Effects of Glycogen Synthase Kinase-3β Inhibitor TWS119 on Proliferation and Cytokine Production of TILs From Human Lung Cancer

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Summary: The canonical Wnt-β-catenin signaling pathway plays an essential role in human thymocyte and peripheral T-cell development.1–3 Wnt pathway preserves the naive T-cell (Tn) phenotype, maintains the “stemness” in mature memory CD8+ T cells, arrests effector T-cell (Teff)-differentiation in human peripheral blood and cord blood-derived T lymphocytes.4,5 As the downstream transcription factors of the Wnt-β-catenin signaling pathway, T-cell-specific transcription factors-1 (TCF-1) and lymphoid enhancer binding factor-1 (LEF-1) are required for normal thymic T-cell development; high expression of TCF-1 in Tn cells and stem-like memory T cell (Tscm) cells;6 TCF-1 expression decreased when the Tn cells differentiate into Teff cells. The 4, 6-disubstituted pyrrolopyrimidine TWS119 is a potent inhibitor of serine-threonine kinase glycogen synthase kinase-3β (Gsk-3β);7 by facilitating the β-catenin accumulation in cytoplasm and translocate into nucleus, TWS119 promotes the expression of TCF-1 and LEF-1 in the nucleus by binding to β-catenin.8–10 Cultured with TWS119, the majority of T cells retained a characteristic of Tn cells phenotype; while in the absence of the TWS119 treatment, native T-cell activation increased, TCF-1 and LEF-1 down-regulation. Research observed that TWS119 maintain the phenotype of Tn cells by promoting the expression of TCF-1 and blocked CD8+ T-cell differentiation, and inhibited the production of IFN-γ.4,11,12

Tumor-infiltrating lymphocyte (TILs) can be expanded from tumor. TILs are heterogeneous cell group, the major population is T T cells.13 Our research illuminated that Tscm cells was found in TILs from non–small cell lung cancer (NSCLC) except Tn cells, Teff, central memory T cells (Tcm), effector memory T cells (Tem),14,15 the frequency and function of Tscm cells and other subset of T cell from TILs is distinct from that of other lymphoid organ.16 Tscm cells was first identified in human peripheral blood; Tscm cells share the phenotype of Tn cells while possess the function of memory T cells; in addition, Tscm cells have a strong antitumor function; Tscm cells also own stem cell-like features and have ability to self-renewal and differentiation.17 Because of these characteristics, Tscm cells are considered to be one of the optimal cell type for adoptive cell transfer (ACT) of cancer immunotherapy.18,19 Tscm cells in TILs expressed CD3+CD4−CD8+CD45RA−CD45RO−CCR7−CD62L−CD122−CD95+, which was different from the phenotype of Tscm cells from the peripheral blood mononuclear cells (PBMCs) and lymph node.13,14 We also observed that Tn cells in TILs possess robust capacity to produce IFN-γ, which varies from the Tn cells from the peripheral blood and lymph node (unpublished data). Whether TWS119 can affect the phenotype and function of Tscm and Tn cells in TILs remain limited. In this study, we analyzed the proliferation and cytokine production of T-cell subsets in TILs from NSCLC with the treatment of TWS119. In terms of proliferation capacity of T-cell subsets, the reactivity to TWS119 was significantly different between TILs from lung cancer and PBMC from healthy donor, and
the effects of TWS119 on CD4+ and CD8+ T cells in TILs are different. TWS119 maintained the proportion of Tn (CD4+ and CD8+ Tn) cells, CD8+ Tscm cells; TWS119 had no effect on the IFN-γ and IL-2 production in Tn and Tscm cells from TILs, but had an increased effect on IL-2 produced by certain other subset of T cells in TILs. Our results showed that the response of T-cell subsets to TWS119 in TILs was different. It is of great significance to select the T-cell subsets in TILs with long-term antitumor effect for ACT in NSCLC, and it was very helpful to understand the potential role of TWS119 in clinical applications.

