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Wnt pathway activator TWS119 enhances the proliferation and cytolytic activity of human γδT cells against colon cancer

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ABSTRACT

γδT cells are a distinct T-cell subset that display unique characteristics regarding T-cell receptor gene usage, tissue tropism and antigen recognition. Adoptive γδT cell transfer therapy has recently been gaining importance as an efficient approach in cancer immunotherapy. However, exploiting γδT cell response for tumour immunotherapy is a challenge due to cell numbers, activities and differentiation states that minimize the clinical therapeutic effects. Previous studies have indicated that the wnt/β-catenin signalling pathway plays a crucial role in the differentiation, survival and enhancement of the immune response of T lymphocytes. In this study, we sought to evaluate whether the activation of the wnt/β-catenin pathway through inhibition of glycogen synthase kinase-3β (GSK-3β) using 4,6-disubstituted pyrrolopyrimidine (TWS119) could be an efficient strategy to improve the proliferation, differentiation and cytolytic activity of γδT cells against colon cancer cells. Remarkably, we found that TWS119 significantly enhanced the proliferation and survival of γδT cells via activation of the mammalian target of rapamycin (mTOR) pathway, upregulation of the expression of the anti-apoptotic protein Bcl-2 and inhibition of cleaved caspase-3 in addition to the Wnt pathway. Our results also showed that enhancement of the cytolytic activity of γδT cells against human colon cancer cells by TWS119 was chiefly associated with upregulation of the expression of perforin and granzyme B *in vitro* and *in vivo*. Additionally, TWS119 can induce the expression of CD62L or CCR5 to generate a population of $CD62L^+$ γδT or $CCR5^+$ γδT cells in a dose-dependent manner. These findings suggested that TWS119 could be a useful complementary agent for improving γδT cell-based immunotherapy.

1. Introduction

Most current adoptive cell therapy (ACT) using autologous αβT cells, which is dependent on major histocompatibility complex (MHC), can effectively treat different types of human cancer. However, some cancer cells evade the cytotoxic effect of αβT cells by downregulating the expression of MHC class I molecules. γδT cells are a component of innate immunity and account for approximately 1–5% of the total T cell population. They can rapidly be activated upon encountering nonpeptide phosphoantigens or pathogen-derived antigens. Importantly, γδT cells can recognize their antigens without processing by antigen presenting cells (APCs) [1,2]. Additionally, activated γδT cells can produce abundant levels of interferon-γ (IFN-γ), perforin and granzyme B and exhibit potent cytotoxicity against autologous tumuor cells [3–5]. Therefore, γδT cells have become an important option for cancer immunotherapy [6–9]. Although it seems that utilizing γδT cells could be a promising cancer therapeutic approach, there is still significant amount of work to be done to optimize cell culture conditions, improve anti-tumuor efficacy, and importantly, increase the persistence of transferred cells.

The evolutionarily conserved wnt/β-catenin signalling pathway has been shown to promote haematopoietic stem cell self-renewal and

Abbreviations: ACT, Adoptive cell therapy; APCs, Antigen presenting cells; CAR, Chimeric antigen receptor; CCR5, CC-chemokine receptor 5; CFSE, Carboxyfluorescein succinimidyl ester; CRC, Colorectal cancer; GSK-3β, Glycogen synthase kinase-3β; IFN-γ, Interferon-γ; Lactate dehydrogenase, LDH; MHC, Major histocompatibility complex; mTOR, Mammalian target of rapamycin; PBMCs, Peripheral blood mononuclear cells; PVDF, Polyvinylidene difluoride; rhIL-2, Recombinant human interleukin-2; T_{SCM} cells, T memory stem cells; TWS119, 4,6-disubstituted pyrrolopyrimidine

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multipotency and regulate the progression of thymocyte development at different stages [10–13]. Importantly, Gattinoni et al. [14] reported that induction of the wnt signalling pathway by glycogen synthase kinase-3β (GSK-3β) inhibitor 4,6-disubstituted pyrrolopyrimidine (TWS119) in mouse tumour-specific anti-gp100 $CD8^+$T cells resulted in the generation of T memory stem (T_{SCM}) cells. These T_{SCM} cells preserved a $CD44^{low}CD62L^{high}$ naive phenotype even after undergoing several cycles of cell division and had superior proliferative and anti-tumour activity in vivo compared with central or effector memory T cells. Recently, the authors generated a clinical-grade CD19-specific chimeric antigen receptor (CAR)-modified CD8+ T_{SCM}, which exhibited superior anti-tumour responses compared with CD19-CAR T cells for the treatment of human B cell malignancies. Currently, this strategy is being tested in clinical trials [15]. However, the role of the wnt/β-catenin signalling pathway in γδT cells is not yet clearly understood.

In this study, we sought to evaluate the effects of activation of the wnt/β-catenin pathway, through inhibition of GSK-3β using TWS119, on the proliferation, differentiation and cytolytic activity of γδT cells against colon cancer cells. Here, we demonstrated that the activation of the wnt signalling pathway with a low concentration of TWS119 (< 4 μM) significantly enhanced the proliferation and survival of γδT cells via activation of the mammalian target of rapamycin (mTOR) pathway, upregulation of the expression of anti-apoptotic protein Bcl-2 and inhibition of cleaved caspase-3. Concurrently, the enhancement of γδT cell cytolytic activity by TWS119 against human colon cancer cells was associated with the upregulation of perforin and granzyme B expression. On the other hand, high concentrations of TWS119 (> 4 μM) appeared to be optimal for generating a population of $CD62L^+$γδT or $CCR5^+$γδT cells, but the cells proliferated poorly at this concentration. Our results suggest that TWS119 could be a useful complementary agent for improving γδT cell-based immunotherapy.

