than Vδ2 T cells (27,28). Vδ1 T cells are also preferentially persistent *in vivo*; this could be the reason why Vδ1 T cells are predominant in the PB of patients with lung cancer.

In the present study, γδ T cells were also detected in the pleural effusion of patients with advanced NSCLC, in whom the frequency of $V\delta2^+$ T cells was even lower than in the PB, and Vδ1 or $V\delta1^-V\delta2^-\gamma\delta$ T cells acted as the predominant

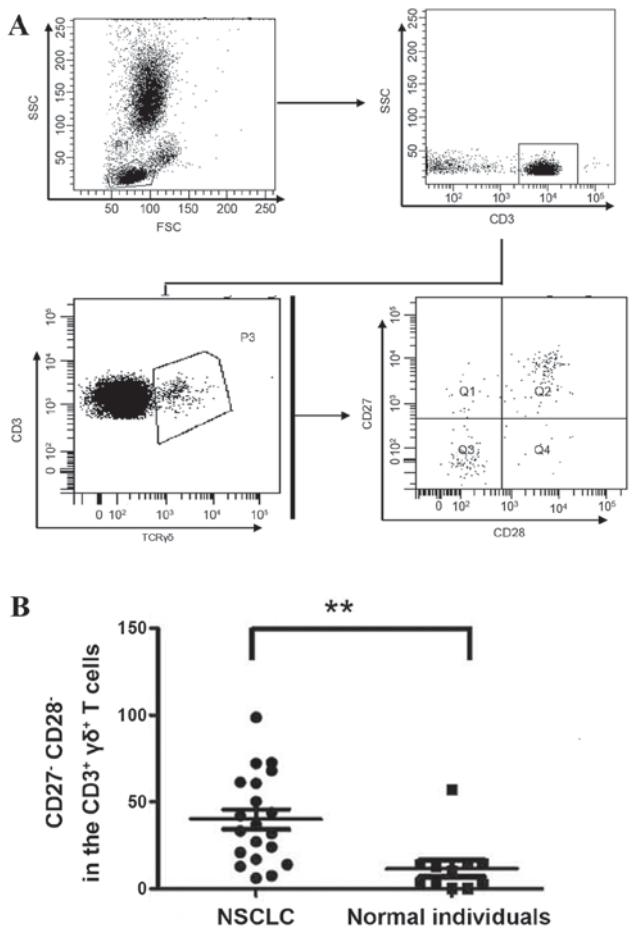

Figure 5. Identification of the co-stimulatory markersCD27 and CD28 on the surface of $CD3^+\gamma\delta^+$T cells in patients with advanced NSCLC. (A) Cells were gated as lymphocytes and then $CD3^+\gamma\delta^+$T cells. Subsequently, the presence of $TCR\gamma\delta^+$cells was assessed for in $CD3^+$ T cells. In the population of $CD3^+\gamma\delta^+$T cells, the expression of CD27 and CD28 was further gated and analyzed. (B) There was a significantly increased frequency of $CD27^-CD28^-CD3^+\gamma\delta^+$T cells in the peripheral blood of NSCLC patients compared with healthy controls (40.2±5.7 vs. 11.9±4.8%, respectively; P<0.01). **P<0.01. NSCLC, non-small cell lung cancer; TCR, T-cell receptor; CD, cluster of differentiation.