**MATERIAL AND METHODS**

**Study Participants**

In total, 5 NSCLC patients from the First Affiliated Hospital of Sun Yet-San University of Guang Zhou, China were enrolled in this study. These patients included 2 women and 3 men, the age range from 41 to 78 years. The final diagnosis of lung cancer was based on pathologic evidence (detected by histologic staining). Patients whose serology tested positive for human immunodeficiency virus, hepatitis B virus, and hepatitis C virus were excluded from the study. None of the patients received cancer-related chemotherapy or radiation during the period of collecting samples. Four healthy donors were recruited for collecting blood (the age range from 18 to 40 yrs). The sex of the healthy donor control group and lung cancer group samples are matched.

**Ethics Statement**

Written informed consent was obtained from all patients and healthy donors. This study was approved by the ethics committees of the Zhong Shan School of Medicine, Sun Yat-Sen University (Guangzhou, China) and First Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China).

**Isolation of PBMCs**

The PBMCs were isolated from sodium heparin–treated blood obtained from healthy donors by Ficoll-Hypaque (cat. LTS1077; Tian Jin Hao Yang Biological Manufacture Co. Ltd, China) gradient centrifugation. The erythrocytes were lysed using an ammonium chloride solution.

**TILs Culture**

Lung cancer tissues were kept in cold Hanks’ buffer and brought to the laboratory within 2–4 hours after surgery, and then remove the blood, adipose tissues, and connective tissues of the tumor tissues. The tumor tissues were cut into 1–2 mm³ pieces and plated into 24-wellplate, containing complete X-VIVO 15 medium (cat. 04-418Q; Lonza, Walkersville, MD 21793) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Grand Island, NY), 100 U/mL penicillin (cat. 15071163), 100 mg/mL streptomycin (cat. 15071163), and 1000 U/mL IL-2 (cat. 200-02; Peprotech, Rocky Hill, NJ 08553). Change the medium every 3 days, and expand the cells after the medium turned yellow.

**Flow Cytometry Analysis**

**Phenotypic Characterization**

The TILs from lung cancer patients and HD-PBMCs from healthy donors were incubated in 24-well plates precoated with 1 µg/mL α-CD3 (cat. 317315; Biolegend) in complete X-VIVO 15 medium contained 1 µg/mL α-CD28 (cat. 30923; Biolegend) and 1000 U/mL IL-2, with or without 7 µM TWS119 (cat. S1590; Selleckchem) for 9 days. TILs and HD-PBMCs cells were harvested on days 3, 5, 7, 9, and then stained for flow cytometry. The following panel of mouse anti-human mAbs, all purchased from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA), were used: anti-human CD3-APC.Cy7 (BD, 557832, SK7), anti-human CD4-Perp.Cy5.5 (BD, 560650, RPA-T4), anti-human CD45RA-FTTC (11-0458-42, HI100; eBioscience), anti-human CCR7-APF700 (BD, 561143, 150503), anti-human CD62L-PE-CF594 (BD, 562301, Dreg-56), anti-human CD95-PE.Cy7 (BD, 561633, DX2), and anti-human CD122-PE (BD, 554525). The cell data were acquired using a BD LSRFortessa analytical flow cytometer. Unstained and single fluorochrome-stained cells were served as controls to provide accurate compensation and data analysis. Cells were recorded per sample and the data were analyzed with FlowJo (version 10).

**Intracellular Staining**

The TILs and HD-PBMCs were cultured in α-CD3 precoated 24-well plates in complete X-VIVO 15 medium contained 1 µg/mL α-CD28 and 1000 U/mL IL-2, with or without 7 µM TWS119 for 5 days. The TILs and HD-PBMCs were then harvested and incubated in 24-well plates at 2×10⁶ cells per well in RP10 media (Roswell Park Memorial Institute, 10% heat-inactivated fetal bovine serum) alone or with phorbol 12-myristate13-acetate (PMA) (20 ng/mL) plus ionomycin (1 µg/mL) for 4–6 hours at 37°C in the presence of brefeldin A (10 µg/mL). The cells were harvested, washed with phosphate-buffered saline, stained on the surface phenotype markers, and fixed at room temperature with 4% Paraformaldehyde. The cells were then permeabilized (0.01% saponin), and the intracellular cytokines were stained using anti-human IFN-γ-V450 (BD, 560371, B27), anti-human IL-2-APC (BD, 554567, MQ1-17H12). All samples were tested using a BD LSRFortessa instrument. The data were analyzed using FlowJo software. PMA (cat. 16561-29-8; Sigma), ionomycin (cat. 10634; Sigma), brefeldin A (BFA) (cat. B7651; Sigma), bovine serum albumin and NaN₃ were all purchased from Sigma-Aldrich (St. Louis, MO).

