Cholangiocarcinoma-derived exosomes inhibit the antitumor activity of cytokine-induced killer cells by down-regulating the secretion of tumor necrosis factor-α and perforin[*]

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Received Nov. 4, 2015; Revision accepted Jan. 22, 2016; Crosschecked June 18, 2016

Abstract: Objective: The aim of our study is to observe the impact of cholangiocarcinoma-derived exosomes on the antitumor activities of cytokine-induced killer (CIK) cells and then demonstrate the appropriate mechanism. Methods: Tumor-derived exosomes (TEXs), which are derived from RBE cells (human cholangiocarcinoma line), were collected by ultracentrifugation. CIK cells induced from peripheral blood were stimulated by TEXs. Fluorescence-activated cell sorting (FACS) was performed to determine the phenotypes of TEX-CIK and N-CIK (normal CIK) cells. The concentrations of tumor necrosis factor-α (TNF-α) and perforin in the culture medium supernatant were examined by using an enzyme-linked immunosorbent assay (ELISA) kit. A CCK-8 kit was used to evaluate the cytotoxic activity of the CIK cells to the RBE cell line. Results: The concentrations of TNF-α and perforin of the group TEX-CIK were 138.61 pg/ml and 2.41 ng/ml, respectively, lower than those of the group N-CIK 194.08 pg/ml ($P<0.01$) and 3.39 ng/ml ($P<0.05$). The killing rate of the group TEX-CIK was 33.35%, lower than that of the group N-CIK (47.35% ($P<0.01$)). The population of $CD3^+$, $CD8^+$, NK ($CD56^+$), and $CD3^+CD56^+$ cells decreased in the TEX-CIK group ((63.2±6.8)%, (2.5±1.0)%, (0.53±0.49)%, (0.45±0.42)%) compared with the N-CIK group ((90.3±7.3)%, (65.7±3.3)%, (4.2±1.2)%, (15.2±2.7)%), $P<0.01$. Conclusions: Our results suggest that RBE cells-derived exosomes inhibit the antitumor activity of CIK cells by down-regulating the population of $CD3^+$, $CD8^+$, NK ($CD56^+$), and $CD3^+CD56^+$ cells and the secretion of TNF-α and perforin. TEX may play an important role in cholangiocarcinoma immune escape.

Key words: Cholangiocarcinoma, Tumor-derived exosomes, Cytokine-induced killer cells, Immune escape
http://dx.doi.org/10.1631/jzus.B1500266   **CLC number:** R73-37

1 Introduction

Cholangiocarcinoma (CCA) is a devastating malignant disease that is proverbially difficult to diagnose and is associated with a high mortality. Although CCA accounts for only 3% of all gastrointestinal cancers, CCA represents the second most frequent primary hepatic malignant tumor after hepatocellular carcinoma, and a progressively increasing incidence rate was observed worldwide during the last decades (Khan *et al.*, 2005; Welzel *et al.*, 2006; Ewald *et al.*, 2013). Adoptive cellular immunotherapy is becoming an important application for cancer therapy. In recent decades, the application of cytokine-induced killer (CIK) cells has evolved from experimental observations into early clinical studies (Morse *et al.*, 2005; Wang *et al.*, 2011).

CIK cells are a population of cells derived from human peripheral blood mononuclear cells (PBMCs) after ex vivo expansion with interferon-γ (IFN-γ), monoclonal antibody against cluster of differentiation 3 (CD3), and interleukin-2 (IL-2) (Lu and Negrin,

1994). CIK cells have a higher proliferation rate, increased cytolytic activities, and a non-major histocompatibility complex (MHC)-restricted killing of tumor cells by secreting tumor necrosis factor-α (TNF-α) and perforin. CIK cells-based immunotherapy has proved to be effective in patients with a variety of malignancies, including CCA. Perforin and TNF-α, which are secreted by CIK cells, play a critical role in killing tumor cells and in immunotherapy against tumors. CIK cells have a potent cytotoxicity against tumor cells in vitro (Hongeng et al., 2003), but the curative effect of immunotherapy based on CIK cells is not that satisfying (Morse et al., 2005). Thus, we suspect that some substances secreted by tumor cells inhibit the activities of CIK cells.

