Lentiviral delivery of CTLA-4 shRNA improves the expansion of cytokine-induced killer cells and enhances cytotoxic activity in vitro

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Abstract. Cytokine-induced killer (CIK) cells are in vitro-expanded cells harboring potent toxicity against tumor cells. Recently, it was identified that the cytotoxicity and proliferation of CIK cells are restricted by a prolonged CIK cell culture period. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) serves a negative role in T cell activation and proliferation. This study aims to determine whether CTLA-4 expression is associated with the inhibition of CIK cells. CIK cells were generated from peripheral blood mononuclear cells (PBMCs), and CTLA-4 shRNA (shCTLA-4) lentivirus was applied to knockdown CTLA-4 expression in CIK cells. The proliferation of CIK cells was evaluated following shCTLA-4 lentiviral transduction, and the cytotoxicity of CIK cells was investigated using the CytoTox 96 Non-Radioactive Cytotoxicity assay. The expression of CTLA-4 in CIK cells was significantly increased, compared with that in PBMCs. The shCTLA-4 lentivirus efficiently knocked down the expression of CTLA-4 in CIK cells. The shCTLA-4 lentivirus transduction of CIK cells promoted the proliferation of CIK cells in vitro (3.18±0.19-fold vs. 2.42±0.29-fold). Furthermore, the cytotoxicity of shCTLA-4 lentivirus-transduced CIK cells was significantly improved compared with that of control shRNA lentivirus-transduced CIK cells (54.5±2.13% vs. 30.5±1.67%). Thus, the suppression of CTLA-4 expression increases cytotoxicity and ex vivo expansion of CIK cells, which indicates a clinical significance for CTLA-4 blockade in CIK cell therapy.

Introduction

Cancer immunotherapy exerts beneficial effects through mediating tumor cell regression (1), which relies on activating antitumor T cells. Co-stimulatory molecules, such as CD80 (B7-1) and CD86 (B7-2), expressed on the surface of dendritic cells (DCs) or other antigen presenting cells (APCs), regulate T cell activation and proliferation by interacting with CD28 (2,3). Conversely, upon activation, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is expressed on the T cell surface, and also interacts with CD80 and CD86. However, contrary to CD28, CTLA-4 serves an inhibitory co-stimulatory role in regulating T cell activation and proliferation (4,5). It was reported that CTLA-4 downregulates the activation of T cells by inhibiting IL-2 production and cell cycle progression (6). It has been verified that CTLA-4 competes with CD28 and acts as an inhibitory receptor to influence T cell function (7).

Adoptive cell therapy (ACT) is a promising anticancer therapy option (8). Cytokine-induced killer (CIK) cells, a heterogeneous subset of expanded lymphocytes, exhibit notable cytotoxic activity against tumor cells (9-11). CIK cells are generated from peripheral blood mononuclear cells (PBMCs), with a cytokine cocktail including interferon (IFN)-γ, anti-CD3 monoclonal antibody, interleukin (IL)-2 and IL-1α (12). Clinically, a limited expansion rate and low in vivo toxicity are significant shortcomings of CIK cell therapy. The present study hypothesized that CTLA-4 expression increases when PBMCs are induced into CIK cells during in vitro culture. In the present study, lentivirus-mediated RNA interference was employed to knock down CTLA-4 expression in CIK cells and explore the effects of CTLA-4 inhibition on the proliferation and toxicity of CIK cells.

Materials and methods

Cell culture. The A549 human lung carcinoma cell line (no. 59239596; American Type Culture Collection, Manassas, VA, USA) was cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.). PBMCs from healthy adults were collected by cell
applied to the manufacturer's protocol. Briefly, shCTLA-4 lentiviral particles (Hanbio Biotechnology Co., Ltd., Shanghai, China), containing a 29-mer shRNA sequence 5'-GGA ATG AGT TGA CCT TCC AGT AGT TGA -3' and reverse, 5'-ACT GTC ACC CGG ACC TCA GTT T-3', were used to knockdown the expression of CTLA-4 in CIK cells. On day 10, CIK cells were transduced with shCTLA-4 lentiviral particles at multiplicity of infection of 10. A total of 96 h later, the transduction efficiency was estimated by detecting CIK cells expressing green fluorescence protein under a fluorescence microscope.

Polymerase chain reaction (PCR). Total RNA was isolated using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany), and reverse transcribed as single-stranded cDNA. cDNA was then used as the template to amplify CTLA-4 via PCR with the following primers: Forward, 5'-GACCTGGCCCTGCACTCT CCTGTIT-3' and reverse, 5'-ACTGTCACCCGGACCTCA GTGGCTT-3'. GAPDH was employed as the internal control. Primers for the amplification of GAPDH are as follows: Forward, 5'-TGCTTCTTGACACCATCACT-3' and reverse, 5'-CCGGTCAGCTCAGGGATAGA-3'. PCR was carried out at 94°C for 30 sec, 55°C for 30 sec and then 72°C for 30 sec.