subsets in all subjects studied. To the best of our knowledge, no previous study has reported on the characteristics of γδ T cells and their subsets in the pleural effusion of NSCLC. Tumor-infiltrating leukocytes (TILs) represent a wide range of variety of immune cells that have been found in invading solid tumors, and the extent of infiltration of γδ T cells is reported to enable a variety of prognostic predictions, for example; in the earlier stages of melanoma there are higher percentages of intra-tumoral γδ T cells, particularly V2γδ T cells, compared with advanced stage melanoma, which serves as a biomarker of good prognoses; in contrast, a higher level of intra-tumoral V1 γδ T cells has been reported as a poor prognostic factor in breast cancer (29,30). In a study of γδ T cells in the TILs of melanoma, γδ T cells were detected in 76% of tumor specimens using immunohistochemistry. Vδ1 was the major subset presenting in 52% of samples (31,32). In a study of γδ T cells in human colon cancer, it was found that the majority (80%) of IL-17^+γδ T cells expressed Vδ1 (γδ17), and those cells thatinfiltratedγδ17 T cells in the tumor were associated with advanced tumor grades and progression. As a result, these T cells may serve as prognostic markers in human colon cancer (33). However, Peng *et al* (34) reported that the Vδ1 subtype was observed to be the dominant subtype among tumor infiltrating lymphocytes in a group of breast cancer patients, and that it appeared to exhibit a broad range of immune suppression activities, via suppressing the production of IL-2 by $CD4^+$ and $CD8^+$ T cells, and the maturation of dendritic cells. The present study indicates that γδ T cells derived from the pleural effusion could be a source of adoptive γδ T cell immunotherapy in lung cancer. Unlike for Vδ2 T cells, which respond to phosphoantigens, there is no protocol concerning the expansion of $V\delta1^+$ populations *ex vivo*, although a recent study indicated that PHA and IL-7 may be candidates to facilitate expansion of the Vδ1 subtype in circulating γδ T cells (13). However, the present authors have been unable to obtain a satisfactory result on the *ex vivo* expansion of Vδ1 or Vδ2 cells, either using PHA and IL-7 stimulation or zoledronic acid when using the γδ T cells isolated from pleural effusion in advanced NSCLC (Bao *et al*, unpublished data). This is likely to be due to the low frequency of γδ T cells in the pleural effusion, and the fact that the majority γδ T cells are Vδ1 T cells. Moreover, it remains unclear whether there are similar antitumor effects of Vδ1 T cells derived from PB or from pleural effusion in lung cancer patients.

In the present study, Vδ2 T cells appeared to be a population of γδ T cells with a $CD3^{bright}TCR\gamma\delta^{dim}$ phenotype; by contrast, Vδ1 T cells were characterized by a $CD3^{dim}TCR\gamma\delta^{bright}$ phenotype. A previous study suggested that TCRγδ-deficient mice display reduced tumor growth compared with wild-type animals (35). The different levels of CD3 and TCRγδ expression may indicate that in these two subsets, a variety of co-stimulatory signaling pathways could be involved in the regulation of activation and proliferation.

The present study found a decrease in the expression of CD27 and CD28 molecules on the surface of circulating γδ T cells in NSCLC patients. A previous study showed a majority of Vδ2 cells in PB are $CD27^+$γδ T cells (36). Another prior study reported evidence supporting an absolute requirement of CD27 for the expansion of γδ T cells by employing a CD27-deficient mice model. CD27 and its ligand CD70 are involved in a signaling pathway that is required to promote the survival of primed T cells (17,37). In the thymus of mice, CD27 is likely to regulate γδ T cell differentiation, and $CD27^+$γδ T cells produce interferon (IFN)-γ, whereas $CD27^-$γδ T cells produce IL-17 (38). In αβ T cells, 'signal 1', which refers to the mature T cells recognizing and binding to a major histocompatibility complex (MHC) molecule carrying a peptide antigen through their antigen-specific receptors (TCR), together with a co-stimulatory pathway, CD28/B7, provides a 'second signal' that is required for the activation and proliferation of T cells (39); this CD28/B7 pathway involves the interaction of co-stimulatory molecule CD28 with its ligands B7-1 (CD80) and B7-2 (CD86) on the antigen presenting cells, and is critical for T-cell activation, proliferation, and survival (40); however, the requirement of the additional CD28/B7 signal for the activation of γδ T cells remains controversial (41). In studying lymphocytes isolated from the lymph node, it was apparent that CD28 is expressed on the surface of γδ T cells and has the capability to promote the activation of γδ T cells, as well as their proliferation and survival, mainly by

stimulating γδ T cells to produce and secrete IL-2 (42,43). Data obtained from a study of CD28-deficient mice suggested that the population of IFN-γ^+ and IL-17^+γδ T cells failed to expand during infection (43,44), indicating that CD28 co-stimulation is also necessary for γδ T cell expansion. Downregulation of CD27 and CD28 molecules in circulating γδ T cells in NSCLC patients suggests an impaired capacity to activate and proliferate γδ T cells in those patients, and indicates that they have a different cytokine profile to healthy individuals (42).

The present data suggested the presence of a dysregulated repertoire of γδ T cells, including impaired activation and a reformed cytokine releasing profile of γδ T cells in NSCLC. Although the *ex vivo* expansion of γδ T cells may be a prospective therapeutic strategy in NSCLC patients, it remains necessary to clarify which subsets (Vδ1 or Vδ2) should be expanded and the sources from which these γδ T cells should be generated.

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