2. Materials and methods

2.1. Reagents and antibodies

TWS119 (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 8 mM and stored at −80 °C. 0, 0.5, 1, 2, 4, and 8 μM of TWS119 were used in this study, and the group (0 μM) was treated with DMSO (0.00625%) as a control, which has no effect on γδ T cell proliferation. Pamidronate disodium was obtained from Neptunus Pharmaceutical (Shenzhen, China). Recombinant human IL-2; fluorochrome-conjugated monoclonal antibodies against γδTCR, δ1TCR, δ2TCR, granzyme B, perforin, CCR5, CD62L, and CD45RA; and isotype controls were from BD Biosciences (San Jose, CA, USA). The primary antibodies against GAPDH, Caspase-3, Bcl-2, p-mTOR (Ser2448) and p-Akt (Ser473) were purchased from Epitomics Inc (Burlingame,CA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

The following human colon cancer cells lines, HCT116, SW620 and SW480, were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). Tumour cells were cultured in RPMI 1640 complete medium (10% heat-inactivated foetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin) at 37 °C in a 5% CO_2 incubator.

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood, which was obtained from healthy donors or cancer patients with the approval of the local ethics committee including written informed consent. PBMCs were separated using Ficoll-Hypaque gradient centrifugation. For γδT cell expansions, 5×10^5 cells/ml of fresh PBMCs were activated using pamidronate disodium (5 μg/ml) and recombinant human interleukin-2 (rhIL-2) (200 U/ml) in RPMI 1640 complete medium supplemented with 5% human AB serum in 5% CO_2 at 37 °C. Complete medium was added every 2 days. After 8 days, cultured cells were harvested, stained with FITC-conjugated γδTCR and analyzed by FACS Calibur analyzer (BD Biosciences).

2.3. Purification of γδT cells

After 8 days of culture, the cells were resuspended in phosphate buffer saline (PBS) supplemented with 2% FBS and 2 mM EDTA. Subsequent positive selection was carried out using magnetic beads coated with anti-γδTCR Ab according to the cell selection protocol (Miltenyi Biotec Inc. Auburn).

2.4. Proliferation assay

PBMCs were cultured with pamidronate disodium for 8 days and then cells were labelled with or without 1.5 μM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) according to the manufacturer's instructions and CFSE-labelled cells were then seeded in 6-well plates (2.5×10^6 cells/well) followed by treatment with various concentrations of TWS119 for 72 h. The total number of cultured cells was evaluated using an automated cell counter (Inno-Alliance Biotech, USA) and the γδT cell proliferation was examined by flow cytometry.

2.5. Flow cytometry

The phenotypic profile of cultured cells was characterized by flow cytometry using the following monoclonal antibodies: FITC/APC-conjugated γδTCR, FITC-conjugated δ1TCR, FITC-conjugated δ2TCR, PerCP-Cy5.5-conjugated CD45RA, PE-conjugated CCR5, PE-conjugated CD62L, PE-conjugated perforin and PE-conjugated granzyme B (BD Biosciences). Surface staining was performed at 4 °C in the dark for 30 min in the staining buffer. For intracellular staining, cells were treated with fixation/permeabilization kit (BD Biosciences) according to the kit instructions. Isotype-matched fluorescent antibodies were used as negative controls. The apoptosis of γδT cells was assessed by Annexin V-FITC and 7-AAD staining following the manufacturer's instruction (BD Biosciences). Finally, cells were analyzed by FACS Calibur analyzer (BD Biosciences).

The data were analyzed by FlowJo software (Tree Star).

2.6. Cytotoxic assays

Expanded γδT cells were positively purified and incubated with various concentrations of TWS119 (0.5, 1.0, 2.0, 4.0, and 8.0 μM) for 72 h. Effector cells (γδT cells) were pretreated with or without 100 nM of concanamycin A (Tocris Bioscience) for 2 h at 37 °C to block the perforin-granzyme pathway and followed by incubation with target cells (HCT116, SW620 and SW480 cells), respectively and separately, at an effector-target ratio of 10:1. After 6 h of cell culture, the supernatants were collected for cytotoxic assays using a lactate dehydrogenase (LDH) release assay kit as previously described [16]. All tests were performed in triplicate (N = 3), and the percentage of LDH release was calculated according to the following formula: % cytotoxicity = (OD experimental release group − OD effector natural release group) / (OD target cell maximum release group − OD target cell natural release group) × 100%.

2.7. Western blotting

The proteins samples were extracted from positively purified γδT cells of different treatment groups. After mixing with 5 × loading buffer and heating for 5 min at 95 °C, proteins were separated by 10% SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare). Before separate incubations with the primary antibodies: GAPDH, β-catenin, Bcl-2, caspase-3, p-Akt or p-mTOR overnight, the membranes were blocked with 5% BSA in TBST for 1 h.