Western blotting. For protein analysis, total protein was extracted from 1x10⁷ CIK cells using RIPA lysis buffer (BestBio, Shanghai, China) and then quantified using a BCA kit (Pierce; Thermo Fisher Scientific, Inc.). The band signals were analyzed using Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc.). The protein samples were separated using SDS-PAGE on a 10% gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was blocked using 2.5% non-fat milk for 1 h at room temperature and then incubated with rabbit monoclonal antibody against human CTLA-4 (cat. no. SC-9094; 1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or rabbit polyclonal antibody against human GAPDH (cat. no. SC-25778; 1:1,000; Santa Cruz Biotechnology, Inc.) or rabbit polyclonal antibody against human GAPDH (cat. no. SC-25778; 1:1,000; Santa Cruz Biotechnology, Inc.) for 2 h at 25°C followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (cat. no. SC-2004; 1:2,000; Santa Cruz Biotechnology, Inc.) for 1 h at 25°C prior to detection with chemiluminescence (FluorChem™ HD2 system; ProteinSimple; Bio-Technne, Minneapolis, MN, USA). The expression of CTLA-4 was normalized to that of GAPDH.

Cytotoxicity assay. CIK cell-mediated cytotoxicity was assessed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. Briefly, shCTLA-4 lentiviral particle-transduced CIK cells, control shRNA (shControl) lentiviral particle-transduced CIK cells and the CIK cells without lentivirus transduction were suspended in RPMI-1640 medium, supplemented with 5% FBS, at a density of 2x10⁴ cells/ml. The control and experimental wells were set up using a round-bottom 96-well culture plate. The wells which only contained CIK cells served as the control for the spontaneous LDH release effector cells; the experimental wells contained CIK cells and A549 cells at a ratio of 20:1. Cells were centrifuged at 250 x g for 4 min at 20°C after incubation at 37°C for 4 h. For target cell maximum LDH release control wells, the lysis solution was added 45 min prior to supernatant harvest. A total of 50 µl supernatant from each well of the assay plate was transferred to a flat-bottom 96-well plate that was pre-loaded with 50 µl/well reconstituted substrate mix. Following incubation at room temperature while protected from light for 30 min, 50 µl stop solution was added to each well followed by reading the absorbance at 490 nm. The cytotoxicity of CIK cells was calculated as follows: [(Experimental-effector spontaneous-target spontaneous)/(target maximal-target spontaneous)]x100.

To further confirm the cytotoxicity of CIK cells, a more convenient and intuitive cell counting assay was used to detect the cytotoxicity of the shCTLA-4 lentiviral particle-transduced CIK cells. As aforementioned, the CIK cells transduced with shCTLA-4 lentiviral particles or shControl lentiviral particles, and the CIK cells without lentivirus transduction were co-cultured with A549 cells at a ratio of 20:1 (effector cells:target cells). For the negative group, only A549 cells were seeded and cultured for 24 h. According to the morphology of adhesive A549 cells and suspended CIK cells, the residual A549 cells from all groups were observed under a microscope and counted following trypan blue dye exclusion. The cytotoxicity of effector cells could be deduced from the summation of A549 cells that survived the CIK cells.

Statistical analysis. All statistical analyses were performed using SPSS software (version 19.0; IBM Corp, Armonk, NY, USA). The band signals were analyzed using Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc.). The data are presented as the mean ± standard error of the mean. Two or multiple group comparisons were performed using a Student's t-test or one-way analysis of variance with Fisher's least significant difference post hoc test, respectively. P<0.05 was considered to indicate a statistically significant difference.

Results

The expression of CTLA-4 increases with the induction of CIK cells. To compare the expression levels of CTLA-4 between CIK cells and the corresponding PBMCs, CIK cells and PBMCs were used to prepare total RNA and protein. As evaluated using RT-PCR and western blot analysis, the mRNA and protein expression levels were much higher in CIK cells than those in PBMCs (Fig. 1A and B). These observations suggested that CTLA-4 expression increases during the induction of CIK cells.

shCTLA-4 lentivirus efficiently inhibits CTLA-4 expression. The shCTLA-4 lentiviral particles were employed to knock out the expression of CTLA-4.
down CTLA-4 expression in CIK cells. As determined by PCR, shCTLA-4 lentiviral particles significantly decreased CTLA-4 mRNA levels in CIK cells when compared with the shControl lentiviral particles (Fig. 2A). As analyzed by western blotting, shCTLA-4 lentiviral particles significantly reduced the expression levels of CTLA-4 protein in CIK cells when compared with the shControl lentiviral particles (Fig. 2B). These data indicate that shCTLA-4 lentiviral particles can successfully silence the expression of CTLA-4 in CIK cells.