Exosomes are small vesicles, about 40–100 nm in size, that are secreted by a variety of cell types, such as B-cells, T-cells, dendritic cells, and tumor cells (Raposo et al., 1996; Zitvogel et al., 1998; Wolfers et al., 2001). Tumor-derived exosomes (TEXs) have been shown to play an important role in the immune system, by mediating antigen and cytokine secretions (Bretz et al., 2013; Thompson et al., 2013).

In this study, in order to confirm the impact of TEX to CIK cells, we used the RBE cell line (human CCA cell line) to generate TEX. CIK cells were stimulated by TEX to evaluate the antitumor activities to the RBE cell line and to probe into their mechanisms, which caused CIK therapy to be dissatisfactory.

2 Materials and methods

2.1 Cell lines and culture

The RBE cell line, which is one of the human CCA cell lines, was obtained from the Committee of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). RBE cells were grown in the Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, USA) and antibiotics (50 µg/ml each of penicillin, streptomycin, and gentamicin; Gibco, USA) at 37 °C in a humidified 5% CO_2 and 95% of air atmosphere.

2.2 Preparation of CIK cells

We collected 15 ml of peripheral blood in evacuated tubes that contained heparin. PBMCs were isolated by using Ficoll density-gradient centrifugation and incubated in tissue culture flasks at 37 °C for 2 h, supplemented with an RPMI-1640 medium. On Day 0, for the non-adherent PBMCs we added 1000 U/ml recombinant human IFN-γ (Genentech, USA). After 24 h, 500 U/ml IL-2 (Chiron, USA), and 50 µg/ml CD3 monoclonal antibody (OKT3; Ortho Biotech, USA) were added and then these cells were cultured for another 7 d. Half of the old medium was replaced every 2 d with fresh CIK medium and 500 U/ml IL-2, respectively.

2.3 Preparation and identification of TEX

2.3.1 Cell culture

The RBE cells were cultured in an RPMI-1640 medium, supplemented with 10% FBS and antibiotics, as described previously, until the culture flask was almost full. Then, the culture media were discarded, and the cells were washed three times with phosphate-buffered saline (PBS; Gibco, USA). Next, new media supplemented with RPMI-1640 medium, without FBS, and the antibiotics mentioned above were added to the media, and the cells were cultured for another 72 h.

2.3.2 Isolation of TEX

After that, the cell culture media were collected and sequential centrifugations were performed. The cell culture media were centrifuged at 300g for 3 min at 4 °C to remove floating cells. These supernatants were then centrifuged at 2000g for 15 min at 4 °C and at 12 000g for 35 min at 4 °C to remove all cell debris. These supernatants were then passed through a 0.22-µm filter (Millipore, USA). The filtrates were ultracentrifuged at 120 000g for 3 h at 4 °C to collect exosomes using an Optima TLX Ultracentrifuge (Beckman Coulter, CA, USA). Exosome pellets were resuspended in PBS and were further ultracentrifuged at 120 000g for 3 h at 4 °C. Exosome protein quantification was performed using a BCA protein assay kit (KeyGen Biotechnologies, China). The final exosome pellets were stored at −80 °C until use (Chiba et al., 2012).

2.3.3 Nanoparticle tracking analysis

Isolated TEX from RBE cell lines were analyzed using the nanoparticle tracking analysis (NTA) Version 2.3 Build 0034. Analysis of the data was done

using the software supplied with the machine. Graphical analysis shows the size distribution of the TEX.

2.3.4 Western blot analysis

TEX was isolated by the procedure mentioned above from the RBE cell lines. A total of 20 µg TEX and RBE cell samples were solubilized in NP40 buffer (Boston BioProducts, MA, USA) and were run on a Western blot according to standard protocols. The following antibodies were used: mouse anti-CD63 (1:500, Abcam, UK), mouse anti-tumor susceptibility gene 101 (TSG101) (1:500, Abcam, UK), mouse anti-ALIX (1:500, Abcam, UK), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam, UK) as the loading control.