**Statistical Analyses**

GraphPad Prism software version 5 was used for the statistical analysis. The Mann-Whitney test (2 tailed) and nonpaired Student’s t test were performed to identify significant differences. A value of P≤0.05 was considered statistically significant.

**RESULT**

**TWS119 Inhibits the IL-2 Production in Tn Cells in Blood from Healthy Donors**

Previous reports had shown that TWS119 preserve the naive-phenotypic molecular expression on mouse CD8+ T cells and induce Tn cells to produce the IFN-γ and IL-2 rapidly, but inhibit the proliferation of T cells by reducing IL-2 utilization. ¹⁴ ²⁰ Our previous researches indicated that the features of CD3⁺CD4⁺CD45RA⁺CD45RO⁻CCR7⁺ Tn cells in TILs from lung cancer were different from that of Tn cells in the PBMCs and lymph nodes. Tn cells in TILs had strong ability to produce IFN-γ and TNF-α, whereas Tn cells from PBMCs and lymph nodes produced a small amount of cytokines. ¹⁵ To investigate whether TWS119...
have different effect on Tn cells in TILs, we cultured TILs derived from NSCLC and PBMCs derived from healthy donors with TWS119 (7 μmol/mL) treatment, gated on the CD3+CD4+/CD3+CD8+CD45RA+CD45RO−CCR7− naive Tn-cell population (Table 1), detected the number of Tn cells at different time points (days 0, 3, 5, 7). The number of CD4+ Tn cells from PBMCs was comparable between the 119 treated and untreated groups; the number of CD4+ Tn cells in TILs increased slightly from days 5 to 7 after TWS119 treatment (Figs. 1A, B). IFN-γ (P = 0.0098) and IL-2 (P = 0.0026) production of CD4+ Tn cells in PBMCs were sharply reduced under TWS119 treatment; by contrast, TWS119 had no effect on the IFN-γ and IL-2 production of CD4+ Tn cells in TILs (Figs. 1C, D).

The frequency of CD8+ Tn cells was less than CD4+ Tn cells from TILs; after 7 days of treatment with TWS119, the number of CD8+ Tn cells in TILs and PBMCs was similar to that of the untreated group (Figs. 1E, F); the capacity of IFN-γ (P = 0.0263) and IL-2 (P = 0.0106) secretion in CD8+ Tn cells from PBMCs was inhibited by TWS119 (Figs. 1G, H). CD8+ Tn cells from TILs had analogous capacity to produce IL-2 in TWS119 treatment group and nontreatment group (Figs. 1G, H). These findings showed that TWS119 treatment does not affect the cytokine production of Tn cells in TILs.

