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Modification of cytokine-induced killer cells with folate receptor alpha (FRα)-specific chimeric antigen receptors enhances their antitumor immunity toward FRα-positive ovarian cancers

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ABSTRACT

Folate receptor alpha (FRα) is aberrantly expressed in ovarian cancers but largely absent in normal tissues, and therefore represents an attractive target for immunotherapy. In recent years, modification of T cells with chimeric antigen receptor (CAR) targeting FRα has been reported to improve antitumor immunity of T cells. However, there are limited data regarding CAR-modified cytokine-induced killer (CAR-CIK) cells. In the present study, we modified CIK cells with FRα-specific CARs and investigated their antitumor immunity against ovarian cancers. We found that both non-transduced and mock CAR-transduced CIK cells showed only low antitumor activity against either FRα-positive ($FR\alpha^+$) or FRα-negative ($FR\alpha^-$) targets. However, all three generations of CAR-modified CIK cells showed enhanced antitumor activity against $FR\alpha^+$ targets, but not $FR\alpha^-$ targets. First-generation ζ-CAR-CIK cells increased production of IFN-γ, enhanced short-term cytotoxicity against $FR\alpha^+$ ovarian cancer cells, and showed modest and short-term suppression of established tumors; while second-generation 28ζ- and third-generation 28BBζ-CAR-CIK cells showed significant proliferation, enhanced secretion of IL-2, eliminated the $FR\alpha^+$ ovarian cancer cells in long-term co-culture, and showed dramatic and long-term inhibition of tumor growth and prolonged survival of xenograft-bearing mice. It is noteworthy that the 28BBζ-CAR was more potent in the modification of CIK cells than 28ζ-CAR both *in vitro* and *in vivo*. Moreover, CAR-CIK cells showed more efficient anticancer activity compared with CAR-T cells *in vitro*, but less efficient than CAR-T cells *in vivo*. According to these results, we conclude that modification of CIK cells with FRα-specific CARs enhances their antitumor immunity to $FR\alpha^+$ ovarian cancers. The third-generation 28BB-ζ CAR containing 4-1BB co-stimulation was more efficient in modification of CIK cells than either first-generation ζ-CAR or second-generation CD28-ζ-CAR.

Abbreviations: ACT, adoptive cellular immunotherapy; AICD, activation induced cell death; ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; BsAb, bispecific antibody; CA125, cancer antigen-125; CARs, chimeric antigen receptors; CBMCs, cord blood mononuclear cells; CEA, carcinoembryonic antigen; CFSE, carboxyfluorescein succinimidyl ester; CIK, cytokine induced killer; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; FRα, folate receptor-alpha; GPI, glycosylphosphatidylinositol; GVHD, graft-versus-host disease; HSV-TK, herpes simplex virus thymidine kinase; ICOS, inducible costimulator; IFN-γ, interferon-gamma; IL, interleukin; LAK, lymphokine-activated killer; LDH, lactate dehydrogenase; mAb, monoclonal antibody; MHC, major histocompatibility complex; MIC, MHC class I chain-related molecules; MOI, multiplicity of infection; NK, natural killer; NKG2D, natural killer group 2 D; NKT, natural killer-like T; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI, polyvinylidene difluoride; scFv, single chain variable fragment; SD, standard deviation; shRNA, short hairpin RNA; TIL, tumor-infiltrating lymphocytes; TM, transmembrane; ULBP, UL16 binding protein.

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1. Introduction

FRα is a GPI-anchored membrane protein that binds folate with high affinity and mediates folate uptake by receptor-mediated endocytosis (Rijnboutt et al., 1996). FRα is overexpressed in many different types of human malignancies including ovarian, breast, lung, colorectal, renal and other solid cancers, but is largely absent in normal tissues (Lu and Low, 2003). Notably, overexpression of FRα is associated with tumor progression and poor prognosis in ovarian and breast cancers (Kelemen, 2006). These features make it a reasonable target for antitumor immunotherapy. Several anti-FRα monoclonal antibodies (*i.e.*, MOv18, MOv19, LK26 and the LK26-derived antibody (Ebel et al., 2007; Rettig et al., 1985; Miotti et al., 1987)) have been developed for the treatment of ovarian cancers. However, a considerable number of FRα-positive tumors do not respond to FRα monoclonal antibodies (Vergote et al., 2016). CAR-engineered T cells that target FRα have been generated and investigated in *ex-vivo* experiments and in animal models of FRα-overexpressing malignancies (Hwu et al., 1993; Song et al., 2011; Song et al., 2012); and two phase-I clinical trials have been conducted to assess the safety of the FRα-targeted CAR-T cells (Kershaw et al., 2006; Kandalaft et al., 2012). In one phase-I study, T cells redirected with a MOv18 scFv-based CAR demonstrated safety and feasibility in the treatment of patients with advanced ovarian cancer. However, therapy efficiency was limited by the inability to generate long-term persistence of transferred CAR-T cells (Kershaw et al., 2006).