2.4 Identification of CIK cells

An amount of 1×10^6 CIK cells was harvested and washed twice with PBS. These cells were resuspended in 150 µl of PBS, labeled with 20 µl of antibodies against CD3/4/8 (PerCP-conjugated anti-CD3, FITC-conjugated anti-CD4, PE-conjugated anti-CD8; BD Biosciences, USA) and 10 µl of anti-CD56 antibody (APC-conjugated anti-CD56; BD Biosciences, USA), and placed in the dark for 30 min at 4 °C, and then washed twice with PBS. Fluorescence-activated cell sorting (FACS) was applied by using a FACSCalibur flow cytometer (BD Biosciences, USA) and CellQuest software (BD Biosciences, USA).

2.5 Detection of TNF-α and perforin

An amount of 1×10^6 CIK cells was added to a new 6-well flat-bottomed plate. These CIK cells, labeled as group TEX-CIK, were then treated with TEX at a ratio of 1×10^6 CIK cells:20 µg of TEX for 24 h. Another batch of 1×10^6 CIK cells, labeled as group N-CIK, was added to a new 6-well flat-bottomed plate for 24 h. After 24 h, the concentrations of TNF-α and perforin in the culture medium supernatant, with or without TEX, were examined by using an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, USA). Each group was tested in triplicate.

2.6 Cytotoxic examination of CIK cells

The RBE cells in the logarithmic growth phase were added to a 96-well plate (10 000 cells/well) as a target group. On the second day, TEX-CIK and N-CIK cells were added as an effector to the target ratio of 10:1 and tested in triplicate. Mixed incubation was done for 24 h. The supernatant was removed and all cells were washed twice with PBS. Then, CCK-8 was added, followed by incubation for another 3 h. The blank control was set with only PBS and CCK-8. The absorbance (optical density (OD)) was measured at 450 nm using a microplate reader. The killing rate was calculated as follows: killing rate=$(OD_{experiment}-OD_{blank})/(OD_{negative}-OD_{blank}) \times 100\%$.

2.7 Statistical analysis

Data were expressed as the mean±standard deviation (SD). A *t*-test was used for analysis of comparisons, correlation, and variance using SPSS 19.0 software (IBM, USA). $P<0.05$ was regarded as statistically significant.

3 Results

3.1 Characterization of TEX

A Philips 208 transmission electron microscope (FEI Company, the Netherlands) was utilized to analyze our TEX preparation for the RBE cell supernatants from a morphological point of view. The electron microscopy indicated that TEX was accorded with the characteristics of the exosomes (Fig. 1a). The size distribution of TEX was confirmed by using the Nanoparticle tracking analysis (Version 2.3 Build 0034), which was consistent with the exosomes (Fig. 1b). The exosome identity markers ALIX, CD63, and TSG101 were confirmed by using a Western blot analysis (Fig. 1c).

3.2 Phenotypic analysis of CIK cells

To determine the immunological effects of TEX-CIK and N-CIK cells, the phenotypes were characterized. The population of CD3$^+$, CD8$^+$, NK (CD56$^+$) and CD3$^+$CD56$^+$ cells decreased in the TEX-CIK group ((63.2±6.8)%, (2.5±1.0)%, (0.53±0.49)%, (0.45±0.42)%) compared with the N-CIK group ((90.3±7.3)%, (65.7±3.3)%, (4.2±1.2)%, (15.2±2.7)%) ($P<0.01$; Fig. 2). The population of CD4$^+$ cells in the TEX-CIK group was (10.1±2.5)%, not statistically significant when compared with that (12.3±2.7)% in the N-CIK group ($P>0.05$).
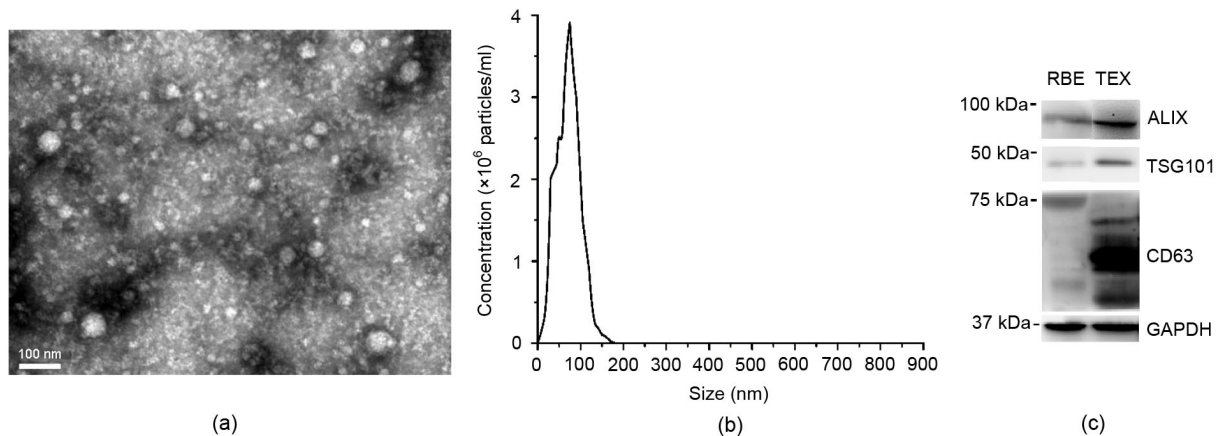
Fig. 1 Characteristics of TEX