**TWS119 has No Effect on the Proliferation of Tscm Cells in TILs From Human Lung Cancer**

Tscm cells are long-lived memory T cells, and share the characteristic features with stem cell, Tscm cells own the capacity for self-renewal and differentiating into Tcm, Tem, and Teff.1-4 Tscm cells are considered to be the most effective therapeutic cell type for tumor clearance in term of survival and effect function.1,8,21,22 Luca’s research identified that human CD95+CD122+ Tn-cell treated with TWS119 upregulated the expression of BCL-2 and CXCR3 which were associated stem cell marker. TWS119 could maintain the phenotype of Tscm cells in blood from healthy donors.6 Our research found the distinct function of Tscm cells in lung cancer.13 To investigate the clone expansion of Tscm cells in TILs with the presence of TWS119, we analyzed the proliferation and function of CD3+CD4+/CD3+CD8+CD45RA−CD45RO−CCR7−CD95−CD122− cells population in TILs (Table 1). We observed that the frequency of CD4+ and CD8+ Tscm cells in TILs between the TWS119-treated and untreated group was comparable (Figs. 2B F); although in the PBMCs group, there was a rapid expansion of Tscm cells in absence of TWS119; with the TWS119 treatment group, the proliferation of Tscm cells was not observed (Figs. 2A, E). TWS119 inhibited the IFN-γ production in CD4+ Tscm (Fig. 2C, P < 0.0001) and CD8+ Tscm cells (Fig. 2G, P = 0.0056) from PBMCs, but it did not affect the production of IL-2 (Figs. 2D, H). There was no change about the number of IFN-γ-secreting and IL-2-secreting Tscm cells from TILs with or without TWS119 treatment (Figs. 2C, D, G, H). Together, these results indicated that TWS119 has no effect on the proliferation and cytokine production of Tscm cells in TILs from human lung cancer.

**TWS119 Induces the IL-2 Production in Tcm Cells From TILs**

We identified the compartment and function of the subsets of T cells in TILs from lung cancer, we investigated that CD4+ T cells with memory phenotype cells take up nearly 77.9%, of which 20.4% are Tcm cells; 49.5% of CD8+ T cells is memory T cells, of which 11.8% are Tscm cells.16 TWS119 regulates the proliferation and cytokine production on the Tn cells and Tcm cells.6,13 We found that TWS119 can also induce the proliferation of CD4+ Tcm cells in PBMCs (Fig. 3A) and the expansion of Tcm cells (CD4+ and CD8+ Tcm) derived from TILs (Figs. 3B, F), but there is no significant difference compare to non-TWS119 treatment control group; TWS119 maintained a stable number of CD8+ Tcm cells in PBMCs from healthy donors (Fig. 3E); IFN-γ production in CD4+ Tm and CD8+ Tcm in PBMCs were inhibited by Tws119 (Fig. 3C, P = 0.028; Fig. 3G); TWS119 promoted the production of IL-2 in CD4+ Tcm cells (Fig. 3D, P = 0.04); and CD8+ Tcm cells (Fig. 3H) from TILs. These findings showed that TWS119 enhanced IL-2 production in Tcm cells from TILs.

**IL-2-secreting CD4+ Tem Cells Increased in TILs With TWS119 Treatment**

CD4+ Tem cells dominated in the CD4+ T cells in TILs, and 38% of CD8+ T cells are Tem cells.15 We have found that TWS119 affect the function and proliferation of Tcm cells in TILs. To understand whether TWS119 has a similar effect on Tem cells from TILs, we treated PBMCs and TILs with TWS119 at 7 μmol/mL, and we observed that the number of CD4+ Tem cells in PBMCs have a sharp decline at day 3 (Fig. 4A); but at day 5, the number of CD4+ Tem cells begun to increase (Figs. 4A, B) both in PBMCs and TILs. In CD8+ Tem cells, TWS119 maintained the relatively stable quantity in the process of cultivation (Fig. 4E, P = 0.04; Fig. 4F). TWS119 promoted Tem cells in TILs to produce IL-2 (Fig. 4D, P = 0.0078; Fig. 4H); but do not contribute to the production of IFN-γ (Figs. 4C, G). On the contrary, TWS119 inhibited CD4+ Tem cells to produce IFN-γ in PBMCs; it did not affect the IL-2 secretion in CD4+ Tem cells. These findings suggest that TWS119 has the opposite effect on the IL-2 production in Tem cells from TILs and PBMCs.