Besides T cells, cytokine-induced killer (CIK) cells and nature killer (NK) cells are also selected as immune effector cells for CAR modification. CIK cells, also known as natural killer-like T (NKT) cells, are polyclonal T lymphocytes that share properties of both T and NK cells (Gutgemann et al., 2007). CIK cells are efficiently generated *in vitro* from peripheral blood mononuclear cells (PBMCs) or cord blood mononuclear cells (CBMCs) by the addition of anti-CD3 monoclonal antibody (mAb), interferon-gamma (IFN-γ), interleukin (IL)-1 and IL-2 (Schmidt-Wolf et al., 1991). CIK cells are endowed with a potent MHC-unrestricted tumor cell-killing activity, and, advantageously, can recognize and kill tumor targets without prior priming or activation (Gammaitoni et al., 2013). The MHC-unrestricted cytotoxicity of CIK cells is principally mediated by the interaction between natural killer group 2 D (NKG2D) molecules on CIK cells and MHC class I chain-related molecules (MIC) A/B or UL16 binding protein (ULBP) molecules on tumor cells (Sangiolo, 2011). CIK cells are of particular interest in the eradication of residue cancer cells and the prevention of recurrence due to the properties of easy availability, high proliferation rate, and widely MHC-unrestricted antitumor activity (Lowdell et al., 2001). Over the past 25 years, CIK cells have been explored for their antitumor activity toward a variety of tumors *in vitro* and *in vivo*, and have been shown to be effective in the treatment of both hematologic and solid malignancies (Schmeel et al., 2014). However, due to their low cytolytic activity against a wide range of tumor cells and their lack of antigen specificity, the clinical application of CIK cells is limited. Moreover, since CIK cells are terminally differentiated effector T cells, they might have only limited survival *in vivo* (Franceschetti et al., 2009). Therefore, novel strategies need to be developed to improve the antigen specificity and *in-vivo* persistence of CIK cells after adoptive immunotherapy.

To enhance the killing capacity and antigen specificity against tumor cells, several strategies have been generated to modify CIK cells. The most common method entails using artificial bispecific antibodies (BsAbs), which are typically designed to recognize receptors on effector cells and tumor-associated antigens on tumor cells simultaneously (Tita-Nwa et al., 2007; Fanger and Guyre, 1991). Previous studies have shown that BsAbs against cancer antigen-125 (CA125) or Her-2 can successfully redirect and enhance the cytotoxicity of CIK cells against ovarian tumor cells (Zhang et al., 2013). Besides BsAbs, chimeric antigen receptors (CARs) were also used to redirect the specificity of CIK cells against tumor cells in recent years (Hombach et al., 2013; Pizzitola et al., 2014). CARs are usually composed of a single-chain variable fragment (scFv), a CD3-signaling domain and one or more co-stimulatory signaling modules, such as CD27, CD28, CD137 (4-1BB), CD134 (OX40) and/or ICOS (Song et al., 2012; Cartellieri et al., 2010; Milone et al., 2009; Sadelain et al., 2009). CAR-engineered T cells have been tested for their anti-tumor activities against a wide variety of tumor-specific antigens, such as CD19, Her-2 and FRα in animal models (Song et al., 2011; Milone et al., 2009; Zhao et al., 2009); and some of these have proceeded to clinical trials (Kalos et al., 2011; Porter et al., 2011). Importantly, the results of CAR-T cells specific for CD19 have shown dramatic antitumor activity against B-cell leukemia and lymphoma, including long-term tumor remission (Porter et al., 2011). Similarly, CD19 CAR-redirected CIK cells render them highly cytolytic toward CD19-expressing B cell and pre-B-cell ALL cells (Marin et al., 2006; Oelsner et al., 2016). Most recently, CIK cell activity against AML has been improved by genetic modification with a CAR specific for the myeloid antigens CD33 and CD123 (Pizzitola et al., 2014). These results indicated that immunotherapy for cancer using genetically modified CIK cells might constitute a novel and promising approach.

In the present study, we modified CIK with all three generations of FRα-specific CARs. The antitumor immunity of the CAR-CIK cells against ovarian cancers was systematically investigated by using both *in-vitro* and *in-vivo* methods, including cytotoxicity assay, cytokine release assay, and xenograft tumor mouse models. We compared the antitumor efficacy of CAR-CIK cells modified with first-, second-, or third-generation CARs against $FR\alpha^+$ cancer; and the antitumor activity between CAR-CIK cells and CAR-T cells was also compared.

2. Materials and methods

2.1. Construction of CARs and preparation of lentivirus

The DNA sequence of anti-FRα scFv was derived from the MOv19 antibody (Miotti et al., 1987). A $(G_4S)_3$ flexible linker was added between the heavy- (VH) and light- (VL) chains of the scFv. The full-length cDNA fragment of 28BBζ-CAR was synthesized by TaKaRa (Dalian, China). The DNA fragments of scFv-hinge-TM, scFv-hinge-TM-CD28 and CD3ζ were amplified from the synthesized cDNA of 28BBζ-CAR by PCR, and then the cDNA fragments containing the various constructs (ζ-, Δζ-, and 28ζ-CAR) were generated by overlap extension PCR and sub-cloned into a lentiviral vector with an elongation factor 1 alpha (EF-1α) promoter. To produce lentiviral particles, the constructed vectors, the packaging plasmid psPAX2 (Addgene), and the envelope plasmid pMD2.G (Addgene) were co-transfected into 293T cells (Cell Bank of Chinese Academy of Sciences, Shanghai, China) by using jetPRIME® transfection reagent (Polyplus transfection, France). After 24 and 48 h, supernatants containing the lentiviral particles were harvested, centrifuged at 500*g* for 10 min to remove the cellular debris, and further clarified using a 0.45-μm filter; the filtrate was then stored at −80 °C until use.

2.2. Human subjects

Human blood samples and fresh tumor specimens were obtained from healthy donors or patients under Independent Ethics Committee-approved protocols of the HuaiHe Hospital of Henan University.