(a) Electron microscopy of TEX (bar=100 nm). The culture supernatants yielded pellets consisting of a similar population of vesicles 30–120 nm in diameter. (b) The size distribution of TEX. (c) Western plot showing the expressions of exosomal markers ALIX, TSG101, and CD63 in TEX, with GAPDH as the loading control
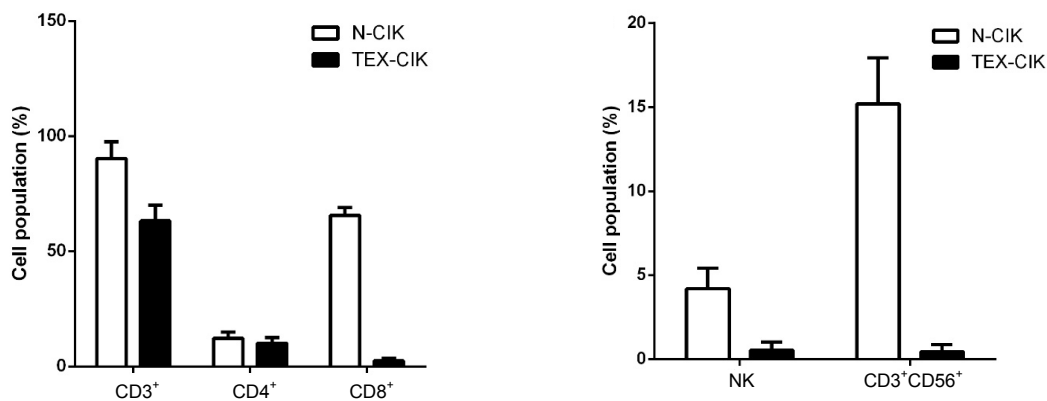

Fig. 2 Characteristics of cell phenotypes

The populations of $CD3^+$, $CD8^+$, NK ($CD56^+$), and $CD3^+CD56^+$ cells decreased in the TEX-CIK group compared with the N-CIK group ($P<0.01$). However, the population change of $CD4^+$ cells was not statistically significant ($P>0.05$)

3.3 TEX down-regulates the secretion of TNF-α and perforin

The concentrations of TNF-α and perforin of the group TEX-CIK were (138.61±46.01) pg/ml and (2.41±1.10) ng/ml, respectively, lower than those of the group N-CIK (194.08±35.14) pg/ml and (3.39± 1.39) ng/ml ($P<0.05$; Fig. 3).

3.4 TEX inhibits the antitumor activity of the CIK cells

The killing rate of the group TEX-CIK was (33.35± 10.05)%, significantly lower than that of the group N-CIK (47.35±12.74)% ($P<0.01$; Fig. 4).