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**TABLE 1. Phenotype of Human Primary T-Lymphocyte Subsets for Flow Cytometry**

| Subsets | Surface Marker |
|---------|----------------|
| CD4+/CD8+ naive | CD3+CD4+/CD3+CD8+CD45RA+CD45RO−CCR7− |
| CD4+/CD8+ Tcm | CD3+CD4+/CD3+CD8+CD45RA−CD45RO−CCR7− |
| CD4+/CD8+ Tem | CD3+CD4+/CD3+CD8+CD45RA−CD45RO−CCR7− |
| CD4+/CD8+ Tscm | CD3+CD4+/CD3+CD8+CD45RA+CD45RO+CCR7+ |

Four human lymphocyte subsets in peripheral blood lymphocytes from healthy donors (n = 4), tumor-infiltrating lymphocytes from lung cancer patients (n = 5) gated by various surface marker combinations.

Tcm indicates central memory T cell; T effector T cell; Tem, effector memory T cells; Tscm, stem cell-like memory T cells.

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FIGURE 1. TWS119 inhibits the IL-2 production in naive T cells in blood from healthy donors. PBMCs were extracted from the blood of healthy donors and TILs were isolated from the lung cancer tissue which analyzed by flow cytometry. The frequency of CD3+CD4+CD45RA+CD45RO−CCR7+ T cells in PBMCs (A), CD3+CD4+CD45RA+CD45RO−CCR7+ T cells in TILs (B), CD3+CD8+CD45RA+CD45RO−CCR7+ T cells in PBMCs (E), CD3+CD8+CD45RA+CD45RO−CCR7+ T cells in TILs (F) treated with DMSO or 7 μM TWS119 for 0, 3, 5, 7, and 9 days (healthy donor, n = 4; lung cancer patients, n = 5). Flow cytometry analysis of cytokine production in naive T cells from PBMCs and TILs with DMSO or 7 μM TWS119 treatment for 5 days. Percentage of IFN-γ (C) and IL-2 (D) secretion in CD4+ Tn in response to phorbol 12-myristate13-acetate/ionomycin stimulation, expressed as the mean ± SEM. The events of IFN-γ-expressing (G) and IL-2-expressing (H) CD8+ Tn cells in the blood from healthy donors and TILs from non-small cell lung cancer patients, expressed as the mean ± SEM. *P < 0.05; **P < 0.01; Mann-Whitney test (2 tailed) and nonpaired Student t test. DMSO indicates dimethyl sulfoxide; PBMC, peripheral blood mononuclear cells; TILs, tumor-infiltrating lymphocytes.
FIGURE 2. TWS119 has no effect on the proliferation of Tscm cells in TILs from human lung cancer. Time course of proliferation in Tscm cells treated with DMSO or 7 μM TWS119 for 0, 5, 7, and 9 days and analyzed by flow cytometry; CD4+ Tscm cells from PBMCs (A) and from TILs (B); CD8+ Tscm cells from PBMCs (E) and from TILs (F). TILs from non–small cell lung cancer patients and PBMCs from healthy donors were stimulated for 4–6 hours with phorbol 12-myristate13-acetate+ionomycin at day 5 after cultured with DMSO or 7 μM TWS119. The mean frequency (±SEM) of the IFN-γ-expressing T cells gated on the CD4+ Tscm (C) and CD8+ Tscm cells (G); IL-2 expression in CD4+ Tscm cells (D) and CD8+ Tscm cells (H). **P < 0.01; ***P < 0.001; Mann-Whitney test (2 tailed) and nonpaired Student t test. DMSO indicates dimethyl sulfoxide; PBMC, peripheral blood mononuclear cells; TILs, tumor-infiltrating lymphocytes.