**Silencing CTLA-4 promotes the proliferation of CIK cells.** To examine whether silencing CTLA-4 with shCTLA-4 lentivirus affects the proliferation rate of CIK cells, CIK cells were transduced with shCTLA-4 lentivirus or shControl lentivirus. A total of 96 h after viral transduction, cell counting was performed. Morphologically, the shCTLA-4 lentivirus-transduced CIK cells presented with more agglomeration than the shControl lentivirus-transduced CIK cells did (Fig. 3A). Compared with control transduction of shControl lentivirus, the transduction of shCTLA-4 lentivirus promoted the ex vivo expansion of CIK cells (Fig. 3B). These findings indicated that transduction of shCTLA-4 lentivirus enhances the proliferation of CIK cells.

**Silencing CTLA-4 improves the cytotoxicity of CIK cells.** As determined by a CytoTox 96 Non-Radioactive Cytotoxicity Assay, the shCTLA-4 lentivirus-transduced CIK cells caused more LDH release against A549 cells compared with the shControl lentivirus-transduced CIK cells did (Fig. 4A). A further cell counting assay indicated that, compared with the shControl lentivirus-transduced CIK cells, a decreased number of A549 cells survived under the cytotoxic activity of shCTLA-4 lentivirus-transduced CIK cells (Fig. 4B and C). These findings indicate that the cytotoxicity of CIK cells against A549 cells can be enhanced through the inhibition of CTLA-4 expression in CIK cells.

**Discussion**

As a potential anti-tumor ACT, CIK cells have been extensively tested in clinical practice (13-16). The ex vivo expansion rate and cytotoxicity are two key factors that determine the therapeutic...
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Efficacy of CIK cells. CIK cells specifically kill tumor cells via the secretion of IFN-γ, tumor necrosis factor-α or perforin (16). However, the activation of immune suppression pathways induces T cell anergy (17). By competing with CD28, CTLA-4 serves anegative role in the regulation of T-cell activation. CTLA-4 inhibits T cell activation through the inhibition of signal transduction of the T cell receptor and transmitting the inhibitory signal to T cells (17-19). These maybe associated with...
the regulation of CTLA-4: CD28/CD80 axis. It was reported that CD80 serves a pivotal role in immune cell-mediated cytotoxicity. Chambers et al (20) indicates that NK cell-mediated cytotoxicity is a result of interactions between triggering signals and the inhibitory receptors and ligands, during which CD80 is the triggering signal for tumor cell lysis.

In the present study, we demonstrated that the expression of CTLA-4 on CIK cells is upregulated compared with that on the corresponding PBMCs. This is concordant with the results of previous reports, in that the responsiveness of IL-2 led to the induction of CTLA-4, particularly for the IL-2-expanded immune effector cells (21,22). It was hypothesized that PBMCs, when induced with a CIK cell cytokine cocktail, are activated and this triggered the activation of inhibitory signal to balance the immune response of CIK cells represented by the concurrent upregulation of CTLA-4 and CD28 (23,24).

It was further demonstrated that the suppression of CTLA-4 expression by lentiviral vector-mediated RNA interference (RNAi) on CIK cells promoted proliferation and the killing efficiency of CIK cells, which confirmed CTLA-4's inhibitory effect on CIK cells. Since regulatory T cells (T\text{regs}) are crucial for the maintenance of immune tolerance, especially for autoimmunity and the tumor microenvironment (25-27), it was hypothesized that the effect of inhibiting CTLA-4 expression on CIK cells may alter the balance of T\text{regs}. T\text{regs} express a high level of CD25 (the IL-2 receptor \(\alpha\) chain) and compete with other effector cells to bind IL-2, resulting in cytokine-mediated immune suppression (6,28). Furthermore, it has been reported that the interaction of CTLA-4 on T\text{regs}, with CD80 and CD86 on APCs can block and even downregulates the expression of CD80 and CD86 (29). It was also reported that blocking CTLA-4 has an impact on the balance of T\text{regs} and downregulates T\text{regs}-mediated suppression, but the mechanisms are still unclear (23,30).

Although the results have revealed the immune suppression of CIK cells mediated by CTLA-4, they were unable to...
determine the exact mechanisms by which CTLA-4 regulates the proliferation and activation of CIK cells, which is the distinct limitation of this study.

To conclude, the present study demonstrated that CTLA-4 expression increased when PBMCs were induced into CIK cells by cytokine cocktail, RNAi mediated by lentiviral particles efficiently inhibited the expression of CTLA-4 on CIK cells, suppression of CTLA-4 expression significantly promoted the proliferation of CIK cells in vitro and enhanced the cytotoxicity of CIK cells against lung cancer cells. These findings indicate that the blockade of CTLA-4 signaling has potential therapeutic significance for CIK cell therapy and further clinical study is warranted to verify these results.

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