4 Discussion

CCA was first reported by Durand-Fardel in 1840 (Olnes and Erlich, 2004). The disease is notoriously difficult to diagnose and is usually fatal because of its late clinical identification and the lack of effective non-surgical therapeutic modalities (Khan et al., 2005). Over the past two decades, the development of CIK cells for the treatment of carcinoma has received considerable attention. CIK cells have a major histocompatibility complex (MHC)-independent antitumor ability in both solid and hematologic malignancies. The antitumor activity is primarily related
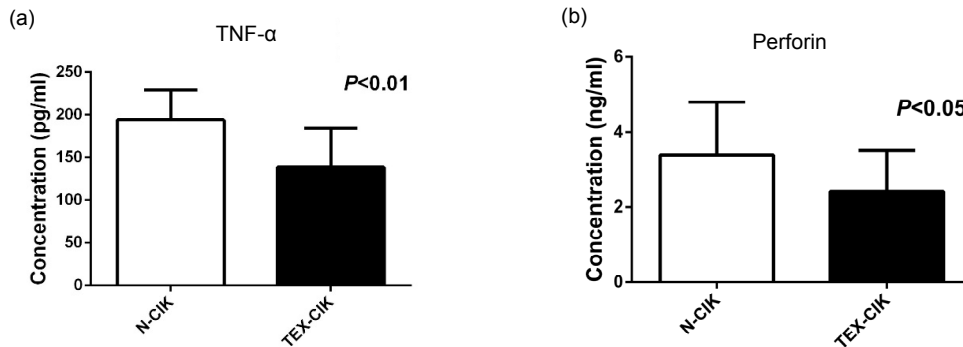
(a)

TNF-α

(b)

Perforin

Fig. 3 Concentrations of TNF-α and perforin

(a) The concentration of TNF-α of the groups TEX-CIK and N-CIK were (138.61±46.01) pg/ml, (194.08±35.14) pg/ml, respectively. (b) The concentration of perforin of the groups TEX-CIK and N-CIK were (2.41±1.10) ng/ml, (3.39±1.39) ng/ml, respectively. The secretion of TNF-α ($P<0.01$) and perforin ($P<0.05$) was down-regulated by TEX in the CIK cells
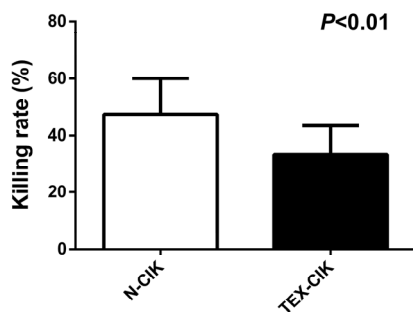

Fig. 4 Killing rate of CIK cells

The killing rate of the group TEX-CIK was (33.35±10.05)%, significantly lower than that of the group N-CIK (47.35± 12.74)% ($P<0.01$)

to a high percentage of the CD3$^+$CD56$^+$ subset (Ma et al., 2012; Nakano et al., 2012). The exact mechanism is still unclear, but primarily relates to the secretion of cytokines to inhibit the growth of tumor cells (Pievani et al., 2011; Wang et al., 2011; Yang et al., 2012). A large number of in vitro studies had confirmed the ability of CIK cells to kill various types of solid tumors. The CD3$^+$CD56$^+$ fraction of CIK cells is considered the crucial effector for the antitumor activities. Proofs of antitumor activities were clearly provided but the clinical responses were much lower than expected (Mesiano et al., 2012).

Exosomes are microvesicles released from various cells into the extracellular space, including tumor cell lines (Thompson et al., 2013). TEXs have been shown to play an important role in the immune system (Sun et al., 2012) by mediating apoptosis of the T lymphocytes and down-regulating the cytokine's presentation (Wolfers et al., 2001). CD3 as a T-cell co-receptor is an important part of the T-cell receptor (TCR) complex. CD8 also serves as a co-receptor for the TCR and binds it to an MHC class I molecule (Garcia et al., 1996). The importance of the MHC class and TCR in cancer immunology is greater than expected when compared with previous studies (Slansky et al., 2000; Xue et al., 2013).

The results in this study showed the imporatance of TEX in down-regulating the population of CD3$^+$, CD8$^+$, NK (CD56$^+$), and CD3$^+$CD56$^+$ cells in the TEX-CIK group when compared with the N-CIK group ($P<0.01$). The concentrations of TNF-α and perforin in the group TEX-CIK were lower than those in the group N-CIK ($P<0.05$). The down-regulation of CD3 and CD8 may attenuate the ability of antigen in presenting, thus meditating the tumor immune escape. Taylor et al. (2003) suggested that FasL associated with TEXs was responsible for apoptosis of the T lymphocytes and a decrease of CD3$^+$ cells in patients with ovarian carcinoma. Wieckowski et al. (2009) showed that tumor-derived microvesicles induce apoptosis in CD8$^+$ T lymphocytes. The subset of the CD3$^+$CD56$^+$ cells, which expresses both the T-cell marker CD3 and the natural killer cell marker CD56, is termed non-MHC-restricted T cells. These cells are capable of killing both autologous and allogeneic tumor targets, and show the greatest cytotoxicity (Schmidt-Wolf et al., 1993). CD3$^+$CD56$^+$ lymphocytes play a critical role in the antitumor process by secreting TNF-α and perforin. TNF-α is a cytokine able to induce hemorrhagic necrosis of tumors and is regarded as a promising approach for cancer therapeutics (Carswell et al., 1975). TNF-α is able to