FIGURE 3. TWS119 induces the IL-2 production in central memory T cells from TILs. Cell events of CD4+ (A), CD8+ Tcm (E) cells from PBMCs and CD4+ (B), CD8+ Tcm (F) cells from TILs cultured with DMSO or 7 μM TWS119 for 0, 3, 5, 7, 9 days. Central memory T cells were gated on TILs and PBMCs, which were cultured for 5 days with DMSO or 7 μM TWS119 and stimulated for 4–6 hours with phorbol 12-myristate13-acetate-ionomycin, cytokine production was assessed by intracellular cytokine staining. The graphs show that relative frequencies of the IFN-γ-producing CD4+ Tcm (C), CD8+ Tcm cell (G), and IL-2-producing CD4+ Tcm (D), CD8+ Tcm cell (H) from human lung cancer patients and healthy donors. *P < 0.05; Mann-Whitney test (2 tailed) nonpaired Student t test. DMSO indicates dimethyl sulfoxide; PBMC, peripheral blood mononuclear cells; TILs, tumor-infiltrating lymphocytes.
FIGURE 4. IL-2-secreting CD4+ effector memory T cells increased in TILs with TWS119 treatment. Evaluation of Tem cells proliferation with DMSO or 7 μM TWS119 treatment at different time points (0, 3, 5, 7, 9 d); cell counts of CD4+ Tem cells (A) and CD8+ Tem cells (E) from PBMCs and CD4+ Tem cells (B) and CD8+ Tem cells (F) from TILs were analyzed by flow cytometry. Analysis of IFN-γ-secreting CD4+ Tem cells (C) and CD8+ Tem cells (G); IL-2-secreting CD4+ Tem cells (D) and CD8+ Tem cells (H) from TILs and PBMCs after culture with DMSO or 7 μM TWS119 for 5 days and stimulated with phorbol 12-myristate13-acetate-ionomycin. *P < 0.05; **P < 0.01; Mann-Whitney test (2 tailed) nonpaired Student t test. DMSO indicates dimethyl sulfoxide; PBMC, peripheral blood mononuclear cells; TILs, tumor-infiltrating lymphocytes.
FIGURE 5. TWS119 promotes the proliferation of CD8+ effector T cells in TILs from human lung cancer. Assessment of the growth of CD4+ effector T cells (A), CD8+ effector T cells (E) from PBMCs and CD4+ effector T cells (B), CD8+ effector T cells (F) from TILs cultured with DMSO or 7 μM TWS119 for 0, 3, 5, 7, 9 days. Expression of IFN-γ in CD4+ effector T cells (C), CD8+ effector T cells (G), and IL-2 in CD4+ effector T cells (D), CD8+ effector T cells (H) cultured with DMSO or 7 μM TWS119 for 5 days and activated with phorbol 12-myristate13-acetate-ionomycin. *P < 0.05; Mann-Whitney test (2 tailed) nonpaired Student t test. DMSO indicates dimethyl sulfoxide; PBMC, peripheral blood mononuclear cells; TILs, tumor-infiltrating lymphocytes.
TWS119 Promotes the Proliferation of CD8+ Teff Cells in TILs From Human Lung Cancer

Expression level of TCF-1 in Teff cells is lower than in Tn cells, and TWS119 can reverse the downregulation of TCF-1 and LEF-1 in activated T cell. Wnt signaling pathway induced by TWS119 severely limited the differentiation of activated T cell. We found that TWS119 do not affect the growth of CD4+ Teff cells (Figs. 5A, B) both in PBMCs and TILs group, and the expansion of CD8+ Teff cells from PBMCs was not affected (Fig. 5E); but significantly promoted the proliferation of CD8+ Teff cells in TILs, the frequency of CD8+ Teff cells in TILs increased from 40% at day 0 to 60% at day 5 with TWS119 treatment (Fig. 5F, P = 0.0245). The effect of TWS119 on Teff cells from PBMCs was observed, we found that TWS119 inhibit the production of IFN-γ in Teff cell from PBMCs (Fig. 5C, P = 0.0111; Fig. 5G), and similar inhibition was observed in IL-2 production (Fig. 5D, P = 0.0154; Fig. 5H); by contrast, IFN-γ and IL-2 production increased in CD8+ Teff cells from TILs with TWS119 treatment (Figs. 5G, H). These data suggest that TWS119 promotes the proliferation and cytokine production of CD8+ Teff cells in TILs.

DISCUSSION

Wnt signaling has an important role during the thymocyte development and peripheral T-cell differentiation, as the downstream of Wnt signaling, TCF-1 also participates in the formation of lymphoma as a tumor suppressor. ACT based on the TILs in cancer therapy-mediated tumor regression. Our previous research has elucidated the composition of different cell subgroups in TILs and Tn cells from TILs in human lung cancer have distinct cytokine-producing capacity compared to the Tn cells from blood and lymph node, whether the effect of TWS119 on T cells, Tscm cells and other subsets in TILs is unclear. In this study, we show that GSK-3β inhibitor TWS119 reduced expansion of T-cell subsets except CD8+ Teff cells from TILs, TWS119 significantly induced CD8+ Teff-cell proliferation. To further determine whether TWS119 treatment impaired the effect function of TILs, we stimulated TILs with polyclonal stimulation and measured IL-2 and IFN-γ production. Our data demonstrated that TWS119 do not affect the IFN-γ production on T cells in TILs compared with the control group, but IFN-γ expression in TWS119-treated CD4+ T cells (except CD4+ Tscm cells) was higher than in TWS119-treated CD4+ T cells from peripheral blood in healthy donors. TWS119 induced the IL-2 production in all subsets of CD8+ T cell from TILs, there is similar capacity to produce IL-2 in CD4+ Tn cell, Tscm and Teff cells compared with control groups. Effects of TWS119 on Tscm cells in TILs, the Forget research showed that TWS119 preserve the phenotype of CD8+ Tscm cells, promote IL-2 production, inhibit the IFN-γ production in the presence of TWS119 on TILs, about the IFN-γ production by the Tscm cells in TILs, which was different from our findings. These findings reveal that TWS119 have distinct effect on the proliferation and cytokine production of TILs, and provide new insights into the clinical application of TILs with TWS119 treatment for the adoptive immunotherapy.

We observed that TWS119-treated CD8+ Teff cells in TILs has obvious proliferation and amplification compared with control group, whereas TWS119 treatment limited the expansion of other T-cell subsets in TILs. The expansion in CD4+ Tcm both in PBMCs from healthy donors and in TILs increased (from 60% increase to 75%). Research show that TWS119 impairs T-cell activation, proliferation and differentiation; TWS119-treated cells retained low expression of T-cell activation marker CD69 and CD25; with the polyclonal nonspecific stimulation, there is no effect on the IL-2 production both on TWS119-treated T-cell group and control group, the mechanism of T-cell proliferation arrested by TWS119 induced is that TWS119 inhibit the IL-2R expression; but in our research, we observed that IL-2 production in Tn cells and CD4+ Teff cells from PBMCs with TWS119 treatment was significantly reduced, that was not consistent with published; in contrast, our data show that the frequency of IL-2 production on TWS119-treated CD4+ Tcm and Tem cells in TILs was significantly increased; we observed the proliferation of CD4+ Tcm cells after treatment with TWS119 for 9 days, not in CD4+ Tem cells, the reason needs to be confirmed by further research, we can check the expression of IL-2R on CD4+ T cells. TWS119 treatment also induced the IL-2 production on CD8+ T-cell subsets in TILs, and promote the significant expansion on CD8+ Teff cells in TILs. Our hypothesis is that the cell subgroups in TILs are different from those in PBMCs, and Wnt signaling has distinct effect on the CD4+ Tem cells and CD8+ Teff cells in TILs, the expression of IL-2R might not be block with TWS119 treatment. TWS119 treatment also inhibited IFN-γ production on T cells in response to PMA/ionomycycin stimulation. Our results show that TWS119-treated T-cell subsets in TILs did not effect on IFN-γ production compared with group, but TWS119 inhibits the IFN-γ production on T cells from PBMCs, that is consistent with the published research.

Our unpublished data indicate that TWS119 inhibits the growth of tumor cell line A549 (data not shown); in this study, we observed that TWS119 significantly promote the proliferation of CD4+ Tcm in TILs, and has a tendency to promote IL-2 secretion of CD8+ Teff, CD8+ Tem and CD8+ Tcm cells and has no effect on the IFN-γ production; at same time, TWS119 can maintain Tn-cell population in TILs, Tn cell in TILs may be Tscm cells (unpublished data). By observing the effects of TWS119 on T-cell proliferation and function in TILs, TWS119 can be predicted to have potential clinical application in the treatment of cancer.

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Conflicts of Interest/Financial Disclosures

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