induce fever, apoptotic cell death, cachexia, and inflammation, and to inhibit tumorigenesis and viral replication. Perforin is a cytolytic mediator produced by killer lymphocytes. The protein is partially homologous to the terminal components of the membrane attack complex, which complements and produces pores of up to 20 nm in diameter in the target membranes (Liu *et al.*, 1995). Upon degranulation, perforin inserts itself into the target cell's plasma membrane, forming pores (Tschopp *et al.*, 1986). This protein creates transmembrane tubules and is capable of lysing non-specifically a variety of target cells. This protein is one of the main cytolytic proteins of cytolytic granules, and it is also known to be a key effector molecule for T-cell- and natural killer-cell-mediated cytolysis. The data above showed that TEX down-regulated the population of $CD3^+$, $CD8^+$, NK ($CD56^+$), and $CD3^+CD56^+$ cells, especially the population of $CD3^+CD56^+$ cells, resulting in the secretion decreases of TNF-α and perforin, thus the antitumor ability of the TEX-CIK group was lower than that of the N-CIK group. An increasing number of literature show that transforming growth factor-β (TGF-β), Wnt signaling components, phosphatase and tensin homolog (PTEN), epidermal growth factor receptor (EGFR), and Fas ligand may play important roles in exosome-meditated tumor immunosuppression (Apte *et al.*, 2013). Thus, the further mechanism underlying the depletion of $CD3^+CD56^+$ CIK cells upon exosome treatment needs additional research.

5 Conclusions

From this discussion, one may conclude that RBE cells-derived exosomes (TEXs) inhibit the antitumor activity of CIK cells by down-regulating the population of $CD3^+$, $CD8^+$, NK ($CD56^+$), and $CD3^+CD56^+$ cells and the secretion of TNF-α and perforin. TEX may play an important role in CCA immune escape.

Compliance with ethics guidelines

Jiong-huang CHEN, Jian-yang XIANG, Guo-ping DING, and Li-ping CAO declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. All participants or their guardians gave written consent for their blood samples to be used for scientific research. The research protocol was reviewed and approved by the Research Ethics Committee of Zhejiang University, Hangzhou, China.

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中文概要

题　目：胆管癌外泌体通过抑制肿瘤坏死因子 α 与穿孔素分泌使细胞因子诱导的杀伤细胞抗肿瘤活性下降

目　的：探索胆管癌来源外泌体（TEX）对细胞因子诱导的杀伤细胞（CIK）抗肿瘤活性的影响，并初步探讨其作用机制。

创新点：首次通过体外实验证明 TEX 可引起 CIK 抗肿瘤活性下降，且此作用与肿瘤坏死因子 α（TNF-α）和穿孔素表达抑制相关。

方　法：采用超速离心法提取人胆管癌细胞（RBE）来源的外泌体，同时 CIK 通过人外周血培养获得。将 TEX 负载到 CIK 培养体系中作为 TEX-CIK 组，

不加 TEX 的 CIK 作为 N-CIK 组。流式细胞仪检测两组 CIK 细胞表型变化，酶联免疫吸附法（ELISA）检测两组培养基上清液中 TNF-α 和穿孔素的浓度，CCK-8 法检测 CIK 对 RBE 细胞的杀伤活性。

结　论：TEX 能降低 CIK 细胞 CD3$^+$、CD8$^+$、NK（CD56$^+$）以及 CD3$^+$CD56$^+$比例，并且抑制 TNF-α 和穿孔素表达，从而降低 CIK 细胞的抗肿瘤效应。

关键词：胆管癌；肿瘤来源外泌体；细胞因子诱导的杀伤细胞；免疫逃逸